

BIOLOGICAL STUDIES OF DISTRACTION OSTEOGENESIS

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谨 以 此 论 文 献 给
我 的 妻 子 文 利
和 我 的 父 母

**To my wife Wenli
and my parents**

知其然
并
知其所以然

**know the how and endeavour to
know the why**

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Biological studies of distraction osteogenesis

A thesis presented in candidature for the degree of doctor of philosophy

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Abstract

The development of limb lengthening techniques has made possible the treatment of many congenital and acquired disorders of the adult and developing skeleton by generating new bone tissue at appropriate sites. The studies described in this thesis have attempted to elucidate some of the biological aspects of this process which is called distraction osteogenesis.

A rabbit model of distraction osteogenesis has been used in the present study. Lengthening at four different distraction rates (0.3, 0.7, 1.3 and 2.7 mm/day) has been investigated radiographically and histologically. The most satisfactory histology and radiology results were observed in the group distracted at a rate of 0.7 mm/day. The present study indicated that the proliferation of osteoprogenitor cells during distraction osteogenesis was affected by the rate of distraction. The rate of cell proliferation in the regenerating tissues was found to increase as the rate of lengthening increased from 0.3 to 0.7 mm/day. However, a further increase of cell proliferation did not occur at higher distraction rates.

In-situ hybridization techniques using non-radioactive labelling probes and immunohistochemistry using specific collagen antibodies have been used in the study. The localization of the mRNAs and proteins for types I and II collagen in the distraction regenerates indicates that bone is formed mainly in an intramembranous manner during distraction osteogenesis. However, higher rates of distraction resulted in increased chondrogenesis in the regenerating tissues. The osteoblasts and chondrocytes within the regenerate originate from the same pool of progenitor cells and their differentiation appeared to be altered by the rate of distraction.

In addition, the present study has characterized the localization of BMP-4 mRNA expression in the regenerating tissues produced by distraction osteogenesis. The BMP-4 gene was expressed mainly by less differentiated osteoprogenitor cells in the regenerating tissue, indicating that BMP-4 may play an important role in controlling the early differentiation and proliferation of osteoprogenitor cells during distraction osteogenesis.

The demonstration of a mixture of proliferative and apoptotic cells in the regenerating tissues, suggested that apoptosis and cell proliferation may be regulated closely during the rapid cell or tissue turnover seen in distraction osteogenesis. The changes of cells undergoing apoptosis and proliferation in the different regions of the distraction regenerate, suggested that apoptosis is an important mechanism controlling cell or tissue turnover and bone remodelling during distraction osteogenesis.

The neovascularization process is affected by the rate of distraction during distraction osteogenesis. A slow rate of distraction (0.3 mm/day) did not maximally stimulate angiogenesis, while the highest rate (2.7 mm/day) of distraction inhibited angiogenesis. The angiogenic response was the best at distraction rates of 0.7 and 1.3 mm/day.

The clinical relevance of the present findings has been discussed and future work are suggested.

Abbreviations

AP	Alkaline phosphatase
APAAP	Alkaline phosphatase anti-alkaline phosphatase
APES	3-aminopropyltriethoxy silane
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BMP	Bone morphogenic protein
bp	Base pair
BrdUrd	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
BSP	Bone sialoprotein
cDNA	Complementary DNA
DAB	Diaminobenzidine
DEPC	Diethylpyrocarbonate
DEXA	Dual-energy x-ray absorptiometry
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNase I	Deoxyribonuclease I
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FZ	Fibrous zone
HE	Haematoxylin and eosin
hr	hour
In vitro	Biological phenomena outside the living body
In vivo	Biological phenomena inside the living body
KSPG	Keratan sulphate proteoglycan
MF	Mineralization front
mg	Milligram
Min(s)	Minute(s)
ml	Millilitre

mRNA	Messenger RNA
n	Number
NBT	Nitro blue tetrazolium
NBZ	New bone zone
nmol	Nanomolar
NZW	New Zealand white
PAP	Peroxidase-anti-peroxidase
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCNA	Proliferating cell nuclear antigen
PMF	Primary mineralization front
PSI	Positive labelling index
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
TBS	Tris buffered saline
TRAP	Tartrate-resistant acid phosphatase
Tris	Tris (hydroxymethyl) aminomethane
tRNA	Transfer RNA
TUNEL	Transferase mediated non-radioactive labelled dUTP nick end labelling

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PART I

INTRODUCTION

Chapter 1 Introduction

1. 1 The development, anatomy and growth of long bones

1. 1. 1 Development of long bones

A long bone is characteristically tubular with expanded ends and is strong for its weight. The shaft is the diaphysis and the ends the epiphyses with the zone adjacent to the epiphyseal plate, the metaphysis, which is the most metabolically active area and has the greatest blood supply.

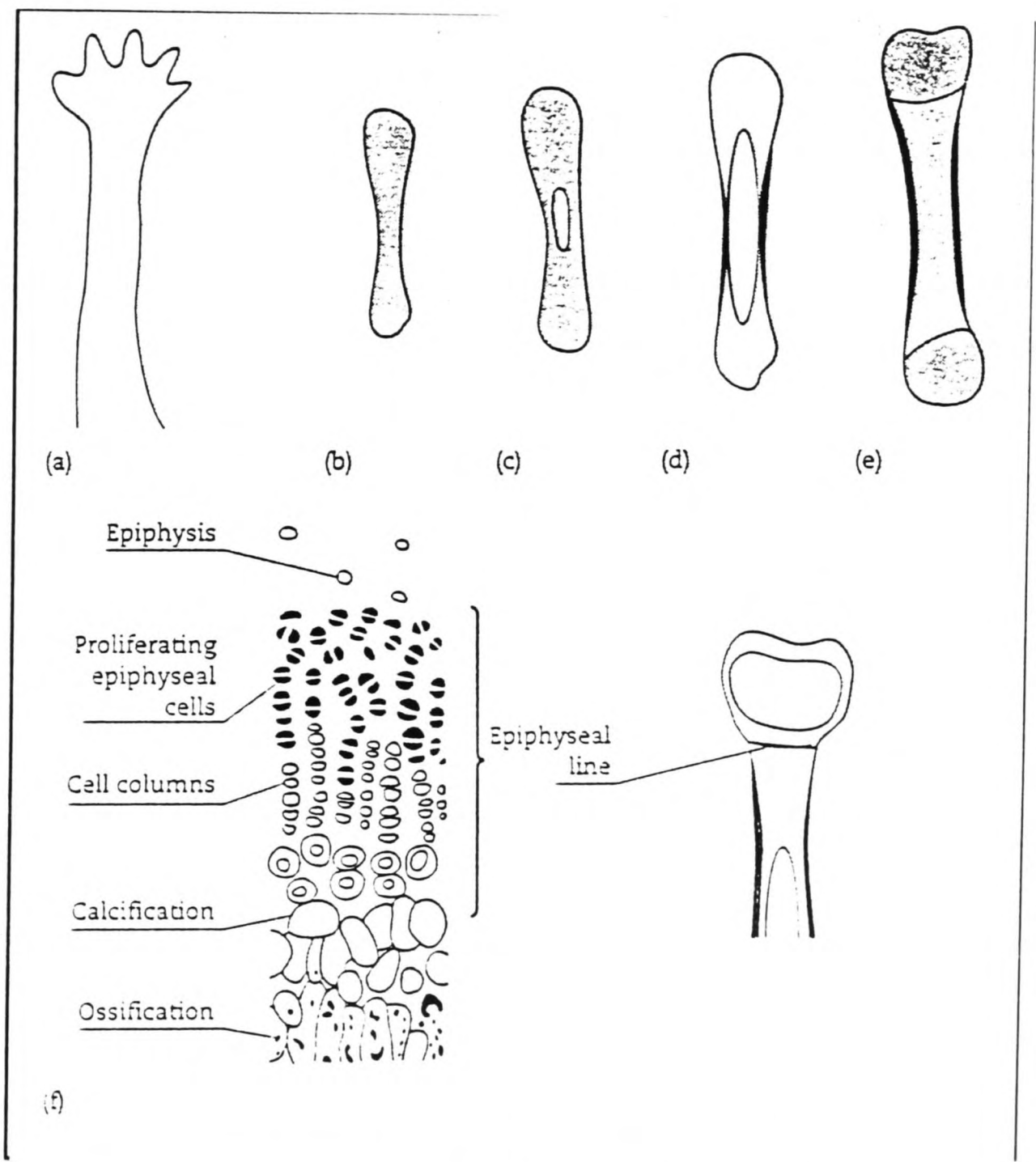


Fig.1.1. Illustrations showing bone growth and development.

Bone development begins in early intra-uterine life as a condensation of mesenchymal tissue in the axis of the limb (Fig 1.1a). By the 6th week the connective tissue cells have started to lay

down cartilage to form the shape of the future bone (Fig. 1.1b). At the centre of the cartilage mass the cells hypertrophy and, apparently die and with the ingrowth of vascular connective tissue, the matrix eventually ossifies (Fig. 1.1c). The ossification process spreads along the bone (Fig. 1.1d) so that it eventually consists of a bony shaft with cartilaginous ends (Fig.1.1e) which become the sites of secondary ossification centres (Figs. 1.1f and 1.2). The epiphyseal cartilage remains until maturity as the growth point for bone length. The proliferating chondrocytes on the diaphyseal side form into columns and undergo a series of changes, eventually calcifying as the zone of vascularization reach them. This results in gradual growth of the epiphysis away from the centre of the shaft. Growth in width occurs by deposition of non-cartilaginous sub-periosteal bone and the whole bone is constantly remodelled as the individual grows (Marks and Hermey, 1996).

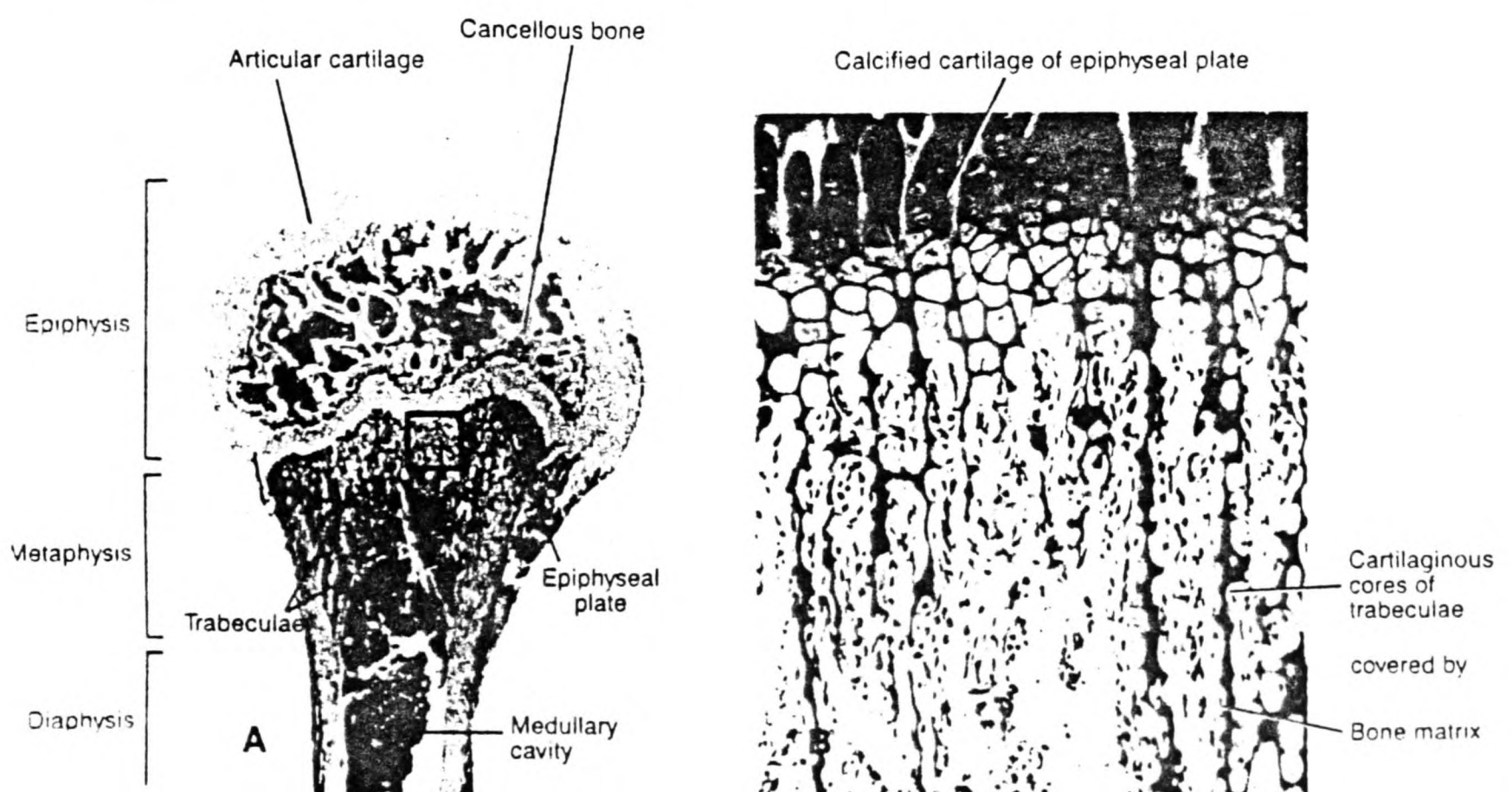


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The earliest bone to be laid down is often called “woven bone”. Its histological structure shows the fibrils to be randomly distributed, and it is also known as immature bone. A feature of immature bone is that it contains a relatively higher proportion of osteocytes. The matrix of woven bone tends to be tinged with blue in haematoxylin and eosin stained sections, possibly indicating that it has a higher proteoglycan content than mature bone (Cormack, 1987). Mature

bone, alternatively described as lamellar bone, is characterized by some microscopic features: (1) the comparatively even acidophilic staining of the matrix, (2) the comparatively regular arrangement of its lamellae and (3) the presence of fewer osteocytes which are more evenly arranged and are present in flat lacunae (Cormack, 1987).

Bone growth involves the coordination of a variety of cellular activities at specific sites whose onset and rates vary among bones and even within a single bone during its development. These activities are under the influence of a variety of humoral and local factors whose relative concentrations, sites and sequences of appearance vary during development (Marks and Hermey, 1996).

1. 1. 2 Bone structural units

Each bone can be seen histologically to be composed of small units that have been formed or remodelled at different times and these have been described variously as bone metabolic units, bone remodelling units or bone structural units (Dickson, 1993). In adult human cortical bone, the typical structural units are the Haversian systems, or osteons, about 250 μm in diameter (Fig.1. 3).

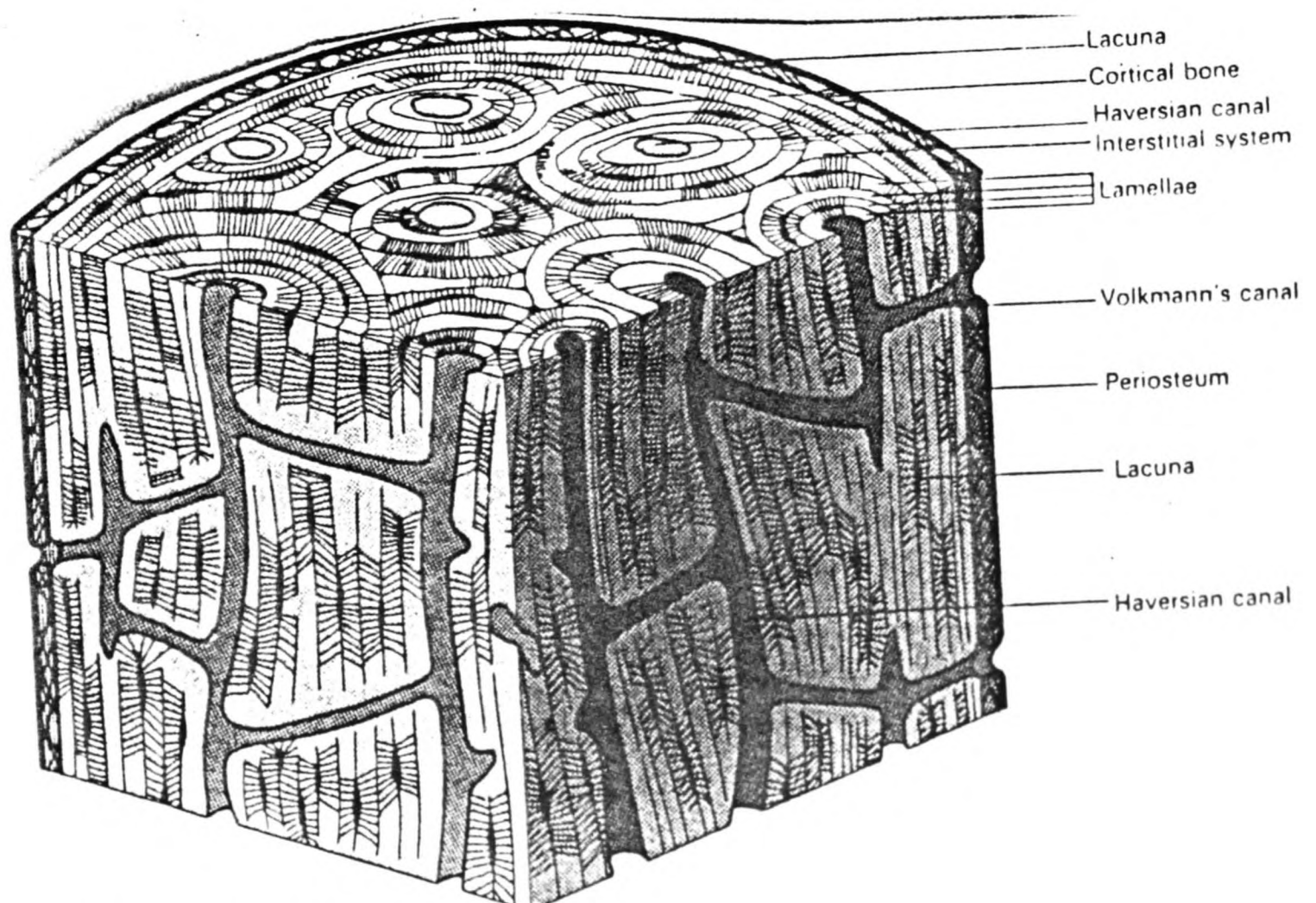


Fig.1.3. Bone structural unit.

Intact osteons comprise about two-thirds of the bone in the young adult, and between these osteons are interstitial lamellae representing the remnants of previous osteons. The central Haversian canals contain blood, lymphatic vessels and nerves and are surrounded by concentrically arranged lamellae; they generally run longitudinally in the bone and are connected transversely by Volkmann's canals (Fig. 1. 3).

1. 1. 3 Blood supply of a long bone

According to Rhinelander (1974) the basic components of the afferent vascular system of a long bone are: (1) a principal nutrient artery, traversing the cortex without giving off branches and joining to the medullary arteries; (2) the metaphyseal arteries (proximal and distal) which anastomose at each end of the medulla and form the medullary arterial supply; (3) the periosteal arterioles which are a minor component and supply blood only to the outer layer of the cortex in very limited areas. The medullary arteries supply most of the blood for the cortex of a long bone, and the metaphyseal arteries are the main contributors for medullary arteries (Bahn, 1982). Physiologically, the regional and local blood supply is probably the most absolute requirement for new bone formation. Vascularization is always an early feature in osteogenesis, and angiogenic factors released by the endothelial cells of blood vessels are important for bone formation (Pfeilschifter et al., 1990; Aubin, et al., 1990; Villanueva and Nimni, 1990). For more details, see section 1. 10.

1. 1. 4 Structure of periosteum and endosteum

Periosteum consists of two main layers, an outer fibrous limiting membrane and an inner cellular osteogenic layer, known as the cambial layer (Wlodarski, 1989). The outer cambial layer is composed of fibroblast-like preosteoblasts and the inner layer of osteoblasts. Cells on both the outer and the inner layer of the periosteum have potential to proliferate and differentiate into osteoblasts (Tonna and Cronkite, 1961). Normal periosteum can be activated to proliferate by trauma, infection and inflammation (Wlodarski, 1989). Activated periosteum produces cartilage and bone and, when appositional bone growth at the periosteal surfaces is complete, osteoblasts of the periosteum revert to become quiescent cells.

The endosteum lies on the inner surface of the bone marrow cavity, and is a thin layer of highly vascular, condensed fibrous tissues. The endosteal cells line the inner surfaces of bone, and are made up of osteoblasts, osteoclasts or bone lining cells. The endosteal cells have similar functional characteristics to the periosteal cells. However, the endosteum appears to be less important for bone repair compared to the periosteum once it has been removed (Kojimoto, et al., 1988).

1. 1. 5 Structure and function of the growth plate

The growth plate consists of cartilage, bone and fibrous tissue, which work in harmony to induce longitudinal bone growth (Brighton, 1978). However, the production of new cartilage matrix does not increase the thickness of the growth plate, as cartilage formation within the plate is only sufficient to keep pace with its replacement by bone tissue on its diaphyseal aspect. The epiphyseal plates grow on one side and become replaced by bone on the other, and they are gradually shifted farther apart. Bony replacement eventually catches up with cartilage production and the growth plate fuses (Cormack, 1987). The growth plate can be divided into 4 zones, each of which has a special function (Figure 1. 4): reserve zone, proliferative zone, hypertrophic zone and zone of vascular invasion (Brighton, 1984).

The reserve zone is also known as the resting zone, germinal zone, or zone of small cartilage cells and consists of rounded cells, which exist singly or in pairs in abundant matrix and are relatively few in number (Brighton, 1978; 1984). They are metabolically active, and they contain more lipid bodies and vacuoles than do cells in other zones (Brighton, et al, 1973). The chondrocytes in the reserve zone do not proliferate or do so only sporadically (Kember, 1960; Rigal, 1962).

In the proliferative zone, flattened chondrocytes are aligned in longitudinal columns and the majority of dividing cells in the growth plate are seen here (Kember, 1960). The matrix of the proliferative zone contains collagen fibrils, distributed at random. The main functions of the proliferative zone are matrix production and cellular proliferation. The combination of these two functions results in linear or longitudinal growth (Marks Hermey, 1996).

In the hypertrophic zone, the chondrocytes undergo massive hypertrophy which may increase their volume five times by the time the average chondrocyte reaches the bottom of the hypertrophic zone (Brighton, 1984). It is generally believed that hypertrophic chondrocytes represent degenerating cells whose ultimate fate is death (Brighton, 1978; 1984). Recent findings, however, suggested that some hypertrophic chondrocytes maybe capable of becoming bone-forming cells (Cancedda, et al., 1995). The functions of the hypertrophic zone are: (1) to prepare the matrix for calcification and (2) to calcify the matrix (Brighton, 1978; 1984; Roach, et al., 1995).

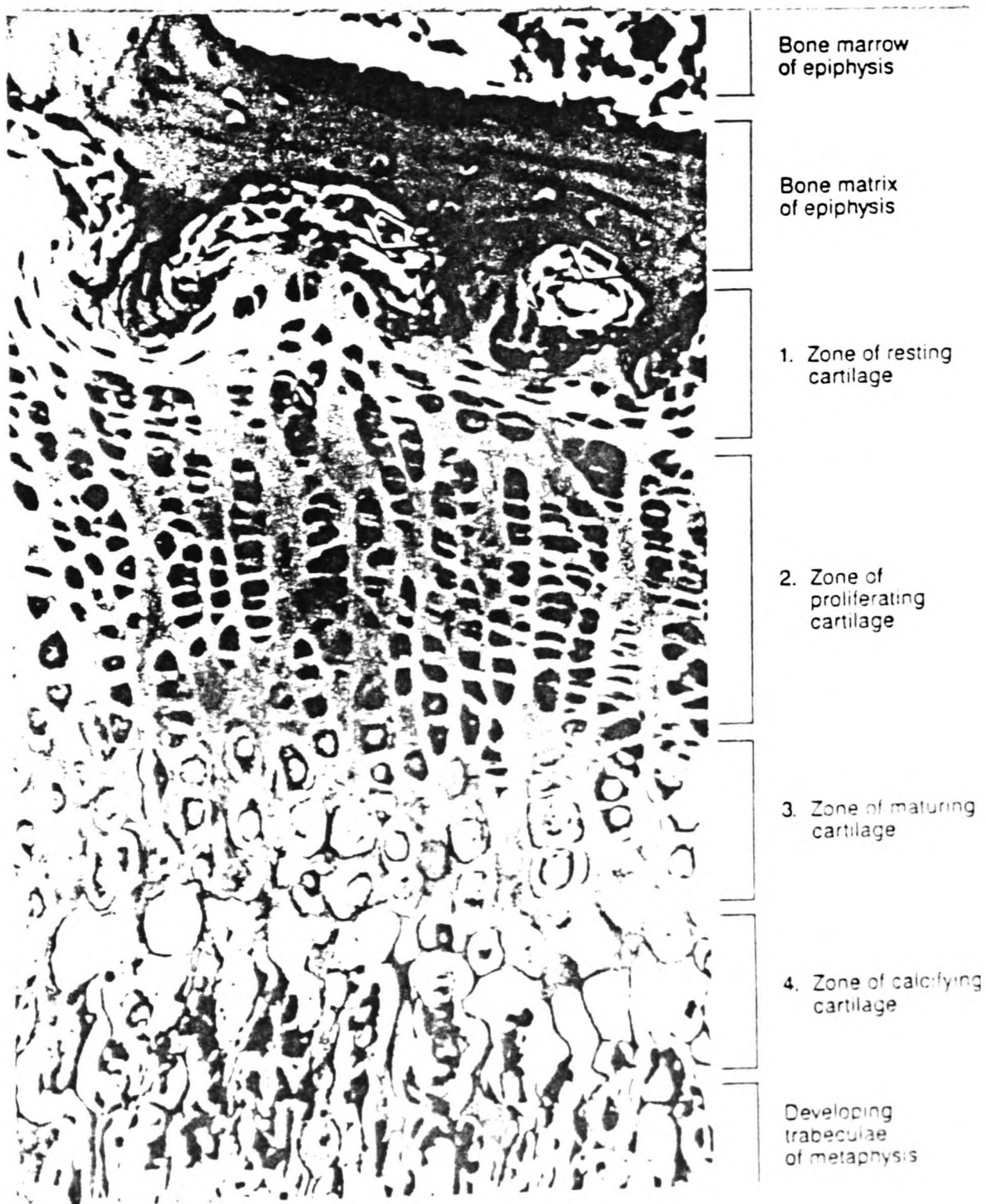


Fig. 1.4. The structure of the growth plate.

In the zone of vascular invasion, capillaries from the metaphysis invade the transverse septae

around the chondrocytes and the process of replacement of calcified cartilage by bone begins. The farther down into the metaphysis, the more bone is formed on the calcified cartilage bars. The functions of the zone of vascular invasion together with metaphysis are (1) vascular invasion at the bottom of cartilaginous portion, (2) bone formation and (3) remodelling, removal of calcified cartilage bars (Brighton, 1978; 1984).

1. 1. 6 Endochondral and intramembranous ossification

Skeletogenesis in the developing embryo involves two major process of cellular differentiation leading to the formation of cartilage and bone (Marks and Popoff, 1988): (1) the endochondral pathway, as in the long bone of the limbs, and (2) the intramembranous pathway, which takes place predominantly in the craniofacial bones. Both endochondral and intramembranous ossification require a solid base and a well-developed vascular supply for elaboration and mineralization of the extracellular matrix.

Bones that participate in joints and bear weight form by endochondral ossification. In endochondral ossification, mesenchymal cells condense and differentiate into cartilage, which form an anlage for the future skeletal components. The cartilage anlage subsequently matures, undergoes hypertrophy, mineralizes and is then invaded by blood vessels and osteoprogenitor cells (Owen, 1980; Hunziker, 1994). These invading cells subsequently become osteoblasts and form bone by the production of an osteoid matrix (Sandberg, 1991).

Intramembranous bone formation involves direct differentiation of mesenchymal cells into osteoblasts (Bernard and Pease, 1969; Marvaso and Bernard, 1977). Bone is directly formed from the fibrous tissue, no cartilage intermediate is found in the intramembranous bones. This type of ossification is restricted to the cranial vault, some facial bones, and parts of irregular bones, such as mandible and clavicle. The mechanism controlling the progression of embryonic mesenchyme along the endochondral or intramembranous pathway is still unknown (Tuan, 1992).

1. 2 Cells of bone and bone marrow

There are numerous cell types associated with bone and bone marrow and of these, five are of specific importance: osteoblasts, bone lining cells, osteocytes, osteoclasts, and bone marrow osteogenic stem cells (Fig. 1. 5).

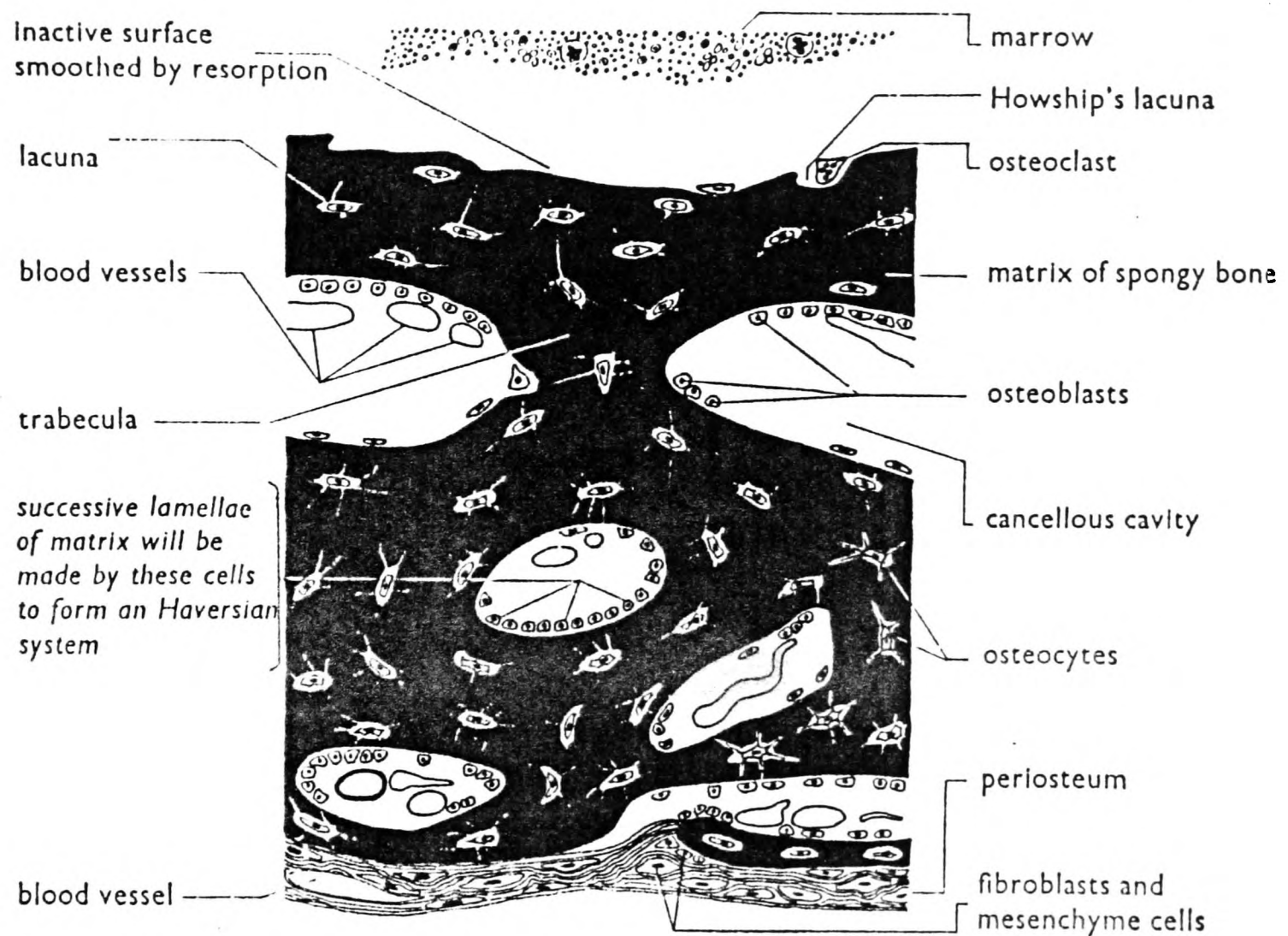


Fig. 1. 5. Diagram showing the structure and cells of bone.

1. 2. 1 Osteoblasts

Osteoblasts are responsible for bone formation. They synthesize and regulate mineralization of the extracellular matrix of bone. Based on morphological studies, osteoblastic cells are categorized in a presumed linear sequence progressing from osteoprogenitor to preosteoblasts, to osteoblasts, and then to lining cells or osteocytes (Nijweide and Mulder, 1986; Marks and Popoff, 1988; Wlodarski, 1990). Earlier morphological definitions of the active osteoblasts define these cells as cuboidal, rounded to polygonal shape, with basophilic cytoplasm, and eccentric nuclei with 1-3 nucleoli (Cameron, 1968; Holtrop, 1975). They are seen as a single

layer of cells attached to the unmineralized osteoid of forming bone. These cells have typical features of active secretory cells including abundant rough endoplasmic reticulum and a prominent Golgi apparatus (Marks and Popoff, 1988). Osteoblasts secrete collagen, as well as the glycoprotein-based ground substance. Mineral is deposited within the matrix and exists as a highly substituted microcrystalline hydroxyapatite. Active osteoblasts give a strong histochemical reaction for alkaline phosphatase that disappears when cells cease their synthetic activity (Doty and Schofield, 1976). The products of osteoblasts are type I collagen (Leblond, 1989), osteocalcin (Hauschka, et al., 1989), osteopontin (Butler, 1989), bone sialoprotein and a number of other matrix components (Sodek, et al., 1992; Bianco, et al., 1993), IGF-1 (Lazowski, et al., 1994), and c-fos (Machwate, et al., 1995).

1. 2. 2 Bone lining cells

Bone lining cells are elongated, flat cells directly apposed to bone surfaces and are a quiescent population of cells (Vaughan, 1981). They are considered to arise by terminal differentiation of osteoblasts, they are metabolically inactive and have few cytoplasmic organelles (Parfitt, 1984). They may be resting osteoblasts which are not secretory cells, but at least some have the capability of dividing as shown by uptake of tritiated thymidine (Bowman and Miller, 1986). The exact function of bone lining cells is unknown. They may be involved in the regulation of bone resorption by releasing chemotactic factors or exposing the bone surface to osteoclasts; or they may be precursors for osteoblasts (Parfitt, 1984; Marks and Hermey, 1996).

1. 2. 3 Osteocytes

When completely surrounded by osteoid or bone matrix, the osteoblast becomes an osteocyte. Osteocytes are in contact with one other by cytoplasmic projections within channels (canaliculi) through the mineralized matrix (Menton, et al., 1984). The projections join in gap junctions and may provide communication across the bone (Rodan, 1992). The size, shape and organization of the osteocytes and their lacunae vary with the type of bone. Tetracycline-labelling studies show that the half life of osteocyte can be 25 years or longer (Marotti et al., 1990).

The exact function of the osteocyte is not clearly established. These cells are possibly responsible for the exchange of ions with the bone matrix and the rapid release of calcium and phosphorus from mineralized bone (Nijweide, et al., 1996). These cells have the capacity not only to synthesize, but also to resorb matrix to a limited extent. The process of “osteocytic osteolysis” proposed by Belanger, 1969 has been discounted. It has been suggested that, because of their location within bone and their communication network, osteocytes are capable of detecting and transforming changes in mechanical stress, act as mechanosensor cells, and causing bone remodelling in response. Several studies have indicated that osteocytes are sensitive to stress applied to the intact bone tissue (Pead, et al., 1988; Skerry, et al., 1989). Over the last few years a number of studies have suggested that osteocytes sense mechanical loading by interstitial fluid flow in bone (Salzstein and Pollack, 1987; Salzstein, et al., 1987; Reich et al., 1990; Turner, et al., 1994). Theoretical as well as experimental studies in vivo and in vitro (see review Nijweide, et al., 1996) agree that the network of osteocytes, perhaps in conjunction with the bone lining cells, provides the cellular structure that allows bone organs to determine local needs for bone augmentation or reduction in response to mechanical demands.

1. 2. 4 Osteoclasts

Osteoclasts are multinucleated cells largely responsible for bone resorption. Active osteoclasts are present in small depressions, called Howship’s lacunae, at the bone surface. Osteoclasts are mainly localised on endosteal bone surfaces and fewer are on periosteal surfaces (Suda, et al., 1992). The cells are 40-100 μm in diameter with an average of 10 to 15 nuclei, though the number may range from 2 to as many as 100 (Marion-Gurly and Roth, 1992). Another characteristic morphological feature of the active osteoclast is the presence of the ruffled border in contact with the bone surface. Adjacent to this border is a clear zone lacking organelles which anchors the osteoclast to the bone surface.

The two enzymes most characteristic of osteoclasts are carbonic anhydrase and an acid phosphatase that retains its activity in the presence of sodium tartrate (tartrate-resistant acid phosphatase), in contrast to that of macrophages, which does not (Dickson, 1993). The mechanism by which osteoclasts resorb bone is not well defined, but their activity appears to

require mineral, and unmineralized tissue is rarely resorbed. Bone resorption by osteoclasts probably occurs in two connected stages: (1) the mineral is first dissolved under the acid conditions created by carbonic anhydrase, which converts metabolically produced carbon dioxide to carbonic acid (Kenny, 1985), and (2) the low-pH environment then provides suitable conditions for the subsequent degradation of organic matrix by lysosomal acid hydrolases, lysosomal cysteine proteinases, and matrix metalloproteinases (MMPs) (Väänänen, 1996). In vitro studies have shown that an osteoclast can go through more than one resorption cycle (Lakkakorpi and Väänänen, 1991). There is in vitro evidence which suggests that multinucleated cells can continuously generate mononuclear cells, which retain proliferative capacity (Solari, et al., 1995). Most osteoclasts, however, are removed by apoptosis after one resorption cycle (Hughes, et al., 1995).

1. 2. 5 **Bone marrow osteogenic stem cells**

Bone marrow is contained within the space of bone and contains numerous cell types, such as haemopoietic stromal cells, haemopoietic cells, endothelial cells and osteoprogenitor cells (mesenchymal cells) or osteogenic stem cells (see review Triffitt, 1996).

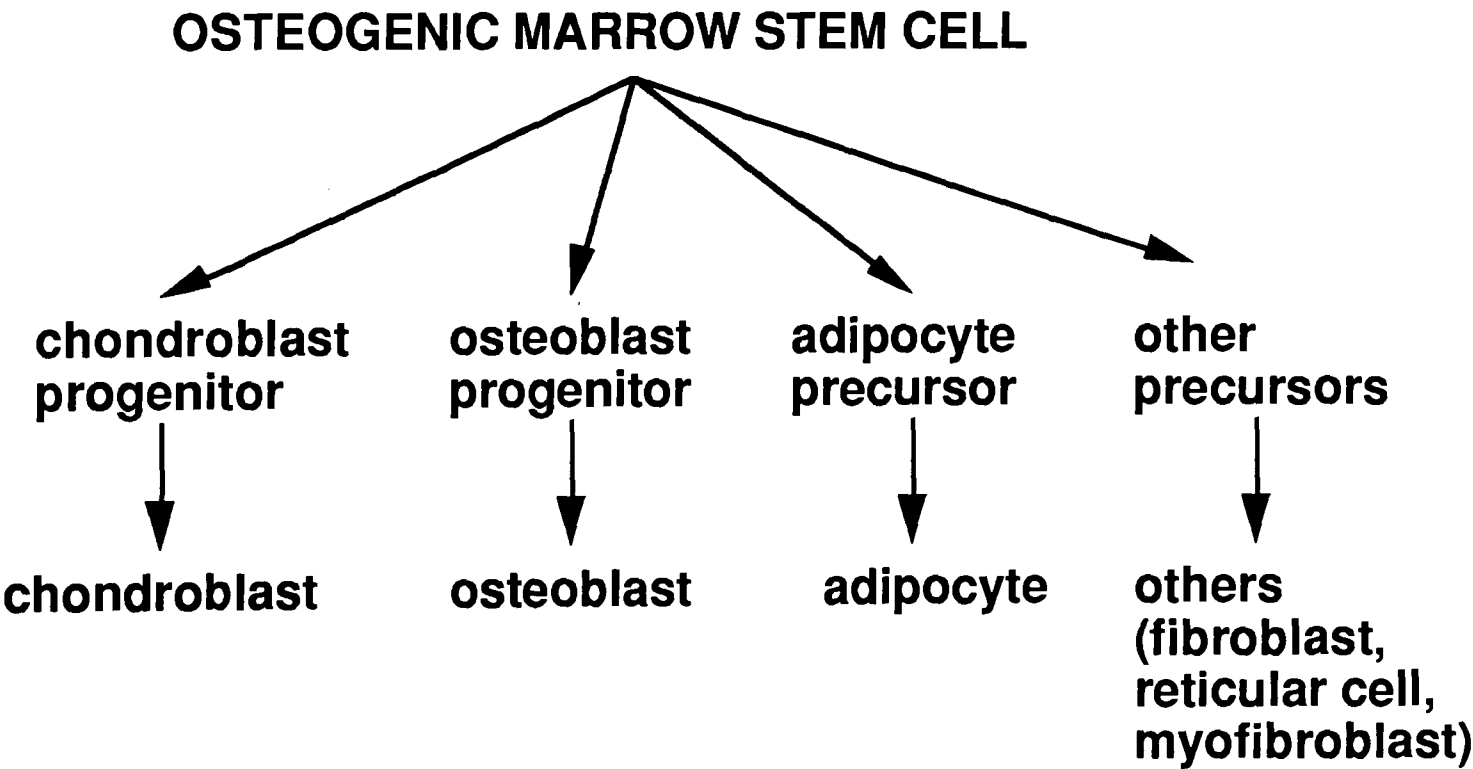


Fig. 1.6. The differentiation of marrow stem cells.

In the postnatal animal, osteogenic and chondrogenic stem cells are components of bone marrow (Friedenstein, 1976; Owen, 1980). Friedenstein (1976) showed that bone marrow

stromal fibroblasts were capable of generating bone when implanted in diffusion chambers in vivo. Fibroblastic cells derived from other tissues did so only in the presence of an osteogenic inducer (Owen, 1988). By definition, stem cells have a high capacity for self renewal and the potential to undergo differentiation into more than one cell type (Fig.1.6). These cells include chondroblasts, osteoblasts, adipocytes, fibroblasts, myofibroblasts and reticular cells (Triffitt, 1996). Progression from the marrow stem cell to the fully differentiated cell phenotype involves many stages and marrow stromal cells lose their proliferative capacity as they become increasingly differentiated. A stem cell can differentiate into a number of tissues, and abnormal patterns are seen in osteoporosis and aging where there is an increase in fat content (Burkhardt, et al., 1987; Martin, et al., 1989). Following the observation that marrow adipocytes could re proliferate and potentially differentiate in an osteogenic direction, came the hypothesis that the differentiation of these two cell types under certain conditions could be mutually exclusive (Beresford, et al., 1992).

Clinical use of marrow in composite bone graft to promote healing of skeletal defects has been recognised and used for many years (Einhorn, 1995). Connolly (1995) reviewed the earlier work and has evaluated methods of obtaining concentrated osteoblast stem cells to serve as injectable grafts in a number of orthopaedic procedures. These include delayed fracture union, nonunion, arthrodeses and bone defects.

1. 3 Cell proliferation in bone development

1. 3. 1 Cell proliferation and the cell cycle

Cell proliferation is defined as the increase in cell number resulting from completion of the cell cycle (Pardee, 1989). Growing cell populations contain cells that repeatedly pass through a well-defined sequence of events from one cell division to the next. This ordered progression of events is termed the cell cycle and has been defined by Baserga (1981) as the interval and events between the midpoint of mitosis in the parent cell, and the midpoint of the subsequent mitosis in one daughter cell. Lajtha (1957) described 5 stages describing the events in the cell cycle which are still used today (Fig. 1. 7).

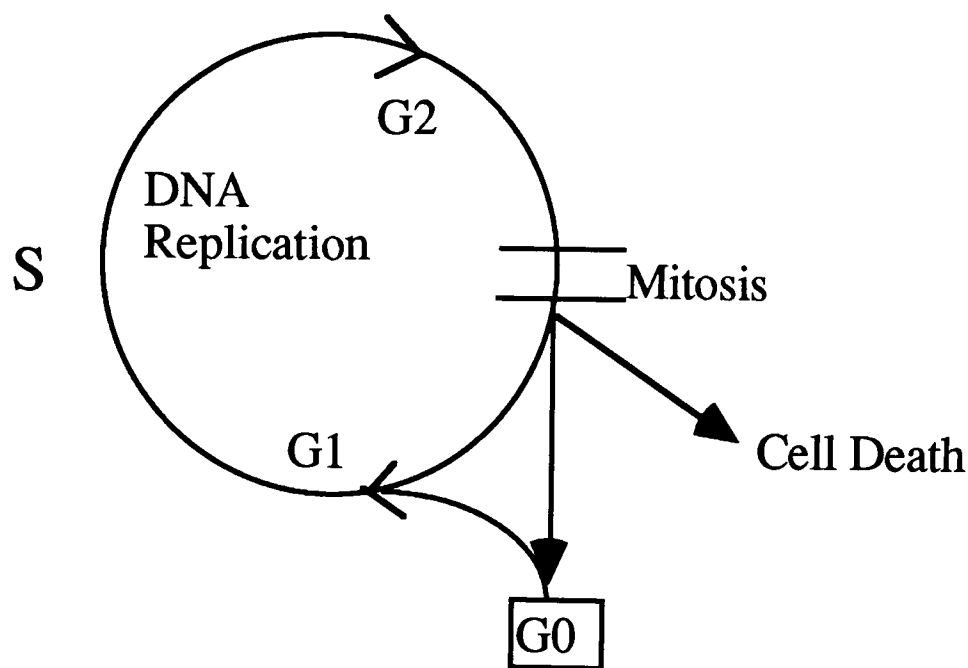


Fig. 1. 7. The cell cycle (from Baserga, 1981).

The microscopically visible period of mitosis is termed the M (mitosis) phase, the detectable period of DNA synthesis is termed the S-phase and the interphase periods before and after S-phase that represent gaps are termed G₁ and G₂. Thus, a newly divided cell leaves M and enters G₁; some time later it enters S-phase, moves on to G₂ when DNA synthesis is complete and then goes through the cycle again (Stubblefield, 1981). In general, a mammalian cell takes 8 hours to synthesize DNA (S-phase), 2.5 hours for G₂, and 0.5 hours to complete mitosis, with the cell cycle averaging about 10 -12 hours (Stubblefield, 1981). These are only average values and different cells show a range of values. Once a cell begins to replicate its DNA complement, it is committed to an irreversible process towards cell division (Stubblefield, 1981). The S-phase begins when the first DNA specific precursor molecules e.g. thymidine or its analogues are incorporated into newly synthesized DNA. Mitosis is the biological process by which one somatic cell divides into 2 daughter cells in a way that ensures accurate perpetuation of genetic information. Although mitosis is a continuous process, it is classically subdivided into four sequential phases as prophase, metaphase, anaphase and telophase (Cormack, 1987).

While the dividing cells go through the cell cycle, there are cells "out of cycle" and there are non-dividing cells. The non-dividing cells are of two types: G₀ state cells or fully differentiated cells (Baserga, 1985). The G₀ state cells may be triggered into the proliferative

phase by an appropriate stimulus, such as trauma, distraction or mechanical loading (Baserga, 1985; Apte and Kenwright, 1994; Skerry, 1997). Cells also leave the cell cycle when they undergo terminal differentiation, and differentiated cells do not re-enter the cell cycle and eventually die (Boyce, 1996).

1. 3. 2 The importance of cell proliferation

The growth and ageing of an organism are both inextricably linked to the ability of cells to divide. Cell proliferation thus has a fundamental role in the existence of life. Although increase in cell size and matrix production play a role in the growth of organs and tissues, an increase in the number of cells is far more important for both normal and abnormal growth (Boyce, 1996). Differentiation is also based on the proliferation and or the migration of undifferentiated cells. In postnatal life, growth continues in the process of controlled proliferation and differentiation with or without simultaneous cell loss (death) until the mature form is achieved. The capacity for cell division in adulthood means the ability to repair tissue damage as in the healing of wounds and fractures is retained. Inadequate cell proliferation may affect the ability of a tissue or organ to grow normally, to repair damage, or both. Uncontrolled excessive cell reproduction occurs in hyperplasia and in both benign and malignant tumours (Wright, 1984). In the musculo-skeletal system, cell proliferation plays a prominent role in development and growth. Longitudinal bone growth initiates in the growth plate, and the growth plate chondrocytes contribute to longitudinal bone growth by the production of more cells (cell proliferation), production of extracellular matrix and by cell hypertrophy. The proliferation of chondrocytes contributes directly to long bone growth as well as providing additional cells which produce matrix and undergo hypertrophy. The hypertrophy is then "fixed" by calcification. The longitudinal growth rate is proportional to the rate of cell proliferation of the chondrocyte column within the growth plate (Kember, 1985).

From a biological perspective, cell proliferation is regulated by molecular parameters (Stein, et al., 1996). It is becoming increasingly evident that developmental control and homestatic regulation of cell and tissue function is mediated by the integration of a complex series of physiological regulatory signals (see review Stein, et al., 1996).

1. 3. 3 **Methods to investigate cell proliferation**

The main methods of studying cell proliferation in skeletal research are: counts of cell numbers and mitotic figures, tritiated thymidine autoradiography, mRNA analysis, incorporation of bromodeoxyouridine, fluorochrome labels, ^3H -proline, and immunohistochemical detection of cell proliferation markers. Some methods relevant to the present studies are discussed briefly below.

Counts of cell numbers: Assessment of the changes in the cellularity of a tissue have been used in the past as evidence of altered proliferative activity. However, cell counts alone fail to provide dynamic information about proliferative activity and have a limited scope in skeletal research.

Study of mitotic figures: The presence of mitotic activity is unequivocal proof of cell division. Counting of mitotic figures should provide the most direct and natural method of measuring proliferative activity (Kember, 1983). This method has been used to locate sites of proliferating cells in the growth plate and articular cartilage (Mankin, 1964). However, because the duration of mitosis is extremely short in the growth plate, it is time-consuming task to collect sufficient numbers of mitotic cells to provide statistically useful information (Kember, 1983). In heterogeneous cell populations (e.g. bone marrow), cell morphology is less clear and changes during mitosis are less easily recognised.

Tritiated thymidine autoradiography: Thymidine is a nucleotide that is specific for DNA synthesis, it is not utilized for any other metabolic function of the cell (Tonna, 1979). There is hardly any turnover of thymidine once it has been incorporated into DNA. Tritiated thymidine (^3H -TdR) was first employed to study cell proliferation in the mouse by Hughes, et al. in 1958. Once administered to an animal, the availability of ^3H -TdR lasts about 10 min. If it does not incorporate into cells within this time it is converted to tritiated water and beta isobutyric acid which are excreted rapidly (Rubini, et al., 1960). ^3H -TdR contains tritium at a stable position within the thymidine molecule so that once it is incorporated into DNA, radioactivity is not lost or diluted unless the cell divides (Tonna, 1979). Autoradiography is the

localization of a radioactive substance (e.g. tritium) within a solid specimen by placing it in close contact with a detector layer. The autoradiography permits the distribution of radioactivity to be studied in relation to the detailed structure of the specimen. The drawbacks of [³H]-TdR autoradiography are: (1) the complex metabolism of thymidine may introduce variables that can affect [³H]-TdR uptake; (2) interpretation of autoradiography may be complicated by background and the subsequent need for grain counting to differentiate labelled cells from non-labelled ones; (3) radiation from incorporated [³H]-TdR may injure or kill labelled cells and [³H]-TdR is potentially dangerous to users.

Immunohistochemical detection of the markers for cell proliferation:

Immunohistochemical methods using antibodies to cell cycle-related antigens may be used as a means of assessing various aspects of proliferation in tissue, and have the important advantage of preserving the spatial orientation of proliferating cells in histological sections. Currently, the most widely available antibodies for this purpose are those to bromodeoxyuridine (BrdUrd), Ki67 and to proliferating cell nuclear antigen (PCNA). Ki67 demonstrates a nuclear antigen expressed in all phases of the cell cycle, except G₀ and early G₁, but it can only applied to frozen tissue (Yu, et al., 1992), thus limiting its use in skeletal research. The antibodies to BrdUrd and PCNA are applicable to formalin-fixed paraffin-embedded tissue. The following sections provide a brief background introduction of antibodies to PCNA and BrdUrd.

1. 3. 4 Proliferating cell nuclear antigen (PCNA)

PCNA is a highly conserved, 36 KD acidic nuclear protein, which is expressed in the late G₁ and S phases of the cell cycle, and is directly involved in DNA synthesis (Bravo and MacDonald-Bravo, 1987; Morris and Mathews, 1989). At least two forms of PCNA are present in cells: one at low levels in quiescent cells and the other at higher levels in proliferating cells. The reliability of using the antibody to PCNA, such as a monoclonal antibody PC10, as a cell proliferation marker has been shown to vary in different tissues and with tissue preparation (Yu, et al., 1992; Yu and Filipe, 1993), and use of PC10 as a widespread marker for cell proliferation is controversial. PCNA has a long half-life of approximately 20 hours,

and thus may be immunologically detectable in cells which have recently left the cell cycle (Bravo and MacDonald-Bravo, 1985; Prelich, et al., 1987). Previous studies have suggested that PCNA is an auxiliary protein for DNA polymerase- δ (Toschi and Bravo, 1988) and in the presence of PCNA, polymerase- δ initiates leading-strand DNA synthesis. The amount of PCNA present in the nucleus of cycling cells appears to be greater than that required for DNA synthesis (Miyachi, et al., 1978). Furthermore, PCNA may be expressed in association with DNA repair as well as with proliferation (Scott, et al., 1991; Shivji, et al., 1992). There is also some evidence that growth factors such as PDGF can induce increased PCNA mRNA stability and thus induce PCNA protein accumulation in normal cells without necessarily inducing DNA synthesis (Bravo and MacDonald-Bravo, 1985; Jaskulski, et al., 1988). Taken together these observations indicate that use of the antibody to PCNA, such as PC10 may tend to over-estimate the numbers of dividing cells.

In addition, conditions of fixation and section preparation have an effect on PCNA detection. Hall et al. (1990) found that prolonged fixation can reduce PCNA immunoreactivity in normal human and rat colon. However, this may not necessarily apply to all types of tissue (Yu and Filipe, 1993). There were studies showing no correlation between PCNA immunoreactivity and other indices of proliferation, such as S-phase fraction and Ki67 immunostaining (Yu et al., 1992) in neoplastic tissues. As immunofluorescence studies and other data indicate that PCNA is present at low but detectable levels in at least some non-cycling cells, this suggests that an artificial "cut-off" has to be employed in assessing the number of proliferating cells using PC10 (Yu and Filipe, 1993). As there appear to be many variables affecting immunostaining for PCNA, including the type of fixative, fixation time and immunohistochemistry technique, it is recommended that any user of the antibodies to PCNA should carefully consider all these factors before drawing any conclusions from the results. For the detailed method used for PCNA immunohistochemistry, see section 4. 2. 3.

1. 3. 5 Immunohistochemical detection of BrdUrd

5-bromo-2'-deoxyuridine (BrdUrd) was synthesised by Beltz and Wisser in 1955 in their search for thymidine analogues that could be used as anti-cancer drugs. The BrdUrd (C_9H_{11}

BrN₂ O₅) is a colourless crystalline compound which is solid at room temperature, with a molecular weight of 307 (the molecular weight of thymidine is 242) and a melting point of 187.9 °C. It is soluble in water to the extent of 20 mg/ml at pH 7.0, but solubility is increased at an alkaline pH. Approximately 90% of the parenterally administered BrdUrd undergoes degradation in the liver within the first 10 minutes while only a small amount is available unaltered for incorporation into the DNA of dividing cells (Kriss and Revesz, 1962). The BrdUrd competes with thymidine for incorporation into the DNA during the S-phase of DNA synthesis (Goz, 1978), and the BrdUrd may be preferentially incorporated into the condensed chromatin associated with the nuclear envelope and nucleolus during early S-phase, and the BrdUrd is stable after the incorporation into the cells (Riccardi, et al., 1988).

Since BrdUrd is not a normal cell constituent, tissues or cells must be labelled with BrdUrd prior to the immunohistochemical detection of BrdUrd uptake. BrdUrd can be labelled either by in vivo administration (intravenous injection) or by in vitro cell or tissue culture. The doses used for intravenous injection ranged from 5 to 200 mg/kg for human tumour treatments and for the rabbit 40 mg/kg dose is recommended (Apte and Kenwright, 1994). In the present study, each of the animals (rabbits) were injected with BrdUrd (40 mg/kg) through an ear vein 1 hour before killing (Appendix 8).

After the introduction of numerous polyclonal antibodies against BrdUrd, Gratzner (1982) developed a monoclonal antibody (B44) which was highly specific for BrdUrd. Magaud et al. (1989) further developed a monoclonal anti-BrdUrd antibody (Bu20A), which is used in the present study. The BrdUrd is originally inaccessible to the anti-BrdUrd monoclonal antibody as it is in the form of a native DNA double helix. Therefore partial denaturation of the DNA is required in order to expose the BrdUrd to the antibody (Moran, et al., 1985; Miller et al., 1986). Apte (1990) has validated the use of BrdUrd immunohistochemistry in decalcified tissues by comparison of double labelling with tritiated thymidine. Many studies have indicated that BrdUrd uptake and its immunohistochemical detection is a reliable technique to assess cell proliferation (Alberty and Pelton, 1993; Matsunaga, et al., 1993; Apte and Kenwright, 1994). A two step method was used for the immunohistochemical detection of BrdUrd uptake using Bu20A antibody in this thesis (see section 4. 2. 2). However, BrdUrd is toxic, some

experiments preclude its use and it is not applicable for human patient investigations.

1. 4 Bone extracellular matrix proteins

About 20-30% (by weight) of cortical bone is organic, 10% is water and remainder is mineral (Fig 1. 8). The organic matrix is predominantly collagen (about 90 -95 %) and the remainder 5-10 % consists of various non-collagenous proteins.

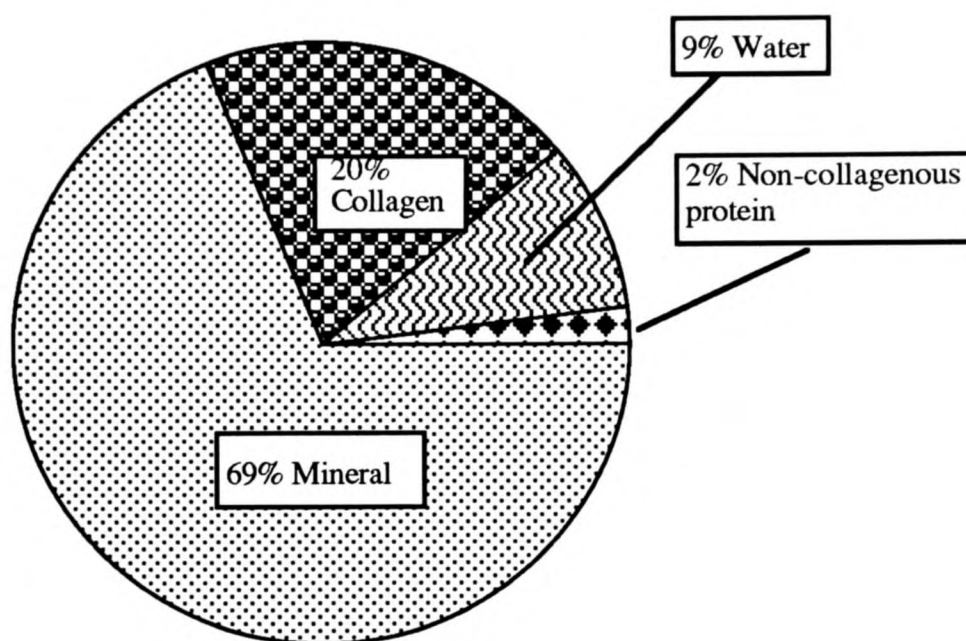


Fig. 1.8. The composition of cortical bone.

1. 4. 1 Bone mineral

Mineral constitutes about 70% of the weight of cortical bone. The major components are calcium and phosphate, with small amounts of carbonate, citrate, sodium, magnesium, fluoride and other ions. Most minerals are present in the form of poorly crystalline carbonate containing calcium phosphate which is an analogue of hydroxyapatite with the basic formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ (Boskey and Posner, 1984; Posner, 1987).

The deposition of mineral crystals in bone and calcified cartilage is regulated by cells and mineral-matrix interactions. The affinity of extracellular matrix constituents for hydroxyapatite may control the formation of initial mineral deposits and regulate the size, morphology, and orientation of resulting crystals (Boskey, 1992). The mineralization of bone possibly takes

place via two distinct mechanisms: (1) mineralization via matrix vesicles (Bonucci, 1967), such as cartilage calcification; (2) mineralization associated with collagen fibrils, such as intramembranous bone mineralization.

1. 4. 2 Collagens

The word "collagen" has been in the English language since at least 1865, derived from the Greek word "glue" (Kielty, et al., 1993). Collagen can be defined as " structural proteins of the extracellular matrix which contain one or more domains harboring the conformation of a collagen triple helix' (Rossert and Crombrughe, 1996). Collagen is the most abundant protein in the animal world and it constitutes about 30% of the protein in the human body (McKusick, 1972).

Collagenous proteins make up approximately 90-95 % of the organic matrix in bone and most of it is type I collagen. Modern research on collagen at the molecular level began in the 1950s, by using developing physiochemical techniques such as X-ray crystallography and electron microscopy, and models for the triple-helical structure of collagen fibrils were established (Ramachandran, 1967). Further advances in protein separation techniques led to the discoveries of genetically distinct types of collagens (Miller and Matukas, 1969; Epstein, 1974). Studies over the last two decades have now led to the identification of approximately 25 different collagenous polypeptides (Vuorio and de Crombrughe, 1990) and at least 19 different collagen types (Kielty, et al., 1993) (see Table 1.1).

Collagen proteins comprise three polypeptide chains which fold to form triple-helical domains, determined by the high glycine and amino acid contents in repeating triplets of - Gly-X-Y-, where X is often proline and Y is hydroxyproline (Bornstein and Traub, 1979). As the number of collagen types has increased, they have been seen to comprise certain groups. Vertebrate collagens can be classified on the basis of their size and self-assembly, and divided into fibrillar collagens, types I, II, III, V and XI, and non-fibrillar collagens, types IV, VII, VIII, IX, X, XII and XIII. The non-fibrillar types can be subdivided into basement membrane collagens, types IV and VII, and short chain collagens, types VIII and X (Hulmes, 1992).

Table 1. 1. Some types of collagens and their distribution

Collagen Type	Chains	Distribution
I	$\alpha 1(I)$, $\alpha 2(I)$	Widespread, skin, bone, tendon, ligament, cornea, etc.
II	$\alpha 1(II)$	Cartilage, vitreous, annulus fibrosus
II	$\alpha 1(III)$	Foteal skin, tendon; scar
IV	$\alpha 1(IV)$, $\alpha 2(IV)$, $\alpha 3(IV)$ $\alpha 4(IV)$, $\alpha 5(IV)$	All basement membranes
V	$\alpha 1(V)$, $\alpha 2(V)$, $\alpha 3(V)$	Widespread, skin, bone, tendon, etc.
VI	$\alpha 1(VI)$, $\alpha 2(VI)$, $\alpha 3(VI)$	Skin, bone, cornea, tendon, ligament, cartilage, widespread.
VII	$\alpha 1(VII)$	Skin, oral mocosa, cervix
VIII	$\alpha 1(VIII)$, $\alpha 2(VIII)$	Embryonic heart, descemet's membrane
IX	$\alpha 1(IX)$, $\alpha 2(IX)$, $\alpha 3(IX)$	Cartilage, vitreous
X	$\alpha 1(X)$	Hypertrophic and mineralized cartilage
XI	$\alpha 1(XI)$, $\alpha 2(XI)$, $\alpha 3(XI)$	Cartilage
XII	$\alpha 1(XII)$	Collagen I-containing connective tissues
XIII	$\alpha 1(XIII)$	Widespread, human fetal epidermis

(Adapted from Kielty , et al., 1993)

Types I , II and III collagens account for 70% of the total body collagens (Kuhn, 1987). These collagens are characterized by an uninterrupted helical domain of 300 nm long and 1.5 nm wide. Type I collagen has two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain; in types II and III collagens, the three α -chains are identical (Sykes, 1990). Collagen types I , II and III are deposited in the connective tissue as discrete organised fibres. They are synthesised and secreted as high molecular weight precursors, the procollagens. Procollagens were discovered in the 1970s (Schofield and Prockop, 1973; Martin, et al 1975) and the procollagens of types I , II , III all have molecular masses about 50% larger than their corresponding α -chains, because of the presence of peptide extensions at both the N-and C-terminals (Olson, 1981).

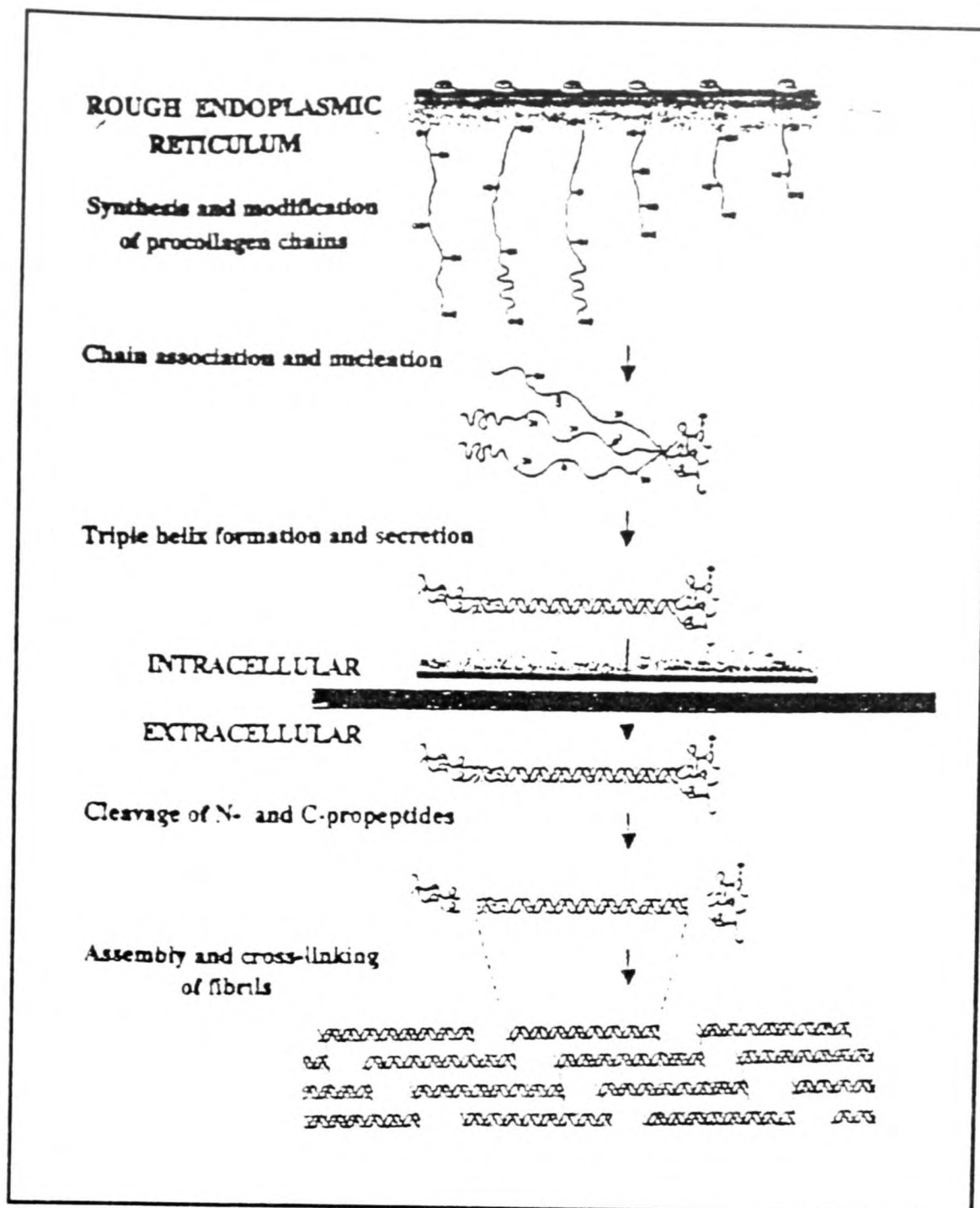


Fig. 1.9. The synthesis and modification of collagen. Schematic representation of the intracellular and extracellular steps involved in the synthesis and modification processes, and assembly of collagen molecules into fibrils. (from Kielty et al., 1993)

The procollagens are synthesized on the ribosomes of the rough endoplasmic reticulum and secreted into the lumen, where they undergo complex enzymic modifications before triple helix formation. The newly formed procollagen molecules are then secreted to the outside of the cells. Once secreted, the procollagens undergo further modifications by specific proteinases which cleave off the N- and C- propeptides (Fig. 1. 9). These proteinases may help control the deposition of collagen molecules into collagen fibrils and fibres by the formation of collagen crosslinks (Francis and Duksin, 1983). It is well known that most collagen fibrils are composed of two or more different collagen types, in skin there are types I and III, while in cartilage the fibrils contain types II, IX and XI (Hulmes, 1992).

Type I collagen is secreted as a propeptide, but the N and the C telopeptide are rapidly cleared by specific proteases (see review Rossert and Crombrughe, 1996). Type I collagen genes are known as COL1A1 and COL1A2, and are located on chromosomes 17q21-22 and 7q21-22 respectively and their sequences are known (Sykes, 1989; Byers, et al., 1991). Expression of these genes produces pro- α 1(I) and pro- α 2(II) collagens. Both the pro- α 1 chain and the pro- α 2 chain undergo hydroxylation and glycosylation, and these modifications are essential for the assembly of type I collagen chains in a triple helix. Type I collagen is produced by the combined expression of these two genes, but there are quantitative and qualitative differences in bone and skin (Byers, 1990). A serious consequence of genetic mutation on type I collagen formation is osteogenesis imperfecta. Osteogenesis imperfecta is a genetic disease characterized by an extreme fragility of bone, which is due to a mutation in the coding sequence of either the pro- α 1 (1) or pro- α 2(1) gene (for review see Kuivaniemi, et al., 1991). Different cytokines, hormones, vitamins, and growth factors can modify type I collagen synthesis by the osteoblast and / or fibroblast. Some factors can act directly on type I collagen synthesis but also act indirectly by modifying the secretion of other factors, which will themselves affect type I collagen synthesis (for review see Rossert and Crombrughe, 1996).

Collagen type II is the major collagen of cartilage, composing more than 50 % of the extracellular matrix of cartilage. The triple helix is assembled from three identical α 1-chins encoded by the gene COL2A1 which is located on chromosome 12q12-14 (Mundlos, 1994). Recent investigations have revealed that alternative splicing of exon 2 can generate two RNA populations. RNA with exon 2 expression was detected in immature chondrocytes and RNA without exon 2 expression was in mature chondrocytes (Sandell, et al., 1991).

Collagen type III was found to be composed of three α 1(III) chins and it has widespread distribution, accompanying type I collagen in many connective tissue and being the main component of the thin "reticulin" fibres present in the loose collagenous networks of organs like liver and lung. With the recent development of recombinant DNA technology, the discovery of the complete amino acid sequences of collagens types I, II, III, and the description of their gene structures have provided powerful tools to investigate the relationship between these and other collagen types during histogenesis and osteogenesis (Kielty, et al., 1993).

The process of collagen breakdown caused by collagenase is similar to that first described in the tadpole (Harris and Krane, 1974). Both cysteine proteases, which work at low pH and neutral metalloproteases, which include collagenase, gelatinase, and stromelysin, are believed to actively participate (Delaisse, et al., 1993). These enzymes split collagen molecules transversely into two fragments which denature spontaneously above 32 °C. There are then degraded further by extracellular neutral proteases or by lysosomal enzymes after phagocytosis. Hydroxyproline and hydroxylysine formed by this means are oxidized or excreted in urine as peptides. Hydroxyproline in urine thus gives an estimate of collagen turnover (Smith, 1979; Eyre, 1996).

1. 4. 3 Non-collagenous proteins

5-10 % of the organic matrix of bone is non-collagenous protein. These are chiefly glycoproteins and proteoglycans (for review see Robey-Gehron, 1996). Glycoproteins are short branched molecules containing sugars, which may include galactose, glucose, galactosamine, glucosamine, mannose and sialic acid. Proteoglycans have long side arms with regular repeating disaccharide units which may be sulphated and carbonylated, thus rendering the proteoglycans acidic. Glycoproteins may be synthesized in bone (e.g. sialoprotein) or resident in bone but synthesized elsewhere (e.g. alpha 2 HS-glycoprotein), and plasma proteins will also found in bone (Herring, 1968; Leaver, et al., 1975; Vaughan, 1975; Triffitt, et al., 1976). Recently there has been intense research into the structure and function of the non-collagenous matrix proteins of bone. A dozen or so proteins have been identified over the last ten years accounting for about 75% of the non-collagenous protein mass (Fisher, 1992). Some of these proteins come from the blood, possibly due to a high affinity for hydroxyapatite, others are secreted products of osteoblasts. Because of the space limitation of this thesis, the major non-collagenous proteins and their possible functions are summarised in Table 1.2.

Table 1. 2 Bone Noncollagenous Proteins and Their Possible Functions

PROTEIN	TISSUE/SITES OF SYNTHESIS	POSSIBLE FUNCTION	REFERENCES
GLYCOPROTEINS			
Alkaline phosphatase	Bone, liver, kidney	Bone and cartilage mineralization; cell interactions.	Rodan, et al.,1992; Henthorn, 1996.
Osteonectin	Bone, skin, tendon, lagment, platelets, Bone	Controlling cell shape, proliferation and mineralization; binding to growth factors.	Wasi, et al., 1984; Tung,et al., 1985; Zung, et al., 1986; Tracy, et al., 1988; Lane and Sage, 1994; Robey Gehron, 1996
Bone acidic glycoprotein	Bone	Mineral crystallization	Young, et al., 1992.
Tetranectin	Bone	Mineralization ?	Wewer, et al., 1994.
PROTEOGLYCANS (PG)			
Chondrotin sulphate PG (Aggrecan and Versican)	Bone and cartilage	Occupies connective tissue spaces destined to become bone.	Fisher, 1992; Roughley and Lee, 1994.
Heparan sulphate PG	Bone, cartilage and skin	Faciliates interaction of osteoblast with matrix and heparin-binding growth factors	Beresford, et al., 1987; David, 1992.
Biglycan (PG-I)	Bone, cartilage and aorta	Mineralization, cell-cell or protein interactions.	Bianco, et al., 1991; Fisher, 1992.
Decorin (PG-II)	Eye, tendon and bone	Regulates collagen fibre and matrix formation	Kresse, et al., 1994; Robey-Gehron, 1996.
GLA-PROTEINS			
Matrix Gla protein	Lung, kidney, heart, bone, dentine, cartilage	Mediates cell attachment, inhibit cartilage mineralization, chondrocyte differentiation.	Hale, et al.,1988; Luo, et al., 1995; Ducy and Karsenty, 1996.
Osteocalcin (Bone Gla protein)	Dentine, bone	Osteoclast recruitment and activity, mineralization, bone remodelling, inhibotor of bone formation.	Lian and Heroux, 1979; Price, 1992; Ducy, et al., 1996.
BONE SIALOPROTEINS			
Osteopontin (BSP -I)	Bone, epithelium, placenta	Osteoclast attachment & regulation, inhibition of uncontrolled mineralization; cell migration.	Denhardt, et al., 1995; Giachelli, et al., 1995; Butler, et al., 1996.
Bone sialoprotein (BSP-II)	Bone, kidney	Early osteogenesis, matrix erosion and attachment; osteoblast differentiation.	Triffitt, 1987; Young, et al., 1992; Bianco, et al., 1991; 1993; Robey-Gehron,1996.
ADHESION PROTEINS			
Integrins	Cells of various tissues	Cell membrane protein, cell-cell and cell-matrix interaction.	Garrod, 1993; Horton, et al., 1996.
Fibronectin	Fibroblasts, osteoblasts, endothelial cells and others	Cell attachment, formation of a fibrin clot	Kleimman, et al., 1987; Potts and Campbell, 1994.
Vitronectin	Many cell types	Cell attachment and cell migration	Grzesik and Robey, 1994.
Thrombospondin	Platelet, variety of mesenchymal cells, bone	Cell attachment and cell migration	Lawler and Hynes, 1986; Gehron Robey, et al., 1989; Young,. et al., 1992; Grzesik and Robey, 1994.
PLASMA PROTEINS			
Albumin	Liver	Calcification, bone matrix formation	Dickson, 1974; Owen and Triffitt, 1976; Triffitt and Owen, 1977; Garnett and Dieppe, 1990.
α 2HS-glycoprotein	Liver	Recruitment of osteoclast, bone turnover	Ashton, et al., 1976; Triffitt, et al., 1976; Triffitt, 1987.

1.5 Control of bone function

1. 5. 1 Bone growth factors and cytokines

Over the last two decades, a number of growth factors and cytokines have been isolated from bone matrix. These include insulin-like growth factors (IGF-1 and IGF-2) (Conver, 1996), transforming growth factors- β (TGF- β) (Bonewald, 1996), acidic and basic fibroblast growth factors (aFGF and bFGF) (Hurley and Florkiewicz, 1996), platelet-derived growth factor, (PDGF) (Canalis and Rydziel, 1996), and bone morphogenetic proteins (BMPs) (Rosen, et al., 1996).

These factors are present in bone at higher concentrations (20 times higher) than that in serum (Sawyer, et al., 1991) and most are products of bone cells, postulated to have effects on their neighbouring cells (paracrine action), or on themselves (autocrine action) (Termine 1990, Mohan and Baylink 1991). A number of growth factors and cytokines are stored in bone extracellular matrix and are released during bone resorption or during mechanical disruption, to act as paracrine agents to stimulate or enhance local bone formation (Meikle, et al., 1991; Conver, 1996). The properties and effects of a number of growth factors and cytokines on bone metabolism are briefly summarized in Table 1. 3.

Table 1.3. Effects of growth factors and cytokines on bone cells in vitro

Abbreviation Names	Relative molecular size	Osteoblast proliferation	Matrix synthesis	Osteoblast differentia- -tion	Bone resorption
IGF-I	7700	increase	increase	increase	increase
IGF-II	7500	increase	increase	increase	
TGF- β 1, β 2	25000	increase/ decrease	increase/ decrease	increase/ decrease	increase/ decrease
PDGF	32000	increase	increase	increase	increase
FGF-b	16000	increase	decrease	decrease	
FGF-a					
EGF	6000	increase	decrease	decrease	increase
TNF-a, b	17000	increase	decrease	decrease	increase
IFN- γ	25000	decrease	decrease	increase	decrease
BMPs		increase	increase	increase	
IL-1	18000	increase	increase/ decrease	decrease	increase

1. 5. 2 Prostaglandins

Prostaglandins are produced by a vast number of tissues and are a family of 20 carbon fatty acids with many effects (Pilbeam, et al., 1996). The earliest reported effect of prostaglandins on bone was their ability to stimulate bone resorption in organ culture (Klein and Raisz, 1970). The most potent stimulators of bone resorption are PGE₂ and PGE₁, which are 10 to 100 fold more active than prostaglandins of the A, B or F series (Eilon and Raisz, 1978). They were locally produced and rapidly degraded; and they were the local regulators of bone cell function (Raisz and Martin, 1983; Bergmann and Schoutens, 1995). A large number of cytokines, growth factors and hormones were found to be able to increase bone prostaglandin production (Kawaguchi, et al., 1995).

Prostaglandins administered over a prolonged period cause increased bone formation in vivo. Increased periosteal bone formation has been observed in infants (Sone, et al., 1980; Ueda, et al., 1980; Woo, et al., 1994), and rats (Jee, et al., 1985; Ueno, et al., 1985; Ma, et al., 1994) after a period of systemic administration of PGE₂. Prostaglandins may also accelerate callus formation in fracture repair (Keller, et al., 1993). The administration of prostaglandins in vivo has also been reported to stimulate bone resorption (Tashjian, et al., 1972; Santoro, et al., 1977; Bergmann and Schoutens, 1995) and increased bone resorption was also associated with the increase production of prostaglandins in inflammation (Robinson, et al., 1975), mechanical stimulation (Somjen, et al., 1989; Lanyon, 1992; Chow and Chambers, 1994) and tumors (Seyberth, et al., 1975). There is evidence that prostaglandins can influence osteoblast activity by inhibiting the synthesis of collagens, but not non-collagenous proteins (Raisz, et al., 1990; 1993). The mechanisms by which prostaglandins act on bone cells involves the stimulation of osteoblast proliferation and differentiation, collagen synthesis, osteoclast differentiation and bone resorption (see review by Pilbeam, et al., 1996).

1. 5. 3 Systemic control of bone function

A number of hormones and other systemic factors are also involved in bone metabolism. Their effects on bone cells are summarized in Table 1.4.

Table 1. 4. Hormones and other systemic factors acting on bone cells

Name	Bone Target Cell	<u>Actions</u>		Effects <i>in vivo</i>	Reference
		Bone formation	<i>in vitro</i> Bone resorption		
1,25-dihydroxy-vitamin D ₃	Osteoblast Osteoclast	increase	decrease	Necessary for bone mineralization. At high doses bone formation decreases; bone resorption increases.	Beresford, et al., 1986; Marks and Popoff, 1988; Franceschi and Young, 1990; Darwish and Deluca, 1993; Holick, 1996.
Parathyroid Hormone	Osteoblast	decrease	increase	At low doses, bone formation increases; at high doses, bone resorption increases.	McSheehy and Chambers, 1986; Perry et al., 1987; Marks and Popoff, 1988; Silver and Kronenberg, 1996.
Calcitonin	Osteoclast		decrease	Bone resorption decreases in acute response.	Farley, et al., 1991; Azria and Avioli, 1996.
Cortisol	Osteoblast	decrease or increase	decrease	At high doses, bone formation decreases and loss of bone occurs.	Ganong, 1983; Leboy, et al., 1991; Beresford, et al., 1992; Harris, et al., 1996.
17-β-estradiol	Osteoblast Osteoclast	increase		Estrogen deficiency leads to bone loss.	Avioli, 1983; Richelson, et al., 1984; Etinger, et al., 1985; Harris, et al., 1996.
Retinoic acid / Retinol	Osteoblast Osteoclast	decrease	increase	At high doses, bone resorption increases.	Dickson, 1993.
Insulin	Osteoblast	increase		Effects not well defined	Raisz and Kream, 1983; Yano, et al., 1996.
Growth Hormone	Osteoblast chondrocyte	increase	decrease	Promotion postnatal longitudinal bone growth.	Russell and Spencer, 1985; Brixen, et al., 1990.
Vitamin C	Osteoblast	increase	decrease	Vitamin C deficiency leads to bone loss.	Aronow, et al., 1990; Whitson, et al., 1992.
Thyroxine /Tri-iodothyronine	Osteoblast	decrease	increase	High doses lead to increased bone turnover and loss of bone.	Sato, et al., 1987; Raisz and Kream, 1983; Perry, 1989.

All of these hormones and systemic factors act on other target tissues besides bone to alter either mineral metabolism, or circulating levels of the other bone-active hormones and factors, or both. Their effects on bone formation and bone resorption therefore result from the combination of both direct and indirect effects on bone cells (Dickson, 1993).

1. 6 Bone remodelling

The terms "modelling" and "remodelling" of bone are specifically defined. In cortical bone, modelling refers to shaping, and is the result of localized apposition and resorption by the periosteal and endosteal envelopes. Remodelling is due to the internal substitution of bone by the Haversian envelope without changes of the external shape. In cancellous bone, modelling and remodelling continue after growth has ceased. The mechanical situation is known to have great influence the processes of bone modelling and remodelling (Schenk, 1987; Skerry, 1997; Frost, 1997; Mosley, et al., 1997).

1. 6. 1 Cortical bone remodelling

An important advance in our understanding of bone remodelling was the observation that bone resorption and bone formation were coupled in space and time, and occur in "bone metabolizing units" (BMU) (Frost, 1964). First a resorption canal was formed by osteoclasts, the resorption canal was well vascularized, and the vessels were accompanied by perivascular cells, possibly including osteoclast and osteoblast precursors. Shortly thereafter, osteoblasts started refilling this canal with concentric lamellae, until it was narrowed to the final diameter (50 -80 μm) of the vascular canal of the newly formed osteon or Haversian system (Enlow, 1962). The dynamics of the remodelling units have been measured by sequential fluorochrome labelling. The osteoclastic resorption rate measured in the radius and tibia of dogs was 50-60 μm daily and the average daily osteoblastic apposition rate was 1.5 - 2.0 μm (Jaworski, 1972; Schenk, 1987). In dogs, the average diameter of osteons is 140 - 170 μm and the wall thickness is about 70 μm . The formation of a new osteon (BMU) takes 5 -6 weeks to complete. In the human, the wall of osteons is about 90 - 100 μm and osteoblastic apposition rate is about 1 μm / day. The life span of a BMU is estimated to be at least 3 -4 months (Frost, 1964).

1. 6. 2 Cancellous bone remodelling

The remodelling of cancellous bone has been studied quantitatively in human iliac crest biopsies (Schenk, 1987). Cancellous bone exposes an enormous surface to the bone marrow and uneven bone resorption and formation influence its overall density. The remodelling of cancellous bone results for the most part in the functional adaptation to the mechanical loading. Remodelling replaces the old scaffold with newly formed bone and enlarges the surfaces for ion exchange. Cancellous bone remodelling begins with the recruitment of osteoclasts, which "dig" a cavity in the trabecular surface over a diameter of about 0.5 - 1 mm to an average depth of 50 μm . When the resorptive phase is completed, the osteoclasts are replaced by mononuclear cells. Thereafter, osteoblasts lay down new lamellae and fill the cavity completely (Fig. 1. 10), this process exhibits similar dynamics to the BMUs in cortical bone (Parfitt, 1979; 1982; Skerry, 1997).

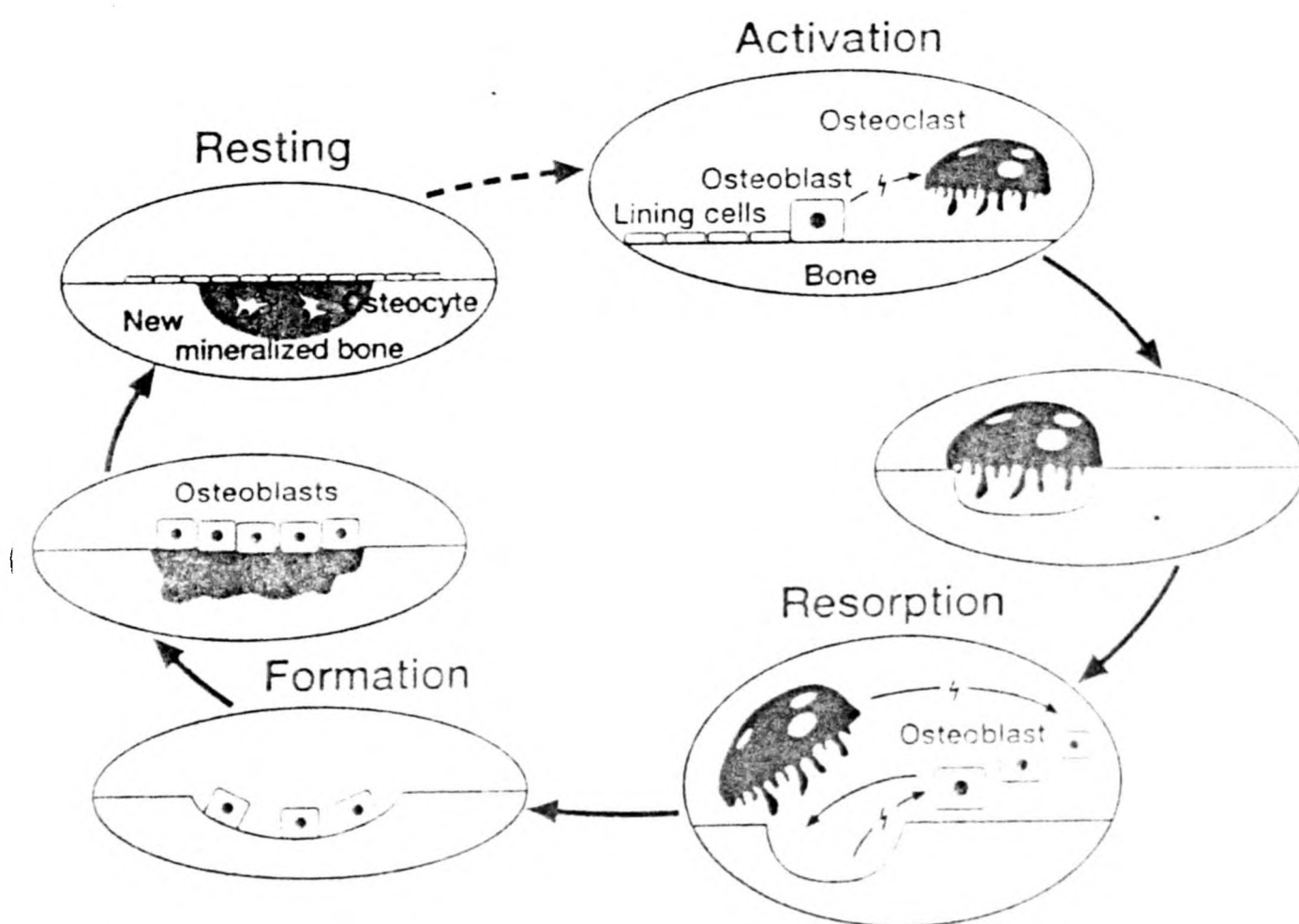


Fig. 1. 10. Schematic illustration of cancellous bone remodelling

1. 6. 3 Regulation of bone remodelling

The coupling of resorption and formation is mediated by the local collaboration of osteoblasts

and osteoclasts. This sequence includes (1) osteoclast activation, possibly mediated by lining cells; (2) factors released from the bone matrix by the resorbing osteoclasts induce osteoblastic proliferation and differentiation, and (3) factors produced by other local cells act to stimulate all these processes (Martin, et al., 1989). The recruitment and activation of the various cells during remodelling are balanced with respect to each other. The amount of bone resorbed must be matched by the same volume of new bone formation (Frost, 1964; Martin et al., 1989). The imbalance of this process maybe an important mechanism for the pathogenesis of osteoporosis.

The skeleton is a complex association of metabolically active cells, attached to, or embedded in a mineralized matrix. The complex cellular interactions during development, maturation and remodelling of the skeleton are largely unknown. It is widely accepted that a multitude of factors, from systemic hormones to local regulatory factors such as the members of the TGF- β superfamily, cytokines and prostaglandins, must act in concert to regulate the continuous coupling between bone formation and bone resorption (Heersche and Aubin, 1990; Hruska, et al., 1993). Recent studies using an in vivo mouse model have found that, an oncogene, c-fos, is a key player in regulating the differentiation and activity of osteoblast and osteoclast populations, both in normal development and in bone disease (Grigoriadis, et al., 1996). Modulation of c-fos expression disrupts the normal balance between bone formation and resorption, which is essential for normal bone remodelling and skeletogenesis. Overexpression of c-fos leads to development of chondrosarcomas and osteosarcomas. C-fos knock-out mice develop osteopetrosis because of a lack of osteoclasts and their precursors (Grigoriadis, et al., 1995).

Mechanical effects on bone also contribute to the remodelling process and play a very important role (Skerry, 1997; Forst, 1997). The adaptive responses to changes in load and physical activity may have an even greater influence on bone mass and bone architecture than that the metabolic aspects of bone (Marcus, 1996; Frost, 1997; Skerry, 1997). The process of bone remodelling is a key phenomenon in bone cell biology and understanding the mechanisms do control this process will clarify not only local controls of osteoclast and osteoblast functions, but also the pathophysiology of age-related bone loss and osteoporosis (Marcus, 1996).

1.7 Biology of fracture repair

The bone healing process normally proceeds in the fracture, through granulation, callus formation and modelling / remodelling stages. Under the influences of local and systemic agents, these mediators determine whether new local osteoclasts and osteoblasts will appear, in addition to when, where, how many, what kind, and for how long (Frost, 1989 a, b). Errors in these functions can then lead to abnormal bone healing.

Frost (1989a) has divided bone healing problems into 3 groups: (1) technical failures, the treatment has impaired normal biologic potential; (2) biologic failures, biologic malfunctions have made the correct treatment ineffective; (3) combinations of the two. Biologic failures include inadequate callus formation, abnormal modelling or remodelling, or maldifferentiation of the healing tissues, plus combinations of these. Accumulating evidence suggests that most biologic failures are due to problems attributable to mitogens, growth factors, and other labile biochemical and biophysical messengers in the region of the fracture (Frost, 1989b). The causes of most biologic failures probably act within the first weeks after the fracture, although it may take months to show their effects radiographically. The ability of bone to heal can differ in different parts of the bony skeleton at a given time (Einhorn, 1995).

1. 7. 1 Vascular repair

The early vascular response is essential for the normal progress of fracture healing. Angiogenesis is an important step in this response as new vessel formation vascularizes the fracture hematoma and the gap. Increases in serum levels of a low molecular weight endothelial stimulating angiogenic factor have been noted in the first 14 days after the fracture in patients (Kurdy, et al, 1996). Chidgey et al. (1986) have demonstrated a direct and predictable relationship between the phase of vascular reorganization and the rigidity of fracture healing. The microangiographic patterns were correlated with the calculated fracture rigidity values.

There has been a long-standing debate as to whether medullary or periosteal blood flow is the dominant vascular supply during the healing of diaphyseal fractures. Strachan et al. (1990)

used radioactive microspheres to quantify blood flow in the canine tibia two weeks after an osteotomy. They found that there was a significant contribution from the periosteum to the blood supply of healing cortical bone after nutrient artery ligation, with a reversal of flow from a centrifugal to a centripetal direction. This has confirmed the qualitative observations of Trueta (1974) regarding the significant recruitment of vessels from surrounding soft tissue during fracture healing.

Marked hypertrophy and hyperplasia of the periosteal cells in the rat tibia bone were induced when blood flow of the tibia was completely arrested for 4.5 hours (Svindland et al., 1995). This response was similar to the initial formation of external callus during fracture healing and transient ischemia may be an important factor in initiating fracture healing. Wallace et al. (1994) reported that in an ovine tibia osteotomy model, a 25% difference in axial interfragmentary displacement caused a fourfold change in corticomedullary blood flow at two weeks after osteotomy. These results suggested that the early vascular response is very sensitive to the initial mechanical environment, and appears to precede and determine the organization of osteogenesis.

Grundnes and Reikeras (1992) measured the blood flow and mechanical variables in the healing bones at 4, 8, and 12 weeks following femur osteotomy in a rat model. Osteotomy reduced total bone blood flow by about 50 %, and cortical flow in the diaphysis by approximately 40 %. Reaming of the osteotomized bones did not lead to any further flow reduction. At 4 weeks, total bone blood flow was more than doubled; increases were found in every segment of the fractured bone, and a more than 10-fold increase in the callus area was seen. At the end of the experiment, the femurs had regained 83 % of their normal strength, and 88 % of normal rigidity. At this time total bone blood flow was marginally increased, flows in the proximal and the distal diaphyses were almost normalized, while a nearly 3-fold increase was still found in the callus area. Flow in the callus area gradually decreased during healing, and regression analysis demonstrated a negative correlation between callus flow and mechanical properties.

1. 7. 2 Growth factors, cytokines and fracture repair

Understanding of the basic events in fracture healing constitutes a foundation for the development of new approaches to stimulate bone healing. Fractured bones heal by a cascade of cellular events in which mesenchymal cells respond to local or systemic regulators by proliferating, differentiating, and synthesizing extracellular matrix. Current concepts are that growth factors and cytokines regulate different steps in this cascade (Bolander, 1992; Einhorn, et al., 1995). Regulatory roles for PDGF, aFGF, bFGF, and TGF- β in the initiation and the development of the fracture callus have been recently reviewed (Elind, 1996). Fracture healing begins immediately following injury, when growth factors, including TGF- β 1 and PDGF, are released into the fracture hematoma by platelets and inflammatory cells (Slater, et al., 1995). TGF- β 1, bFGF and PDGF appear to have an influence on the initiation of fracture repair and the formation of cartilage and intramembranous bone in the initiation of callus formation, they are detectable in osteoblasts and chondrocytes throughout the healing process (Joyce, et al., 1990; Nash, et al., 1994). Fracture healing was inhibited by administration of recombinant human tumor necrosis factor alpha (TNF- α) over a period of time. TNF- α inhibited cartilaginous callus formation and this effect may be due to the inhibition of differentiation of mesenchymal cells into chondroblasts by TNF- α (Hashimoto, et al., 1989). Acidic FGF is synthesized by chondrocytes, chondrocyte precursors, and macrophages. It appears to stimulate the proliferation of immature chondrocytes or precursors, and indirectly regulates chondrocyte maturation and the production of the cartilage matrix (Blander, 1992).

Nakasa et al. (1994) reported the temporal and spatial distribution of bone morphogenetic protein 4 (BMP-4) gene during fracture repair. The BMP-4 mRNAs were present in cells distributed in the proliferating periosteum, the medullary cavity, and the muscles near the fracture site. The BMP-4 gene is expressed by less differentiated osteoprogenitor cells, not by differentiated osteoblasts. Bostrom et al. (1995) used monoclonal antibodies to BMP 2 and 4 to study the early stages of fracture healing. Within several days after the fracture, periosteal cells and osteoblasts near to the fracture site exhibited intense staining for BMP 2 and 4. As the woven bone was replaced by mature lamellar bone, the staining decreased. The results suggest that BMP 2 and 4 are important regulators of cell differentiation during fracture healing.

Many growth factors and cytokines have been isolated from platelets and these are thought to be released at local sites in response to injury. Grundnes and Reikeras (1993a) have studied the importance of the hematoma during fracture repair. In bilateral femoral fractures in rats, they removed the hematoma on one side after 30 min, 2 days and 4 days, and the fractures were mechanically tested at 4 weeks. Removal of the hematoma at day 2 or 4 impaired fracture healing more than at 30 mins, as bending rigidity and fracture energy were greatly decreased compared to the control fractures. The results indicated that the fracture hematoma favours healing; removal of the hematoma some days after fracture was harmful to fracture healing.

Cytokines, a group of proteins known to regulate hemopoietic and immune functions, are also involved in inflammation, angiogenesis, and bone and cartilage formation. Einhorn et al. (1995) confirmed that cytokines are also involved in fracture healing. Calluses or bone specimens were collected from a rat femoral fracture model on days 3, 7, 14, and 21 postfracture, and explanted to organ culture and treated with either media alone or media containing the inducing agents such as lipopolysaccharide or concanavalin A. A titration of conditioned medium from these cultures was then added to factor-dependent clonal cell lines that are known to be specifically responsive to interleukin-1, interleukin-6, granulocyte-macrophage colony stimulating factor or macrophage-colony stimulating factor. To confirm the identities of each of these cytokines, neutralizing antibody studies were performed. The results showed that interleukin-1 was expressed at very low constitutive levels throughout the period of fracture healing but can be induced to high activities in the early inflammatory phase (day 3). Granulocyte-macrophage colony stimulating factor showed no constitutive activity but could also be induced to high activities with lipopolysaccharide. Interleukin-6 showed high constitutive activity early in the healing process (day 3), and treatment with inducing agent did not increase the activity of this cytokine at this timepoint. Lipopolysaccharide did increase interleukin-6 activity in day 7 and 14 fracture calluses. None of the samples of unfractured bone showed constitutive or inducible activities for any of these cytokines. These findings suggest that the occurrence of a fracture results in a cascade of events that includes the production of cytokines. The expression of specific cytokine activities are time-dependent and are differentially regulated, and the process of fracture healing may be partially mediated by these cytokines (Einhorn, et al., 1995).

1. 7. 3 Changes of extracellular matrix during fracture repair

The expression of several collagens, cartilage proteoglycan core protein (aggrecan), and osteonectin was investigated during fracture healing in mice (Hiltunen et al., 1993). Total RNA was extracted from the fracture calluses and normal bone, and analyzed by Northern hybridization using specific cDNA probes. Based on the predominant fibrillar collagen types expressed, three phases of fracture healing were observed. Type III collagen expression characteristic for the inflammatory stage increased first, followed by type II collagen expression indicative of the cartilaginous reparative phase, and by type I collagen production, characteristic for the ossification and remodelling stages. Type IX collagen and aggrecan expression coincided with Type II collagen expression during the chondrogenesis. The mRNA for Type X collagen, a marker for hypertrophic chondrocytes during endochondral ossification, occurred somewhat later than that of other cartilage-specific genes. Osteonectin mRNA was present throughout the healing process and peaked during rapid new bone growth.

Jingushi et al (1992) have characterized gene expression in the fracture callus in the rat. The callus was divided into regions of bone formation (hard callus) and cartilage formation (soft callus), and gene expression was examined separately in each region. mRNAs for osteonectin, alkaline phosphatase and type I procollagen were detected in the hard callus at maximal levels during endochondral ossification and bone remodeling (day 15) and at 50% maximal levels during intramembranous bone formation (day 7). mRNAs for these proteins in the soft callus were detected at low levels during chondrogenesis (day 9), but increased to 80% of maximal levels with chondrocyte hypertrophy and mineralization of the cartilage matrix (day 13). mRNAs for type II procollagen and proteoglycan core protein were detected at maximal levels in the soft callus during chondrogenesis (day 9). Osteocalcin gene expression was detected in the hard callus during endochondral ossification and remodeling but not during intramembranous bone formation or at any time in the soft callus. Osteonectin mRNA was detected in both the hard and soft callus throughout the entire course of fracture repair. Expression of cartilage and bone-related genes correlated with the temporal sequence of histologic changes, suggesting transcriptional regulation of gene expression during repair. Differences in gene expression between hard and soft callus and in each of these regions as

repair progressed suggest local regulation of gene expression during cell differentiation and matrix synthesis.

Joerring et al. (1994) have examined collagen turnover after tibial fractures in patients. Sequential changes in serological markers of turnover of types I and III collagen for up to 26 weeks after fracture were measured. The turnover of type III collagen reached a maximum after 2 weeks. The synthesis of type I collagen reached a maximum after 2 - 6 weeks. The degradation of type I collagen increased after 4 days and reached a maximum at 2 weeks. The authors suggested that delayed healing of tibial diaphyseal fractures was accompanied by an early increase in the turnover of types I and III collagen.

1. 7. 4 Mechanical stability and fracture healing

Kenwright et al. (1986) reported that axial micromovement can improve fracture healing in fracture patients. Axial micromovement (30 minutes per day, starting 1-3 weeks after injury and continuing until partial weight-bearing) reduced the overall time to independent weight-bearing compared with the patients only with rigid fixation; and more cases of delayed union occurred in patients without micromovement treatment. The time taken to reach fracture stiffness levels equivalent to clinical union was significantly shorter in patients with micromovement treatment (Kenwright, et al., 1986). Grundnes and Reikeras (1992) also reported that a certain level of instability favours fracture healing as compared to absolute rigidity. Osteotomies that were stably pinned healed with less callus than those unstably pinned; mechanical testing revealed that osteotomies treated by rotational stability and axial telescoping healed better than stably fixed and rotational unstable osteotomies.

The production and distribution of types I, II, III, V and IX collagens in healing fractures under stable and unstable mechanical conditions have been investigated by immunohistochemical methods (Page, et al., 1986). In mechanically stable fractures, cancellous bone forms over the entire periosteal surface by 5 - 7 days; type I collagen was laid down within the previous fibrous matrix. The bone marrow cavities contain a matrix of types III and V collagens. Small nodules of cartilage may be present between 7 and 14 days; these contain types II and IX

collagens. In mechanically unstable fractures, cancellous bone was initially formed away from the fracture gap. The fibrous tissue over the gap was replaced by cartilage; types II and IX collagens were laid down on the pre-existing fibrous matrix; and the cartilage was replaced by endochondral ossification. At the ossification front, type I collagen was found around the chondrocyte lacunae of the spicules of cartilage. The new trabeculae contained a core of cartilage which was surrounded by a bone matrix of types I and V collagens.

The glycosaminoglycans secreted into the matrices associated with fractures have also been characterized histochemically in both stable and unstable mechanical conditions (Page and Ashhurst, 1987). Under stable mechanical conditions, the glycosaminoglycans detected in the cortical bone were located mainly around the osteocyte lacunae where chondroitin and keratan sulphates have been found. The newly formed callus contained chondroitin and keratan sulphates, but as it became mature, these glycosaminoglycans were no longer present. The cartilage in the callus of mechanically unstable fractures contains chondroitin, chondroitin-4- and 6-sulphates and keratan sulphate, though their distributions were variable. The authors concluded that the mechanical stability of the fractures appears to affect the amount and degree of sulphation of the glycosaminoglycans, rather than the types of glycosaminoglycan produced.

1. 8 Limb-lengthening techniques

Current methods of limb lengthening (via skeletal traction) date back to Alessandro Codivilla, who published the first report of surgical limb-lengthening in 1905. He described the limb lengthening technique in four stages: (1) division of the bone; (2) stabilisation with external fixation; (3) gradual lengthening and (4) a period of callus consolidation.

Magnuson (1913) carried out the first experimental work to find ways of making the operative technique of lengthening safer and more reliable. However, problems were encountered in stabilising the fragments and postoperative complications were frequent. Putti (1921) reported a method of femoral elongation by "Z" osteotomy between two transfixion pins which were connected by a spring-loaded device providing a constant distraction force. Abbot, in 1927, described a tibial lengthening method that became a standard operation in the United States. It

was modified by Carrell (1929) who was the first to emphasize that taut leg muscles were responsible for the angulation of the tibial fragments.

The basic method of externally lengthening a surgically created osteotomy has not changed much over the last 80 years. Until the 1960s and 70s, the attention of orthopaedic surgeons had focused mainly on finding new types of external fixators and lengtheners. Kawamura et al (1968) were the first to investigate the biochemical responses of the body to bone lengthening in an animal model. Their research emphasised the concept that the body had the potential to regenerate tissues.

In the early 1960s and 70s, Wagner described a new limb-lengthening technique known as the Wagner technique, which soon became popular in Europe and North America. Wagner's method consisted of an open osteotomy, unilateral external fixation and an initial gap of 0.5-2.0 cm. Gradual lengthening started immediately after osteotomy at a rate of 1.5 mm/day, in one or two steps per day. Once the desired lengthening was achieved, cancellous bone graft was used to fill the distraction gap and the bone ends were stabilised with a plate (Wagner, 1978). In Wagner's procedure, new bone usually does not form spontaneously to fill the lengthening gap, and the method depends on bone grafting for its success. In clinical practice, Wagner lengthenings had a high complication rate, such as non-union and poor bone formation (Mosca and Moseley, 1986; Herzog and Hefti, 1992).

Professor G.A. Ilizarov started his remarkable research into the biology of distraction osteogenesis in the late 1950s, in Kurgan, USSR, where he had developed a fixation system using circumferential rings secured with transfixion wires (Ilizarov, 1991). His pioneering studies investigated the importance of the rate and rhythm of elongation, and the effect of preservation of the endosteum and periosteum on distraction osteogenesis. He examined the mechanisms of bone and soft tissue (muscle, fascia, blood vessels, nerves) regeneration and established the concept of the "tension-stress" effect on tissue regeneration (Ilizarov, 1989 a, b). Ilizarov's results have subsequently been confirmed and expanded upon by investigators in Europe, Japan, and North America (Kojimoto, et al, 1988; Aronson, et al., 1989; 1990; Delloye, et al, 1990; Steen, et al., 1990; Kenwright, et al., 1990; Lokietek, et al., 1991;

Aronson and Shen, 1994; Aronson, 1994 a, b). The results show that under appropriate conditions, bone responds to gradual mechanical distraction by forming new bone, a process now termed *distraction osteogenesis*.

1. 8. 1 The principles of limb-lengthening

The quality of osteogenesis in limb lengthening is determined to a large extent by the surgical management. Wagner's technique (transverse midshaft osteotomy, intraoperative lengthening of 1 cm and postoperative distraction of 1-1.5 mm/day) resulted in much less osteogenesis than the currently used Ilizarov methods. Patients operated on using the Ilizarov technique have improved osteogenic reactions . According to Ilizarov (1990), the factors important for osteogenesis are:

- 1) maximum preservation of extraosseous and medullary blood supply;
- 2) stable external fixation;
- 3) a delay period prior to distraction of 5-10 days;
- 4) a distraction rate of 1 mm per day in frequent small steps;
- 5) a period of stable neutral fixation (consolidation) after lengthening;
- 6) physiologic use of the elongating limb.

For successful fixation during this process, the apparatus must be applied with consideration given to the number, size, and location of the rings, the placement of and tension on the wires, the technique of wire insertion, the effect of soft-tissue transfixion on limb use, and the prevention of bone and joint deformities caused by the resultant countertension generated in the soft tissues.

1. 8. 2 Osteotomy and corticotomy

Corticotomy, a surgical technique of cutting bone with minimal damage to the surrounding soft tissues, was first advocated by Ilizarov (1989 a,b). It was suggested that corticotomy improves bone healing compared to conventional osteotomy. The corticotomy technique is a

combination of an open, subperiosteal, partial cortical osteotomy, followed by manual breaking of the remainder of the opposite bone cortex. Corticotomy is a more complicated procedure than simple osteotomy with complications which include: fractures extending from the level of cortocotomy; displacement of the fragments after corticotomy; and incomplete corticotomy (Schwartzman and Schwartzman, 1992).

A comparison of corticotomy and osteotomy techniques was performed by Frierson, et al in dogs (1994). Five dogs were assigned to each of three separate groups: (I) corticotomy; (II) osteotomy with multiple drill holes and an osteotome; (III) osteotomy with an oscillating saw. Distraction began on the seventh postoperative day and continued at a rate of 0.25 mm every six hours for 26 days using an Ilizarov external fixator. The regenerate segments were evaluated by weekly radiographs and dual energy photon x-ray (DEPX). Barium perfusion studies and histologic preparations were also performed after the dogs were killed. Perfusion studies demonstrated blood vessels bridging across the regenerate gap in Groups I and II, but not in the oscillating-saw Group III. No histologic differences were seen in Groups I and II. Three of the four tibiae cut with the oscillating saw did not consolidate at ten weeks. The authors concluded that a simple transverse osteotomy yielded regenerate bone that was indistinguishable from the technically more difficult corticotomy. Use of the oscillating saw however may lead to delayed consolidation. An experimental study of distraction osteogenesis comparing a metaphyseal with a diaphyseal site showed that there was no significant difference in the healing process between the two sites (Aronson and Shen, 1994).

1. 8. 3 Timing of distraction

Excessive or premature loading and motion of repair tissue can inhibit or stop healing (Buckwalter, 1996). The importance of delay before distraction of the osteotomy has been studied by White and Kenwright (1991). They showed that experimental osteotomies subjected to immediate distraction resulted in the production of a small volume of callus with deficient vascularity. A delay period altered this response, leading to a more cellular osteogenic reaction with proliferative vascularization during lengthening. Their results clearly showed that a delay period between osteotomy and starting lengthening is associated with

enhanced bone healing. There were two possible explanations for this effect : 1) immediate distraction may inhibit the recruitment of osteogenic precursor cells from surrounding tissues; 2) the lack of stability associated with immediate and repeated traction may frustrate the local repair of damaged blood vessels (Kenwright and White, 1993).

A delay period is now advocated in all gradual limb-lengthening procedures. The period of delay should be carefully determined according to each individual case. Morphologic studies have shown that the optimal delay period should be from seven to ten days (Schwartzman and Schwartzman, 1992). The benefit of delayed distraction after transverse osteotomy was demonstrated by microangiographic studies, which showed that even though intramedullary vessels were interrupted by osteotomy at surgery, blood circulation recovered during the waiting period before distraction (Yasui, et al., 1993).

1.8.4 Effect of stability of fixation on distraction osteogenesis

Ilizarov (1989 a, b) described an experiment in which dogs were divided into five groups according to the different degree of stability of fixation during distraction. He showed that the level of osteogenic activity depended upon the degree of stability. The most stable fixation led to direct bone formation within the distraction zone, whilst less rigid or unstable fixation led to cartilage and fibrous tissue formation.

A key parameter determining the mechanical environment created during distraction osteogenesis was the axial stiffness of the external fixator (Waanders, et al., 1993). Paley, et al. (1990) tested four external fixator systems currently used for limb lengthening to determine the fixator stiffness. The Ilizarov system, with its tensioned wires supported on rings, combined excellent shear resistance with preservation of axial dynamization. The Orthofix unilateral fixation device was found to offer the best overall biomechanical stability and the capability for dynamization. Limb-lengthening using the Orthofix device has been reported to have some advantages in comparison with the Ilizarov methods: (1) the application was relatively simple and required less operative time than that of the circular frames; (2) there was less risk of neurovascular injury with the use of half-pins, and radiographic evaluation was

simplified (Paley, 1990). In clinical practice, bone formation using the Orthofix device was the same as using the Ilizarov device. Compared to the Wagner technique the need for bone grafting and internal fixation was greatly reduced (Price and Mann, 1991).

1. 8. 5 Rate and rhythm of distraction

Ilizarov (1989 b) investigated the influence of both the rate and the rhythm of distraction on osteogenesis during limb elongation. A canine tibial lengthening model was used with various combinations of distraction rates (0.5 mm, 1.0 mm, or 2.0 mm per day) and distraction rhythms (1 step, 4 steps, 60 steps per day). It was observed that distraction at a rate of 0.5 mm/day in four steps often led to premature consolidation of the regenerating bone, whilst with a distraction rate of 2.0 mm/day in 4 steps or 1 mm/day in 1 step, a large proportion of the regenerate zone was filled with fibrous connective tissue. A distraction rate of 1.0 mm/day in four steps or auto-distraction at 0.017 mm every 24 minutes led to the best bone formation. New bone forming in parallel columns extended in both directions from a central growth zone. With small increments of distraction, better bone formation occurred, and distraction at a rate of 1.0 mm/day in 4 steps was considered most favourable for osteogenesis and is commonly used clinically.

1. 8. 6 Bone formation

The primary mechanism of bone formation with Ilizarov's method was observed to be intramembranous ossification. Direct bone formation occurred in the central fibrous interzone, and quickly remodelled to match the normal macro and microstructure. Bone columns formed along longitudinally oriented collagen bundles. As the distraction gap increased, the bone columns increased in length and in diameter, while the fibrous interzone remained about 4 mm long (Tetsworth and Paley, 1995). Radiodensity increased gradually from the central fibrous interzone towards each osteotomy surface. Mineral density, as determined by calcium quantification, reflects the microscopic geometry and radiographic appearance (Aronson, et al., 1990).

Aronson (1994a) reported the data from experimental tibial lengthenings in more than 125 animals (dogs, rabbits, and rats), the data showed that distraction osteogenesis provided new bone that remodelled at rates ranging from 200 to 400 microns per day. In over 100 clinical cases, patients ranging in age from 18 months to 49 years have regenerated bone at an average rate of 213 microns per day in adults and 385 microns per day in children. Approximately 10% of these cases required supplemental bone grafts. The volume of callus and rate of bone consolidation has been reported to depend on the condition of the periosteum and endosteum, and also by factors such as age, nutrition, systemic disease, bone diameter, cortico-medullary ratio, site of osteotomy, and previous injuries of the surrounding tissues (Hamanishi, et al 1992). Maffulli, et al (1996) reviewed 240 patients undergoing distraction osteogenesis for congenital, post-traumatic or post-infective lower limb length discrepancy. They found that femoral osteotomies healed faster than tibial osteotomies. The healing index in post-traumatic and post-infective limb length discrepancy was significantly lower than that in congenital conditions. Bifocal lengthenings healed significantly faster than single level ones.

The quantity and rate of new bone formation during lengthening can be monitored by dual-energy X-ray absorptiometry (DEXA), ultrasonography and radiography (Eyres, et al., 1993). Whereas new bone was detected by both DEXA and ultrasonography within 1 to 2 weeks of distraction, it was not visible on the radiographs until 4 to 8 weeks. Ultrasound can be used to identify defects in mineralisation and to determine when to dynamise the fixator system, and DEXA can measure the quantity and rate of bone formation throughout the lengthening period (Eyres, et al., 1993).

Malde, et al. (1993) reviewed and suggested that ultrasound imaging in bone was superior to conventional radiography in the following areas: (i) detecting early new bone formation, (ii) establishing the extent of cortical and medullary canal remodelling, (iii) detecting soft tissue complications at the distraction site and (iv) determining the presence of fluid collections at the distraction site. Conventional radiographs are more accurate in determining the distance between the two distracted bone ends, and thus the degree of distraction achieved. An ideal assessment of events at the distraction site could be achieved by a combination of conventional radiography and ultrasound .

There was a direct relationship between the percentage of intact cortex seen on ultrasound imaging and the mechanical properties of the lengthened bone (Hamdy, et al., 1995). It has been suggested that the fixator may be removed until the echo reflection value has reached 70 percent of the value of the control cortical bone (Hamanishi, et al., 1994c).

1. 8. 7 The difficulties of limb-lengthening

Lengthening a long bone may be associated with many complications, especially in a congenitally short bone. Compere (1936) classified the serious complications of limb-lengthening into three groups, which are due to (1) over-stretching, (2) interference with the blood supply to the fragments, and (3) insufficient fixation of the fragments. Paley (1990) subclassified the difficulties that occur during limb lengthening into problems, obstacles, and complications. Problems required no operative intervention to resolve, while obstacles required an operative intervention. These difficulties included muscle contractures, joint displacement, axial deviation, neurological injury, vascular injury, premature consolidation, delayed consolidation, nonunion, pin site problems, and pain and difficulty in sleeping. Late complications were loss of length, late bowing, and refracture. Sproul and Price (1992a) have proposed a simple classification dividing complications into major and minor ones:-

Major Complications :

1. Bone or joint infection
2. Permanent loss of functional joint range of motion
3. Arthritis
4. Knee or hip subluxation / dislocation
5. Delayed union or nonunion of bone
6. Residual limb length inequality
7. Malalignment of anatomic limb axis greater than five degrees
8. Upward migration of lateral malleolus
9. Vascular or nerve injury :
 - a. stretch related
 - b. pin penetration
10. Chronic oedema
11. Loss of muscle strength
12. Poor limb function (worse than amputation and prothesis)

Minor complications :

1. Pin-track inflammation / infection
2. Pin loosening
3. Premature consolidation of lengthened fibula
4. Temporary malalignment of anatomic axis
5. Neuropraxia (temporary)

In Dahl et al's series of 1994, major complications were frequent in patients with severe deformities. There was, however, a significant decrease in complications as experience was gained (Dahl, et al., 1994). Soft tissue contractures rarely occur if the lengthening is less than 10% of the initial bone length. Loss of joint motion occurs more often during femoral lengthening than tibial lengthening, due to transfixation of the quadriceps (Paley, 1990). Knee articular cartilage injury has been observed during leg lengthening (Nakamura, et al., 1993). It has been suggested that increased mechanical stress on the articular cartilage might alter glycosaminoglycan metabolism, and increasing the frequency of distraction may prevent or reduce damage to articular cartilage (Nakamura, et al., 1995).

Despite good results, the Ilizarov technique still produces an overall complication rate of approximately 30% (Garcia-Cimbrelo, et al., 1992). In the past, leg lengthening has been a procedure of last resort because of its high complication rate and significant morbidity with a long period of disability even without complications (Moseley, 1989). An increased understanding of the biology of distraction osteogenesis, as well as technical advances, has made lengthening more feasible. New techniques of lengthening enable orthopaedic surgeons to achieve more length with fewer complications. The modern era of limb-lengthening has therefore brought two things: new technical versatility to correct complex and coexisting deformities, and new concepts of the biology of lengthening, which are not device specific and can be applied to all lengthening procedures.

1.9 Biology of distraction histogenesis

The most important advance in limb-lengthening was the recognition that under appropriate

conditions, bone and soft tissues will regenerate within the resultant gap of a controlled separation of osteotomized bone ends. There has been much recent research into the basic science of distraction histogenesis.

1. 9. 1 Distraction osteogenesis

The sequence of events associated with distraction osteogenesis has been monitored radiographically, and the corresponding histology has been studied in animal models. In a distraction osteogenesis model of the rabbit, x-ray analysis showed that on completion of the distraction the distracted gap was organized into three zones, which comprised two sclerotic zones (mineralization fronts) and a central radiolucent zone (fibrous zone). These zones became indistinguishable from the adjacent pre-existing cortical bone by 10 weeks after distraction. However, corticalization of the lengthened bone segment occurred gradually after several months (Komuro, et al., 1994).

The central fibrous zone is a fibroblastic network that contains spindle-shaped cells oriented parallel to the direction of the applied distraction force. This network develops in the haematoma which forms after osteotomy and eventually connects the two bone ends (Tetsworth and Paley, 1995). Peltonen (1993) showed that collagen bundles together with large polymorphic fibroblast-like cells were present in the fibrous zone. These collagen bundles were organized in the direction of distraction, and formed a template for the developing bone. The fibroblast-like cells in the central part of the fibrous zone remain functionally active as long as distraction proceeds (Karaharju, et al., 1993). During lengthening, mineralization started at the osteotomized ends of the bone in the form of conical bony columns about 200 microns thick, which grew towards each other until the central fibrous zone had completely mineralized (Giebel, 1995). The different stages of distraction osteogenesis are illustrated in Fig. 1. 11.

Tajana, et al. (1989) analysed 64 biopsies from patients undergoing tibial lengthening by the Ilizarov method. Using conventional and polarized light microscopy, direct bone formation was seen with only a few cartilaginous areas. Shearer et al. (1992) reported the histological

appearance of a lengthened tibia, which has been removed from a patient who had undergone leg lengthening following trauma, but had subsequently required amputation 6 months after lengthening. No cartilage was found in this specimen at this stage, and new bone was seen to form directly around the collagen fibres.

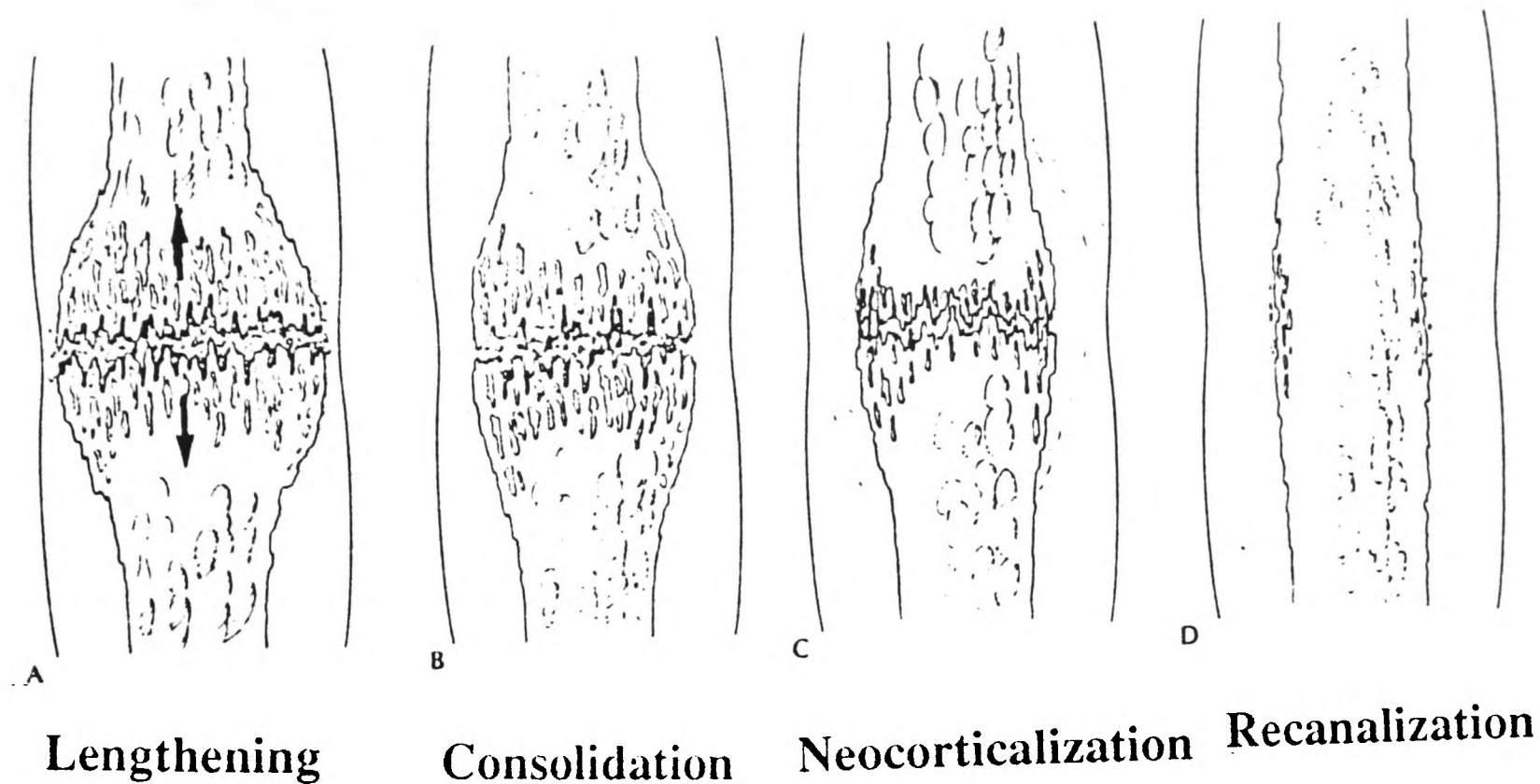


Fig.1.11. The stages of distraction osteogenesis. A. The lengthening phase; longitudinally oriented trabeculae formed either side with a central fibrous zone. B. The consolidation phase; the new bone is allowed to mature and ossification of the central fibrous interzone begins. C. Corticalization phase; sufficient neocorticalization allow safe removal of the fixator. D. Recanalization; the medullary canal is remodelled and the new cortex is formed during this phase. (Modified from Tetsworth and Paley, 1995)

Karaharju et al (1992) studied distraction osteogenesis in the sheep mandible model; their results indicated that the process of bone formation by distraction osteogenesis in the mandible is principally the same as in long bones. Four phases can be observed during the process: (1) formation of collagen bundles aligned in the direction of distraction; (2) bone formation over the collagen template; (3) bone union across the distraction gap, and (4) remodelling of the formed bone. At the end of distraction, the consolidation period begins. This is the period during which the newly formed bone matures and remodels to re-establish a cortex. The period of neo-corticalization is necessary for the regenerate bone to increase its strength prior to removal of the external fixator.

1. 9. 2 Enhancement of distraction osteogenesis

Mechanical stimulation, such as weight-bearing, is an important factor in the promotion of bone formation (Meadows, et al., 1990). It has been found that axial dynamization can improve bone healing. Dynamized osteotomies were significantly stiffer under torsional mechanical testing than the controls (Egger, et al., 1993). Hamanishi et al. (1994b) reported that the callus was stimulated to consolidate by axial shortening. In their experiment, the tibiae of rabbits were lengthened by 10 mm in 2 weeks and then shortened by 2 mm in 3 days. In the shortened callus, marked histological changes were observed in the central fibrous zone where the number of osteoblasts increased 4 fold compared to the control group.

Van-Roermund et al. (1990) have studied the effect of direct current stimulation on bone growth after distraction epiphysiolyis in the rabbit tibia. Their data showed that direct current stimulation had effects in the early phase of a limb-lengthening procedure, but had no effect on the extent of bone formation in the regenerate. Hamanishi, et al (1995) evaluated the effect of pulsed direct current stimulation on callus formation during leg-lengthening in a rabbit model. A pulsed direct current at 20 mA was applied for 12 hours daily from the day of osteotomy until 40 days after the completion of lengthening. The values of bone mineral density (measured by DEXA) of the electrically stimulated callus were significantly increased compared to the control group. Pulsed direct current electrical stimulation may stimulate the poorly mineralized callus and shorten the time course of leg-lengthening.

Niedzwiedzki (1993) reported that implantation of fresh autologous bone marrow cells can speed up the healing process. The technique was especially useful in the treatment of delayed unions. His results were supported by the work of Hamanishi et al. (1994a), who transplanted autologous bone marrow cells into the primitive callus of lengthened tibia in rabbits. Transplantation of fresh suspended bone marrow cells increased the bone mineral density significantly. However, transplantation of cultured colony-forming cells did not increase the bone mineral density.

There have been many attempts to use drugs to enhance the process of bone formation during

distraction osteogenesis. Yamamoto (1995) studied the effect of 25-dihydroxyvitamin D3 on callotasis in rabbits. The bone mineral content in the animals which received 25-dihydroxyvitamin D3 was significantly higher than those receiving no treatment, and this result correlated with the radiographical and histological findings.

1. 9. 3 Distraction histogenesis of soft tissues

Ilizarov has investigated the effects of distraction on skin, muscle, tendon, fascia, blood vessels, and peripheral nerves. Although different tissues react in different ways, there are two common mechanisms involved: (1) reorganization of collagen in response to stretch and (2) neohistogenesis. Controlled mechanical distraction of skin is a familiar technique to plastic surgeons, who use inflatable skin expanders. Distraction histogenesis of soft tissues however, is far less understood than that of distraction osteogenesis.

Effects on muscle: Ilizarov (1991) suggested that soft tissues react to distraction by developing some of the characteristics of embryonic tissue. Muscle responds initially to stretching (less than 10% original length) without cell proliferation. An increase of satellite cells contributes to neohistogenesis and growth in the length of the muscle. A mixed cellular response was seen with additional stretching. Sun, et al. (1994) reported an increase in cellularity with inflammatory cell infiltration near the myotendinous junction of the lengthened muscles in a rabbit model. Neuromuscular injury during limb-lengthening in rabbits was seen frequently, and needle electromyography (EMG) has shown denervation in the elongated legs even at a low magnitude of lengthening (Chuang, et al., 1995). Simpson, et al. (1995) used an experimental rabbit model of leg lengthening to study the morphology and function of muscle after distraction at different rates. New contractile tissue did form during lengthening, but some damage to the muscle fibres was seen even at rates of less than 1 mm per day. Abnormalities increased with higher rates of lengthening. At distraction rates of over 1 mm/day there was proliferation of fibrous tissue between the muscle fibres.

Effects on nerve: New Schwann cells and active myelinization were observed in peripheral nerves in dogs (Ilizarov, 1989 a, b). The effects of lengthening on the sciatic nerve of rats

were reported by Battiston, et al. (1992). They found that if distraction was performed gradually, the nerve did not undergo any electrophysiologic functional changes, but underwent some morphologic changes, such as a decrease in both the areas of the axons and the myelin sheaths, an increase in the distances between the nodes, and proliferation of Schwann cells. Ippolito, et al. (1994) studied the effects of lengthening of the metacarpal bone on peripheral nerves in calves. After a lengthening of 8%, moderate degenerative changes in the myelin sheath of the palmar nerve were observed. Axoplasmic changes were evident after a lengthening of more than 20%. Clinical observation confirmed that some patients after limb-lengthening treatment developed persistent weakness in the lengthened limb. Young et al. (1993) have examined patients at completion of tibial lengthening, and found specific conduction abnormalities. Their data indicates that a mild subclinical sensorimotor axonal neuropathy may be common in limb-lengthening patients.

1. 10. Angiogenesis and osteogenesis

1. 10. 1 Structure and function of blood vessels

Bone is a highly vascularised tissue and it has been estimated that a bone cell is no more than 200 μm away from a blood vessel (Ham and Cormack, 1979). The vascular supply of bone consists of main nutrient arteries which are mainly from medullary arteries and the rich vasculature of the periosteum. The blood supply feeds complex sinusoidal networks within the bone and drains to venous channels leaving through all bone surfaces not covered by articular cartilage.

The surfaces of blood vessels are lined with a single layer of endothelial cells which adhere to an encircling basal lamina (Fig. 1. 12). The basal lamina provides rigidity to the vessel wall, serving as an anchor for the endothelial cell layer and may also act as a filter. Larger vessels also have surrounding smooth muscle cells and connective tissue arranged in layers. Variations exist in the laminar organisation according to the vessel function. Exchange of gases, water, inorganic ions, vitamins, hormones and other regulatory factors, antibodies and defensive cells takes place between blood and tissue via the walls of small blood vessels (capillaries and

sinusoids) (Ham and Cormack, 1979).

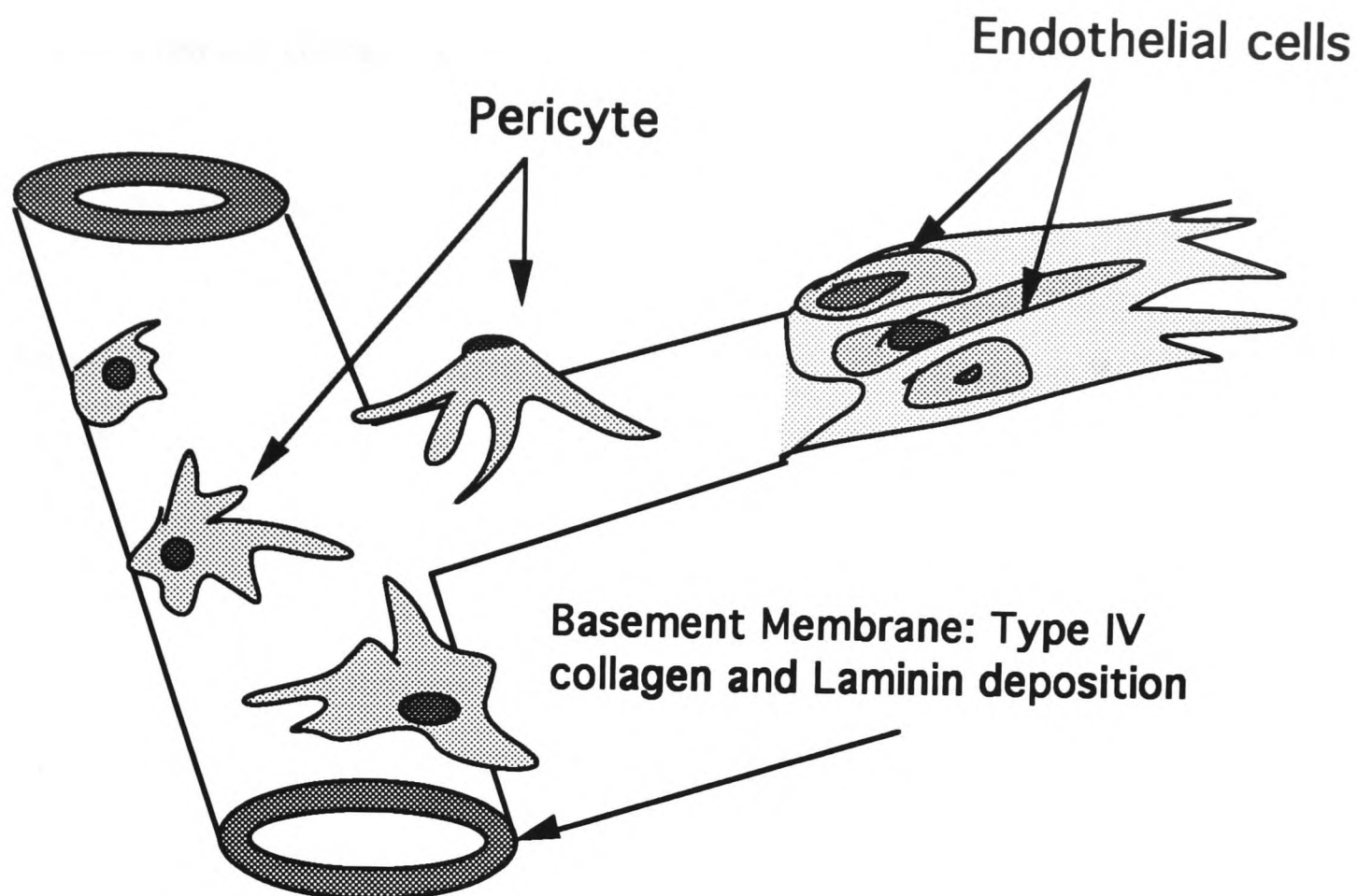


Fig. 1. 12. Schematic representation of microvascular system.

Until recently endothelial cells were considered to play a mainly structural role within blood vessels. Endothelial cells act as a selective permeability barrier and a non-adhesive surface for blood cells. The endothelial cells are now known to have much more active roles in events such as angiogenesis, inflammation and immune response, coagulation and wound repair (Madri and Pratt, 1986). The pericyte is a polymorphic cell of mesenchymal origin that surrounds the endothelial cell in the microvessel and shares a common basement membrane or basal lamina with the endothelial cell (Brighton, et al., 1992). The possible functions of pericytes are : (1) to maintain rigidity of microvessel walls; (2) to regulate blood flow in microvasculature; (3) to act as resting stem cells for smooth muscle cells; (4) to regulate capillary regeneration during wound healing and (5) to act as a supplementary source of osteoprogenitors (Brighton, et al., 1992; Allsopp, 1994).

1. 10. 2 Sequence of events in angiogenesis

Angiogenesis, the growth of new blood vessels, occurs normally during osteogenesis, fracture repair, luteinisation, the development of the embryo, and in pathological states such as chronic

inflammation, certain immune reactions and neoplasia. Furthermore, solid tumours have been reported to secrete a diffusible factor which promotes the growth of new capillaries towards a growing tumour (Zetter, 1980).

Light and electron microscopic studies of early angiogenic reactions showed that endothelial cells migrated away from existing capillaries to form sprout and loops (Cliff, 1963). These sprouts and loops were directed toward the source of the angiogenic stimulus. According to Furcht (1986), the process of angiogenesis can be divided into a number of distinct yet overlapping steps which include:

1. The initial vascular response. Vessels become dilated and permeable; retraction of endothelial cells with an increased gap in endothelial junctions.
2. Degradation of the endothelial basement membrane caused by activation of a variety of proteases, including type IV collagenase.
3. Migration of endothelial cells from the vascular wall, through perivascular connective tissue toward the angiogenic stimulus.
4. Proliferation of endothelial cells in the sites of migration.
5. Formation of capillary tubes and further development of capillary loops and sprouts. The newly formed blood vessels develop further and lay down a basement membrane consisting of type IV collagen, laminin and proteoglycans.
6. Migration of pericytes and fibroblasts to the sites of the capillary loops; these cells associate with mature capillaries.
7. Perfusion of the capillaries with blood.
8. Further remodelling, regression and rearrangement of the newly formed capillary in response to the presence or absence of angiogenic stimuli.

1. 10. 3 Factors influencing angiogenesis

Angiogenesis is a very tightly controlled process that rarely occurs under normal conditions, except for instances of wound healing, embryonic development and development of the corpus luteum. Otherwise, vascular turnover is very low. It has been demonstrated that the potential

doubling time of capillary endothelium from normal tissue is in the range of 47-20,000 days, as opposed to 2-13 days for tumour capillary endothelium (Hobson and Denekamp, 1984). The vascular endothelium is responsible for maintaining the liquidity of blood, and the endothelial cells synthesise many active substances, such as fibronectin, heparin sulphate, interleukin-1, prostacyclin, nitric oxide and endothelin-1 (Vance, et al., 1990). The production of these substances is modulated by changes in the concentrations of intracellular messengers and by interaction between the endothelium and white cells or platelets.

Studies of Schor et al. (1979, 1980) showed that the extracellular matrix, principally collagen, dictated the ability of the endothelial cells to respond to angiogenic stimuli. In those studies, angiogenic tissue extracts promoted proliferation of endothelial cells cultured on native type I collagen, but not on denatured collagen or uncoated tissue culture dishes. Type IV collagen, but not types I and III collagen, was capable of inducing a phenotype of endothelial cells with a more differentiated state whereby it produced type IV collagen and laminin (Madri and Williams, 1983; Madri and Pratt, 1986). Fibronectin, a non-collagenous adhesion molecule is also responsible for cell behaviour in angiogenesis. McAuslan et al. (1980) proposed that fibronectin serves as a signal for endothelial cell migration and subsequent capillary formation. This was confirmed by the studies of Clark et al. (1982) and Bowersox and Sorgente (1982). The role of proteoglycans in angiogenesis is not clear.

Heparin derived from mast cell supernates could stimulate the migration of capillary endothelial cells (Azizkham, et al., 1980). The heparin effect on angiogenesis was not due to its coagulation effect, but perhaps was due to its ability to bind endothelial growth factor or angiogenic factors (Maciag, et al., 1984). A variety of soluble factors have been found to promote angiogenesis, among them, fibroblast growth factor (FGF), transforming growth factor- α , β (TGF- α , β), platelet derived growth factor (PDGF) and epidermal growth factor (EGF) are particularly capable of promoting angiogenesis *in vitro* and *in vivo* (Furcht, 1986).

Angiogenesis is a crucial event during endochondral ossification. Blood vessels grow into the hypertrophic cartilage and erode it to produce a scaffold on which osteoblasts settle to produce woven bone. Baron et al (1994) reported that bFGF infused directly into the rabbit proximal

tibial growth plate, accelerates vascular invasion and ossification of growth plate cartilage. They suggested that bFGF couples osteogenesis to chondrogenesis by attracting endothelial and bone cell invasion from the adjacent metaphyseal bone. Brown et al. (1990) studied the influence of dexamethasone on vascular invasion of the rabbit growth plate. The growth plate of the rabbit treated with dexamethasone underwent major changes in the pattern of capillary invasion. Many narrow channels were formed which were filled with debris or red blood cells, but did not contain capillaries. Dexamethasone treatment disrupted the normal control of capillary invasion.

Alini, et al. (1996) demonstrated for the first time the production of an angiogenic molecule by hypertrophic chondrocytes of the growth plate. Chondrocytes from bovine growth plates were separated into distinct subpopulations. The hypertrophic chondrocyte phenotype was defined by the synthesis of type X collagen and matrix calcification. The culture medium collected from the hypertrophic cells contains a chemoattractant molecule for bovine endothelial cells which can induce angiogenesis in vivo in the rabbit cornea model. The production of this molecule by hypertrophic cells was enhanced by both 1, 25-(OH)₂ vitamin D₃ and 24, 25-(OH)₂ vitamin D₃ treatment. The results demonstrated an important role for vitamin-D₃ in regulating hypertrophy and angiogenesis during normal skeletal growth.

Harada, et al. (1994) examined the role of vascular endothelial growth factor (VEGF), a secreted endothelial cell-specific mitogen, in PGs stimulation of bone formation. They found that in rat calvaria-derived osteoblasts PGE₂ and E1 increased VEGF mRNA and protein levels. The increased expression of VEGF mRNA produced by PGE₂ was rapid (maximal at 1 hr) and transient (declined by 3 hrs). The upregulation of VEGF mRNA by PGE₂ was inhibited by dexamethasone treatment. In addition, Northern blot analysis showed that VEGF mRNA was expressed in adult rat tibia. Stimulation of VEGF expression by PGs and its suppression by glucocorticoids implicate the involvement of VEGF in bone metabolism.

Oxygen tension also played a role in regulating angiogenesis. Kourembanas et al. (1990) reported that hypoxic conditions (0 -3% oxygen environments) significantly increased PDGF-B mRNA expression in cultured human vein endothelial cells. This increase was inversely

proportional to oxygen tension and was reversible upon reexposure of cells to a 21% oxygen atmosphere.

Mechanical environment and stimulation are both important in osteogenesis and angiogenesis. Angiogenesis couples osteogenesis, and cells involved in angiogenesis could also participate in osteogenesis (for review see Clemens, 1996). Diaz-Flores, et al. (1992) demonstrated that pericytes of blood vessels took part in both angiogenic and osteogenic processes under appropriate mechanical stimuli, in which periosteum strip of the rat femur led to rapid new bone formation and angiogenic response, and both pericytes and endothelial cells of the postcapillary venules were activated.

1. 10. 4 Angiogenesis and distraction osteogenesis

Ilizarov (1990) suggested that there is an increase in blood supply during distraction, and this is the theoretical guidance for the treatment of fracture non-union and osteomyelitis using the Ilizarov method. Aronson (1994b) has confirmed Ilizarov's theory by showing that temporal and spatial increases in blood flow occur during distraction osteogenesis. Quantitative technetium scintigraphy was used to measure regional blood flow during distraction. The flow increased to nearly ten times control, peaked at two weeks postoperatively, then decreased to four to five times the control for the remainder of the distraction period. During the consolidation period, increased flow persisted at levels of two to three times control. Aronson (1994b) also found that each column of new bone was completely surrounded by large vascular sinusoids, osteoblasts at the tip or on the surfaces of each column were in close relation to these sinusoids. These vessels paralleled the bone columns and the distraction force. Technetium scintigraphy of the bone forming area was intensely hot, with a central cool area corresponding to the fibrous interzone. A schematic representation of the blood supply to the regenerate is illustrated in Fig. 1. 13.

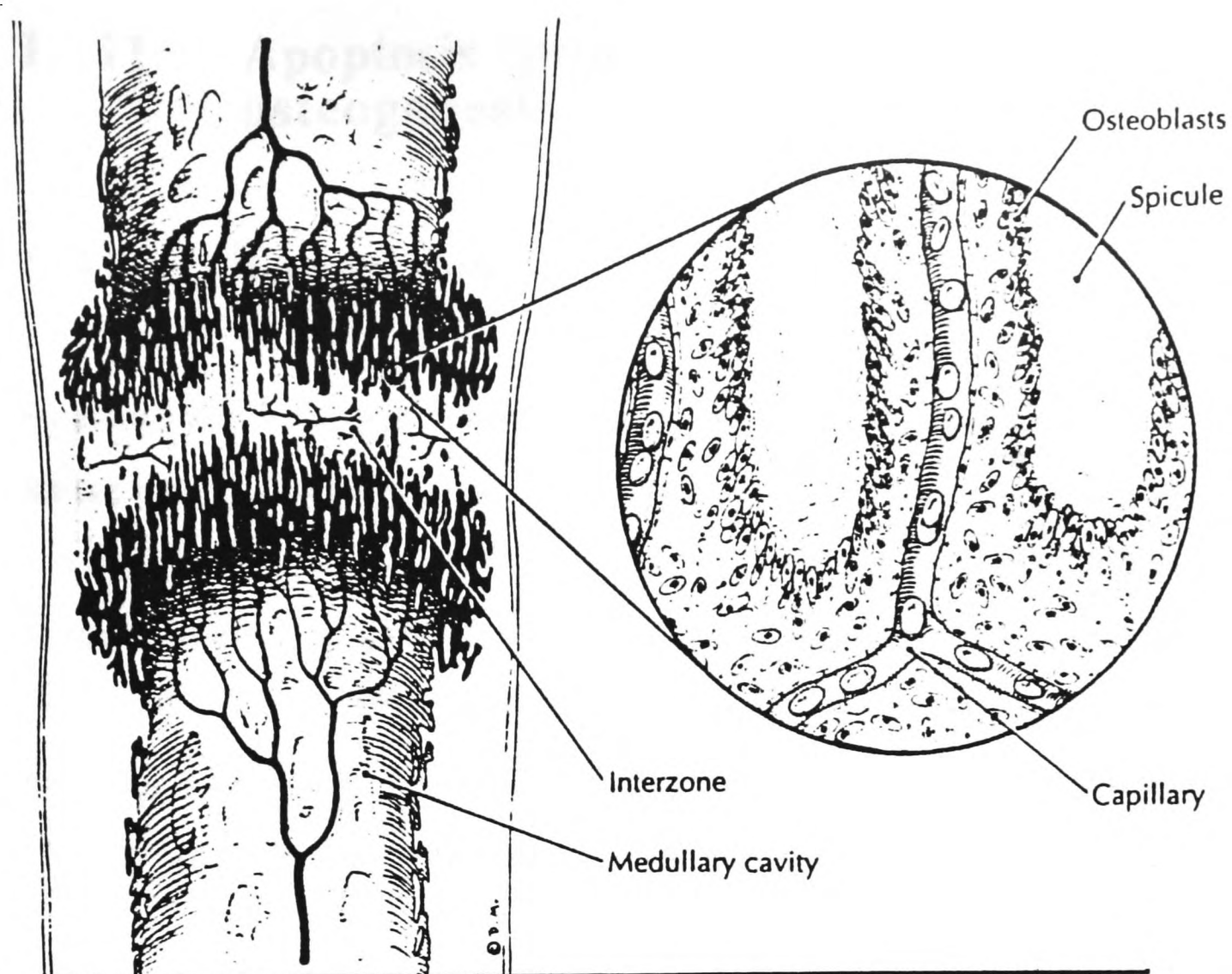


Fig. 1. 13. Illustration of the blood supply to the regenerate during distraction osteogenesis. Continuity exists between the nutrient artery and new microvessels in newly formed trabeculae. The interzone is relatively hypovascular compared with the new bone region. (*From Tetsworth and Paley, 1995*)

Delloye et al. (1990) have studied the effect of bone marrow cavity plugging on bone regeneration during distraction osteogenesis. When an osteotomy was performed, the marrow cavity was in some cases plugged with either resorbable bone wax or nonresorbable material. They found that the morphological sequence of bone regeneration was similar with or without bone-wax plugging.

Einhorn (1996) has examined expression of molecular markers during distraction osteogenesis in a rat leg-lengthening model. The mRNA for vascular endothelial growth factor (VEGF) was expressed at low to moderate levels on day 7 before distraction and on day 19 during distraction. However, at eight hours after distraction on day 7, and at 30 minutes after distraction on day 19, VEGF mRNA was expressed at very high levels. These findings suggest that each change in the mechanical environment may trigger the activity of VEGF and possibly other factors, which may drive the angiogenic processes.

1. 11 **Apoptosis (programmed cell death) and osteogenesis**

1. 11. 1 **Introduction**

Programmed cell death or apoptosis is a process whereby developmental or environmental stimuli activate a genetic program to implement a specific series of events that result in the death and efficient disposal of a cell (White, 1996). Death of a cell may occur by one of two processes: necrosis or apoptosis. Necrosis is usually an accidental, unplanned death caused by severe cell injury due to factors external to the cell such as hypoxia, toxins, extremes of temperature and attack by bacteria or viruses. By contrast, apoptosis is a genetically controlled cellular response to specific developmental and environmental stimuli.

Necrosis is characterised by cytoplasmic organelle destruction and loss of plasma membrane integrity and is generally accompanied by an inflammatory response. The main features of apoptosis are cell shrinkage, in contrast to the swelling seen in necrosis, and chromatin condensation, nuclear DNA fragmentation, and apoptosis does not usually induce an inflammatory response (Wyllie, 1980). Apoptosis can be regarded as a neat and tidy process to kill cells with minimal damage to surrounding cells or tissue. While the pathways for apoptosis and necrosis may be distinct, there is likely to be overlap between the pathways in vivo as shown in Fig. 1. 14.

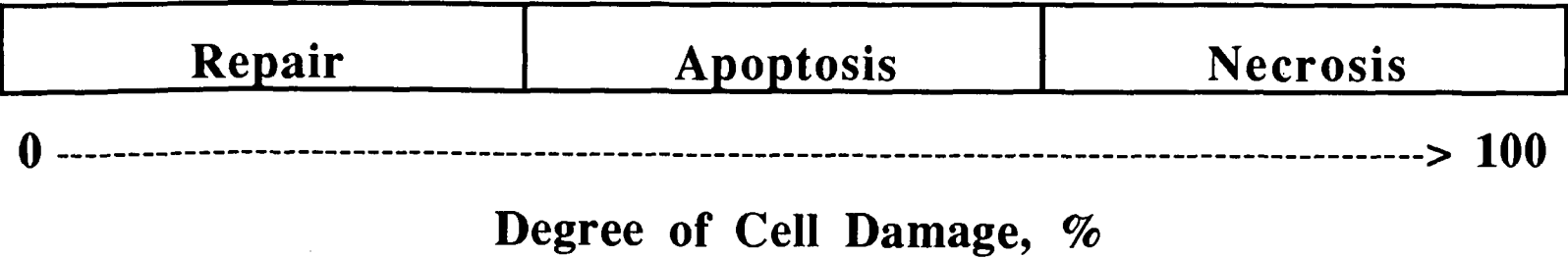


Fig. 1. 14. The link between apoptosis and necrosis. Increasing cell damage can overcome the ability of the cell to repair itself. Apoptosis is one way to remove these damaged cells in a controlled manner. If the damage is too severe, necrosis, rather than apoptosis, is usually the result.

According to Ellis, et al. (1991) from studies of programmed cell death in *C. elegans*, the apoptotic process can be divided into four distinct stages. These include (1) the decision

whether a cell will die or survive; (2) the death of the cell; (3) the engulfment of the dead cell by phagocytes, and (4) the degradation of the engulfed corpse. There are specific genes involved in all stages of cell death, some of them promote or prevent the process of cell death. Mutations of these genes will influence the balance between cell proliferation and cell death. Diseases such as cancer, autoimmune disorders and some viral infections develop as a result of the inhibition of apoptosis; while others, such as AIDS, neurodegenerative disorders, aplastic anaemia, ischemic injury or toxin-induced diseases, develop as a result of increased apoptosis (Thompson, 1995).

1. 11. 2 Regulation of apoptosis

There are multiple triggers of apoptosis, which work through a variety of signalling pathways. According to Norris (1995), the inducing factors of apoptosis may be:

- (1) DNA and protein damage by radiation, reactive oxygen species, or enzymatic damage;
- (2) Survival factor withdrawal, such as growth factor withdrawal during development and differentiation;
- (3) Ligand binding to some special receptors, such as receptors of Fas, tumour necrosis factor, or transforming growth factor- β .

The signalling pathways for these triggers are quite varied, but eventually they result in activation of bax, one principal molecular inducer of apoptosis (Norris, 1995). The production of a protease related to the interleukin-1-converting enzyme (ICE) or other proteases such as calpains or deoxyribonuclease I (DNase I), which are factors leading to apoptosis, are activated by bax (Polzar, et al, 1993; Lazebnik, et al, 1994). The ability of bax to induce apoptosis is regulated by the formation of a number of proteins; among them, bcl-2, p53 and c-myc are of particular interest.

bcl-2 is an oncoprotein, originally discovered in follicular B cell lymphomas (Tsujimoto, et al, 1984). The physiological function of bcl-2 turned out to be different from that of any other known oncogene product. Its expression does not induce proliferation and differentiation but

allows cells to survive, even in the absence of essential growth factors (Vaux, et al, 1988). bcl-2 was then shown to have the ability to inhibit apoptosis in response to many different death-inducing signals (Thompson, 1995). This may be achieved by an antioxidant pathway through suppression of lipid peroxidation (Hockenberry, et al, 1993).

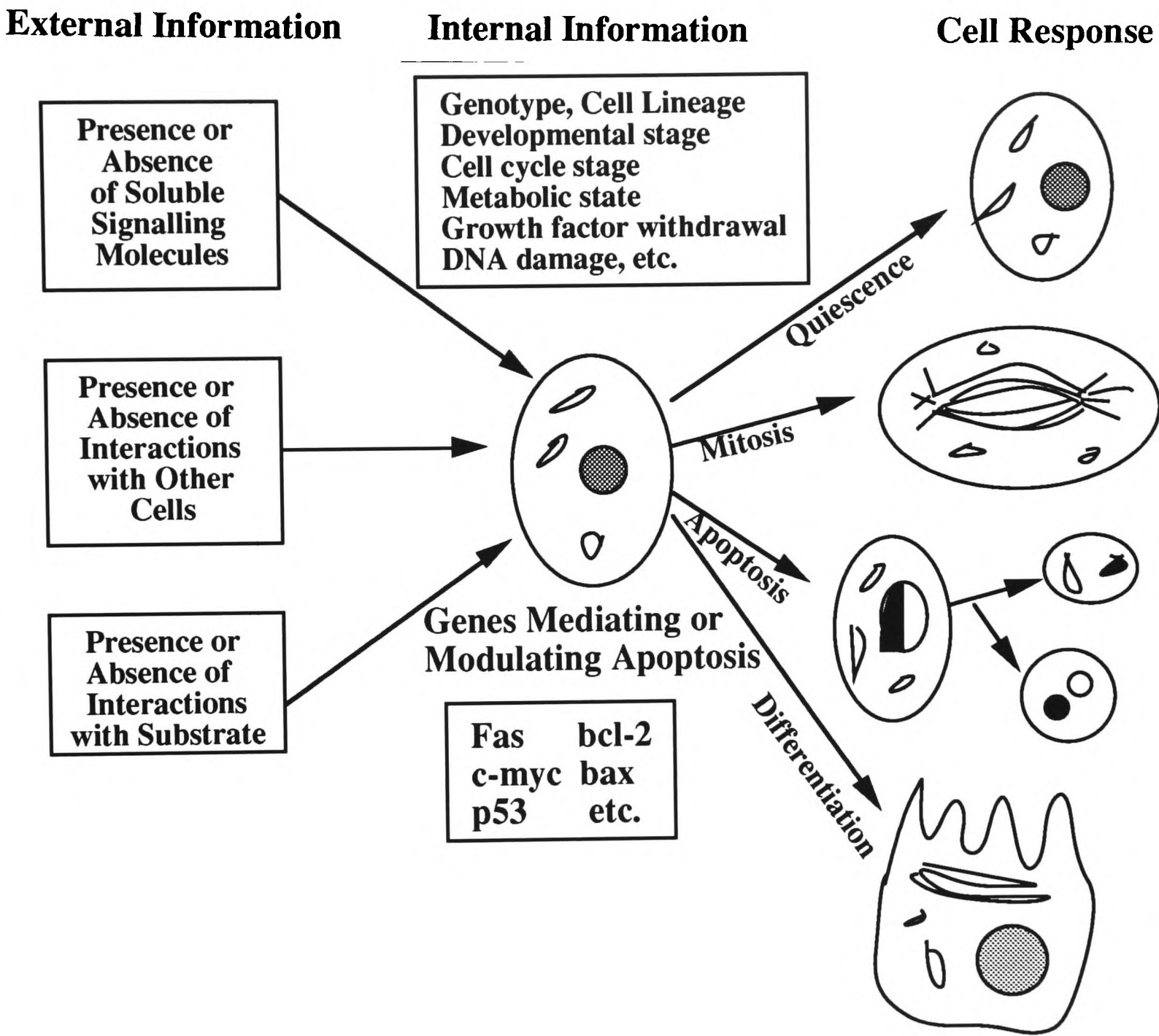


Fig. 1. 15. Regulation of apoptosis. (Adapted from Williams and Smith, 1993)

p53 protein acts opposition to bcl-2, and induces apoptosis (Yonish-Rouach, et al, 1991). A striking feature of wild type p53 expression in normal and neoplastic tissues is that it is inversely related to that of bcl-2 and it is expressed mainly in tissues undergoing apoptosis (Pezzella and Gatter, 1995; Ikeda, et al, 1995). p53 is required for the apoptotic process triggered by agents damaging DNA (Lowe, et al, 1993). Milliman and Korsmeyer (1993) have reported that apoptosis was controlled by the coordinate regulation of the intra-cellular level of the proapoptotic molecule bax and bcl-2. Miyashita et al. (1994) confirmed that p53

induces bax and decreases bcl-2 gene expression. Upregulation of bcl-2 expression protects cells dying of apoptosis.

c-myc, like p53, is an important element in both proliferation and apoptosis control (Wyllie, 1995). c-myc induces a state in which both cell proliferation and apoptosis become possible, the crucial choice between them depending upon additional factors, such as interactions with surrounding cells and growth factors (Evan, et al, 1992). The function of c-myc, therefore, emphasises the existence of a high turnover state, in which cell proliferation and cell death are likely to coexist, their relative quantities being determined by the microenvironment.

1. 11. 3 Measurement of apoptosis

There are numerous ways to measure apoptosis; probably none alone are sufficient criterion but several in combination are usually definitive. White (1996) has reviewed the current methods to measure apoptosis. In general, they are:

- (1) Loss of cell viability; failure to exclude vital dye.
- (2) Cell and nuclear morphology; microscopy to visualise chromatin condensation, DNA organisation, and cytoplasmic integrity.
- (3) DNA fragmentation; assayed by agarose gel electrophoresis, pulsed field gel electrophoresis, in situ terminal transferase labelling of DNA breaks (such as the TUNEL method).
- (4) Cysteine protease activity as demonstrated by immunohistochemistry.

1. 11. 4 Apoptosis in osteogenesis

Apoptosis in bone cells has been reported increasingly over the last three years (see review Boyce, 1996). Osteoclasts have been found undergoing apoptosis in vivo (Boyce, et al., 1995) and in vitro (Hughes, et al., 1995a). Apoptotic changes were also found in osteocytes in normal and pathological human bone, suggesting a functional relationship between bone turnover and controlled cell death of osteocytes (Noble, et al., 1997). The role of apoptosis in

the regulation of osteoblast activity is much less clear because of limited data at present. However, increased osteoblast apoptosis as a consequence of aging could lead to reduced matrix deposition and thinner bone trabeculae, which are characteristic of age-related bone loss. Furthermore, apoptosis of osteocytes within bone could impair the proposed mechano- and/or trauma-receptor function of these cells, and thus delay the removal of damaged bone and so increase the risk of fracture (Boyce, 1996).

Gibson et al. (1995) have studied endochondral ossification of chick sterna. Light microscopy of the developing chick sterna demonstrated chondrocyte condensation at the interface with the invading vasculature. Electron microscopy showed cytoplasmic and nuclear condensation of chondrocytes. In situ detection of DNA fragmentation showed that the apoptotic chondrocytes were restricted at the interface with invading blood vessels. They concluded that terminal differentiation of chondrocytes resulted in death by apoptosis prior to resorption of the tissue and invasion by blood vessels. Thus, apoptosis may be an initiating event in tissue remodelling and revascularization.

Lewinson and Silbermann (1992) reported an ultrastructural study of condylar cartilage of rat. They demonstrated that the terminal hypertrophic chondrocytes undergo apoptosis and are phagocytosed by chondroclasts. The chondroclasts were followed by tube-forming endothelial cells which collaborated with chondroclasts to extend the invading capillaries into the evacuated lacunae. They suggested that the osteoprogenitor cells were being brought to the evacuated lacunae of the calcified cartilaginous scaffold by invading capillaries, and this environment allowed osteoprogenitor cells to differentiate into osteoblasts and to build up trabecular bone. Similar evidence of apoptosis in hypertrophic chondrocytes was also found in the avian growth plate (Hatori, et al., 1995). By end labelling of the fragmented DNA, pulsed field electrophoresis, and electron microscopy examination, they found that most of the hypertrophic chondrocytes undergo apoptosis. In addition, they used flow cytometry to analyse chondrocytes isolated from the growth plate and found about 8% of cells in the growth plate were apoptotic.

Previous studies of osteogenesis in vitro have revealed the participation of cell death in the

course of calcification (Roberts and Chase, 1981). These observations were confirmed by a recent study of ossification in fetal mouse calvariae (Zimmermann, 1994). Abundant necrotic osteoblasts were found at the mineralization border and in the osteoid of long bone cortices from fetal mice. Cell degeneration of osteoblasts was studied by electron microscopy and was characterised by swelling of the endoplasmic reticulum and loss of the plasma membrane. Mineralization of the osteoid was accompanied by necrosis of the osteoblasts and cell debris was incorporated with the mineral. The number of necrotic osteoblasts increased markedly as the ossification of the long bone cortices proceeded. The importance of cell death in the process of mineralization, however, is not yet clear. Possibly, the cells act as calcium/phosphate stores, which are liberated by cell death to supply mineral constituents at sites of mineralization (Zimmermann, 1994).

Iwama et al (1983) have reported that limb deformity was induced in chick embryos by hydroxy-urea administration. This was the result of an unbalance of cell proliferation and cell death during early limb development. Limb deformity was observed soon after a dose of 800 mg hydroxy-urea was administered to day-4 chick embryos. There was histological evidence for the cell death and the depression of cell proliferation just after the treatment with hydroxy-urea, and the retardation in periosteal ossification during the process of recovery from cell death. It was concluded that the retardation in chondrogenesis and osteogenesis during the recovery from cell death caused by the hydroxy-urea was responsible for the limb deformities, such as: shortening, thinning and bending of the limbs.

1. 12 The aims of the present studies

The introductory text has emphasized the importance of cell proliferation, extracellular matrix production, growth factor and cytokine expression, angiogenesis and apoptosis in the process of bone formation and bone repair. The development of limb-lengthening techniques has made generating new bone tissue at appropriate sites possible. However, many complications resulting from the limb-lengthening technique have indicated the importance of understanding the biological mechanisms of distraction osteogenesis, which are still poorly understood. The

main aims of this thesis were to study some of the important biological aspects of distraction osteogenesis.

The first aim of the present studies was to set up an animal model of leg-lengthening. The surgical procedure was standardised and a reproducible rabbit model of tibial lengthening was refined. The distraction regenerates at 4 different distraction rates (0.3 mm/day, 0.7 mm/day, 1.3 mm/day and 2.7 mm/day) were studied histologically and radiographically, and this provided the basic knowledge of the structure of the distraction regenerate on which the further studies were based.

The second aim was to investigate the effects of various distraction rates on cell proliferation in the regenerating tissues during distraction osteogenesis. At first, cell proliferation was assessed in the tibial osteotomy fixed by external fixator for 1 or 2 weeks, but without distraction. Secondly, cell proliferation was assessed in the regenerating bone during distraction osteogenesis at 4 different distraction rates. BrdUrd labelling and its immunohistochemical detection were used to measure the bone cell proliferation. In addition, a comparison was made between the assessment of cell proliferation either by expression of PCNA and by the uptake of BrdUrd in the present rabbit model of leg-lengthening.

The third aim was to study the effects of distraction rates on the changes in the extracellular matrix during distraction osteogenesis. Protein expressions and specific mRNAs for collagen types I and II were localised by immunohistochemistry and by in-situ hybridization techniques. Markers for bone mineralization and bone resorption were also examined.

The fourth aim was to examine the hypothesis that BMP-4 may act as a mediator of induction of bone formation during distraction osteogenesis. The expression of BMP-4 mRNA in the regenerating tissue was localized by in-situ hybridization methods.

The fifth aim was to investigate the role of cell death (apoptosis) during distraction osteogenesis. The TUNEL method was used for detecting DNA fragmentation, and a method of simultaneous (dual) labelling of cell death and cell proliferation was developed.

The final aim of this thesis was to examine the angiogenic responses to the rates of distraction. Angiogenic markers, such as endothelial cell antigen and type IV collagen, were localised by immunohistochemical methods in the regenerates at 4 different distraction rates.

PART II

Experimental Methods

Chapter 2 Experimental Methods

2. 1 Experimental animal model

2. 1. 1 Adult male New Zealand White rabbit

The life-span of New Zealand White (NZW) rabbits is from 5 to 8 years. The NZW rabbit grows very rapidly and may weigh 1.8 kg as early as 8 weeks of age. The adults weigh from 4 to 6 kg and a body weight ranging from 2.5 to 3.0 kg is satisfactory for most surgical procedures (Kaplan and Timmons, 1979). Longitudinal growth of the NZW rabbit is complete at 28 weeks with 91% of total longitudinal growth being achieved at 16 weeks. Mean tibial length is 94% of the adult value at 16 weeks (Masoud et al., 1986).



Fig. 2. 1. A 26 week old male NZW rabbit.

To minimise the effect of sex and potential growth activity, adult male rabbits were used in the experiments described in this thesis. The age of the rabbits at the time of surgery ranged from 22 to 26 weeks, and the body weight was 3.0 -3.5 kg (Fig. 2. 1). The tibial epiphyseal growth plate at this time is completely fused determined by radiography.

2. 1. 2 Anatomy of the rabbit hind leg -- tibia and fibula

The tibia and fibula are completely fused distally in the rabbit for nearly half of their length, though proximally there is a space between them except at the very end. The tibia is thick and strong, the fibula slender. The fibula is proximally fixed to the posterior rim of the lateral tibia condyle (Fig. 2. 2).



Fig. 2. 2. The tibia (T) and fibula (F) of the NZW rabbit.

2. 1. 3 Review of rabbit leg-lengthening models

The rabbit leg-lengthening (callotasis) model was first induced by Kojimoto et al. (1988). They used a unilateral dynamic external fixation (Orthofix M-100) to fix the tibia osteotomy of the rabbit. The operation was performed under general anaesthesia. A longitudinal skin incision

was made and the periosteum was cut and retracted carefully. Four self-tapping screws were placed at right angles to the longitudinal axis of the shaft. Transverse osteotomy was performed just below the tibio-fibular junction using a hand saw. The osteotomy was held in a neutral position for 10 days and then distracted at 0.25 mm/12 hours (0.5 mm/day). Radiographs and histological examinations showed callus formation within the distraction gap with three distinct zones.

The same animal model was used by Yasui, et al. (1993), again with a ten-day waiting period before distraction, to examine the appropriate rates of distraction. The animals were divided into three groups according to the rate of distraction (0.35 mm/12 hours, 0.7 mm/12 hours, 1.4 mm/12 hours). The process of callus formation was monitored by x-ray. Bone lengthening regenerate histology was satisfactory when distraction was carried out at rates of 0.35 mm/12 hours or 0.7 mm/12 hours. The callus filling the distraction gap showed a characteristic zone structure, with a central radiolucent zone and two adjacent sclerotic zones.

Hamanishi et al. (1994, a, b; 1995) adapted a similar rabbit model of leg-lengthening to study the enhancement of distraction osteogenesis by axial compression, direct electrical current stimulation and fresh bone marrow transplantation. In their model, the waiting period was one week, the lengthening rate was 1mm /day and total lengthening was 1 cm. Simpson et al (1995) used the rabbit leg-lengthening model to study the limitation of the muscles on the lengthening process. They used a 1 week waiting period, lengthening at various rates from 0.3 mm/day to 4 mm/day, with a total lengthening of 20% or 30% of the original tibia length.

Thus, leg-lengthening in the rabbit using unilateral external fixators has been validated as an experimental model in studies of distraction osteogenesis, and has been adapted in this thesis. All the animal experiments described in this thesis were carried out under the guidelines for animal experiments in the faculty of medicine, the University of Oxford, and were performed under animal license and supervision of the British Home Office.

2. 1. 4 Anaesthesia

Rabbits were anaesthetised by intramuscular injection of Hypnorm (0.2 ml/kg, Janssen Animal Heath, High Wycombe, England), combined with intravenous injection of Midazolam (Hypnovel, 0.5 mg/kg, Roche, Welwyn Garden City, England). In addition, the tibial osteotomy site was locally infiltrated with 0.25 % Marcain (Astra Pharmaceutical Ltd, England), to reduce the quantities of intravenous agents required. The rabbits were in deep sleep during the operation and gradually recovered after the operation.

2. 1. 5 Operative procedure

The left leg of the rabbit was shaved and prepared for sterile isolation. After local infiltration with 0.25% Marcain, a 5 cm skin incision was made over the medial aspect of the left tibia, the periosteum was carefully protected and kept with the bone as much as possible while the tibia was exposed (Fig. 2. 3).

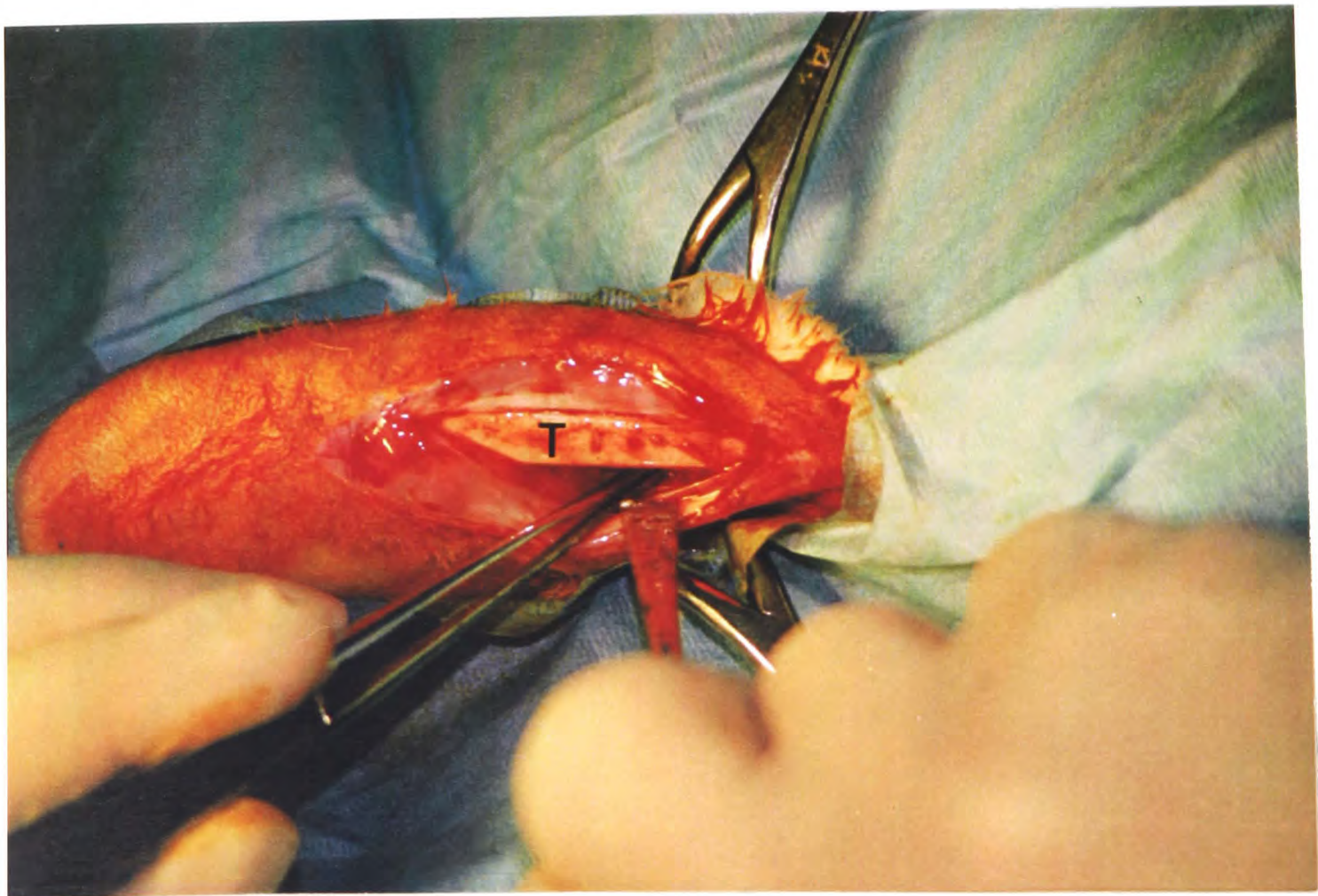


Fig. 2.3. Operative procedure: exposure of the tibia (T).

Rabbit bone is brittle and predrilling is required. The Orthofix M-100 lengthener (Orthofix,

Italy) was used to position the screws before making the first drill hole. A 1.8 mm Kirschner wire was used to drill both cortices of the most distal screw site under saline irrigation. The cortices were then overdrilled using a 2 mm drill. The distal screw (self-tapping tapered screw, Orthofix M300, total length 40 mm, thread length 15 mm, thread diameter 2/2.5 mm) was then inserted. Following this, the M100 fixator was positioned and the most proximal screw applied. A metal sleeve was used to guide the Kirschner wire and the 2 mm drill to ensure the screw sat evenly in the clamp of the fixator. (If the clamp was tightened over a screw that did not sit in line with the other screws in the fixator, the brittle bone may crack through the screw holes). The remaining two screws were then inserted in a similar manner (Fig. 2. 4). A MacDonald's dissector was passed deep to the bone to protect the soft tissues (Fig. 2. 4).

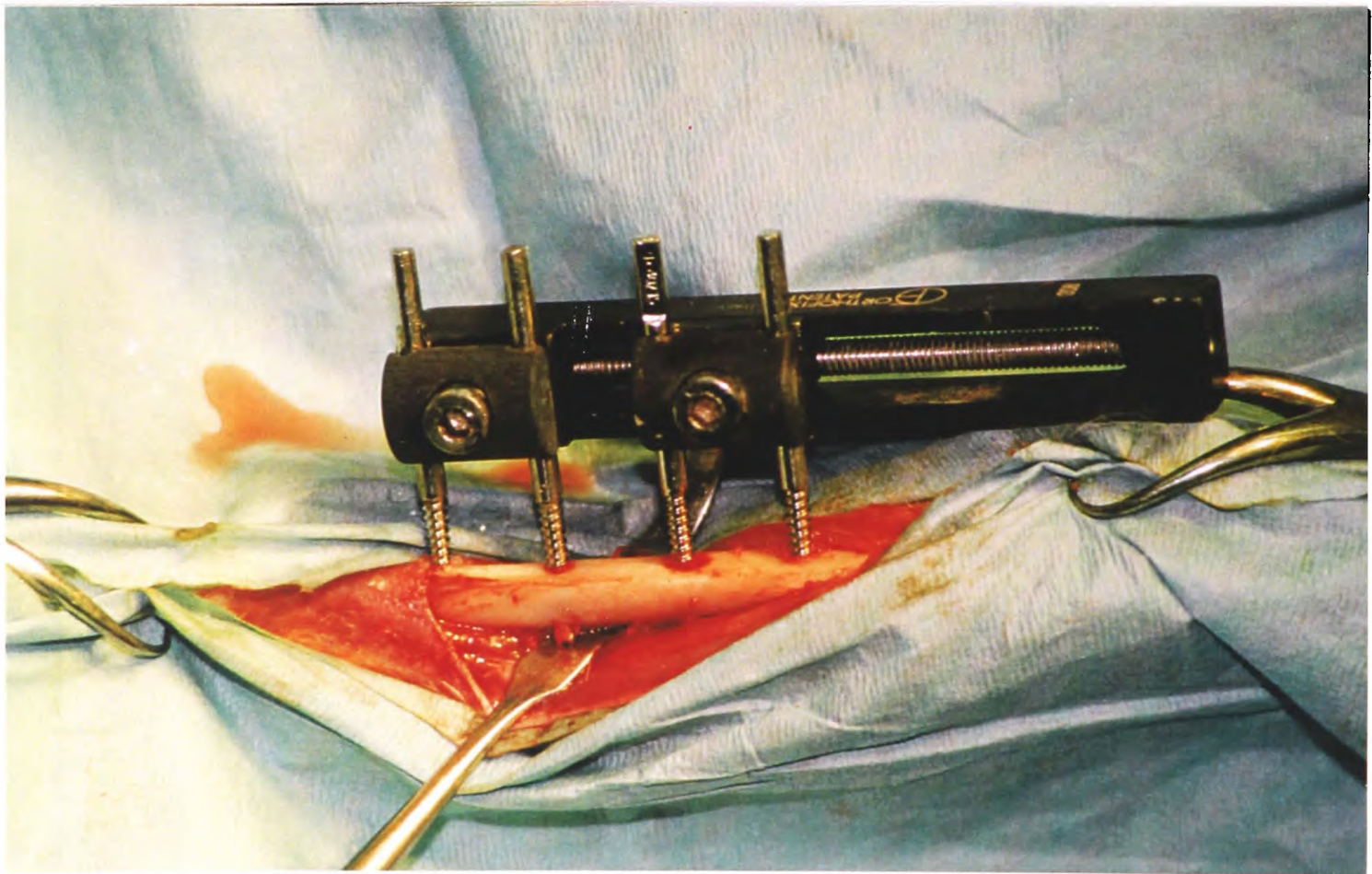


Fig. 2.4. M-100 fixator was fixed with four screws inserted in the tibia.

The tibia was osteotomized at the tibiofibular junction between the 2 inner screws using a hand saw. The blade of a small fret saw was passed underneath the tibia, and then connected to the saw. The tibia was cut transversely from deep to superficial with the fixator applied (Fig. 2. 5). This method produced minimal damage to the soft tissues and a reproducible

osteotomy (Fig. 2. 6). Penicillin powder was then applied to the region of each pin, and the deep tissue and skin were closed using an absorbable suture. The operated leg was dressed and a plastic plaster was applied to protect the fixator (Fig. 2. 7).

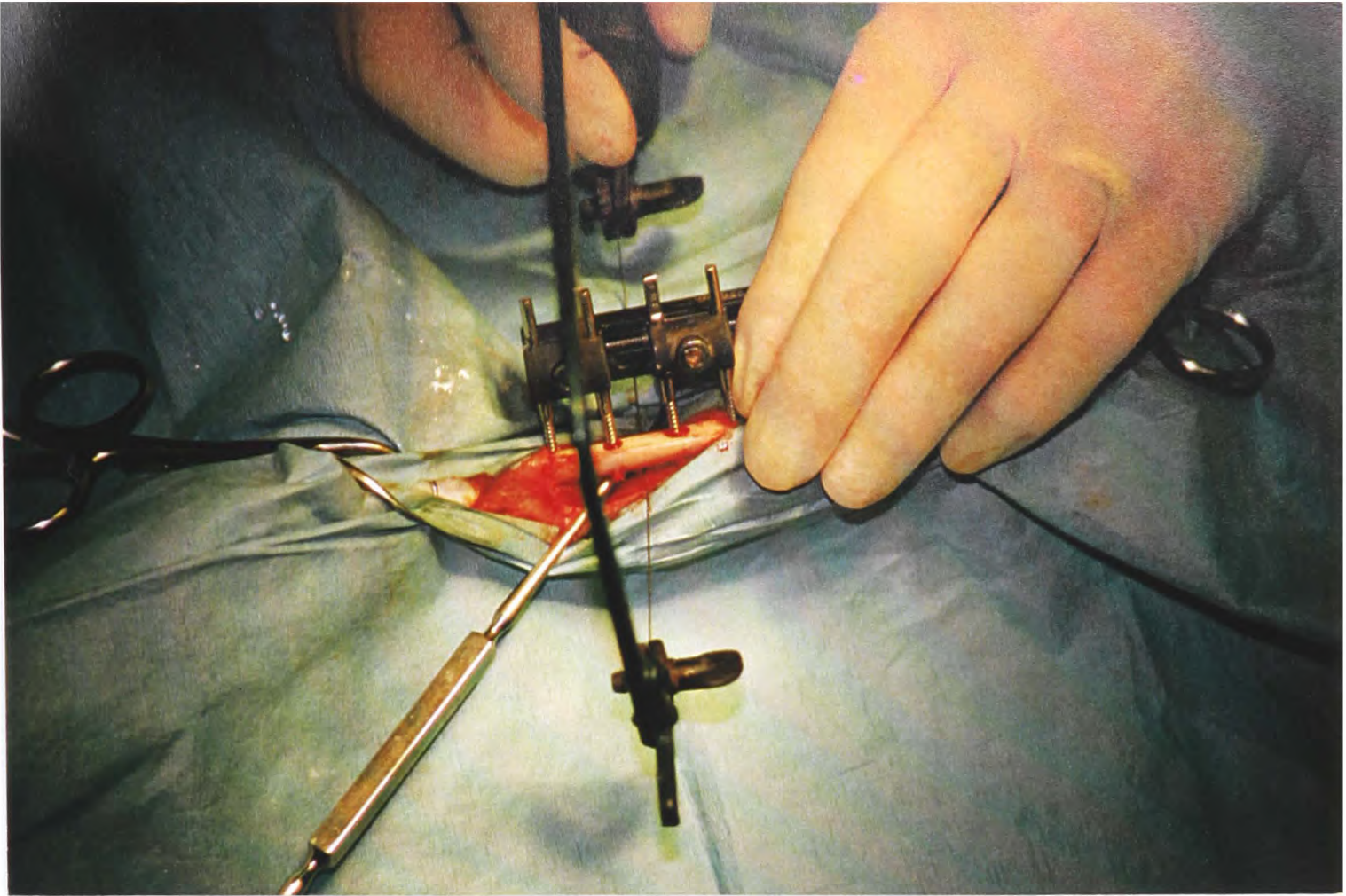


Fig. 2. 5. The tibia was carefully osteotomied with a hand saw.

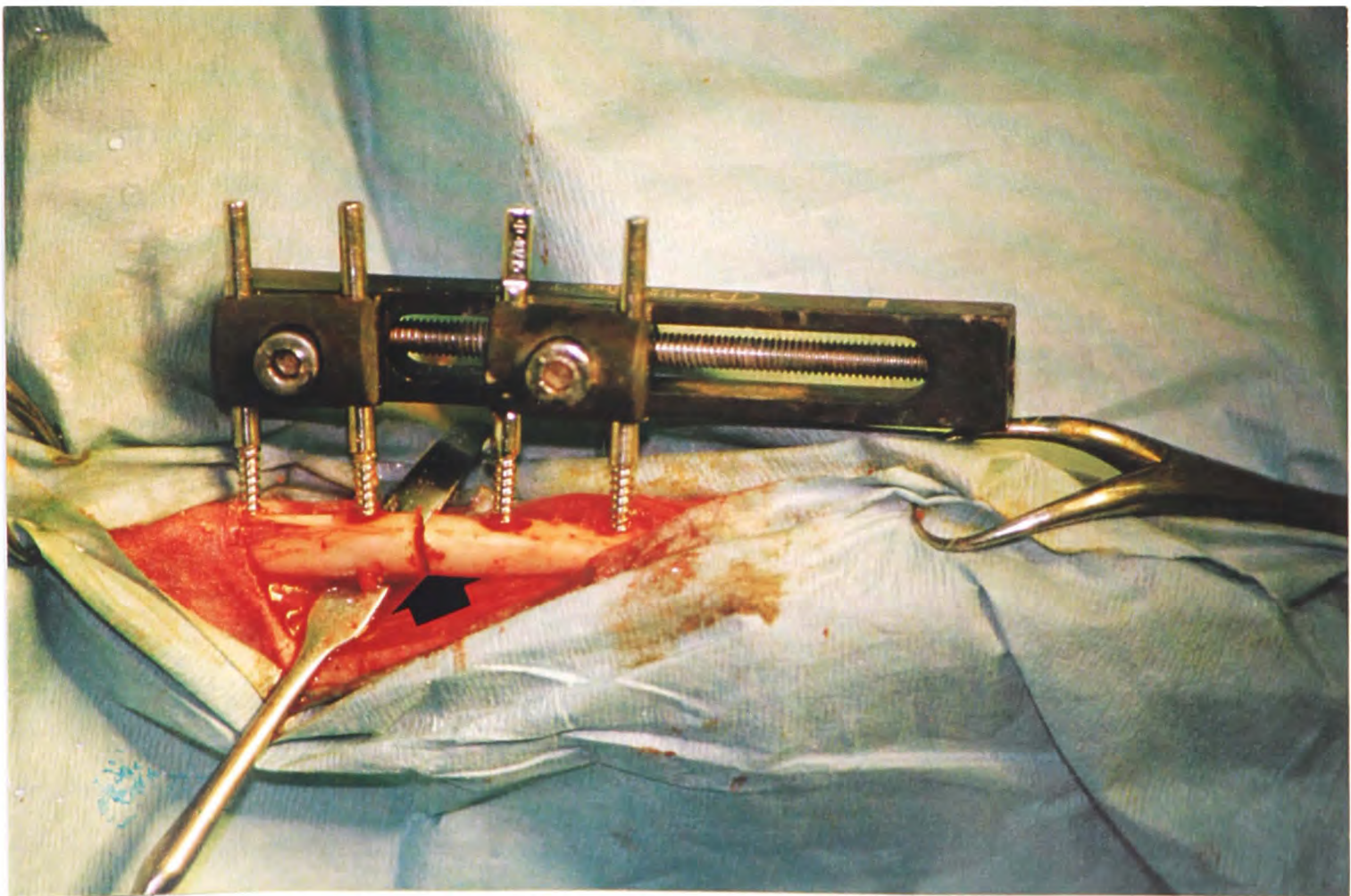


Fig. 2. 6. The tibia has been divided (arrow).



Fig. 2. 7. The dressing of the operated leg after operation.

2. 1. 6 Post-operative care

Rabbits were housed in an animal house supervised by the British home office. Each animal was housed in a separate cage with free access to food and water under the supervision of an animal care specialist. After the operation, body movement was unrestricted and the animals were able to bear weight upon the operative leg upon recovery about 1 -2 hours after the operation. Radiography of the operated leg was taken weekly.

2. 1. 7 Experimental groups and lengthening protocols

60 male adult NZW rabbits (24-26 weeks, body weight 3.0-3.5 kg) were randomly divided into 6 experimental groups. Osteotomies were made in the left tibiae of all the experimental animals and fixed using an Orthofix M100 fixator in a standard fashion (see 2. 1. 5). The animals were subdivided into four lengthening groups, and two sham non-lengthening groups. For the lengthening groups, after a seven-day latency period, distraction was initiated at rates of 0.3 mm / day (a quarter turn / 12 hours) for approximately 8 weeks, 0.7 mm / day (a half turn / 12 hours) for approximately 4 weeks, 1.3 mm / day (1 turn / 12 hours) for about 2

weeks and 2.7 mm / day (2 turns / 12 hours) for 1 week. In all animals the distraction was accomplished in equal steps every twelve hours. Distraction was stopped when lengthening to 20% (approximately 2 cm) of the original tibia length was achieved, and at this point the animals were killed. During the distraction period, the animals were free to weight-bear on the operated leg, and weekly radiographs were taken to monitor the lengthening. There were two sham groups in which the external fixators were applied under the above conditions and the osteotomy was created, but no lengthening initiated. The sham animals were killed 1 week or 2 weeks after the osteotomy.

Among the 60 experimental animals, 8 rabbits died or were killed during the experimental period because of a various reasons, such as anaesthetic accident, pin hole fracture and infection. These latter animals have been excluded from the experimental groups. The details of the experimental groups were summarised in Table 2.1.

Table 2.1. Details of the experimental animal groups

Experimental Groups	Distraction Rate (mm / day)	No. of rabbit	latency (day)	Dis. Period *(day)	Lengthening (%) Mean ± SE
Sham	0 (non-distracted)	8	7	-	0
Sham	0 (non-distracted)	6	14	-	0
Lengthening	0.3 (0.25 turn/12 hrs)	9	7	56	19.3 ± 2.3
Lengthening	0.7 (0.5 turn /12 hrs	9	7	28	20.4 ± 2.4
Lengthening	1.3 (1 turn /12 hrs)	12	7	14	21.2 ± 2.8
Lengthening	2.7 (2 turn /12 hrs)	8	7	7	20.9 ± 1.7
Others	Died or Killed	8	7	Varied	

* The sacrifice day was determined by the lengthening rate and the amount of lengthening, and varied inversely with the distraction rate.

2. 1. 8 Monitoring the lengthening by radiographs

Immediately after the operation radiographs were taken of the operated leg to examine the position of the screws and the fixator, and to check the tibia diaphysis. Radiographs were taken before lengthening to measure the original tibia length, and the planned 20% lengthening was calculated according to the original tibia length. Weekly radiographs were taken thereafter

to monitor the increase of the distraction gap and to confirm the lengthening rate. For taking radiograph, sedation was given to the rabbit by injecting Hypnorm intramuscularly at 0.15 ml / kg. The rabbit was placed in a standardised position and the X-ray was taken using an Atomscope HF100 X-ray machine (Seer Green, Buckinghamshire, England) at optimal conditions (46 KV, 20mA, 0.06 seconds) (Fig. 2. 8). The anteroposterior and lateromedial radiographs were taken on each occasion including knee and ankle joints. The initial tibia length and the length of the distraction gap were measured from the radiographs. The length of the tibia was measured from the lateral condyle of the proximal tibia to the lateral portion of the articular surface of the distal tibia. The percentage of lengthening was calculated by the formula : $\text{Lengthening (\%)} = \frac{\text{The distance of the osteotomized ends}}{\text{the total length of the tibia (the initial tibia length + the length of the distraction gap)}}$, and these were summarised in Table 2. 1.

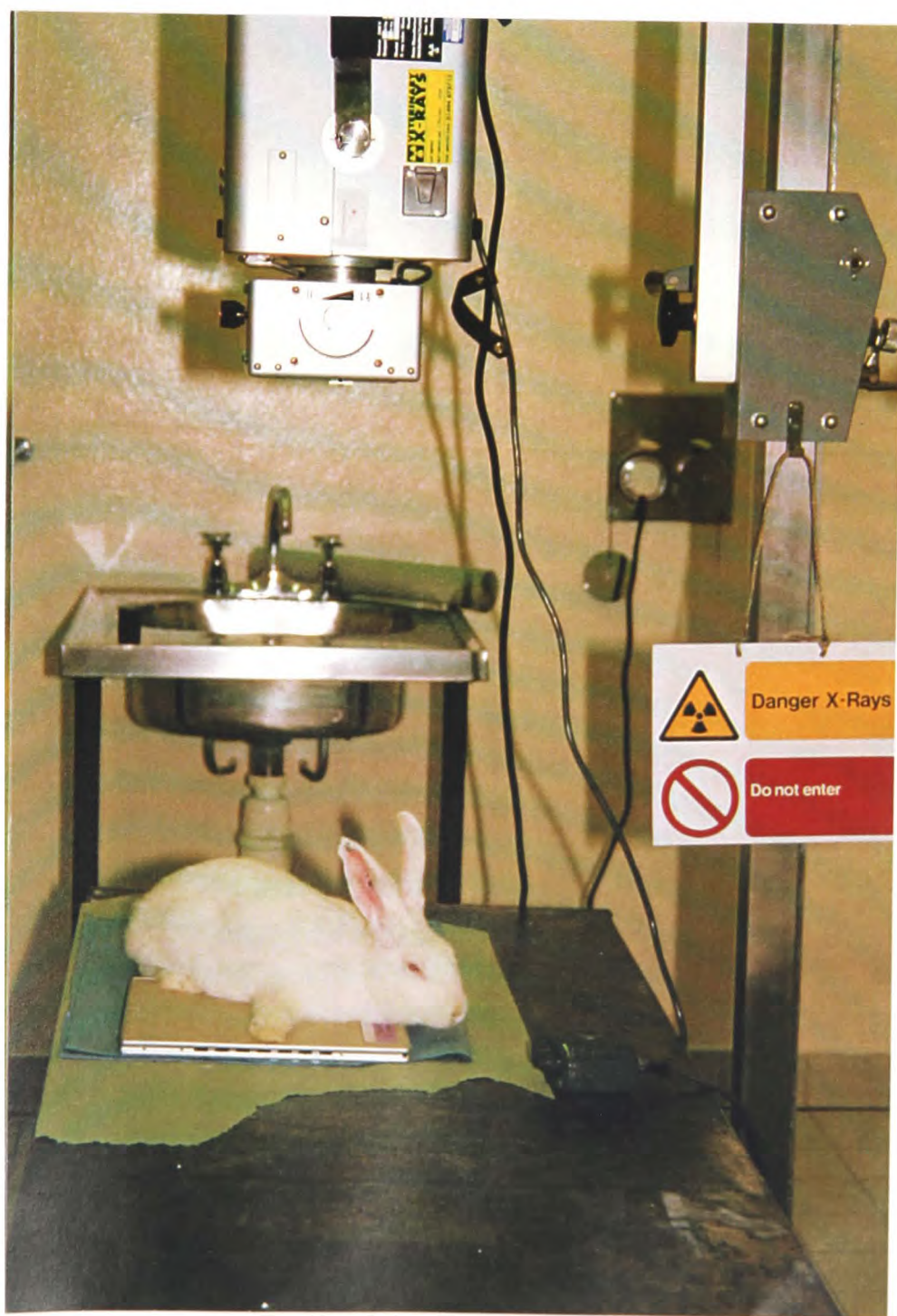


Fig. 2. 8. The radiographs were taken weekly to monitor the lengthening.

2. 1. 9 Killing of the rabbits

All the experimental animals were killed immediately upon conclusion of the particular experimental protocol by an intravenous overdose injection of barbiturate (2 mg/kg). For labelling dividing cells, 32 animals were injected intravenously with bromodeoxyuridine (Brdurd, Sigma, Poole, UK) at a dose of 40 mg/kg (8 mg/ml in saline) one hour before killing.

2. 2 Histology Methods

2. 2. 1 Fixation

When considering the practical application of fixatives, it is necessary to be clear as to the purpose of the investigation, the size, type and freshness of the tissue, the sectioning process and the staining technique it is desired to employ. No fixative is ideal for all tissues and all techniques. The volume of the fixative used should be at least 10 times that of the tissue, and the duration of fixation also needs consideration; normally it should not exceed 48 hours. Otherwise, it may result in loss of cellular detail, binding ability to certain dyes, antibodies and accessibility to hybridization probes. In the present study, the preservation of the tissue structure and cell details is essential for histology, immunohistochemistry and in situ hybridization studies, so that micro-anatomical fixatives, such as 10 % formalin, 4% paraformaldehyde, Carnoy's fluid (a rapidly penetrating fixative, which preserves the cell well and requires short time for fixation), and industrial methylated spirit were chosen for use.

After killing the animal, the central portion of the distraction gap, the regenerate, including 2-5 mm in length of both the neighbouring cortical bone ends was excised and sliced into half longitudinally in the median plane of the tibia using a band saw, each bone slice was approximately 1.5 cm (length) x 1 cm (width) x 0.5 cm (thickness). In all animals, one half of the specimen was fixed in 4% paraformaldehyde (pH 7.4) or 10% formalin (pH 7.0) for 24 hours. In some animals, the other half of the specimen was fixed in either 95% ethanol for 24 hours or Carnoy's fluid for 6 hours. After complete fixation, the tissues were rinsed in distilled water or saline before being transferred into decalcifying solutions.

2. 2. 2 Decalcification

To study bone tissue, the removal of the hard mineral is required to prepare thin sections for the study of cellular detail. For general diagnostic purposes, 5 -7.5% nitric acid was effective. But decalcification with nitric acid results in rapid loss of calcium salts and this is followed by swelling and hydrolysis of the bone matrix collagens (Jeffers and Vinnicombe, 1976). Thus, decalcification using nitric, hydrochloric or acetic acids is usually unsatisfactory if histochemical or immunohistochemical methods are proposed.

Since the present study involves investigations using histochemical methods, immunohistochemistry and in-situ hybridization methods, a more general and less harsh decalcifying reagent is required. Neutral ethylenediaminetetraacetic acid (EDTA) (pH 7.0 -7.5) is used for decalcification purposes in this thesis. EDTA is a known gentle decalcifying reagent, which preserves most of the proteins and enzymes of the bony tissue during the decalcification process. In the current study, 14.5% (w/w) EDTA (pH 7.2-7.4) (Appendix 5) has been used for decalcification purposes. Fixed bone slices were decalcified in 14.5% EDTA solution (pH 7.4) for 3-4 weeks at room temperature. The EDTA solution was changed twice a week and the decalcification process was monitored by radiography. Once completion of the decalcification was confirmed, the samples were rinsed in distilled water and prepared for paraffin wax embedding.

2. 2. 3 Paraffin and glycol methacrylate (plastic) embedding

Soft tissue samples and decalcified bone were embedded in paraffin wax. Some undecalcified bone tissues were embedded in glycol methacrylate. For paraffin embedding, samples were taken through 70% (3 hrs) , 90% (1 hr) and 100% alcohol (3 changes, 2 hrs for each step), transferred to xylene (2 changes, 1 hr for each step) and 3 changes paraffin wax at 60 °C (1 hr for each step), and finally embedded in paraffin wax manually.

For glycol methacrylate embedding, all reagents were supplied by British Drug House (BDH, Poole, Dorset, England), and the following solutions were made:-

Solution A	2-hydroxyethyl methacrylate	91.4	w/w
	2-butoxyl ethanol	8.2	w/w
	polyethylene glycol 400	0.4	w/w
Solution B	2-butoxyethanol	95	w/w
	Benzoyl peroxide	5	w/w
Solution C	polyethylene glycol 400	93.5	w/w
	N-N dimethyl aniline	6.5	w/w

The samples were fixed in 100% alcohol for 24 hours and then infiltrated in a solution made of 50 parts of solution A and 5 parts solution B for 2 changes, 24 hrs per change. The embedding solution is made by mixing the three solutions in the following proportions by weight A: 25 g, B: 2.2 g, and C: 1.0 g. A plastic mould was first placed on ice, and embedding solution was added to the mould. The sample was then placed into the mould and covered by a metal or plastic chuck on top. Polymerisation of the embedding solution was allowed overnight in the fridge. Once the polymerisation was complete, the chuck, together with the embedded sample, was removed from the plastic mould, and allowed to air dry for 2 - 4 days before sectioning.

2. 2. 4 Sectioning and slide preparation

5 -7 μm thick paraffin sections or 3 - 5 μm thick glycol methacrylate sections were cut using a microtome (Jung 200, West Germany). Paraffin sections were placed on glass slides and heated at 60 °C for 15 minutes to allow adhesion. In order to reduce the section detachment, poly-L-lysine (Sigma, Poole, UK) coated slides were used for histology and immunohistochemistry. To coat the slides, clean slides were immersed in 0.5 % (w/v) poly-L-lysine in distilled water for 1 min, and air dried. For in-situ hybridization, 3-aminopropyltriethoxy silane (APES) (Sigma, Poole, Dorset, UK) coated slides were used. For details of slide coating with APES, see Appendix 3. The paraffin sections were stored at 37 °C in an oven prior to use. The glycol methacrylate sections were stored at room temperature.

2. 2. 5 Histological staining

2. 2. 5. 1 Haematoxylin and eosin staining

Haematoxylin and eosin (HE) staining is the most commonly used staining for histological studies. Haematoxylin stains the cell nuclei (blue) and eosin stains the extracellular matrix (pink red) (Fig. 2.9).

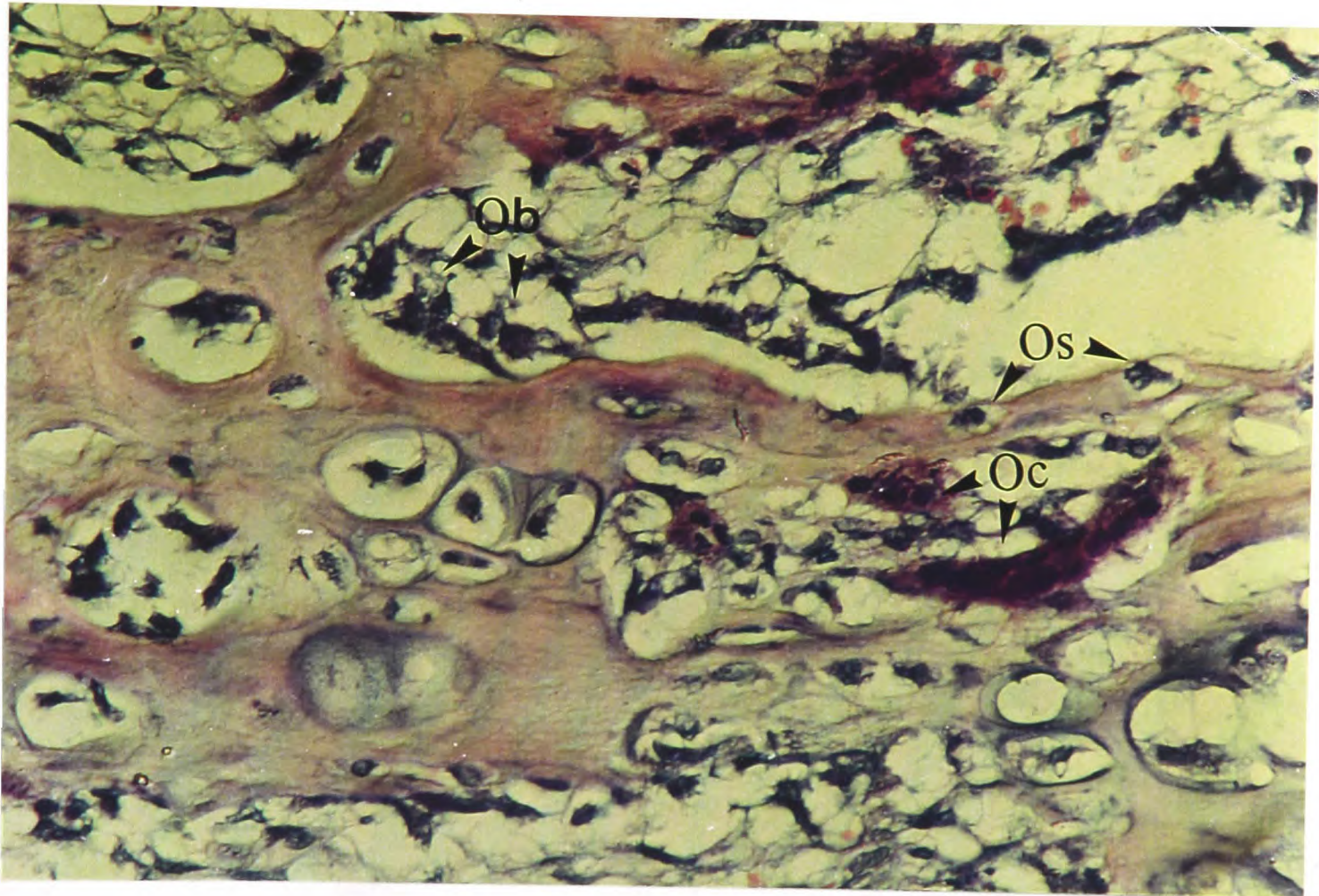


Fig. 2. 9. An example of HE staining of bone section. Osteoblast (Ob), osteocyte (Os) and osteoclast (Oc) were seen, X100.

Protocol for HE staining for paraffin sections:

1. Dewax in Xylene for 10 minutes.
2. Rehydrate through 100% (twice), 90% and 70% alcohol, each step for 5 minutes.
3. Wash in running water for 5 minutes.
4. Stain in Ehrlich's haematoxylin for 20 minutes.
5. Wash in running tap water for 5 minutes.
6. Differentiate in 1% acid alcohol for 5 - 10 seconds and wash in running tap water.
7. Blue in 2% sodium hydrogen carbonate or 5 -10 seconds and wash in running tap water.
8. Stain in 1% eosin for 5 minutes and wash in running tap water.
9. Dehydrate through 70%, 90%, 100% (twice) alcohol, cleaned in xylene & mount in DPX.

2. 2. 5. 2 Weigerts haematoxylin / alcian blue / sirius red staining

Weigerts haematoxylin / alcian blue / sirius red staining (Appendix 6) may be used to distinguish bone matrix from cartilage matrix (Lison, 1954). Alcian blue stains proteoglycans, especially the cartilaginous matrix a deep turquoise colour. At the interface between hard and soft callus, this stain clearly demonstrates the cartilaginous remnants within struts of endochondral bone. Sirius red stains type I collagen fibres, such as bone matrix and fibrous periosteum. The bone matrix often stains a very bright red, particularly in younger, trabecular bone. When viewed with polarised light, sirius red enhances the birefringence of bone matrix (Junquiera, et al, 1979). This helps to identify the orientation of bone fibres, particularly in lamellar bone. Calcified cartilage also stains with sirius red, but with a pinkish-red colour. Older (or dead) bone loses the capacity to stain bright red and appears orange. Fibrocartilage appears slightly paler red (Fig. 2. 10).

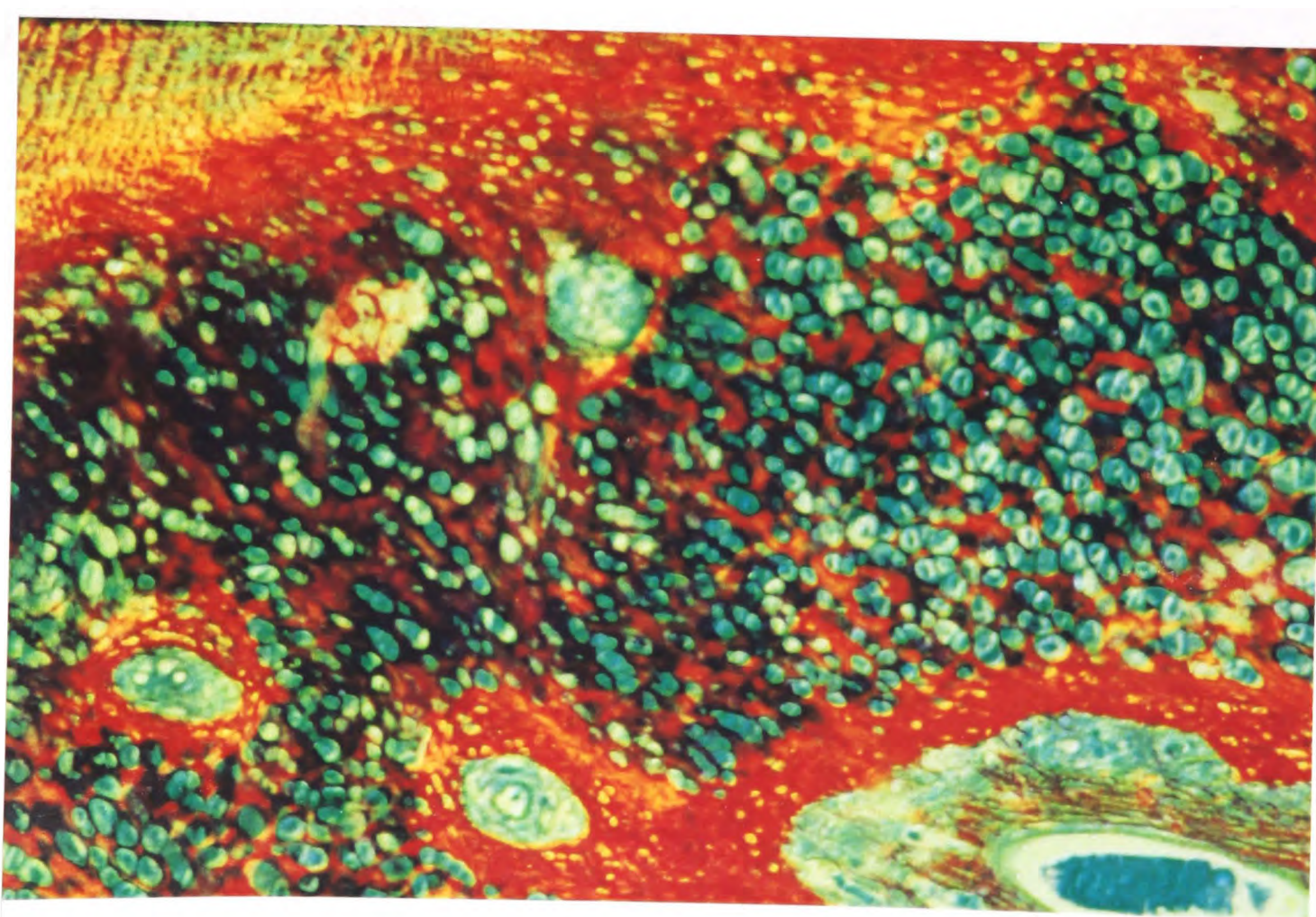


Fig. 2. 10. An example of Weigerts haematoxylin / alcian blue / sirius red staining. Bone matrix: bright red; cartilage matrix: blue; calcified cartilage/fibrocartilage: orange. Original magnification, X 100.

2. 2. 5. 3 Von-Kossa staining on plastic sections

Von Kossa staining is commonly used for detection of calcium phosphate as it stains phosphate salts black. This staining requires undemineralised bone sections. For the procedure, undecalcified plastic (glycol methacrylate) thin sections are cleared in xylene and rehydrated through 100%, 90% and 70% alcohols, and taken to distilled water. The sections are placed in 5% silver nitrate in bright sunlight, or under an ultra-violet lamp until blackened. Sections are then washed in two changes of distilled water, rinsed with 2% sodium thiosulphate for 1-2 min and washed in water again. In this step, if the sections go brown, wash well in distilled water and replace in silver nitrate until they are black again. The sections are usually counterstained with Mayer's haemotoxylin or toluidine blue for 5 mins, washed in several changes of distilled water, dehydrated through graded alcohols and mounted in DPX (Fig. 2.11).

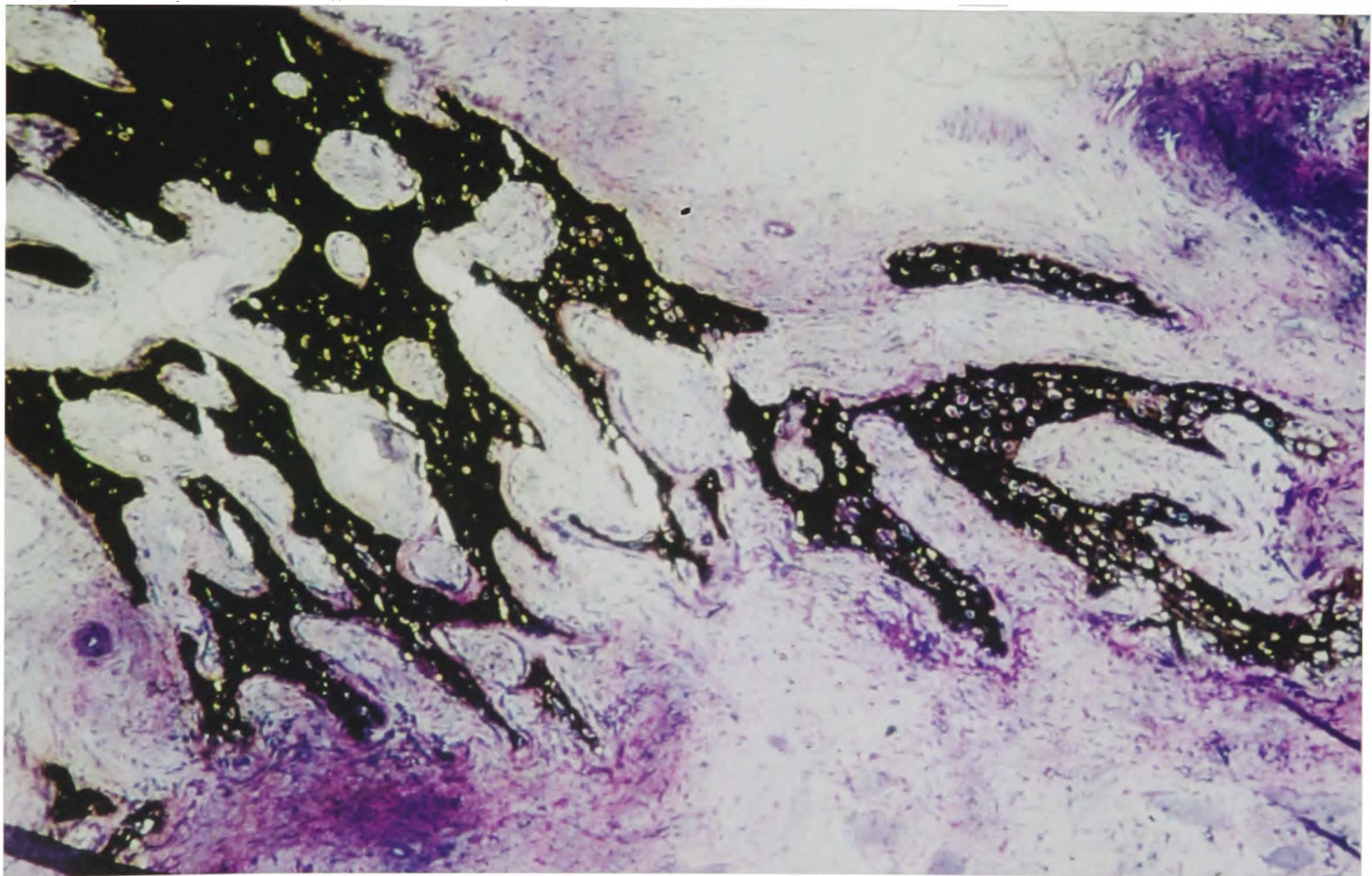


Fig. 2. 11. An example of Von Kossa staining on a plastic section of the regenerate of the 0.7 mm/day group, showing the mineralized bone (black). Original magnification, X 100.

2. 2. 6 Detection of enzymes: alkaline and acid phosphatases

Alkaline phosphatase is an important phenotypic marker for osteoblasts and preosteoblasts.

Alkaline phosphatase activity was detected on the plastic sections of bone using a commercially available kit according to the manufacturer's instruction (Sigma, Poole, Dorset, UK). In brief, plastic sections were incubated with substrates (5mg Naphol AS B1 phosphate in 1 ml dimethylformalin; 50 ml 0.05 M pH 8.3 Tris-maleate buffer; 40 mg diazonium salt, fast red RC) at room temperature for 20 minutes in dark. Sections were then rinsed in distilled water and counterstained with Mayer's haematoxylin, and mounted in glycerin jelly (Fig. 2.12).

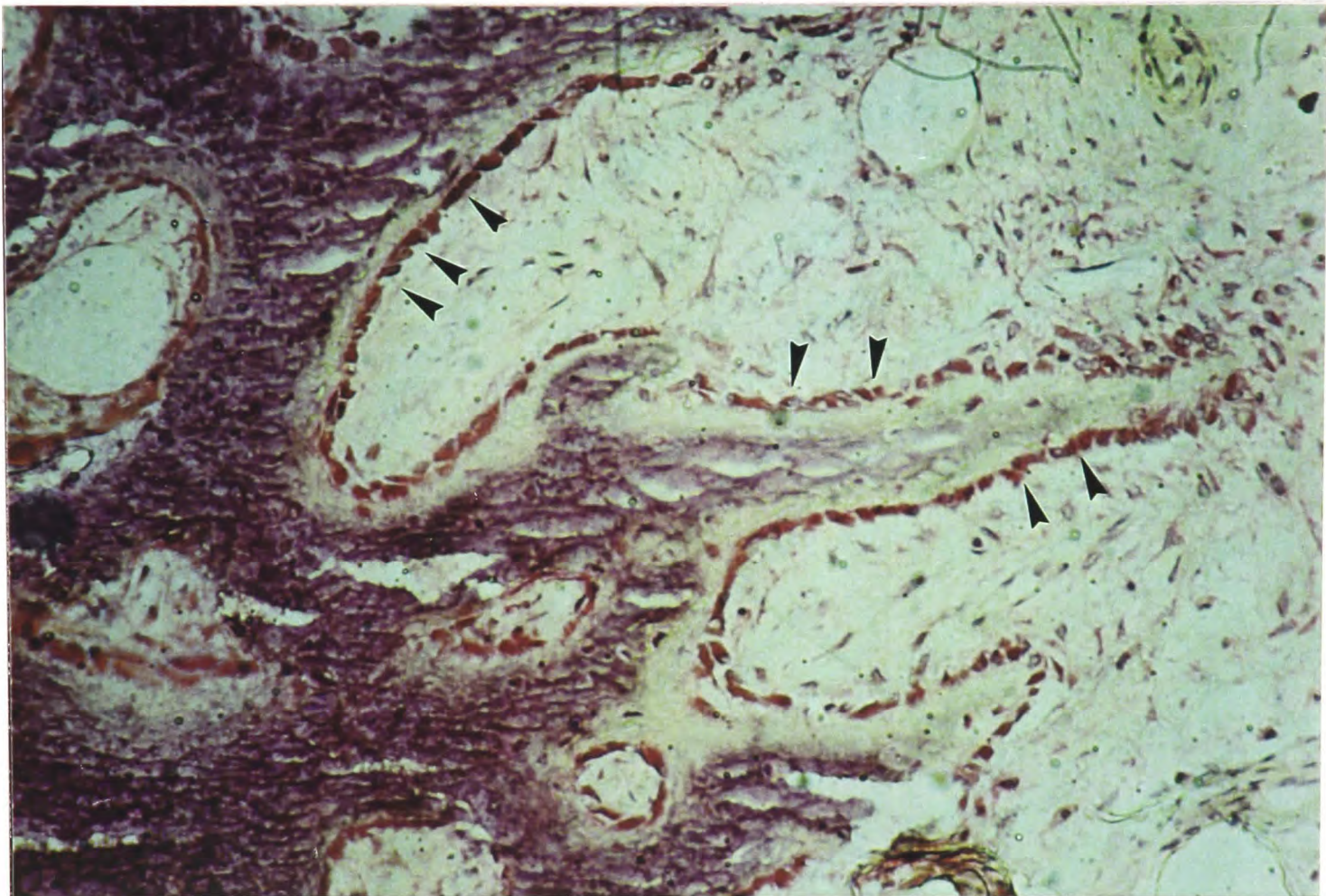


Fig. 2. 12. An example of alkaline phosphatase staining of a regenerate bone of the 0.7 mm/day group, showing the cells on the bone surfaces were alkaline phosphatase positive (arrows). Original magnification, X 25.

For identifying bone-resorbing cells, tartrate-resistant acid phosphatase (TRAP) staining was performed (Appendix 7). In brief, paraffin sections were dewaxed and rehydrated through graded ethanol, washed in Tris-HCl buffer (pH 9.0) and incubated with acetate buffer (0.5% w/w sodium acetate, 0.83% v/v acetic acid, pH 5.0) for 1 hour. The substrate mixture, consisted of 15 mg Naphthol AS B1 phosphate (Sigma, Poole, Dorset, UK), 75 mg sodium tartrate, 1 mg pararosaniline, 0.8 mg sodium nitrite in 26 ml acetate buffer (pH 5.0). The substrate mixture was filtered before use and sections were incubated with the mixture at 37 °C for 1 hr. Sections were rinsed in running water for 5 mins followed by HE staining or without counterstaining, and mounted in Aquamount (BDH, poole, England) (Fig. 2. 13).

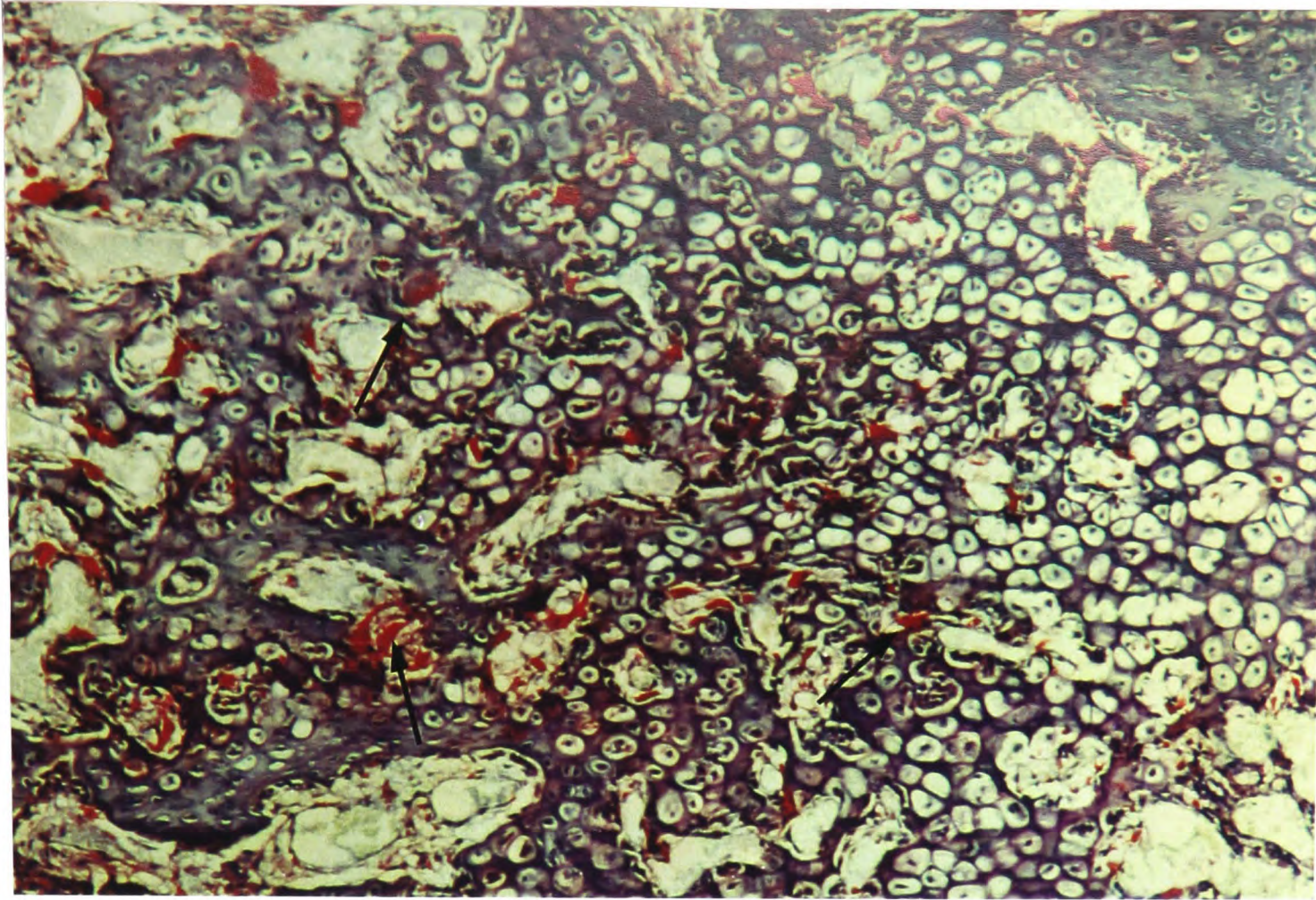


Fig. 2. 13. An example of TRAP staining on part of a regenerate of the 1.3 mm/day group, showing the bone resorbing osteoclastic cells (red, arrows) in the cartilage-bone interface. Original magnification, X 25.

2. 3 Immunohistochemistry methods

2. 3. 1 Immunohistochemistry

Immunohistochemistry or immunocytochemistry is the union of microscopy and immunology. Immunohistochemistry exploits the very specific binding exhibited by an antibody for its antigen. Protein, carbohydrates, nucleic acids, lipids, and many other naturally occurring and synthetic compounds can act as successful antigens for raising antibodies. Microscopical analysis by immunohistochemistry can therefore be used to obtain a unique view of macromolecular changes within individual cells (Caterson, 1991; Beesley, 1993). If the antibody is specific, immunohistochemistry can be performed on virtually any biological specimen provided: (i) the antigen remains intact and able to bind the antibody and (ii) the specimen preparation technique exposes the antigen for immunolabelling by the antibody. Immunohistochemistry is widespread in use since it is very simple to perform once an antibody

has been identified and requires little specialised laboratory equipment.

2. 3. 2 The Antibody

The antibody is central to the success of immunohistochemical labelling. A good antibody is highly specific, possessing a high titre (amount of antibody activity), high affinity (the exactitude of stereochemical fit of an antibody-combining site to its complementary antigenic determinant). Many antibodies are available commercially and are fully characterized for use. Both polyclonal and monoclonal antibodies can be used for immunohistochemistry, the choice is dependent on antibody performance.

2. 3. 2. 1 Polyclonal antibody

Polyclonal sera contain a mixture of high affinity antibodies, each active against different epitopes in the antigen. If all these epitopes are not destroyed by fixation or decalcification, immunolabelling remains possible. Polyclonal antisera are produced by injecting an antigen into a host animal, usually a rabbit, sheep, or goat and, after booster injections, the serum is periodically collected and tested for antibody. Antibodies are produced in the spleen of the host animal by B lymphocytes and plasma cells, the latter being the terminal stage in the differentiation of B lymphocytes. Sites on the complex antigenic molecule that are recognised by antibody are called epitopes and a polyclonal antiserum is a mixture of antibodies to different epitopes on the immunogen. Antibodies to collagen types I and II used in this thesis are polyclonal antibodies.

2. 3. 2. 2 Monoclonal antibody

A monoclonal antibody is a "pure" preparation representing only one of the constituents of the polyclonal antiserum. A monoclonal antibody has narrow specificity and the activity of a monoclonal antibody is susceptible to fixation of the antigen. Since monoclonal antibodies are specific to a single epitope on the antigen, if this is destroyed all reactivity is lost. A relatively weak fixation, as achieved with formalin or paraformaldehyde, is usually necessary.

The initial phase of preparation of a monoclonal antibody is to induce synthesis of a polyclonal antiserum in the host animal. The plasma cells are removed from the host animal and each fused with a malignant myeloma cell to form hybridoma cells (Kohler and Milstein, 1975). Hybridoma cells are isolated and cultured and theoretically each will produce unlimited monoclonal antibodies against a specific epitope. These hybridoma cells are either cultured in vitro, in which case the culture supernatant fluid contains antibody, or in vivo in the peritoneal cavity of a suitable host and then the resultant ascites fluid contains high levels of the antibody. Antibodies used in this thesis are all monoclonal antibodies except antibodies to collagen types I and II.

2. 3. 3 Immunolabelling techniques

The antigen, the antibody, and the microscopic marker, which is developed to a coloured reaction product, must be linked by successive incubations. For labelling the antibody, enzymatic labels, such as peroxidase or alkaline phosphatase, or fluorescent labels and non-enzymatic, non-fluorescent labels such as ferritin and colloidal gold have been used at the light microscopic level (Sternberger, 1986). Horseradish peroxidase (HRP)-conjugated or alkaline phosphatase-conjugated antibodies remain the most widely used in immunohistochemistry and were used in this study. For the histochemical demonstration of HRP, 3, 3'-diaminobenzidine (DAB) is the most commonly used substrate, and its oxidation forms a intensely coloured and highly insoluble polymer. Once colour is developed, it remains for a long period without a tendency to fade (Nakane and Pierce, 1966). Similar results can obtained for the histochemical demonstration of alkaline phosphatase by fast blue or fast red, which are sensitive, stable and relatively insoluble in organic solvents. The reaction for colour development is performed in the dark and the reaction time is determined by the signal to noise ratio, which should be examined microscopically during the enzyme reaction.

2. 3. 3. 1 Reducing non-specific background labelling

Non-immunological attachment of antibodies and dye reagents to the specimen can introduce immunostaining background, and this can be reduced by reagents added to the buffers. Some of these reagents are applied during pre-incubations while others are included in the buffers

used for washing the section and diluting the antibodies and substrates. These reagents are of three types, all of which are used in this thesis:

- (a) Blocking reagents, such as bovine serum albumin, rabbit and goat normal serum, contain proteins which compete with the immunological reagents for non-immunological protein binding sites on the specimen.
- (b) Surfactant reagents, such as Tween 20 and NaCl, reduce surface charges on the specimen, thereby reducing electrostatic attachment of molecules to the specimen.
- (c) Chemical reagents, such as 0.02 M glycine, or 1% sodium borohydride, block free aldehyde groups on the tissue thereby preventing fixation of proteins to the specimen.

Buffers used in the present studies include phosphate-buffered saline (PBS, 0.01 M, pH 7.2) or Tris-buffered saline (TBS, 0.05 M, pH 7.2) (Appendix 9). PBS or TBS buffers can be made to contain 0.1% (v/v) Tween 20 for washing purposes. Excess immunological reagents are removed by washing with these buffers.

2.3.3.2 Controls for immunostaining

As described above, it is important to reduce non-specific labelling of the specimen during immunostaining. However, the absence of immunolabelling does not always mean the absence of antigen. Adequate controls are therefore absolutely essential and should be devised specifically to verify each experiment. Immunological controls should be performed before immunostaining in a test system to determine the exact specificity of the antibody. Methodological controls assure the quality of the immunolabelling technique.

Positive controls test the reactivity of the antigen, the antibody and the substrates used. The antigen in the specimen should be tested with an alternative antibody to confirm that specimen preparation procedures have not destroyed the antigen. The efficacy of the antibody should be confirmed by immunolabelling a known antigen-positive specimen. For example, a human artery specimen may be used as a positive control for an antibody to the human endothelial cell antigen. The secondary antibody and its substrates should also be tested in a known positive system.

Negative controls assess the non-specificity of the immunostaining technique employed. Non-specificity can be assessed by using a specimen in which the antigen is known to be absent, or by omitting the antibody or using an unrelated antibody.

2. 3. 3. 3 Tissue section pretreatment

In general, formalin-fixed, paraffin-embedded material provides satisfactory results for immunodetection of the majority of antigens. However, the demonstration of many antigens can be significantly improved by treatment with proteolytic enzymes such as trypsin and proteinase K (Jackson and Blythe, 1993) and microwave treatment (Hopwood, 1993). The enzyme digestion and microwave oven treatment help to break the protein cross-links formed during fixation and thereby to expose hidden antigenic sites and improve the sensitivity of immunolabelling. Not all antibodies require section pretreatment and care must be taken not to create "false" antigenic sites, or to alter existing antigenic sites. For general use of proteolytic enzyme digestion, the time for incubation is critical. Longer treatment with proteolytic digestion may compromise tissue morphology, and to cause section detachment from slide. The optimal times and concentrations of proteolytic digestion for paraformaldehyde-fixed, paraffin sections have been tested out in the preliminary experiments. In the present study, proteinase K and protease type XXIV (Sigma, Poole, Dorset, UK) have been used to pretreat the paraffin sections for immunostaining using certain antibodies, and for in-situ hybridization. The detailed conditions are given in the relevant chapters.

2. 3. 3. 4 Immunostaining techniques for light microscopy

There are numerous immunohistochemical techniques which may be used to localize antigens. All immunostaining techniques can be reduced to a number of constitutive steps. The selection of a suitable technique should be based on parameters such as the type of antibody and specimen, and the degree of sensitivity required. The immunostaining techniques include direct methods and indirect methods. In the direct method, a labelled antibody reacts directly with the antigen in the histological preparation. This method employs only one antibody and can be quickly completed. The technique, however, provides little signal amplification and is rarely used on paraffin sections (Beesley, 1993).

The most commonly used immunostaining technique is the indirect two step technique in which an unlabelled specific primary antibody (first layer) is visualised by a labelled secondary antibody (second layer), which is against the immunoglobulin of the animal species in which the primary antibody has been raised. The indirect technique is widely used because a labelled secondary antibody can be used to localize a number of different unlabelled primary antibodies. This method is more sensitive than the direct method because several secondary antibodies may react with different antigenic sites on the primary antibody. Consequently more labelled secondary antibody binds to the antigenic sites, and amplifies the signal (Fig. 2. 14).

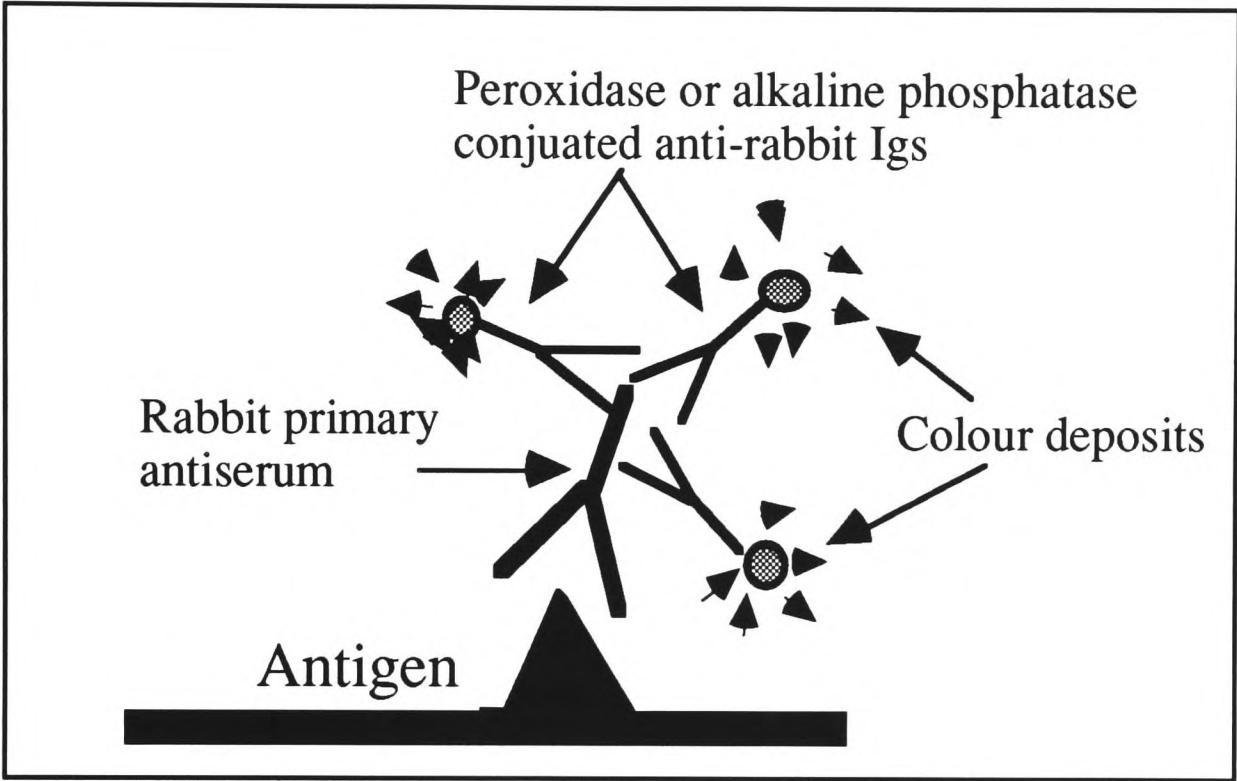


Fig. 2. 14. Schematic representation of two step immunostaining method.

Only the two step immunoenzymatic technique has been used in the immunohistochemical staining in this thesis. The details of various primary and secondary antibodies, and the immunostaining procedure are given in the relevant chapters.

2. 4 In-situ hybridization techniques

2. 4. 1 Introduction

In situ hybridization -- the localization of specific mRNA or DNA species in tissues and cells using nucleic acid probes, has become an increasingly valuable research technique since it was

first described in 1969 for the detection of multiple copy ribosomal RNA genes (Gall and Pardue, 1969; John, et al., 1969). Nucleic acid hybridization techniques rely upon the fact that complementary sequences of single-stranded nucleic acid species spontaneously re-anneal, under appropriate conditions, to form a double-stranded hybrid. Since it is possible to incorporate isotopic or non-isotopic markers into nucleic acids, either enzymatically or photochemically, these labelled nucleic acid molecules can be used as probes to study the distribution or level of expression of specific genes. Nucleic acid hybridization for the detection of specific DNA and RNA sequences are now extensively used in research (Singer, et al., 1986; Warford, 1988; Stahl, 1993; Wilcox, 1993).

Whilst conventional immunocytochemical techniques provide information on the spatial patterns of protein distribution, they do not allow the changes in mRNA levels to be studied. A protein may persist in the cell long after its translation and long after the concentration of the mRNA encoding it has declined. In situ hybridization, in conjunction with immunohistochemical analyses provides information on both the spatial and temporal patterns of expression of transcription and translation, and on the mechanisms of gene regulation. Current techniques of in situ hybridization enable the localization of specific nucleic acid species in wide range of tissues from ultrastructural to macro-tissue level. Sensitivity of in situ hybridization, always considered a problem in the past, is now extremely good in optimized systems and the localization of single-copy genes and mRNA transcripts present at less than ten copies per cell are now possible (Harper and Marselle, 1986). Since mRNA species present at low levels are often expressed in only a few cells, the sensitivity of in situ hybridization may exceed that of Northern hybridization where the target mRNA is necessarily diluted by the relatively large scale homogenization of tissue.

2. 4. 2 Hybridization theory

The extent and rate of the re-annealing process (hybridization), in which single-stranded species combine to form double-stranded species is primarily determined by the relative concentration of the reacting species, the temperature, and the ionic strength. Under conditions of low stringency (low temperatures, ionic strength > 0.3 M) double-stranded hybrids may be formed

between species of low complementarity (having little homology). Under highly stringent conditions (higher temperature, ionic strength < 0.3 M), however, only those hybrids formed between species with almost exact complementarity (100% homology) will remain as hybrids.

The maximal rate of nucleic acid hybridization in solution (at ionic strength above 0.4 M NaCl) occurs at the temperature 25 °C below the T_m of the duplex, where T_m is the temperature at which half of the hybrids are dissociated. For probes longer than 50 nucleotides an empirical formula has been derived to calculate the T_m (Meinkoth and Wahl, 1984): $T_m (^{\circ}\text{C}) = 81.5^{\circ}\text{C} + 16.6 \log M + 0.41 (\%G + \%C) - 500/N$ 0.61 (% formamide). M : the ionic strength of the hybridization solution (molar); N : the length of the probe (bp); $\%G + \%C$: the molar percentage of guanine plus cytosine. 1°C hybridization temperature difference will cause 1% mismatched base pairs. The incorporation of haptens, such as biotin, into nucleic acid also affects hybrid stability and reduces the T_m by 5 °C. It is expected that hybridization should take place at a temperature as close to the expected T_m of the hybrid as possible. However, a more common approach is to perform the hybridization under conditions of low stringency (low temperature, high salt concentration), promoting hybrid formation, and then to wash off non-specifically bound probe in a series of post-hybridization washes of progressively higher stringency. Washing at low salt and higher temperatures dissociates hybrids formed between the labelled probe and a target which is not homologous. The temperature of hybridization is usually about 16 - 32 °C below T_m .

2. 4. 3 General guide lines for in situ hybridization procedures

Compared to immunohistochemistry, in situ hybridization is a more difficult procedure to optimize, owing to the large number of variables involved in each new tissue type, fixation, probes and hybridization conditions must be re-assessed accordingly. With in situ hybridization, unlike conventional filter hybridization, we have no control over the target concentration. We are able, however, to control a number of variables affecting both the rate and extent of hybrid formation, these include the temperature, probe concentration, probe size, NaCl concentration, formamide concentration, and the presence of dextran sulphate. An in situ hybridization protocol has the following general outline:

- Fixation of material and slide preparation
- Pretreatment of material on slides
- In situ hybridization
- Post-hybridization washings
- Immunocytochemistry and microscopy

These steps are discussed below in more detail. In addition, the effects of various components of hybridization solutions used in the present study are briefly discussed.

2. 4. 3. 1 Sample preparation

The samples used for in-situ hybridization in this thesis were fixed in 4% buffered paraformaldehyde or 10% buffered formalin, decalcified in 14.5% EDTA (pH 7.4) and paraffin embedded. It should be noted that the DNA or RNA target sequences are surrounded by proteins and extensive cross-linking will mask these targets, therefore, permeabilization procedures are generally needed (see section 2. 3. 3. 3). Slide preparation is important to prevent mechanical loss of sections during the procedures. Glass slides for use of in-situ hybridization procedures were coated with 3-aminopropyltriethoxy silane (APES, Appendix 3).

2. 4. 3. 2 Pretreatment of materials and sections

RNase-free environment: RNA molecules, whether as probe or target, are extremely labile and very susceptible to degradation by RNase. RNase A is found in skin secretions. It is virtually indestructible and it is not destroyed by autoclaving. Therefore, gloves are worn all the time during the in situ hybridization procedures. Equipment to be used for in situ hybridization experiments should be sterile and treated to ensure complete absence of RNases. Solutions may be rendered RNase-free by treatment with the strong acylating compound diethylpyrocarbonate (DEPC) (Appendix 15). Tris salts, and other primary amines react with DEPC and should not be treated; Tris solutions should be made up in water previously treated with DEPC. All the glassware in the present study was baked overnight at 200 °C. RNase-free micropipette tips and microcentrifuge tubes are commercially available and used. Any apparatus which can not be baked, or autoclaved in the presence of DEPC, should be rinsed

with a freshly prepared solution of 3% hydrogen peroxide followed by a methanol rinse.

Endogenous enzyme inactivation: With an enzyme as label, the endogenous enzyme activity requires inactivation. For peroxidase this is done by treating the sections with 1% H₂O₂ in methanol for 30 min. For alkaline phosphatase, levamisole (Sigma, Poole, UK) may be added to the enzyme substrates. However, there was no residual (endogenous) alkaline phosphatase activity detectable in the specimens prepared for in situ hybridization in the present study.

HCl treatment: Hydrochloric acid treatment maybe helpful for extraction of proteins and partial hydrolysis of the target sequences, and may reduce background binding.

Protease treatment: This treatment increases accessibility by digesting protein surrounding the target RNAs. It is usually performed by incubating the sections in Proteinase K (20 µg/ml) in Tris-HCl buffer (pH 7.4) for 30 min at 37 °C (Critchlow, et al., 1995).

2. 4. 3. 3 Notes for in situ hybridization

Temperature and pH: The maximum rate of renaturation (hybridization) of DNA is at about $T_m - 25$ °C (see 2. 4. 2 for detail). In the pH range 5 to 9, the rate of renaturation is fairly independent of pH. In this thesis, buffers with pH 6.5 - 7.5 are used.

Choice of probe: The essential requirement is that the probe must have a sequence complementary to the sense sequence of the target mRNA. Four types of probes are usually used for in situ hybridization.

(1) Oligonucleotide probes are prepared from deoxynucleotides by automated synthesis and are commercially available. They are resistant to RNases, small (20 - 40 bases) and they penetrate cells easily, but they tend to give high background (non-specific binding), and require high stringency hybridization conditions (Stahl, 1993).

(2) Single-stranded DNA probes can be produced by reverse transcription of RNA or by amplified primer extension of a PCR-generated fragment in the presence of a single antisense primer.

(3) Double-stranded DNA probes are usually made by bacterial amplification. The probe must be denatured prior to use, since only one strand hybridizes with mRNA. Double-stranded probes are less sensitive than single-stranded DNA, because of the tendency of the two strands to re-anneal.

(4) Riboprobes are complementary RNAs prepared by RNA polymerase-catalyzed transcription of mRNA in the 3' to 5' direction. The advantage using riboprobes is that RNA-RNA hybrids are very thermostable and resistant to RNase attack. This allows post-hybridization digestion by RNase to remove non-hybridized RNA, thereby reducing background. Only riboprobes are used in the present study.

Choice of probe label: Because of advances in non-radioactive labelling and detection techniques, in situ hybridization is now more widely used. The use of non-radioactive probes avoids the handling of hazardous radioactive reagents, reduces backgrounds, and increases resolution. Non-radioactive labels include biotin, digoxigenin, alkaline phosphatase, fluorescein, texas red and rhodamine. In the present study, all the probes were labelled with digoxigenin. The digoxigenin labels can be detected by an anti-digoxigenin antibody, which is conjugated to alkaline phosphatase (Fig. 2. 15).

Controls of in situ hybridization: Is the probe hybridizing to mRNA or to other components of the cell ? This question can be answered by incubating a specimen with RNase prior to hybridization. The absence of binding after RNase treatment will indicate that without RNase the binding was indeed to RNA. If it hybridizes to mRNA, is it only to the target mRNA ? Any sequence that has a similar GC content, a similar length and no homology to the sequence of interest could be used as a control for non-specific binding; the sense sequence of the antisense probe, therefore, can be used. A negative result after the hybridization with the sense probe would prove that there was no indication of non-specific binding of the probe.

Tissue known to contain the mRNA of interest can serve as a positive control to test the conditions for tissue preparation and in situ hybridization.

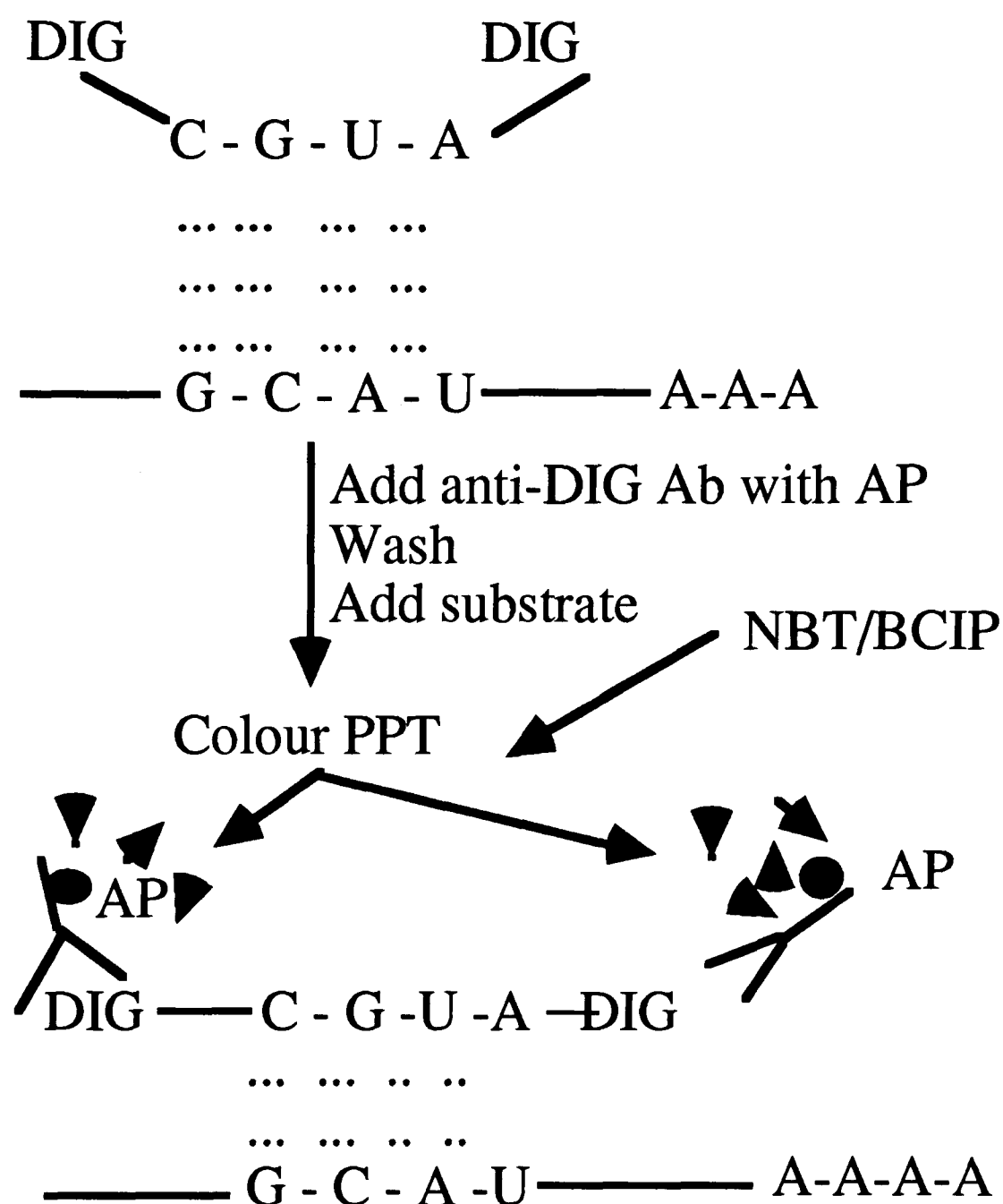


Fig. 2. 15. Diagram showing the process of in-situ hybridization. A labelled digoxigenin (DIG) probe is hybridized with mRNAs in a specimen. DIG binds to an anti-DIG antibody conjugated to alkaline phosphatase (AP). AP catalyzes conversion of a substrate, NBT/BCIP, to a coloured precipitate (PPT).

Examination of the mRNA preservation: The amount of mRNA preserved in the sample can be examined by using polyd(T) as a probe to detect mRNA poly (A) tails. If only a weak signal is obtained, it indicates insufficient mRNA preservation. Acridine orange (a nucleic acid intercalating dye) staining can also be used for the purpose of detecting total nucleic acids (Appendix 10). After incubation with acridine orange, the sections should be mounted in non-fluorescent mountant and viewed under blue (470 nm) excitation fluorescence. DNA fluoresces yellow/green, RNA fluoresces orange/red (Fig.2. 16).

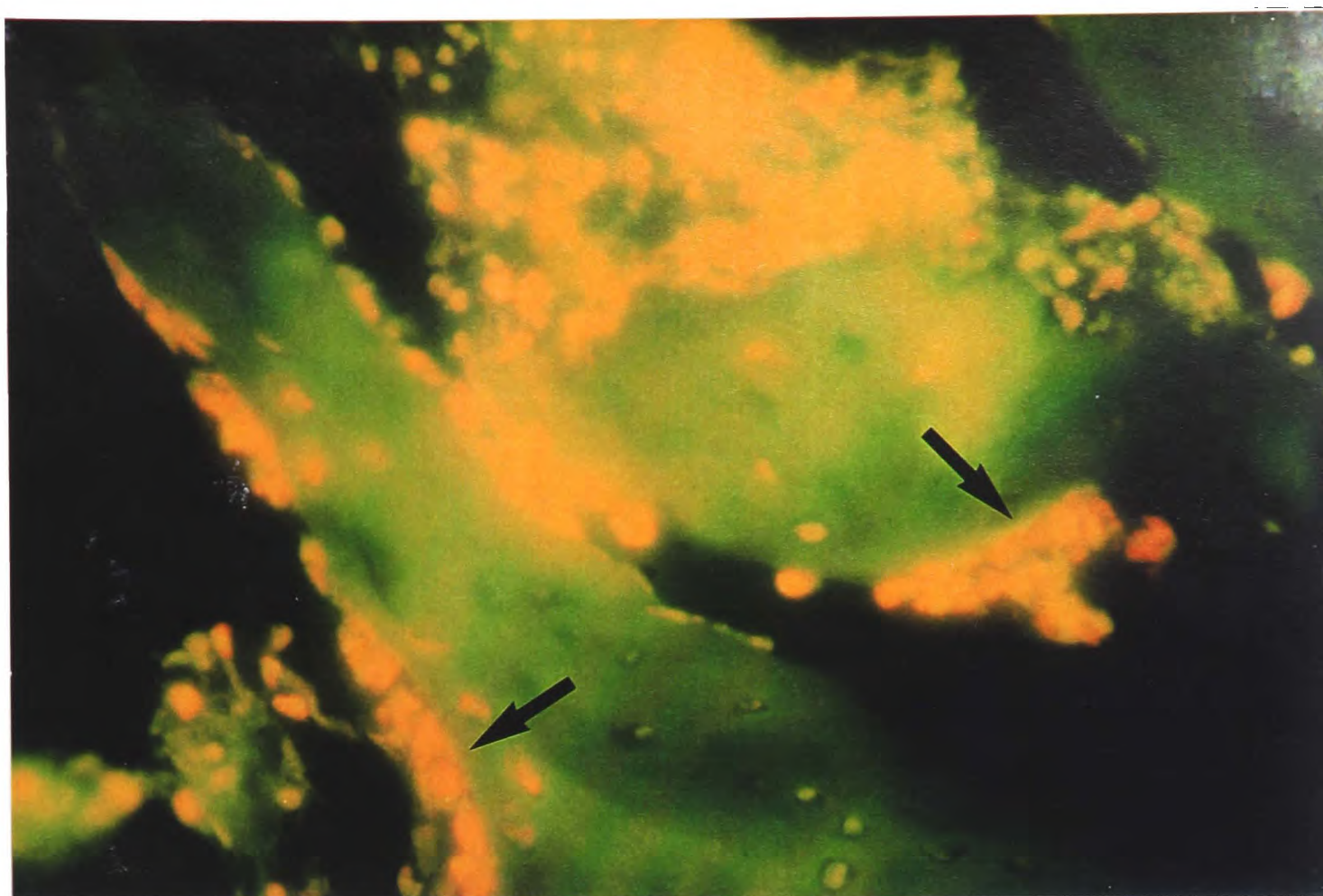


Fig. 2. 16. An example of acridine orange staining of nucleic acids in a bone section. Viewed under 470 nm fluorescence, RNA fluoresces orange/red (arrows), indicating sufficient RNA preservation in the bone tissue sections after fixation, decalcification and paraffin embedding processes. X 400.

2. 4. 3. 4 Post-hybridization washings and immunocytochemistry

Labelled probes may hybridize aspecifically to sequences which bear homology, but not identical complementarity to the probe sequence. Such hybrids are less stable than the perfectly matched hybrids. They can be dissociated by performing washings of various stringencies (see 2. 4. 2). The stringency of the washings can be manipulated by varying formamide concentration, salt concentration and hybridization temperature. In the present study, for in situ hybridization using types I and II collagen sense and antisense riboprobes, a wash in 50% formamide and 2 x SSC buffer suffices under the high stringency conditions employed (see Appendix 14 and 15 for detail).

Two step immunocytochemical procedures were used to detect the digoxigenin-labelled probes. As illustrated in Fig. 2. 15, an anti-digoxigenin (anti-DIG) antibody conjugated to alkaline phosphatase was used to visualise the digoxigenin-labelled probes. The results for in situ hybridization were all examined under bright-field microscopy.

2. 5 Detection of apoptosis

2. 5. 1 Introduction

The role of programmed cell death (apoptosis) in osteogenesis has been discussed in section 1. 11. There are several established methods to detect apoptosis (see 1. 11. 3). The morphological criteria of apoptosis were different from necrosis, and originally defined by electron microscopy (Wyllie, 1980). During the early stage of apoptosis, nuclear chromatin condenses and DNA is digested into nucleosome-size fragments. The cell volume decreases and small bodies containing nuclear debris and cellular organelles surrounded by plasma membrane are extruded from the cell. The morphological features of apoptosis are easily recognised ultrastructurally, but it is only detected with difficulty under light microscopy. Validation of the detection of apoptosis on a DNA level requires extraction of DNA, and analysis by agarose gel electrophoresis. However, assay systems of this type are difficult to quantitate and fail to evaluate apoptosis on a cell-by-cell basis.

Since extensive DNA fragmentation is an important characteristic of the apoptotic process, visualization of DNA breaks could greatly facilitate the identification of apoptotic cells. Kishimoto and colleagues (1990) and Fehsel et al. (1991) used in situ nick-translation (ISNT) to detect DNA strand breaks in cultured cells. Gold, et al. (1993) showed that ISNT with fluorescein-12-dUTP or digoxigenin-11-dUTP allows easy and reliable quantification of DNA fragmentation in cell suspensions and on tissue sections. Apoptosis can be detected in routine histological sections. Wijsman et al. (1993) described a new method to detect apoptosis in formalin-fixed paraffin-embedded tissue sections that involves an in situ end-labelling (ISEL) procedure. After the tissue sections were treated with protease, biotinylated nucleotides were incorporated into DNA breaks by DNA polymerase. Incorporated biotin was subsequently detected by immunolabelling via peroxidase-conjugated avidin. A high correlation was found between the fractions of ISEL-labelled cells and the fractions of apoptotic cells that were morphologically determined (Wijsman, et al., 1993).

2. 5. 2 TUNEL method for detection of apoptosis

Gavrieli et al. (1992) have established the terminal deoxynucleotidyl transferase (TDT)-mediated dUTP-biotin nick end-labelling (TUNEL) method that allows detection of apoptosis at the single-cell level. This method uses TDT to label 3'OH ends of DNA fragments generated during the process of apoptosis. The TUNEL method preferentially labels cells undergoing apoptosis, but it can also label DNA fragments generated in the late stage of necrosis. The TUNEL staining has been used in the present study to detect DNA fragments of the apoptotic cells in histological sections. The cell death detection kit was purchased from Boehringer Mannheim (East, Sussex, UK, Cat. No. 1684809). This kit was designed for detection and quantification of apoptosis at a single-cell level, and was used to label of DNA strand breaks, which can be seen under light microscopy.

2. 5. 2. 1 Protocols for the TUNEL staining

The quality of the TUNEL staining was affected by the methods used for tissue fixation. Tornusciolo et al. (1995) found that background staining was minimal in paraformaldehyde-fixed tissue, and the background was decreased significantly by fixing the tissue at 4 °C rather than at room temperature. Frozen sections exhibited a higher background than paraffin-embedded sections regardless of the fixatives used. The tissue samples for the TUNEL staining in this study were fixed in 4% (w/v) paraformaldehyde (pH 7.4) for 24 hours at 4 °C and paraffin-embedded. Bone tissue was decalcified in 14.5 % (w/v) EDTA prior to embedding as described before.

The protocol for the TUNEL staining was provided with the cell death detection kit (Catalogue No: 1684809, Boehringer Mannheim, East Sussex, UK). It was a modified version of that developed by Gavrieli et al. (1992). Slides were de-paraffinized, rehydrated and washed in TBS. The sections were permeabilized for 10 min using 0.5% (w/v) Triton X-100-PBS or for 5 min using proteinase K (5 µg/ml) at room temperature. Following 2 X 5 min TBS washes, TUNEL reaction mixture (40 µl / slide) was added, the section was covered with a glass coverslip and incubated for 60 min at 37 °C. To make 500 µl the TUNEL reaction mixture, the

following solutions were added: 50 µl terminal deoxynucleotidyl transferase (TDT) from calf thymus in storage buffer as provided with the kit; 450 µl label solution containing fluorescein-labelled dUTP and nucleotide mixture in reaction buffer provided with the kit. The reaction was terminated by immersing the slides in Tris-EDTA buffer (pH 8.0; Appendix 15) for 15 mins. The slides were rinsed with distilled water and subsequently with PBS, and then incubated with a sheep anti-fluorescein antibody conjugated with alkaline phosphatase (Boehringer Mannheim, Poole, UK). Following washes with TBS, the slides were developed with a substrate solution containing 10 ml TBS (pH 7.4), 10 mg Fast red TR or Fast blue (BDH, Poole, UK), and 400 µl Naphthol AS MX buffer (Sigma, Poole, UK) for 15 min in the dark at room temperature. The slides were rinsed with distilled water, mounted with water soluble mountant (Aquamount, BDH, Poole, UK), and analyzed by light microscopy. The TUNEL-positive staining appears to be pink/red (Fast red TR) or dark blue (Fast blue).

2. 5. 2. 2 Critical steps of the TUNEL staining

Permeabilization treatment: The method used for tissue permeabilization also affected the quality of the TUNEL staining. Tornusciolo et al. (1995) reported that they initially used proteinase K to permeabilize the tissue but found that Triton X-100 provided more reliable results. Both reagents provided similar results when the proteinase K was used at a low concentration for a short period at room temperature. However, at higher concentrations, proteinase K caused increased tissue damage, increased non-specific staining and destruction of some antigenic sites. This was confirmed by Roach et al. (1995).

In the present study, the concentrations of proteinase K, Triton X-100, and the temperatures used for incubation, were tried in several different combinations in the preliminary experiments. Sections permeabilized for 10 mins using 0.5% (w/v) Triton X-100 in PBS at room temperature were found to provide the most reliable and reproducible results, with minimum compromise of the morphology of the sections. These conditions were used as a standard for the permeabilization treatment for the TUNEL staining in this thesis.

Specificity controls: For methodological positive control, sections were treated with

DNase I (10 µg/ml) for 30 mins at RT. Strong positive labellings were seen in all the sections treated in this way. For a negative control, slides were incubated only with the solution containing fluorescein-labelled dUTPs, but without TDT, or with TBS buffer instead of the anti-fluorescein antibody. No reaction was observed in negative controls, even when DNA breaks had been created with DNase I as described. In addition, some tissues are known to have low cell turnover, such as brain tissue, peripheral nerve and muscle tissue, were processed through the TUNEL staining along with the tested sections, as negative controls.

2. 5. 3 Dual-labelling method for simultaneous detection of apoptosis and proliferation

2. 5. 3. 1 Introduction

Immunohistochemical multi-labelling of apoptotic cells was first performed by Soriano et al. (1993). They labelled the pyknotic cells with BrdUrd, and immunohistochemistry for BrdUrd was performed followed by DNA breaks staining. Subsequently, Mundle et al. (1994) reported a double-labelling method to simultaneously investigate proliferation and apoptosis. They gave BrdUrd to patients with acute myeloid leukaemia or myelodysplastic syndromes, and S-phase cells were detected using a monoclonal anti-BrdUrd antibody, and apoptosis was measured by in situ end-labelling of DNA. Double labelling revealed that large numbers of S-phase cells in myelodysplastic syndrome were simultaneously undergoing apoptosis.

2. 5. 3. 2 Dual labelling method

Cell birth and cell death are critical biological processes during bone regeneration. In order to study these two events, a dual labelling technique for simultaneous detection of cell proliferation and apoptosis was developed in the present study. The dual labelling of cell proliferation and apoptosis provides additional information for a better understanding of the bone regeneration and remodelling process. This method also applies to the other purposes, where two or more markers can be detected simultaneously in a single histological section.

Apoptosis was first detected by the TUNEL staining as described above, and then followed by

immunodetection of cells that labelled by BrdUrd (Fig. 2. 17). For detail of the dual labelling procedure, see Appendix 16.

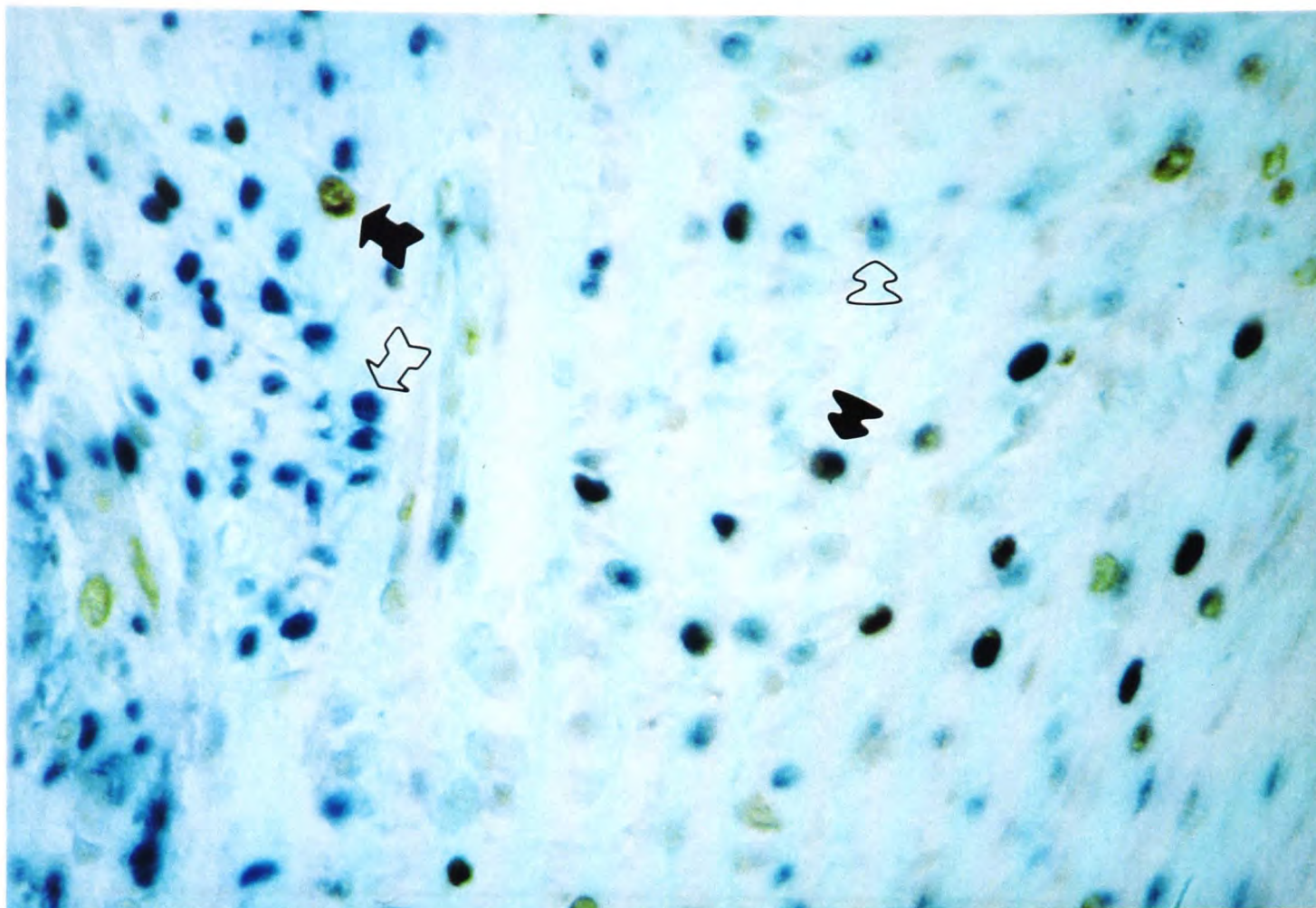


Fig. 2. 17. An example of dual-labelling of cell proliferation and death. In the mineralization front of a regenerate of the 0.7 mm/day group, apoptotic cells (dark blue, empty arrows) were labelled by the TUNEL staining, while S-phase proliferating cells (brown, solid arrows) were labelled by Bu20A immunostaining. Original magnification, X 100.

The dual labelling procedure is a combination of the TUNEL staining and the BrdUrd immunostaining. It is a lengthy procedure involving multiple-steps, and loss of sections from the glass slide was a major concern. Careful sample, slide and section preparation were essential (see section 2. 4. 3. 1) and the tissue sections should be treated with great care during the entire procedure.

PART III

RESULTS

Chapter 3 Radiology and Histology Results

3. 1 Introduction

The first aim of the present studies was to set up an animal model of leg-lengthening and to study the morphological and radiographical changes of the regenerates at various distraction rates. In this chapter, the distraction regenerates from four different experimental groups (distraction at rates of 0.3, 0.7, 1.3 and 2.7 mm/day, see Table 2. 1 for detail) were examined radiographically and histologically. The radiology and histology results in this chapter formed the basis on which subsequent experimental studies were performed.

3. 2 Methods

Male NZW rabbits (age 24 weeks, body weight 3.0 -3.5 kg) were distributed into 5 groups and standardised open transverse osteotomies of the right tibial diaphysis were performed as described before (section 2. 1. 5). The experimental variables included: (1) age of the animal; (2) amount of lengthening; (3) rate of lengthening; (4) rhythm of distraction; (5) latency period before lengthening; (6) time after lengthening before killing. In order to examine the effect of rate, the other variables were kept constant. However, by keeping the amount of lengthening constant, at different rates the time taken to reach this length varies inversely to the rate. After a seven-day latency period, distraction was initiated at rates of 0.3 mm/day for 8 weeks, 0.7 mm/day for 4 weeks, 1.3 mm/day for 2 weeks and 2.7 mm/day for 1 week, in two steps per day, until lengthening to 20% (approximately 2 cm) of the original tibia length. During the distraction period, the animals were free to weight-bear on the operated leg, and weekly radiographs were taken to confirm the lengthening (see 2. 1. 8). There was also a sham group which underwent external fixation and osteotomy but no lengthening.

Once the animal was killed, the material in the distraction gap, together with 5 mm of the neighbouring cortical bone from both ends was excised and sliced in half longitudinally in the

median plane of the tibia. One half was fixed in 95% ethanol and then embedded in glycol methacrylate (section 2. 2. 3) and the other half was fixed in 4% paraformaldehyde (pH 7.4) for 24 hours before decalcification in 14.5% EDTA (pH 7.2) for 3-4 weeks. The specimens were then embedded in paraffin wax, 5 μ m sections were cut by microtome and placed on poly-L-lysine coated slides. For histological examination, the paraffin sections were stained with haematoxylin and eosin (H&E staining) and weigerts haematoxylin / alcian blue / sirius red staining. 5 μ m thick sections were also cut from glycol methacrylate embedded tissues and the alkaline phosphatase activity was detected (see section 2. 2. 6). To identify bone-resorbing cells, TRAP staining was performed in paraffin sections (Appendix 7).

3. 3 Radiography results

3. 3. 1 Experimental groups

In the undistracted osteotomy group, periosteal callus was not visible radiographically in the vicinity of the osteotomied bone ends at the end of first postoperative week. Periosteal callus was visible on x-ray by the end of the 2nd postoperative week (not shown).

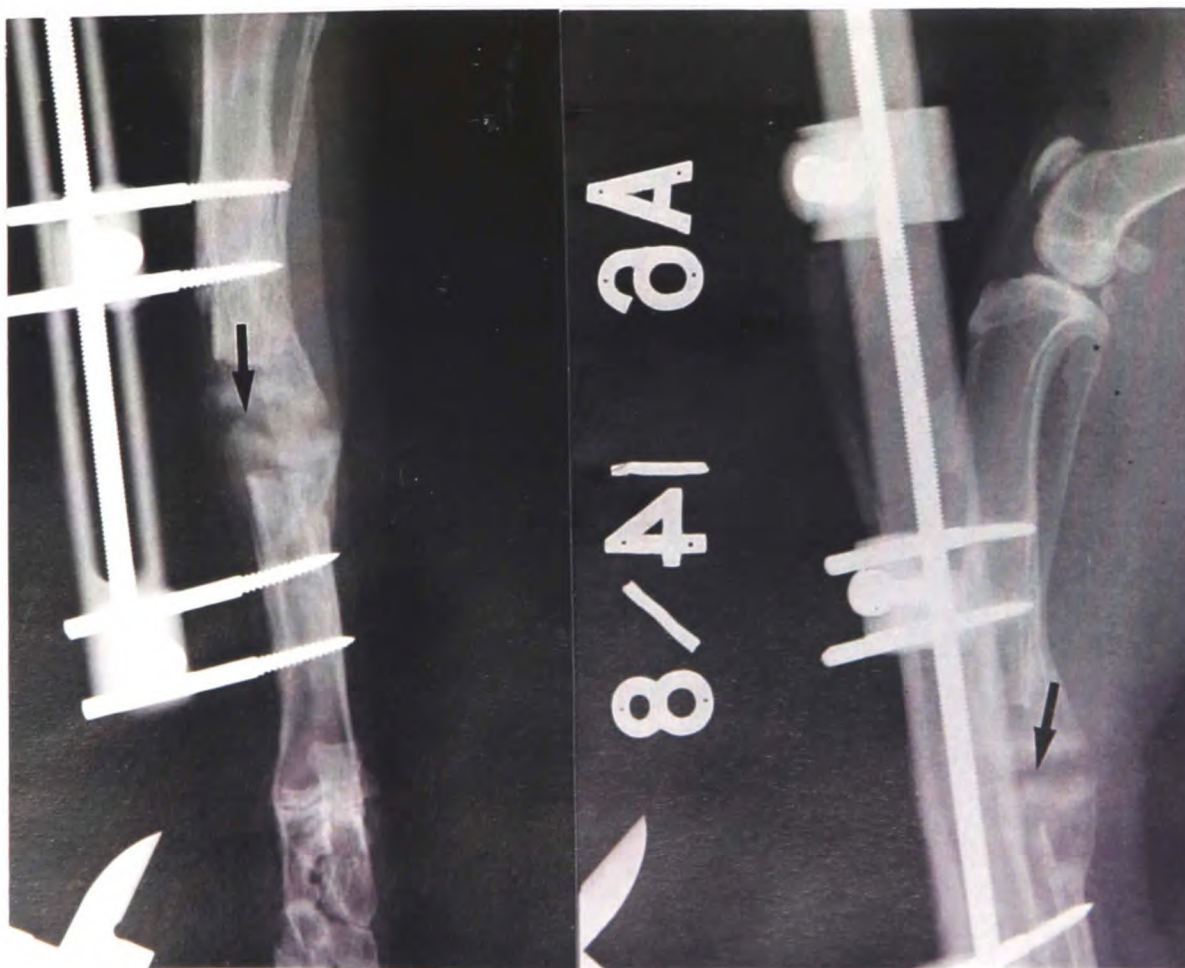


Fig. 3. 1. Radiographic appearance of the regenerate at a distraction rate of 0.3 mm/day, the two sclerotic zones became fused (arrows).

At a distraction rate of 0.3 mm/day, at the end of the lengthening the two sclerotic zones became fused, and the central radiolucent zone was barely seen (Fig. 3. 1).

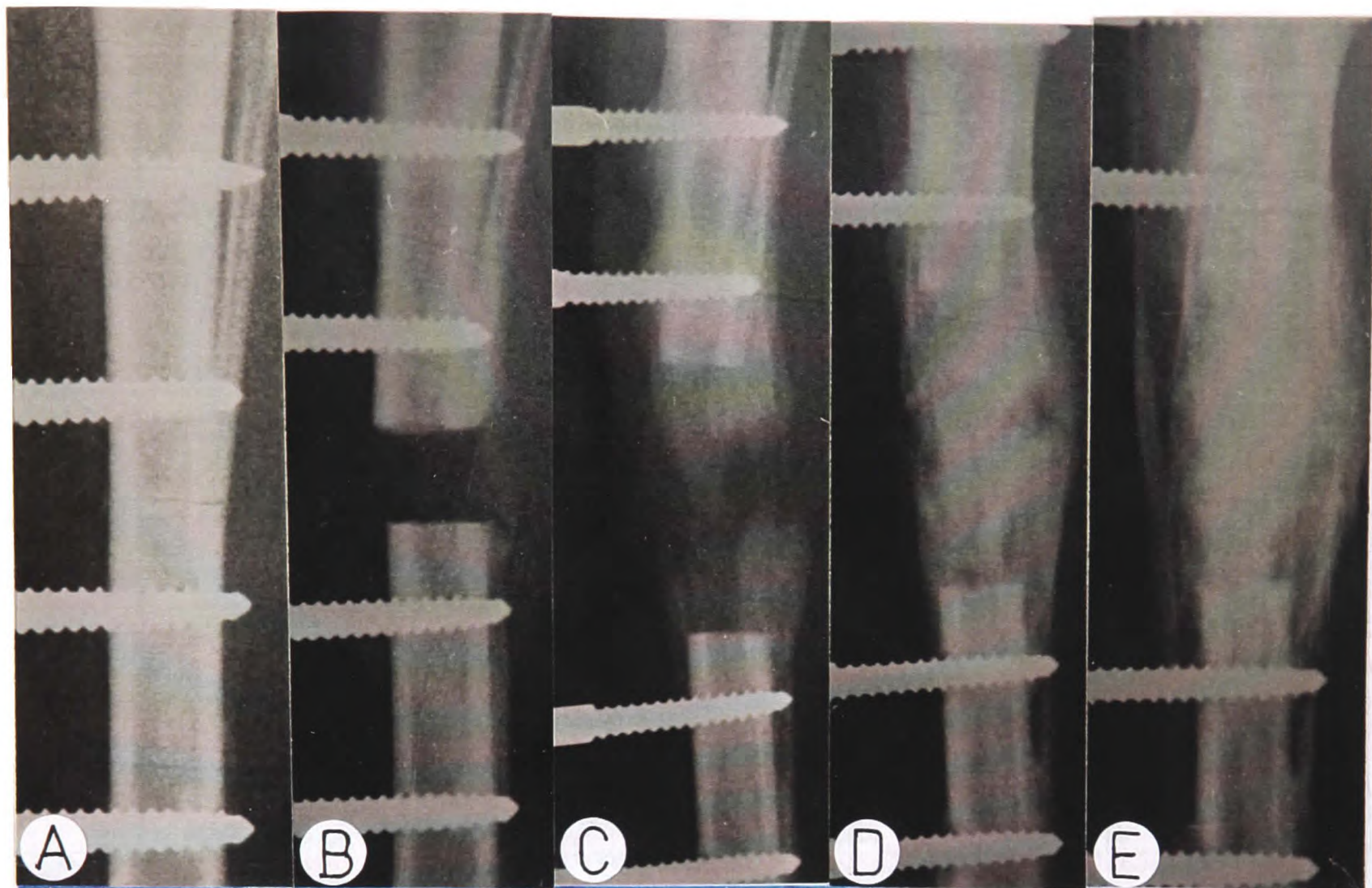


Fig. 3. 2. The radiographic changes during distraction at a rate of 0.7 mm/day. A: 1 week after osteotomy. B: 1week after distraction. C: 2 weeks after distraction. D: 3 weeks after distraction. E: 4 weeks after distraction (at this time, the animal was killed).

At the group lengthened at 0.7 mm/day, callus was not seen at the osteotomied site at the end of first week (Fig. 3. 2A). Sparse callus was seen in the distraction gap during the first week of distraction, most of the distraction gap was radiolucent (Fig. 3. 2 B). Callus was apparent during the second week of distraction, approximately half of the distraction gap was occupied by callus (Fig. 3. 2C). During the third week of distraction, the callus formation was so rapid that it filled the entire distraction gap, with only a few scattered radiolucent areas (Fig. 3. 2 D). At the end of lengthening during the fourth week, the radiograph showed the entire distraction gap was bridged by callus (Fig. 3. 2E), but in some animals the central radiolucent zone was still visible at this time (Fig. 3. 3).

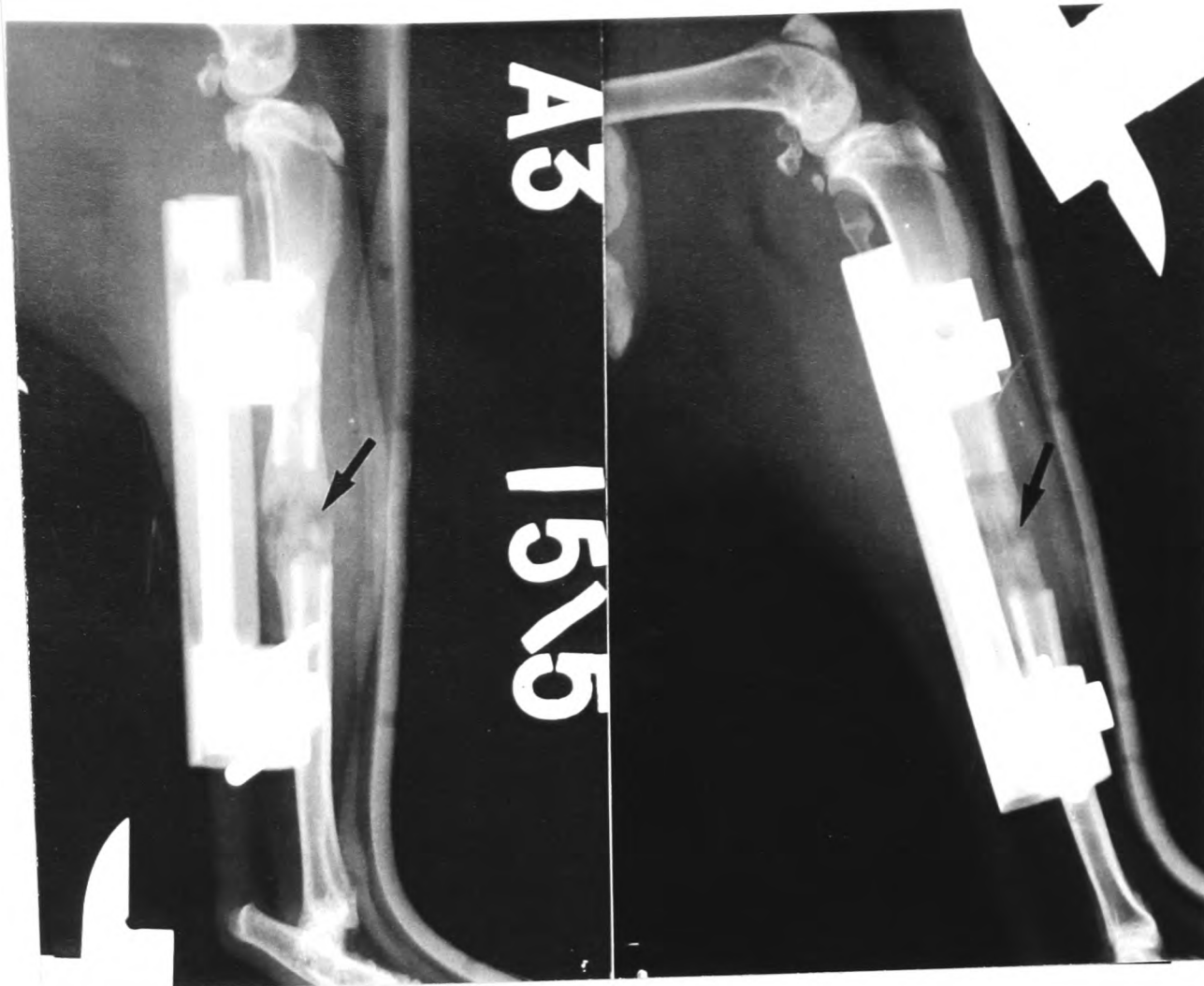


Fig. 3. 3. The radiographic appearance of a regenerate lengthened at a rate of 0.7 mm/day. Central radiolucent zone was still visible (arrow).

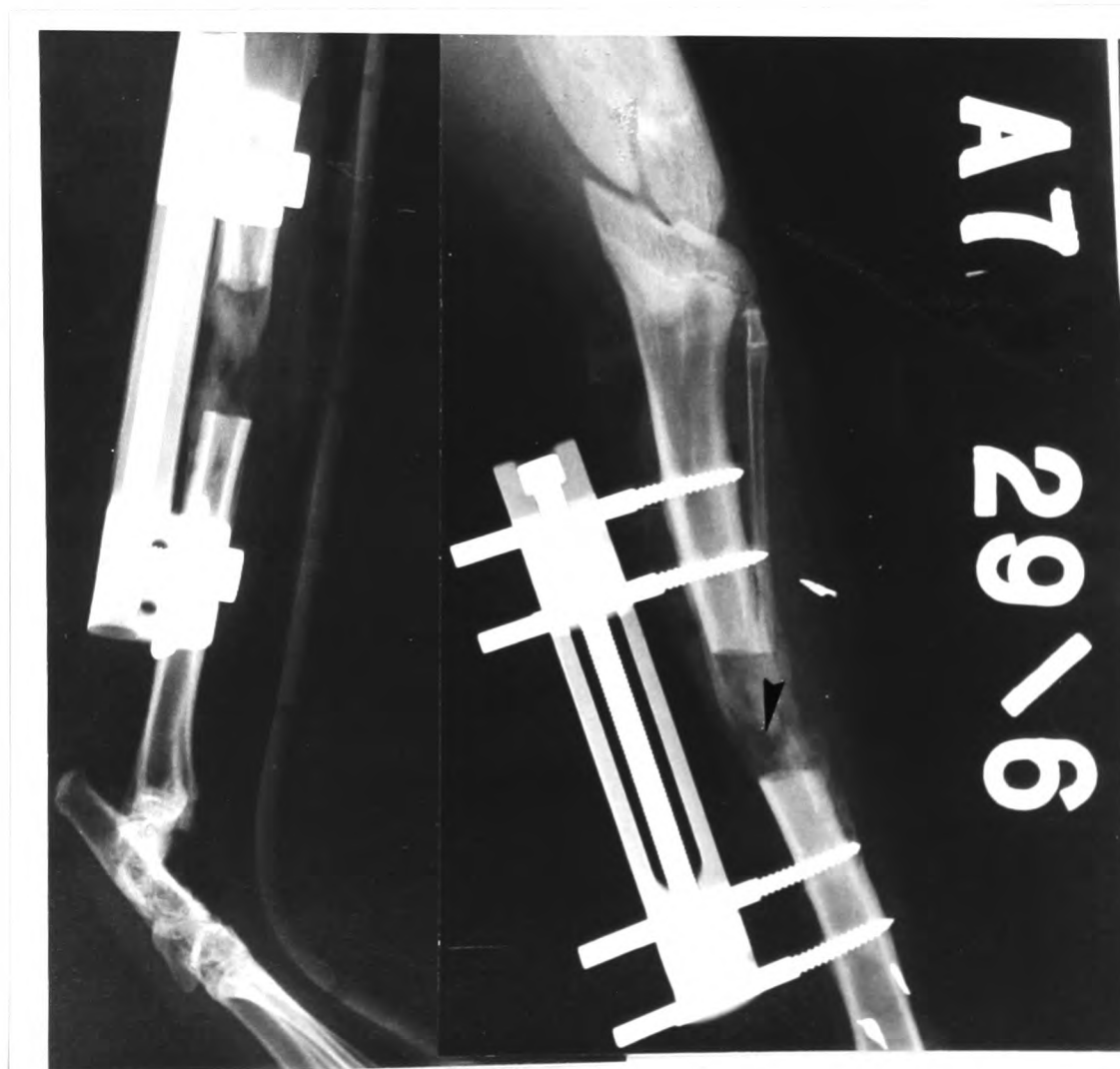


Fig. 3. 4. The radiographic appearance of a regenerate lengthened at a rate of 1.3 mm/day. The regenerating callus in the distraction gap still did not bridge the distraction gap.

At a distraction rate of 1.3 mm/day, as distraction began, the bridging callus separated into proximal and distal segment with a central radiolucent zone which was bounded by proximal and distal sclerotic zones. The central radiolucent and adjacent sclerotic zones were maintained at a relatively constant thickness during the distraction period. By the end of 20% lengthening (about 2 weeks after lengthening) the callus still did not bridge the distraction gap (Fig. 3. 4).

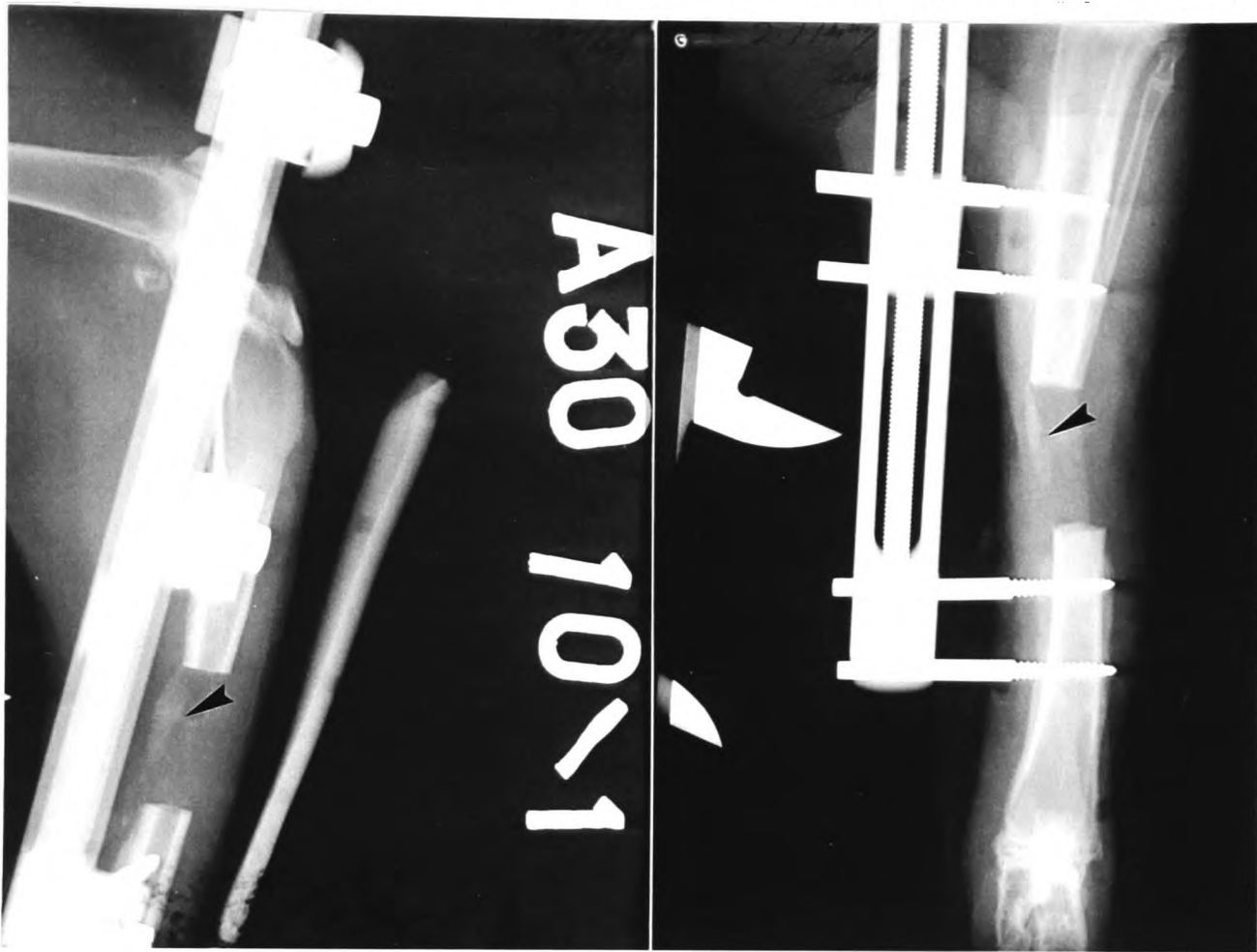


Fig. 3. 5. Radiographic appearance of the distraction gap at a distraction rate of 2.7 mm/day. Most of the distraction gap was radiolucent and there was sparse callus at the proximal and posterior aspect (arrow).

At a distraction rate of 2.7 mm/day, 20 % lengthening (about 2 cm) was achieved after about 1 week of lengthening. With this rapid distraction rate, there was little callus formed by the end of lengthening, and this was mainly localised at the proximal and posterior aspect of the distraction gap (Fig. 3. 5).

3. 3. 2 Complications

In general, the animals tolerated the surgery and lengthening well. There was a small loss of weight (approximately 0.3-0.5 kg) during lengthening period. Lose of ankle joint movement was evident at the end of lengthening, and this was more marked at the faster distraction rates. Eight out of sixty rabbits (13. 3 %) died before the experiments were complete, the reasons for this were fractures through pin holes (5/8), anaesthetic sensitivities (2/8) and serve pin tract

infection (1/8). These animals have been excluded from the experimental groups.

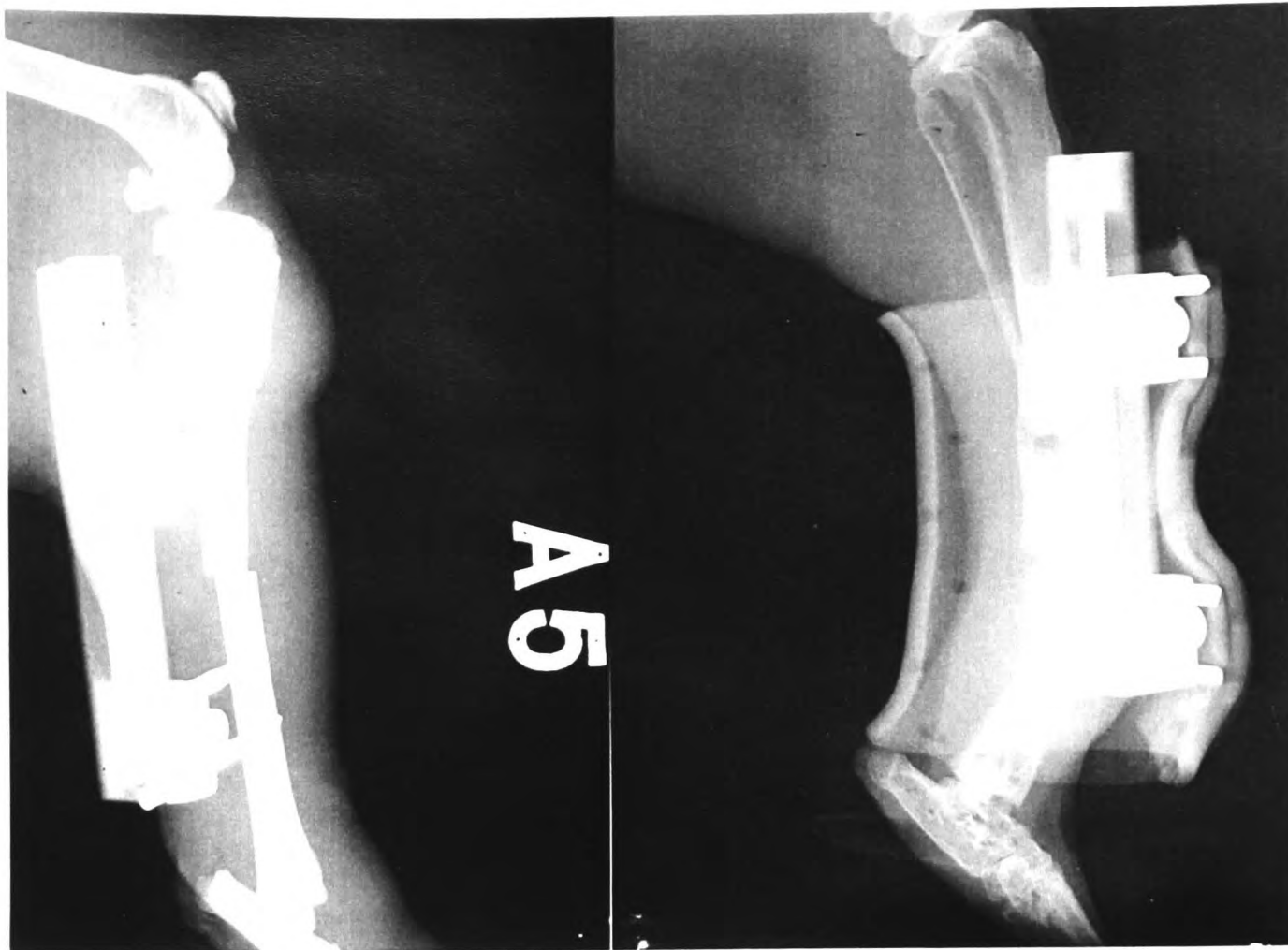


Fig. 3. 6. Radiograph showing fracture through pin hole, which was the most common complication during leg-lengthening in the rabbit.

Fracture through pin holes was most common problem (Fig. 3. 6), this may be due to inappropriate pin alignment, or as a result of the fixator catching against the cage. To reduce the fracture, the operative protocol was standardised (see section 2. 1. 5) to make sure that the pins were positioned optimally. A thermoplastic splint was applied to protect the fixator immediately after surgery. Two rabbit died during anaesthetic procedure, which highlighted that some rabbits were extremely sensitive to anaesthetics. The general anaesthesia was therefore always started with lowest possible dose and combined with local anaesthesia (see section 2. 1. 4). Pin tract infection is a common complication in patient during leg-lengthening, however, it is less common in rabbits. As well as performing the surgical procedures under sterile conditions, penicillin powder was applied to the surgery site and the rabbits lived in a clean, disinfected environment, and these may account for the low rate of pin tract infection.

3. 4 Histology results

3. 4. 1 Gross sample appearances

The gross appearance of the regenerates of the four distraction groups is shown in Fig. 3. 7.

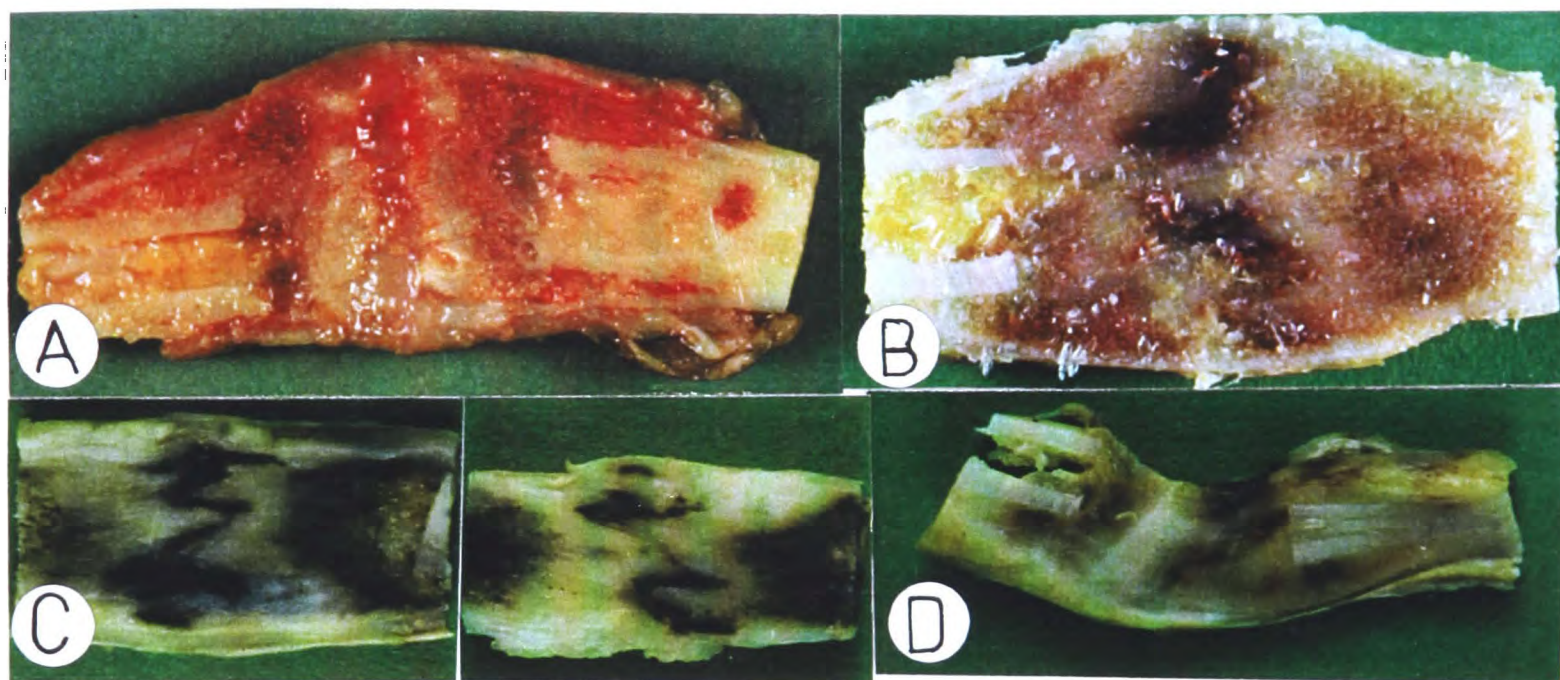


Fig. 3. 7. Representatives of the the samples of the regenerates. A: 0.3 mm/day group; B: 0.7 mm/day group; C: 1.3 mm/day group and D: 2.7 mm/day group.

In general, the material in the distraction callus was longitudinally oriented in the direction of distraction force. The volume of the total callus decreased as the rate of distraction increased. At distraction rates of 0.3 and 0.7 mm/day, the callus was wider than the original bone (Fig. 3. 7A, B), but at 0.7 mm/day, a few haemorrhage (black areas in Fig. 3. 7B) were found in the middle of the regenerate. At a distraction rate of 1.3 mm/day, the callus was the same width as the original bone and the areas of haemorrhage were larger compared to the 0.7 mm/day group (Fig. 3. 7C). At 2.7 mm/day, the callus was sparse and the fibrous tissue zone in the middle of the distraction gap was predominant (Fig. 3. 7D).

3. 4. 2 Sham groups

7 days after osteotomy, organised blood clot was still seen within the osteotomy gap, and there was little periosteal callus bridging the osteotomy gap (Fig. 3. 8 A). The periosteum near the

osteotomy was thickened, with invasion of blood vessels from the surrounding soft tissues (Fig. 3. 8 B).

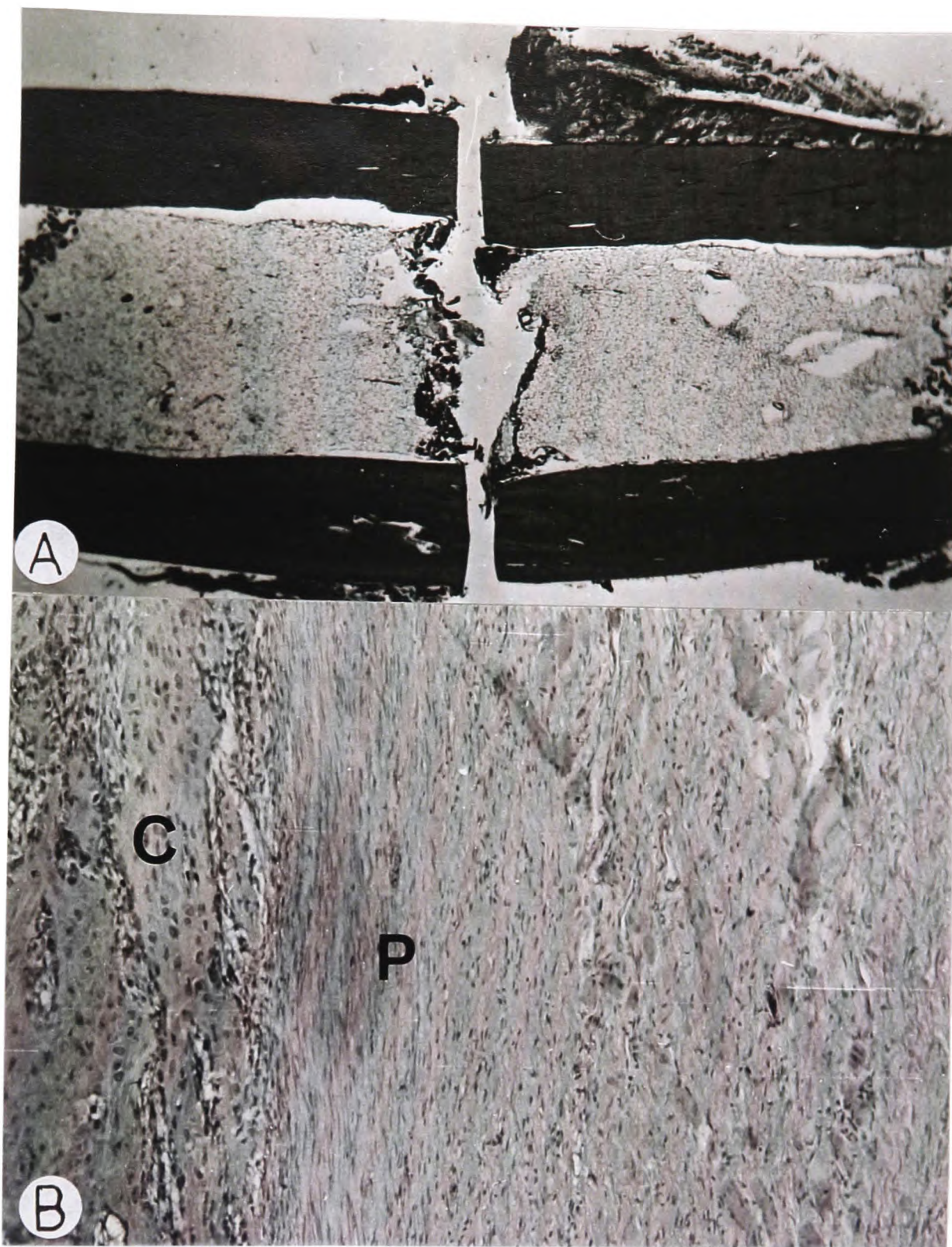


Fig. 3. 8. In the sham group 7 days after osteotomy, there was little callus formation. A: Morphological appearance of the fracture gap. B: The periosteum near the osteotomy was thickened. P: periosteum, C: callus. HE staining, A X 8, B X 25.

The external callus was formed mainly by intramembranous ossification directly from the inner layer of the periosteum (Fig. 3. 9). There was a layer of osteoid tissue formed along the endosteal surface, showing activation of the endosteum (Fig. 3. 10).

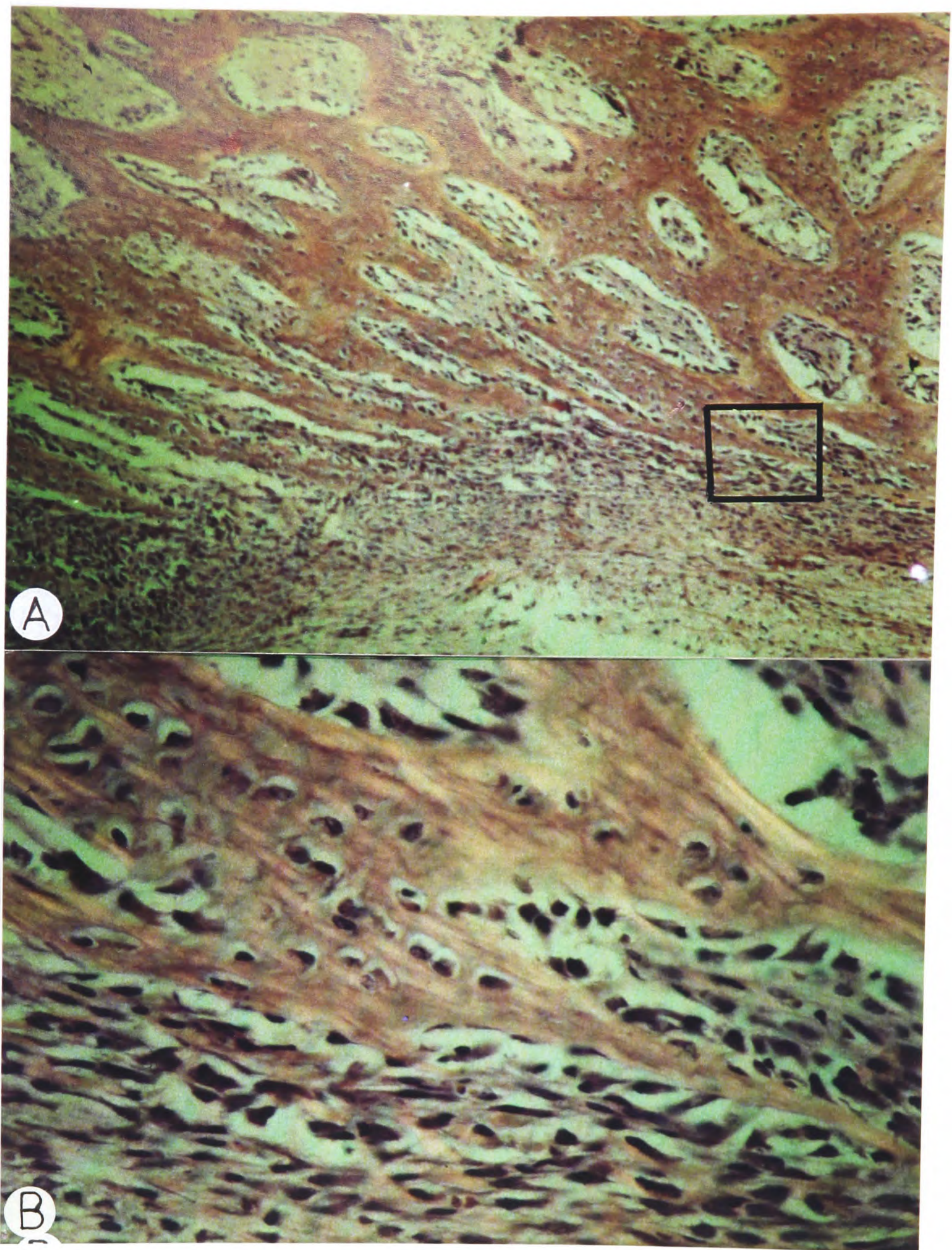


Fig. 3. 9. A: Direct periosteal callus formation from the inner layer of the periosteum (arrow) in the sham group 7 days after osteotomy. B: Close up of boxed area in A. A and B, HE staining, A X 25, B X 100.

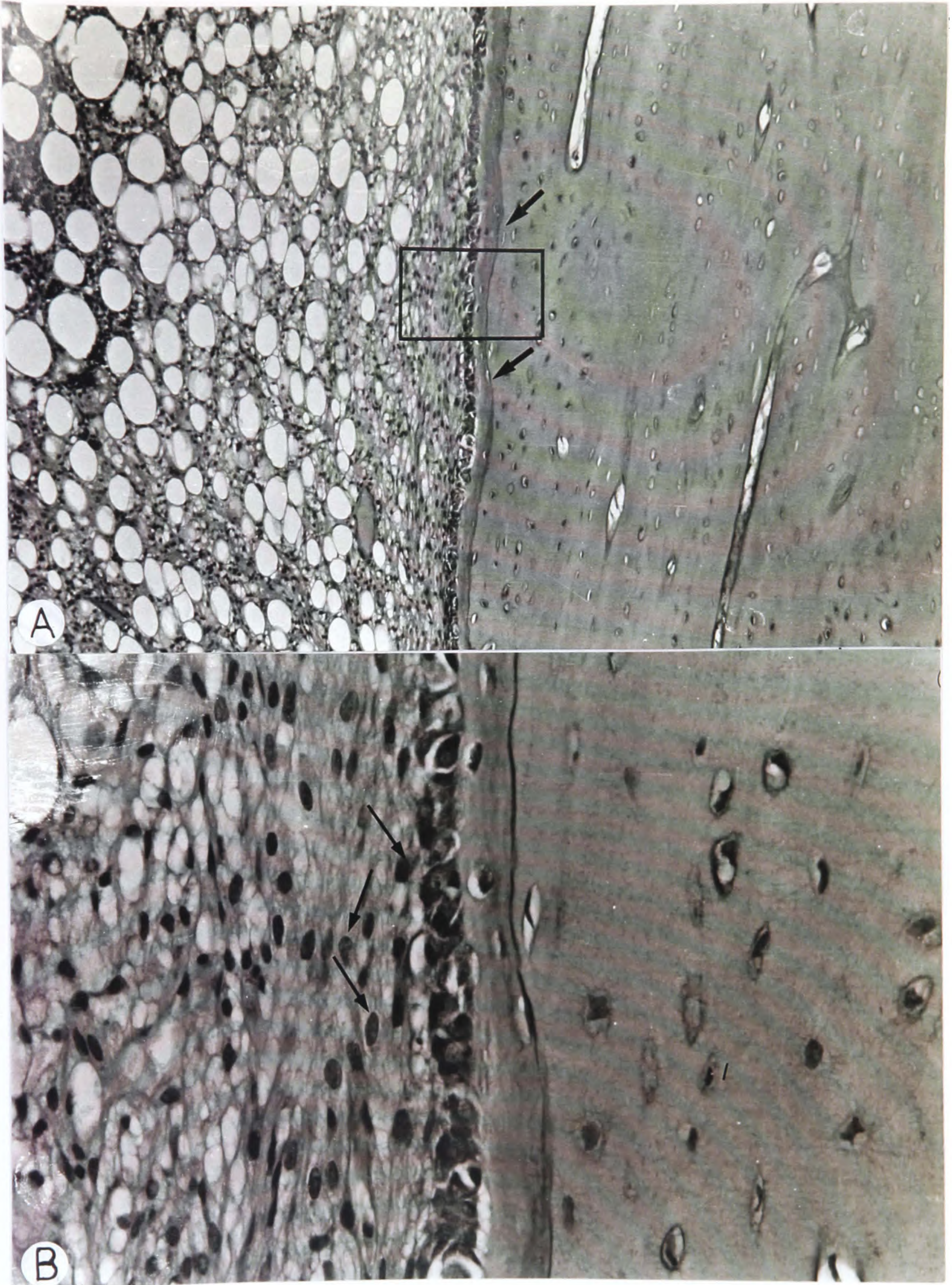


Fig. 3. 10. 7 days after osteotomy. A: The endosteum was activated with an increased cell numbers and a layer of osteoid tissue formed along the endosteal surface (arrows). **B:** Close-up of the boxed area in A, which shows that the cells from bone marrow were contributing to endosteal callus formation (arrows). A and B, HE staining, A X 25; B X 100.

In another sham group, 14 days after osteotomy, the osteotomy gap was bridged by adequate external callus as well as endosteal callus (Fig. 3. 11A). The osteotomy gap was still visible filled with loose connective tissues, and adjacent to which a few cartilage islands were present (Fig. 3. 12). Bone remodelling was seen in the cortices adjacent to the osteotomy ends. The haversian channels of the cortex widened and appeared to merge with adjacent canals, and began to be modelled into a trabecular pattern (Fig. 3. 11B). The endosteal callus formation was very extensive and in 2 animals, the entire bone marrow cavity of the osteotomized tibia was filled with endosteal callus.



Fig. 3. 11. Histological appearance of the sham group 14 days after osteotomy. **A:** The osteotomy gap (arrow) was bridged by periosteal and endosteal callus. HE staining X 8. **B:** Close-up of the osteotomy gap (arrow), showing the periosteal callus (PC) and endosteal callus (EC), and active bone remodelling, X 12.

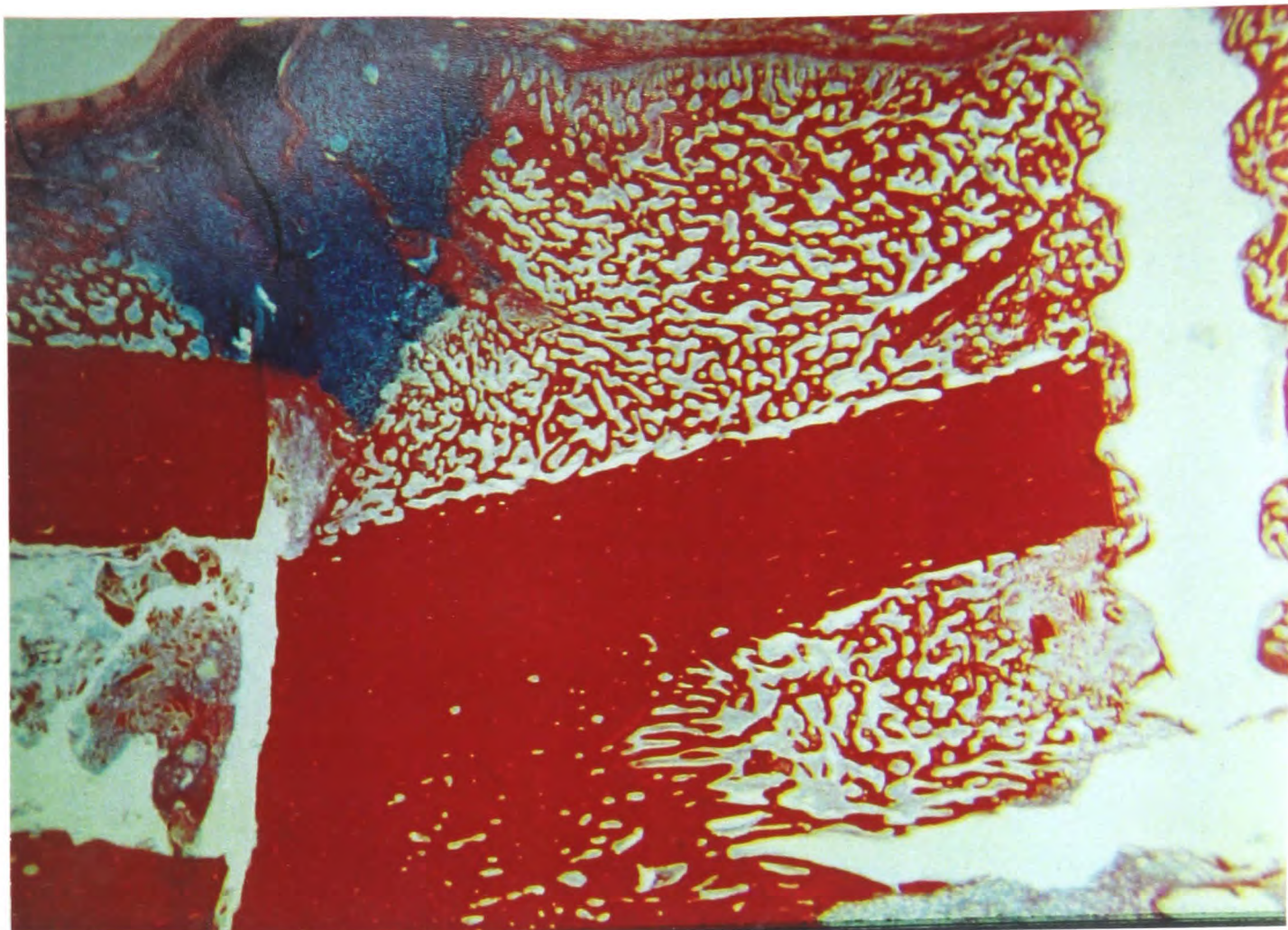


Fig. 3. 12. Cartilage islands (in blue) were found adjacent to the osteotomy gap in some of the sham group, 14 days after osteotomy. Sirius red / alcian blue staining, X25.

Cross sections of the osteotomied tibiae (14 days after osteotomy) showed that the callus was mainly localised posteriorly (Fig. 3. 13).

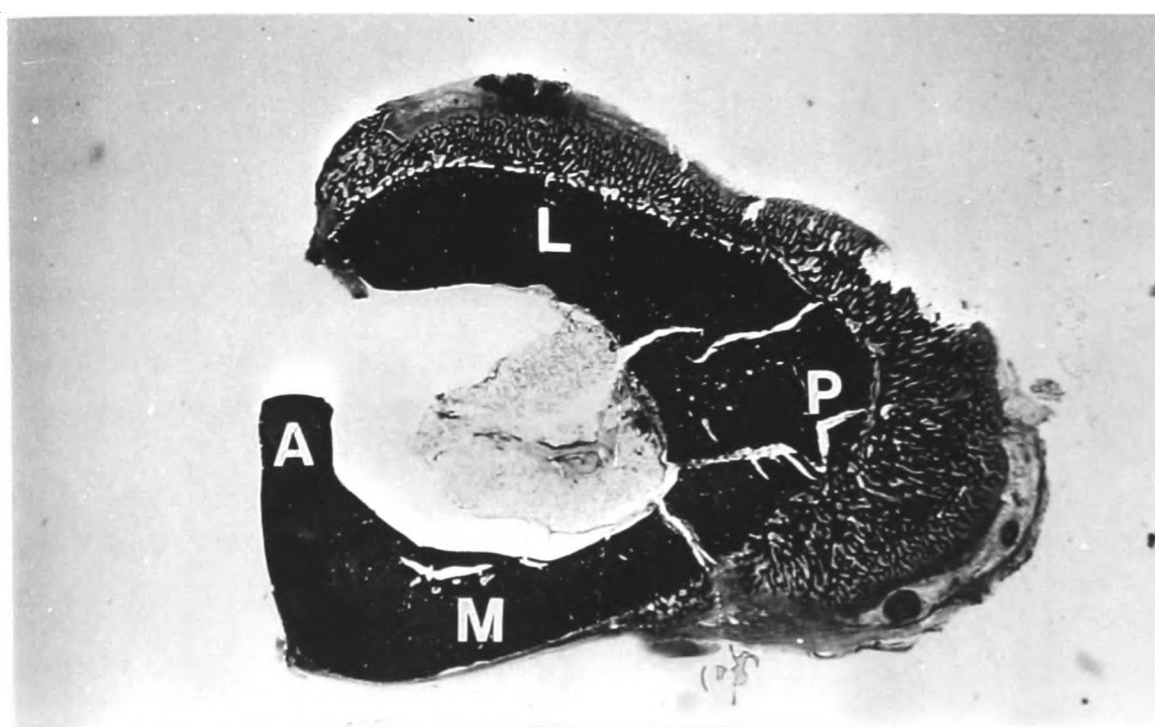


Fig. 3. 13. A cross section of the osteotomied tibia showed asymmetry of callus formation 14 days after osteotomy. A: anterior aspect; P: posterior aspect; M: medal aspect; L: lateral aspect. HE staining, X 8.

3. 4. 3 0.3 mm/day group

The distraction gap consisted almost entirely of trabecular bone and a few areas of fibrocartilage tissues. The new bone was formed in a fashion which was highly oriented to the direction of distraction force. The central region of fibrous tissue was almost closed and replaced by the newly formed bone. In the new bone zone, consolidation was so advanced that distraction appeared to fracture the new bone trabeculae in 4 out of 9 animals (Fig. 3. 14).

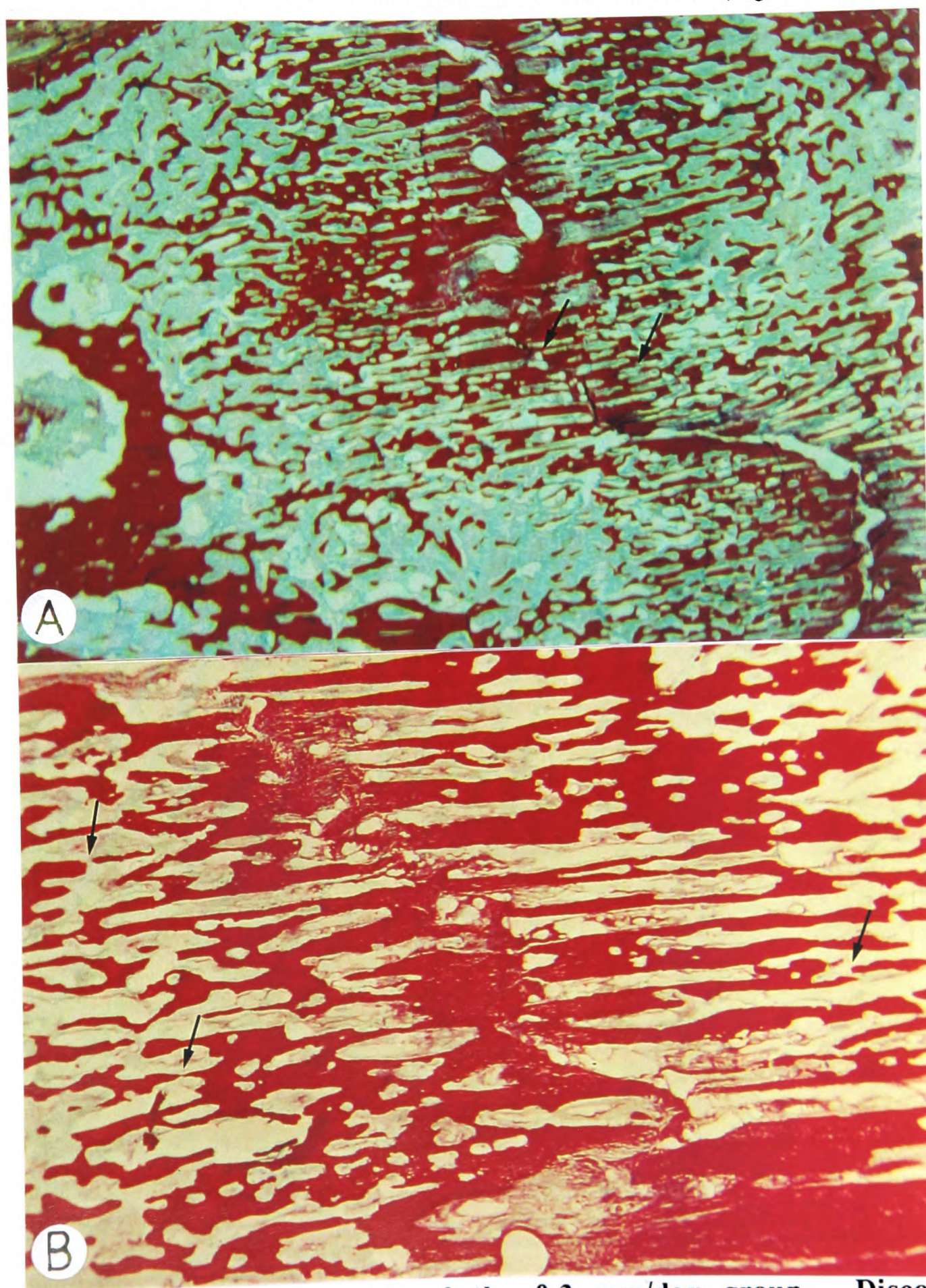


Fig. 3. 14. A regenerate of the 0.3 mm/day group. Discontinuity of trabeculae was found in the regenerate (arrows). Sirius red / alcian blue staining, A X 8; B: X 12.

3. 4. 4 0.7 mm / day group

The central region of fibrous tissue enlarged and became distinct from new bone tissue. New bone was formed and oriented in the direction of distraction forces (Fig. 3. 15A). There were a few cartilage islands in the regenerate demonstrated by sirius red / alcian blue staining (Fig. 3. 15B). Small areas of haemorrhage, necrosis and cyst formation were occasionally seen.

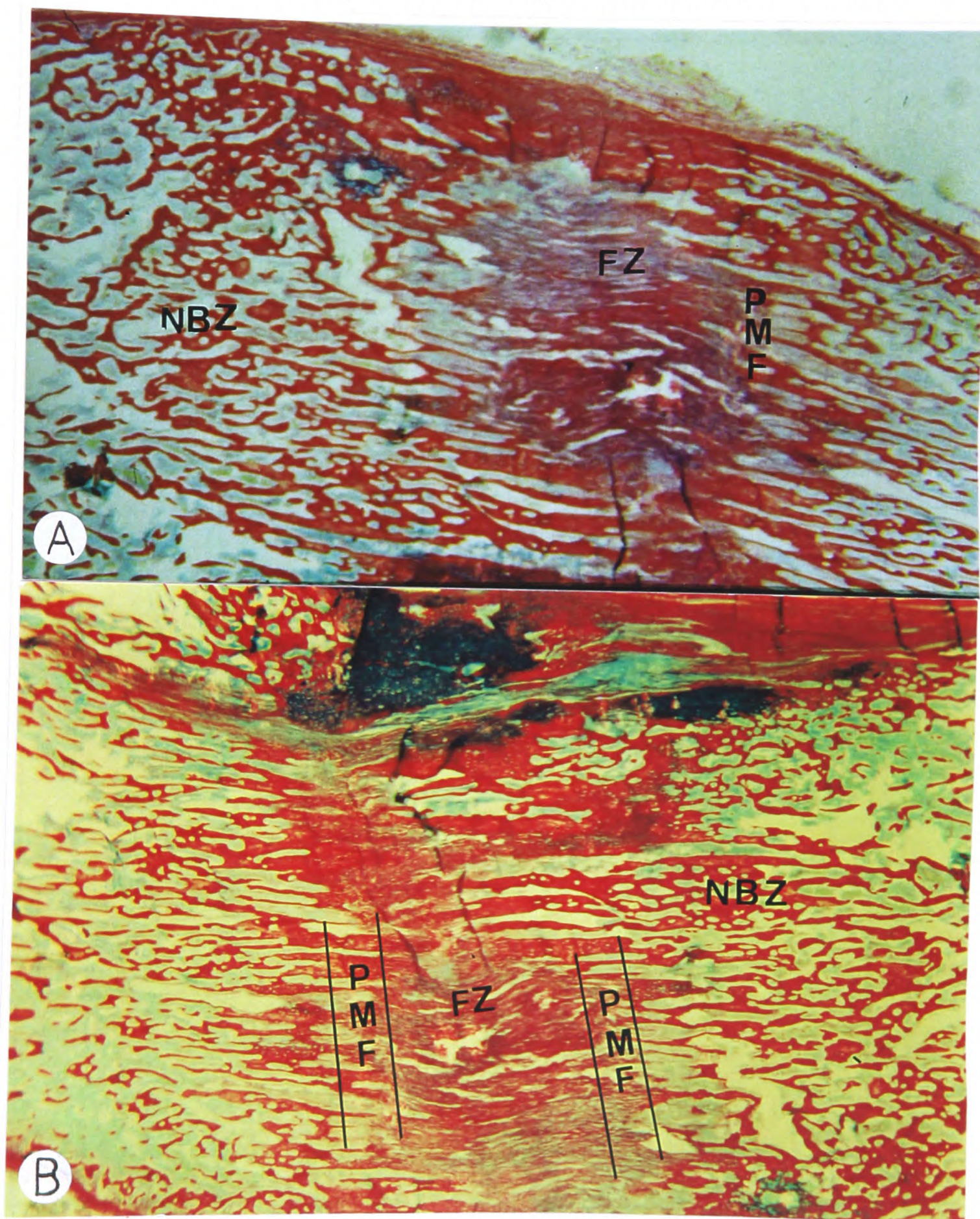


Fig. 3. 15. A: longitudinal section of a regenerate of the 0.7 mm/day group, X 12. B: Close-up of the central fibrous region, boxed areas representing primary mineralization front, cartilage islands (in blue) were present, X 25. FZ: fibrous zone; PMF: primary mineralization front; NBZ: new bone zone. Alcian blue / sirius red staining.

3. 4. 5 1.3 mm / day group

A considerable number of cartilage and necrotic areas, and cyst formation were seen in the centre of the regenerate. These occupied nearly one third of the whole regenerate (Fig. 3. 16).

The new bone was highly oriented to the direction of distraction force, it mainly consisted of woven bone under polarised microscopy examination (Fig. 3. 17).

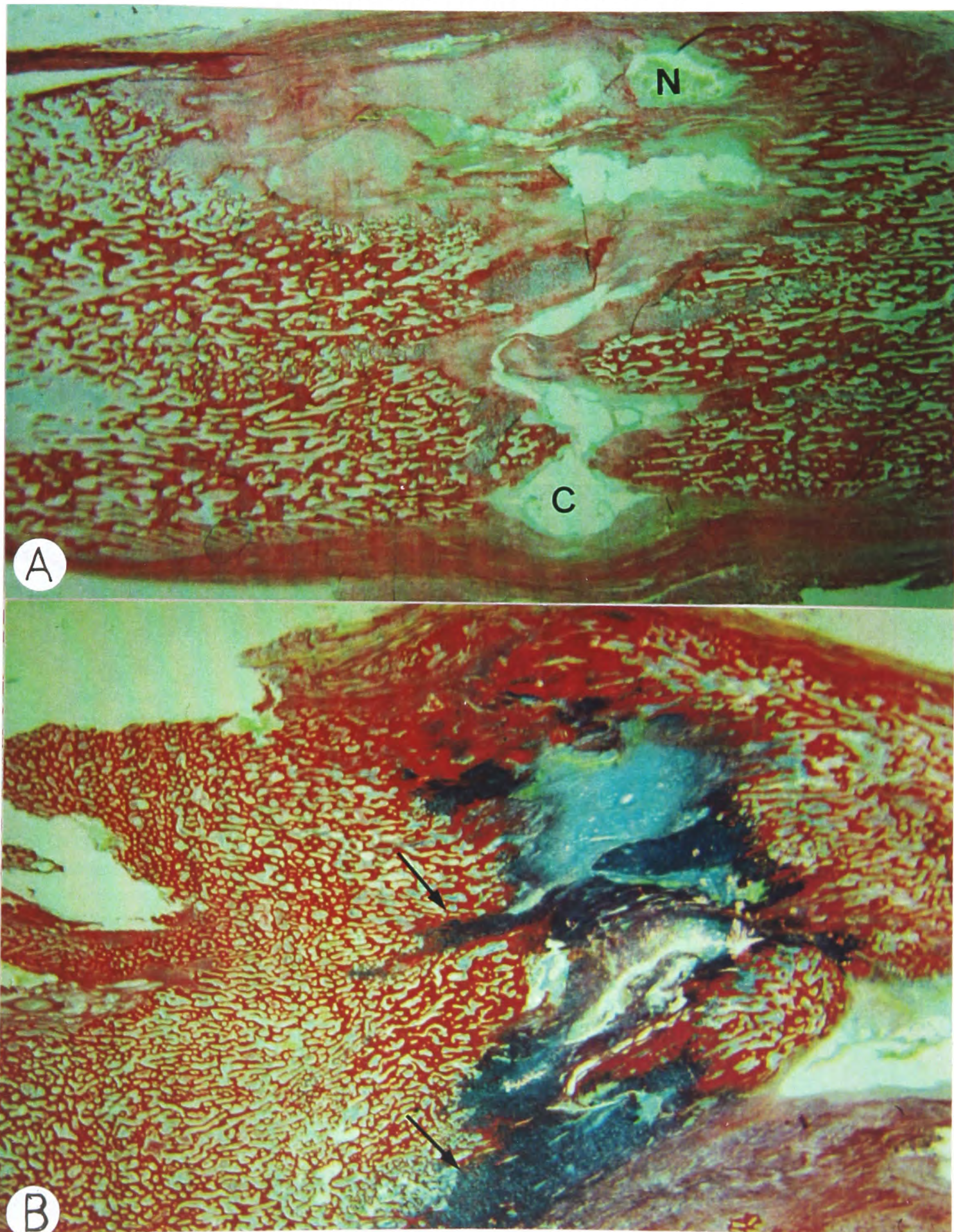


Fig. 3. 16. Morphological appearance of a regenerate of the 1.3 mm/day group. A: The necrotic areas (N) and cyst formation (C) were present in the regenerate. B: Cartilage areas (arrows) were seen. Alcian blue/ sirius red staining, A and B, X 25.

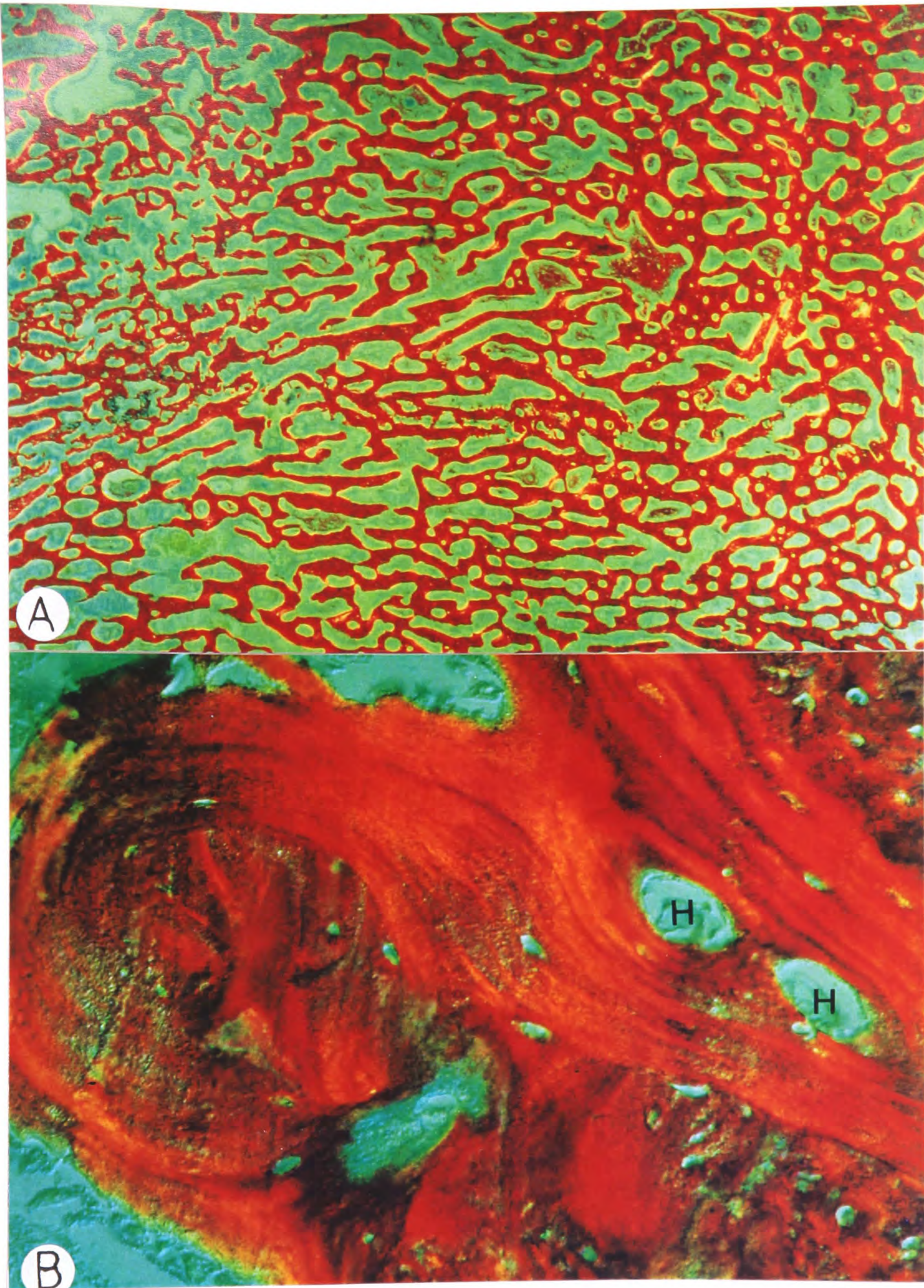


Fig. 3. 17. A: The newly formed bone was oriented to the direction of distraction and in trabecular pattern. B: Polarised microscopy showed that the pattern of the new bone was mainly woven bone, haversian canals (H) were seen. A and B, sirius red / alcian blue staining, A X 25, B X 100.

Moderate amounts of cartilage within the regenerate were found and the cartilage arose directly from the central fibrous tissue in some regions (Fig. 3. 16B). At some of the transitional

regions of cartilage-bone, it was noticed that there was no distinct boundaries between cartilage tissues and new bone trabeculae (Fig. 3. 18A). Cartilage remnants were often seen in the new bone trabeculae, where chondrocytes were buried into bone matrix as demonstrated by alcian blue and sirius red staining (Fig. 3. 18B).

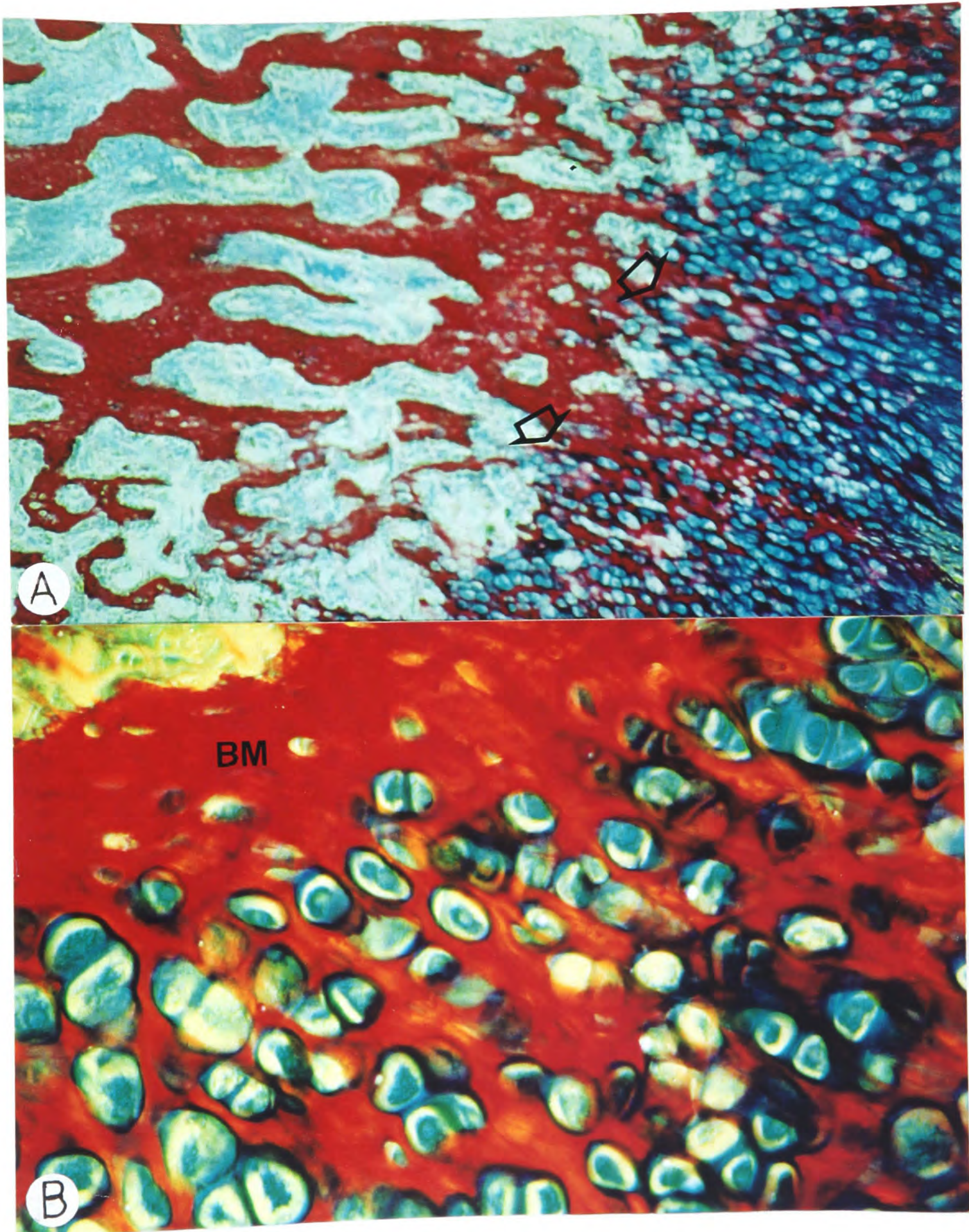


Fig. 3. 18. A: The cartilage-bone junction (arrows) in a regenerate of the 1.3 mm/day group. The cartilage tissue (blue) merged into new bone trabeculae (red). B: Close-up of the cartilage-bone interface, chondrocytes (blue) were buried into bone matrix (red). BM: bone matrix. Alcian blue / sirius red staining, A X 100; B X 400.

For identifying bone-resorbing cells, TRAP staining was performed. TRAP-positive-cells were seen in most areas of the cartilage-bone junction (Fig. 2. 13), these were multi- or mon-nuclear cells. The TRAP-positive staining was also present in some of the chondrocyte lacuna at the cartilage-bone transitional interfaces (Fig. 2. 10).

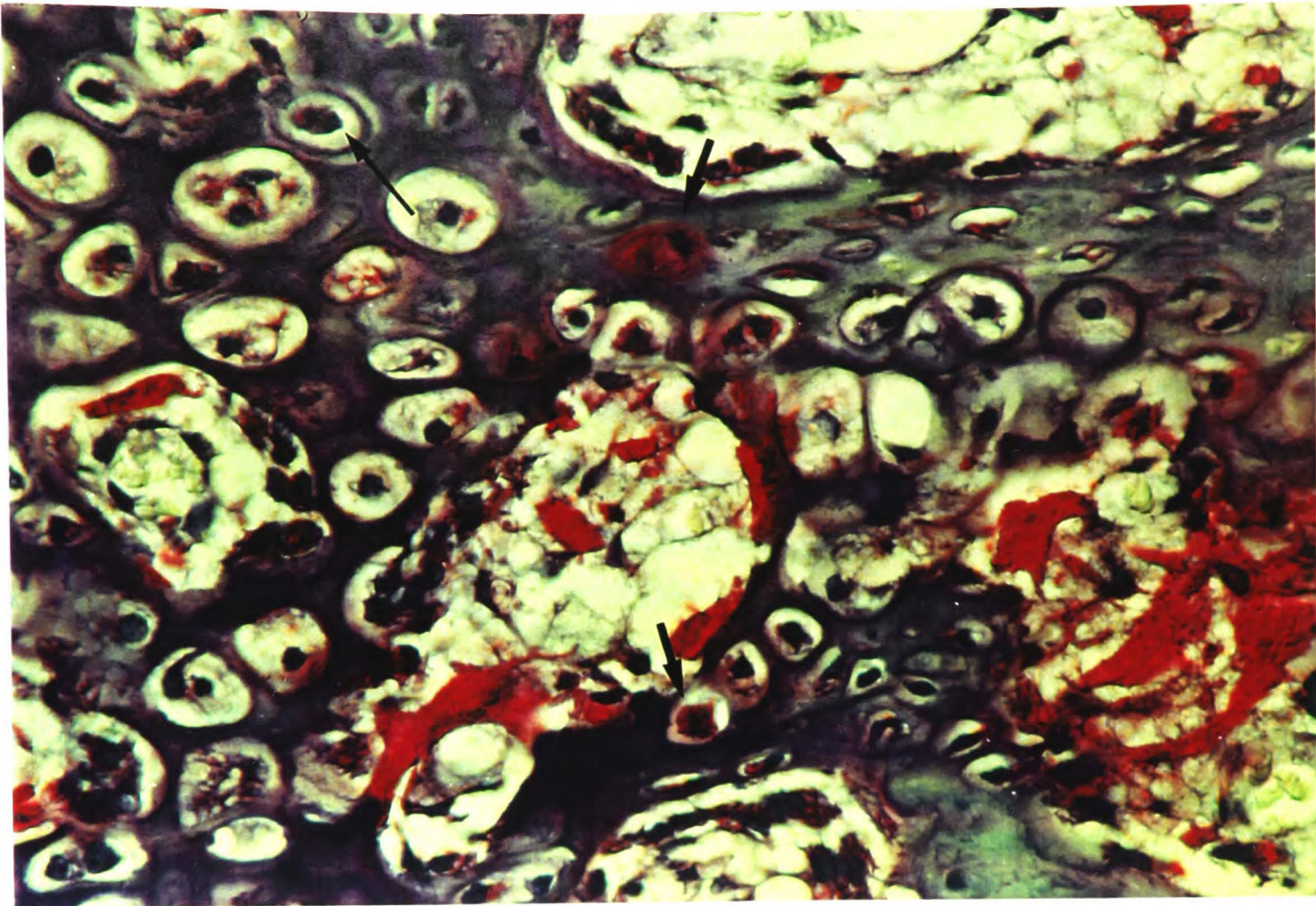


Fig. 3. 19. In a regenerate of the 1.3 mm/day group, TRAP staining showed that the TRAP-positive cells (in red) were in the cartilage-bone junction, TRAP-positive staining were also found in some of the chondrocyte lacuna (arrows), X 100.

3. 4. 6 2.7 mm / day group

Almost 70-80% of the regenerate was fibrous tissue, which consisted of highly oriented collagen bundles parallel to the direction of distraction (Fig. 3. 20). The new bone was formed from both proximal and distal ends of the central fibrous regions, and also oriented to the direction of distraction. Compared to the 0.7 and 1.3 mm/day group, the bone formation was sparse, and fewer cartilage areas were present within the regenerate, some small areas of haemorrhage, necrosis and cyst formation were present, which were mainly localised near the distal end of the regenerate.

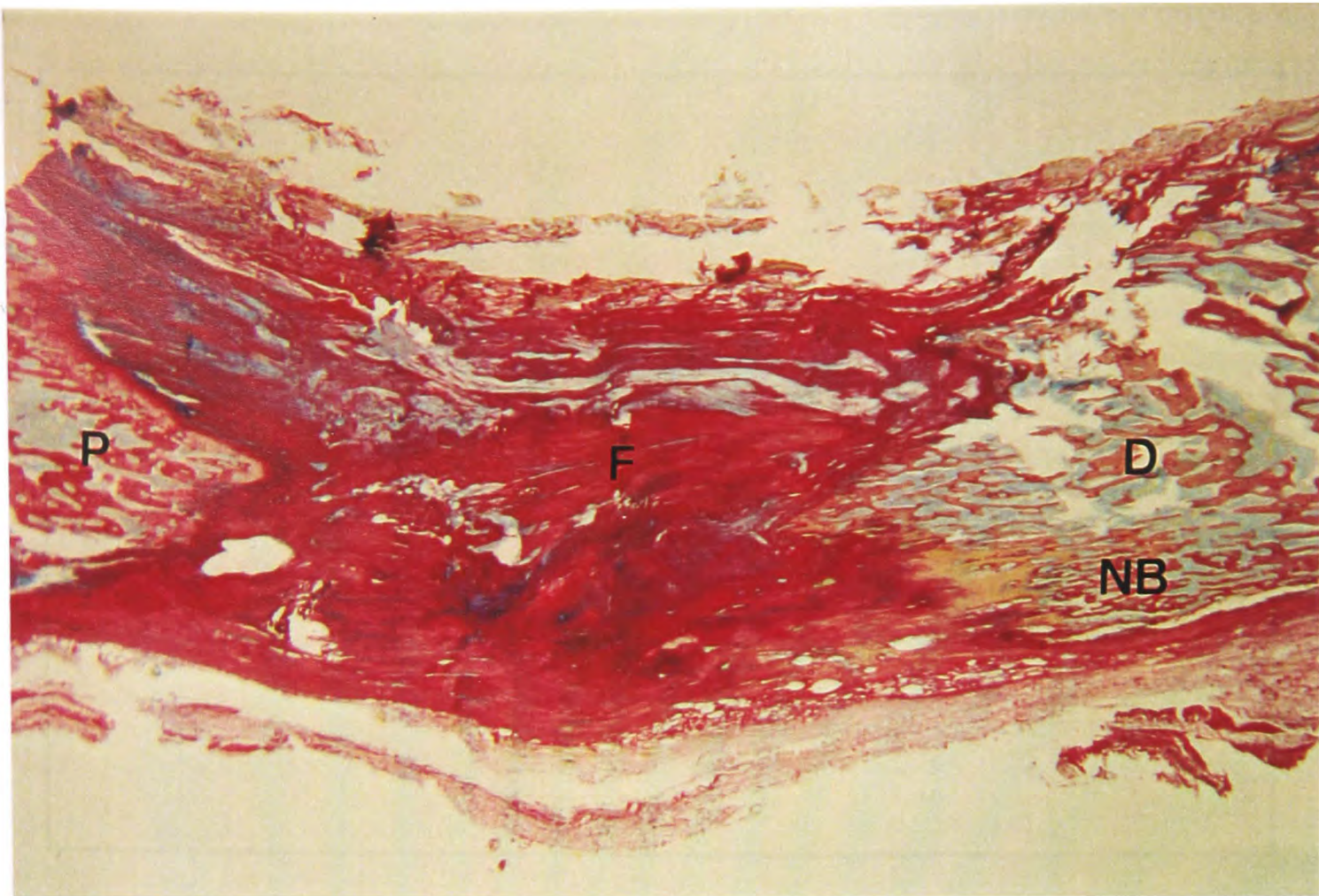


Fig. 3. 20. Representative of the morphological appearance of the regenerate of the 2.7 mm/day group. P: proximal end; D: distal end; F: fibrous tissue; NB: new bone. Alcian blue / sirius red staining, original magnification, X 12.

3. 5 Definition of the zones of the regenerate

The morphological and radiographic appearances of the distraction regenerates have similarities in all the distraction groups, which can be classified and divided into three distinct zones as showed in Fig 3. 21.

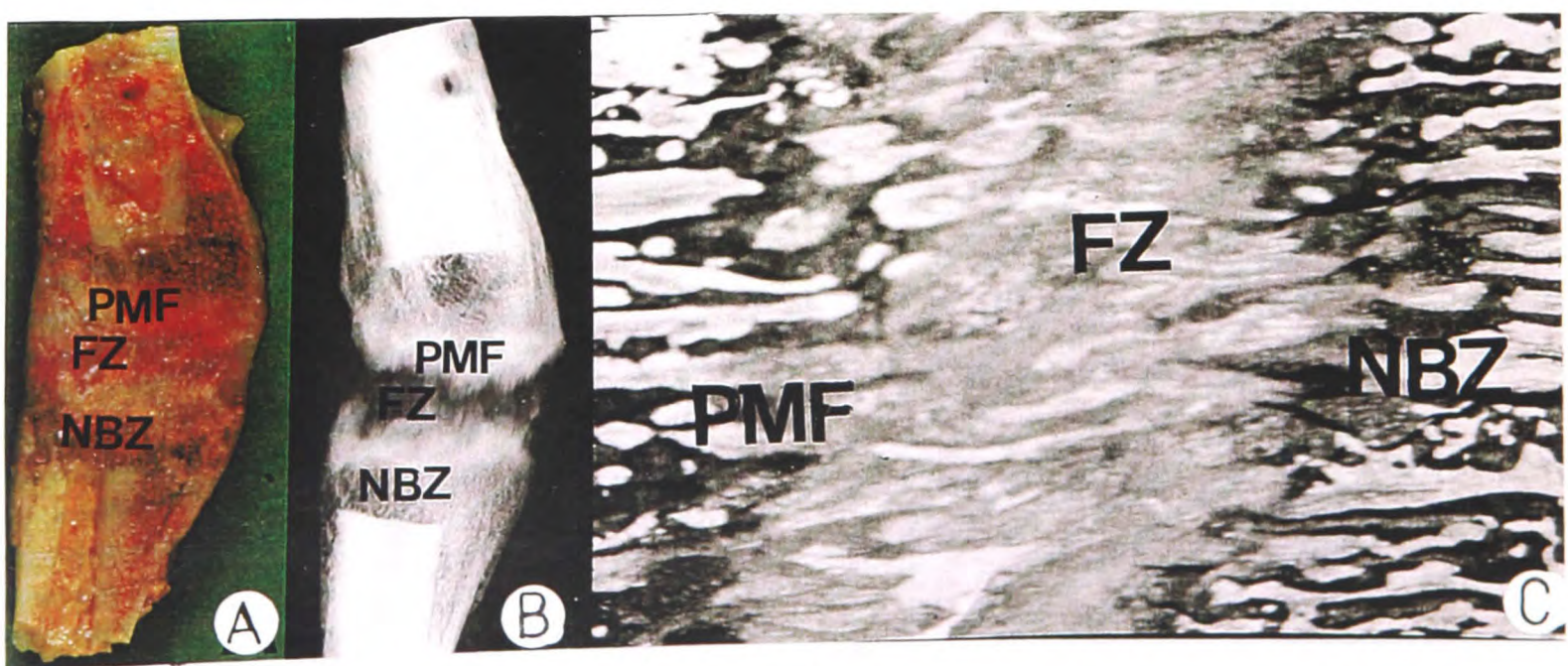


Fig. 3. 21. A: Gross sample appearance of the 0.7 mm/day group. B: Microradiography of the regenerate, showing the central radiolucent zone (FZ) and two adjacent sclerotic zones (PMF). C: The regenerate was divided into three zones: the central fibrous zone (FZ), the primary mineralization front (PMF) and the new bone zone (NBZ). HE staining, X 8.

Zone I: the central fibrous zone (FZ) localised in the middle of the distraction regenerate (Fig. 3. 21 A, C). The FZ on radiography appears as a radiolucent zone (Fig. 3. 21 B), which gradually elonged in length as the distraction rate increased. The FZ contains mainly fibroblast-like cells.

Zone II: the primary mineralization front (PMF) was adjacent to the central fibrous zone (Fig. 3. 21 C), and contained longitudinally arranged, well-vascularized collagen bundles, which were undergoing mineralization. New bone trabeculae were formed in this zone. The PMF on radiography appeared as a sclerotic band (Fig. 3. 21 B).

Zone III: the new bone zone (NBZ) was observed between the PMFs and the osteotomied bone ends, and contained mineralized woven and lamellar bone. Rapid bone remodelling occurred in this zone. The NBZ on X-ray appeared as a zone of relative radiolucency adjacent to the sclerotic band of the PMF (Fig. 3. 21 B).

3. 6 The length of zones of the regenerates

The lengths of zones of the regenerates of the different groups were measured from the radiographs by ruler and from the histological sections under light microscopy. The mean length of the zones and standard errors were summarised in Table 3. 1. As the distraction rate increased, the length of the FZ increased and that of the NBZ decreased, but the PMF remained relatively constant at 2-4 mm in length at all distraction rates (Fig. 3. 22)

Table. 3. 1. The length of zones of the regenerate (Means $\pm$ SE)

Rate (mm/d)	FZ		PMF		NBZ		Total (mm) Lengthening
	FZ (mm)	SE (mm)	PMF (mm)	SE (mm)	NBZ (mm)	SE (mm)	
0.3	3.47	± 0.84	1.60	± 0.20	14.70	± 0.44	19.77 $\pm$ 1.48
0.7	5.27	± 0.47	2.17	± 0.26	13.07	± 1.11	20.51 $\pm$ 1.84
1.3	7.98	± 0.48	1.90	± 0.10	11.00	± 0.38	20.88 $\pm$ 0.96
2.7	11.25	± 1.75	3.00	± 0.50	5.25	± 0.25	19.50 $\pm$ 2.50

FZ: fibrous zone; PMF: primary mineralization front; NBZ: new bone zone; SE: standard error.

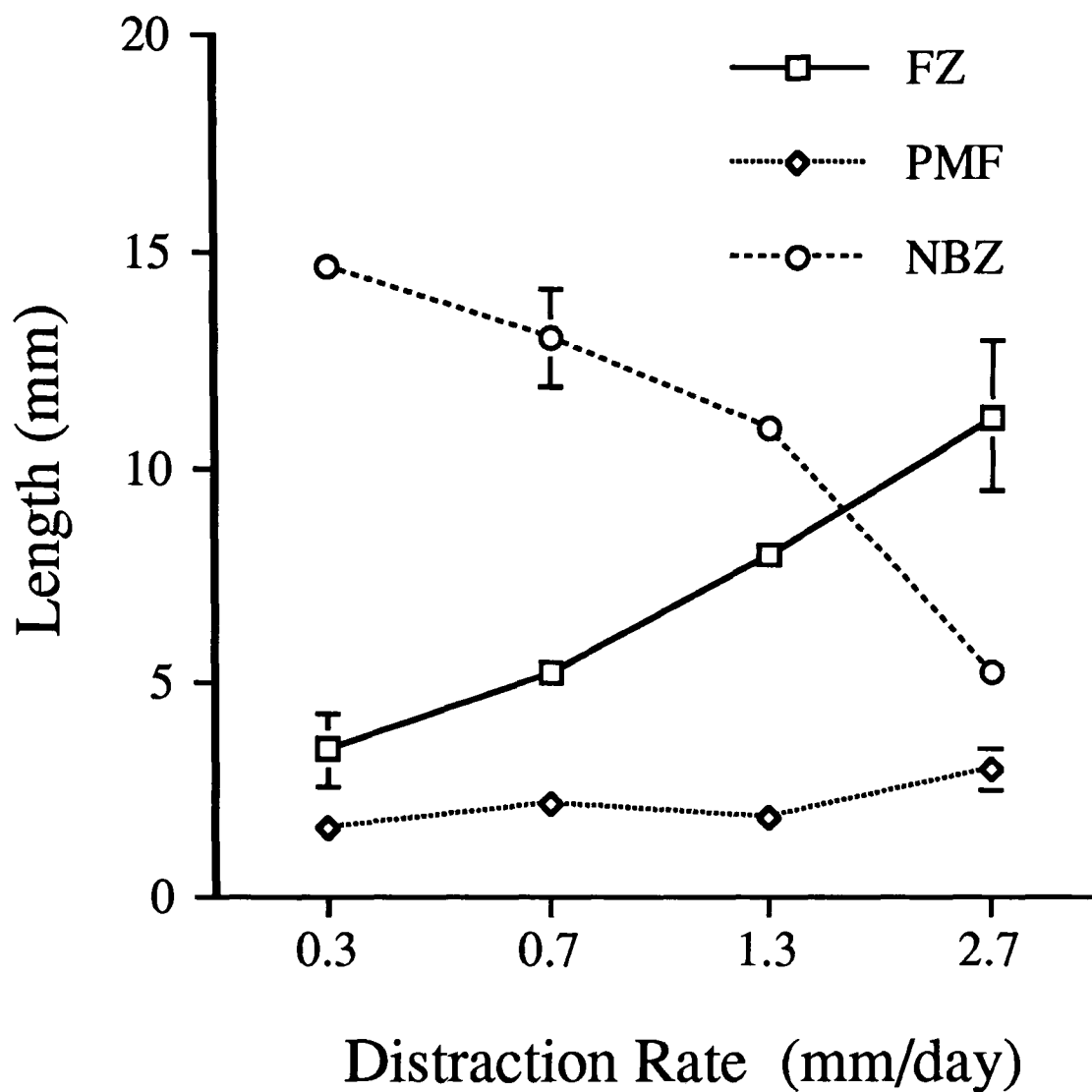


Fig. 3. 22. The length of different zones of the regenerates. As the distraction rate increased, the length of the FZ increased and the length of the NBZ decreased, but the PMF remained relatively constant at 2-4 mm in length at all distraction rates. Means and standard errors were plotted.

3. 7 Summary and discussion

In the present study, the process of distraction osteogenesis at 4 different distraction rates has been investigated radiographically and histologically in a rabbit model of leg-lengthening. To investigate the effects of distraction rates on distraction osteogenesis, the variables (such as rhythm and the amount of lengthening) have been kept constant. By keeping the amount of lengthening constant and varying the distraction rate, an inevitable sequel is that the tissue is obtained at different times post-osteotomy. The magnitude of lengthening was 20% and this was constant in all lengthening groups, but the rate of distraction varied from 0.3 to 2.7 mm/day, so that the sacrifice time of different groups post-surgery varied from 8 weeks (0.3 mm/day) to 1 week (2.7 mm/day). This needs to be born in mind when considering the results.

The events following the osteotomy consisted of an initial waiting period, in which periosteal and endosteal callus had begun to form, followed by a distraction period, and finally a consolidation phase. During the distraction period, histological examinations showed new trabeculae emanated from both ends of the osteotomied bone, and terminated in a central fibrous interzone. The central fibrous interzone consisted of spindle-shaped fibroblastic cells oriented parallel to the distraction force, these cells appeared gradually to differentiate into osteoblasts, and to produce new bone tissue. The PMF was the zone of turning the fibrous tissue into bone tissue, where highly oriented collagen bundles were mineralized to form new bone. The new bone was formed parallel to the distraction forces, initially in a pattern of woven bone, and later replaced by lamellar bone.

Slow distraction rates are sometimes employed clinically because of poor adaptation of the soft tissues. In this model, at a distraction rate of 0.3 mm/day, direct bone formation was achieved. In areas the regenerate appeared to have healed and fractured, which would support the clinical observation that premature consolidation can occur at slow rates of distraction (Shearer, et al. 1992). At a distraction rate of 0.7 mm/day, most bone formed in an intramembranous manner, with a few small cartilaginous areas and a few small areas of haemorrhages and cysts, but the overall morphological appearance was satisfactory. Small areas of endochondral ossification were also observed, which concurs with the findings of several previous studies (Kojimoto, et al., 1988; Delloye, et al., 1990; Masamizu, et al., 1994). Ilizarov (1989a, 1990) suggested that the presence of cartilage was the result of mechanical instability and found that maximum stability led to intramembranous bone formation, whereas less rigid or unstable fixation led to endochondral bone and fibrous tissue formation. Aronson, et al. (1989) agreed that the instability during the lengthening process could be the main reason for cartilage formation during lengthening. The device used in this study was a strong rigid unilateral fixator, which was applied in a controlled manner and the mechanical environment was likely to be similar at all rates of distraction. However, it is possible that at the higher distraction rates, the large daily increment and resultant displacement between the osteotomy ends at the time of lengthening may disturb the vascularization and local blood supply to the regenerating tissues.

As a vascular-dependent process, the rapidity of new bone formation during the distraction

process required the maintenance of an adequate blood supply. At distraction rates of 1.3 mm/day and 2.7 mm/day, in the centre of the regenerate, there were considerable numbers of cartilage islands, haemorrhages, cysts and regions of necrotic tissues, which suggested lack of blood supply to the central regenerate at higher rates of distraction.

Aronson (1994b) studied the blood supply of the distraction regenerate by using technetium scintigraphy. The newly formed bone region showed intense radioactivity indicating an extensive blood supply, whereas the central fibrous region showed relatively little vascularization. At the higher rates of distraction (> 0.7 mm/day) in the present study, the daily increments might have exceeded the rate of vascularization, resulted in the growth of blood vessels failing to match the extent of lengthening, and blood supply to the centre of regenerate being greatly reduced. In addition, the excess stretch applied to the tissues may cause spasm and damage of blood vessels and hence cause haemorrhage and reduced the blood supply. Lack of blood supply would cause low oxygen tension in the tissue, hence the differentiation of osteoprogenitor cell maybe switched towards the chondrogenic pathway to produce cartilage. Necrosis and cyst formation have occurred as a result of prolonged lack of blood supply.

Aronson, et al. (1990) divided the distraction regenerate into three zones according to morphology and the calcium content. The zones were defined as the fibrous interzone (30% of normal bone calcium content); the primary mineralization front (40% of normal bone calcium content) and the new bone formation zone (60% of normal bone calcium content). In agreement with Aronson, the distraction regenerate was divided into the FZ, the PMF and the NBZ in the present study. Each of these zones has a distinct morphological and radiographic appearance, and they are referred in the further studies in this thesis.

In the present study, it was observed that the newly formed bone tissue underwent active remodelling throughout the distraction period. However, in the experiments reported here, the animals were killed at completion of the lengthening period and consolidation of the regenerate was not complete at this point.

In conclusion, fast rates of distraction diminish the quality of the regenerating bone, and at slow rates there is a risk of premature union. Based on the radiography and histology, the distraction regenerate can be divided into three distinct zones: the central fibrous zone, the primary mineralization front and the new bone zone. In the present rabbit model of leg-lengthening, radiographic and histological examinations show that a rate of distraction at 0.7 mm/day appears to be most satisfactory for bone formation during distraction osteogenesis.

Chapter 4 Assessment of cell proliferation during bone regeneration

4. 1 Introduction

Codivilla (1905) first introduced the concept of limb lengthening nearly a century ago. Limb lengthening, by means of osteotomy and distraction of callus under the control of an external fixator, is still a developing technique of wide-spread clinical application. However, little is known about the biological mechanisms involved in distraction osteogenesis and in particular the responses of the osteogenic precursor cells to distraction. To generate bone tissue, adequate proliferation of bone-forming cells is of basic importance. The proliferative response of osteogenic cells to varying distraction rates during distraction osteogenesis has not previously been studied.

Labelling with bromodeoxyuridine (BrdUrd) as a method to assess cell proliferation, has been established and validated in skeletal tissue (see section 1. 3. 5). Monoclonal antibodies, such as Bu20A, are commercially available and may be used to display localisation of BrdUrd following cellular uptake on the histological section (Albery and Pelton, 1993; Apte and Kenwright, 1994). The expression of PCNA can also be served as a marker of cell proliferation. Previous studies in a variety of human tissues, have shown that a monoclonal antibody (PC10) may be employed to detect PCNA in formalin-fixed, paraffin-embedded tissues (see section 1. 3. 4 for detail). Recently, the PCNA has been used as a marker to study cell proliferation during rat fracture repair (Iwaki, et al., 1997).

The prime aim of this chapter was to examine the hypothesis that the rate of cell proliferation was affected by the rate of distraction during distraction osteogenesis. BrdUrd labelling and its immunohistochemical detection were used to assess cell proliferation in the present study. First, cell proliferation was assessed in the sham groups, in which the tibial osteotomies were fixed by external fixator for 1 or 2 weeks, but without distraction. Second, the effect of four different distraction rates on cell proliferation during the distraction period was assessed. A

subsidiary aim of this chapter was to compare the assessment of cell proliferation by PCNA expression and by the uptake of BrdUrd in the rabbit tissues.

4. 2 Methods

4. 2. 1 Experimental methods

Open transverse osteotomies of the right mid-tibial diaphysis were performed and fixed with external fixators (Orthofix M100, Italy) on 19 male NZW rabbits (age 24 weeks, body weight 3.0-3.5 Kg) as previously described. After a latency period of seven days, distraction was started at 0.3 mm / day (n=3) for 8 weeks, 0.7 mm / day (n=4) for 4 weeks, 1.3 mm / day (n=3) for 2 weeks and 2.7 mm / day (n=3) for 1 week, in two steps per day, until lengthening to 20% (approximately 2 cm) of the original tibia length, and as soon as the lengthening had been achieved, the animals were killed. There was also a sham group who underwent osteotomies and external fixation for 1 week (n=3) and 2 weeks (n=3), but did not undergo lengthening. BrdUrd (Sigma, Poole, Dorset, UK) 40mg/kg was injected intravenously by an ear vein one hour before killing.

The regenerate was excised carefully and halved longitudinally in the median plane of the tibia using a band saw, and one half were fixed in 4% paraformaldehyde (pH 7.4) for 24 hours, and the other half was fixed in Carnoy's fluid for approximately 6 hours, decalcified in buffered 14.5% EDTA (pH 7.2) for 3-4 weeks at room temperature and embedded in paraffin wax. 5 µm thick sections were cut and placed on poly-L-lysine-coated slides for immunohistochemistry.

For immunodetection of BrdUrd uptake and PCNA expression, sections were dewaxed in xylene and taken through graded ethanol (100%, 90%, 70%, 50%). Endogenous peroxidase activity was blocked by immersion in 80% methanol containing 0.5% (v/v) hydrogen peroxide for 30 min. Sections were then incubated with monoclonal antibodies, either PC10 (gift from K. Gatter, Department of Pathology, John Radcliffe Hospital, Oxford) at a dilution of 1:10 or

Bu20A (gift from K. Gatter) at a dilution of 1:5 for one hour, in a humid chamber at room temperature. For BrdUrd detection by Bu20A, the sections were treated with 1N HCl at 60°C for 15 min prior to incubation with the antibody. Normal rabbit serum (10%) in PBS containing 0.05% Tween 20 (BDH, Poole, UK) was used to block the non-specific binding of the secondary antibody. The sections were then incubated with rabbit anti-mouse peroxidase conjugated antibody (Sigma, Poole, Dorset, UK) at a dilution of 1:50 in PBS for 30 min. Peroxidase activity was visualised by incubation with 0.5mg/ml 3, 4, 3', 4', -tetrabiphenyl hydrochloride (Sigma, Poole, Dorset, UK) in PBS (pH7.2) containing 0.3% (v/v) hydrogen peroxide for approximately three minutes, and the sections were lightly counterstained with Mayer's haematoxylin, dehydrated through graded ethanol, cleared in xylene, and finally mounted with DPX (BDH, Poole, UK).

4. 2. 2 Critical step for Bu20A immunostaining

The critical step for BrdUrd immunodetection is DNA denaturation. Since BrdUrd was incorporated into the double helix during DNA synthesis, it is literally inaccessible to the anti-BrdUrd antibody. Denaturation is a process to separate double-stranded DNA to single-stranded one by rupturing the hydrogen bonds on the DNA double helix. The denaturation may be induced by heat, extremes of pH, a variety of chemicals or by a combination of several methods (Adams et al, 1981). Apte (1990) has examined different conditions for DNA denaturation on the bone sections and he has recommended that denaturation with 1N HCl at 60°C for 15 min is optimal for paraformaldehyde or Carnoy's fluid-fixed, paraffin-embedded bone tissue sections. Longer incubation time with HCl or at higher temperature will compromise the morphology of the sections and increase the risk of section detachment from the slide. Slides coated with poly-L-lysine provide better section adhesion than uncoated slides or slides coated with glycerin-egg albumin.

4. 2. 3 Special notes for PC10 immunostaining

The use of PC10 as a reliable marker for cell proliferation is still controversial. The reliability

of using PC10 as a cell proliferation marker has been shown to vary in different tissues and with tissue preparation (Yu, et al., 1992; Yu and Filipe, 1993). PC10 immunostaining may be altered by different histological fixatives and immunostaining methods. My preliminary experiment of immunostaining using PC10 showed that the number of stained cells increased concurring with the increase of the incubation time for the enzyme substrates. In the present study it was found essential to maintain a standard immunostaining procedure for PC10. PC10 at a dilution of 1:10 and a colour development time of three minutes with freshly prepared DAB containing 0.3% (v/v) hydrogen peroxide, were found to be optimal conditions and the under which the immunostaining results were reproducible.

4. 2. 4 Interpretation of the immunostaining results

A cell was considered positively-stained if there was visible brown nuclear staining when examined by light microscopy, and the cells with blue nuclei (haematoxylin staining) were considered negatively-stained (Fig. 4. 1).

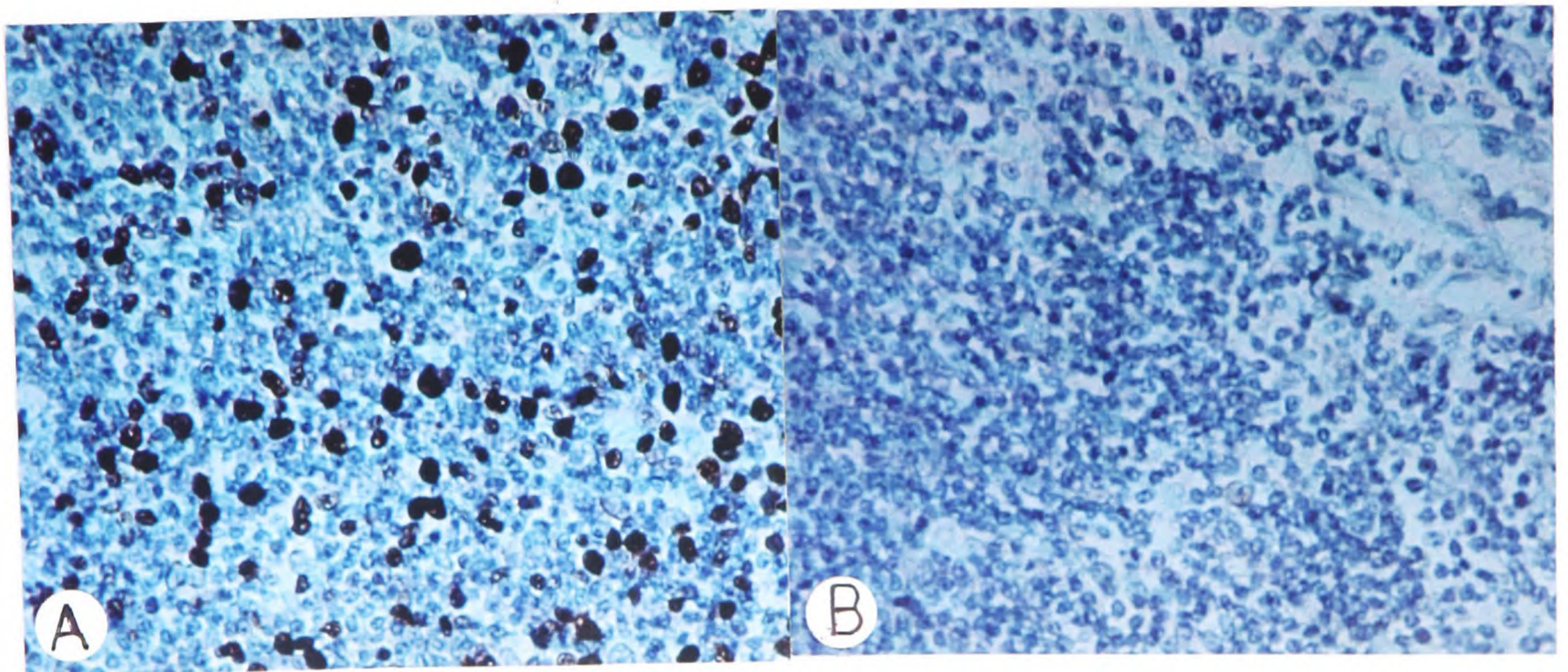


Fig. 4. 1. A: Bu20A immunostaining showing the Bu20A positive-stained cells (dark brown) on a rabbit thymus section. B: When Bu20A antibody was omitted, there was no positive staining. A and B, counterstained with Mayer's haematoxylin, X 40.

4. 2. 4. 1 Cell counting technique

For semi-quantitative analysis of the numbers of cells incorporating BrdUrd or expressing PCNA, the positively-stained cells as well as non-stained cells were counted by using a x 10 eyepiece graticule combined with a x 40 objective lens. The eyepiece graticule is a regular rectangle of 1 square millimetre (1 mm x 1 mm), which was evenly divided into 100 small rectangles by 10 horizontal and 10 vertical lines (Fig. 4. 2).

1									10
11									20
21									30
31									40
41									50
51									60
61									70
71									80
81									90
91									100

Fig. 4. 2. Diagram representing the layout of the eyepiece graticule, which was subdivided into 100 small regular rectangles and these rectangles are numbered as shown in the diagram.

Under light microscopy with x 400 magnification, positively and negatively stained cells were counted in randomly selected fields of similar morphological appearance on the longitudinal sections of the regenerates, according to the definition of the three distinct histological zones (see section 3. 5). Selection of fields for cell counting in the eyepiece graticule was performed using a random number tables (Fig. 4. 3), which contain randomly generated numbers ranging from 1 to 99. For example, when the generated number was 43, the cells in the number 43 small graticule were counted. Cells on the bottom and right hand lines of the graticule were included for the counting, whereas cells on the top and left hand lines were excluded from the counting. On each occasion, the numbers of positively or negatively stained cells of the chosen

graticule were recorded. The positive staining index (PSI) was calculated as: $PSI (\%) = \frac{\text{Number of positively stained cells}}{\text{Number of positively + negatively stained cells}} \times 100$. After obtaining counting data from small graticules, the cumulative mean PSI and the standard error (SE) of the mean PSI were calculated as each of the subsequent counting proceeds.

16	90	82	66	59	83	62	64	11	12	67	19	00	71	74
11	27	94	75	06	06	09	19	74	66	02	94	37	34	02
35	24	10	16	20	33	32	51	26	38	79	78	45	04	91
38	23	16	86	38	42	38	97	01	50	87	75	66	81	41
31	96	25	91	47	96	44	33	49	13	34	86	82	53	91
66	67	40	67	14	64	05	71	95	86	11	05	65	09	68
14	90	84	45	11	75	73	88	05	90	52	27	41	14	86
68	05	51	18	00	33	96	02	75	19	07	60	62	93	55
20	46	78	73	90	97	51	40	14	02	04	02	33	31	08
64	19	58	97	79	15	06	15	93	20	01	90	10	75	06
05	26	93	70	60	22	35	85	15	13	92	03	51	59	77
07	97	10	88	23	09	98	42	99	64	61	71	62	99	15
68	71	86	85	85	54	87	66	47	54	73	32	08	11	12
26	99	61	65	53	58	37	78	80	70	42	10	50	67	42
14	65	52	68	75	87	59	36	22	41	26	78	63	06	55

Fig. 4. 3. Example of random sampling numbers, based on which the field for counting on the eyepiece graticules were selected.

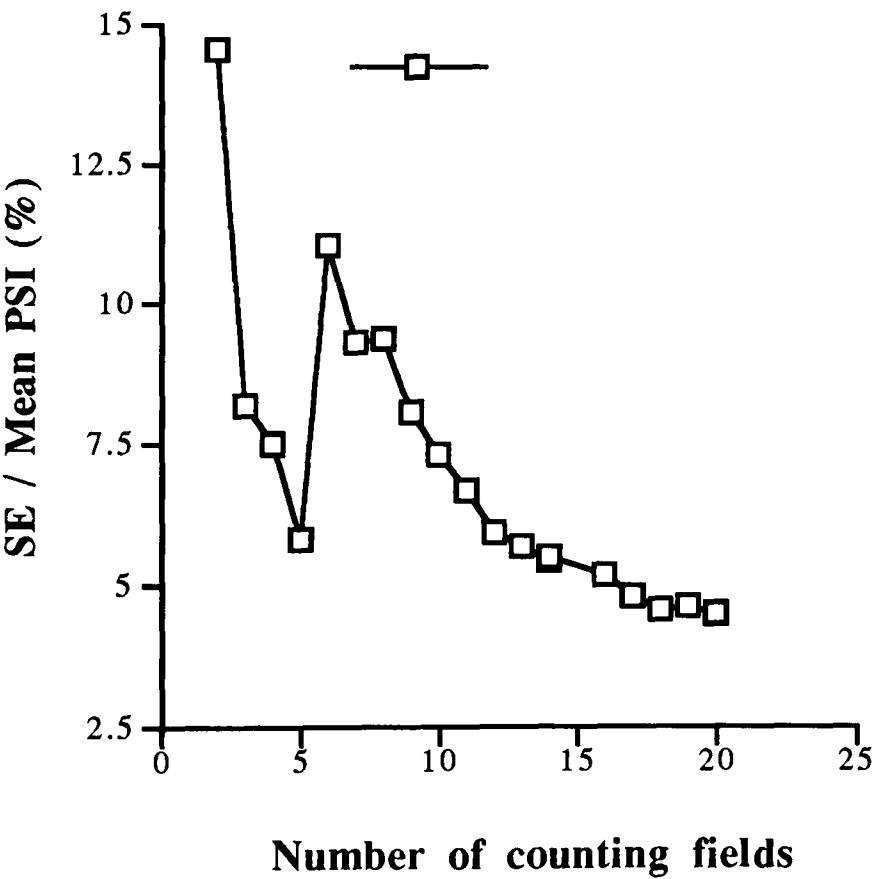


Fig. 4. 4. An example showing the relationship between the sample size (the number of counting fields) and the value of SE/Mean PSI. The SE/Mean PSI became constantly less than 10% after counting in 15 randomly selected fields, and more cell counting made no change of the cumulative SE/Mean PSI.

Counting was continued until the cumulative SE of the PSI was less than 10% of the cumulative PSI, this was determined by a stereological technique which ensures a reproducible result (Weibel, 1979). The sample size (the number of counting fields) determines the value of SE / Mean PSI, as demonstrated in Fig. 4. 4. To maintain the cumulative SE / Mean PSI being less than 10%, a minimum of 500 cells in each morphological zone were counted. About 2000 cells in total in each section were counted and for each sample a minimum of 2 sections were examined.

4. 2. 4. 2 Statistical analysis

The mean values of PSI and their SE were calculated for 3 different morphological zones in each specimen. Since the values of PSI of 3 different zones in 4 different experimental groups were unpaired data, unpaired one-way analysis of variance (ANOVA) was used for a comparison of the values of PSI within or between groups (Harper, 1982). F value at $P = 0.05$ or $P = 0.01$ can be found from F value tables (Hayslett, 1985). In this study: If $V_R < F_{0.05}$, then $P > 0.05$, and there was no statistically significant difference. If $V_R > F_{0.05}$, then $P < 0.05$, and there was a statistically significant difference.

4. 3 Studies of cell proliferation during bone regeneration: results and discussion

4. 3. 1 Study of cell proliferation in the osteotomy groups

The events that occur during fracture repair include inflammatory reaction, chondrogenesis, intramembranous ossification, and endochondral ossification. In each event, various types of cells proliferate and differentiate. The cellular proliferation of osteogenic progenitor cells during fracture healing is still poorly defined. In order to study the cell proliferation during distraction osteogenesis, it would be of benefit to know the degree of cellular proliferation during the latency period prior to distraction. This section was to investigate cell proliferation during fracture repair 7 and 14 days after the tibial osteotomies.

4.3.1.1 1 week post tibial osteotomy

At this stage there was noted to be a marked periosteal response and newly dividing fibroblastic cells were seen on the inner or deep surface of the periosteum and on the bone surface of forming callus (Fig. 4. 5). The dividing cells were seen throughout the area where periosteum had been stripped about 2 cm each side from the osteotomy, and were evenly distributed. A few Bu20A-positive cells were seen in the fracture gap including the intracortical area, the endosteal area and the area of the medullary cavity. There was a concentration of dividing cells seen around blood vessels in all the areas, particularly, in the medullary cavity near the osteotomy (Fig. 4. 6).

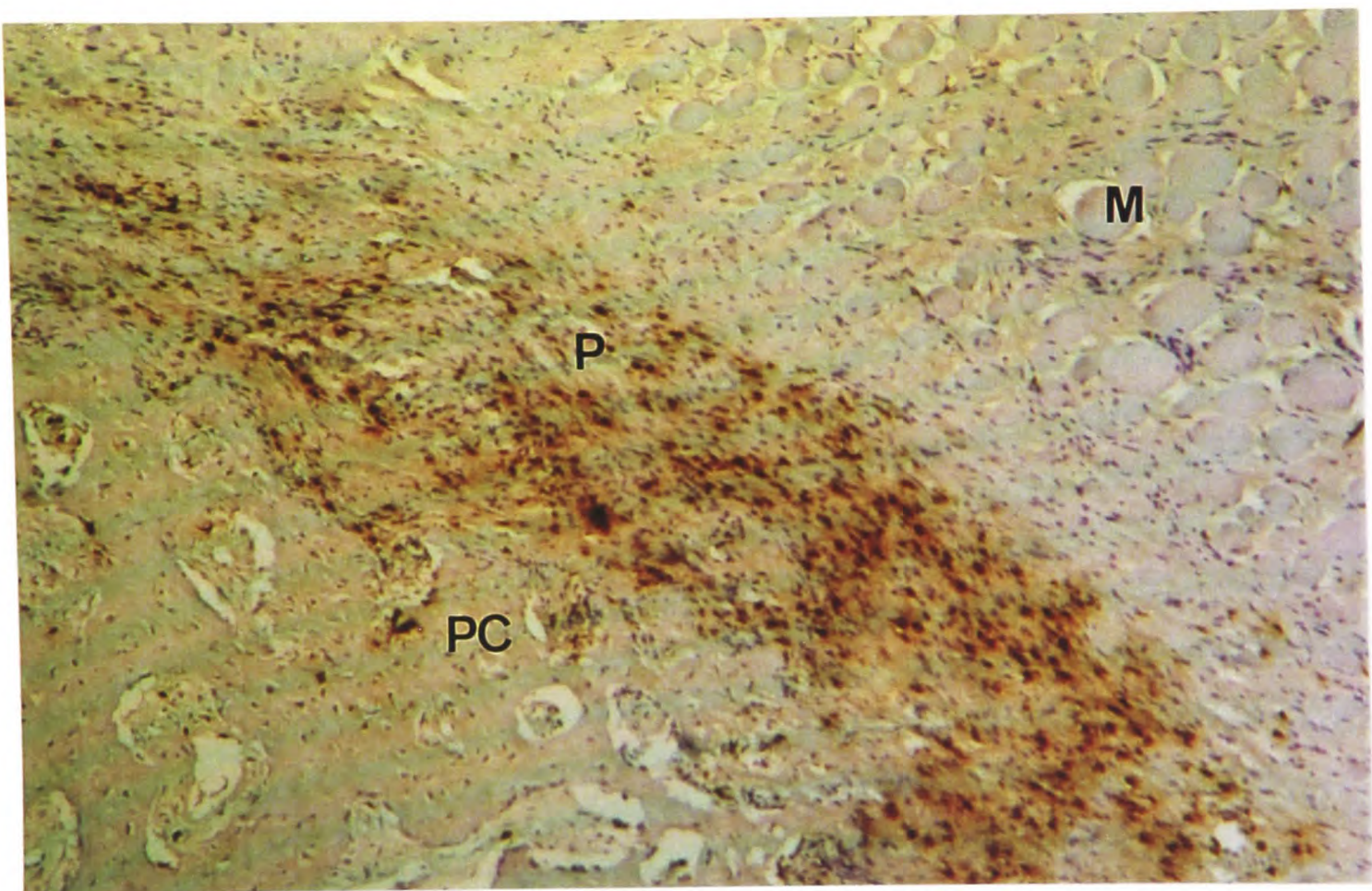


Fig. 4. 5. 7 days post osteotomy, the periosteum was thickened and Bu20A-positive cells (brown) were seen in the inner layer of the periosteum and close to the bone surfaces in the periosteal callus. PC: periosteal callus; M: muscle; P: periosteum. Bu20A immunostaining, X 25.

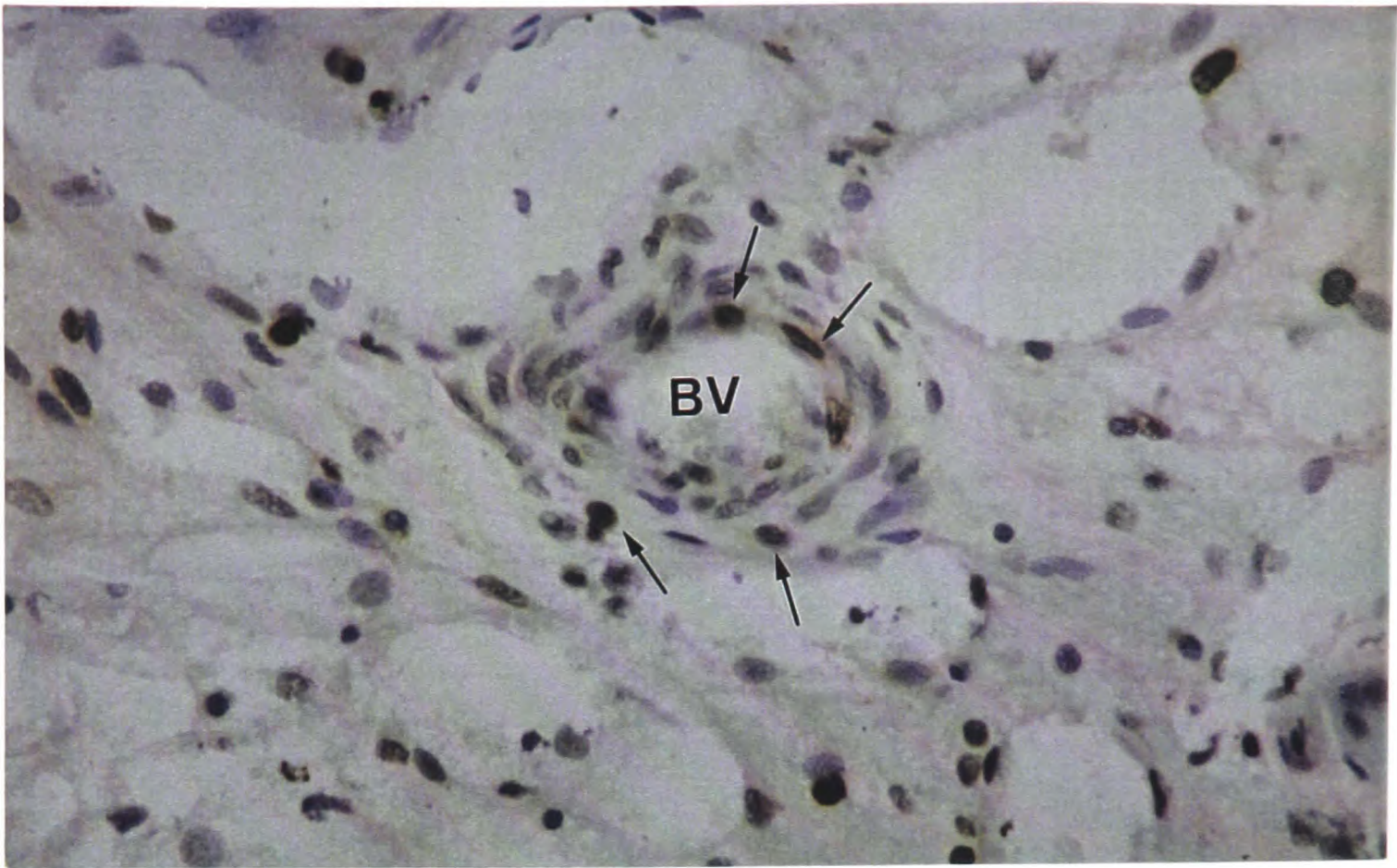


Fig. 4. 6. At 7 days post osteotomy, an example showing that there was a concentration of dividing cells (brown, arrows) around the blood vessel (BV) within the medullary cavity. Bu20A immunostaining, X 100.

4. 3. 1. 2 2 weeks post tibial osteotomy

It was noted that the periosteal dividing cells had slightly decreased at this time point. Bu20A-positive cells were seen predominantly in the periosteum as well as on the bone surfaces of the newly formed callus (Fig. 4. 7A).

There was a marked increase in the number of proliferating primitive fibroblastic cells to be seen in the gap of osteotomy (Fig. 4. 7 B), in the endosteal region and in the region of the medullary cavity (Fig. 4. 8 A), and in many instances this proliferative response extended by more than 3 cm from the osteotomy. Bu20A-positive cells were also found in some chondrocytes within the cartilage islands near the osteotomy gap (Fig. 4. 8 B).

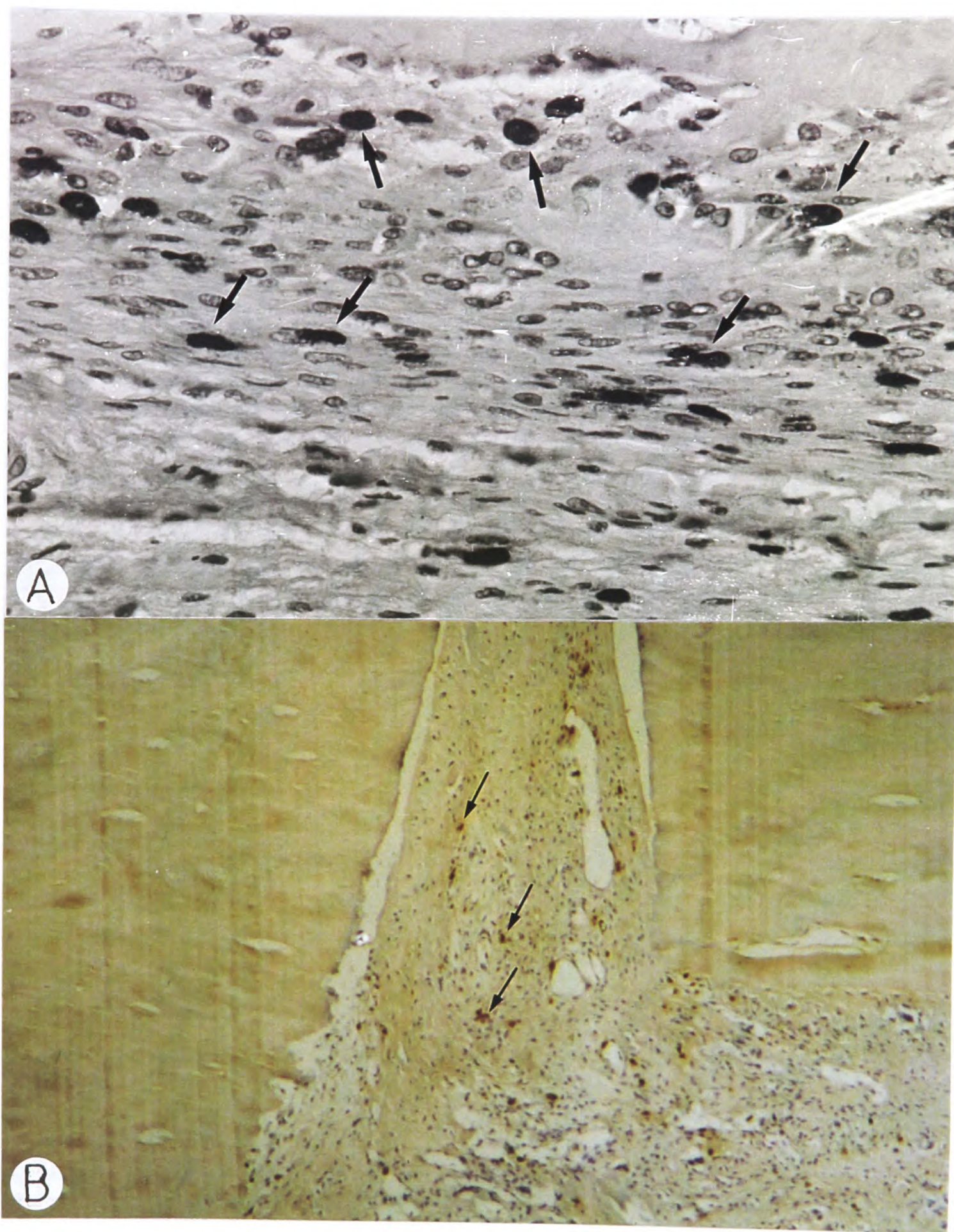


Fig. 4. 7. 2 weeks post tibial osteotomy. A: Bu20A-positive cells were seen in the periosteum and on the bone surfaces of the periosteal callus (arrows). B: there was a marked increased in the number of proliferating cells (brown nuclei) in the gap of osteotomy. Bu20A immunostaining, A and B, X 25.

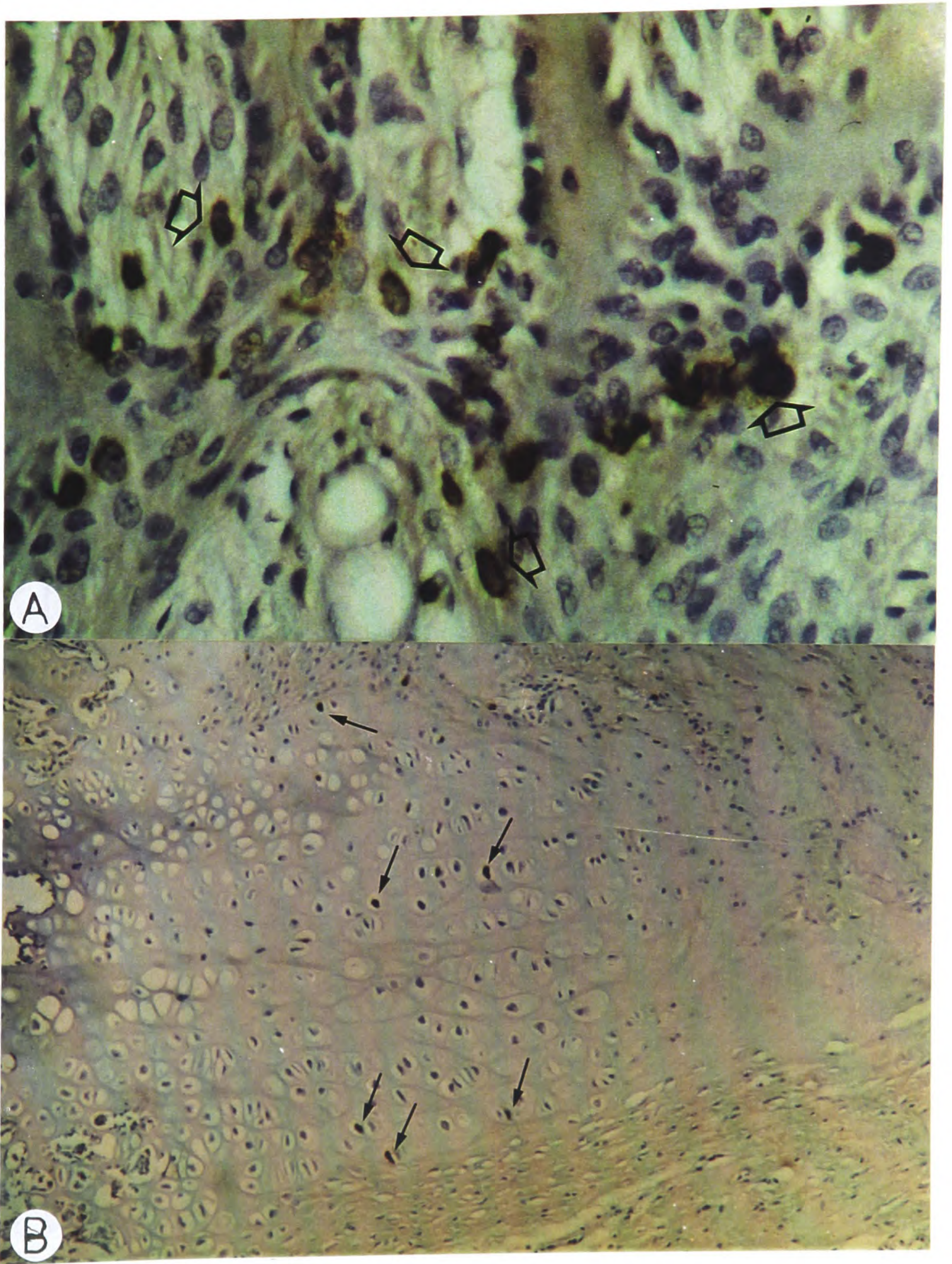


Fig. 4. 8. 2 weeks post tibial osteotomy. A: the number of Bu20A-positive cells (arrows) was markedly increased in the region of the medullary cavity. B: some chondrocytes within the cartilage island were Bu20A positive (arrows). A and B, X 25.

4.3.1.3 Summary and discussion

The present model was one in which external skeletal fixation was used, the fixator was attached remote to the osteotomy and therefore with little interference at the osteotomy site. This is a different type of fixation from that described in previous studies in which fractures have not been stabilised (Tonna and Cronkite, 1961) or have been stabilised using internal fixation (Ashhurst, 1985; Iwaki, et al., 1997). It has therefore been possible to make a comparison of the effects of the mechanical conditions on fracture healing, and these observations in the present study have served as a control group for further investigation of cell proliferation in distraction osteogenesis.

The findings described above may be summarised as follows: (1) Proliferating cells which take up BrdUrd were detected by immunohistochemistry. The proliferative response was observed in the whole callus, but with different distributions at different times. (2) The importance of the periosteal contribution in the early phases (1 week post osteotomy) of fracture repair has been confirmed. The late contribution (2 weeks post osteotomy) of cell division from the endosteal region, and from the medullary cavity, has also been defined. (3) Proliferating cells were found predominantly adjacent to new blood vessels. The localization of these dividing cells has not been defined in previous studies.

The first reaction to a fracture is proliferation of mesenchymal cells from thickened periosteum on the periosteal surface of the bone. The fractured area is gradually invaded by capillaries, which bring the cells include fibroblasts, macrophages, monocytes and osteoprogenitor cells. The source of these cells is not clearly known yet, but there is evidence that some may migrate from the surrounding connective tissues while others are blood-borne (Ashhurst, 1986; Frost, 1989 a, b). Cell proliferation in the early periosteal reaction was reported to peak at 32 h after a fracture in rat femur using ^3H -thymidine incorporation (Tonna and Cronkite, 1961). This finding was recently confirmed by Iwaka et al. (1997) using immunohistochemistry with anti-PCNA antibody on a similar rat fracture model. They found that the peak of cell proliferation was 36 hrs after a fracture. In both studies, the labelling index at the peak was approximately 10 times that of the control, and the labelling index decreased as fracture healing progressed.

However, 14 days after fracture, the labelled population of periosteal cells at the fracture site was still above the value of the controls (Tonna and Cronkite, 1961).

The present study confirmed the previous findings that cellular proliferation in the periosteal region was the most important contributor in the early phases of fracture repair (1 week post osteotomy), and the cellular response was not limited at the fracture site where the inflammatory reaction had occurred, but extended in the periosteum along almost the entire tibial diaphysis where intramembranous ossification was initiated. At later stage, 2 weeks post osteotomy, the periosteal reaction was reduced but the late contribution of cell proliferation from the endosteal region, and the medullary cavity has become apparent. This indicates that osteoprogenitor cells in the bone marrow might contribute to fracture healing in a late stage, and bone marrow cells may serve as a pool of osteogenic cells. Endochondral ossification appeared on days 14 and some premature chondrocytes were Bu20A-positive. Bu20A-positive cells were found predominantly adjacent to blood vessels, which indicated the importance of the blood supply and the involvement of the endothelial cells for the development of new bone.

The present study reinforces the importance of periosteum and blood supply in the early stage fracture repair and the marked contribution of bone marrow cells from the medullary cavity in late stages fracture healing. The degree of cell proliferation varied according to the time lag after the fracture, suggesting that local regulatory factors such as growth factors, cytokines, and the vascularization of the fractured bone are important for fracture healing.

4. 3. 2. Cell proliferation during distraction osteogenesis

Assessment of cell proliferation in the regenerating bone tissues during distraction osteogenesis at 4 different distraction rates, was performed using the technique of BrdUrd uptake and its immunohistochemical detection. Semi-quantitative results of cell proliferation in the three different morphological regions on the histological section of the regenerates were obtained by cell counting according to a stereological technique (see section 4. 2. 4. 1). For each specimen the means and standard errors of the PSI were calculated from the different morphological regions, and the results were compared between the different groups.

4.3.2.1 0.3 mm/day group

At the rate of distraction of 0.3 mm/day, the distraction gap consisted almost entirely of trabecular bone and some fibrocartilagenous tissue with a narrow central fibrous zone. The FZ and the PMF overlapped in some of the animals. The Bu20A-positive cells were seen mainly near the bone surfaces (Fig. 4. 9A), which were also positive for alkaline phosphatase (Fig. 4. 9 B). These cells were considered to be preosteoblasts or osteoblasts as they were polygonal, plump cells with large round nuclei and a basophilic cytoplasm (Fig. 4. 9 C).

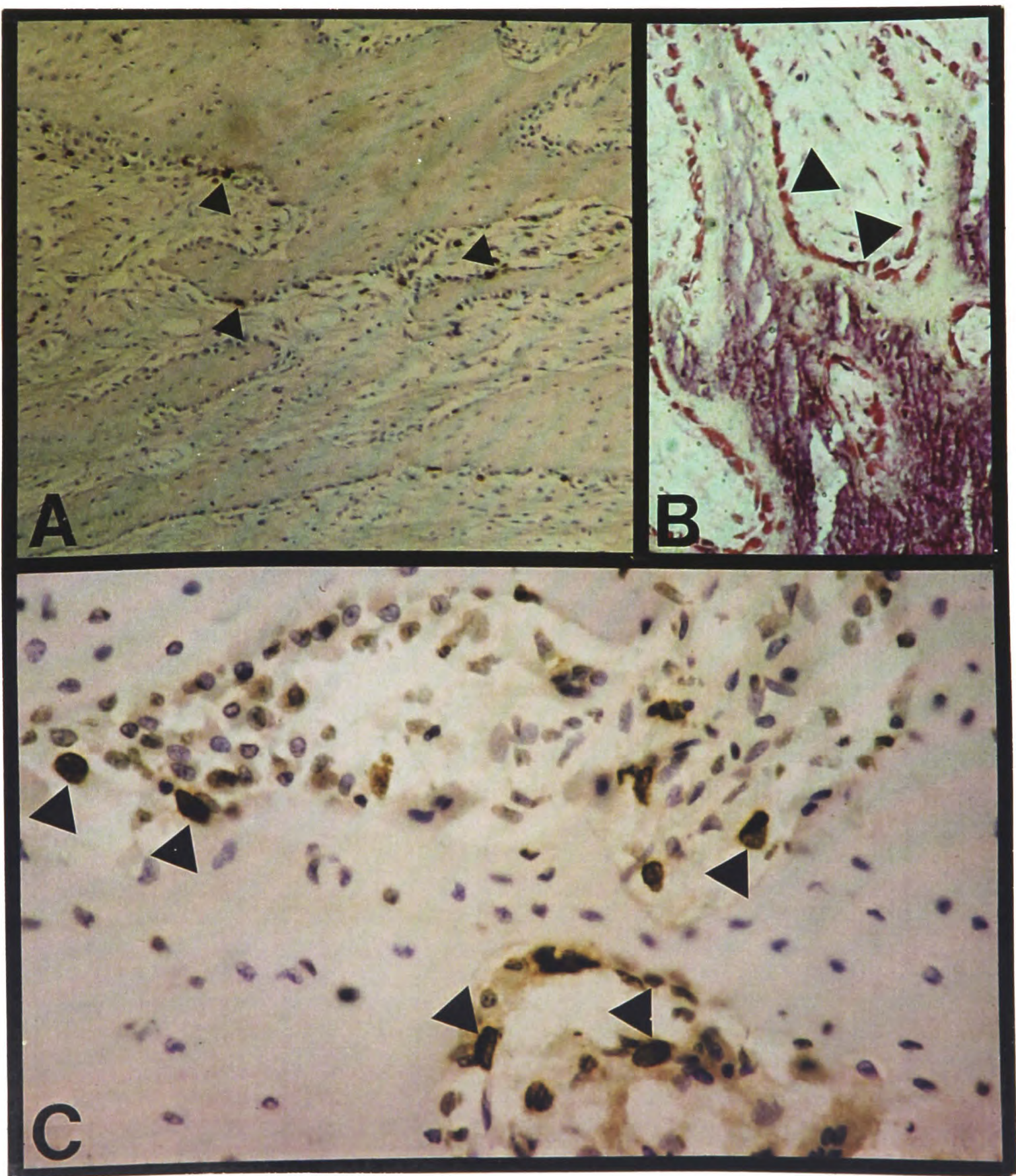


Fig. 4. 9. A regenerate of the 0.3 mm/day group. A: Bu20A-positive cells were seen on the bone surfaces (arrows). B: These bone surface cells were alkaline phosphatase positive (arrows). C: Close-up of the bone surface Bu20A-positive cells (arrows). A-C, X 100.

The Bu20A-positive cells were evenly distributed through the distraction gap, with a slight increase in the PMF. The values of PSI of the FZ, the PMF and the NBZ were calculated in 3 animals and there was no statistical difference of the PSI values in any of the zones within this group (Fig. 4. 10).

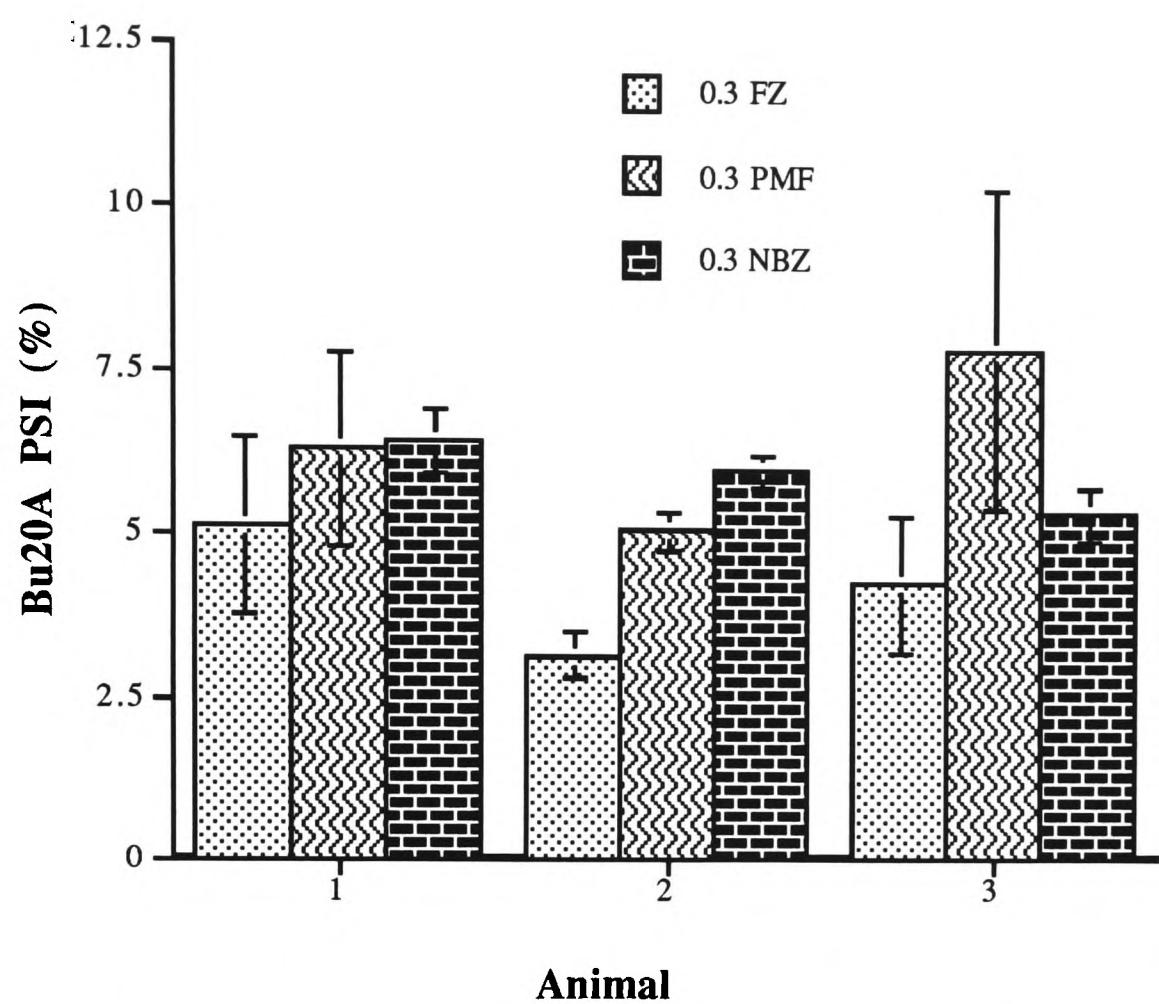


Fig. 4. 10. Diagram showing the Bu20A PSI of the FZ, the PMF and the NBZ in 3 animals in the 0.3 mm/day group. There was no significant difference of the PSI values in the three zone in this group ($P > 0.05$, ANOVA). Means and standard errors were plotted.

4. 3. 2. 2 0.7 mm/day group

In this group, the three zones were distinct. In the FZ, the Bu20A-positive cells were oriented to the direction of direction, and most of them were round-nuclei cells (Fig. 4. 11).

Most Bu20A-positive cells were at the junction between the PMF and the NBZ (Fig. 4. 12). The Bu20A-positive cells in the NBZ were close to bone surfaces (Fig. 4. 13). The greatest Bu20A PSI was obtained in the PMF in 3 out of 4 animals (Fig. 4. 14).

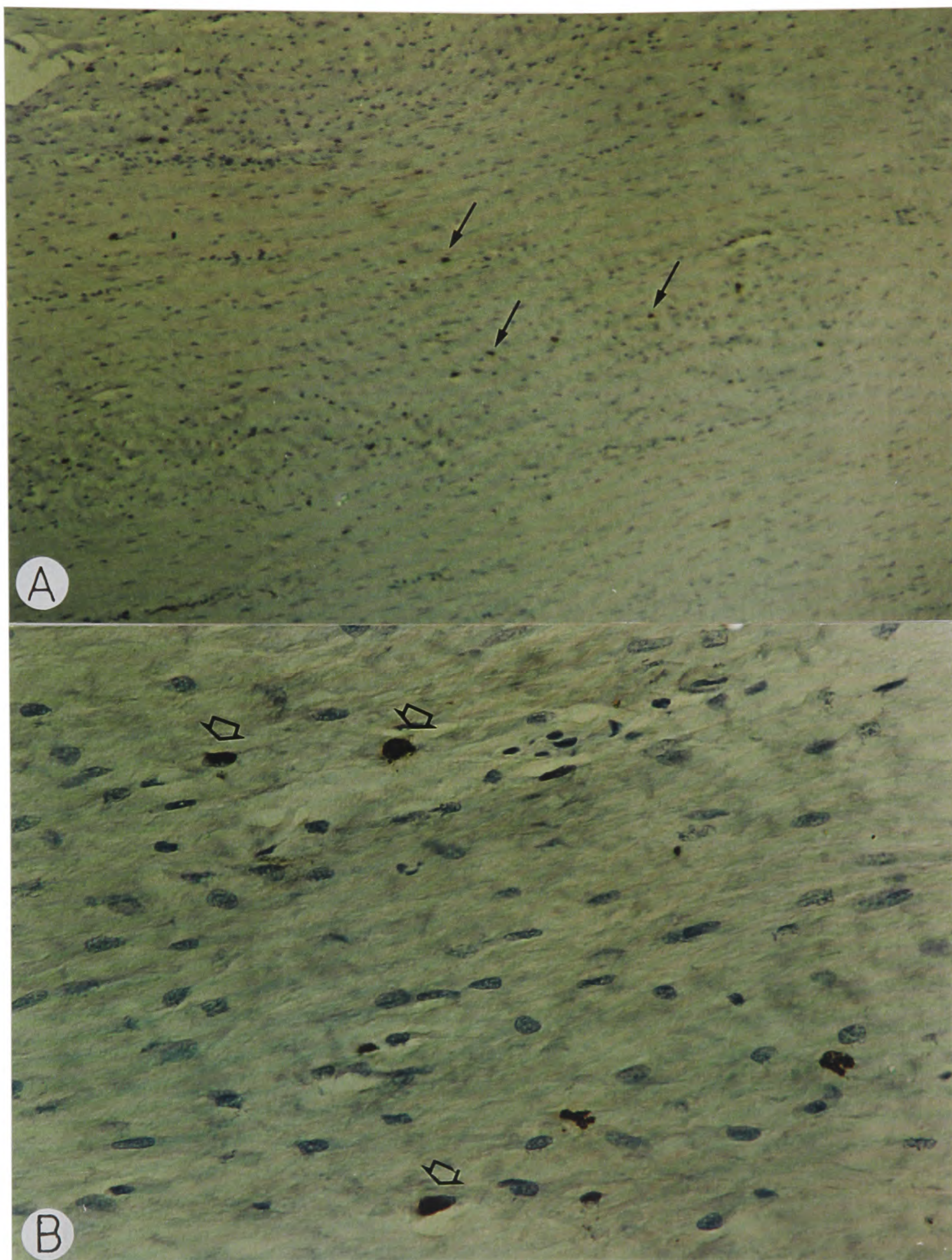


Fig. 4. 11. An example of Bu20A immunostaining in the fibrous zone of the 0.7 mm/day group. Bu20A-positive cells were round-nuclei cells (arrows), parallel to the distraction direction. A X 25, B X 100.

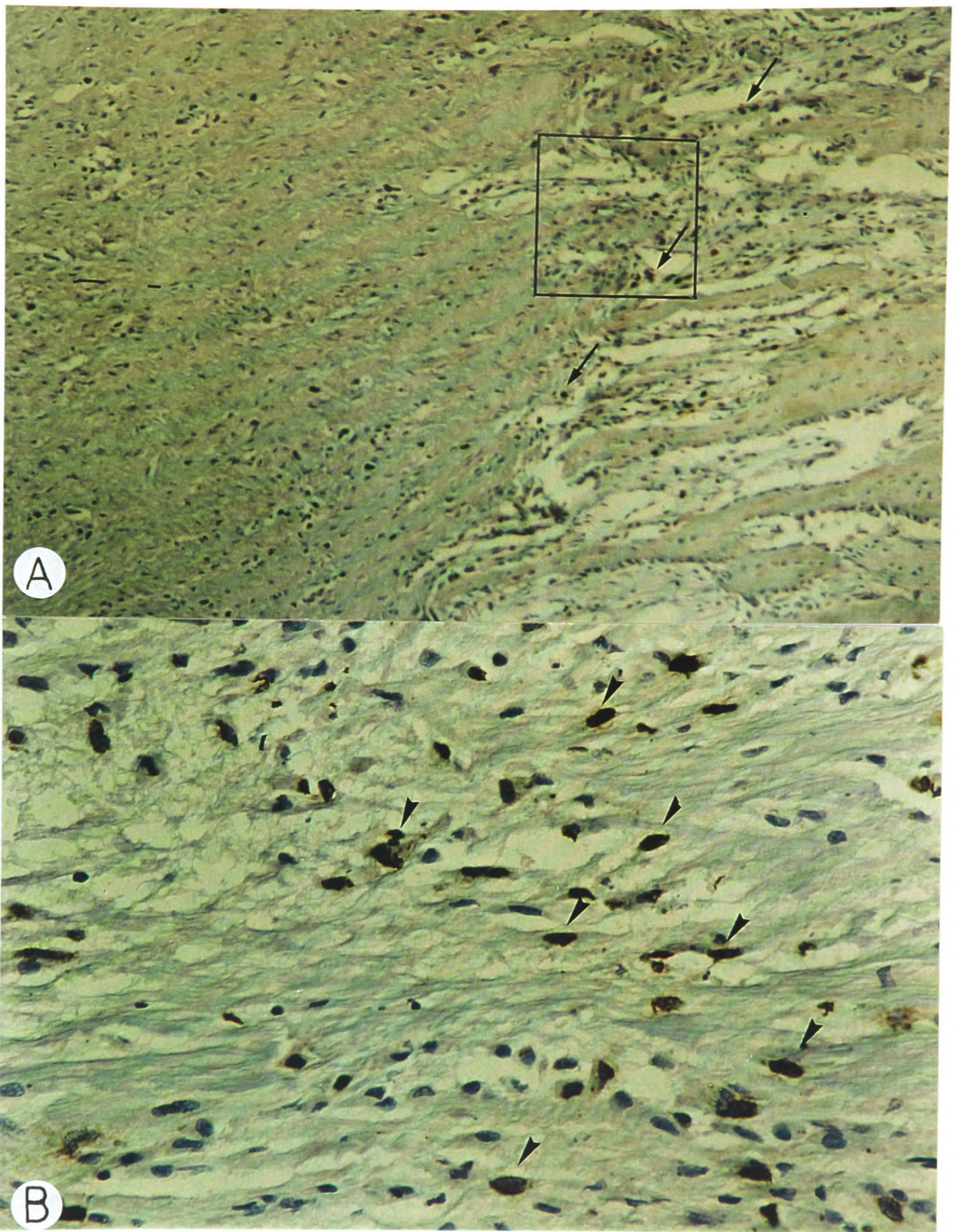


Fig. 4. 12. An example of Bu20A immunostaining in the mineralization front in the 0.7 mm/day group. **A:** The new bone was being formed at the junction between the FZ and the PMF, and the greatest number of Bu20A-positive cells (arrows) was observed in the PMF. **B:** Close-up of the boxed area in A, showing Bu20A-positive cells (arrows) laying between the collagen bundles (CB). A X 25, B X 100.

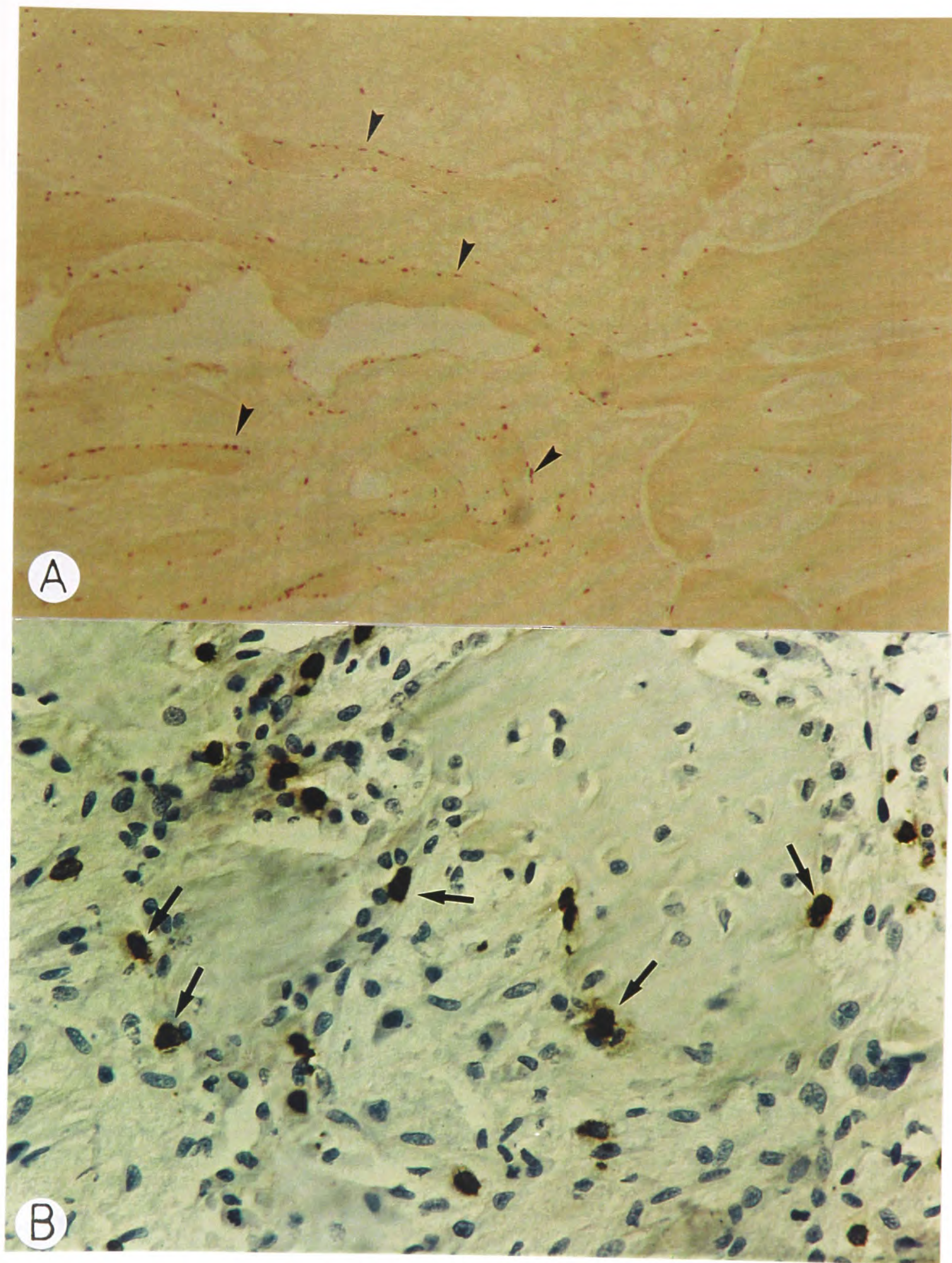


Fig. 4. 13. Bu20A immunostaining in the new bone zone in a regenerate of the 0.7 mm/day group. Most of the Bu20A-positive cells in the new bone zone were on or close to bone surfaces (arrows), and they are preosteoblasts or osteoblasts. A X 25, B X 100.

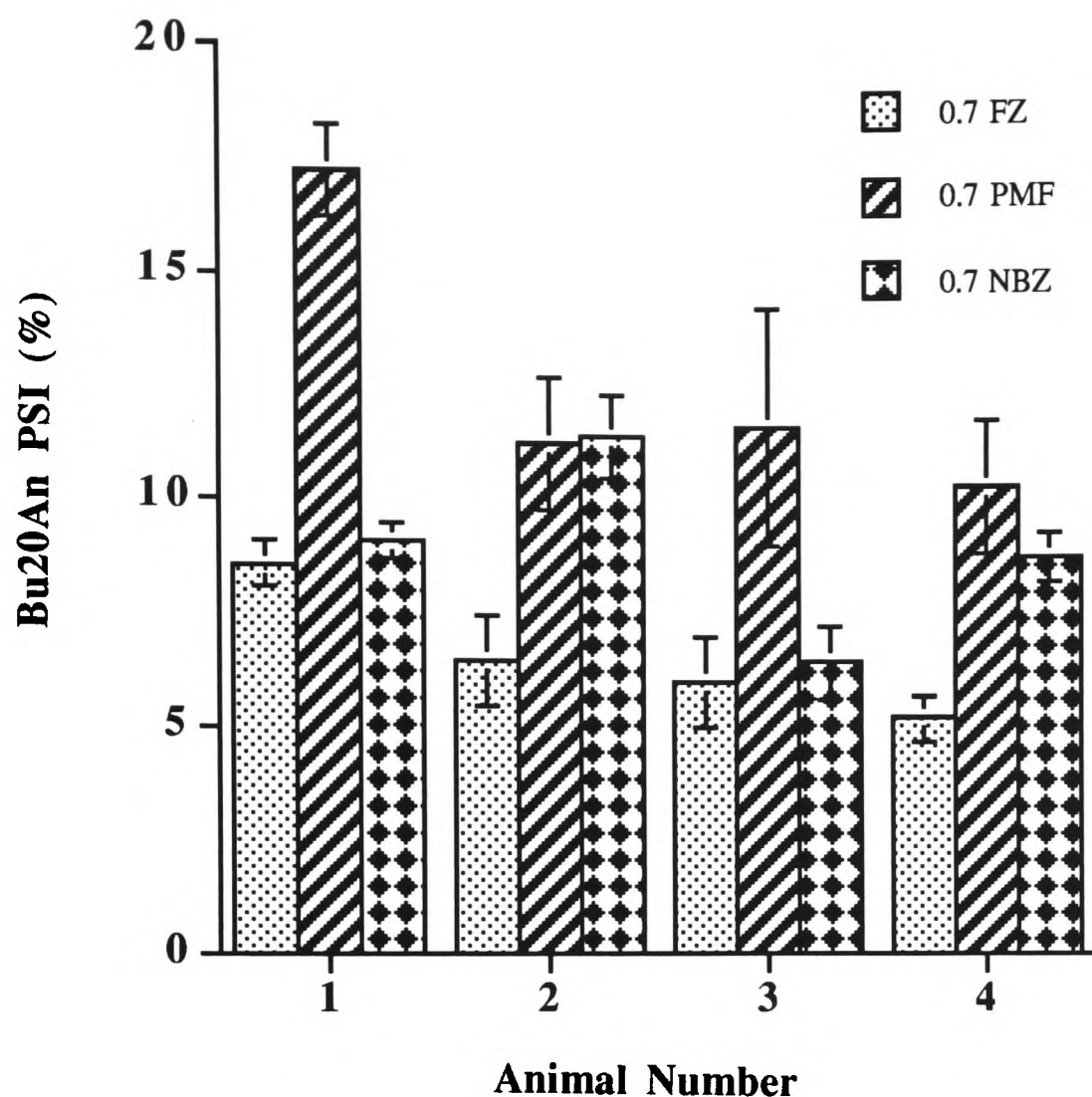


Fig. 4. 14. Diagram showing the Bu20A PSI of the FZ, the PMF and the NBZ in 4 animals in the 0.7 mm/day group. The PSI value of the PMF within the three zones was the highest in this group ($P < 0.05$, ANOVA).

4. 3. 2. 3 1.3 mm/day group

In this group, a considerable number of cysts and necrotic areas were found in the regenerate, which were only occasionally seen in the 0.7 mm/day group (see section 3. 4. 4 and 3. 4. 5), and these areas were excluded for cell counting. The distribution of Bu20A-positive cells in this group was of a similar pattern to that in the 0.7 mm/day group. The Bu20A-positive cells in the FZ and the PMF were oriented toward the distraction force (Fig. 4. 15), and the highest concentration of the Bu20A-positive cells was in the PMF. It was noticed that bone marrow cells were also activated, Bu20A-positive labelling were found in some areas of the newly formed bone marrow tissue within the new bone region (Fig. 4. 16). The values of Bu20A PSI of the three zones were calculated in 3 animals in this group, the Bu20A PSI of the PMF was significantly higher ($P < 0.05$) than that of the FZ and the NBZ (Fig. 4. 17).

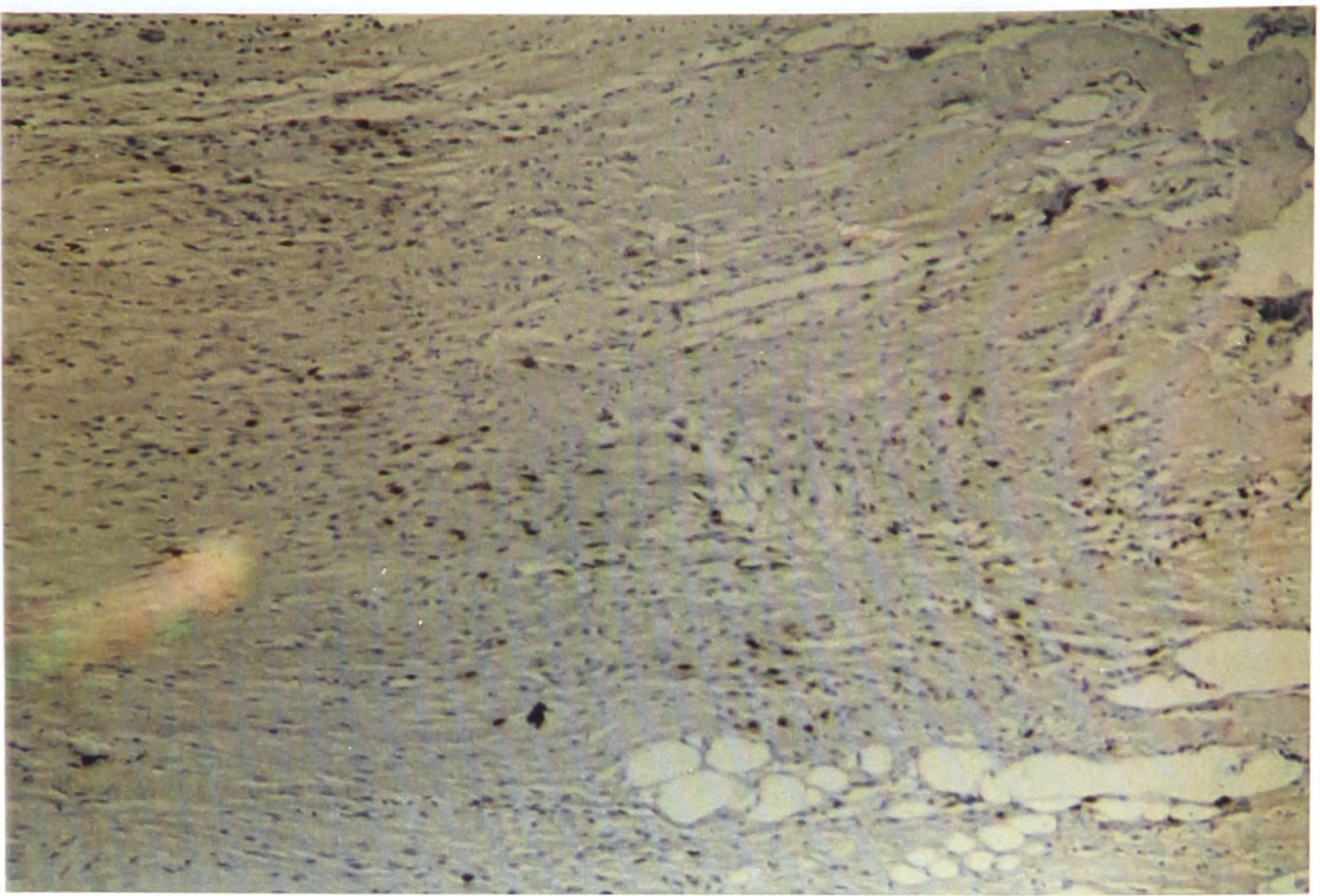


Fig. 4. 15. In a representative regenerate of the 1.3 mm/day group, the highest concentration of the Bu20A-positive cells (brown) was seen in the PMF, these cells were oriented parallel to the distraction forces. Bu20A immunostaining, X 25.

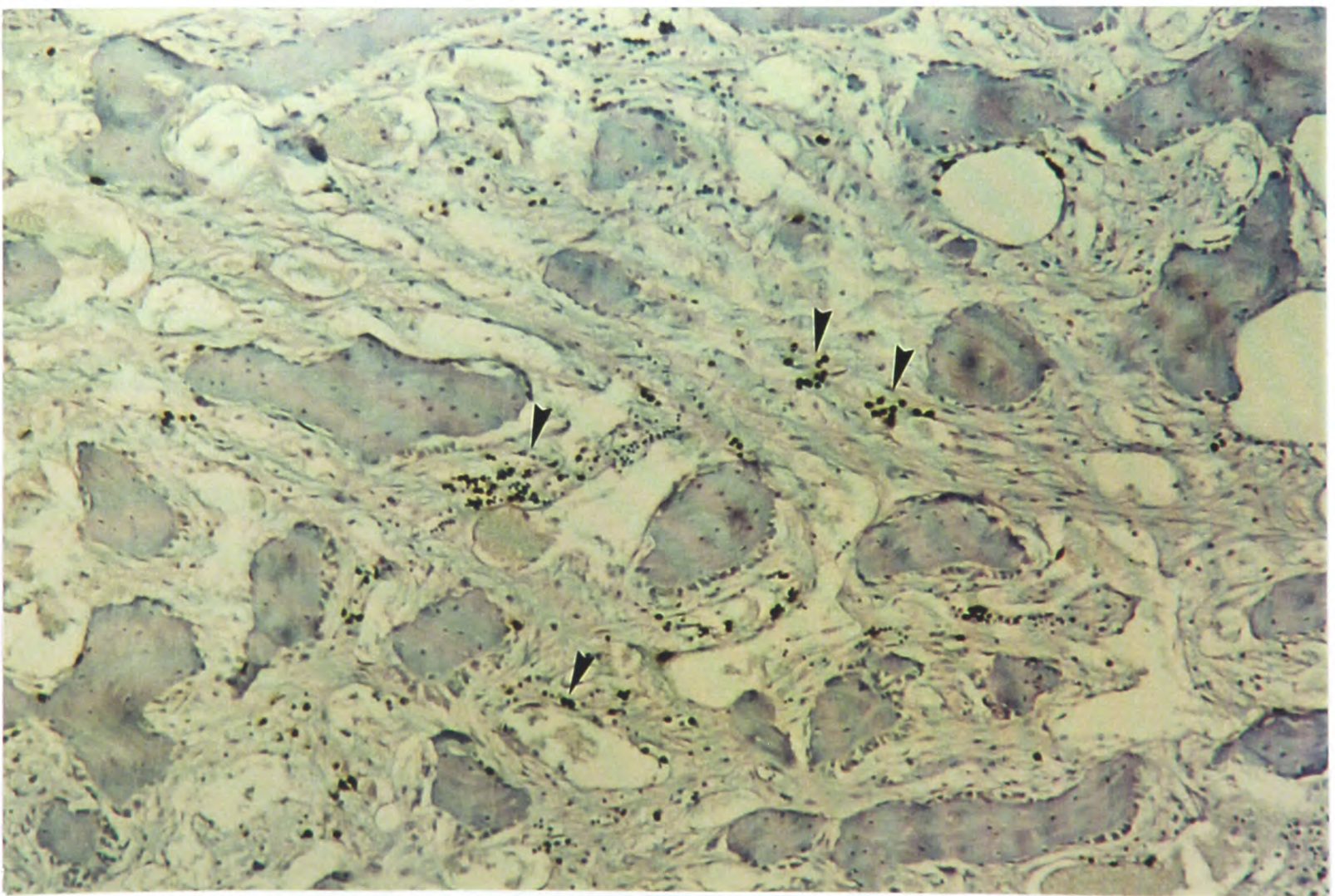


Fig. 4. 16. An example of Bu20A immunostaining in the new bone zone of a regenerate of the 1.3 mm/day group. Bu20A-positive cells (brown dots, arrows) were seen in the newly formed bone marrow tissues, X 25.

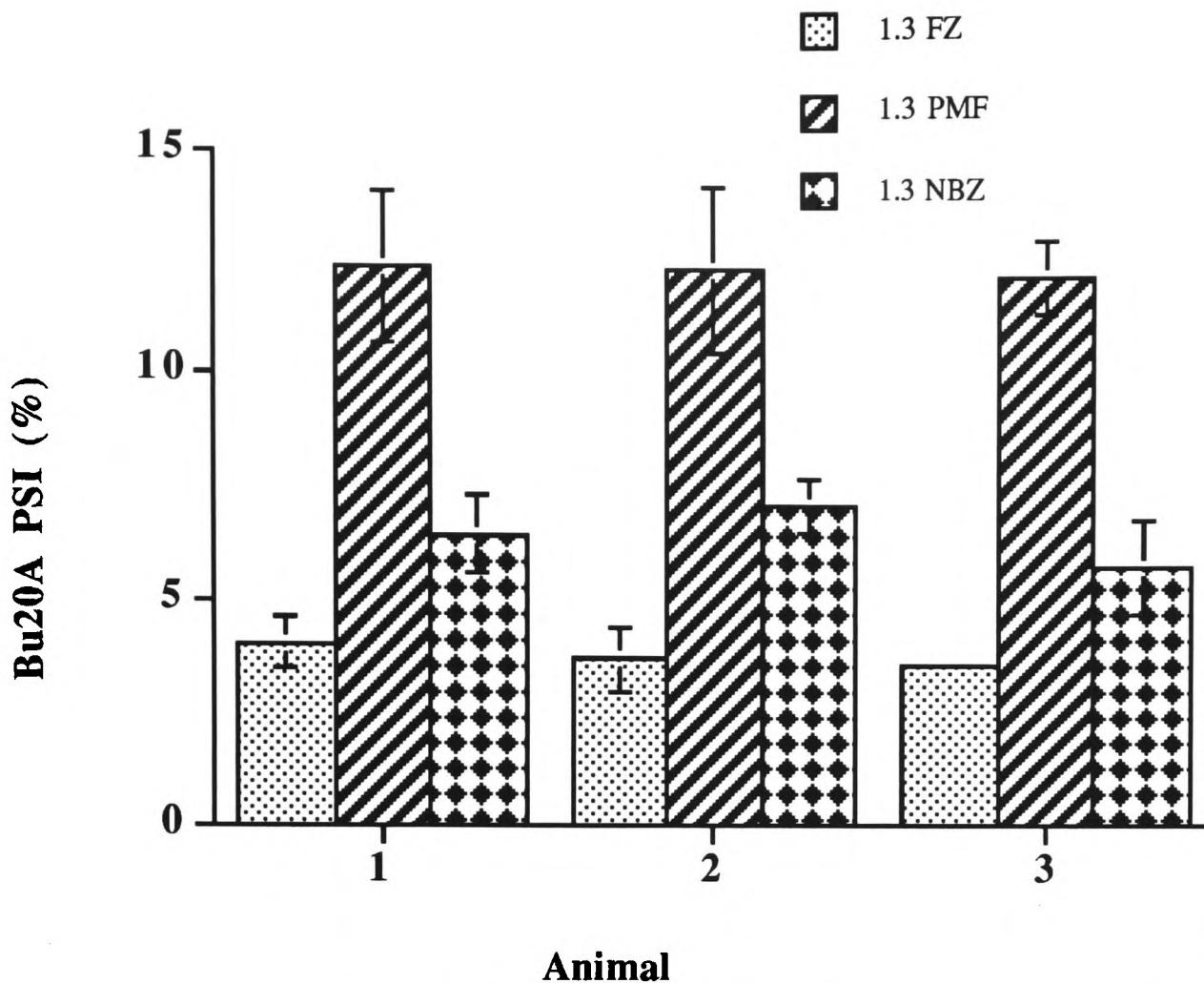


Fig. 4. 17. Diagram showing the Bu20A PSI of the FZ, the PMF and the NBZ in 3 animals in the 1.3 mm/day group. The PSI value of the PMF within the three zones was the highest in this group ($P < 0.05$, ANOVA).

4. 3. 2. 4 2.7 mm/day group

In this group, bone formation was sparse and most of the distraction gap was fibrous tissue (see section 3. 4. 6). The majority of the Bu20A-positive cells were observed within the FZ (Fig. 4. 18), the Bu20A-positive cells in the FZ were parallel to the direction of distraction.

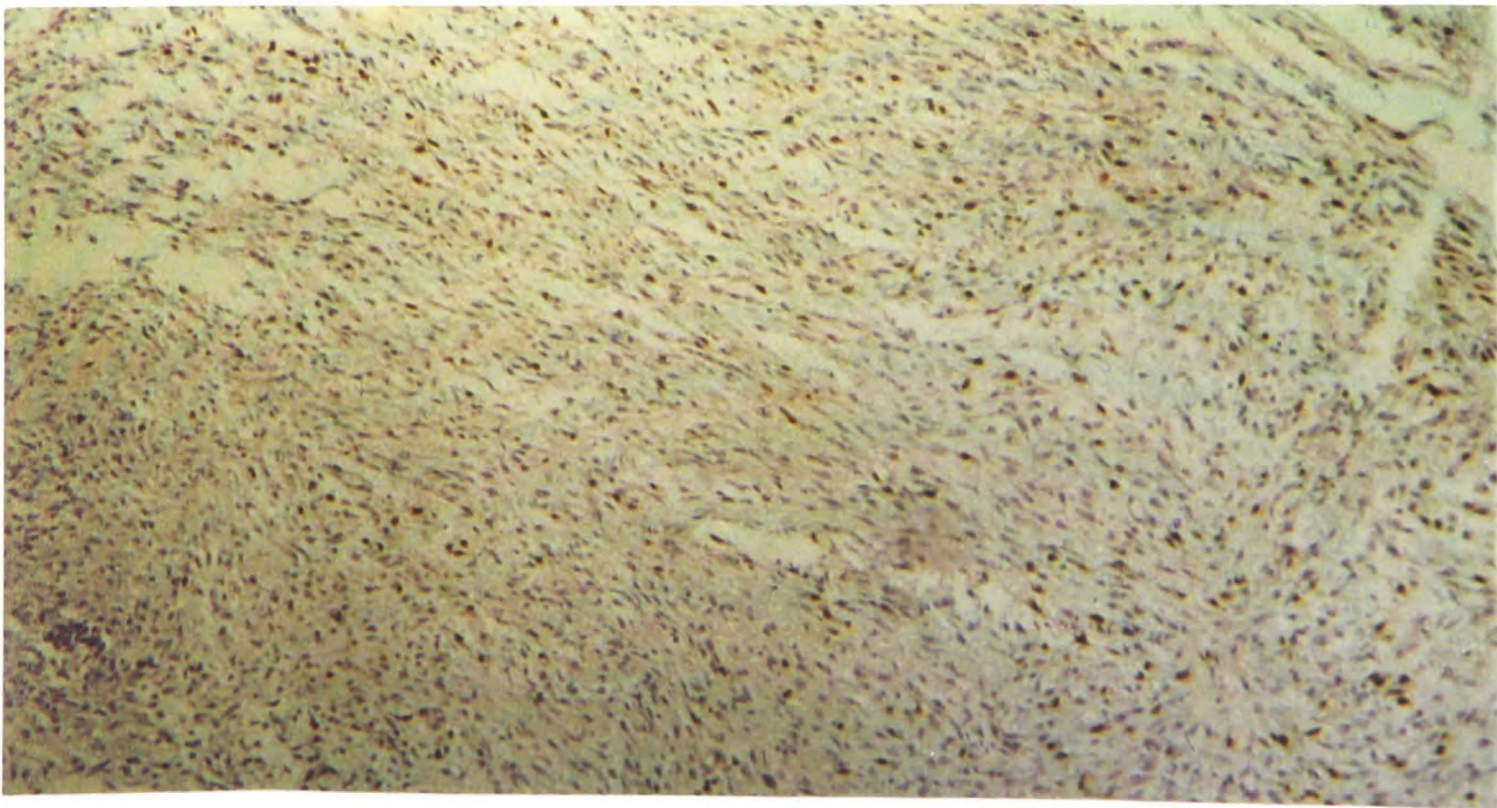


Fig. 4. 18. In a representative of the regenerate of the 2.7 mm/day group, Bu20A-positive cells (brown dots) were predominately seen in the FZ, X 25.

In the PMF and the NBZ, the distribution of Bu20A-positive cells was similar to that at distraction rates of 0.7 and 1.3 mm/day. The Bu20A-positive cells were localised on the bone surface of the newly formed bone column (Fig. 4. 19). The values of Bu20A PSI of the three zones were calculated in 3 animals in this group, the Bu20A PSI of the PMF was also significantly higher ($P < 0.05$, ANOVA) than those of the FZ and the NBZ (Fig. 4. 20).

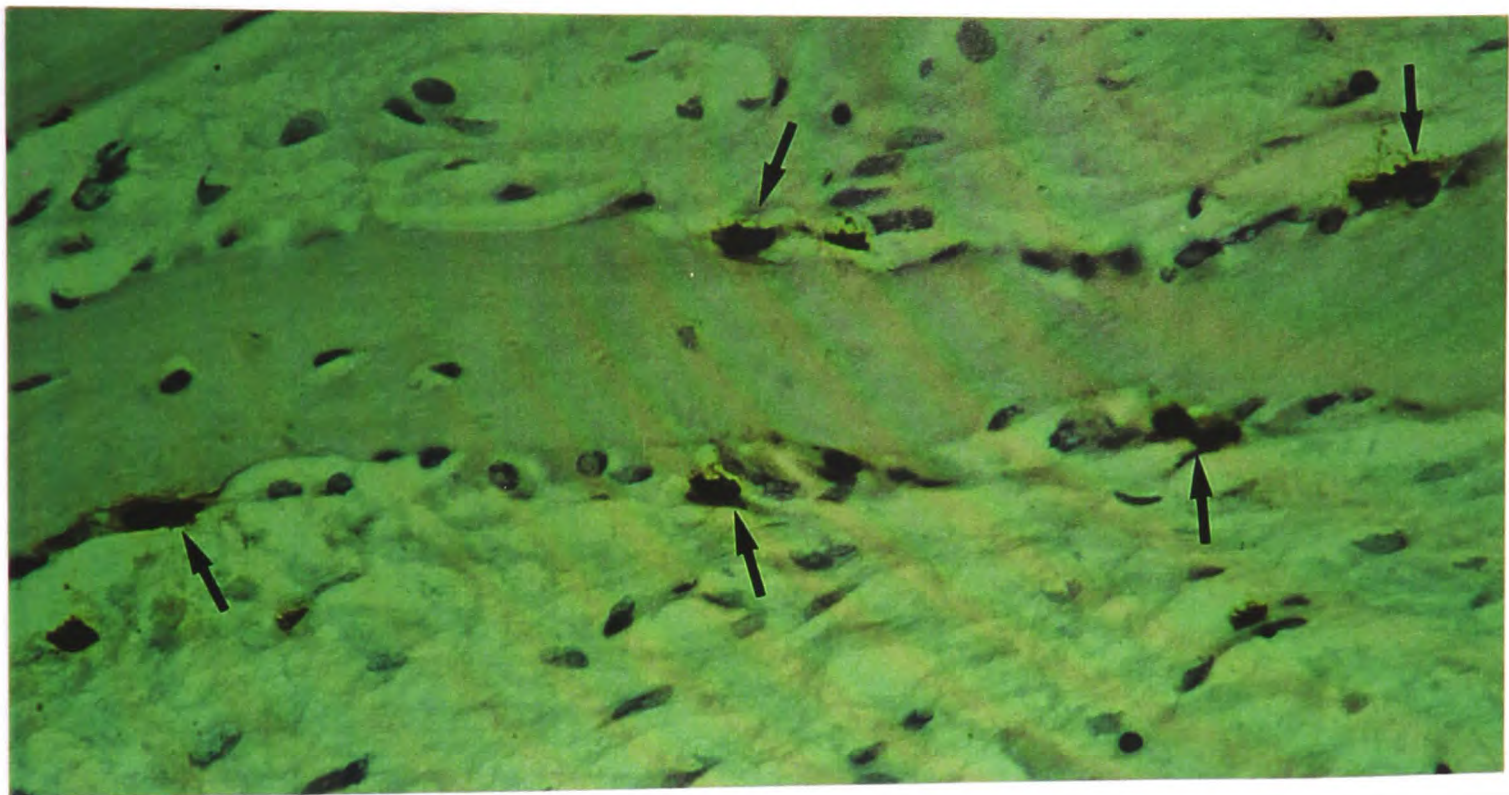


Fig. 4. 19. In a regenerate of the 2.7 mm/day group, Bu20A-positive cells (arrows) were seen on the bone surfaces in the NBZ, similar to that in other groups, X 25.

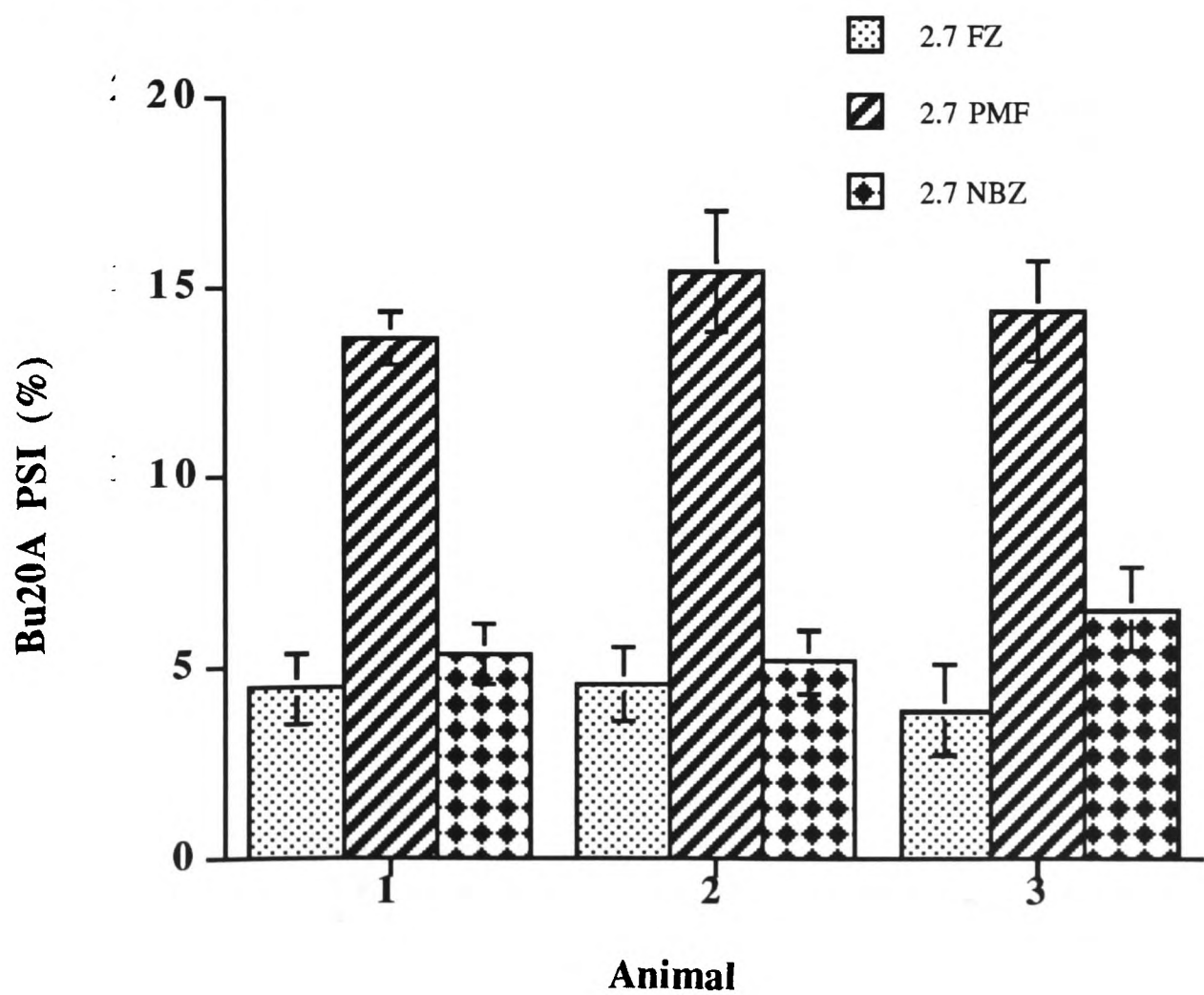


Fig. 4. 20. Diagram showing the Bu20A PSI of the FZ, the PMF and the NBZ in 3 animals in the 2.7 mm/day group. The Bu20A PSI value of the PMF within the three zones was the highest ($P < 0.05$, ANOVA) in this group.

4.3.2.5 Summary

To quantitate the number of dividing cells in the specific zones, the Bu20A PSI was calculated for each zone at the different distraction rates. In all distraction groups except the 0.3 mm/day group, the Bu20A PSI values in the PMFs were significantly higher than those of the FZs and the NBZs (Fig. 4. 23). There was no statistical differences between the Bu20A PSI values seen in the FZ or the NBZ in any of the distraction groups. There was a significant increase of the Bu20A PSI value in the PMF as the distraction rate rose from 0.3 mm/day to 0.7 mm/day ($P < 0.05$, ANOVA), and this was maintained but not increase further at the higher distraction rates.

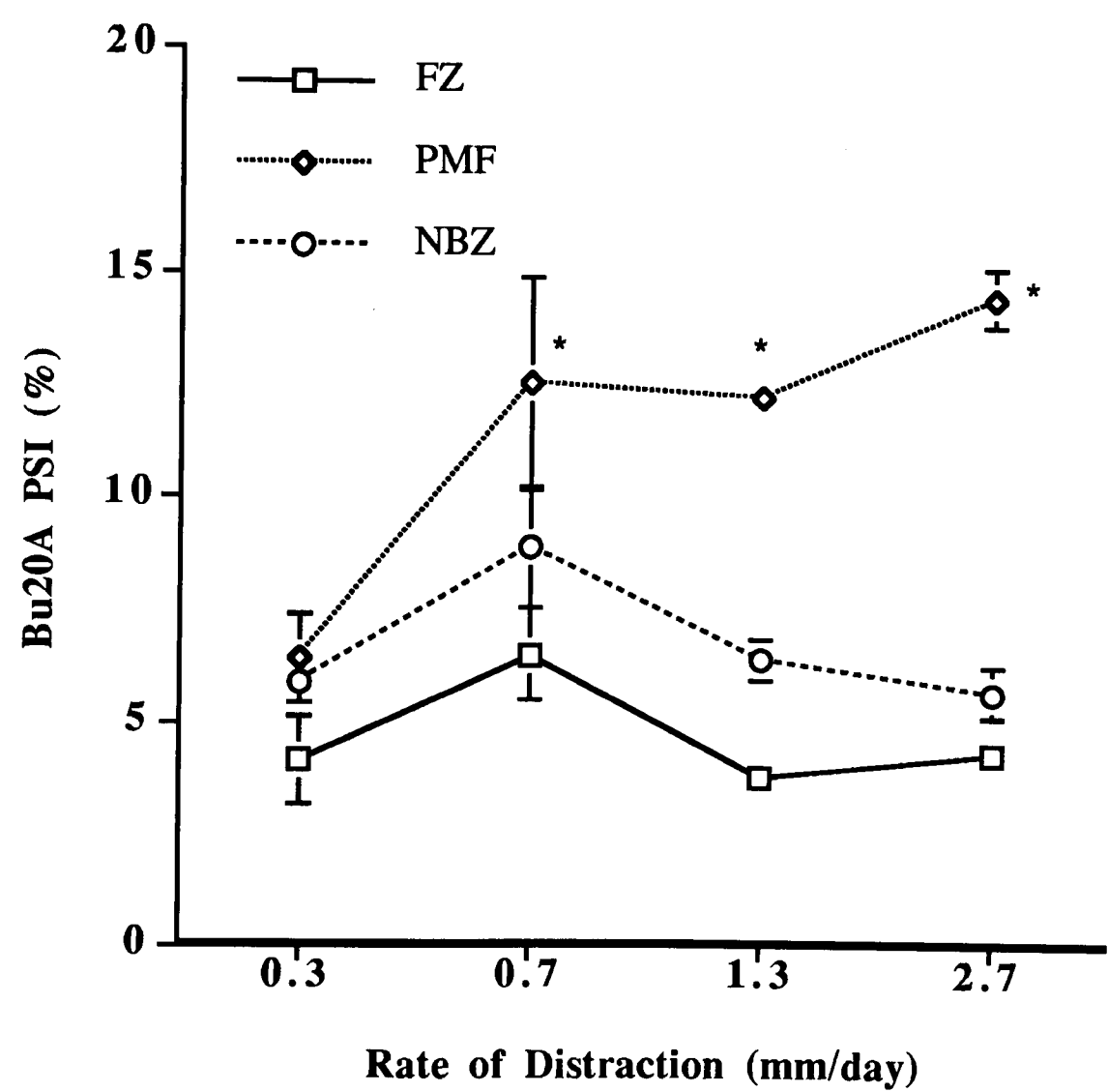


Fig. 4. 21. Bu20A PSIs of the three zones of the regenerate at the 4 different distraction rates. There was no statistical difference in the Bu20A PSI values between the FZs and the NBZs of any distraction group. The Bu20A PSI values of the PMFs were the highest (*) among the three regions of all groups except the 0.3 mm/day group. There was a significant increase of the Bu20A PSI value in the PMF as the distraction rate increased from 0.3 to 0.7 mm/day (*, $P < 0.05$, ANOVA). The Bu20A PSI values for the 1.3 and 2.7 mm/day groups were also significantly higher than the 0.3 mm/day group (*). Means and standard errors have been plotted.

4. 3. 2. 6 Discussion

Some of the most important variables during limb-lengthening are the rate, the rhythm and the amount of lengthening. In our experimental model, the rate of distraction has significant effects on optimal tissue regeneration. In this experimental system of leg-lengthening, the magnitude of lengthening was 20% and this was kept constant in all lengthening groups, as a consequence of the rate of distraction varying from 0.3 to 2.7 mm/day, the sacrifice time of different groups was different. It varied from 8 weeks (0.3 mm/day) to 1 week (2.7 mm/day) postsurgery. However, continuous distraction at all the rates used in the present experiments prolonged the process of fracture repair as seen in the osteotomy control groups. Therefore, it seems reasonable to compare the cellular proliferation occurring in the regenerating tissues at the end of distraction.

The cells in the FZs were oriented to the direction of distraction. These cells were highly proliferative, with osteogenic potential and may have been recruited from the surrounding soft tissues (periosteum, fascia, muscle, blood vessel) or from bone marrow stromal cells (Ilizarov, 1989a; Iwamoto, et al., 1993). The values of the Bu20A PSI in the FZs and the NBZs did not change significantly with increases of the rate of distraction, indicating that the rate of cell proliferation within the FZs and NBZs may be constant. The distribution of the Bu20A-positive cells in the FZ and the NBZ of the distraction groups was similar to that seen in the osteotomy control groups, which suggested that the process of distraction osteogenesis may be a prolonged process of fracture repair.

The PMF was a narrow zone, with the highest rate of cell proliferation of the three zones in all distraction groups except the 0.3 mm/day group. The osteoblasts started making new bone in this region. These results suggest that the PMF was a zone of high metabolic activities because of the fast fibro-bone tissue turnover, and the PMF may be the region most sensitive to the changes of mechanical properties such as rate of distraction in the regenerate. The Bu20A PSI in the PMF increased significantly ($P < 0.05$) as the rate of distraction rose from 0.3 mm/day to 0.7 mm/day, indicating that cell proliferation can be enhanced by an appropriate rate of distraction, and so this could speed up the bone formation in the PMF. However, at distraction

rates higher than 0.7 mm/day, there was no further increase of Bu20A PSI in the PMF, but the areas of cartilage islands, haemorrhages, cyst formation and necrotic tissues were increased. This indicated that higher rates (> 0.7 mm/day) of distraction were biologically unfavourable and should be avoided.

In conclusion, the present study indicates that cell proliferation of osteoprogenitor cells during distraction osteogenesis was affected by the rate of distraction. A slow rate of distraction (0.3 mm/day) does not maximally stimulate osteogenic progenitor cells to generate new bone tissue, and a distraction rate at 0.7 mm/day was optimal for cell proliferation and had the least unfavourable histological characteristics. Higher rates (1.3 mm/day or above) of distraction caused tissue damage without having any beneficial effect on stimulating bone cell proliferation and formation of bone in the regenerate.

4. 4 Comparison of the expression of PCNA or the uptake of BrdUrd in the rabbit tissues

4. 4. 1 Introduction

Many studies have indicated that BrdUrd uptake and its immunohistochemical detection is a reliable technique to assess cell proliferation (see section 1. 3. 5). However, BrdUrd is toxic, some experiments preclude its use and it is not applicable for human patient investigations. In this section, the validity of using the PC10 (a monoclonal antibody to PCNA) immunostaining technique to detect proliferating cells in the experimental system of leg-lengthening was assessed by comparison with cellular uptake of BrdUrd and its detection by Bu20A monoclonal antibody. In addition, the effects of a variety of fixatives and decalcifying solutions, on the detection of proliferating cells by each method, were also investigated.

4. 4. 2 Methods

The same leg-lengthening animal model as described before was used. Once the animal had been killed, small intestine, thymus, liver, normal tibialis anterior muscle and the regenerate

from the distraction gap were collected. These tissues were then divided into several pieces and fixed in one of a number of fixatives. Carnoy's fluid, 10% neutral formalin (pH 7.2), 4% paraformaldehyde (pH 7.2), 95% industrial methylated spirit (IMS) and 1:1 (v/v) methanol/ethanol were employed. Fixation was carried out for 24 hours at room temperature, except for the Carnoy's fluid for which the fixation time was 3 hrs for soft tissues and 10-12 hrs for hard tissues. The effect of demineralisation on detection by the immunohistochemical procedures, was assessed by using small intestine and the regenerating bone tissue. After fixation, the small intestine and the regenerating bone tissues were treated with either 5% nitric acid for 24 hrs or 14.5% EDTA (pH 7.4) solution for 14 days. All fixed samples were embedded in paraffin wax and serial sections (5 µm) were cut and mounted on poly-L-lysine coated glass slides.

Adjacent sections were then subjected to immunostaining using either PC10 or Bu20A antibodies by a standard method, which has been described in detail in section 4. 2. 1, 4. 2. 2 and 4. 2. 3. The immunostaining results were examined under light microscopy, cells were counted by a cell counting technique (see section 4. 2. 4. 1) and the Bu20A PSI or PC10 PSI was calculated and compared by ANOVA (see section 4. 2. 4. 2).

4. 4. 3 The effect of sample processing on PC10 and Bu20A immunodection

As shown in Table 4. 1, tissues fixed in paraformaldehyde or formalin gave the most satisfactory results. Fixation in IMS, methanol/IMS and Carnoy's fluid was not satisfactory, with respect to immunoreactivity or preservation of morphological details. Decalcification with EDTA after fixation with Carnoy's fluid and paraformaldehyde did not significantly affect the expression of PCNA or BrdUrd.

Table 4. 1. Summary of the effects of fixation and decalcification on PCNA and BrdUrd immunodetection in paraffin sections

Fixatives & Decalcifying Solutions	Soft Tissues		Regenerate Bone	
	PC10	Bu20A	PC10	Bu20A
Formalin	++	++	++	++
Paraformaldehyde	+++	+++	+++	+++
Carnoy's Fluid	++	+++*	++ [#]	+++*
Industrial Methylated Spirit	+*	+* [#]	+* [#]	+* [#]
Methanol / IMS	+*	+* [#]	+* [#]	+* [#]
Carnoy's + EDTA	++*	++*	++	++
Carnoy's + HNO ₃	+++*	+++*	+++*	+++*
Paraformaldehyde+ EDTA	+++	+++	+++	++
Paraformaldehyde+ HNO ₃	+++ [#]	+++*	+++ [#]	+++ [#]

Codes:

- +

immunoreactivity present

++

fairly strong

+++

very strong

*

poor morphology

#

excess immunostaining background

Samples of small intestine which are known to be highly proliferative were used to assess the extent of cell labelling with PC10 or Bu20A antibodies under different fixation and decalcification methods. By comparing the Bu20A PSI or PC10 PSI of the samples treated with different fixation and decalcification, it was found that most fixatives did not affect immunostaining results with PC10 or Bu20A monoclonal antibodies with fixation up to 24 hrs. Prolonged fixation altered PCNA expression as shown by a marked reduction in PCNA immunoreactivity in the crypts of the small intestine, thymus and distracted regenerating bone tissue when they were fixed in paraformaldehyde or formalin for more than 48 hours (Fig. 4. 22). Tissues fixed in IMS exhibited a significant change in PC10 immunostaining in small intestine, with the PC10 PSI decreasing ($p<0.01$, ANOVA) compared to other fixatives (Fig. 4. 23).

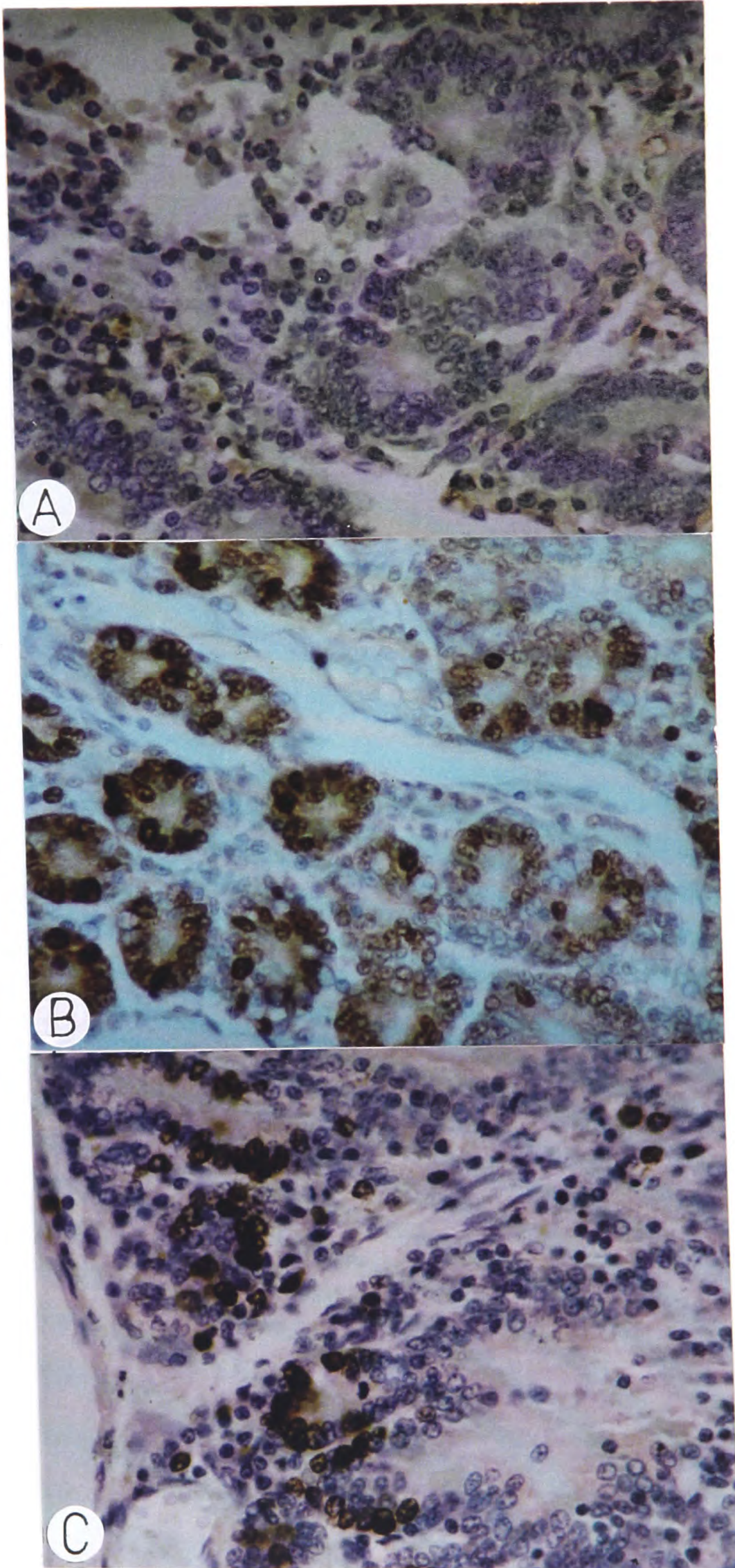


Fig. 4. 22. Prolonged fixation altered PCNA expression as shown by a marked reduction in PCNA immunoreactivity in the crypts of the small intestine. A: negative control; B: sample was fixed in 4% paraformaldehyde (pH 7.2) for 24 hrs; C: sample was fixed in 4% paraformaldehyde (pH 7.2) for 10 days. PC 10 immunostaining, All X 25.

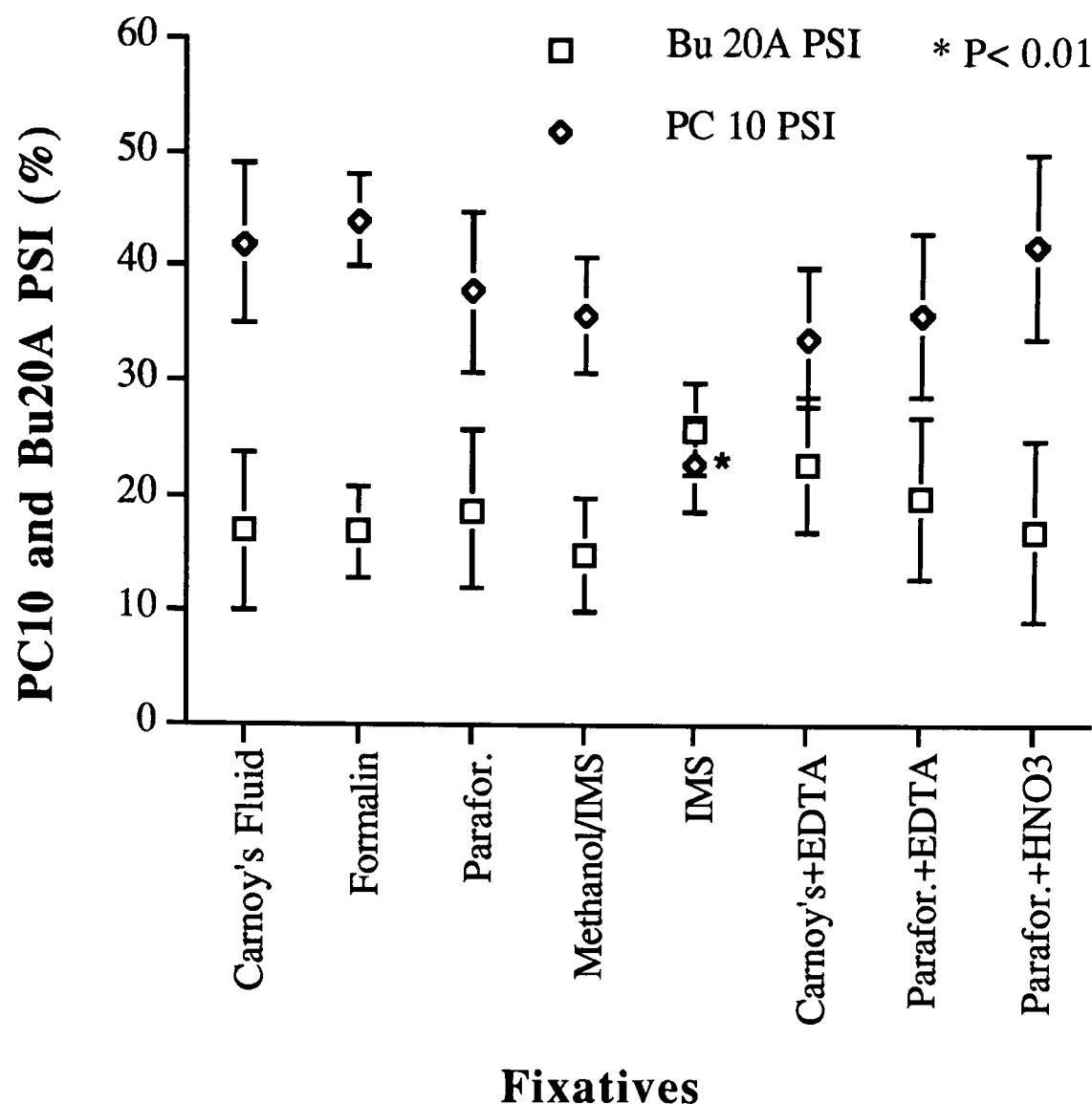


Fig. 4. 23. The effect of fixatives and decalcifying solutions on PC10 and Bu20A immunostaining of samples of small intestine. Most fixatives and decalcifying solutions did not alter the PC10 and Bu20A PSI except IMS, which significantly decreased PC10 PSI (* $P < 0.01$, ANOVA). Parafor. = paraformaldehyde.

4. 4. 4 Immunostaining by PC10 or Bu20A in the rabbit tissues

PC10 and Bu20A immunostaining was seen almost exclusively in the nucleus and showed mainly a granular staining pattern (Fig. 4. 22). In the small intestine, PC10 and Bu20A positive-staining cells were localised mainly in the columnar epithelium of the intestinal villi and the crypts (Fig. 4. 24 A, B), which were known to have a high rate of cell turnover with a high proportion of mitotic figures. In the thymus, the positive staining cells were found in the cortex and medulla, where the cells were of lymphocyte origin with a high potential for division (Fig. 4. 24 C, D). In both tissues, PC10 labelling was almost double that of Bu20A. In the liver and skeletal muscle, few cells showed PC10 immunoreactivity and there was little uptake of BrdUrd (not shown).

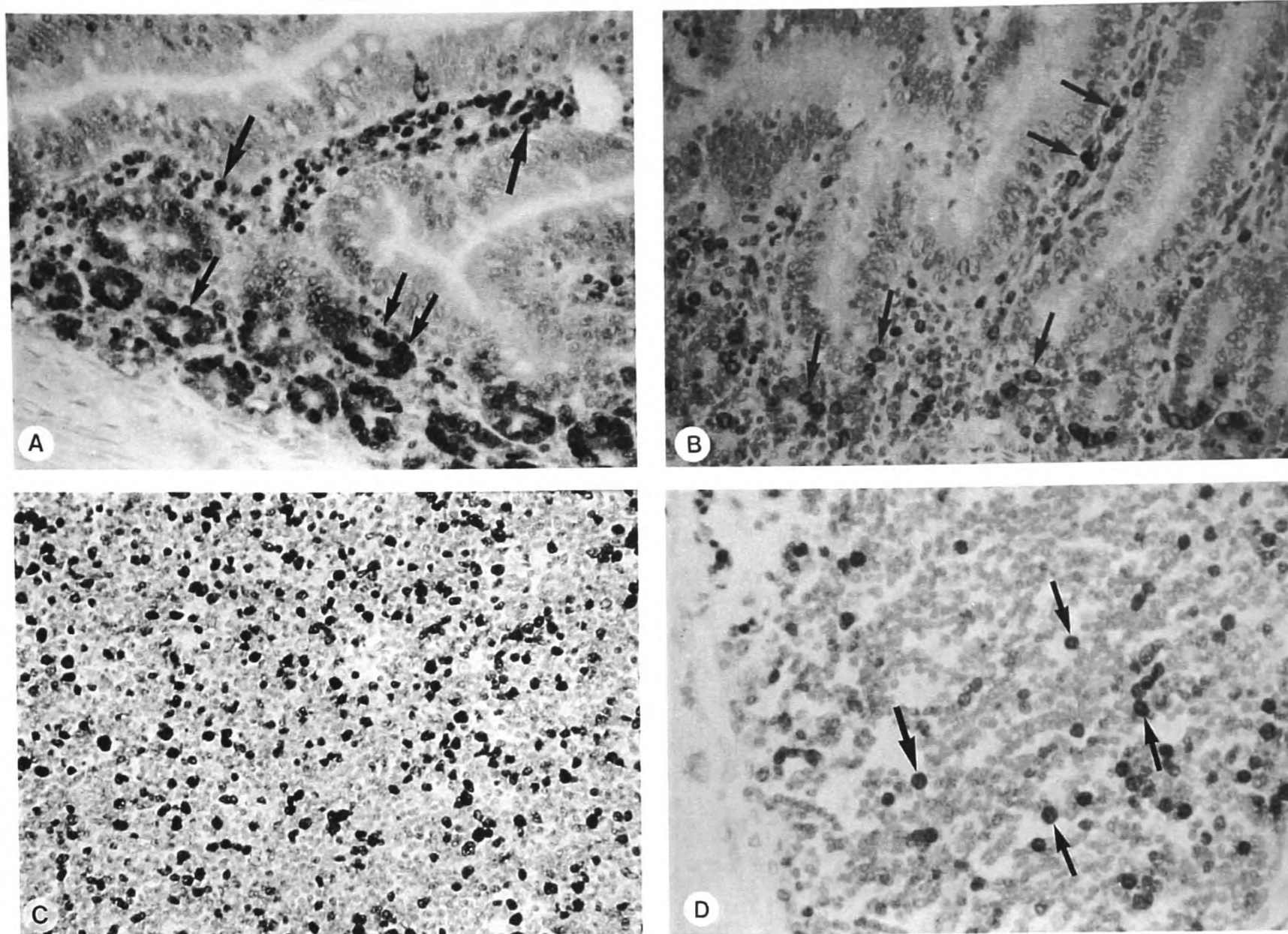


Fig. 4. 24. Immunohistochemical detection of PCNA and BrdUrd by PC10 and Bu20A monoclonal antibodies in different tissues. A: PC10 and B: Bu20A positive-labelling cells in the crypts and villi of small intestine (arrow heads); C: PC10 and D: Bu20A positive-labelling cells in medullar cells of thymus. Original magnification, all X 25.

In the regenerating bone tissue, the distribution of PC10-positive cells was very similar to that of Bu20A, which has been described in detail in section 4. 3. 2. Bu20A and PC10-positive cells were seen in the FZ, the PMF and the NBZ of the regenerates in all the distraction groups. Again, PC10 labelled more cells than Bu20A. PC10 and Bu20A-positive cells in the fibrous zone were fibroblast-like and were oriented parallel to the direction of distraction (Fig. 4. 25 A, B); whereas in the NBZ they were bone-forming cells on or close to the bone surfaces (Fig. 4. 25 C, D). In each of the three zones, the PC10 PSI was greater than Bu20A PSI.

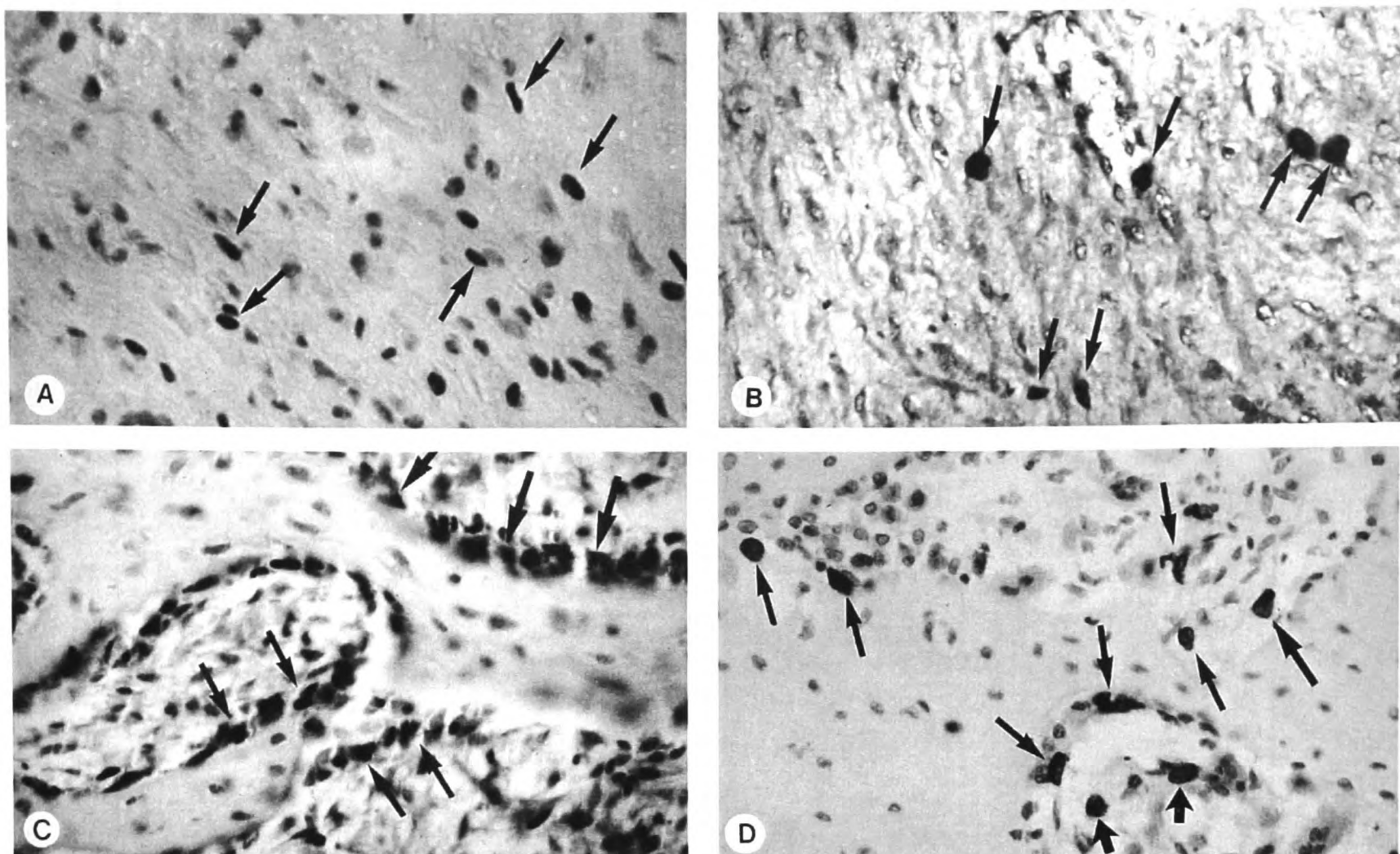


Fig. 4. 25. Cell proliferation of the regenerating bone detected by PC10 and Bu20A. A: PC10 and B: Bu20A positive-labelling cells (arrow heads) in the fibrous zone of a regenerate of the 0.7 mm/day group. C: PC10 and D: Bu20A immunostaining showed that the bone surface cells (arrow heads) were actively dividing in the new bone zone of the distraction gap. PC10 stained more cells than Bu20A. Original magnification, all x 100.

4. 4. 5 The correlation between the PC10 and Bu20A PSI

A general comparison was made between Bu20A PSI and PC10 PSI calculated from all the above specimens (small intestine, thymus, liver, regenerating bone from animals distracted at different rates) from adjacent serial sections immunostained with either PC10 or Bu20A. The Bu20A PSI was significantly lower than the PC10 PSI with the PC10 PSI being approximately twice that of the Bu20A PSI (Fig. 4. 26). As shown in Figure 4. 26, a consistent linear correlation between the PSIs of these two antibodies was found and was defined by the expression $PC10\ PSI = 1.6\ Bu20A\ PSI + 12.9$, with a correlation coefficient of 0.79.

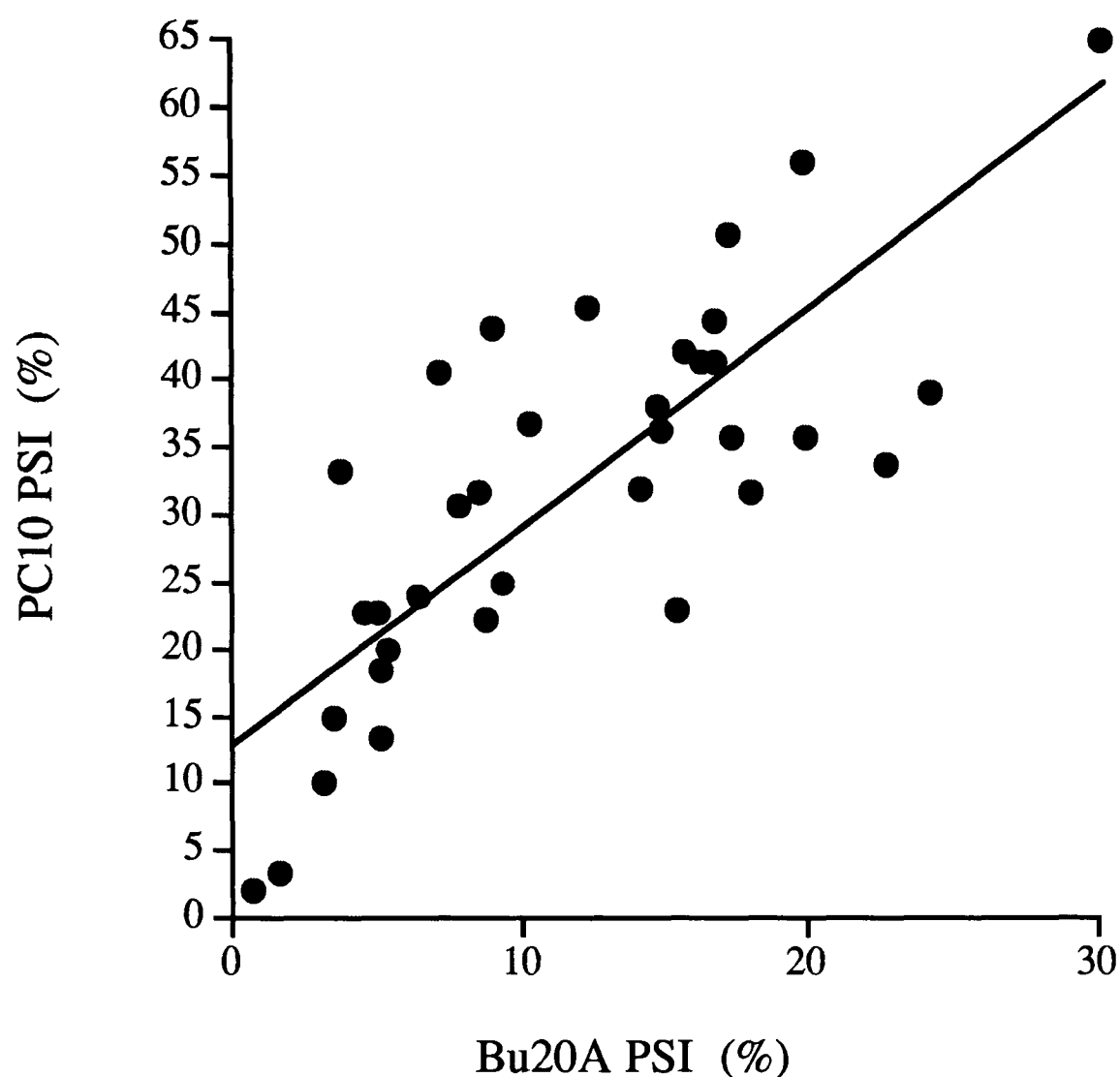


Fig. 4. 26. The correlation between PC10 and Bu20A PSI. PC10 PSI and Bu20A PSI of a variety of samples derived from different tissues were plotted. A linear correlation was found as defined by the expression: $PC10\ PSI = 1.6\ Bu20A\ PSI + 12.9$, with a correlation coefficient of 0.79. The means and standard errors of the PSIs were plotted.

4. 4. 6 Summary and discussion

Hall et al. (1990) found that prolonged fixation may reduce PCNA immunoreactivity in normal human and rat colon. Similar results were found in the present study when small intestine, thymus and regenerating bone tissues were in fixatives for 48 hours or longer. It was important to control the time of fixation and this period should be less than 48 hours. The PC10 immunostaining technique needed special attention and this has been discussed before (see section 4. 2. 3).

In this study, the PC10 PSI was greater than that of Bu20A PSI as has been previously reported (Woods, et al., 1991). There are several possible explanations for this difference.

PCNA has a long half-life of approximately 20 hours, and thus may be immunologically detectable in cells which have recently left the cell cycle (Bravo and MacDonald-Bravo, 1985; Prelich, et al., 1987). Previous studies have suggested that PCNA is an auxiliary protein for DNA polymerase- δ (Toschi and Bravo, 1988) and in the presence of PCNA, polymerase- δ initiates leading-strand DNA synthesis. The amount of PCNA present in the nucleus of cycling cells appears to be greater than that required for DNA synthesis (Miyachi, et al., 1978). Furthermore, PCNA may be expressed in association with DNA repair as well as with proliferation (Scott, et al., 1991; Shivji, et al., 1992). There is also some evidence that growth factors such as PDGF can increase PCNA mRNA stability and thus induce PCNA protein accumulation in normal cells without necessarily inducing DNA synthesis (Jaskulski, et al., 1988). Taken together these observations indicate that PC10 may tend to over-estimate the number of dividing cells.

In vivo labelling with BrdUrd and detection with a monoclonal antibody (Bu20A) is an established method for the assessment of cell proliferation (Gratzner, 1982). Apte (1990) has assessed the validity of BrdUrd immunohistochemistry in decalcified tissues by double labelling with tritiated thymidine. In this report, good correlation was found between the PC10 and Bu20A immunostaining results in rabbit soft tissues (small intestine, thymus, liver and muscle) and regenerating bone tissues (bone, cartilage, connective tissues). These results indicate that PC10 can be used as an alternative marker to assess cell proliferation during the regeneration of skeletal tissues. It may also be used with human materials. However, close control of sample preparation and immunostaining technique is essential in order to obtain consistent PC10 immunostaining results.

To conclude, the numbers of cells expressing PCNA are greater than those accumulating BrdUrd in the rabbit tissues. However, a linear correlation was found according to the expression: $PC10\ PSI = 1.6\ Bu20A\ PSI + 12.9$, with a correlation coefficient of 0.79. The PC10 labelling index may thus be used as an alternative marker of cell proliferation. This is particularly valuable where the use of other markers of cell proliferation which require systemic administration such as BrdUrd, are not possible.

4. 5 **Conclusions**

The present study indicates that cell proliferation of osteoprogenitor cells during distraction osteogenesis is affected by the rate of distraction. The rate of cell proliferation during distraction osteogenesis was found to reach a peak at a distraction rate of 0.7 mm/day, and did not change further at higher rates. A slow rate of distraction (0.3 mm/day) does not maximally stimulate osteogenic progenitor cells to proliferate. Higher rates (> 0.7 mm/day) of distraction caused tissue damage without having any beneficial effect on stimulating bone cell proliferation and formation of bone in the regenerate.

Cell proliferation was also assessed by detection of PCNA using a monoclonal antibody, PC10. The validity of this technique was verified by comparison with BrdUrd uptake. The PSI was calculated for a variety of tissues and the PC10 PSI was greater than that of Bu20A, as described by the expression: $PC10\ PSI = 1.6\ Bu20A\ PSI + 12.9$, with a correlation coefficient 0.79. The results suggest that PC10 may be used as an alternative marker to assess cell proliferation during bone regeneration.

Chapter 5 Studies on changes in extracellular matrix during distraction osteogenesis

5. 1 Localisation of collagen types I and II proteins and mRNAs

5. 1. 1 Introduction

Previous reports on distraction osteogenesis at rates of distraction of 0.5 - 1.0 mm/day in the human (Kawamura, et al., 1968; Tajana, et al., 1989; Shearer, et al., 1992), dog (Aronson, et al., 1989; 1990; Delloye, et al., 1990; Ilizarov, 1989a, b; Orbay, et al., 1992), and sheep (Karaharju, et al., 1993; Vauhkoen, et al., 1990) have shown that the bone forms mainly by intramembraneous ossification and endochondral ossification is not commonly observed. However, in the rabbit model of leg-lengthening, direct cartilage formation from the central fibrous tissue of the regenerate was observed at distraction rates of 0.5 - 1 mm/day (Kojimoto, et al., 1988; Masamizu, et al., 1994), and this was found and confirmed in the present rabbit model of leg-lengthening with distraction rates at or higher than 0.7 mm/day (see chapter 3, radiography and histology results).

To understand more about the mechanisms governing or limiting beneficial distraction osteogenesis, this chapter was to investigate the effects of distraction rates on the changes in extracellular matrix during distraction osteogenesis. Protein expressions of collagen types I and II, bone keratan sulphate proteoglycan (KSPG), and specific mRNAs for collagen types I and II were localised by immunohistochemistry and by in-situ mRNA hybridization methods.

5. 1. 2 Methods

NZW rabbits (n=16, age 24 weeks, body weight 3.0 - 3.5 kg) were anaesthetised and open transverse osteotomies performed and stabilised with unilateral external fixators in the left mid-

tibial diaphysis as previously described. After a 7 day latency period, the osteotomies were distracted twice daily at rates of 0.3, 0.7, 1.3 and 2.7 mm/day , until a 20% lengthening of the tibia was achieved. Rabbits were randomly divided into 4 groups, each group containing 4 rabbits. The rabbits were killed by anaesthetic overdose at the end of the experiment and the central portion of the tibia, including the distraction gap was excised and fixed in 4% (w/v) buffered paraformaldehyde for 24 hours before decalcification in neutral EDTA (14.5%, pH 7.2) for 14-30 days. The end-point of decalcification was determined radiographically. The tissues were embedded in paraffin wax, sections (5 μ m) were cut and mounted on poly-L-lysine coated slides for immunohistochemistry or 3-aminopropyltriethoxy silane (APES) (Sigma, Poole, Dorset, UK) coated slides for in-situ hybridization. For identification of bone-resorbing cells, TRAP staining was performed as described (see Appendix 7).

5. 1. 2. 1 Preparation of type I collagen riboprobes

Riboprobes for type I collagen mRNA were made from a cDNA probe, pHCAL1U (Makela, et al., 1988). The plasmid containing the cDNA probe was a gift from E. Vuorio (University of Turku, Turku, Finland). The cloned fragment of pHCAL1U is 372 bp and covers the C-terminal telopeptide and part of the C-propeptide of human pro α 1(I) collagen. This was subcloned into pGEM-3Z plasmid and named pGM3C1a1 (Critchlow, et al., 1995). To generate the antisense probe, pGM3C1a1 was linerized with Hind III and incubated with T7 polymerase; for the sense probe, it was linerized with EcoRI and incubated with SP6 polymerase (see appendix 11 and 12 for detailed procedure). During the incubation, the digoxigenin-labelled dUTPs were present in the incubation mixture, which was provided with the kit (Boehringer-Mannheim, UK).

5. 1. 2. 2 Preparation of type II collagen riboprobes

The type II collagen riboprobe was made from the plasmid, pRCOL2a1-1 (a gift from E. Vuorio, University of Turku, Turku, Finland), which carries a 400bp cDNA insert that covers the 3'- untranslated region and a small part of the C-propetide of rabbit pro α 1(II) collagen mRNA (Metsaranta, et al., 1996). To generate the probes, pRCOL2a1-1 was linearized with

SmaI and incubated with T3 polymerase to give the antisense probe, or with Hind III and T7 for the sense probe (see appendix 11 and 12). During the incubation, the digoxigenin-labelled dUTPs were present in the incubation mixture, which was provided with the kit (Boehringer-Mannheim, UK).

5. 1. 2. 3 In situ hybridization protocols

A standard procedure was used according to Critchlow, et al. (1995) with minor modifications. In brief, the pretreatments were (1) 0.2 N hydrochloric acid, (2) 6% hydrogen peroxide, (3) 20 µg/ml proteinase K (Sigma, EC 3.4. 21.14) in Tris-EDTA buffer, pH 8.0, for 10 min at 37°C, (4) 4% paraformaldehyde, pH 7.4 for 20 min, (5) 0.1 M glycine (2 X 10 min) and (6) 0.25% acetic anhydride for 10 min. The sections were dehydrated through graded ethanols and 40 µl hybridization solution was applied per section. The hybridization solution contained 50% formamide, Tris-HCl (10mM, pH 7.4), EDTA (1.0 mM), 0.02% polyvinyl and Ficoll, 0.02% bovine serum albumin, 0.5% sodium dodecyl sulphate, NaCl (300 mM), 10% dextran sulphate, 0.5 mg/ml yeast or E. coli tRNA and the labelled antisense, or sense, probe, as appropriate. Hybridization was for 18 hr at 55 °C in a humid chamber. After hybridization, the sections were treated with 20 ug/ ml RNase in Tris-EDTA-NaCl buffer, pH 8.0. The sections were then washed initially with 1 times standard saline citrate (SSC), followed by 0.5 times and 0.1 times SSC; all washes were at 55 °C, except the final wash was at room temperature. The digoxigenin-label was detected using the Boehringer detection kit (Boehringer Mannheim, UK), except that 0.3 % Triton X-100 was added to the antibody solution to block the non-specific antibody-binding. NBT and X-phosphate were used as substrates to visualise the antibody to digoxigenin according to the instruction provided with the kit. The sections were then mounted in glycerine jelly. For the detailed protocol and buffers and solutions for in-situ hybridization, see Appendix 14 and 15.

5. 1. 2. 4 Immunohistochemistry of types I and II collagen

Specific goat antibodies to rabbit types I and II collagens (Page, et al., 1986; gifts from Dr. DE Ashhurst, Department of Anatomy, St. George's Hospital, London) were used for

immunohistochemistry. In brief, the sections were rehydrated in Tris buffered saline, pH 7.3 for 15 mins at room temperature and incubated in 2% hyaluronidase (ovine testicular, Sigma, Poole, UK) in PBS for 1 hr at 37°C. They were then washed in PBS and incubated with heat-inactivated normal rabbit serum for 15 mins at room temperature. The specific goat anti-rabbit type I or II polyclonal collagen antibodies were applied at a dilution of 1:40 in PBS and incubated overnight at 4°C. After washing, the sections were incubated with rabbit anti-goat IgG conjugated with alkaline phosphatase or with peroxidase for 1 hr at room temperature. Sections were treated with visualizing substrates (see section 2. 3. 3) washed in PBS, and mounted in glycerine jelly.

5. 1. 3 Results

The sense riboprobes of types I and II collagen were used as negative methodological controls for in-situ hybridization in each set of experiments. Controls for types I and II collagen immunostaining were performed by omitting the primary antibodies. The controls all showed only minimal background staining. As shown in Fig. 5. 1, a representative of the negative controls, there was no positive-labelling. Because of space limitation, the negative controls for each experiment are not shown separately.



Fig. 5. 1. A representative of the negative controls showing that there was no positive-labelling. In situ hybridization using type I collagen sense probe on a bone section, no counterstaining, original magnification X 12.

5. 1. 3. 1 0.3 mm/day group

The regenerate contained mainly newly formed bone and a few areas of fibrocartilage tissues. New bone arose directly from the central fibrous tissue and the lamellae of newly formed bone were oriented along the axis of the distraction force (Fig. 5. 2). Bone remodelling and new bone marrow cavities were frequently seen in the regenerate.



Fig. 5. 2. Adjacent sections of part of a regenerate of the 0.3 mm/day group. A: Direct bone formation (B) from the fibrous tissue (F), H&E staining. B: In situ hybridization using type I collagen antisense probe showed strong expression of type I collagen mRNA in the new bone (arrows). A and B, X 25.

The fibrous tissue in the centre of the regenerate and the osteoblasts on the bone surfaces strongly express type I collagen mRNA, but the expression was reduced in the osteocytes in the newly formed lamellae (Fig. 5. 3 A). There were detectable amounts of type II collagen mRNAs in a few cells within the fibrocartilage tissue of the regenerate (Fig. 5. 3 B).

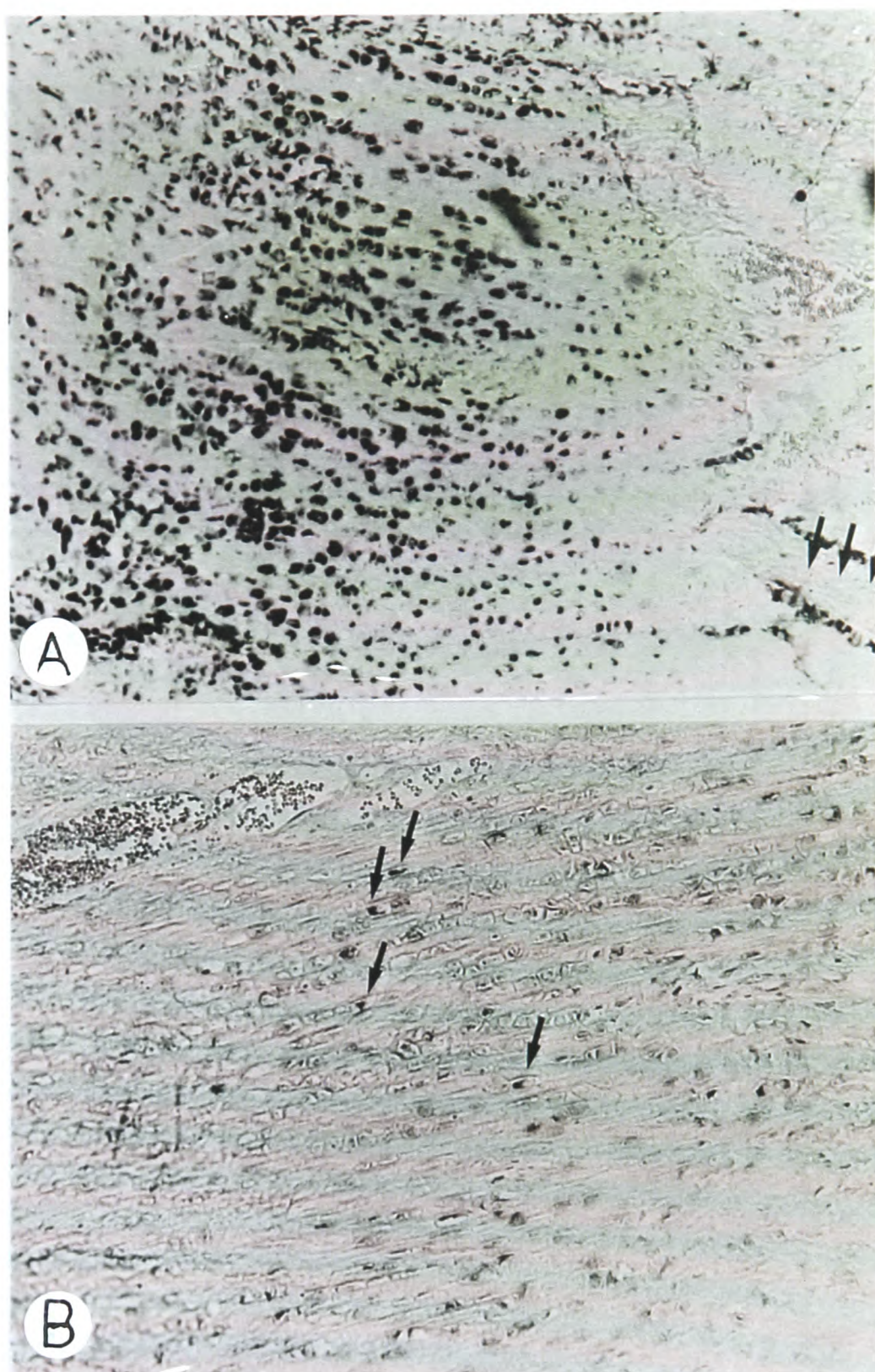


Fig. 5. 3. In situ hybridization in a regenerate at of the 0.3 mm/day group. A: in-situ hybridization using type I collagen antisense probe showed that type I collagen mRNAs (black) were mainly detected in the fibroblasts in the central fibrous tissue and osteoblasts on the bone surfaces. The expression reduced in the osteocytes (arrows), X 25. B: On an adjacent section in a similar area, in-situ hybridization with type II collagen antisense probe demonstrated that type II collagen mRNAs (arrows) were detectable in a few cells within the fibrocartilaginous tissue, X 25.

5. 1. 3. 2 0.7 mm/day group

When the osteotomies were distracted by 0.7 mm / day, new bone grew from the osteotomy ends leaving a central area of fibrous tissue. Small areas of cartilage were found between the central fibrous region and the new bone or adjacent to the periosteal regions. Type I collagen mRNA was detected in the cells on the bone surfaces, the fibroblasts of the central fibrous

tissue, but not in the chondrocytes (Fig. 5. 4 A). Type II collagen mRNAs were only detected in chondrocytes in areas of cartilage in the regenerate (Fig. 5. 4 B). In the region of transition between the fibrous tissue and the cartilage, type I collagen mRNAs still remained in a few marginal chondrocytes (Fig. 5. 4 C). The expression of type I collagen mRNA was diminished in the mature chondrocytes further away from the fibrous tissue (Fig. 5. 4 C), but the expression of type II collagen mRNA was predominant in all the chondrocytes (Fig. 5.4 D).



Fig. 5. 4. In situ hybridization with types I and II collagen antisense probes in a regenerate at a distraction rate of 0.7 mm/day. A: type I collagen mRNAs (black) were attributed to the fibroblasts and bone surface cells. B: On an adjacent section in a similar region, type II collagen mRNAs (arrows) were localised only in the chondrocytes of the cartilage. C: In the region where cartilage was developing from the fibrous tissue, type I collagen mRNAs were still detectable in the developing chondrocytes (arrows), but the expression diminished as the chondrocytes became mature. D: In an adjacent section of this region, type II collagen mRNAs were strongly expressed by the developing chondrocytes. A and B, X 25, C and D, X 100.

In the region of cartilage development from the fibrous tissue, the cartilage remnants in the bone trabeculae were often seen (Fig. 5.5 A). The expression of type I collagen mRNA still remained in the newly developing chondrocytes at the fibrous tissue-cartilage transitional

region, but it became weaker and diminished as the chondrocytes became hypertrophic (Fig. 5.5 B). At some of the fibrous tissue-bone transitional regions, type I collagen mRNA expression was detected in the lacane of some hypertrophic chondrocytes (Fig. 5.5 C, D).

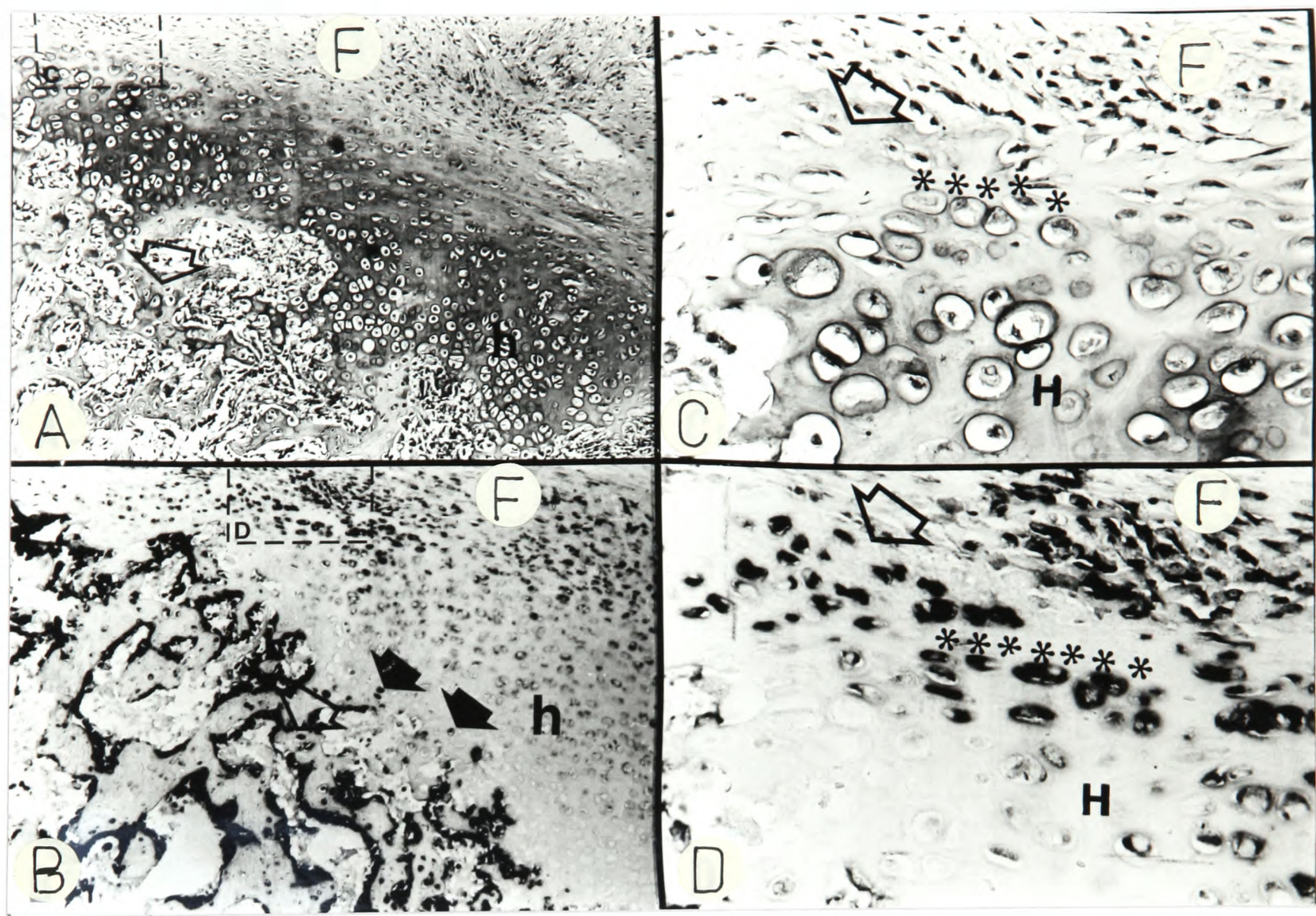


Fig. 5. 5. Adjacent sections of part of a regenerate of the 0.7 mm/day group. **A:** The cartilage was developing from the fibrous tissue (F), the cartilage remnants were seen in the new bone trabeculae (empty arrow head), H&E staining. **B.** In situ hybridization using type I collagen antisense probe. The expression of type I collagen still remained in the developing chondrocytes at the fibrous tissue-cartilage transitional region (bracket), but the expression diminished in the mature chondrocytes. Type I collagen mRNA was detected in the lacunae of a few hypertrophic chondrocytes at the cartilage-bone transitional region (arrow heads). **C.** Close up of the region in bracket of A. The chondrocytes were developing from the fibroblastic cells (empty arrow) in the fibrous tissue, H&E staining. **D.** Close up of the region in bracket of B. Type I collagen mRNA was still expressed in the newly differentiated chondrocytes from the fibrous tissue (empty arrow head). Cells marked "****" in C and D were from very similar regions. F: fibrous tissue, H or h: hypertrophic chondrocytes. A and B, X 25; C and D, X 100.

5. 1. 3. 3 1.3 mm/day group

In this group, moderate amounts of cartilage were found within the regenerate. In some regions cartilage arose directly from the central fibrous tissue, extending to new bone trabeculae

without distinct boundaries (Fig. 5. 6).

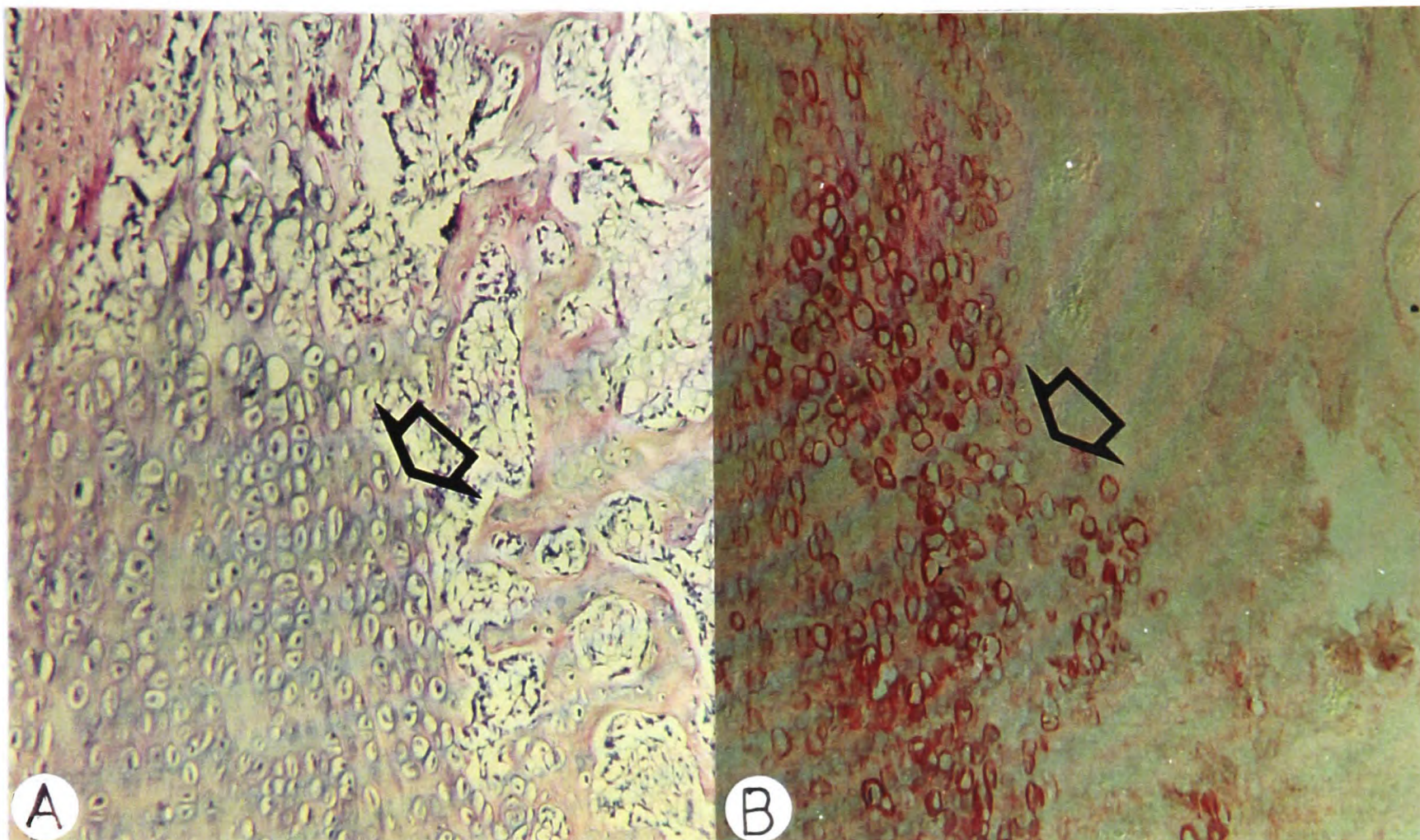


Fig. 5. 6. Adjacent sections of part of a regenerate of the 1.3 mm/day group. A: Cartilage region (arrow) in the regenerate, HE staining, X 25. B: Immunostaining using goat anti-rabbit type II collagen antibody showed that the chondrocytes were positive for type II collagen (arrow), X 25.

Cartilage remnants were often seen in the new bone trabeculae, where chondrocytes were buried in new bone matrix and high power views of the cartilage-bone transitional regions showed that some chondrocytes remain intact in their lacunae within the new woven bone trabeculae. Chondrocytes with their matrix could be seen being replaced by new bone trabeculae as demonstrated by alcian blue and sirius red staining (Fig. 5. 7 A). TRAP-positive cells were seen in most areas of the cartilage-bone junction and TRAP staining was also observed in the cartilage matrix in areas of the cartilage-bone transitional regions (Fig. 5. 7 B).

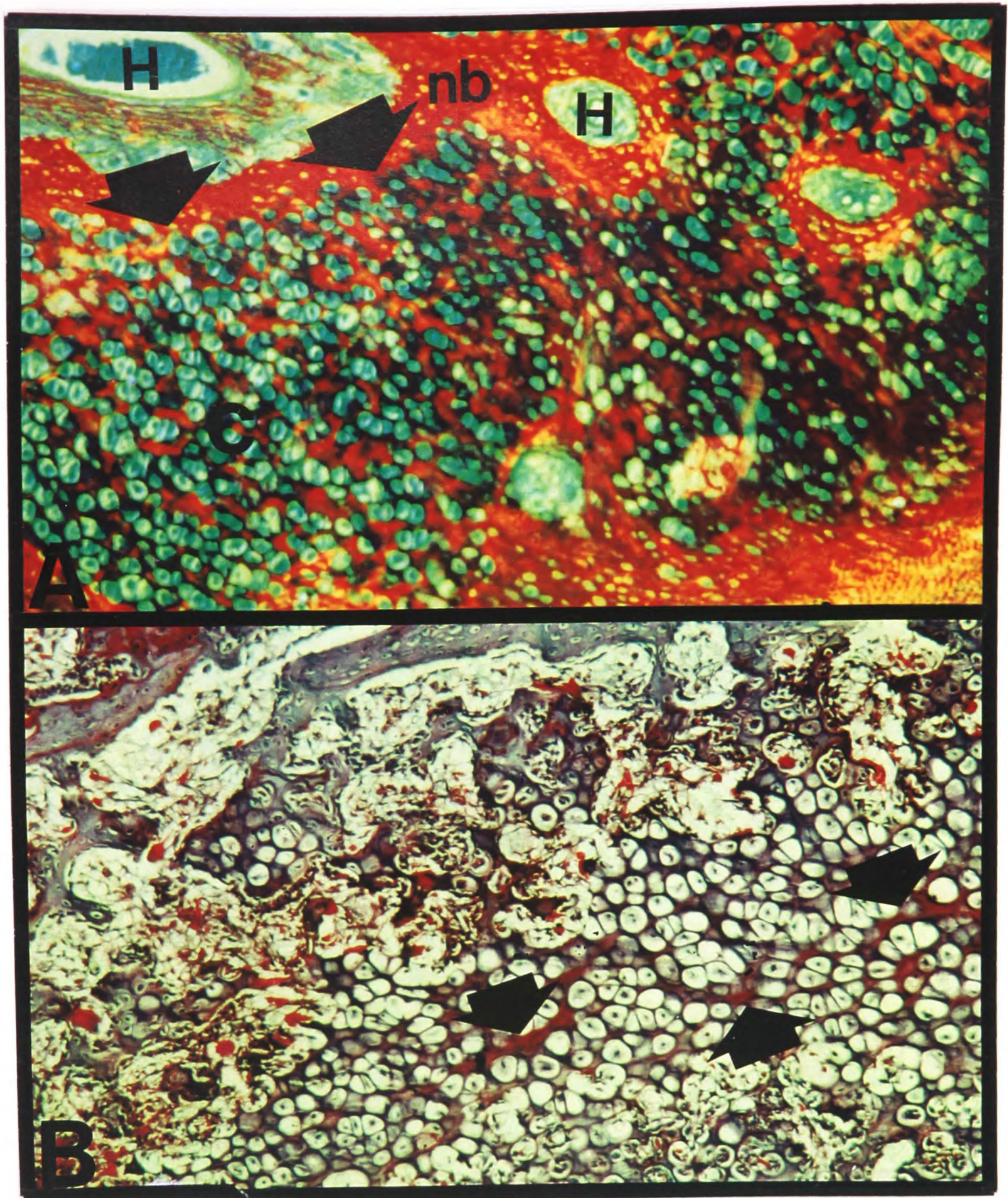


Fig. 5. 7. A: In areas of cartilage-bone transition, chondrocytes appeared to directly transform into new bone matrix (arrows). nb: new bone; C: cartilage; H: Haversian canal. Alcian blue / sirius red staining, X 25. **B:** TRAP staining at the transitional region of cartilage-bone. TRAP positive cells (red) were seen close to the transitional front of cartilage-bone, and nearby cartilage matrix was also showing positive TRAP staining (arrows), X 25.

In-situ hybridization using the type I collagen mRNA antisense probe showed that the cells with the strongest expression of type I collagen mRNA were localised on the new bone surfaces, these cells were flattened and cuboidal multilayered preosteoblasts or osteoblasts. A few newly formed osteocytes in the core of bone trabeculae expressed type I mRNA also, but

the signal was weaker and diminished as the woven bone completed calcification (Fig. 5. 8A). In the region of fibrous-cartilage transition, the cartilage developed from the fibrous tissue where type I collagen mRNA was expressed, and the expression remained in the newly forming chondrocytes at the transitional margin of the fibrous and cartilage tissue. Once the chondrocytes became hypertrophic the expression of type I collagen mRNA was greatly reduced (Fig. 5. 8A). At the transitional region between cartilage and bone, type I collagen mRNA was detected in a small number of cells within the hypertrophic chondrocyte region (Fig. 5. 8B).

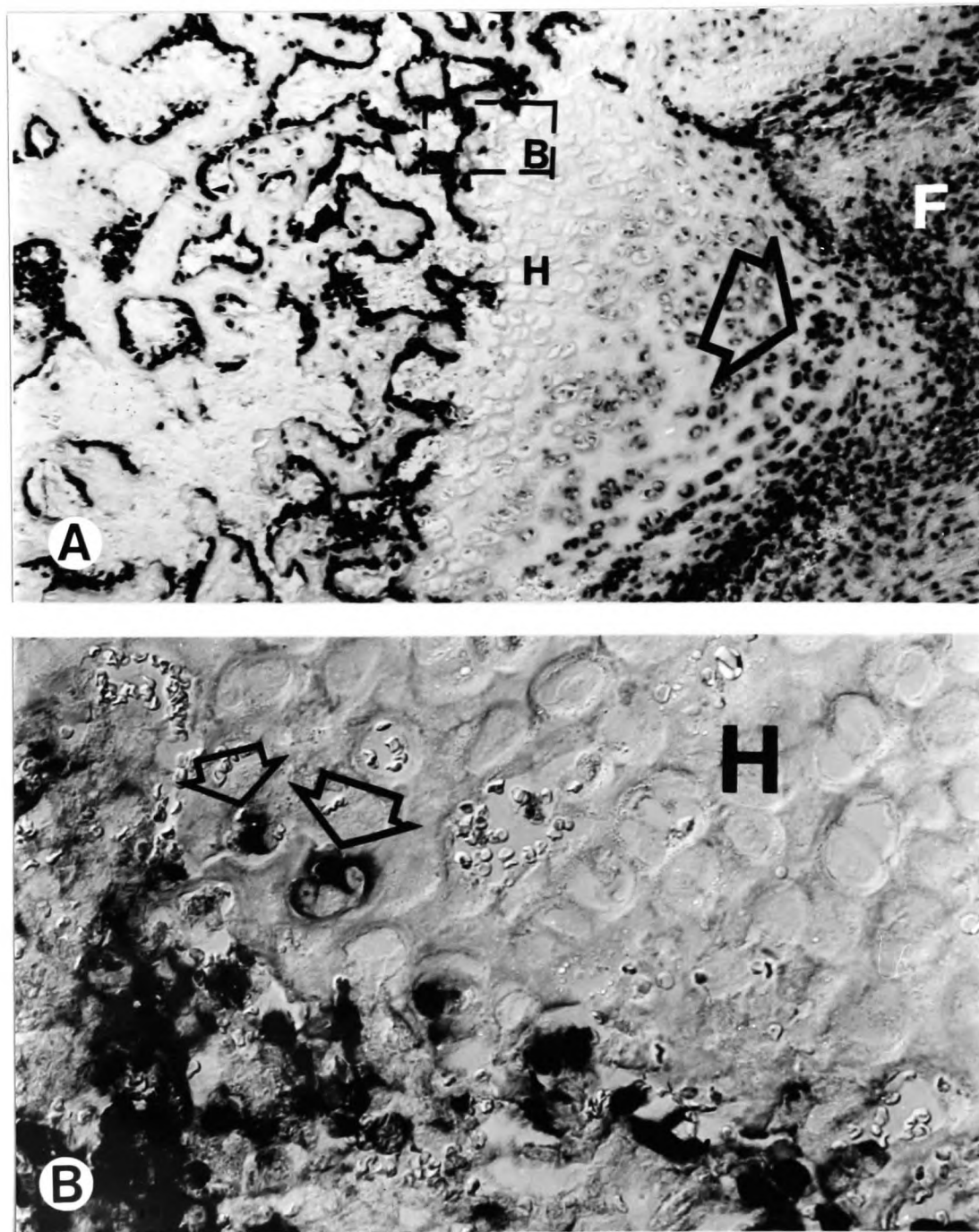


Fig. 5.8. In-situ hybridization using type I collagen antisense probe on part of a regenerate of the 1.3 mm/day group. **A:** Type I collagen mRNA was strongly expressed by the developing chondrocytes at the fibrous tissue-cartilage transitional region (arrow), the expression diminished as the chondrocyte became hypertrophic. **B:** Close up of the boxed area in **A**. Type I collagen mRNA was clearly present in a few hypertrophic chondrocytes lacunae (arrows). **F:** fibrous tissue; **H:** hypertrophic chondrocytes; **A** X 25, **B** X 100.

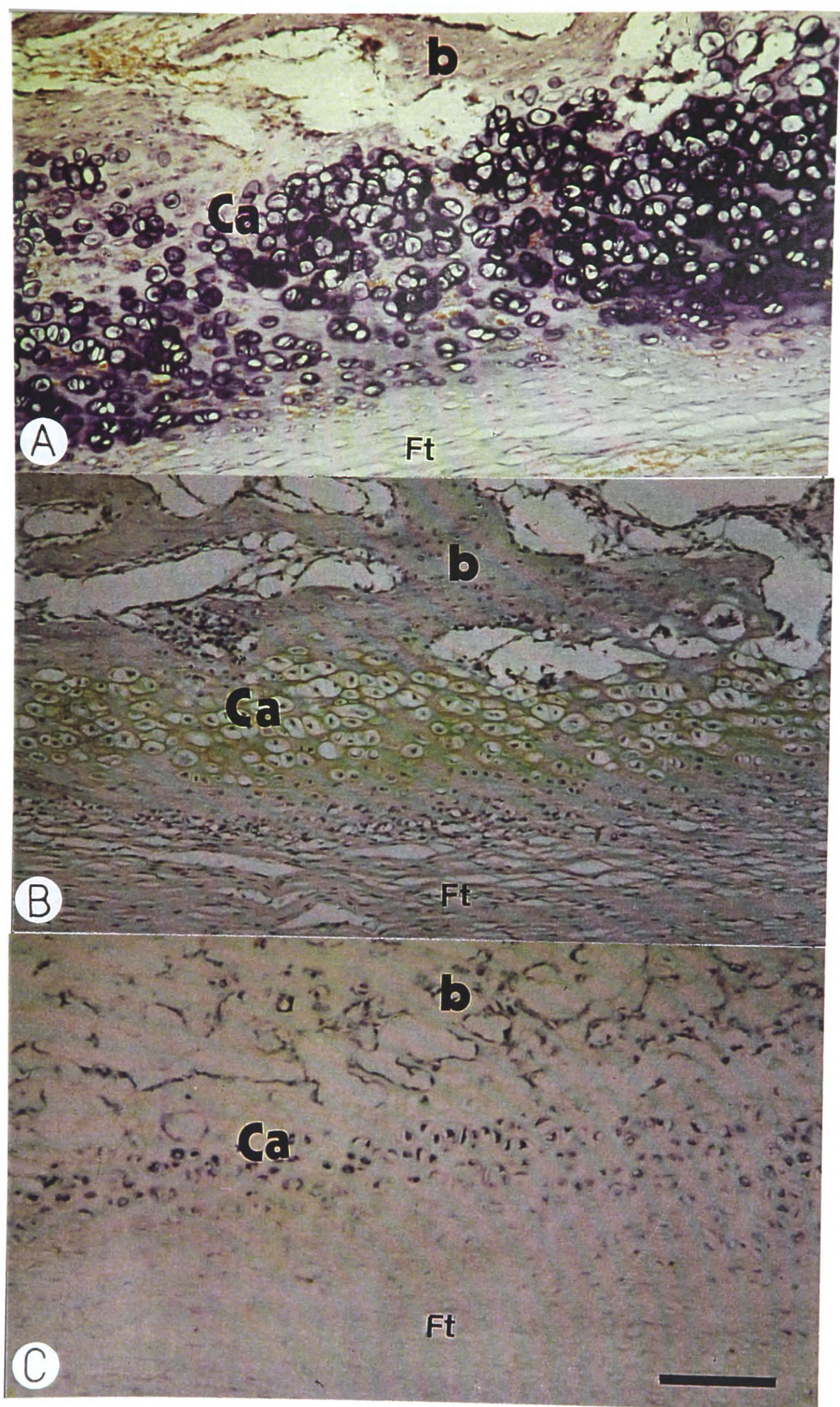


Fig. 5. 9. Adjacent sections of part of a regenerate of the 1.3 mm/day group. A: H&E staining. B: Antibody to type II collagen. C: In situ hybridization with riboprobe for type II collagen mRNA. Cartilage (Ca) is present between the peripheral fibrous tissue (F) and new bone (B). The cartilaginous matrix binds the antibody for type II collagen and the chondrocytes are expressing the mRNA for type II collagen. Bar = 60 μ m.

The presence of type II collagen protein in these cells (Fig. 5. 9 B) confirmed that the marginal cells between cartilage and bone were indeed chondrocytes, and they were expressing type II collagen mRNA (Fig. 5. 9 C).

Blood vessel invasion was observed in some areas of the cartilage region. There was an overlapping of expression of both types I and II collagen in a considerable number of cells in the vicinity of blood vessels within the cartilage region. Type II collagen mRNA was not detected in the tissue closely adjacent to the blood vessels, but it was strongly expressed by the chondrocytes in the surrounding areas of the cartilage (Fig. 5. 10), while the cells of the wall of blood vessels and adjacent cartilage area were expressing type I collagen mRNAs and were positive for immunostaining using specific goat anti-rabbit type I collagen antibody (Fig. 5. 11).

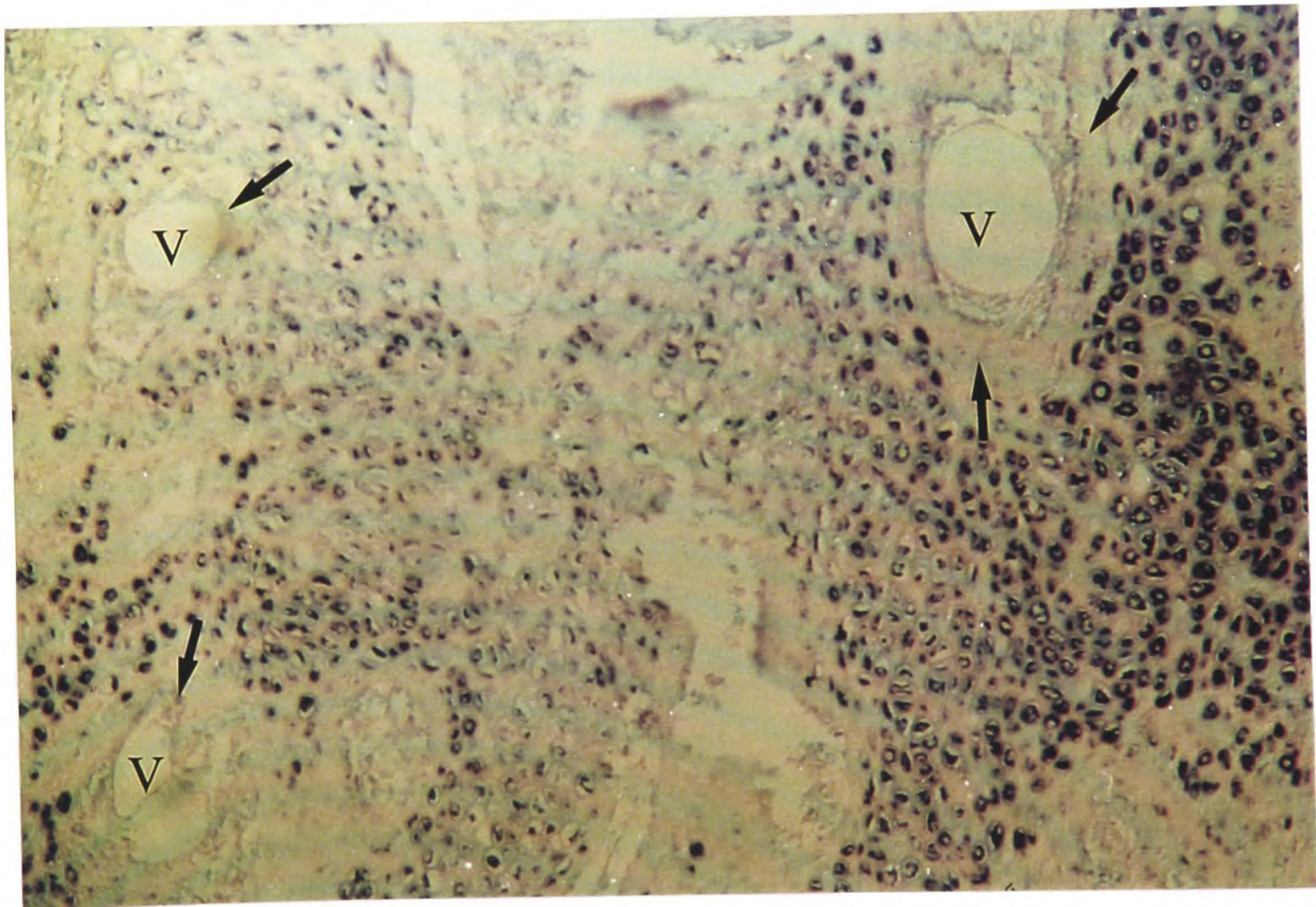


Fig. 5. 10. In situ hybridization using type II collagen antisense probe in the cartilage region of blood vessel invasion in part of a regenerate of the 1.3 mm/day group. Type II collagen mRNAs were present in most of the chondrocytes, but were absent in the vicinity of the invading blood vessels (arrows), V: blood vessel, X 25.

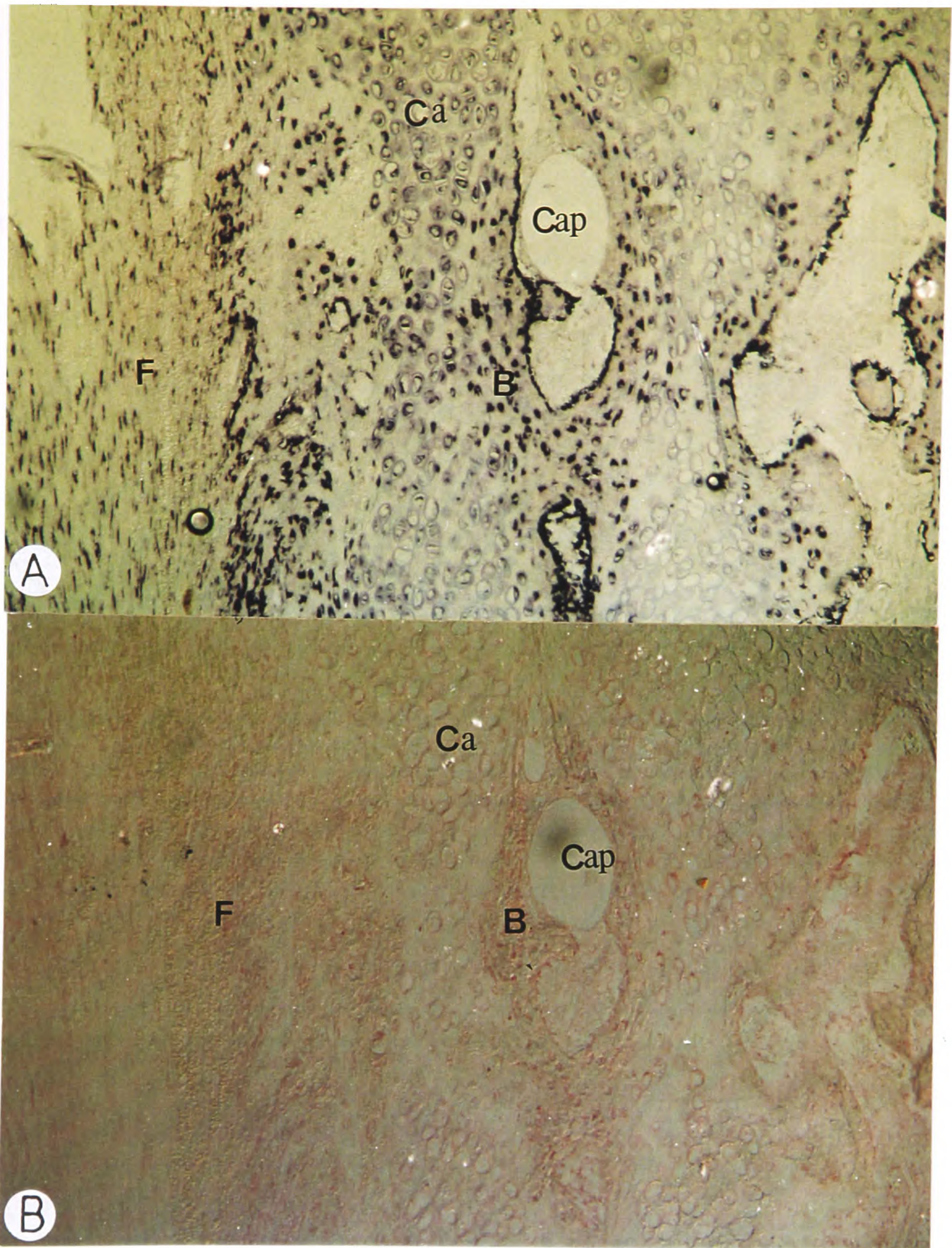


Fig. 5. 11. Adjacent sections of part of a regenerate of the 1.3 mm/day group. A. In situ hybridization for type I collagen mRNA. B. Antibody to type I collagen. This region contains fibrous tissue (F), bone (B), cartilage (Ca) and capillaries (Cap). The capillaries are surrounded by a layer of bone (arrow). The cells in the bone are expressing the mRNA for type I collagen. The bone matrix binds to the antibody to type I collagen. Bar = 60 μm.

5. 1. 3. 4 2. 7 mm/day group

In this group, there was only sparse bone formed and most of the distraction gap was fibrous tissue. Areas of cartilage similar to those found at distraction rates of 0.7 or 1.3 mm/day groups were present, but the number of cartilage areas was less. Type I collagen mRNA was mainly expressed by the fibroblasts in the central fibrous zone (Fig. 5. 12), and type II collagen mRNA was expressed in chondrocytes with the similar patterns as seen in the 0.7 and 1.3 mm/day groups.

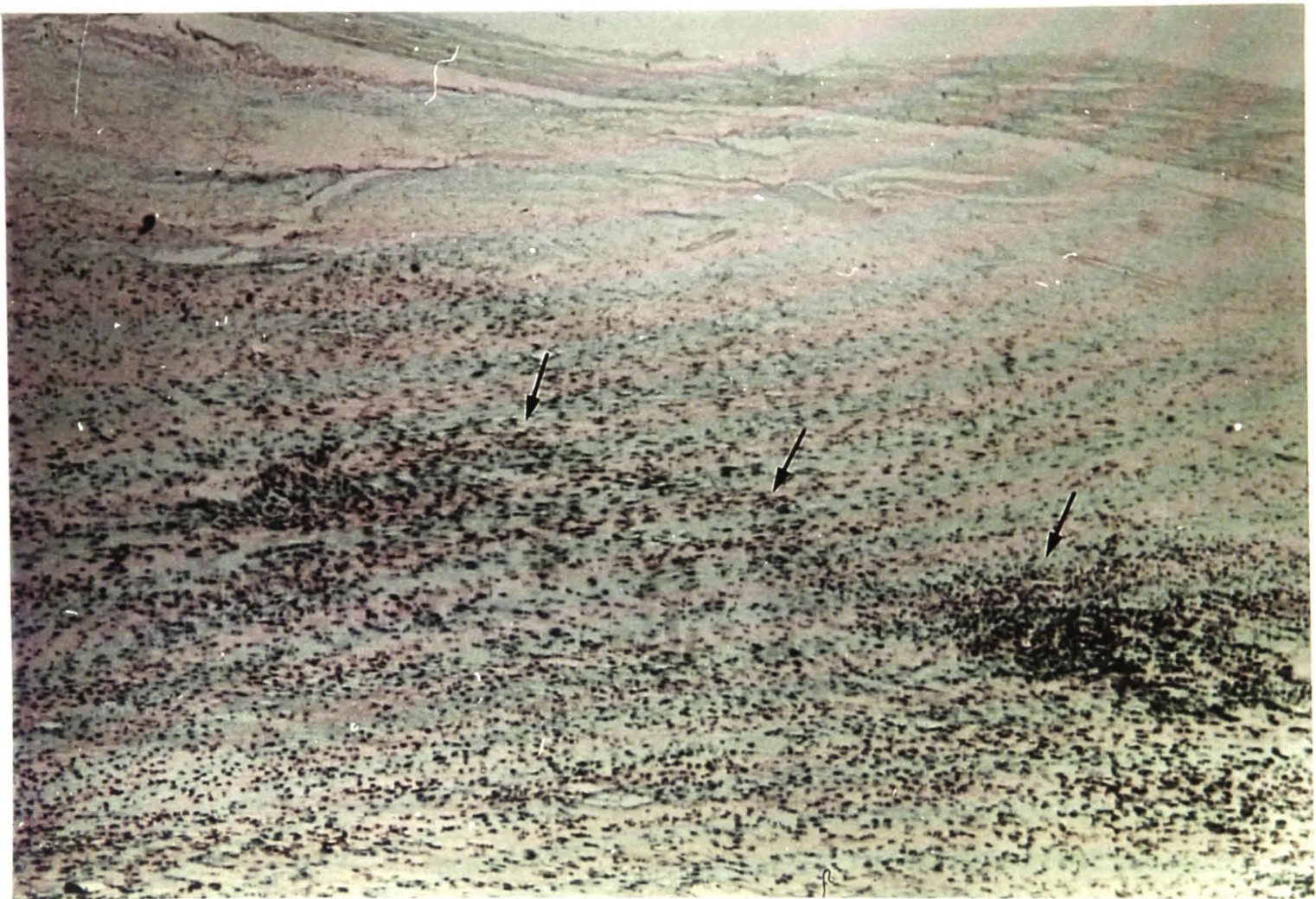


Fig. 5. 12. In part of a regenerate of the 2.7 mm/day group, in situ hybridization using type I collagen antisense probe showed that type I collagen mRNAs were predominantly expressed by the fibroblasts in the central fibrous zone (arrows), X 25.

5. 1. 3. 5 Summary

Type I collagen mRNA was expressed predominantly by the fibroblasts in the fibrous tissue, the bone surface cells and to a reduced extent by the osteocytes. Type II collagen mRNA was expressed by chondrocytes. Type I collagen was present in the matrices around the cells expressing its mRNA and similarly, type II collagen was located around the chondrocytes. The osteoblasts and chondrocytes within the regenerate originate from the same pool of progenitor

cells, and the differentiation of these cells and the subsequent expression of types I and II collagen genes are altered by different rates of distraction during distraction osteogenesis. As the distraction rate increased, the expression of type I collagen in the regenerating tissue decreased, and the expression of type II collagen of the regenerating tissues increased. But, the increase in chondrogenesis ability has a limit and it did not increase further at a distraction rate of 2.7 mm/day.

5. 1. 4 Discussion

5. 1. 4. 1 The distraction rate determines the types of tissues formed during distraction osteogenesis

Several experimental studies of distraction osteogenesis have used different animal models (Kojimoto, et al., 1988; Ilizarov, et al., 1989 a, b; Aronson, et al., 1990; Delloye, et al., 1990; Karaharju, et al., 1993) and the results show that at distraction rates of 0.5 - 1 mm/day, intramembraneous bone was formed during distraction osteogenesis, and in addition fibrocartilage tissues were often present. These studies were morphological and there was no attempt to characterize the collagens in the newly formed matrices or to determine the cells that were expressing the mRNAs for these collagens. In the present study, tibial osteotomies were distracted at rates from 0.3 to 2.7 mm/day until the tibiae were 20% longer than their original length. This design offered an unique model to examine the changes of collagen gene expressions at different distraction rates. At the lowest distraction rate (0.3 mm/day), the tissues in the gap were similar to those found in previous studies, and bone formed by an intramembraneous manner with scattered fibrocartilage in the middle of the regenerate. At distraction rates 0.7 mm/day and above considerable amounts of cartilage areas, areas of necrosis and cyst formation were present in the distraction regenerate. In addition, the daily increments in length at higher distraction rates may be greater than the rate of vascularization, so that the blood supply to the centre of the regenerate is compromised and this will produce an anoxic region. Together, these factors may lead to cartilage, necrosis and cyst formation.

The cells in the distraction gap maybe derived from osteogenic progenitor cells in the periosteum, marrow cavity, blood circulation and surrounding tissues (Kojimoto, et al., 1988; Iwamoto, et al., 1993). The differentiation of these osteogenic progenitor cells determines the tissues formed during distraction osteogenesis. These tissues were developing very rapidly and were affected by the local mechanical conditions and vascularity. The results in the present study indicate that the differentiation of osteogenic progenitor cells was affected by the distraction rate. Irrespective of the rate of distraction, bone formation was primarily intramembraneous, that is, the bone matrix was laid down within an existing fibrous matrix. Endochondral ossification was restricted to those areas in which cartilage had developed. In the newly formed bone, the osteoblasts on or close to bone surfaces express type I collagen mRNAs, while the expression of type II collagen mRNAs was restricted to chondrocytes in the cartilage. In regions between bone and cartilage, areas of chondroid bone where the matrix contains both types I and II collagens and the large, chondrocyte-like cells may be expressing type I collagen mRNAs have been reported previously (Page, et al., 1986; Sandberg, et al., 1989; Hughes, et al., 1995b). A similar situation may occur between cartilage and fibrous tissue, where a chondrocyte-like cell may express type I collagen mRNA as was observed in the present model, which also suggests that the chondrocytes within the regenerate originate from the same pool of progenitor cells as osteoblasts. However, from the present in-situ hybridization experiments, it was not possible to determine whether the individual cells are expressing both types I and II collagen mRNAs at the same time.

In conclusion, the distribution of the cells expressing types I and II collagen mRNAs indicates an altered process of tissue regeneration by the different rates of distraction. In this animal model of leg-lengthening, both endochondral and intramembraneous ossification occur in the regenerate at distraction rates higher than 0.3 mm/day. The osteoblasts and chondrocytes within the regenerate originate from the same pool of progenitor cells, their differentiation and the subsequent expression of types I and II collagen genes may be regulated and affected by local factors such as the rate of distraction.

5. 1. 4. 2 The role of chondrocytes in intramembranous and endochondral ossification during distraction osteogenesis in the rabbit

Bone formation by intramembraneous ossification was reported to be the unique characteristic of distraction osteogenesis in previous studies in human, dog and sheep species. However, in rabbits, cartilage was observed during tibial or femoral lengthening by using Orthofix unilateral lengtheners at rates of 0.5 - 1.0 mm/day (Kojimoto, et al., 1988; Masamizu, et al., 1994). This difference may reflect the species differences in response to distraction.

Histological staining techniques, immunohistochemistry, and non-radioactive in situ hybridization using types I and II collagen, have been used to document the presence of an overlapping cartilage-bone phenotype in cells of the cartilage-bone transitional region. In this region, we observed that some chondrocytes appeared to be being transformed directly into bone cells surrounded by bone matrix in the new bone trabeculae. This was clearly shown by alcian blue / sirius red staining, and is termed chondroid bone (Beresford, 1981). When the undifferentiated osteogenic progenitor cells in the newly formed skeletal tissue are stimulated to differentiate, some do not go down a simple pathway to an osteoblast or chondrocyte. Determined by the environment, systemic and local factors, some cells become "ambivalent" and may become hybrid cells producing chondroid bone or fibrocartilage, and may produce matrices with both types I and II collagens (Ashhurst, personal communication).

Another interesting observation was the presence of acid phosphatase within the cartilage matrix in the cartilage-bone transition region. Similar observations have been reported by Scammell and Roach (1996) in a study of fracture healing in the rabbit. They found acid phosphatase activity within intact chondrocytic lacunae and they defined this as "lacunar" bone. They suggested a role for chondrocytes in fracture repair, with endochondral ossification including direct bone formation by former chondrocytes. Robles-Marin et al. (1984) reported an increase of acid phosphatase activity in the chondrocytes next to the vascular invasion during endochondral ossification in the chicken growth plate. They

proposed that vascular invasion will occur only after the secretion of acid phosphatases from the chondrocyte into the cartilage matrix, and disintegration of the cartilage matrix is induced by oxidation following vascularization. However, the possibility that diffusion of acid phosphatase staining may result in a similar pattern was not considered by these authors.

At the cartilage-bone transitional regions, both types I and II collagen mRNAs and proteins were found together in some of the marginal hypertrophic chondrocytes. This could be a result of direct transformation of hypertrophic chondrocytes into osteoblasts or be a result of invasion of the chondrocyte lacunae by osteoblast progenitor cells, but it is impossible to distinguish between these two hypotheses from the current study. Previous studies of chondrocyte differentiation have indicated that hypertrophic chondrocytes *in vitro* may express characteristic proteins for the osteoblast phenotype (Cancedda, et al., 1995). Expression of these proteins by hypertrophic chondrocytes has also been observed *in vivo* in selected cartilage regions. *In-situ* hybridization and/or immunohistochemistry shows that human, rat and chicken hypertrophic chondrocytes may be capable of synthesising some of the markers for the osteoblast, such as type I collagen (Yasui, et al., 1984; Sandberg, et al., 1989; Galotto, et al., 1994; Hughes, et al., 1995), osteocalcin (Stafford, et al., 1994; Hughes, et al., 1995b), osteopontin (Franzen, et al., 1989) and osteonectin (Metsaranta, et al., 1989; Pacifici, et al., 1990). In addition, Galotto et al. (1995) have reported that preosteoblasts and osteocytes share the same membrane proteins as hypertrophic chondrocytes. Nevertheless, none of these published observations conclusively prove that hypertrophic chondrocytes give rise to osteoblasts directly.

An alternative explanation for the presence of type I collagen mRNA in the chondrocytic lacunae is that these lacunae may be invaded by other cells outside the plane of the sections. They are likely to be osteogenic progenitor cells brought into the chondrocyte lacunae by the invading blood vessels.

To conclude, morphological observations, together with the pattern of acid phosphatase activity, expression of types I and II collagen mRNAs and localization of types I and II collagen proteins, indicate a process of endochondral ossification occur at higher rates of

distraction in the rabbit. The terminal fate of hypertrophic chondrocytes is still controversial and it has been suggested that it may be different at different microanatomical sites (Cancedda, et al., 1995). Previous reports regarding chondrocyte and osteoblast turnover, together with the findings in the present study suggest that osteoblasts and chondroblasts originate from a common pool of mesenchymal cells. These differentiated cells could share some plasticity of phenotype. However, in the present study, as in all other studies published to date, this has not been proven. More direct evidence is required before any claims can be made that the hypertrophic chondrocytes can transform into bone-forming cells.

5. 2 Localization of bone keratan sulphate proteoglycan in the regenerate

5. 2. 1 Introduction

A central requirement for any study of cell differentiation is the availability of markers which can be used to distinguish between cells of different lineages and to define the stage of differentiation of a given cell. Joyner et al. (1989) produced a monoclonal antibody, BRL 12 which reacts with a high molecular weight antigen present exclusively in osteogenic tissue. Immunohistochemical studies have shown that the antigen is present throughout the mineralized matrix and in osteoid, but not in other tissues examined such as liver, kidney, spleen thymus, heart, skeletal muscle. Expression of the antigen is restricted to tissues which are either mineralized or are about to mineralize and it is possible that the BRL-12 antibody may be a "late-stage" marker for cells of the osteoblastic lineage (Joyner, et al., 1989). The bone constituent recognized by BRL-12 antibody was characterized later by gel filtration, ion exchange chromatography, protein blotting and immunolocalization (Joyner, et al., 1992). It was found that BRL-12 reacts with an epitope associated with the core protein of rabbit keratan sulfate proteoglycan (KSPG), a molecule which shares considerable homology with the bone sialoprotein present in the bone tissue of other mammalian species.

The aim of study in this section was to investigate the distribution of BRL-12 antibody binding

in the regenerates in the group with a distraction rate of 0.7 mm/day, which was found to be optimal for bone cell proliferation and bone formation. In addition, von Kossa staining was used to study the mineralization process.

5. 2. 2 Methods

Paraformaldehyde-fixed paraffin-embedded and ethanol-fixed glycol methacrylate-embedded specimens of the regenerates from the group at a distraction rate of 0.7 mm/ day were used for the present study. The BRL-12 antibody was a gift from Dr. CJ Joyner (MRC Bone Research Laboratory, Nuffield Orthopaedic Centre, Oxford). For immuno- histochemistry of BRL-12, a standard indirect (two step) method was used. In brief, paraffin sections were dewaxed in xylene and taken through graded ethanol (100%, 90%, 70%, 50%). Endogenous peroxidase activity was blocked by immersion in 80% methanol containing 0.5% (v/v) hydrogen peroxide for 30 mins. Sections were then incubated with BRL-12 antibody (undiluted cell culture supernatant) for 1 hr in a humid chamber at room temperature. Normal rabbit serum (10%) in PBS containing 0.05% Tween 20 (BDH, UK) was used to block the non-specific binding of the secondary antibody. The sections were then incubated with rabbit anti-mouse peroxidase conjugated antibody (Sigma, Poole, Dorset, UK) at a dilution of 1: 50 in PBS for 30 mins. Peroxidase activity was demonstrated with 0.5mg/ml 3,4,3',4',-tetrabiphenyl hydrochloride (Sigma, Poole, Dorset, UK) in PBS (pH7.2) containing 0.3% (v/v) hydrogen peroxide for approximately 5 mins, and the sections were lightly counterstained with Mayer's haematoxylin, and mounted in DPX. For von Kossa staining, 5 µm thick glycol methacrylate sections were used and the von Kossa staining was performed according to the method described in section 2. 2. 5. 3.

5. 2. 3 Results

In undecalcified plastic sections, BRL-12 antibody binding was evident throughout the mineralized bone matrix including the osteoid in a diffused pattern (Fig. 5. 13).

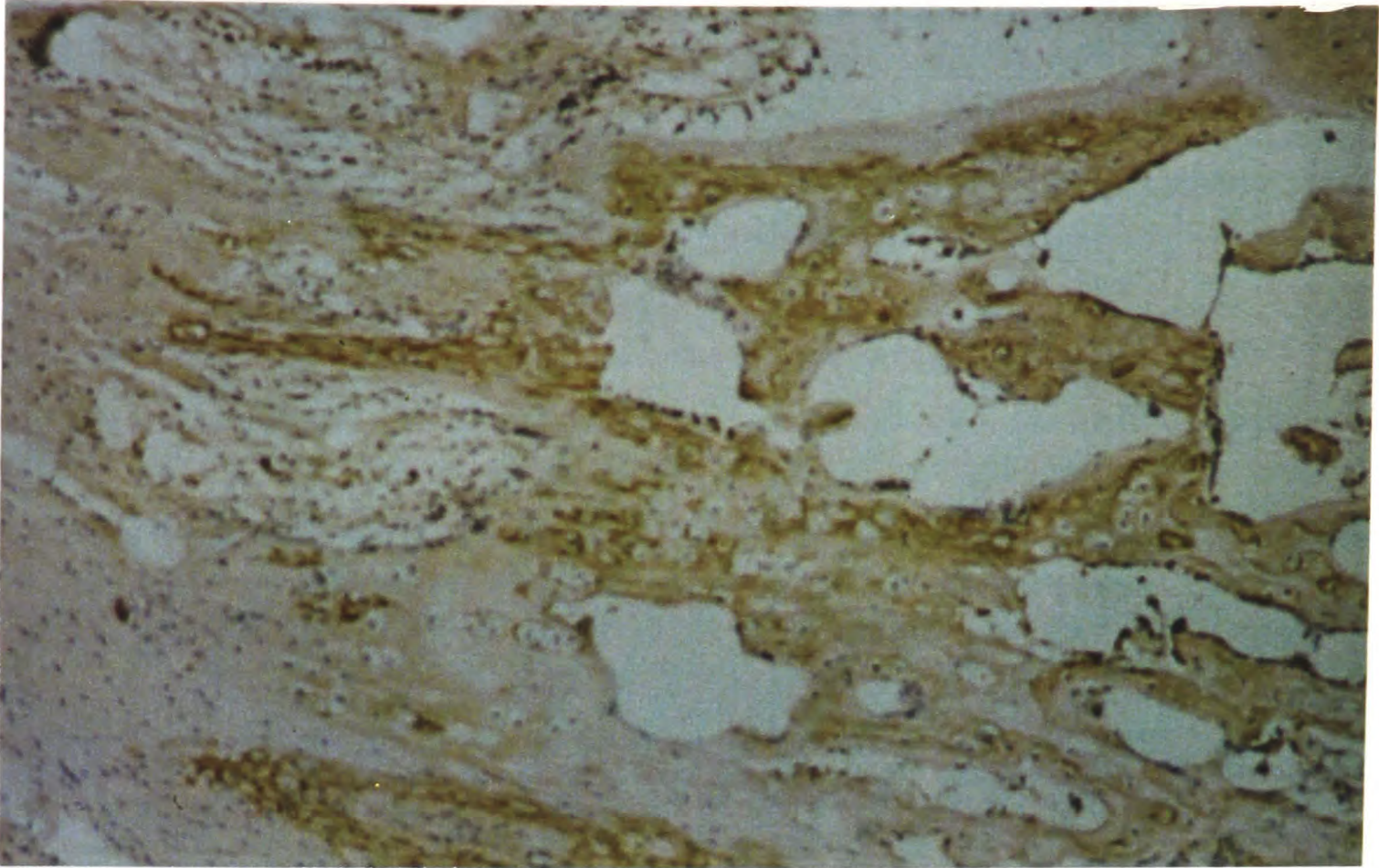


Fig. 5. 13. BRL-12 antibody binding on a plastic section of a regenerate of the 0.7 mm/day group. Bone matrix and the osteoid were positively stained (brown), X 25.

In decalcified paraffin sections, the BRL-12 antibody staining was localised within the cytoplasm of some cells, in a diffuse manner. The BRL-12 positive-staining was observed: (1) in the fibroblastic cells of the central fibrous region, where new bone was developing (Fig. 5. 14 A, B); (2) in a few marginal chondrocytes at the transitional region of fibrous tissue-cartilage (Fig. 5. 14 C) or in the region of cartilage-bone developing (Fig. 5. 14 D), but not in the central region of the cartilage tissue (Fig. 5. 14 A); (3) in the cells on or close to the bone surfaces of the newly formed bone trabecule (Fig. 5. 14 E). There was no binding on the negative control section (Fig. 5. 14 F).

In the cartilage-bone transitional region, BRL-12-positive staining was localised in the cytoplasm of osteoblastic cells at the ossifying fronts, where cartilage matrix was being broken down and new bone matrix was being formed and mineralized (Fig. 5. 15 A). However, the remnants of calcified cartilage within bone trabeculae were unstained (Fig. 5. 15 B). The mineralization process at the cartilage-bone junction was confirmed by von Kossa staining (for phosphate) on undecalcified glycol methacrylate sections (Fig. 5. 16 and Fig. 2. 11).

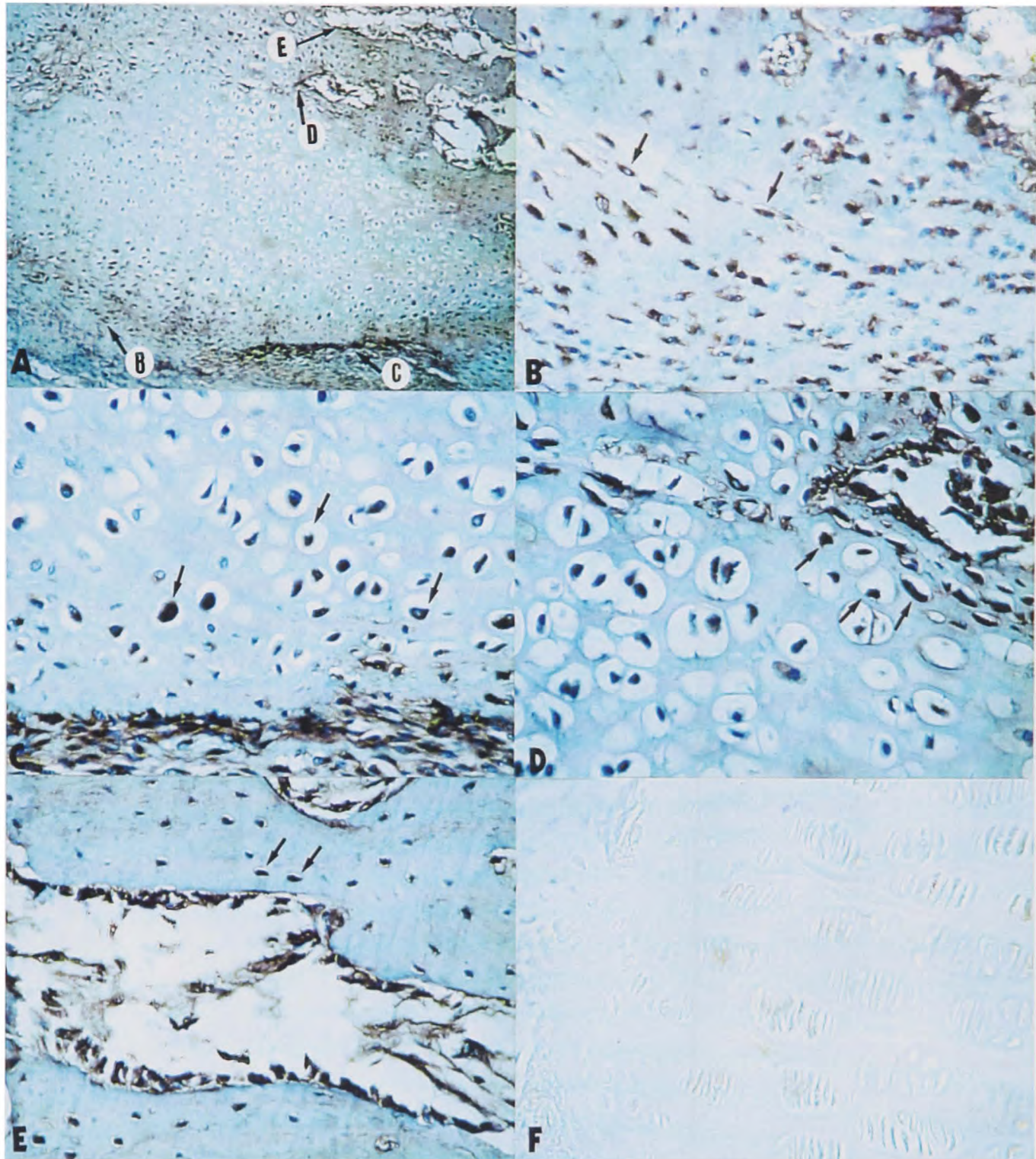


Fig. 5. 14. Localization of BRL-12 antibody binding in a regenerate of the 0.7 mm/day group. A: Orientation of the morphology and the sites of binding. B: Positive-staining was localised in the cytoplasm of fibroblastic cells, from where bone was formed intramembranously (arrows). C: Binding was present in a few marginal chondrocytes (arrows) at the transitional region of fibrous tissue-cartilage. D: Binding was found in chondrocytes (arrows) at the transitional region of the cartilage-bone. E: Binding was also localised in the cells on or close to the bone surfaces (brown colour) of the newly formed bone trabeculae, some osteocytes were also positive (arrows). F: Immunostaining was performed on a section of rabbit growth plate without applying the BRL-12 antibody; no positive-staining was found. A X 25, B-F, X 100.

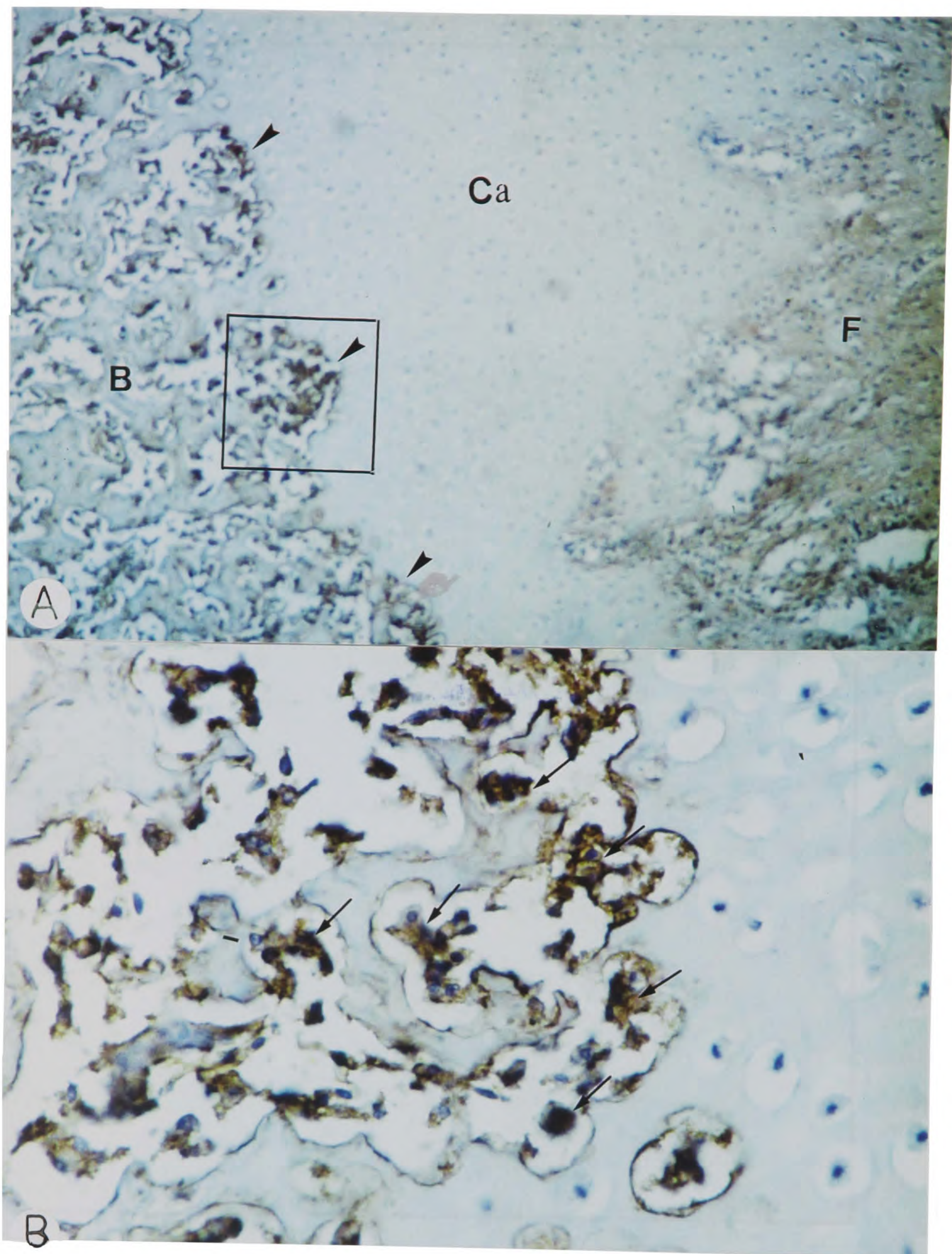


Fig. 5. 15. BRL-12 binding at the mineralization front of the cartilage-bone junction in part of a regenerate of the 0.7 mm/day group. A: Binding was seen at the ossifying fronts (arrows), cartilage tissue was unstained. B: Close-up of the boxed area in A. The osteoblastic cells were constantly stained by BRL-12. F: fibrous tissue; Ca: cartilage; B: bone. A, X 25, B, X 100.

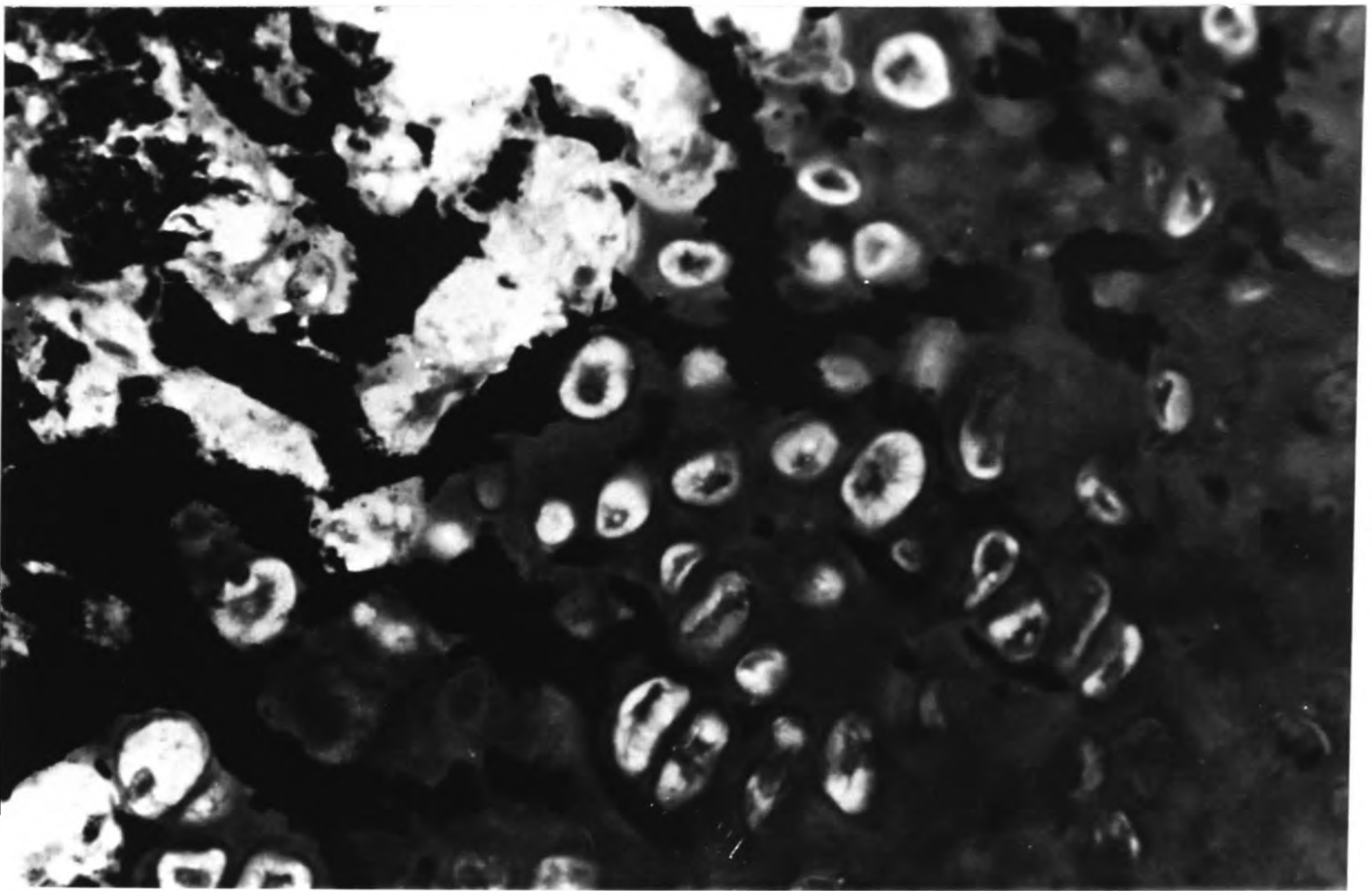


Fig.5. 16. At the cartilage-bone junction in a regenerate of the 0.7 mm/day, the mineralization preceeds as the cartilage matrix calcified and remodelled. Phosphate stains in black, Von Kossa staining, X 100.

5. 2. 4 Discussion

The present study has demonstrated that the BRL-12 antibody can be used on paraformaldehyde-fixed, EDTA-decalcified, paraffin-embedded rabbit bone tissue as well as on the resin-embedded undecalcified bone tissue. However, the pattern of the immunostaining was slightly different. On plastic sections, BRL-12 binds to bone matrix and osteoid, but fails to binds to bone surface cells (Joyner, et al., 1989). On paraffin sections, BRL-12 stains bone matrix as well as cell cytoplasm in a diffuse pattern, and it is possible to identify individual cells stained by BRL-12.

It has been shown that the core protein of rabbit bone KSPG is closely related to that of human bone sialoprotein (BSP). BSP and bone KSPG possess almost identical N-terminal amino acid sequences and a polyclonal antibody to rabbit bone KSPG bound to bovine BSP core protein

(Kinne and Fisher, 1987). BSP is highly concentrated in bone matrix and it is synthesized by osteoblasts (Robey-Gehron, 1996). Likewise, bone KSPG is considered to be a late stage product of differentiated cells of the osteoblastic lineage (Joyner, et al., 1992). The physiological role of KSPG is unknown, but it seems likely that BSP and KSPG have related functions. BSP may play a role in cell attachment at sites of osteogenesis (Sommerman, et al., 1988). The exposure of BSP on the bone surface may be an important factor in osteoclast adhesion and subsequent bone resorption (Bulter, et al., 1996).

The localization of BRL-12 antibody binding to the fibroblastic cells in the transitional region between fibrous zone and the mineralization front suggests that the osteoprogenitor cells in the fibrous tissue have potential to differentiate into osteoblasts and make bone directly. BRL-12 binds to some newly developing chondrocytes at the transitional regions of fibrous tissue to cartilage, and the marginal chondrocytes between the cartilage and bone, but the majority of the chondrocytes in the centre of the cartilage tissue were unstained. These observations indicate possibilities that (1) some chondrocytes may be capable of synthesising KSPG at certain anatomical sites and conditions; (2) chondrocytes and osteoblasts share a common pool of progenitor cells, and may switch phenotype or have overlapping phenotype. This has also been discussed in section 5. 1. 4. 2; (3) potential osteoblastic cells may remain in the chondrocyte lacunae at an early stage of cartilage development; and (4) the osteoblasts may have invaded the chondrocyte lacunae at the cartilage-bone transitional region. It is still not possible to establish whether hypertrophic chondrocytes may be able to change into bone-forming cells in the present study.

5. 3 Conclusions

In-situ hybridization using types I and II collagen riboprobes demonstrated that the expression of type I collagen mRNA was predominant in the fibroblasts of fibrous tissue and bone surface cells, but reduced in the osteocytes; type II collagen mRNA was only expressed by the chondrocytes of cartilage. Some unusual features of endochondral ossification, which occurs during the rapid transition of cartilage to bone in the regenerate, were observed.

Special histological staining together with immunohistochemistry for osteoblast related molecules (eg. bone KSPG), have documented the presence of an overlapping cartilage-bone phenotype in cells of the fibrous tissue-cartilage or cartilage-bone transitional regions. In those particular areas, some chondrocytes appeared to be directly transformed into new bone trabeculae. Acid phosphatase was also found within the cartilage matrix in the region of the cartilage-bone transition, and type I collagen mRNA, bone KSPG protein and type II collagen protein were found together in some of the marginal hypertrophic chondrocytes. This study indicates an unusual but active role of chondrocytes in the process of endochondral ossification during distraction osteogenesis in the rabbit. Further direct evidence is required to prove the hypothesis that the hypertrophic chondrocytes may directly transdifferentiate into bone-forming cells during intramembranous or endochondral ossification.

It is concluded that in the present leg-lengthening system in the rabbit, higher rates of distraction (from 0.7 mm/day to 1.3 mm/day) result in increased chondrogenesis in the regenerating tissues. The osteoblasts and chondrocytes within the regenerate originate from the same pool of progenitor cells, their differentiation and the subsequent expressions of types I and II collagen genes and proteins may be altered and affected by the rate of distraction, and therefore, the rate of distraction determines the types of skeletal tissues formed during distraction osteogenesis.

Chapter 6 Expression of BMP-4 mRNA during distraction osteogenesis

6. 1 Introduction

Bone formation in distraction osteogenesis resembles aspects of both normal fracture healing and embryonic limb development (Ilizarov, 1991; Sproul and Price, 1992b). Shared features include a stimulus for proliferation of mesenchymal cells and an induction of the mature osteoblasts. Urist et al. (1982) has suggested that a common morphogen (bone morphogenic protein) exists in fracture healing, bone graft incorporation and heterotopic bone formation. Similarly, as the process of fracture repair and distraction osteogenesis have many features in common, they may share some common molecular mediators.

Although many cytokines, growth factors, hormones and extracellular matrix components are capable of regulating specific aspects of bone formation and remodelling during bone growth and repair, bone morphogenetic proteins (BMPs) are the most potent osteoinductive proteins identified and the only factors known to date that can induce bone formation in vivo (Urist, 1965; Wozney 1992; Campbell and Kaplan, 1992). BMPs have also been implicated as potential transducers of the mechanobiological influence on bone development, making the proteins appropriate candidates for the mediation of the influence of mechanical forces (Van der Meulen, et al., 1993; Mikic, et al., 1995). The histological (Schenk and Gachter, 1994) and clinical features (Ilizarov, 1990) of distraction osteogenesis have been well documented. However, little is known about the presence of BMPs during distraction osteogenesis.

BMP-4 is a member of the BMPs and TGF- β superfamily, it plays an important role in normal embryogenesis, inducing proliferation and differentiation of mesodermal tissues (Rosen and Thies, 1992; Hogan, 1996). BMP-4 can induce primitive mesenchymal cells to form bone by a direct or by an endochondral process in vitro and in vivo (Wozney, 1993; Hughes, et al., 1995). The hypothesis in this study is that BMP-4 may act as a mediator of induction of bone formation in distraction osteogenesis. To test this hypothesis, the temporal and spatial pattern

of BMP-4 mRNA expression were investigated by using in-situ hybridisation methods in the established rabbit model of leg-lengthening.

6. 2 Methods

6. 2. 1 Animal model and sample preparation

6 NZW rabbits (age 6 months, body weight 3.0-3.5 kg) had mid-tibial osteotomies performed in the left tibiae and stabilised with external fixators (Orthofix M100) under general anaesthesia as previously described. 7 days later, twice daily distraction was initiated at rate of 0.7 mm/day, until a 20% lengthening of the tibia was achieved. The animals were killed by overdosage of anesthetic and the lengthened tibia was surgically excised, sliced longitudinally into 2 pieces and fixed in 4% paraformaldehyde (pH 7.4) for 24 hours. The samples were decalcified for 14-28 days in 14.5 % (w/v) EDTA (pH 7.4) which was changed weekly and completeness of decalcification was determined radiographically. The tissue was dehydrated in graded ethanols, cleared in xylene and embedded in paraffin wax. 5 µm thick sections were cut and mounted on APES-coated slides for in-situ hybridization purpose as previously described.

6. 2. 2 Cell culture of rabbit and human bone marrow cells and human osteosarcoma (SaOS2) cells

Human bone marrow cells were obtained from the operating theatre during bone operations at the Nuffield Orthopaedic Centre, Oxford. Only bone which would otherwise be discarded was used with the approval of the hospital medical staff committee. Rabbit marrow stromal cells were obtained from the non-operated femurs of NZW rabbits at the time of killing. Primary cultures of bone marrow cells were established as previously described (Green et al, 1986). Saos2 cells are human osteosarcoma cells, which are known to express human BMP-4 mRNA constitutively (Anderson , et al., 1995). Saos2 cells were thawed from a frozen stock (gift from Clarke Anderson, University of Kansas Medical Center, Kansas City, USA), and resuspended in α -MEM medium (minimal essential medium). Rabbit marrow cells and Saos2 cells were plated in T-175 cm² flasks at a concentration of 10^7 cells / flask. All cells were

cultured in α -MEM supplemented with 10% (v/v) foetal calf serum, and refed every 3 days with fresh medium. Cells were harvested for experimental use when the flasks were confluent.

6. 2. 3 Preparation of BMP-4 riboprobes

Single-stranded RNA probe (riboprobe) for human BMP-4 was made from a cDNA fragment inserted into a plasmid which contained a 301 bp region of the human BMP-4 gene, corresponding to the carboxy-terminal coding region (Gift from J. Wozney, Genetics Institute, Cambridge, MA, USA). This region encompasses the most specific (least homologous) coding domain for human BMP-4 gene (Rosen, et al., 1989). For northern blotting analysis, to generate the antisense probe, human BMP-4 template was linearized with Hind III (Promega, Madison WI, USA) and incubated at 37 °C with SP6 RNA Polymerase (Boehringer Mannheim, East Sussex, UK). The riboprobe was labelled with [³²P] using a standard kit (Boehringer Mannheim, East Sussex, UK). For in-situ hybridisation on paraffin sections, human BMP-4 cDNA fragment was subcloned into a PBS vector from a Pst vector in order to facilitate transcription of a sense probe for use as a negative control. The plasmid was linearized with Hind III and transcribed with T7 for the antisense probe, or linearized with EcoR1 and transcribed with T3 for the sense probe. The riboprobes were labelled with digoxigenin using a standard kit according to the protocols provided by the manufacturer (Boehringer-Mannheim, East Sussex, UK). Detail can be found in Appendix 11 and 12

6. 2. 4 Use of BMP-4 riboprobe for Northern analysis

The sensitivity and cross-reaction of human BMP-4 riboprobe to human and rabbit tissues were examined by northern blotting. The [³²P] labelled human BMP-4 riboprobe was hybridized with mRNAs isolated from human osteosarcoma cells, and rabbit and human bone marrow cells. In brief, total RNA was isolated from cells using acid guanidine thiocyanate-phenol-chloroform as described previously (Chomczynski and Sacchi, 1987). mRNAs were purified from the extracted total RNA using oligo (dT)-coated magnetic microspheres (Dynabeads; Dynal IK Ltd., Wirral, UK) according to the manufacturer's instructions. Equal amounts of purified mRNAs were then separated by electrophoresis on 1% agarose gels before transferring

to a Biodyne nylon membrane (Pall Ultrafine Filtration Corporation, USA) under alkaline conditions. Membranes were prehybridized and hybridized in a buffer of 50% (v/v) deionized formamide, 1% (v/v) SDS, 4 x SSPE (0.18 M NaCl, 0.01 M NaH₂PO₄, 0.001M EDTA, pH 7.7), 0.05% (w/v) non-fat dried milk and 300 ug/ml denatured salmon sperm DNA. [³²P]-labelled riboprobe was used at >1x10⁶ cpm/ml for hybridization. Hybridization was performed overnight at 55 °C. Post-hybridisation washes were at 55 °C in 2 x SSC with 0.1% SDS followed by 1x SSC with 0.1% SDS. The signal was obtained on autoradiographs by exposing the blot to pre-sensitized Kodak X-OMAT film (Kodak Ltd., Hemel Hempstead, England) at -70 °C for 4 to 24 hours. The detailed procedure for Northern blotting is given in appendix 13.

6. 2. 5 Protocols for in-situ hybridization on paraffin sections

For in-situ hybridization on paraffin sections, a standard procedure was used according to Sandberg and Vuorio (1987) with some modifications. In brief, the pretreatments were (1) 0.2 N hydrochloric acid, (2) 6% hydrogen peroxide, (3) 20 µg/ml proteinase K (Sigma, Poole, UK) in Tris-EDTA buffer, pH 8.0, for 10 min at 37°C, (4) 4% paraformaldehyde, pH 7.4 for 20 min, (5) 0.1 M glycine (2 X 10 min) and (6) 0.25% acetic anhydride for 10 min. The sections were dehydrated through graded ethanols and 40 µl hybridization solution was applied per section. The hybridization solution contained 50% formamide, 10mM Tris-HCl, pH 7.4, 1.0 mM EDTA, 0.02% polyvinyl and Ficoll, 0.02% bovine serum albumin, 0.5% sodium dodecyl sulphate, 300 mM NaCl, 10% dextran sulphate, 0.5 mg/ml yeast or E. coli tRNA and the antisense, or sense, probe labelled with digoxigenin. Hybridization was for 18 hrs at 55 °C in a humid chamber. After hybridization, the sections were treated with 20 ug/ ml RNase in Tris-EDTA-NaCl buffer, pH 8.0. The sections were washed initially with standard saline citrate (SSC), followed by 0.5 X, then 0.1 X SSC; all washes were at 55 °C, except the final wash was at room temperature. The digoxigenin-label was detected using a mouse anti-digoxigenin monoclonal antibody (Boehringer Mannheim, East Sussex, England) by a standard indirect method. NBT and X-phosphate (Boehringer Mannheim, East Sussex, England) were used as substrates to visualise the secondary rabbit anti-mouse antibody conjugated with alkaline phosphatase, according to the manufacturer's instructions. The

sections were then washed in distilled water, mounted in glycerine jelly without counterstaining, and examined under light microscopy .

6. 3 Results

6. 3. 1 Northern blotting analysis

Northern blotting using human BMP-4 probes on equal amounts of mRNAs of Saos2 cells, and human and rabbit bone marrow cells showed that the hybridization patterns of Saos2 cells and human bone marrow cells were identical to that of rabbit bone marrow cells (Fig. 6. 1). This indicates cross-reaction of the human BMP-4 probe to rabbit BMP-4 mRNA and validates the use of the human BMP-4 probe in this study.

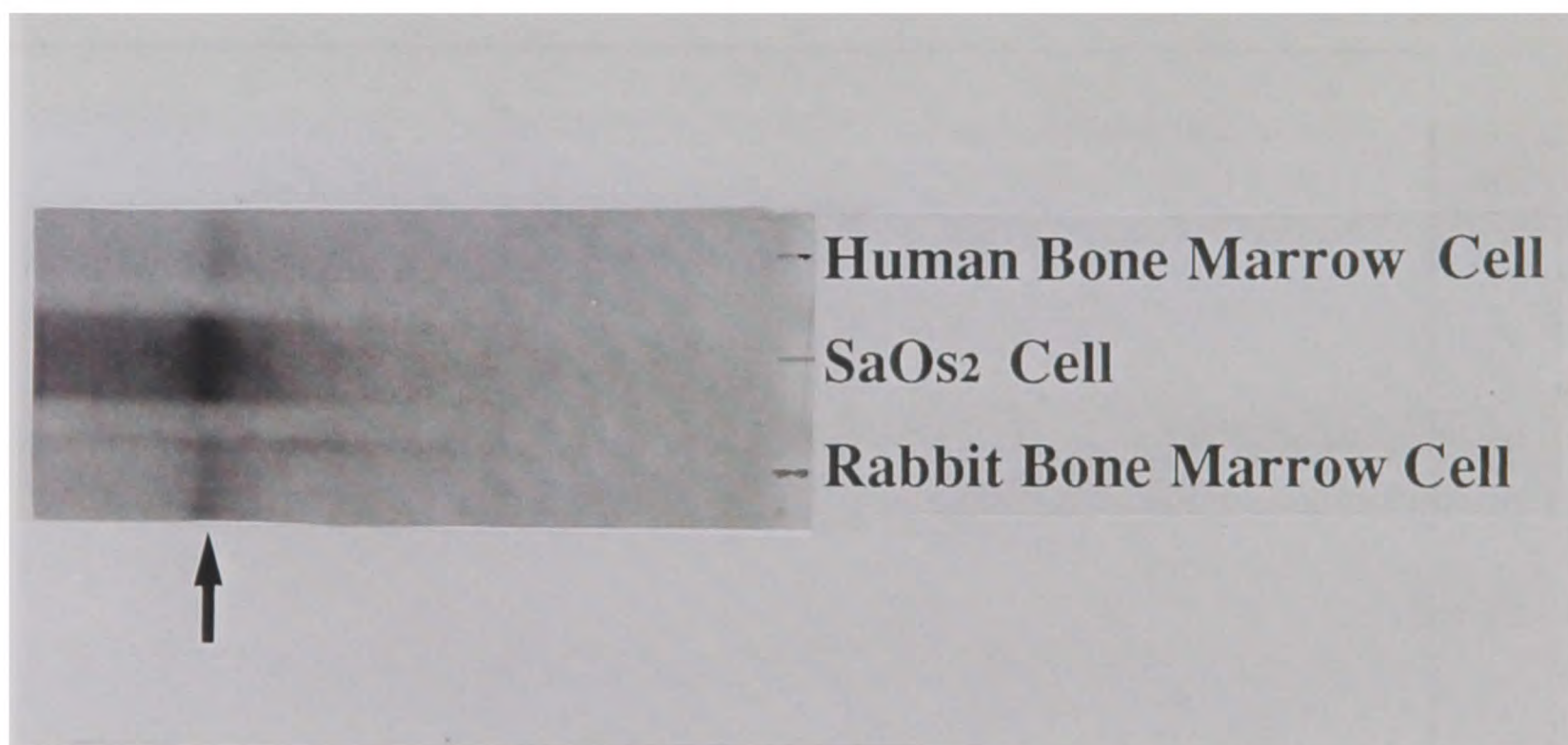


Fig. 6. 1. Northern blotting showing identical patterns of human and rabbit bone marrow cells, and human osteosarcom (SaOs2) cells when hybridized with [³²P] labelled human BMP-4 antisense riboprobe.

6. 3. 2 In-situ hybridization on paraffin sections

After 35-40 days distraction period at the distraction rate of 0.7 mm/day, histological investigation of the regenerates of all animals showed that new bone had developed from the cut surfaces with a central area of fibrous tissue and areas of cartilage present between the central fibrous region and the new bone (Fig. 6. 2. Top left). BMP-4 mRNAs were detected in

the fibroblasts of the central fibrous region (Fig. 6. 2. a), the bone surface cells and osteoblasts of newly formed woven bone (Fig. 6. 2. b), the chondrocytes within the developing cartilage region (Fig. 6. 2. c), the proliferating periosteum surrounding the distraction regenerate (Fig. 6. 2. d) and attached skeletal muscles to periosteum (Fig. 6. 2. e). BMP-4 mRNA was not detected in the osteocytes in the new bone nor in the normal cortical bones (Fig. 6. 2. b). With hypertrophy of the chondrocytes, the expression of BMP-4 mRNA decreased (Fig. 6. 2. c).

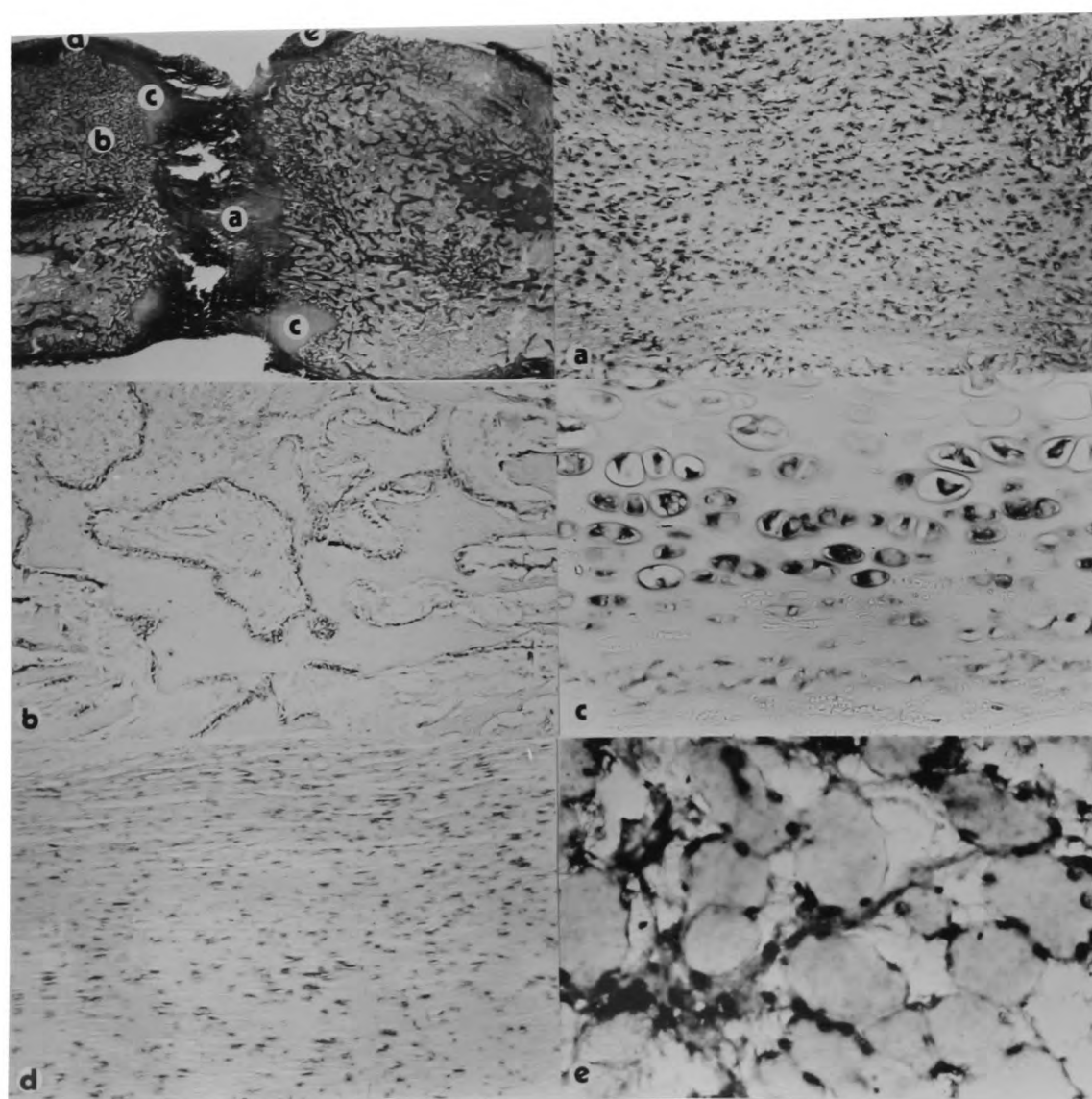


Fig. 6. 2. Top left: the histological appearance of a regenerate of the 0.7 mm/day group. HE staining, X 8. Areas of (a): the central fibrous interzone, (b): the new bone zone, (c): the cartilage tissue, (d): periosteum and (e): skeletal muscle. Photography a-e: in-situ hybridization results using BMP-4 antisense riboprobe, BMP-4 mRNA was expressed in:- a: the fibroblasts; b: bone surface cells (osteoblasts), but not in the osteocytes in the new bone; c: chondrocytes in the area of cartilage developing from the fibrous tissue; d: in the cells of the inner layer of the periosteum; e: skeletal muscles adjacent to the periosteum. Original magnification: a, c, d and e, X 100; b X 25.

Near the mineralization front, osteoblasts on the surfaces of the developing trabecular bone exhibited strong BMP-4 mRNA expression (Fig. 6. 3), but as the new bone extended towards to the osteotomied ends, where lamellar (mature) bone replaced the primitive woven bone, the intensity of the expression decreased (Fig. 6. 4). Control sections hybridized with BMP-4

sense probes showed no specific staining (Fig. 6. 5).



Fig. 6. 3. In the new bone zone near the mineralization front, in-situ hybridization using BMP-4 antisense riboprobe showed that the osteoblastic cells (arrows) on bone surfaces of the primitive woven bone exhibited strong BMP-4 mRNA expression, X 100.

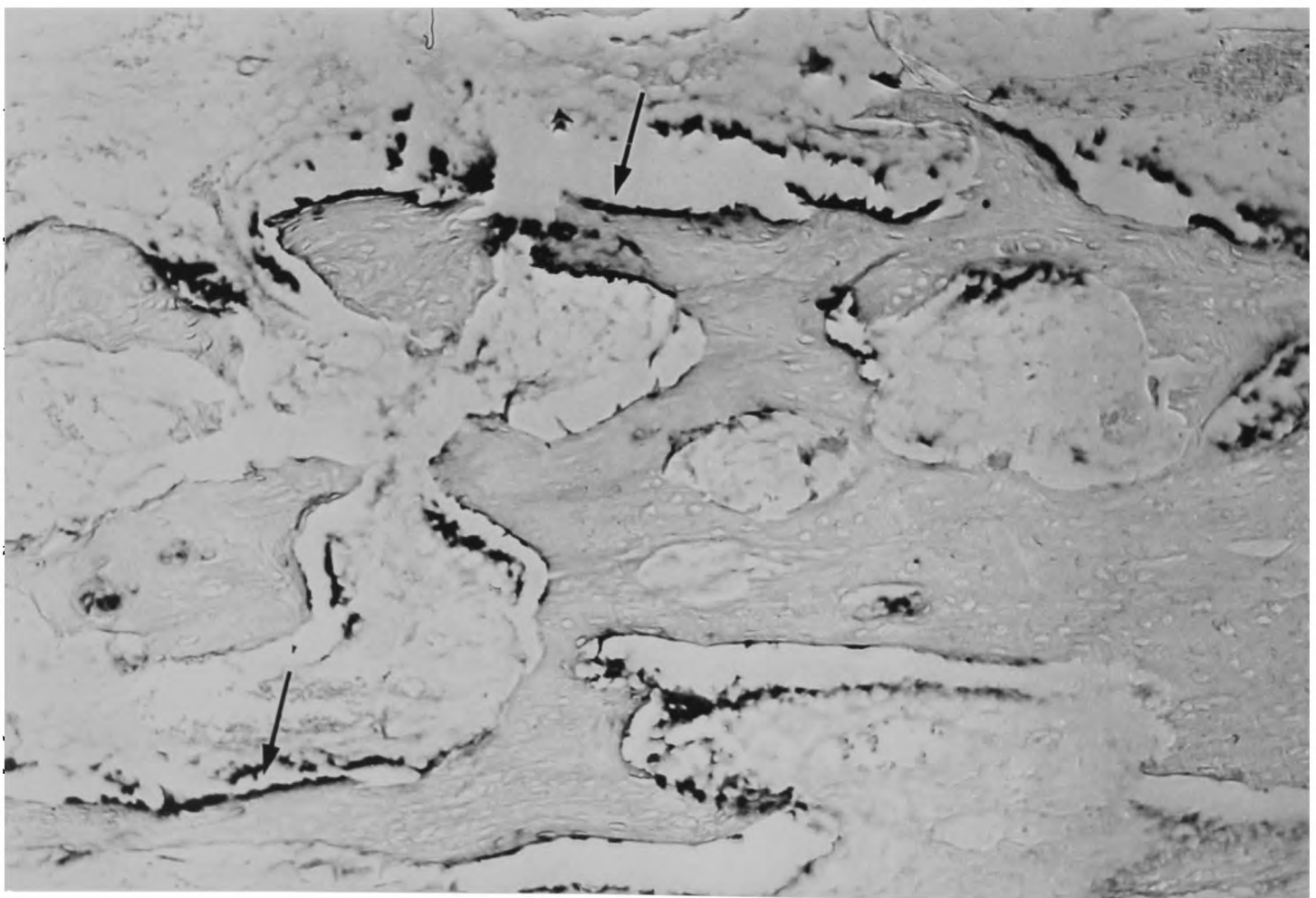


Fig. 6. 4. In the new bone zone away from the mineralization front, in-situ hybridization using BMP-4 antisense riboprobe showed that as the new bone becomes mature, the intensity of the BMP-4 mRNA expression in the bone surface cells decreased (arrows), X 25.

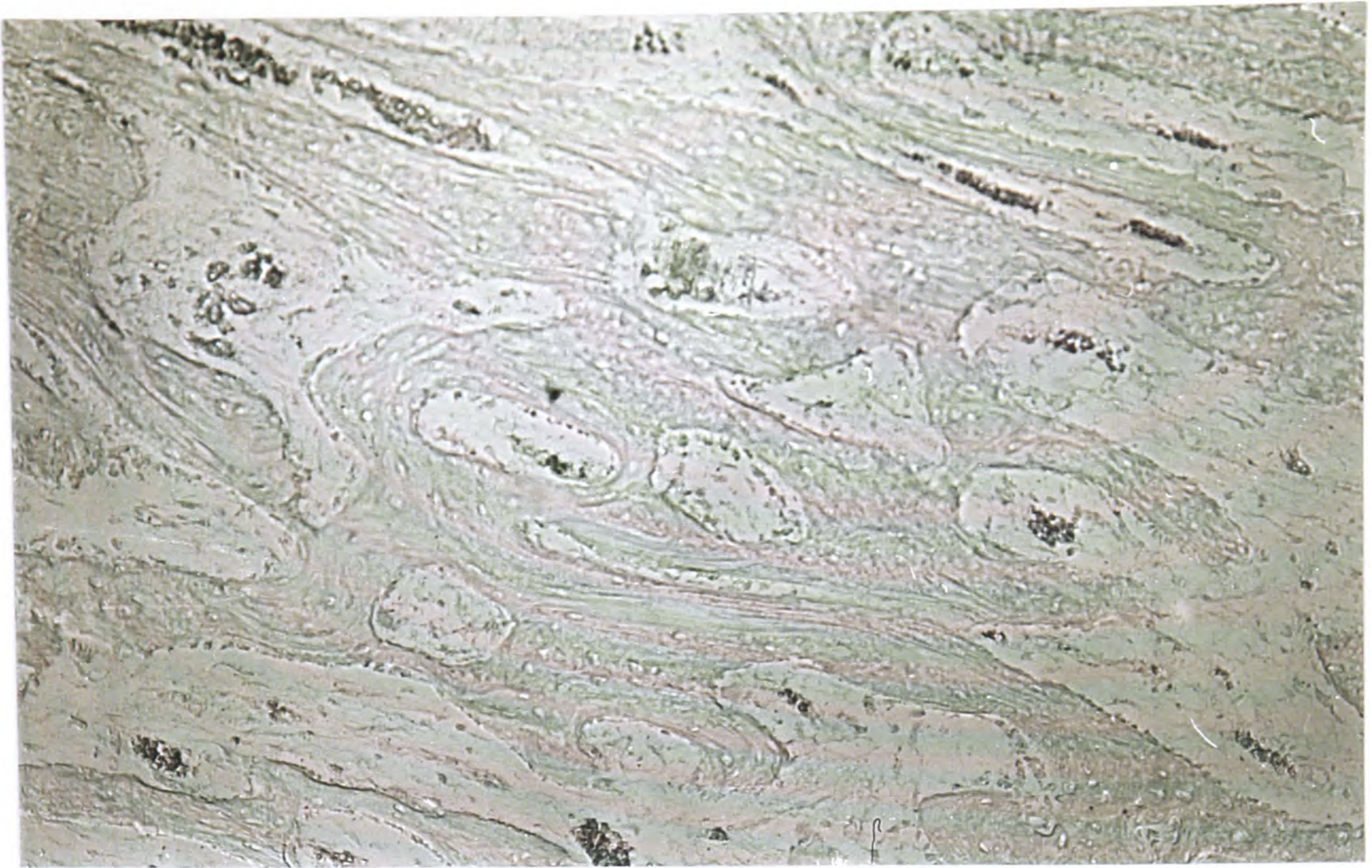


Fig. 6. 5. In the new bone zone of a regenerate, in-situ hybridization using BMP-4 sense riboprobe showed absence of positive-labelling, X 25.

6. 4 Discussion

The results demonstrate that BMP-4 mRNA is expressed in specific regions of the regenerating tissues during the distraction period. This mRNA expression pattern is distinct from that described in a mouse model of rib fractures (Nakase, et al., 1994), in which there was a transient expression of BMP-4 mRNA from 12-72 hours after fracture as detected by in-situ hybridization, prior to the appearance of differentiated osteoblasts at the fracture site.

The early phases of fracture healing involve a recruitment of primitive mesenchymal cells, proliferation of osteoprogenitor cells, and induction of a differentiated osteoblastic phenotype. During distraction osteogenesis, these early phases are prolonged in the regenerating callus, creating a continuous cell proliferation and differentiation similar to that seen in the early fracture healing phase as described in previous chapters. The extended induction of new bone formation is paralleled by the observed prolonged expression (35 days after osteotomy) of BMP-4 during distraction osteogenesis.

BMP-2 and 4 were detected by immunohistochemistry at fracture sites in rats (Bostrom, et al., 1995). The amount of BMP-2 and -4 bindings was found to be highest in primitive mesenchymal cells, early chondrocytes, and early osteoblasts. The level of BMP-2 and -4 proteins decreased as the healing progressed and woven bone was replaced by mature lamellar bone. Parallel results were observed in a rat rib fracture study measuring BMP-4 mRNA expression by in situ hybridization (Nakase, et al., 1994). BMP-4 mRNA was not detected before fracture but was found to be expressed in tissue adjacent to the fracture site. This expression was transient and BMP-4 mRNA levels became undetectable 5 days after fracture.

The spatial pattern of BMP-4 mRNA expression during distraction osteogenesis is similar to the pattern demonstrated in fracture models by in-situ hybridization (Nakase, et al 1994; Hirakawa, et al., 1995). The intense staining of the inner cambium layer of the periosteum suggested that BMP-4 plays a role in the proliferation and differentiation of mesenchymal tissue. In the present study, BMP-4 mRNA expression was also found within peripheral, immature chondrocytes, but the expression was less in the mature chondrocytes. This pattern is similar to that obtained by using immunohistochemical localizations (Yan, et al., 1994; Bostrom, et al., 1995). The source of chondroprogenitor cells appears to be from the active periosteum abutting the cartilaginous portion of the callus. This is the same layer of the periosteum that seems to give rise to primitive osteoblasts elsewhere, showing that the primitive mesenchymal cells are pluripotent, and able to differentiate along either chondroblastic or osteoblastic lineages. These results were consistent with the findings in chapter 5.

BMP-4 mRNA expression was also localized within skeletal muscle cells in the region adjacent to the regenerate, and similar findings has been reported by Nakase, et al. (1994) in the fracture model study. The ability of BMPs to induce osteogenesis in intramuscular sites was used for the early assays of BMPs activity (Urist, 1965). However, in the present study it is not possible to clarify whether or not the multipotential cells from skeletal muscle can differentiate into bone-forming cells and contribute to the bone formation during distraction osteogenesis.

As mRNA expression does not necessarily equate to protein synthesis, the present study would be strengthened considerably if BMP-4 immunohistochemistry were included to determine in

which regions transcription was actually accompanied by translation. However, antibodies to rabbit BMPs are not available at the present time, and immunohistochemical studies await future availability of a suitable antiserum.

6. 5 Conclusion

The present study characterizes the localization of BMP-4 mRNA expression in the regenerating tissues produced by distraction osteogenesis. The findings are consistent with previous reports on BMP-4 expression in fracture healing, that the BMP-4 gene is expressed by less differentiated osteoprogenitor cells (fibroblastic mesenchymal cells and preosteoblasts), and the more mature bone-forming cells have significantly less BMP-4 mRNA expression. BMP-4 gene expression is also localized in nearby callus-forming tissues (periosteum and skeletal muscles) prior to callus formation, and these results suggest that BMP-4 plays an important role in controlling osteoprogenitor cell differentiation and proliferation. Our results suggest that the BMP-4 gene product is one of the local contributing factors regulating bone and cartilage formation. These findings, together with previous studies on the function of BMPs, support the conclusion that application of BMPs may have potential value for treatment of inadequate bone formation during the processes of distraction osteogenesis, such as bone transport and limb-lengthening.

Chapter 7 Study of apoptosis in the regenerating tissue during distraction osteogenesis

7. 1 Introduction

During the process of distraction osteogenesis new bone is formed rapidly and it undergoes remodelling almost immediately to form the medullary cavity and bone cortex. Little is known of the regulatory mechanisms which govern the removal the redundant callus in this process.

Apoptosis is a fundamental property of cells, allowing unwanted cells to be eliminated quickly and neatly. It is intrinsic to the cell and regulated by extracellular signals from other cells (see section 1.11). Tissue homeostasis is achieved by the balance between cell death and proliferation. Previous studies on cell proliferation of this thesis (chapter 4) have indicated that cell proliferation was activated and affected by distraction, and the population of cells within the regenerate was shown to be consistently changing. In such a cell population, the proportion of proliferating to dying cells may be varying, and to understand this process it is important to define the populations of cells undergoing apoptosis, the time course of cell death and the factors influencing this process. However, there is no information on apoptosis during distraction osteogenesis in the literature. The hypothesis in this study is that apoptosis is of major importance during bone regeneration; and that cells in the regenerating tissues undergo apoptosis during distraction osteogenesis.

Apoptosis has generally been detected in cells and in tissues by analyzing genomic DNA. Gavrieli et al. (1992) established the terminal deoxynucleotidyl transferase (TDT)-mediated dUTP-biotin nick end-labelling (TUNEL) method that allows detection of apoptosis at the single-cell level. This method uses TDT to label 3'-OH ends of DNA fragments that are generated during the period of DNA damage. Although the TUNEL method is a useful indicator of apoptosis, it is not sensitive enough to distinguish apoptotic cells from necrotic

cells, and the TUNEL method tends to overestimate the true number of the apoptotic cells (Aizawa, et al., 1997). However, the TUNEL method is a useful tool, it has been widely employed to explore the occurrence of apoptosis (Boyce, 1996), and it was used in the present study.

7. 2 Methods

Mid-tibial osteotomies were performed in 4 adult NZW rabbits, and the tibiae were stabilised with external fixators as previously described. 7 days later, twice daily distraction was initiated at rates of 0.7 mm/day, until the tibiae were lengthened by 20%. At this time, the animals were killed, and the regenerates were processed histologically and embedded in paraffin as previously described. BrdUrd (40 mg/kg) was injected intravenously 1 hour before killing and BrdUrd immunohistochemistry was performed to detect proliferating cells as previously described. The TUNEL staining was used to identify the cells undergoing apoptosis. The reagents used for the TUNEL staining were supplied with a "Cell death detection kit" (Catalogue No.1684809, Boehringer Mannheim, East Sussex, UK). For details of the TUNEL staining, see section 2. 5. 2. In addition, a method of dual labelling for cell proliferation and apoptosis by using BrdUrd immunohistochemistry following the TUNEL staining was performed, see section 2. 5. 2 and Appendix 16 for the procedures. TRAP staining was also performed to identify bone resorbing cells (see appendix 7).

7. 3 Results

7. 3. 1 Controls for the TUNEL staining

For methodological negative controls, slides were incubated (1) only with solution containing fluorescein-labelled dUTPs (without TDT); (2) with TBS buffer instead of the anti-fluorescein antibody. In addition, some tissues that are known to be very homeostatic, such as brain tissue, peripheral nerve and muscle tissue, were used as negative controls. No TUNEL-

positive labelling was observed in any of the negative controls under the experimental conditions used in the present study (Fig. 7. 1).

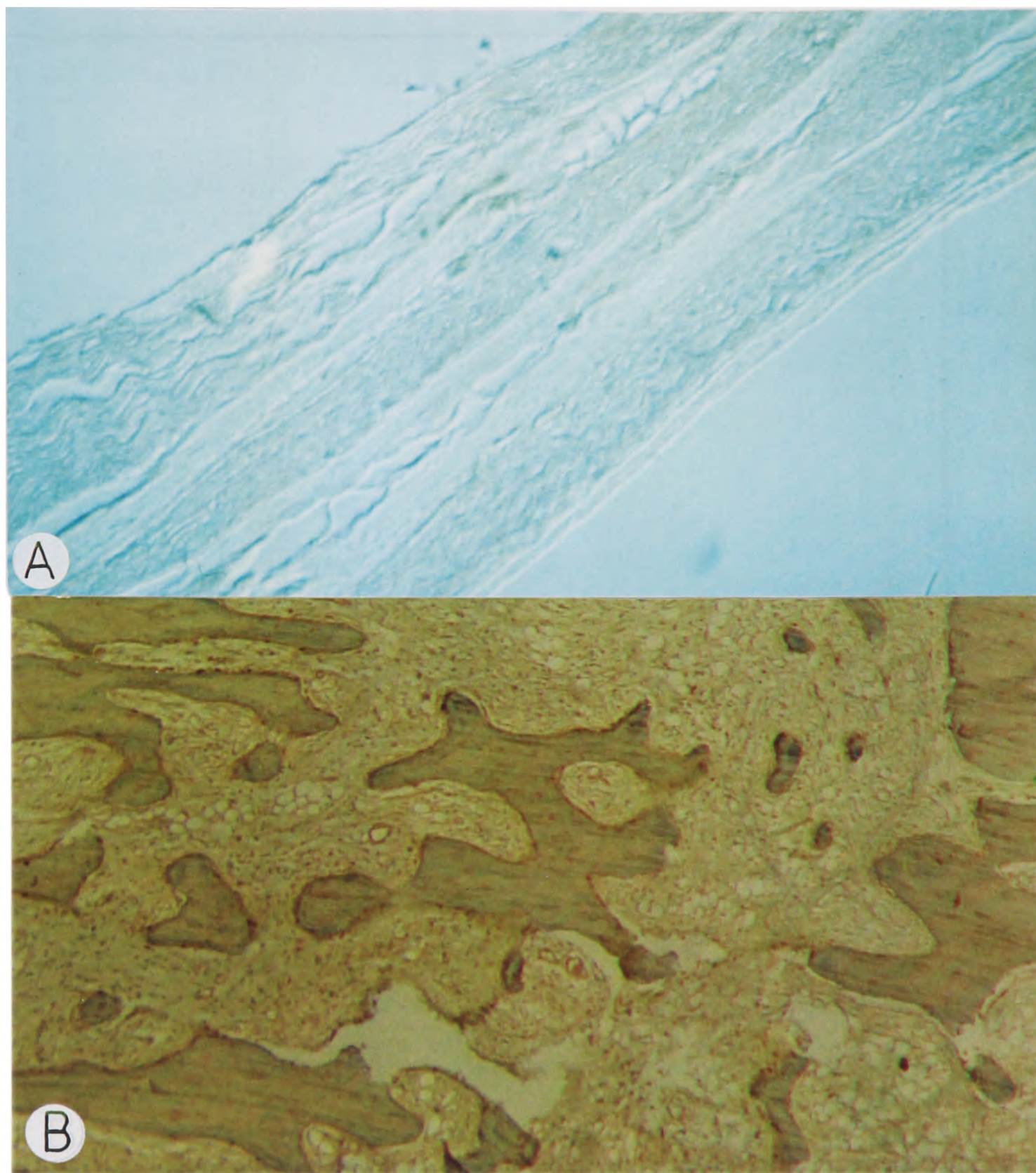


Fig. 7. 1. Negative controls for the TUNEL staining. A: No positive TUNEL-labelling is detected in the rabbit peripheral nerve tissue. B: No positive labelling is seen in the regenerating bone incubated only with solution containing fluorescein-labelled dUTPs (without TDT). A and B, X 25, no counterstaining.

For positive controls of the TUNEL staining, tissues which are known to have a high rate of cell turnover, such as small intestine and thymus, were used. For methodological positive controls, tissue sections were treated with DNase I (10 µg/ml) for 30 mins at room temperature,

to break the DNA strands deliberately. At the end of the TUNEL reaction, a few TUNEL-positive labelling cells were seen in the tissue section of the small intestine (Fig. 7. 2. A) and the thymus (Fig. 7. 2. B). Extensive TUNEL positive labelling was seen in all the tissue sections treated with DNase I (Fig. 7. 2. C, D).

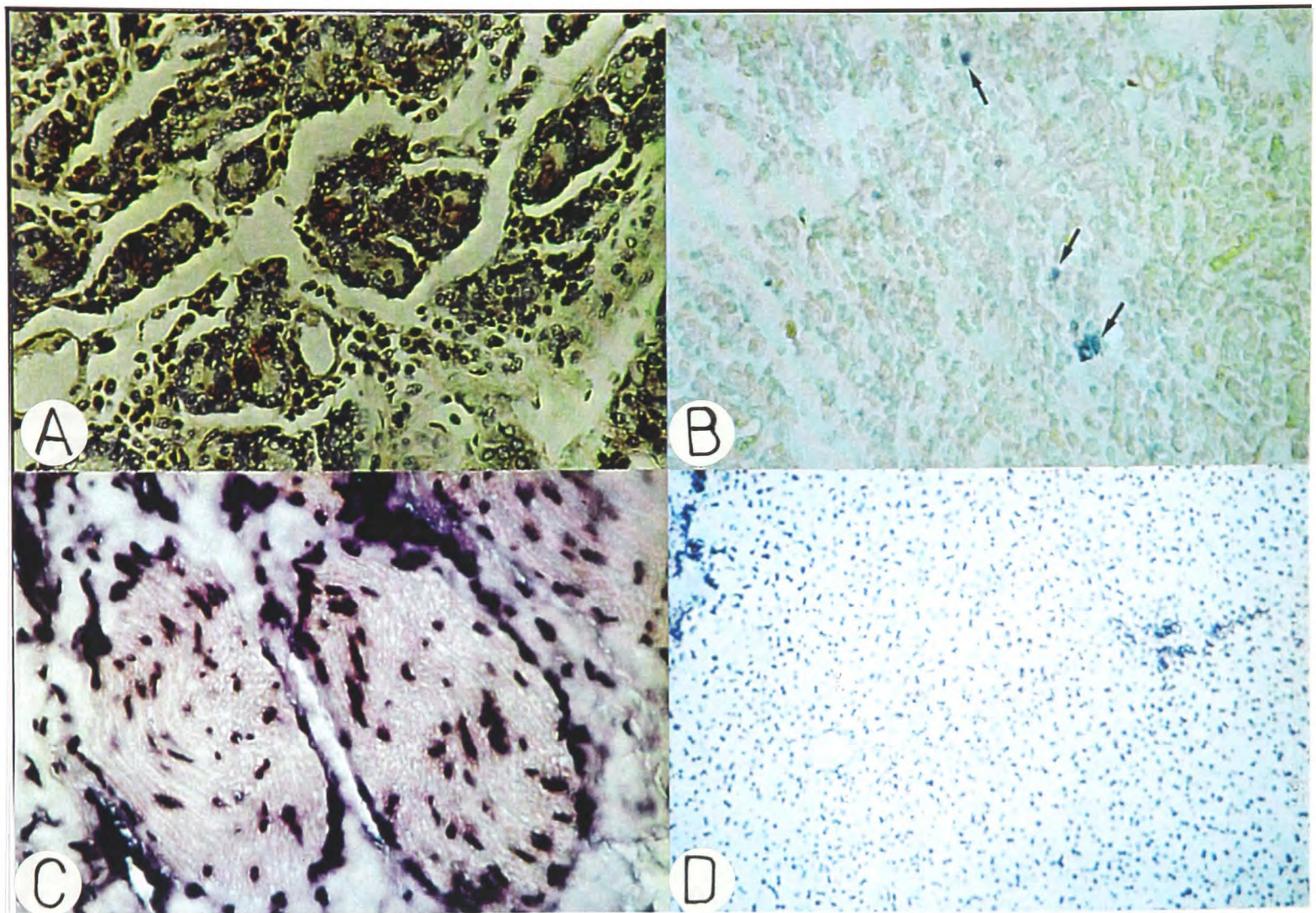


Fig. 7. 2. The positive controls of the TUNEL staining. A few TUNEL-positive labelling cells (arrows) were seen in the tissue sections of the rabbit small intestine (A) and the thymus (B). Extensive TUNEL-positive cells (in black or dark blue) were seen in the tissue sections treated with DNase I: (C) rabbit peripheral nerve, X 100 and (D) the thymus, 25.

7. 3. 2 The TUNEL staining in the regenerate

TUNEL-positive cells were present in all regions of fibrous tissue, new bone, mineralization front and cartilage throughout the regenerate. In the central fibrous zone, nearly half of the cells were TUNEL-positive (Fig. 7. 3. A). In the mineralization front, the majority of the TUNEL-positive cells were present in the transitional region between the fibrous tissue and the new bone (Fig. 7. 3 B, C). The TUNEL-positive cells were also seen in the cartilage region of the regenerate, a dense labelling was seen in the chondrocytes around invading blood vessels (Fig.

7. 3 D).

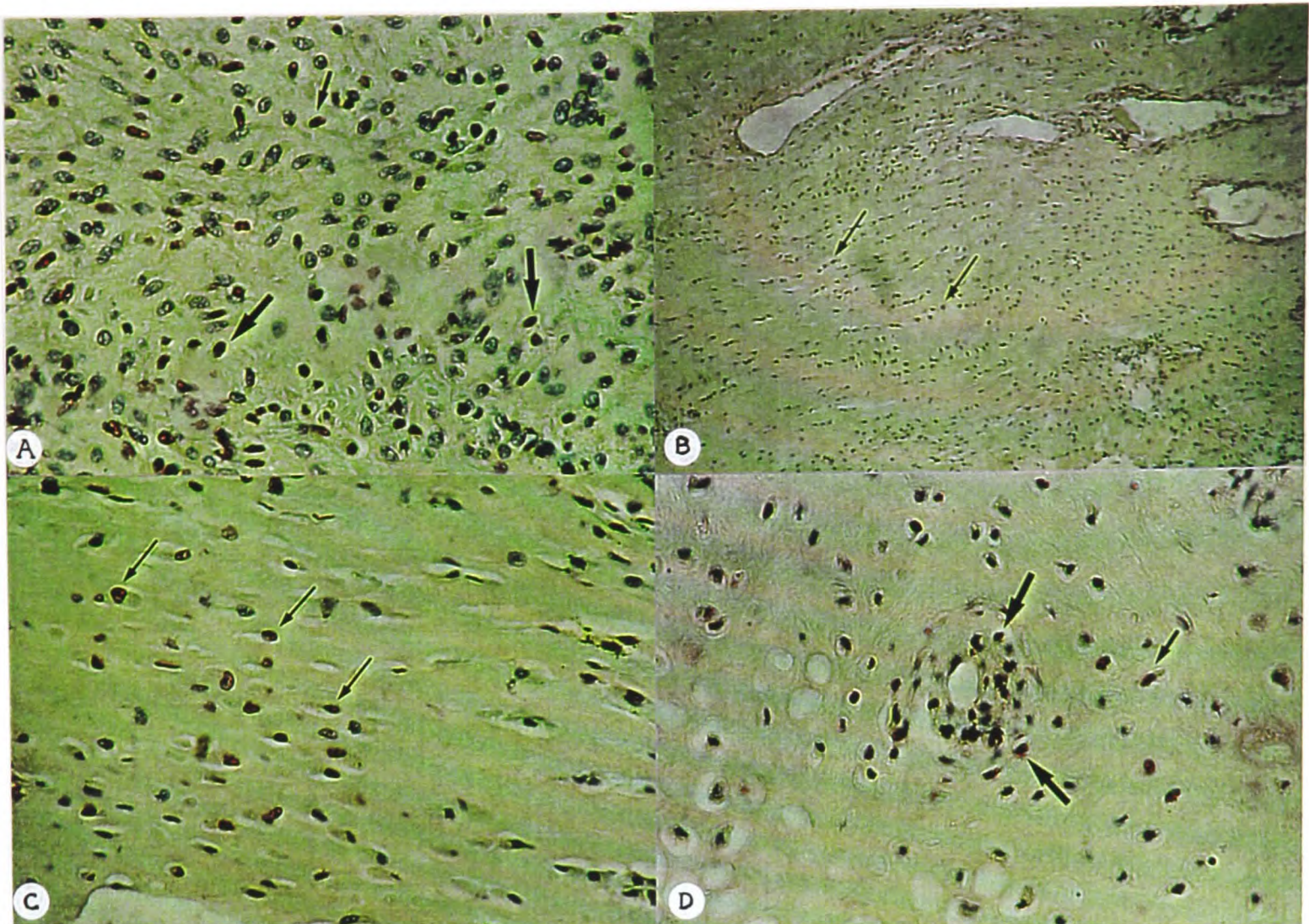


Fig. 7. 3. The TUNEL staining in a regenerate. A: In the fibrous zone, almost half of the cells were TUNEL-positive (in pink-red, arrows). B: In the mineralization front, the majority of the TUNEL-positive cells (arrows) were present in the transitional region between the fibrous tissue and the new bone. C: close up of the arrowed cells in B. D: The TUNEL-positive cells in the cartilage region of the regenerate, with a dense labelling around invading blood vessels (arrows). A, C, D, X 100; B, X 25.

In the new bone zone near the mineralization front, more than half of the cells were TUNEL-positive (Fig. 7. 4 A, B). In this region, the TUNEL-positive cells were close to or on the bone surfaces, and some of the newly formed osteocytes in the new trabeculae were positive (Fig. 7. 4 C). However, the TUNEL labelling was greatly reduced in the new bone close to the osteotomied bone ends, the number of the TUNEL-positive cells in this region was about one third of that in the region near the mineralization front, and most of the TUNEL-positive cells were osteocytes (Fig. 7. 4 D, E). The TUNEL-positive labelling was not detected in the cortical bone of the osteotomied bone ends (Fig. 7. 4. F) and in the adjacent surrounding periosteum.

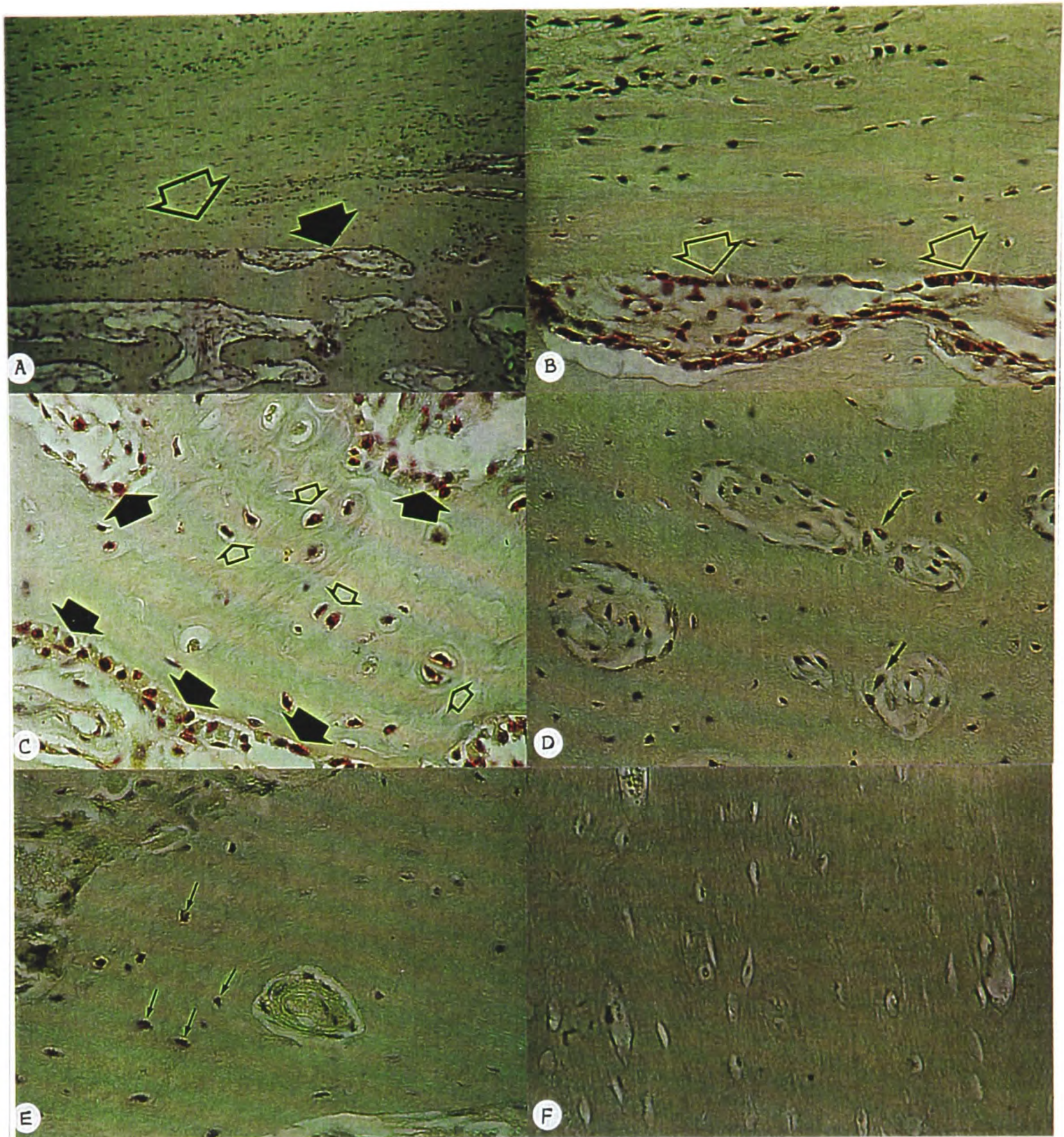


Fig. 7. 4. TUNEL staining in the NBZ of the regenerate. A and B: In the NBZ near the MF, half of the cells were TUNEL-positive (in pink-red, arrows). C: In the NBZ near the MF, the TUNEL-positive cells were close to or on the bone surfaces (dark arrows), and some of the newly formed osteocytes in the new trabeculae were also positive (white arrows). D and E: In the new bone close to the osteotomied bone ends, the TUNEL labelling was greatly reduced, and the majority of the TUNEL-positive cells were osteocytes in the new bone trabeculae (arrows). F: The TUNEL-positive labelling was not detected in the cortical bone of the osteotomied bone ends. A, X 25; B-F, X 100, all without counterstaining.

7. 3. 3 Dual labelling of cell proliferation and cell death

The cellular uptake of BrdUrd was detected by immunohistochemistry using Bu20A antibody as previously described. In adjacent sections, the Bu20A-positive cells and the TUNEL-positive cells were observed to be located in similar regions in the fibrous zone (Fig. 7. 5 A,

B), the mineralization front (Fig. 7. 5 C, D) and on the new bone surfaces. Dual labelling of the TUNEL staining followed by the BrdUrd immunohistochemistry demonstrated a coexistence of the TUNEL-positive and the Bu20A-positive cells in the regenerating tissues (Fig. 7. 5 E, F). The number of the TUNEL-positive cells and the Bu20A-positive cells in the new bone regions close to the mineralization front were higher than that in the new bone region near the osteotomied bone ends .

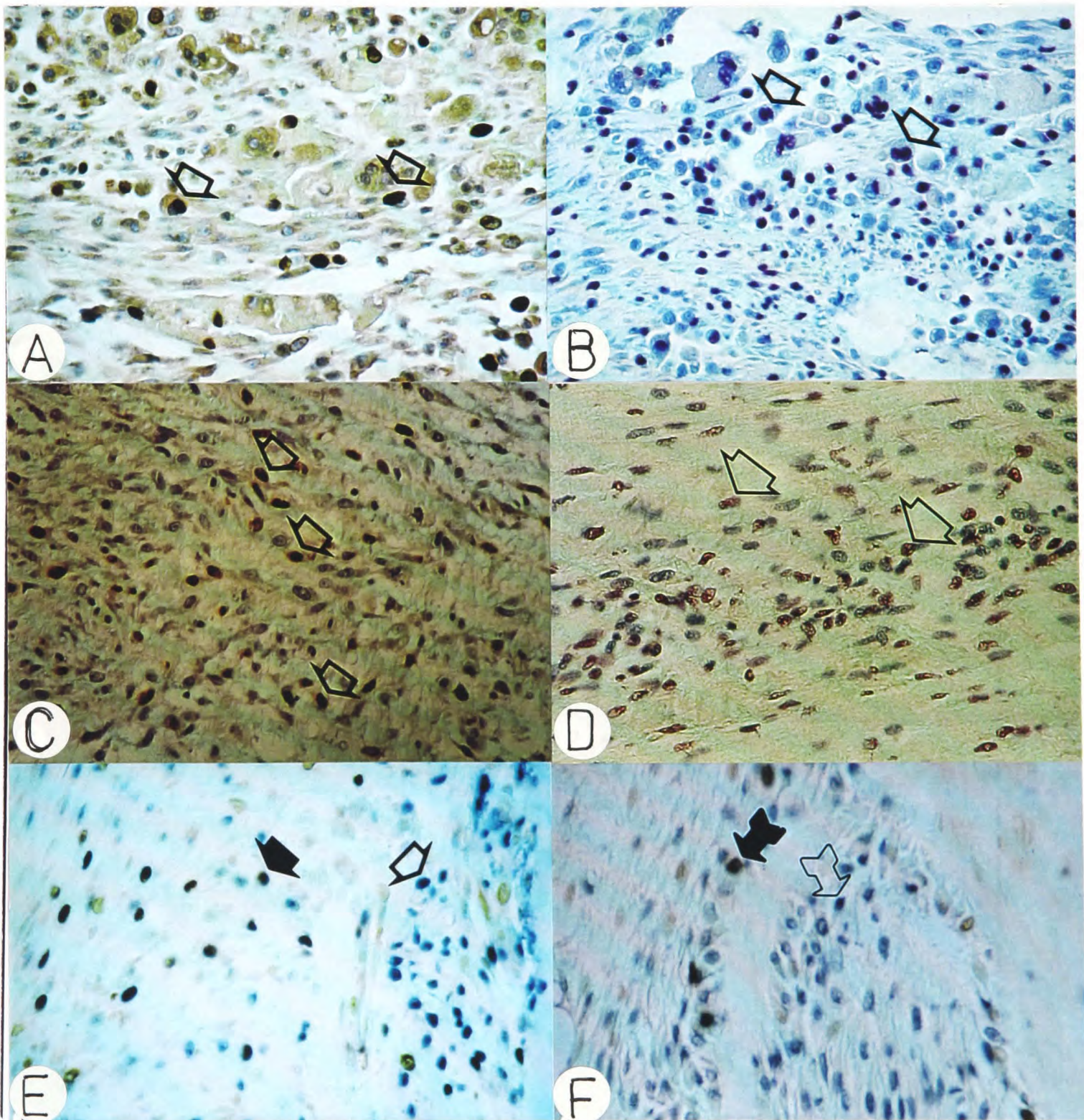


Fig. 7. 5 Bu20A and the TUNEL staining on adjacent sections of a part of a regenerate. A: Bu20A-positive cells (arrows) in the FZ. B: The TUNEL-positive cells (arrows) were localised in a similar area in the FZ. C: Bu20A-positive cells (arrows) in the MF. D: The TUNEL-positive cells in the MF were also localised (arrows). E and F: Dual labelling of BrdUrd immunohistochemistry following the TUNEL staining. E: Dual labelling in the FZ and F: Dual labelling in the NBZ, in those area, proliferative cells (brown nuclei, solid arrow) and apoptotic cells (blue nuclei, empty arrow) were found to coexist. A - F, original magnification X 100.

The distribution of the TRAP staining in the regenerate revealed similar results to the TUNEL staining. There were more TRAP-positive cells in the new bone near the mineralization front than that of the new bone region close to the osteotomied bone ends (Fig. 7. 6).

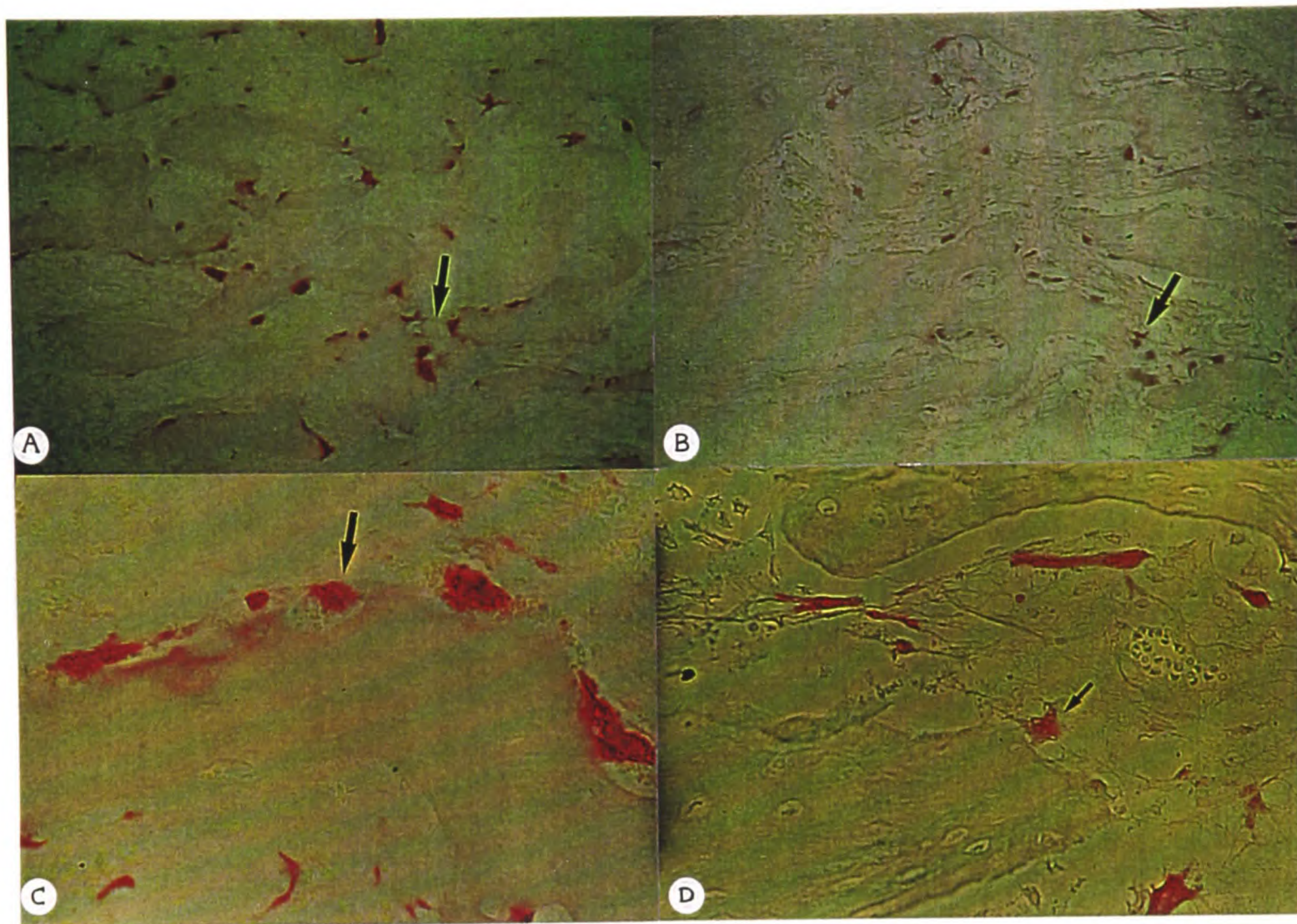


Fig. 7. 6. TRAP staining in the new bone zone of a regenerate, showing that there were more TRAP-positive cells (in red, arrow) in the new bone zone near the mineralization front (A and C) than that of the new bone zone close the osteotomied bone ends (B and D). A and B, X 100; C and D, X 400.

7. 4 Discussion

7. 4. 1 The reliability of the TUNEL staining

The reliability and the quality of the TUNEL staining were dependent upon the methods used for tissue preparation. The non-specific background staining was minimal in paraformaldehyde-fixed tissue (Tornusciolo, et al., 1995). The method used for permeabilization prior to TUNEL staining could also affect the quality of TUNEL staining. Both proteinase K and Triton X-100 provided similar results when the proteinase K was used at a low concentration for a short period of time at room temperature. However, at higher

concentrations, proteinase K increased non-specific staining (Roach, et al., 1995). In contrast, permeabilization treatment with Triton X-100 provided more reliable results and this was confirmed in the preliminary experiments (see section 2. 5. 2. 2).

The conditions for the TUNEL staining used in the present study were optimised to ensure minimum non-specific staining. However, in the present study, the number of TUNEL-positive cells was surprisingly high. Nearly half of the cells in the fibrous zone, the mineralization front and in some areas of the new bone zone were positively labelled. The TUNEL staining method, like most other methods involving in vitro transcription, does not necessarily distinguish between apoptosis and other causes of DNA fragmentation. In a study on the cell death of the chick hypertrophic chondrocytes using the TUNEL staining, Gibson et al. (1995) found that up to 30% of the cells in the developing chick sterna were TUNEL-positive. In another study, Hatori et al. (1995) using flow cytometry estimated that only 8% of chondrocytes of the avian growth plate were apoptotic at one time. These reports suggested that the TUNEL staining tends to overestimate the true number of apoptotic cells by two to three times. However, none of the tissues that were known to be very homeostatic showed significant labelling when examined by the TUNEL staining in the present study, and there was little TUNEL-positive staining seen in the the intact cortical bone tissue. These results indicate that the TUNEL staining results in the present study may reflect the true situation of cell death in the regenerating tissues.

Another consideration of the TUNEL labelling result is that some of the DNA fragmentation could be occurring ex-vivo during the process of sample preparation. To investigate this, samples of rabbit thymus and liver were obtained and immediately snap frozen and frozen sections were examined using the TUNEL staining. It was found that the distribution of TUNEL-positive cells was similar to that of the paraformaldehyde-fixed paraffin-embedded thymus and liver samples. In addition, the potential effects of the decalcifying process on the tissue were examined. Paraformaldehyde-fixed tissues of the rabbit thymus, liver and skeletal muscle were treated with 14. 5 % EDTA (pH 7.4) for 2 weeks, and were then paraffin-embedded, examined by the TUNEL staining. The distribution of TUNEL-positive cells was found to be similar to that of the samples without EDTA treatment. Taken together, these data

strongly suggest that any artificial effect on increasing DNA fragmentation, such as tissue preparation and processing, can be ruled out in the present study. Consequently, the DNA fragmentation identified by the TUNEL staining in the present study in all probability reflects the situation in situ in the regenerating tissues produced by distraction osteogenesis.

7. 4. 2 Apoptosis and distraction osteogenesis

The most striking evidence for cell death (apoptosis) in the regenerating tissues was seen in the central fibrous zone, the mineralization front and the newly formed bone zone near the mineralization front. DNA fragmentation was readily detected in these regions using the TUNEL staining, but the number of cells undergoing apoptosis was surprisingly high. Prior to this finding it was expected that apoptosis might be low in the regenerating tissues during distraction osteogenesis.

Distraction osteogenesis is a process demanding rapid cell proliferation, differentiation and tissue turnover (bone remodelling). During the development of new bone tissue in this process, the number of cells which could differentiate into an osteoblastic cell type probably exceeds the number of cells required to make bone and so the superfluous cells are eliminated through programmed cell death. In general, there are three pathways that could lead to apoptosis: (i) DNA and protein damage by inappropriate physical and chemical conditions; (ii) positive induction by ligand binding to a plasma membrane receptor; and (iii) negative induction by loss of a suppresser signal (see section 1. 11).

A possible explanation for the induction of apoptosis during distraction osteogenesis maybe inadequate vascularization. Gottlieb, et al. (1994) reported that apoptosis can be induced in cardiac myocytes by ischemia and reperfusion. A similar situation might have existed in the central regenerating tissues during distraction osteogenesis, where fast tissue formation and rapid increase in cell number, may cause severe local hypoxia and acidosis. Meanwhile, there was also an increased amount of neovascularization occurring in the centre of the regenerate (see chapter 8). Alternating ischemia (caused by rapid tissue formation) and reperfusion (by neo- or revascularization) in the regenerating tissue, may lead to oxygen radical formation,

which may induce DNA fragmentation and apoptosis in the cells of the newly formed tissues. The distribution and the changes in the number of TUNEL-positive cells in the different regions of the regenerate, concurs with the changes of the angiogenic responses in these regions, which were described in detail in Chapter 8. Furthermore, in the areas of the intact cortical bone where there was little revascularization, there was little TUNEL-positive labelling.

An alternative explanation for the induction of programmed cell death during distraction osteogenesis is loss of growth factor / cytokine-dependent suppression of apoptosis. The viability of many cells is dependent on a constant supply of growth factors and cytokines, and in the absence of these factors, the cells undergo apoptosis. The cell population in the regenerating tissues during distraction osteogenesis changes rapidly, and during this process some cells may be deprived of conditions favouring their survival. This could be, for example, reducing growth factors and cytokines, lack of the nutrition and mineral constituents. It will be valuable to obtain information on the expression and distribution of several proteins known to be mediators of the apoptotic process, such as bcl-2, Fas and p53. Immunohistochemistry using bcl-2 antibody was tried in the preliminary study, but the bcl-2 antigen was not well preserved during the sample preparation for paraffin embedding. Therefore, it was not possible to determine the expression and distribution of bcl-2 in the regenerate in the present study. The expression and distribution of bcl-2, Fas and p53 and other factors controlling apoptosis await future investigation, and frozen sections are required for future immunohistochemistry study using bcl-2 antibody and the antibodies to Fas and c-myc. In the present study, the demonstration of a mixture of proliferative and apoptotic cell populations in the regenerating tissue during distraction osteogenesis, suggests that apoptosis and cell proliferation may be regulated by local growth factors or cytokines or via cell-cell contacts. These findings are consistent with the hypothesis that the process of cell death and cell proliferation are inter-related during distraction osteogenesis.

Gibson et al. (1995) studied endochondral ossification of chick sterna, and suggested that apoptosis may be an initiating event in tissue remodelling and revascularization. Noble, et al. (1997) in their study of apoptotic changes in osteocytes in normal and pathological human bone, suggested a functional relationship between bone turnover and the controlled cell death.

In our studies of distraction osteogenesis in the rabbit, the increased TUNEL-positive labelling in the new bone zone near the mineralization front was accompanied by increased TRAP-positive staining in this region, indicating high osteoclastic activity. This suggests that apoptosis may be an important cellular mechanism by which redundant callus is removed, and local tissue turnover is controlled such as during distraction osteogenesis.

7. 5 Conclusion

In the present study, dual labelling with the BrdUrd immunohistochemistry and the TUNEL staining have been developed and revealed that the BrdUrd-positive proliferating cells and the TUNEL-positive apoptotic cells coexisting in the regenerating tissues. No overlap was seen between BrdUrd-positive proliferating cells and the TUNEL-positive apoptotic cells, and the distribution of each labelling in the dual-labelled section was similar to the distribution in the individually labelled sections. The dual labelling technique may be used as a sensitive method to identify apoptosis and proliferation simultaneously in the skeletal system in future research.

In the process of rapid development of new bone tissues during distraction osteogenesis, the superfluous cells are removed by programmed cell death (apoptosis). The demonstration of a mixture of proliferative and apoptotic cell populations in the regenerating tissue, suggests that apoptosis and cell proliferation may be regulated by local environment during rapid cell or tissue turnover seen in distraction osteogenesis.

The neovascularization of the regenerate and withdrawal of growth factors and cytokines may be responsible for apoptosis occurring in the regenerating tissue. The changes of distribution of apoptotic cells in the different regions of the regenerate, together with the results of TRAP staining, suggest that rapid bone remodelling through attraction of bone-resorbing cells during distraction osteogenesis may be initiated by bone cells undergoing apoptosis. Whether this is similar in other forms of bone regeneration is for future study.

Chapter 8 Study of angiogenesis during distraction osteogenesis

8. 1 Introduction

The formation of new blood vessels (angiogenesis or neovascularization) is associated closely with osteogenesis in vivo. Small blood vessels consist of two cell types: (1) endothelial cells (EC) resting on the basement membrane and lining the lumen, and (2) pericytes embedded in the basement membrane and making contact with the ECs through processes. The latter may be important in the link between angiogenesis and mineralization (Schor, et al., 1995). During angiogenesis in vivo, ECs migrate from the existing vessels and after rapid cell division, a lumen is formed and subsequent anastomosis of adjacent sprouts lead to the onset of blood flow (for detail see section 1. 10).

As osteogenesis appears to be a vascular-dependent process, it is essential that angiogenesis precedes the ossification process during bone development, repair and regeneration. The blood flow in a fracture site has been shown to reach a peak in the first two weeks after injury, and the bone blood flow and mineral deposition in fractured bone are closely related (Paradis and Kelly, 1975). In tibial fractures, Trueta (1974) suggested that the pattern and rate of fracture healing is dependent on the viability of the local circulation and its capacity to elicit a response. Ilizarov (1990) and Aronson (1994b) have shown that there is an increase in blood supply to the regenerating tissues during limb-lengthening.

During the surgical procedure of distraction osteogenesis, new bone is formed between the cut ends of an osteotomy as they are gradually separated mechanically. The neovascularization processes which occur during this procedure, however, are still poorly defined. The aim of this chapter was to examine the vascularization process during distraction osteogenesis, and to test the hypothesis that the angiogenesis is affected by the rate of distraction in distraction osteogenesis. Using the established rabbit leg-lengthening model, the localization and distribution of angiogenic markers, such as endothelial cell antigen and type IV collagen, were

studied using immunohistochemical methods.

8. 2 **Methods**

Mid-tibial osteotomies were performed in 16 adult NZW rabbits, which were randomly divided into 4 groups and the tibiae were stabilised with external fixators as previously described. 7 days later, twice daily distraction was initiated at rates of 0.3, 0.7, 1.3 and 2.7 mm/day, until the tibiae were lengthened by 20%. At this time, the animals were killed, and the regenerates were processed histologically and embedded in paraffin as described previously.

Immunohistochemistry using specific antibodies to type IV collagen and endothelial cell (EC) antigen (CD 31), were performed using standard indirect methods. In brief, 5 µm thick paraffin sections were dewaxed in xylene and taken through graded ethanol (100%, 90%, 70%, 50%). Endogenous alkaline phosphatase of the tissues was blocked by immersion of the sections in TBS containing 0.1 mM levamisole (Sigma, Poole, UK) for 30 min at room temperature. The sections were then enzymatically treated with protease type XXIV (0.125 mg/ml, Sigma P8038, UK) for 20 minutes at 37°C. Following TBS washes, the sections were incubated with monoclonal antibodies, either mouse anti-human type IV collagen (KAKO Ltd, UK, Code: M0785) at a dilution of 1:40 or mouse anti-human EC antigen (CD 31, DAKO Ltd, UK, Code: M0719) at a dilution of 1:50 for one hour in a humid chamber at room temperature. After rinsing in excess TBS, the sections were incubated with a rabbit anti-mouse alkaline phosphatase conjugated antibody (Sigma, Poole, Dorset, UK) at a dilution of 1:50 in TBS for 30 minutes. After this incubation, the sections were washed several times with TBS, then the sections were treated with a visualisation solution containing substrates of 10 ml TBS, 400 µl Naphthol AS MX buffer (Sigma, Poole, Dorset, UK) and 10 mg fast red (BDH, Dorset, UK) for 30 minutes in darkness. The slides were rinsed in running water, counterstained with Mayer's haematoxylin, dehydrated through graded ethanol, cleared in xylene, and finally mounted with Aquamount (BDH, Dorset, UK). As the negative control, the rabbit normal serum was applied instead of the primary antibodies. For the positive control, tissues known to contain type IV collagen or EC antigen, such as human blood vessels or skin tissues, were used. All stained sections were examined under light microscopy.

8. 3 Results

8. 3. 1 Controls

The negative controls, in which the primary antibodies for both type IV collagen and EC antigen were omitted, did not show any positive staining. The positive controls (human skin) showed staining for both type IV and EC antigen (Fig. 8. 1 and Fig. 8. 2). Staining of the type IV collagen antibody showed a diffuse pattern throughout the matrix (Fig. 8. 1). Staining of EC antigen, on the other hand, was localised in the nuclei and cytoplasm of the cells of the vascular endothelium (Fig. 8. 2).

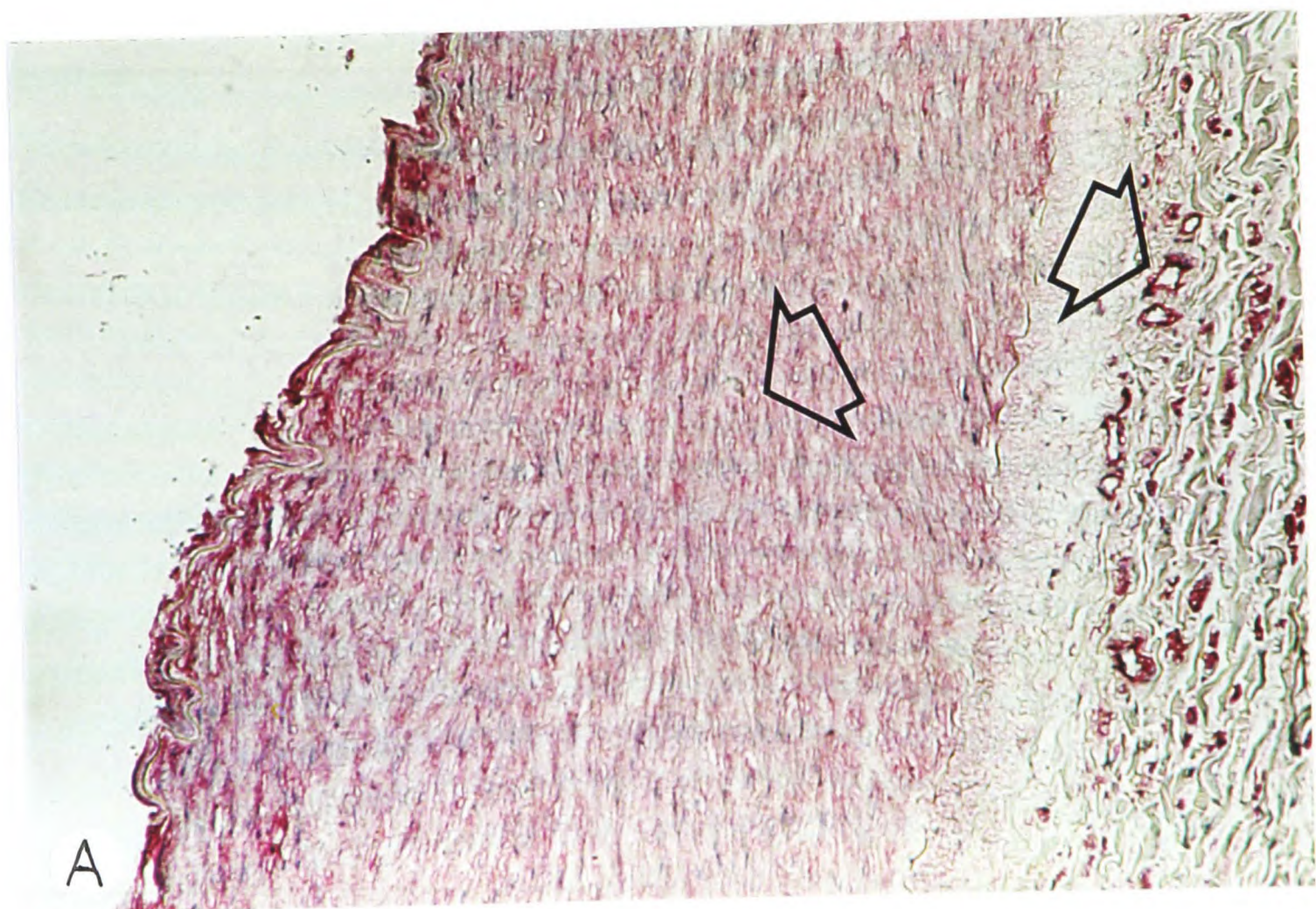


Fig. 8. 1. An example of immunostaining using type IV collagen antibody in a human skin section. **A:** Positive staining was diffuse (arrows) throughout the matrix, original magnification, X 25.

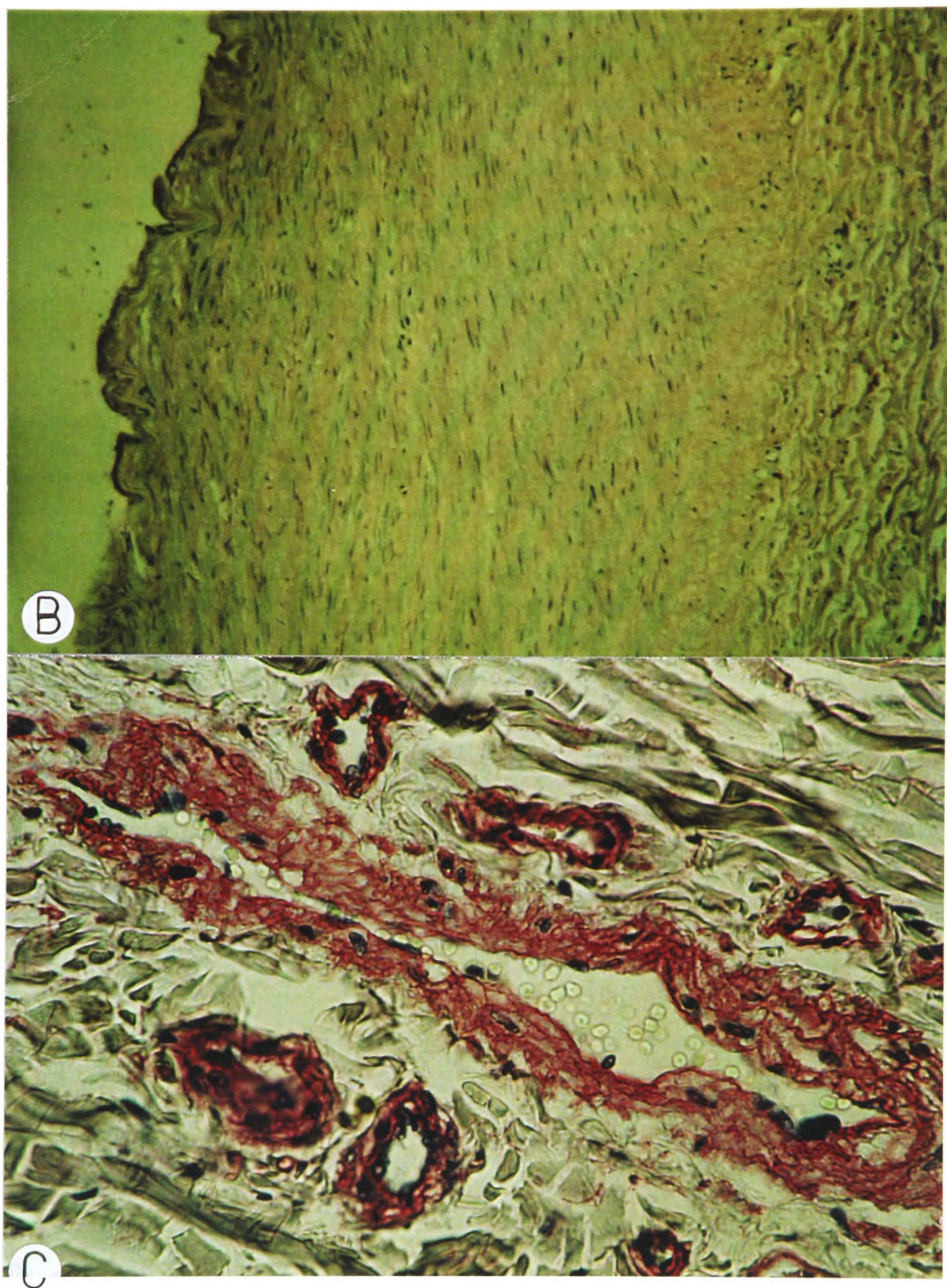


Fig. 8. 1. An example of immunostaining using type IV collagen antibody in a human skin section. B: No positive staining was seen in the similar region on an adjacent section without applying primary antibody, original magnification, X 25. C: Close up of the pattern of type IV collagen positive staining in the vessel lumen, original magnification, X 100.

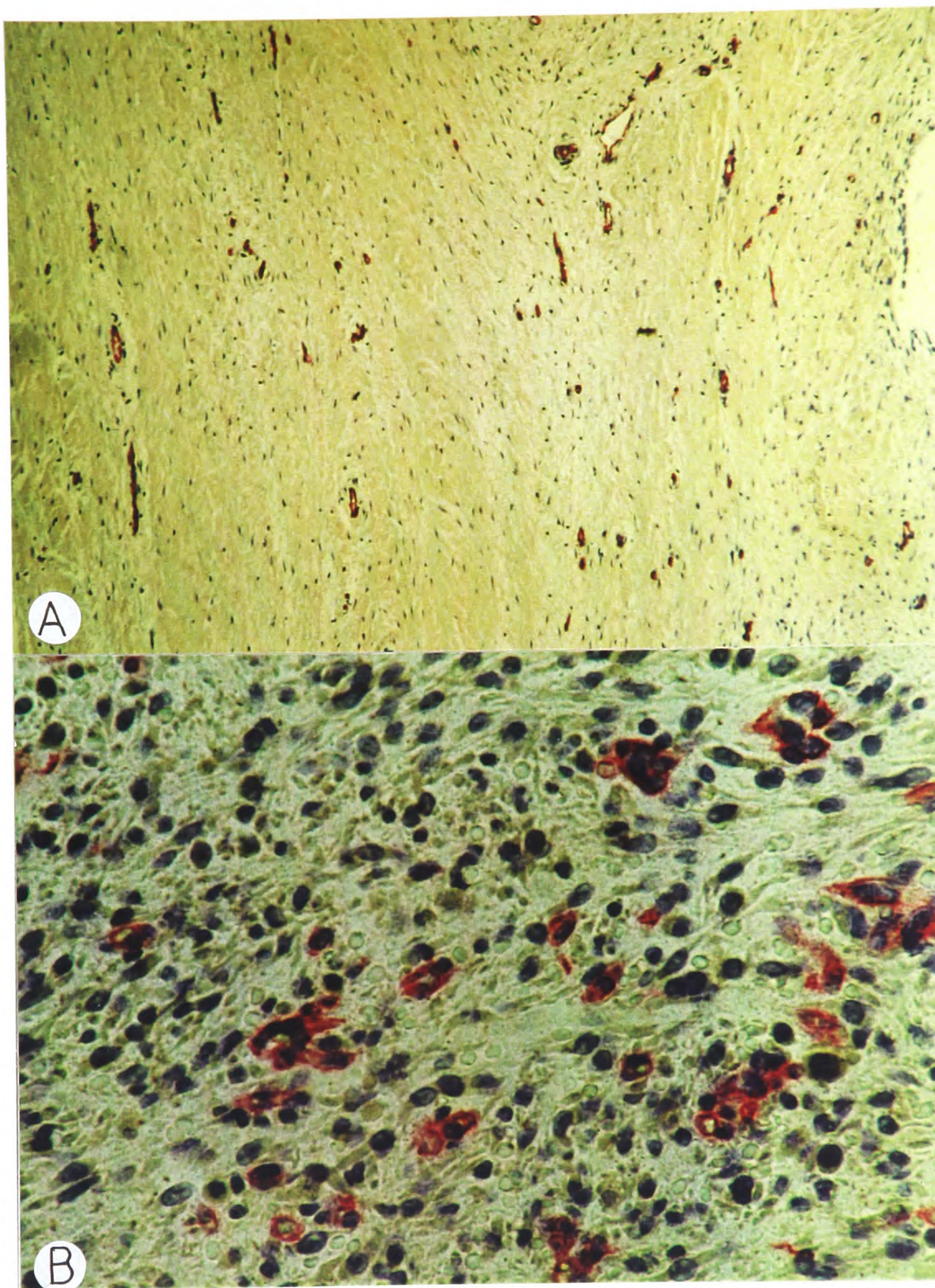


Fig. 8. 2. An example of immunostaining using EC antigen antibody in a human skin tissue section. The positive staining was localised in the nuclei or cytoplasm of the vascular endothelium. A: blood vessels in human skin, X 25. B: Close-up of the positively stained endothelial cells, which were forming the blood vessel buds (in red colour) in the central fibrous region of a regenerate of the 0.7 mm/day group, X 100.

8. 3. 2 0.3 mm/day group

Staining for type IV collagen and EC antigen was evaluated at three separate regions of the regenerate: (1) the central fibrous region; (2) the mineralization front; and (3) the new bone

zone. Staining for type IV collagen and EC antigen was demonstrated in each of the three regions of the regenerate, and was found to have a similar distribution. In the 0.3 mm/day group, the regenerate was almost filled by newly formed bone, with only a narrow central fibrous zone. Staining for type IV collagen and EC antigen was present throughout the regenerate, but was weak in the new bone zone (Fig. 8. 3).

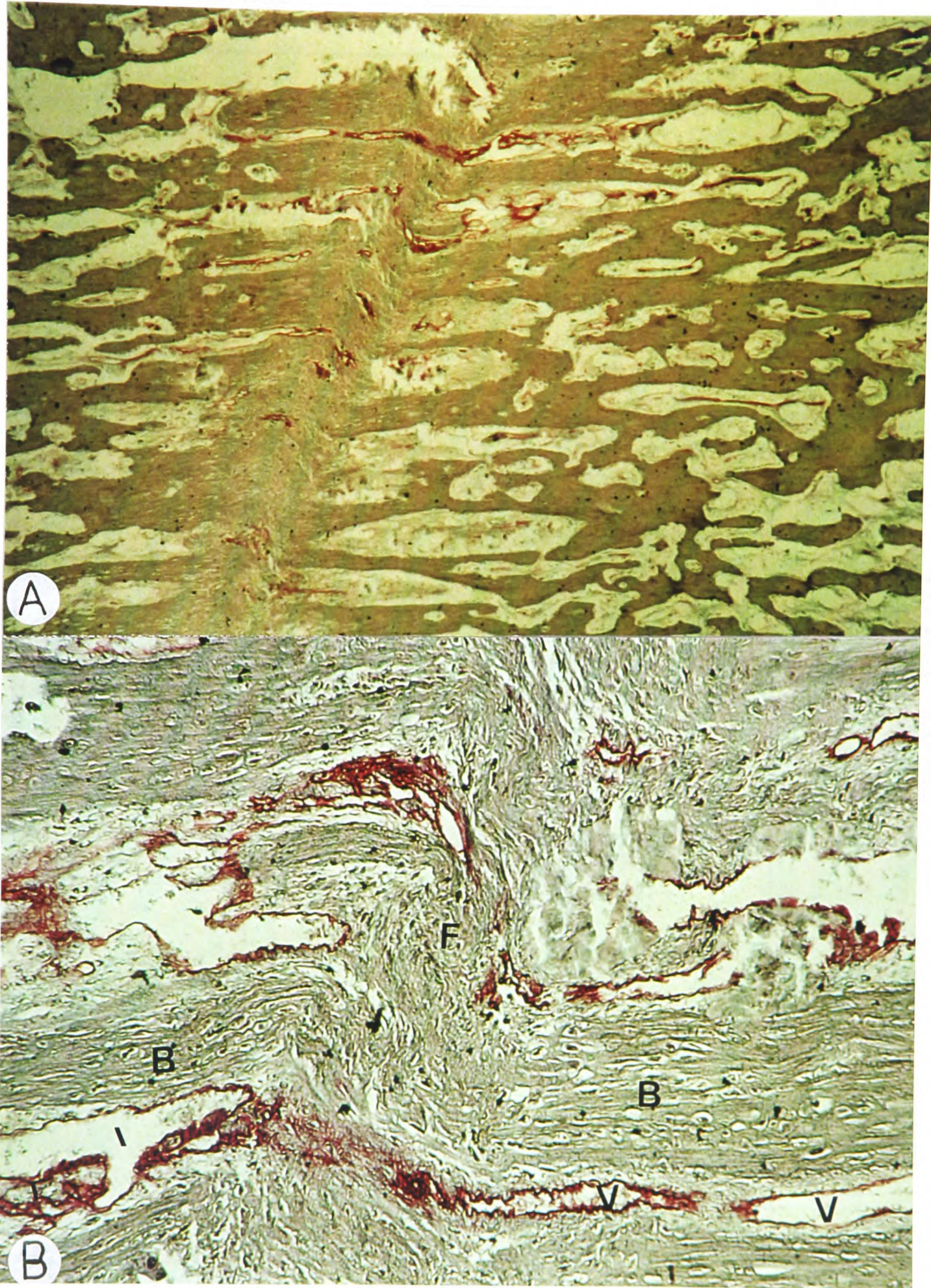


Fig. 8. 3. Type IV collagen binding (in red) in the regenerate of the 0.3 mm/day group was generally weak. A: the low power view, X 25; B: close up of the mineralization front, staining in this region was relatively strong, X 100. B: bone; F: fibrous tissue; V: blood vessel.

8. 3. 3 0.7 mm / day and 1.3 mm / day groups

The immunostaining pattern of the type IV collagen and EC antigen was very similar in the 0.7 mm/day and 1.3 mm/day group. As the rate of distraction increased, the size of the central fibrous zone enlarged, and there was a significant increase of staining for both type IV collagen and EC antigen in the mineralization front and the central fibrous zone (Fig. 8. 4 and Fig. 8. 5) compared to the 0.3 mm/day group.

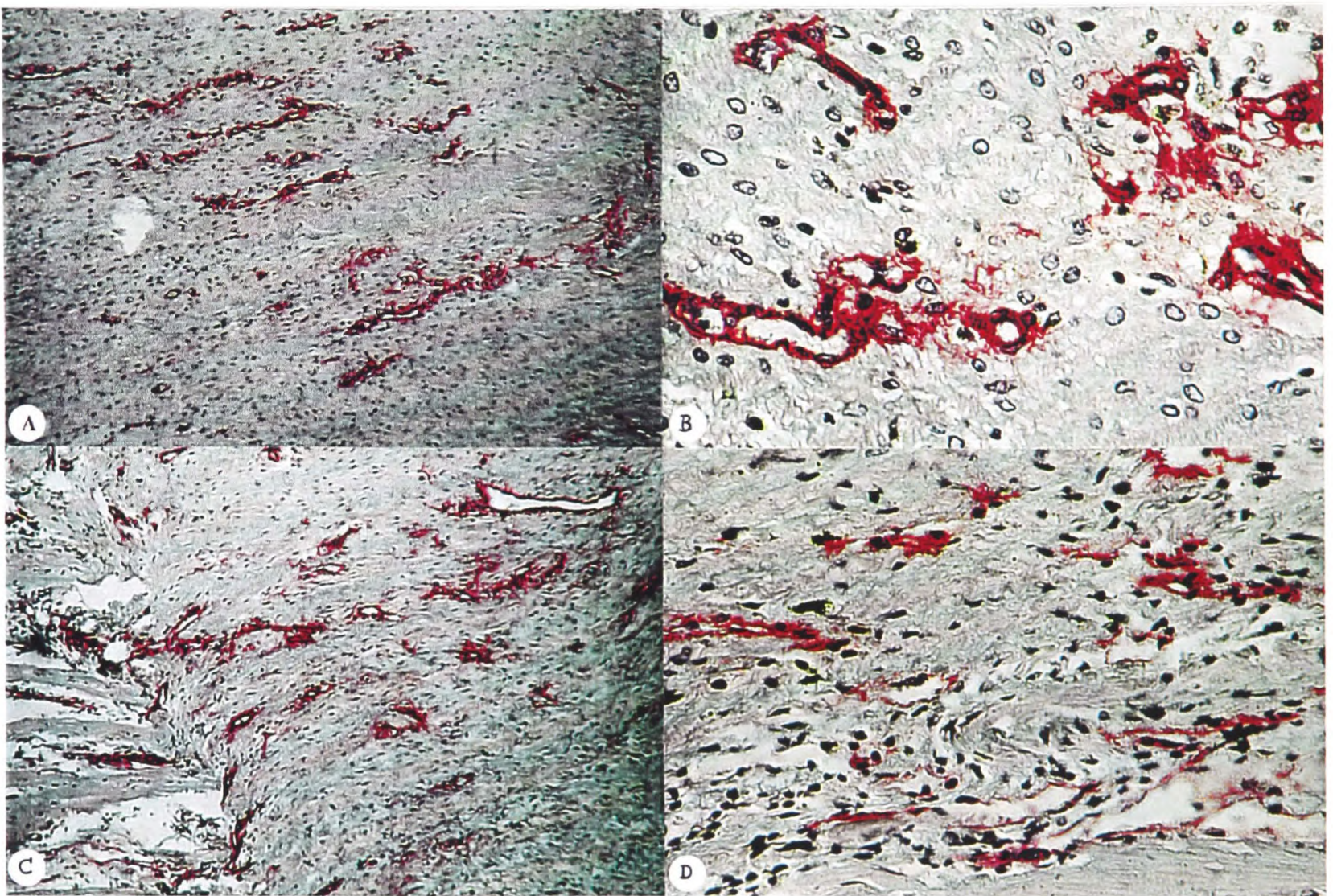


Fig. 8. 4. Compared to the 0.3 mm/day group, immunostaining by type IV collagen antibody showed increased staining in the fibrous zone (A and B), and the mineralization front (C and D) in a regenerate of the 0.7 mm/day group. The new blood vessels were formed parallel to the direction of the distraction. A and C, X 25; B and D, X 100.

The staining of blood vessels was intense in the central fibrous zone and the mineralization front in the regenerates of the 0.7 mm/day and the 1.3 mm/day groups (Figs. 8. 4 A, C; 8. 5 A). Since the shape of the endothelial cells were similar to the fibroblasts in the fibrous region, only by immunostaining using antibody to EC antigen was it possible to identify the endothelial cells from the fibroblasts (Fig. 8. 5) and the formation of the new vessel buds was also clearly

outlined by the staining of EC antigen (Fig. 8. 5).

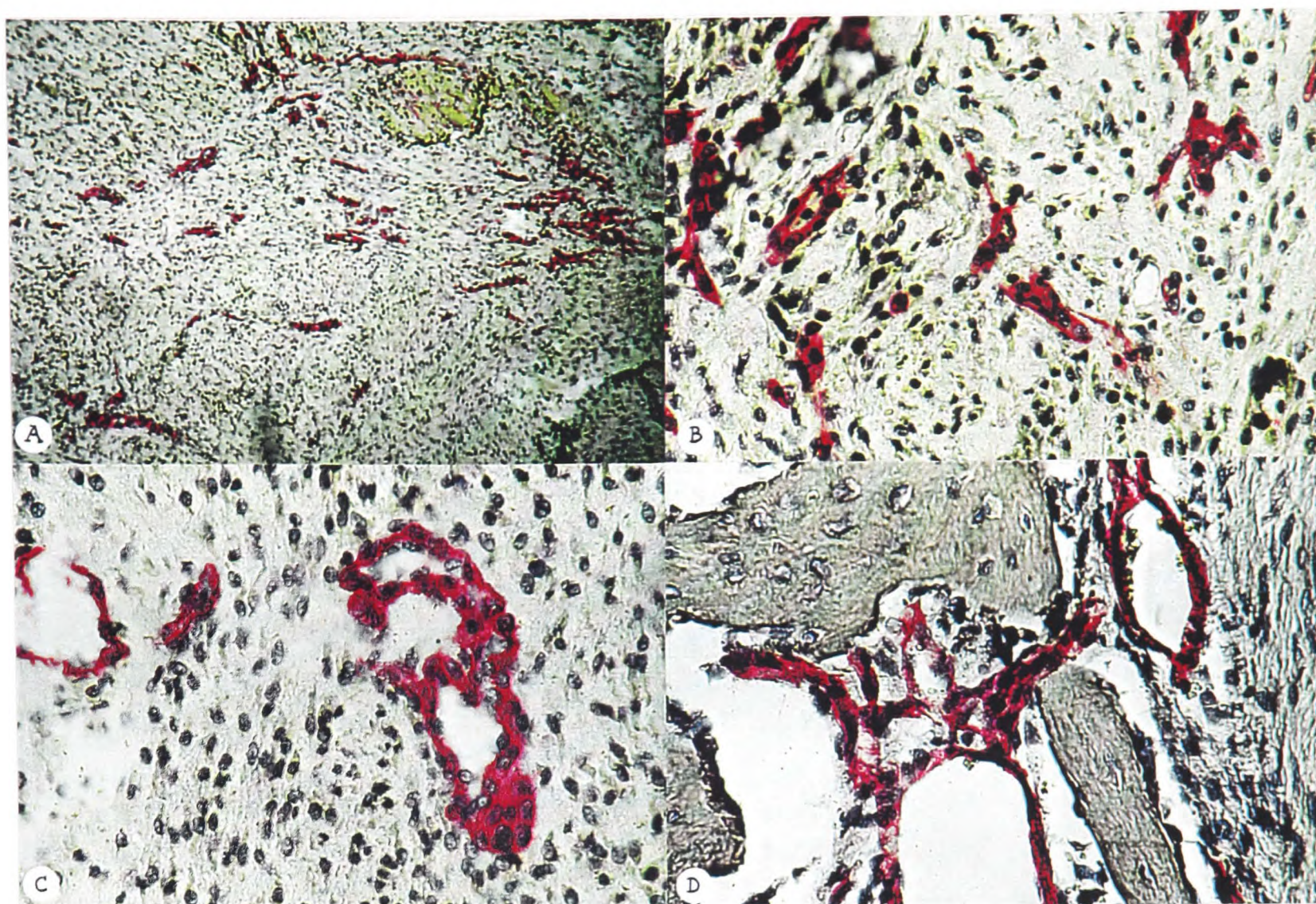


Fig. 8. 5. In comparison with the 0.3 mm/day group, there was an increase in staining of EC antigen in the regenerates of the 1.3 mm/day group. The distribution and staining patterns were similar to those obtained by using antibody of type IV collagen in the 0.7 mm/day group. A: The number of newly formed blood vessels increased in the fibrous zone. B and C: The outline of the new vessel buds in the fibrous region. D: In the new bone zone, the microvessels and new bone were in close contact. A, X 25; B - D, X 100.

In the central fibrous zones and the mineralization fronts of regenerates of both the 0.7 and 1.3 mm/day groups, intense staining for both type IV collagen and EC antigen show that the new blood vessels were formed parallel to the direction of the distraction. In the new bone zone near the mineralization front, newly forming bone and blood vessels were in close contact and the new bone formed where blood vessels lay. This is clearly demonstrated in Fig. 8. 6.

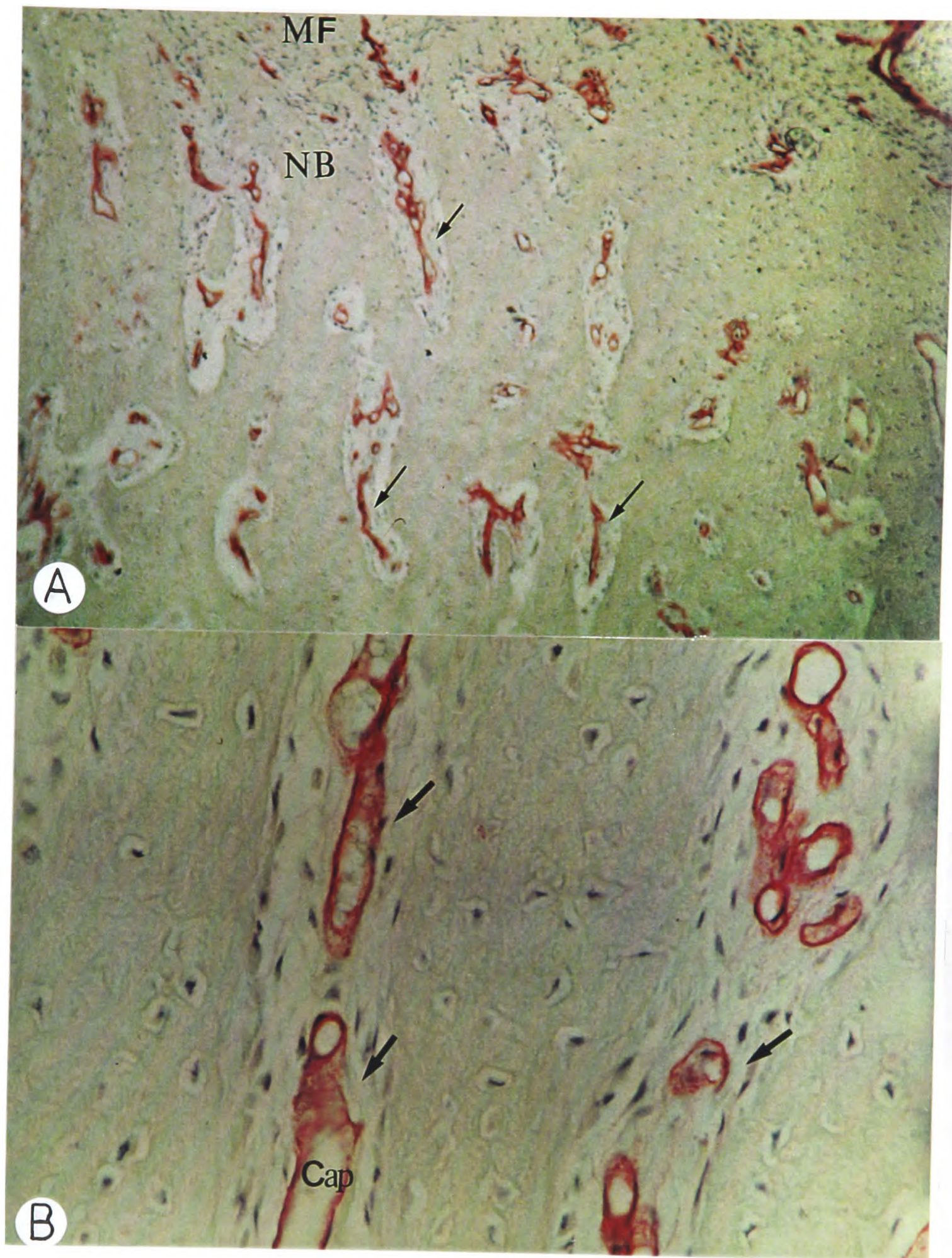


Fig. 8. 6. Type IV collagen immunostaining showing the relationship between vascularization and bone formation in the new bone zone of a regenerate of the 0.7 mm/day group. The new bone was formed in close contact with the new capillaries (arrows). MF: mineralization front; NB: new bone; Cap: capillary. A X 100; B X 400.

The significant contribution of the surrounding soft tissues (periosteum and muscle) to the neovasclarization to the regenerating callus was observed in both the 0.7 and 1.3 mm/day group. There was enhanced new vessel formation in the surrounding thickened periosteum and nearby skeletal muscle (Fig. 8. 7 A, B). New bone was formed directly from the periosteum in proximity to the new blood vessels (Fig. 8. 7 C). Cartilage islands were found in the regenerates of groups with distraction rates higher than 0.7 mm/day. The staining for both type IV collagen and EC antigen was absent in the cartilage region, but present in the vicinity of invading blood vessels in the cartilage region (Fig. 8. 7 D).

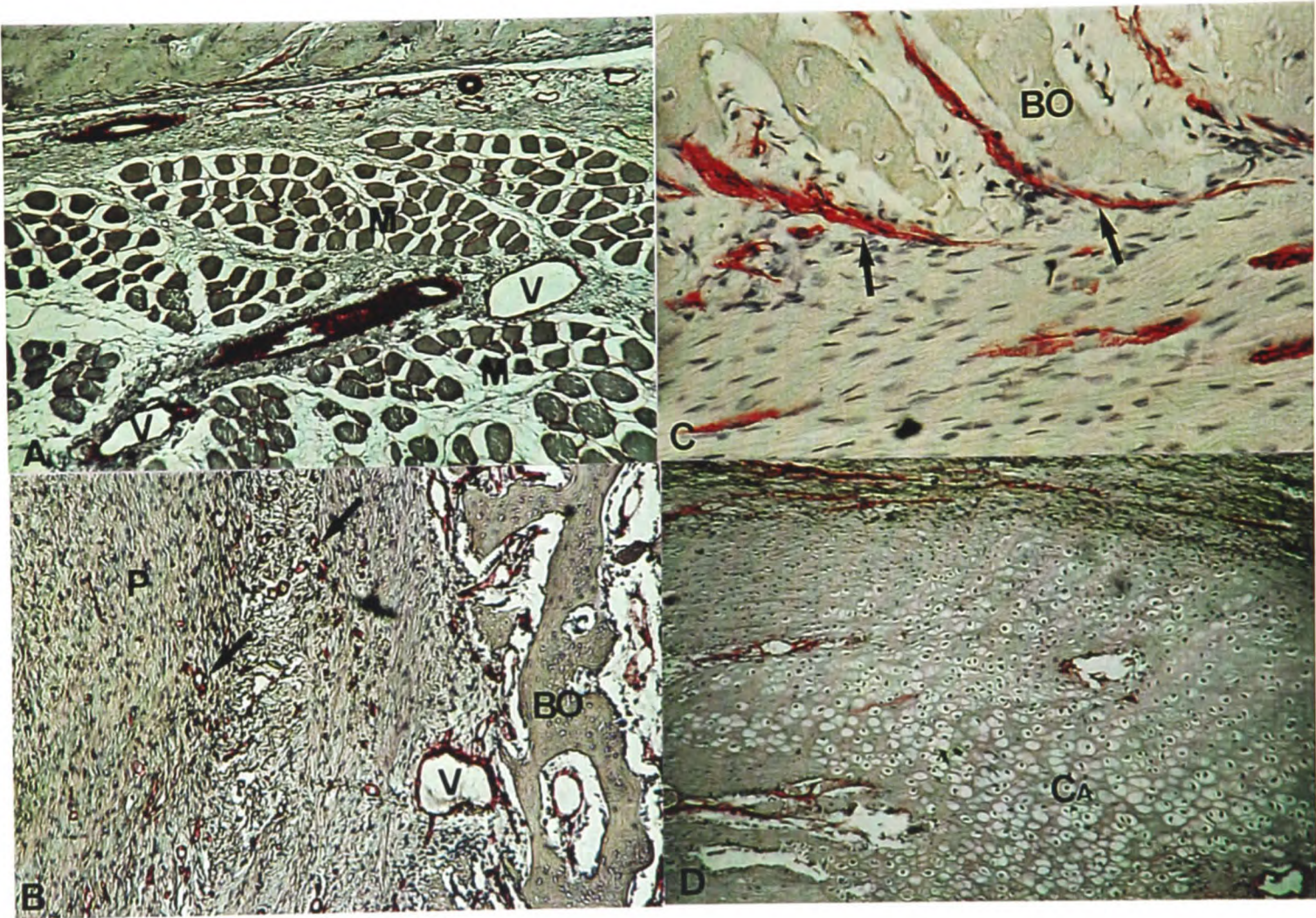


Fig. 8. 7. Examples of the blood vessel distribution in the surrounding soft tissues adjacent to the regenerate in the 0.7 mm/day group. A: blood vessel (V) formation was enhanced in the attached skeletal muscle (M). B: The periosteum (P) was thickened with a rich capillary network (arrows). A and B, X 100. C: Intramembranous bone (B) formation was closely related to the vascular orientation. D: Blood vessels were absent in the region of cartilage (Ca). Type IV collagen staining, all were counterstained with Mayer's haematoxylin, A, B, D, X 40; C X 100.

Staining of type IV collagen and EC antigen was evident throughout the new bone zone, but there was a tendency for the staining to reduce as the new bone zone extended from the mineralization front to the osteotomied ends (Fig. 8. 8). The staining was sparse or absent in the region of intact cortical bone (not shown).

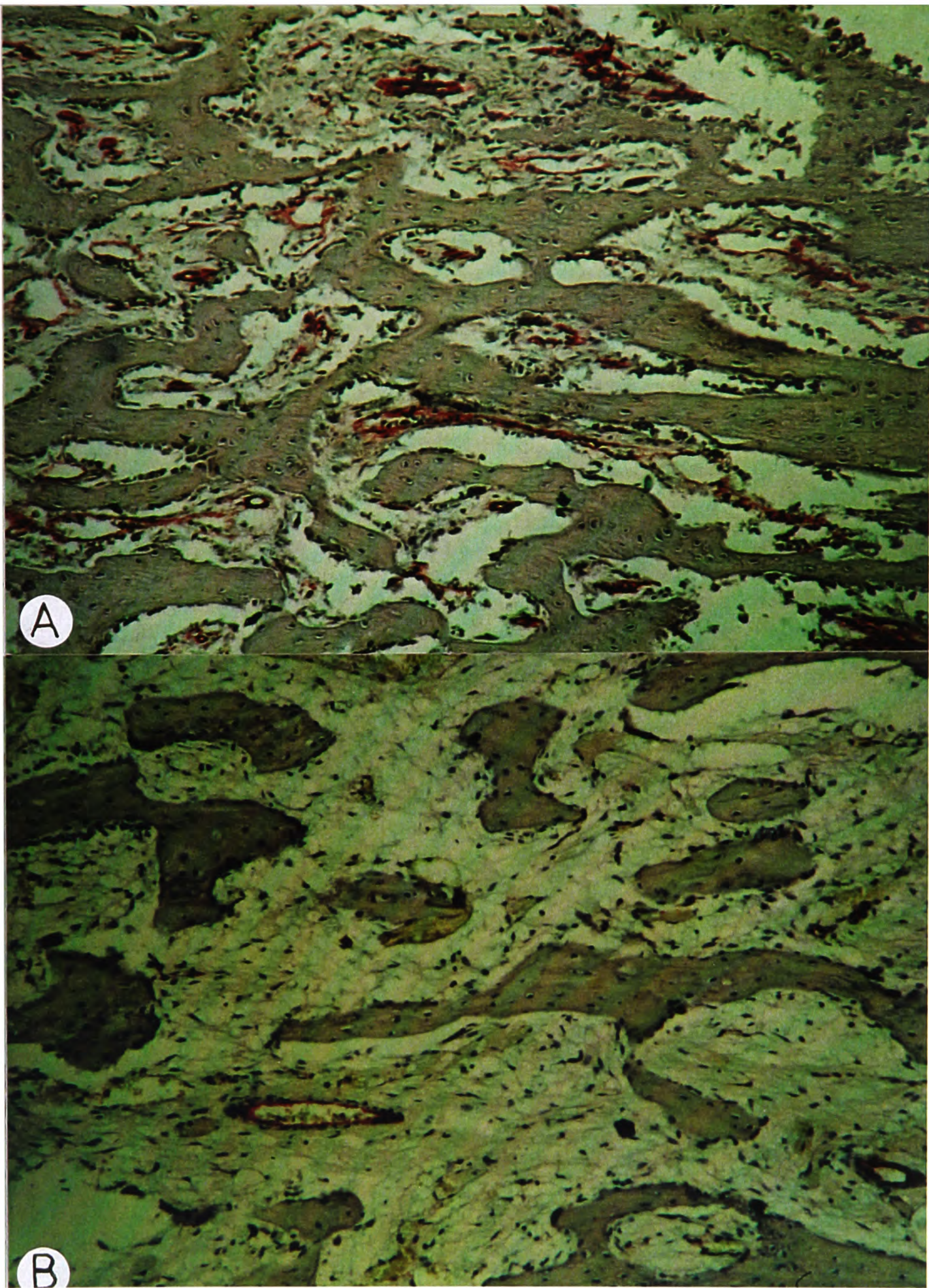


Fig. 8. 8. Type IV collagen immunostaining in the NBZ of a regenerate of the 0.7 mm/day group. A: the staining was strong in the NBZ near the MF. B: the staining became weaker in the NBZ distant to the MF, close to the osteotomied bone ends. A and B, X 100.

8. 3. 4 2.7 mm/day group

At a distraction rate of 2.7 mm/day, most of the distraction gap was fibrous tissue, bone

formation was sparse and type IV collagen and EC staining were greatly reduced in the central fibrous zone, the mineralization front and the new bone zone compared to similar regions in the 0.7 or 1.3 mm/day groups (Fig. 8. 9).

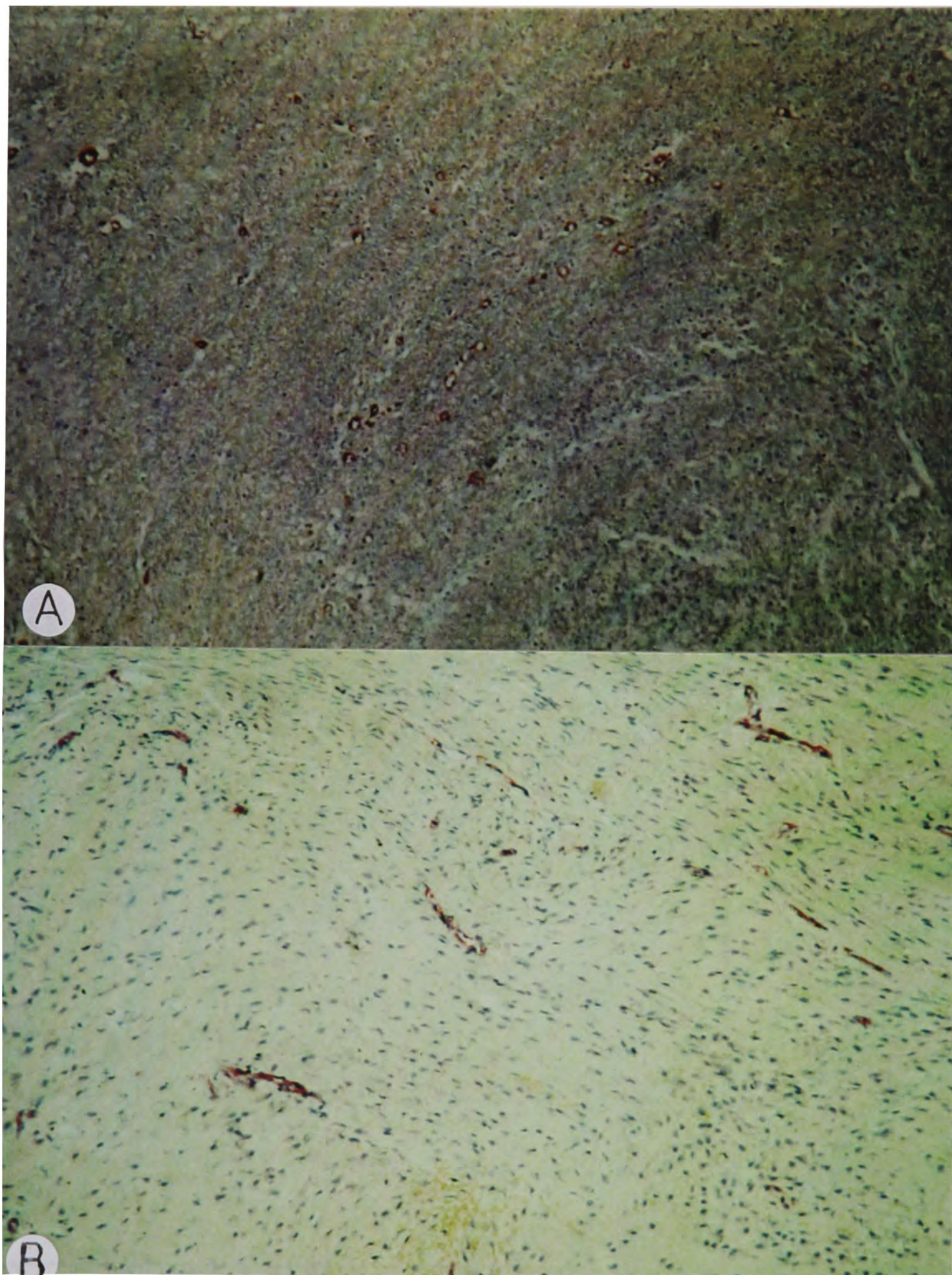


Fig. 8. 9. Compared to the 0.7 or 1.3 mm/day groups, an example showing that the immunostaining for both type IV collagen (A) and EC antigen (B) was greatly reduced in the regenerate (the central fibrous zone) of the 2.7 mm/day group, A and B, X 25.

8. 4 Discussion

Angiogenesis is a complex multi-step process involving extracellular matrix remodelling, endothelial cell migration and proliferation, capillary differentiation, and anastomosis. Angiogenesis is tightly regulated and is observed only transiently during particular circumstances such as in development and wound healing (see section 1. 10). The current model of distraction osteogenesis presents an excellent opportunity to study the angiogenic process.

In agreement with the reports by Ilizarov and Aronson, an enhanced angiogenesis process was observed during distraction osteogenesis at appropriate distraction rates (0.7 and 1.3 mm/day), whilst the slow distraction rate (0.3 mm/day) did not maximise the angiogenic potential of the tissue and an excessive rate of distraction (2.7 mm/day) inhibited or reduced the angiogenesis of the regenerating tissue. Angiogenesis during distraction osteogenesis was enhanced only at appropriate rates of distraction, in the present study 0.7 and 1.3 mm/day. This finding is consistent with the findings of histology and cell proliferation studies described before in this thesis and, taken together, the findings suggest that distraction at 0.7 mm/day was most satisfactory for both osteogenesis and angiogenesis. Higher rates (2.7 mm/day) of distraction create the lengthening gap in less time, but the large increment of each distraction damaged the regenerating tissues, and appeared to inhibit the angiogenesis process at the centre of the regenerating tissue.

In the groups of 0.7 and 1.3 mm/day, the intense staining of both type IV collagen and EC antigen was localised mainly in the central fibrous zone and the mineralization front. Similarly, the most intense staining for cell proliferation was observed in these regions. The results suggest that endothelial cells may play an important role in osteogenic cell differentiation and proliferation. Endothelial cells or pericytes may be a supplementary source of osteoblasts and participate directly in intramembranous bone formation (Diaz-Flores, et al. 1992). Schor et al. (1995) have suggested that pericytes of blood vessels may act as links between angiogenesis and mineralization. Reddi and Cunningham (1993) reported that native BMP-3 and recombinant BMP-4 bind type IV collagen of the basement membrane, and this novel

connection indicates that angiogenesis and vascular invasion may be a prerequisite for bone morphogenesis.

Brandi et al. (1993) investigated the effects of estrogens on bone endothelial cell metabolism. They found that estrogen binding sites were present in the endothelial cell. The presence of functional estrogen receptors in bone endothelial cells suggests a role of estrogen in bone angiogenesis and in the process of bone remodelling. Distraction osteogenesis results in unusual remodelling with collagen bundles and the microvasculature aligned parallel to the distraction force. Ganey et al. (1994a) found a different distribution of membrane protein laminin in response to sustained (distraction) and intermittent tension (development of tibial tuberosity). They suggested that the direction and frequency of mechanical stimulation are both capable of altering angiogenesis.

Furthermore, the demonstration of enhanced angiogenic responses and neovascularization in the different regions of the regenerate of the 0.7 mm/day group, concur with the changes of the TUNEL staining in these regions as described in chapter 7. These results are consistent with the hypothesis that neovascularization and subsequent blood perfusion of the regenerate may initiate programmed cell death as discussed in chapter 7. Taken together, the previous results of cell proliferation, cell death, matrix mineralization and remodelling, and the findings from the present study, suggest that the angiogenic process plays a crucial role in controlling osteogenesis and bone remodelling and in regulating cell proliferation and cell death.

8. 5 Conclusion

(1) A reliable staining method using mouse anti-human type IV collagen and EC antigen antibodies was obtained on paraformaldehyde-fixed paraffin-embedded rabbit tissues.

(2) The angiogenic process was affected by the rate of distraction during distraction osteogenesis. A slow rate of distraction (0.3 mm/day) did not maximally stimulate the angiogenic response, while higher rates (2.7 mm/day) of distraction inhibited the angiogenesis.

(3) Compared to the 0.3 and 2.7 mm/day groups, there was an increased angiogenic response in the regenerating tissues in the groups lengthened at 0.7 mm/day and 1.3 mm/day. Intense staining of collagen type IV and EC antigen was localised in the central fibrous zone, the mineralization front and the new bone zone near the mineralization front. This is consistent with angiogenesis was playing a crucial role in bone formation during distraction osteogenesis.

(4) Neovascularization may also be responsible for initiating bone remodelling by inducing programmed cell death in the regenerating tissues during distraction osteogenesis. Direct evidence for this conclusion is for future study.

PART IV

**Conclusions
Clinical Relevance
Future Work**

Chapter 9 Conclusions, Clinical relevance and Future work

9. 1 Summary of findings in present studies

The introduction to this thesis has emphasised the importance of cell proliferation, extracellular matrix production, growth factors and cytokine expression, angiogenesis and apoptosis in the process of bone formation and bone repair. The development of limb-lengthening techniques has made the treatment of many congenital and acquired disorders of the adult and developing skeleton possible by generating extensive new bone tissue at appropriate sites. However, many complications still result from the limb lengthening technique and the biological mechanisms of distraction osteogenesis are still poorly understood.

The main aims of this thesis were, therefore, to elucidate some of the important biological aspects of distraction osteogenesis. The work in this thesis has concentrated on the following aspects of distraction osteogenesis:

- (1) The animal model of distraction osteogenesis;
- (2) The histology and radiology of the newly formed tissues;
- (3) Cell proliferation;
- (4) Changes in extracellular matrix;
- (5) BMP-4 mRNA expression in the regenerate;
- (6) Apoptosis in the regenerating tissues;
- (7) Angiogenesis during distraction osteogenesis.

This work has generated a number of observations and conclusions, which can be summarised as follows:

- (1) An animal model of leg-lengthening has been refined. The surgical procedure was

standardised and a reproducible rabbit model of tibial lengthening was developed.

(2) The process of distraction osteogenesis at four different distraction rates (0.3, 0.7, 1.3 and 2.7 mm/day) has been investigated radiographically and histologically. The most satisfactory histological and radiological results of the distraction regenerate was observed in the group lengthened at a rate of 0.7 mm/day.

(3) Based on the radiography and histology, the distraction regenerate can be divided into three separate zones: the central fibrous zone, the primary mineralization front and the new bone zone.

(4) BrdUrd labelling was adapted for this model and used to measure cell proliferation.

(5) The importance of the periosteal contribution in the early phases (1 week post osteotomy) of fracture repair was confirmed. The late contribution (2 weeks post osteotomy) of cell division from the endosteal region, and from the medullary cavity, has also been clarified.

(6) The present study indicated that cell proliferation of osteoprogenitor cells during distraction osteogenesis was affected by the rate of distraction. The rate of cell proliferation in the regenerating tissues was found to rise as the distraction rate increased from 0.3 to 0.7 mm/day, but not to rise further as the distraction rate increased to 2.7 mm/day. Higher rates (> 1.3 mm/day) of distraction, which cause tissue damage, have no additional beneficial effect on stimulating cell proliferation in the regenerating tissue and should be avoided.

(7) Cell proliferation was also assessed by the detection of PCNA using a monoclonal antibody, PC10. A comparison was made between the assessment of cell proliferation by expression of PCNA and by the uptake of BrdUrd (Bu20A immunohistochemistry) in the rabbit tissue. A correlation was found between the PSI values of PC10 and Bu20A as: $PC10\ PSI = 1.6\ Bu20A\ PSI + 12.9$, with a correlation coefficient 0.79. The results suggest that PC10 may be used as an alternative marker to assess cell proliferation where the use of BrdUrd

was impossible.

(8) In-situ hybridization techniques using non-radioactive labelling probes have been modified for use in the present study. In-situ hybridization using types I and II collagen riboprobes demonstrated that the expression of type I collagen mRNA was predominant in the fibroblasts of fibrous tissue and bone surface cells, but reduced in the osteocytes; type II collagen mRNA was only expressed by the chondrocytes.

(9) The localization of mRNA for types I and II collagen in the distraction regenerates indicates that bone was formed mainly by an intramembranous process during distraction osteogenesis. However, the presence of type II collagen mRNA and proteins in the regenerates at higher distraction rates (≥ 0.7 mm/day) demonstrated that areas of endochondral ossification do exist in the present model of distraction osteogenesis.

(10) An overlapping of the cartilage and bone phenotypes was noted in cells at the fibrous tissue-cartilage or cartilage-bone transitional regions. In those particular areas, type I collagen mRNA and protein, bone keratan sulphate proteoglycan and type II collagen mRNA and protein were found together in some of the marginal hypertrophic chondrocytes, which suggested an unusual but active role of chondrocytes in the process of osteogenesis at higher rates of distraction.

(11) In the present leg-lengthening system, higher rates of distraction (from 0.7 to 1.3 mm/day) resulted in increased chondrogenesis in the distraction regenerating tissues. The osteoblasts and chondrocytes within the regenerate originated from a pool of progenitor cells. Their differentiation, and the subsequent expressions of types I and II collagen genes and proteins was altered by the rate of distraction. The rate of distraction, therefore, affected the types of skeletal tissues formed during distraction osteogenesis.

(12) The present study is the first to characterize the presence of BMP-4 mRNA expression in the regenerating tissues produced by distraction osteogenesis. The BMP-4 gene was expressed mainly by less differentiated osteoprogenitor cells in the regenerating tissue,

indicating that BMP-4 might play an important role in controlling early proliferation and differentiation of osteoprogenitor cells during distraction osteogenesis.

(13) Simultaneous dual labelling with the BrdUrd immunohistochemistry and the TUNEL staining has been developed. The BrdUrd-positive labelled (proliferating) cells and the TUNEL-positive apoptotic cells were found to coexist in the regenerating tissues. The dual labelling technique can be used to identify apoptosis and proliferation simultaneously in the skeletal system in future research.

(14) The demonstration of a mixture of distinct proliferative and apoptotic cell populations coexisting in the regenerating tissues, suggested that apoptosis and cell proliferation might be regulated at by local factors or via cell-cell contact during the rapid cell or tissue turnover seen in distraction osteogenesis.

(15) The changes of distribution of apoptotic cells in the different regions of the regenerate, together with the results of TRAP staining, suggest that rapid bone remodelling through attraction of bone-resorbing cells during distraction osteogenesis may be initiated by the cells undergoing apoptosis.

(16) Reliable staining using mouse anti-human type IV collagen and EC antigen antibodies was obtained on paraformaldehyde-fixed paraffin-embedded rabbit tissues. The angiogenic process was affected by the rate of distraction during distraction osteogenesis. A slow rate of distraction (0.3 mm/day) did not maximally stimulate angiogenesis, while a high rate (2.7 mm/day) of distraction inhibited angiogenesis. The best angiogenic response was observed in the groups lengthened at 0.7 and 1.3 mm/day. Intense staining of collagen type IV and EC antigen was observed in the central fibrous zone, the mineralization front and the new bone zone near the mineralization front. These results are consistent with angiogenesis have played a crucial role in bone regeneration during distraction osteogenesis.

9. 2 **Clinical relevance of the present studies**

Distraction osteogenesis is used to generate new skeletal tissues during surgical techniques of limb-lengthening. After the bone has been divided, there is an initial waiting period, followed by a distraction period, and finally a consolidation phase. The distraction period continues as long as controlled gradual distraction is being applied. In the present studies, the sequence of events associated with the distraction phase of distraction osteogenesis, have been studied using radiographic, histological, immunohistochemical and in-situ hybridization techniques in a rabbit model of leg-lengthening at distraction rates of 0.3, 0.7, 1.3 and 2.7 mm/day. The studies in this thesis indicate some biological bases for understanding the mechanisms of distraction osteogenesis.

In the clinical situation, the main variables of limb lengthening are the rate, rhythm and the amount of lengthening and, as evident in the present experimental model, the rate of distraction had significant effects on the cellular responses of the regenerating tissue. The present studies suggested that the processes of cell proliferation, matrix synthesis, cell death and angiogenesis during distraction osteogenesis were affected by the rate of distraction. A slow rate of distraction (0.3 mm/day) did not maximally stimulate progenitor cells to undergo osteogenic and angiogenic differentiation and proliferation. High rates (> 1.3 mm/day) of distraction had no beneficial effect on stimulating bone cell proliferation, enhancing angiogenic response, or accelerating bone formation. However, these higher rates of distraction (> 1.3 mm/day) were observed to cause damage to the regenerating tissues, and they should be avoided in clinical practice.

In the present study, the results suggest that a distraction rate of 0.7 mm/day is most favourable for bone regeneration in distraction osteogenesis. These results concur with Ilizarov's clinical and experimental observations that distraction at a rate of around 1.0 mm/day was most desirable for bone regeneration. The distraction rate between 0.5 - 1.0 mm/day is recommended for clinical practice. However, in the clinical situation, this rate often has to be slowed because of poor adaptation of the soft tissues or because of poor bone formation due to other local and systemic factors.

The present study confirmed that bone formation in the distracted area occur mainly by intramembranous ossification. Cartilage formation and endochondral ossification, were seen in the groups at higher rates (≥ 0.7 mm/day) of distraction, and this may be due to insufficient stability caused by fast distraction or local ischaemia resulting from vascular damage caused by excessive distraction. The long-term effect of the presence of cartilage in the regenerate on the total course of distraction osteogenesis has to be clarified. However, this study indicates the possibility of an unusual but active role of chondrocytes in the process of ossification during distraction osteogenesis, suggesting that mechanical conditions such as the rate of distraction could alter the phenotypic differentiation of the osteoprogenitor cells. This observation applies to the clinical situation, in that in the course of distraction osteogenesis, the clinician should act in a reflective way to make sensible adjustments of the frequency and rate of distraction to meet the individual needs of the patients.

In the present study, angiogenesis was enhanced only at appropriate rates of distraction, which were at 0.7 and 1.3 mm/day and confirms previous reports by Ilizarov and Aronson that the blood supply is enhanced during distraction osteogenesis. This offers direct evidence to support the use of distraction osteogenesis techniques to treat bone diseases such as osteomyelitis, fracture non-union or malunion and ischemic bone necrosis, in which an enhancement of angiogenesis and blood supply would be a necessary step for cure.

In addition, the observations of changes in apoptosis occurring in the regenerating tissues, which were closely related to angiogenesis, may explain the mechanism by which rapid bone remodelling coexists with rapid bone formation during distraction osteogenesis. Clinically, the distraction osteogenesis technique may be used to stimulate the bone remodelling process to shorten the treatment of certain diseases, such as fracture mal-union and some congenital bone diseases.

Finally, the present study defines the localization of BMP-4 mRNA expression in the regenerating tissues produced by distraction osteogenesis. The BMP-4 gene is expressed mainly by less differentiated osteoprogenitor cells in the regenerating tissue, indicating that BMP-4 may play an important role in controlling osteoprogenitor cell proliferation and

differentiation during distraction osteogenesis. The findings in the present study together with previous reports on the function of BMPs and clinical application in fracture treatment, support the conclusion that the application of BMPs may have potential value for treatment of inadequate bone formation during limb lengthening and bone transport.

9. 3 Future work

The work covered in this thesis has raised many related questions, some of which have been noted in previous chapters, which suggest the course of future work:

- (1) The cellular events at different time sequences, such as during phases of lengthening, consolidation and corticalization should be examined in future study.
- (2) Further direct evidence is required to test the hypothesis that the hypertrophic chondrocytes may transdifferentiate directly into bone-forming cells in the course of intramembranous or endochondral ossification, as seen in distraction osteogenesis.
- (3) What are the factors controlling cell proliferation and cell death during distraction osteogenesis ? What growth factors and cytokines are involved in distraction osteogenesis ?
- (4) The angiogenic process and its control during distraction osteogenesis needs to be clarified. The angiogenic process during bone transport needs to be studied.
- (5) The link between neovascularization and apoptosis during distraction osteogenesis needs to be proven and the control factors of this process need to be clarified.
- (6) Several possible applications of biological factors such as recombinant human BMP-2 (rhBMP-2) can be investigated in distraction osteogenesis. The ability of rhBMP-2 to accelerate the consolidation phase during distraction osteogenesis along with the most beneficial way of delivering rhBMP-2 into the regenerating tissues should be investigated.

(7) Clinical questions such as the optimum length of treatment and the timing of frame removal remain unanswered. Concerns regarding the effects of metabolic status, drug usage, cigarette smoking and chronic disease need to be addressed.

(8) Development of new models of distraction osteogenesis, such as using rat or mice, could be potentially useful for future study of changes in gene level using molecular biology techniques.

PART V

Appendix & Reference

Chapter 10 Appendix and Reference

Appendix

Appendix 1. Related publications generated from this thesis

1. SJ Gregg-Smith, **G Li**, J Bradley, JT Triffitt and AHRW Simpson. The assessment of cell proliferation in bone during leg-lengthening in the rabbit. J. Bone Joint Surg (Br) 1995; 77B, SuppIII: 282 [Abstract].
2. **G Li**, AS Viridi, AHRW Simpson, DE Ashhurst, JT Triffitt. In-situ localisation of collagen type I and II mRNAs in the regenerating tissue of distraction osteogenesis. Bone, 1995; 16(6): 687 [Abstract].
3. L Zhao, SJ Gregg-Smith, AHRW Simpson, **G Li**, J Bradley and J Kenwright. A histopathological study of the fascia subjected to a variety of distraction rates in a rabbit model. J Bone Joint Surg (Br), 1996; 78B, SuppI: 42 [Abstract].
4. **G Li**, AHRW Simpson, J Kenwright, JT Triffitt. Cell proliferation as assessed by expression of proliferating cell nuclear antigen or the uptake of bromodeoxyuridine in a rabbit model of leg-lengthening. Veterinary and Comparative Orthopaedic and Traumatology (V.C.O.T), 1996; 9: 95-100.
5. E Pålsgård, C Johansson, **G Li**, GW Grime, JT Triffitt. Bone growth and bone development in the presence of implants or after induced bone lengthening studied using the Oxford scanning proton microprobe. Nuclear Instruments and Methods B, 1997 [in press]
6. **G Li**, AHRW Simpson, J Kenwright and JT Triffitt. Assessment of cell proliferation in regenerating bone during distraction osteogenesis at different distraction rates. J Orthop. Res. [in press]
7. **G Li**, S Berven, AHRW Simpson and JT Triffitt. The expression of BMP-4 mRNA during distraction osteogenesis. Bone [submitted]
8. **G Li**, AS Viridi, DE Ashhurst, AHRW Simpson and JT Triffitt. The distraction rate determines the types of skeletal tissues formed during distraction osteogenesis in the rabbit; localization of types I and II collagens mRNA and cells that synthesise proteins. Acta Orthop. Scandia. [submitted]

9. **G Li**, AHRW Simpson and JT Triffitt. Morphological observations on cellular transitions during distraction osteogenesis performed at a high rate: localisation of protein and mRNA for collagen types I and II. *Calcified Tissue International* [submitted]
10. S Berven, **G Li**, NA Athanasou, AHRW Simpson, JT Triffitt. Bone transport over an intramedullary nail: a case report with histologic examination of the regenerate and literature review. *Injury* [submitted]
11. **G Li**, JT Triffitt, AHRW Simpson and J Kenwright. Cell proliferation in early phases of fracture healing following tibial osteotomy and external fixation. *Journal of Orthopaedic Trauma* [submitted]
12. **G Li**, AHRW Simpson, JT Triffitt. Cell death, cell proliferation and angiogenesis during distraction osteogenesis. *Bone* [submitted]

Appendix 2. Peer reviewed conference proceedings

1. **G Li**, SJ Gregg-Smith, AHRW Simpson, J Bradley and JT Triffitt. In-situ assessment of cell proliferation: The use of PC10 monoclonal antibody to proliferating cell nuclear antigen. *Proc. Bone & Tooth Society Winter Meeting. December, 1993, London, UK.*
2. **G Li**, SJ Gregg-Smith, AHRW Simpson, J Bradley and JT Triffitt. Quantitative studies of cell proliferation in bone formed during leg-lengthening. *Proc. Bone & Tooth Society Spring Meeting. March, 1994, Southampton, UK.*
3. SJ Gregg-Smith, **G Li**, J Bradley, JT Triffitt and AHRW Simpson. The assessment of cell proliferation in bone during leg-lengthening in the rabbit. *Proc. The Combined 4th European Orthopaedic Research Society and British Orthopaedic Research Society Meeting. April, 1994, London, UK.*
4. **G Li**, AS Viridi, AHRW Simpson, DE Ashhurst, JT Triffitt. In-situ localisation of collagen type I and II mRNAs in the regenerating tissue of distraction osteogenesis. *Proc. Bone & Tooth Society Autumn Meeting. December, 1994, London, UK.*
5. J.T. Triffitt, G.W. Grime, **G Li**, N.A. Athanasou, A.H.R.W. Simpson. Nuclear microbeam analysis of biomaterial wear particles and associated tissues. *Proc. Perth International Bone Meeting. Bone Fragility in the year 2000: Clinical Measurement. Feb. 14-19, 1995, Melbourne, Australia.*
6. AHRW Simpson, **G Li**, SJ Gregg-Smith, JT Triffitt and J Kenwright. Cell proliferation in regenerating bone during distraction osteogenesis. *Proc. 41st Annual Meeting of American*

Orthopaedic Research Society. Feb 13-16, 1995, Florida, U.S. A.

7. G Li, AHRW Simpson, AS Viridi, JT Triffitt and J Kenwright. The gene expression of collagens types I and II in an experimental system of leg-lengthening. Proc. The 5th EORS Conference. July, 1995, Munich, Germany.
8. L Zhao, SJ Gregg-Smith, AHRW Simpson, G Li, J Bradley and J Kenwright. A histopathological study of the fascia subjected to a variety of distraction rates in a rabbit model. Proc. British Orthopaedic Research Society Meeting. September, 1995, Dundee, Scotland, UK.
9. G Li, A Viridi, H Simpson, J Kenwright, J Triffitt. The gene expression of collagens type I and II during distraction osteogenesis. Finalist of New Investigator Recognition Awards. Proc. The 2nd Combined Meeting of Orthopaedic Research Society of U.S.A, Japan, Canada and Europe. November 5-8, 1995, San Diego, California, U.S.A.
10. G Li, AHRW Simpson, J Kenwright and JT Triffitt. Assessment of cell proliferation in regenerating bone during leg-lengthening. Japanese Travelling Scholarship winner. Proc. SICOT 96 Amsterdam Meeting. August 18-23, 1996, Amsterdam, Holland.
11. G Li, AHRW Simpson, J Kenwright and JT Triffitt. Early proliferative responses following tibial osteotomy. Proc. The 5th Meeting of the International Society for Fracture Repair (ISRF-96). September 18 -21, 1996. Ottawa, Canada.
12. G Li, AS Viridi, JT Triffitt. Apoptosis in bone regeneration during leg-lengthening. Proc. Bone and Tooth Society Autumn Meeting. September 20, 1996, London.
13. E Pålsgård, C Johansson, G Li, GW Grime. Bone growth and bone development in the presence of implants or after induced bone lengthening studied using the Oxford scanning proton microprobe. Proc. The 5th international conference on nuclear microbeam technology and applications. November 1996, Santa Fe, USA.
14. G Li, AHRW Simpson, JT Triffitt. Mechanisms for controlling callus volume during distraction osteogenesis. Proc. British Orthopaedic Research Society Spring Meeting. 3-4 March, 1997, Sheffield.
15. G Li, S Berven, AHRW Simpson and JT Triffitt. The gene expression of BMP-4 during distraction osteogenesis. Proc. British Orthopaedic Research Society Spring Meeting. 3-4 March, 1997, Sheffield.
16. S Berven, G Li, NA Athanasou, JT Triffitt and AHRW Simpson. Bone transport over

an intramedullary nail: A case report with histologic examination of the regenerate. Proc. British Orthopaedic Research Society Spring Meeting. 3-4 March, 1997, Sheffield.

17. G Li, AHRW Simpson, JT Triffitt. Cell programmed to die (apoptosis) and cell proliferation during distraction osteogenesis. Proc. The 7th Conference of European Orthopaedic Research Society. 22-24 April, 1997, Barcelona, Spain.

18. G Li, AHRW Simpson and JT Triffitt. Angiogenesis during distraction osteogenesis is affected by distraction rate. Proc. Bone and Tooth Society Autumn Meeting. September, 1997, Edinburgh, Scotland, UK.

19. G Li, E Palsgard, GW Grime and JT Triffitt. Elemental analysis of rapidly regenerating bone tissues by scanning proton microprobe. Proc. Bone and Tooth Society Autumn Meeting. September 1997, Edinburgh, Scotland, UK.

Appendix 3. Slide coating using APES

1. Degraese slides in 1% acid alcohol, wash in distilled water and allow to dry.
2. Place slides in slide rack and immerse in 2% Decon (or other detergent) for 30 min.
3. Rinse in distilled water thoroughly.
4. Immerse in acetone for 5 min.
5. Immerse in 2 % 3-APES (3-aminopropyltriethoxy silane, Sigma A3648) in acetone for 5 min.
6. Rinse briefly in distilled water, air dry and store the slides in clean boxes.

Appendix 4. Carnoy's fluid

100% Alcohol	60 mls
Chloroform	30 mls
Glacial acetic Acid	10 mls

Appendix 5. 14.5% (w/v) EDTA, pH 7.4.

For 100ml: 14.5g EDTA (disodium dihydrate)
 ~80ml distilled water

Adjust the pH using sodium hydroxide pellets (usually around 20 pellets). This solution is difficult to prepare as the EDTA requires alkaline conditions to dissolve and, as it does, the pH falls again. It is sometimes aided by heating and stirring.

Appendix 6. Weigerts haematoxylin /Alcian blue / sirius red stain

1. Dewax and hydrate - histoclear 15 -20 minutes each. Alcohols (2 min each) to water.
2. Stain the sections with Weigerts haematoxylin (mix solutions A and B in equal proportions immediately before staining) for 10 mins.
3. Blue in running water 10 mins minimum (can be longer).
4. Differentiate in acid alcohol (20 ml HCl in 2l methanol) 3-5 dips.
5. Blue in running water for 5 mins minimum.
6. Stain in alcian blue for 10 mins.
7. Rinse briefly in running water (1 min max).
8. Stain in molybdophosphoric acid 20 mins (fresh each day).
9. Rinse briefly in water (1 min max).
10. Stain in sirius red / picric acid for 60 min.
11. Rinse briefly in water (1 min max).
12. Dehydrate and mount in DPX.

SOLUTIONS:

Alcian blue 8GX

1.5g Alcian blue 8GX in 300 ml of water + 3 ml acetic acid.

Molybdophosphoric acid

3g molybdophosphoric acid in 300 ml of water.

Sirius red F3B

0.3g of sirius red F3B in 300 ml of saturated picric acid.

Haematoxylin Weigerts A

1g haematoxylin
100 ml methanol
leave for 4 weeks to “ripen”

Haematoxylin Weigerts B

6g ferric chloride
500 ml of water
5 ml conc HCl

Appendix 7. Tartrate-resistant acid phosphatase (TRAP) staining

- 1) Dewax paraffin sectionsdewax in xylene and take through graded IMS.
- 2) Incubate sections in Tris-HCL buffer 1 hour.
- 3) Incubate sections in acetate buffer for 1 hour.
- 4) Make 15 mg Naphthol AS-B1 phosphate in 1ml dimethylformamide.(Alternatively, use 1 ml of Sigma acid-phosphatase kit AS-B1 solution, containing 12.5 mg/ml AS-B1 in dimethylformamide.)

- 5) Dissolve sodium nitrite in 5 ml distilled water (fresh each day).
- 6) Make up hexozotized pararosaniline : 0.25 ml of stock pararosaniline, add 0.25 ml of sodium nitrite and mix.
- 6) Make acetate buffer 5 ml of solution B to 6.9 ml of solution A, then add 14 ml water.
- 7) Add AS-B1 solution to (25.9 ml) above acetate buffer, then add 0.1 ml hexozotited pararosaniline and filter the substrate mixture.
- 8) Make acetate buffer as step 6, dissolve 75 mg sodium tartrate in 10 ml acetate buffer.
- 9) Put slides on rack, incubate in sodium tartrate buffer for 30 min.
- 10) Drain off the sodium tartrate solution and then add filtered substrate mixture, incubate at 37 °C for 0.5 - 1 hour.
- 11) Rinse sections in running water for 5 min.
- 12) Counterstain with Gills haematoxylin 1.5 minutes or 0.2 % light green 1 minute.
- 13) Dehydrate through alcohols, histoclear or xylene, mount in DPX.

Tris-HCl buffer (pH 9.0):

Solution A: 12.1 g TRIS in 500 ml distilled water.

Solution B: 1.7 ml conc. HCl to 100 ml distilled water.

Then mix 75 ml A + 6.5 ml B to 300 ml water and check that pH is 9.0.

Acetate Buffer (pH 5.0):

Solution A: 16 ml acetic acid in 500 ml of distilled water.

Solution B: 13.6 g sodium acetate in 500 ml of distilled water.

Make acetate buffer: 25 ml solution B + 34.5 ml solution A, add 70 ml water.

Stock pararosaniline:

Pararosaniline: 0.5 g

Distilled water: 10 ml

Conc. HCl: 2.5 ml store in a dark bottle.

Appendix 8. BrdUrd solution for injection

Dissolve BrdUrd (Sigma Chemical Co., Poole, U.K.) in sterile silane (8 mg/ml, 40mg/kg for rabbit) at 37°C. The solution was prepared on the day of use and stored at 37°C until required.

Appendix 9. Tris buffered saline pH 7.4

720 ml distilled water

7.01g NaCl

80 ml stock Tris solution
(0.5 M, pH 7.6)

Tris stock solution:

Trishydroxymethyl aminomethane 61g

Distilled water 1 liter

Adjust pH to 7.6 by conc HCl

Appendix 10. Acrindine orange staining

1. Dewax sections and bring to water.
2. Incubation with glycine-HCl solution (0.2 M, pH 2.0) for 30 mins.
3. Acrindine orange solution for 10 mins.
4. Wash in glycine-HCl (0.2 M, pH 2.0) solution for 15 mins.
5. Mount in water soluble non-fluorescent mountant (e. g. Citifluor).
6. View under blue (470 nm) excitation fluorescence.

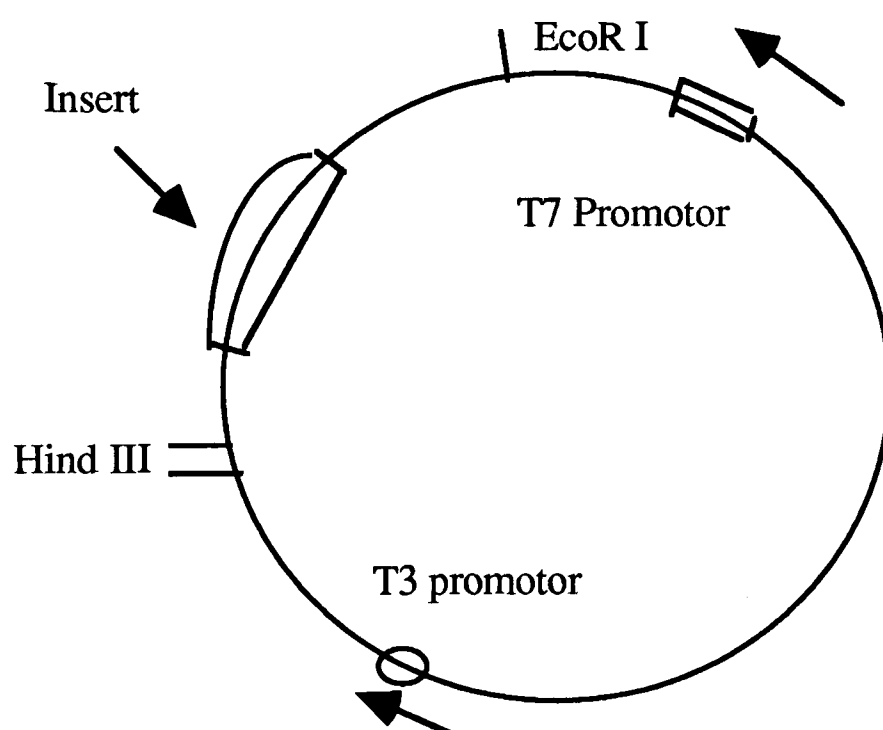
DNA fluoresces yellow/green; RNA fluoresces orange/red.

Acrindine orange solution

Dissolve acridine orange 1 mg in 2ml glycine-HCl (0.2 M, pH 2.0) solution.

Appendix 11. Digoxigenin-labelled riboprobe preparation

The plasmid DNA must first be linearized. To do this restriction enzymes are chosen which will cut downstream of the T7 and T3 promoters but leave the insert intact within the cloning site, the insert must not contain the restriction sites.



A. For labelling purposes approximately 1 μ g of linearised DNA template (plasmid with insert) is required in a final volume of 2 μ l. However, more should be linearised to allow for losses during processing. For digest 10 μ g plasmid from 4° C stocks, use 1 μ l 10 X restriction enzyme buffer; 1 μ l restriction enzyme, digest for 2 hrs.

B. Run an aliquot of digest on a 1% agarose gel to check that plasmid has linearised.

C. Before proceeding to label, it is essential to remove the restriction enzyme by performing a phenol/chloroform extraction as follows:-

1. Make volume up to 100 μ l to minimise losses during extraction.
2. Add equal volume of phenol chloroform ie 100 μ l into mixture.
3. Spin in microfuge 2 mins at top speed.
4. Remove 80 μ l of top layer to clean eppendorf. Add 80 μ l TE buffer (pH 8.0) to make volume to 100 μ l again.
5. Repeat phenol/chloroform extraction (steps 2-4).
6. Add 100 μ l chloroform in 4 ml isoamyl alcohol (24: 1).
7. Spin 2 mins at top speed. Remove 70 μ l from top layer to clean tube.
8. Add 10% the removal volume (7 μ l) sodium acetate, and EtOH 154 μ l.
9. Leave at - 20 °C for 1 hr or on dry ice for 15 mins to precipitate.
10. Spin at top speed in microfuge for 10 mins.
11. Remove the supernatant and discard.
12. Wash pellet twice (do not disturb pellet) with 70% EtOH.
13. Air dry pellet before redissolving it in 10 μ l of TE buffer.
14. Run 1 μ l sample on a 1% agarose gel.

D. Digoxigenin labelling:-

1. Have ready prepared labelled microfuge tubes on ice.
2. Add to tubes the following solutions in order (if the template is added before the buffer is diluted, it would precipitate the DNA).

10 X dig rNTPs (10 mM)	2 μ l
10 X transcription buffer	2 μ l
DEPC water	11 μ l
Linearised template	2 μ l
RNA guard	1 μ l
RNA polymerase (T3 or T7)	2 μ l (40 units)

3. Incubate at 37 °C for 2 hrs.
4. Stop the reaction with 0.2 M EDTA (DEPC, pH 8.0) and precipitate as follows:

To reaction mixture of 20 μ l add:

0.2 M EDTA	2 μ l
2.5 M Na acetate pH 7.5	2 μ l
EtOH at - 20 °C	80 μ l

5. Cool at -70 °C for 1 hr.
6. Spin 15 mins at top speed in microfuge. Remove supernatant and discard.
7. Wash pellet twice (do not disturb pellet) with ice cold EtOH. Spin between washes for 2 mins.
8. Allow pellet to air dry before redissolving it in 100 μ l of TE buffer (pH 9.0).

9. To check success of labelling, use 1 µl of the above solution and 1 µl of RNA guard to run a northern gel.

Appendix 12. Northern gel for determining digoxigenin labelling of riboprobes

1. Preparation of samples. For each probe use:

6 M glyoxal	2.7 µl
DMSO	8.0 µl
0.1 M NaH ₂ PO ₄ (pH 7)	1.6 µl
RNA (up to 20 mg)	1 µl
Ladder sample	3-5 µl

2. Denature sample at 50 °C for 1 hr in tightly closed tubes

3. Prepare gel in medium size slab gel apparatus (Biorad)

1.4 g Agarose in 130 ml 0.01 M NaH₂PO₄ (pH 7)

Allow approx 30 mins to set ready

4. Samples cooled to RT. Add 4 µl sterile loading buffer:

50% glycerol

0.01 M NaH₂PO₄ (pH 7)

0.4 % bromophenol blue

5. Load samples on gel. Run gel at 85 V or 3-4 V/cm (distance between electrodes).

Recirculate the running buffer (0.01 M NaH₂PO₄ , pH 7) using peristaltic pump. If buffer is not recirculated pH may rise and glyoxal dissociates from RNA at ≥ pH 8.0.

6. Cut ladder track from gel at run completion. Stain with ethidium bromide at 5 µg / ml.

7. Blot rest of gel onto nitrocellulose (NC) or nylon (Hybond-N) membrane using 20 X SSC (3 M NaCl, 0.3 M trisodium citrate) as follows:

7. 1. Pre-wet NC first with water then 20 X SSC.

7. 2. Construct blotting apparatus as shown in Figure 10. 1.

7. 3. Blotting overnight at RT. Remove membrane from gel, and allow air dry.

Detection of digoxigenin labelling

1. Wash membrane 1.5 hrs in AP buffer (pH 7.5) with 0.5% Tween 20.

2. Incubate in sealed bag with anti-digoxigenin-APAAP (1/5000) in AP (pH 7.5) buffer with 0.05% Tween 20 (use 5 mls) for 30 mins at RT.

3. Wash 3 times, 5 mins each in AP (pH 7.5) buffer with 0.05% Tween 20.

4. Wash once, 5 mins in AP (pH 9.5) buffer with 0.05% Tween 20.

5. Use 5 mls NBT/BCIP substrate solution in sealed bag, the develop of colour takes about 5 mins, but need to watch.

6. Stop reaction by washing with tap water and check.

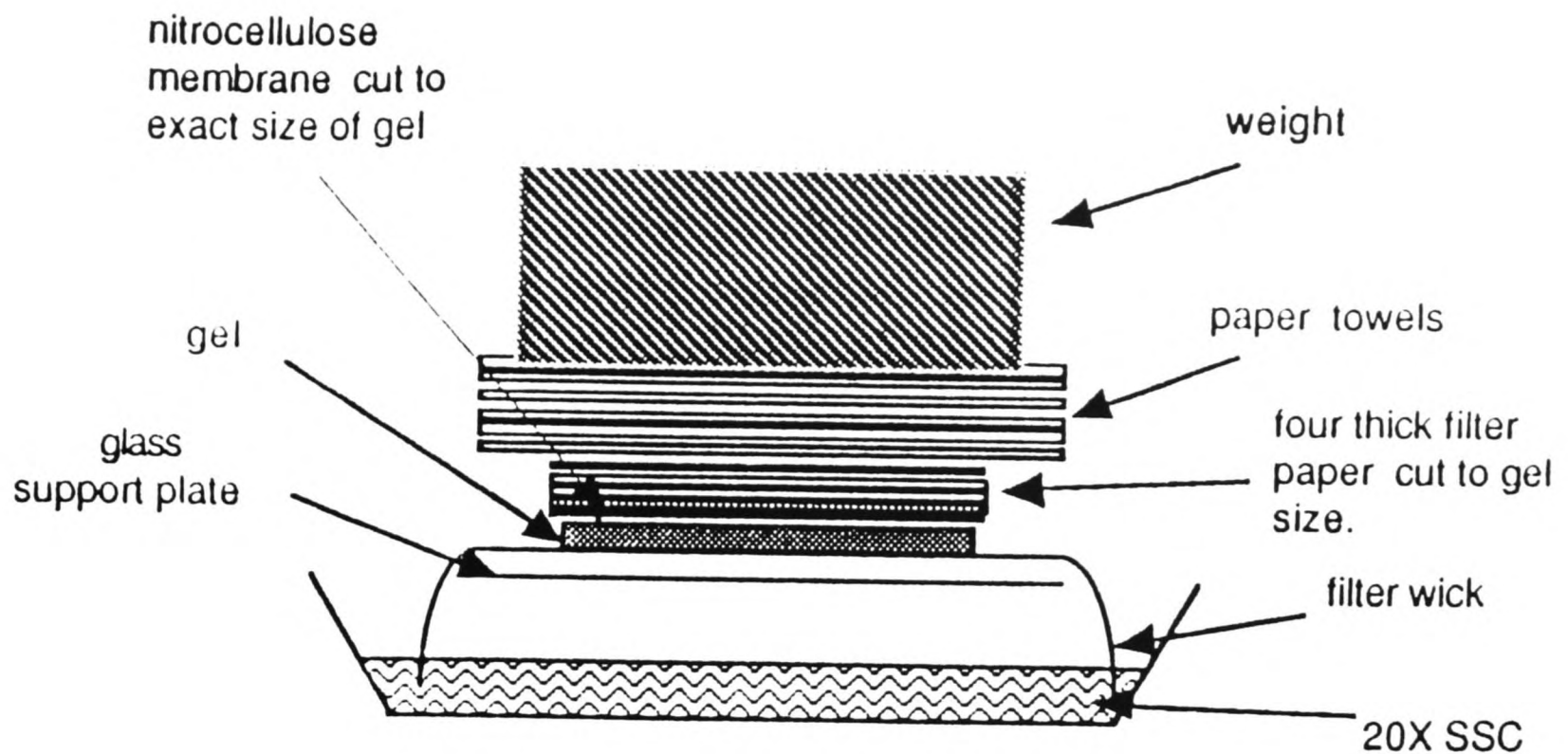


Fig. 10. 1 Diagram of the blotting apparatus.

Stock solutions:

AP buffer (pH 7.5) 10 X

1 M Tris pH 7.5

1M NaCl

30mM MgCl₂

(Dissolve Tris and NaCl, pH, and then add MgCl₂)

AP buffer (pH 9.5) 1 X

0.1 M Tris pH 9.5

0.1 M NaCl

100 mM MgCl₂

NBT/BCIP substrate solution

1. 10mg NBT dissolved in 200 μ l dimethylformamide.
2. Add to 1 ml AP (pH 9.5) buffer and mix.
3. Then add to 29 mls AP (pH9.5) buffer at 37 °C in a dropwise manner.
4. Add BCIP (5mg dissolved in 200 μ l dimethylformamide) slowly to the 30 mls NBT solution.
5. Store in 5 mls aliquots at -20 °C, and warm up to RT before use.

Appendix 13. Northern blotting procedure

This method allows RNA to be separated on a gel under electrophoresis and then transferred to a nylon membrane where it is irreversibly bound. The membrane can then be examined using

radioactively labelled probes which bind to specific message sequences. This enables relative levels of mRNA expression to be determined.

Buffers:

Denaturation Buffer: 60ml DEPC- treated water
10ml 0.2M Na₂PO₄, pH 6.5
100ml DMSO (Dimethyl Sulphoxide)
30ml Deionised Glyoxyl

Loading Buffer: 50% glycerol ("MB" grade)
10mM Na₂PO₄, pH 6.5
0.25% Bromophenol Blue
0.25% Xylene cyanol.

(prepare using DEPC treated water, filter sterilised & store in small aliquots -20°C)

Staining solution: 250ml 10M Sodium Hydroxide (fresh stock)
5ml Ethidium Bromide solution (10mg/ml)
49.745ml Milli-Quf water.

Destaining solution: 0.2M Na₂PO₄, pH 6.5

Alkaline transfer: 999ml Milli-Quf water
1ml 10M sodium Hydroxide.

Brief Protocols:

1. For a total RNA sample 10 - 20 mg should be used. For mRNA the message should be extracted from 22mg of total RNA using dynal beads. An RNA ladder (0.24kb) should be used as a standard and may be used together with the 1Kb DNA ladder. A sample of the total RNA being run on the gel should be chosen to allow location of the ribosomal components (5mg should be sufficient but less can be used for a cleaner sample).
2. Add 1ml of denaturation buffer per mg of RNA in each sample to a maximum volume of ~15ml. Vortex the samples and spin in a microfuge, then place in a water bath at 50°C for 30 min.
3. Prepare gel: Weigh the following into a sterile 200ml flask: 2.0g Sea-Kem Agarose gel, 10ml 0.2M Na₂PO₄, pH 6.5 ,190ml DEPC treated water. Heat in a microwave until the gel is fully dissolved. Place the flask into a water bath at 50°C to equilibrate.

4. After 30 min of sample incubation, re-spin the samples and place back into the water bath for a further 30 min. After this period vortex and spin the samples once more and place on ice.

5. Seal the ends of the gel casting tray with a length of clean autoclave tape. Place the tray on clean cling film on the levelling board. Pour gel gently into tray, taking care to avoid the creation of bubbles in the gel. Any bubbles can be removed immediately (before the gel starts to solidify) using a pipette tip. Remove the comb and autoclave tape.

6. Prepare electrophoresis buffer: To the 1900ml of water in the fridge add 100ml sterile 0.2M Na₂PO₄, pH 6.5. Keep in fridge until just before the samples are ready. Carefully place the gel into position on the platform and add sufficient buffer to just cover the wells

7. Add 0.25 Vol. of loading buffer to the denatured samples (ie 2.5ml per 10ml sample.) Mix thoroughly and centrifuge for 1 min at maximum speed in the microfuge.

8. Carefully load the samples. Be sure to make note of orientation of loading as this will be required later. The samples to be blotted and the standards for staining should be separated by at least one empty well, the more you are able to leave the better as the gel will be sliced later. If there are duplicate samples then try to separate these into two groups divided by one well if possible. The samples should then have been loaded similarly to:

1Kb	total	0.24	Blank	Samples	Blank	Samples
	RNA					

- l..Section to be Stained..ll.....

Section to be blotted.....l
9. Begin electrophoresis at 150V. Electrophorese until both tracking dye fronts have entered the gel (~10 - 15 minutes). After this period, reset the power pack to 150V and switch on the pump to recirculate the buffer.

 10. Check the pH on either side of the gel after 90 min to ensure the pump is circulating the buffer sufficiently.

 11. Prepare papers: Wearing gloves and using a sterile scalpel blade, cut the Pall Biodyne B Nylon membrane and 5 sheets of Whatman 3MM paper to the size of the gel section to be blotted (15cm x width of the gel to be blotted). Cut one corner of the membrane diagonally to be aligned with a diagonal on the gel. With a soft-leaded pencil, label the membrane at the top with which-ever coding system you desire. Prepare a 3MM wick 30 - 35 cm long x 4cm width of the gel to be blotted and four strips of parafilm. Cut 12 sheets of Sigma Quick-dry blotting

paper to the same size as the membrane and three sheets of the 3MM paper cut earlier. Prepare several sheets of blue roll, or equivalent, cut to the same size as the membrane.

12. Continue electrophoresis for 2.5 - 3 hours or until the bromophenol blue dye front is 10 - 12cm from the origin.

13. Place a 20 x 20 cm glass plate on an inverted supporting dish inside the plastic tuppaware box. Place the 3MM wick on it with the ends hanging down approximately 1cm from the bottom of the dish. Add 400 - 500 ml of alkaline transfer solution and allow wick to moisten. (see diagram Fig. 10. 2)

14. When the electrophoresis is almost complete pre-soak membranes etc: soak the pre-cut membrane in sterile DEPC-treated water for 10 - 15 min. After 5 min, place two sheets of the 3MM paper in the alkaline transfer solution (to soak for 5 - 10 min).

15. When electrophoresis is complete, remove the gel tray from the apparatus immediately after the power is switched off and place square on a sheet of graph paper covered with cling-film.

16. Cut gel: Using the cutter supplied for the gel, divide the gel into the portion to be stained and the portion to be transferred using the graph paper as a guide. Slide the gel section along the tray and use the edge of the tray as a guide, using a scalpel, cut 1 cm behind the well, then slide the gel to the other end and cut the excess to give a total length of 15 cm.

17. Cut one corner of the section containing the samples diagonally, and make sure make note of the position of this cut with respect to loading. Using clean gloves and a piece of moistened X-ray film for support if required, transfer the section to be blotted into a dish containing 10 mM NaOH and leave to equilibrate for 10 min.

18. Transfer the section to be stained to a small dish (the lid of a yellow tip box is suitable). Place on the orbital shaker in the fume hood and add sufficient staining solution to cover the gel, leave to stain for 25 - 30 min.

19. Assemble the blotting apparatus: Place two sheets of 3MM, previously soaked in NaOH, onto the centre of the wick. Place strips of parafilm along each edge to ensure the solution blots through the gel and not around it. Lift the gel onto the apparatus and place top side facing down. Place the nylon membrane on top of the gel, align the diagonal on the gel with that of the membrane. The gel and membrane should be lined as squarely as possible. Place the remaining three sheets of 3MM paper onto the membrane and then the twelve sheets of Sigma quick draw paper followed by the sheets of blue roll, the glass plate, some sheets of cling film, overlapping the edges of the container, then the lid of the container and lastly the

lead weight. See diagram.

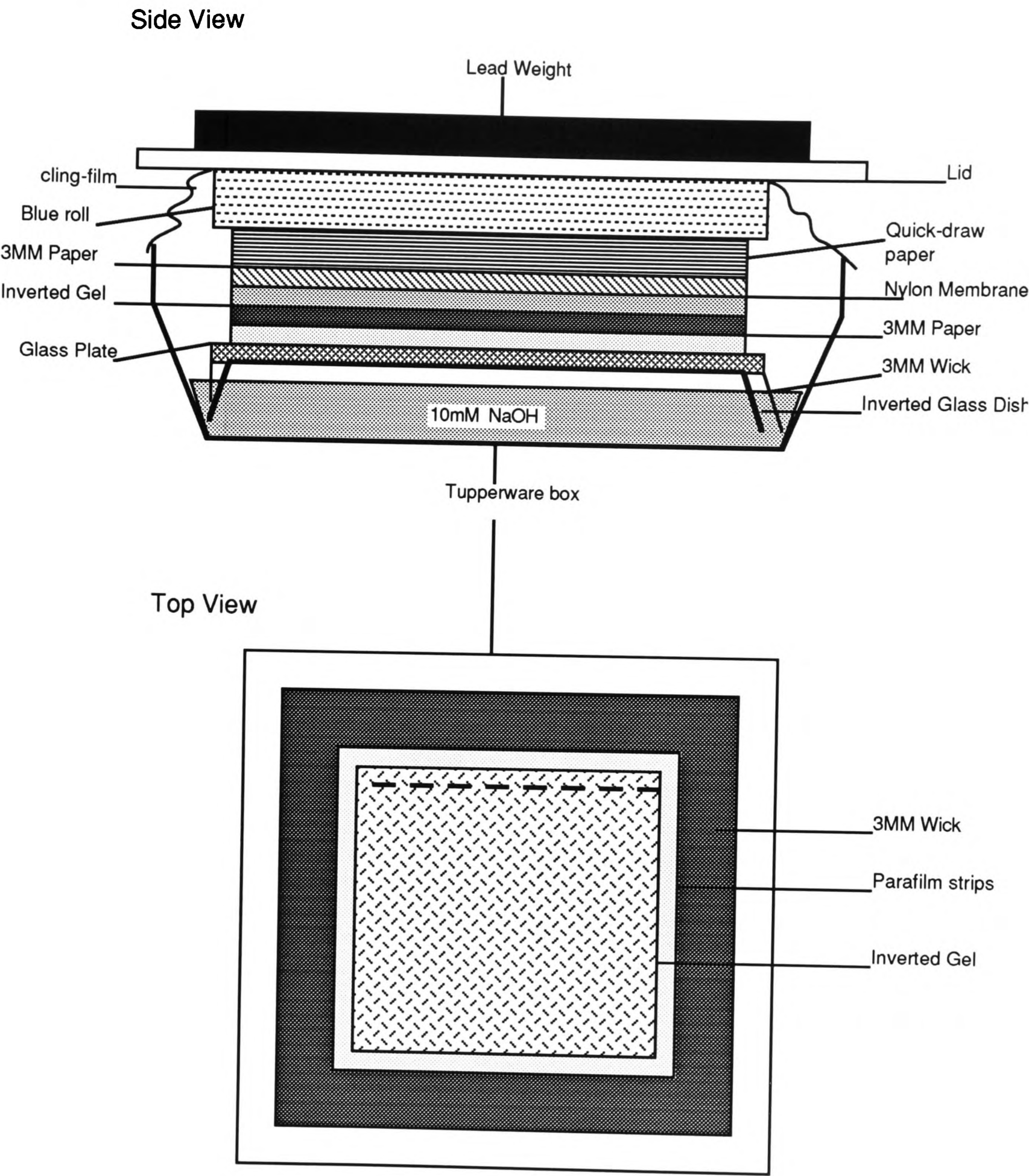


Fig. 10. 2. Diagram of Northern blotting procedure.

20. Once the staining has been on for 25 - 30 min: Destain the section in 0.2M Na_2PO_4 , pH 6.5, for 15 - 20 min. Place on the UV transilluminator, align the fluorescent ruler as 0 cm is in line with the wells and take a photograph.
21. Leave the blot set up overnight.

22. Pre-heat the oven to 80°C and prepare 200ml of 2x SSC (Saline Sodium Citrate).
23. Disassembling the blot: Remove the upper layers of the blot to leave one sheet of 3MM paper on top of the membrane. Lift the remaining sandwich of 3MM paper, membrane, gel and 3MM paper and place upside down onto a fresh piece of cling film. Carefully remove the 3MM paper to expose the gel.
24. Gently remove the membrane and rinse in 100ml 2x SSC for 1 min. Lightly blot the membrane on 3MM paper and then place between two fresh sheets of 3MM paper.
25. Bake the membrane at 80°C for 30 - 45 min.
26. After baking, remove the 3MM paper and rinse the membrane once more in 100ml 2x SSC for 2 min, lightly blot dry and seal in a plastic hybridisation bag with a small quantity of DEPC treated water to keep the blot moist. Store at -20°C for use.

Appendix 14. Procedure for in-situ hybridization on paraffin section

1. Take slides to water.

Dewax :

Xylene I	5 min
Xylene II	5 min

Rehydrate :

100% alcohol (IMS)	2 min
90% alcohol	2 min
70% alcohol	2 min
50% alcohol	2 min

DEPC water

Circles drawn by PAP-pen around each section.

2. Unmask of protein, RNA exposure :

0.2 M HCL	20 min
PBS wash	2 X 5 min

3. Clearing RNA-se from the sections :

6% H2O2 in PBS	30 min
PBS	2 X 5 min

4. RNA exposure :

Proteinase K	10 min at 37°C
PBS	2 X 5 min

5. Tissue fixation :

4% Paraformaldehyde	20 min (freshly prepared and chilled)
---------------------	---

- | | |
|---------|------------|
| PBS | 5 min |
| Glycine | 2 x 10 min |
| PBS | 2 X 5 min |
6. Triethanolamine (TEA) wash briefly
 7. Prevention of non-specific binding of probe to positively charged amine-groups :

Acetic anhydride in TEA	10 min
PBS	2 X 5 min
 8. Dehydrate through graded ethanols 50%-75%-100% with DEPC water
 9. Hybridization:

Place 30-50 µl of hybridisation solution containing the probes on each section

Cover with baked coverslips

Hybridise overnight at 55-56 °C
 10. Remove coverslip by immersion of slides in 2x SSC.
 11. RNase buffer 10 min at RT.
 12. RNase 20 min at 37 °C.
 13. RNase buffer 30 min at 37 °C
 14. Stringency washes :

1 x SSC	2 x 10 min at 55°C
0.5 x SSC	2 x 10 min at 55°C
0.1 x SSC	2 x 10 min at 55°C
0.1 x SSC	5 min at RT
 15. Buffer 1 5 min

Buffer 2	45 min
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 16. Anti-digoxigenin antibody 60 min at RT.
 17. Buffer 1A, 3 x 10 min.
 18. Buffer 3, x 5 min.
 19. Alkaline phosphatase substrate 1-3 hours protected from light (in dark) at RT.
 20. Stop the reaction with a drop of TE buffer, pH 8.0.
 21. Dehydrate through graded alcohols (IMS) 70%-90%-100% for 2-5 min each step.
 22. Mount in non-fluorescent mountant (23%w/v DABCO in 9:1 mixture of glycerol + PBS)

Appendix 15. Buffers and solutions for in-situ hybridization

Sterilisation Procedure:

Water: Add 1 ml of DEPC (diethylpyrocarbonate) to 9 ml absolute ethanol. Mix well. Make up to 1 liter with distilled water. Leave to 2 to 18 hours. Autoclave to deactivate DEPC.

Glassware: All glassware is baked at 200 °C for 2 to 18 hours. Items that can not be heat-treated should be soaked in 6% H₂O₂.

Solutions and buffers required for hybridisation:

1. Standard saline citrate (SSC), 20 X

For 1 litre:

175.3g Sodium Chloride

88.2g Sodium Citrate (or citric acid)

Dissolve in 800ml of DEPC treated water. Adjust pH to 7.0 using a few drops of 10N sodium hydroxide. Adjust volume to 1 litre. Sterilise by autoclaving.

2 X SSC = 1 part stock soln. + 9 parts water

1 X SSC = 1 part stock soln. + 19 parts water

2. Phosphate buffered saline (PBS) pH 7.3

15.6 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ or 13.79g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$

43.8 g NaCl

10 X stock solution

3. 0.5 M EDTA

For 100ml:

18.61g EDTA (disodium ethylene diamine tetraacetic acid)

80ml water (DEPC'd)

Adjust the pH to 8.0 using sodium hydroxide pellets (usually around 20 pellets).

It is sometimes aided by heating and stirring.

Make up to 100ml using DEPC-treated water.

4. 1 M Tris Buffer

121.1g Tris base

800 ml water

conc HCl as given below:

70 ml (pH 7.4)

60 ml (pH 7.6)

42 ml (pH 8.0)

Dissolve tris, adjust pH with HCl, make up to 1 liter with DEPC'd water.

5. Tris-EDTA (TE) buffer, pH 8.0 -- 10 X stock solution

3.03 g Tris base

0.093 g EDTA

Make up to 250 ml DEPC'd water, adjust to pH 8.0m with concn. HCl.

6. Proteinase K solution (pH 8.0)

Stock solution of proteinase K (10 mg/ml) in water

2 µl stock solution proteinase K

100 µl 10 X TE buffer, pH 8.0.

Make up to 1 ml with DEPC'd water.

7. Glycine in PBS (0.1 M)

750 mg glycine (7.5 mg/ml) in 100 ml PBS

8. Acetic anhydride (0.25%) in 0.1 M TEA

18.57 g (0.1M) triethanolamine HCl (TEA)

1 liter water

Take up to pH 8.0 with 5 M NaOH

0.25 ml acetic anhydride

100 ml TEA

Must be freshly prepared immediately before use

9. Dithiothreitol (DTT) (1 M)

3.09g DTT

Dissolve in 20 ml 0.01M Sodium Acetate (pH 5.2)

Sterilise by filtration, dispense into 1 ml aliquots and store -20°C.

DO NOT AUTOCLAVE SOLUTIONS CONTAINING DTT!!

10. Hybridization buffers

(a) High Stringency

1. 50% deionized formamide	100%	10 ml
2. 10 mM Tris-HCl, pH 7.4	0.1 M	2 ml
3. 1.0 mM EDTA	10 mM	
4. Dehardt's solution 1 X	50 X	400 µl
5. 0.5 % SDS	20 %	500 µl

6. 150 mM NaCl	5. 0 M	600 µl
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To give stock solution volume of 13.5 ml
Store in aliquots of 135 µl at - 20 °C (total salt conc. is 0.161 M, 161 mM).

(b) Medium Stringency

1. 50% deionized formamide	100%	10 ml
2. 10 mM Tris-HCl, pH 7.4	0.1 M	100 µl
3. 1.0 mM EDTA	10 mM	100 µl
4. Dehardt’s solution 1 X	50 X	400 µl
5. 0.5 % SDS	20 %	500 µl
6. 300 mM NaCl	5. 0 M	1. 2 ml

To give stock solution volume of 12.3 ml
Store in aliquots of 123 µl at - 20 °C (total salt conc. is 0.311 M, 311 mM).

(c) Low Stringency

1. 50% deionized formamide	100%	10 ml
2. 10 mM Tris-HCl, pH 7.4	0.1 M	200 µl
3. 1.0 mM EDTA	10 mM	100 µl
4. Dehardt’s solution 1 X	50 X	400 µl
5. 0.5 % SDS	20 %	500 µl
6. 300 mM NaCl	5. 0 M	2.4 ml

To give stock solution volume of 13.6 ml
Store in aliquots of 136 µl at - 20 °C (total salt conc. is 0.611 M, 611 mM).

Add the following to the aliquot of the chosen buffer:

7. 10% aq. detran sulphate	40%	50 µl
8. 0.5 mg/ml yest / E.coli tRNA	25 µg / µl	4 µl
9. labelled probe		5 µl
10. DEPC’d water to give total volume of		200 µl

50 x Denhardt’s solution:

For 250ml:

- 2.5g Ficoll
- 2.5g Polyvinylpyrrolidone
- 2.5g Bovine serum Albumin

Make upto 250ml using DEPC-treated water. Sterilise by filtration.

Aliquot and store at -20°C.

11. RNase solutions

- (a) Buffer
- | | |
|-----------------|---------|
| 0.5 M NaCl | 14.61 g |
| 10 mM Tris base | 0.606 g |
| 1.0 mM EDTA | 0.186 g |
- to 500 ml with water, adjust to pH 8.0 with concn. HCl.
- (b) RNase solution -- 20 µg / ml RNase A in above buffer
(Stock RNase A solution conc. is 2 mg/ml, use 2 µl stock solution in 1 ml buffer)

12. Solutions for digoxigenin-labelled probes detection

1. Buffer 1: 0.1 M Tris-HCL + 0.15 M NaCl, pH 7.5.
2. Buffer 1A: Buffer 1 plus 0.05 % Tween 20.
3. Buffer 2: 0.5% Boehringer's Blocking Reagent (BBR) in Buffer 1.
4. Buffer 3: 0.1 M Tris-HCl, 0.01 NaCl, 0.05 M MgCl₂, pH 9.5.
5. Anti-digoxigenin antibody solution: antibody 1 in 500 in buffer 2, plus 0.3 % Triton-X 100.
6. Substrate solution: 4.5 µl NBT and 3.5 µl X-phosphate are added to 1 ml buffer 3.

Appendix 16. Dual labelling of cell death and cell proliferation

1. Sections were first processed through the TUNEL staining as described in section 2. 5. 2.
2. After the TUNEL procedure, sections were then rinsed thoroughly in 0.5 M TBS and treated with 1N HCl at 60 °C for 15 mins.
3. Endogenous peroxidase activity was blocked by immersion of the slides in 80% (v/v) methanol containing 0.5% (v/v) hydrogen peroxide for 30 mins.
4. Following TBS washes, normal rabbit serum (10%) in phosphate buffered saline (PBS) containing 0.05% (v/w) Tween 20 (BDH, poole, UK) was added and incubated for 15 mins to block the non-specific binding of the secondary antibody.
5. Sections were then incubated with anti-BrdUrd antibody, Bu20A (Gift from Dr. K Gatter, Department of Pathology, John Radcliffe hospital, Oxford) at a dilution of 1: 5 for 1 hr, in a humid chamber at room temperature.
6. The sections were then incubated with rabbit anti-mouse peroxidase conjugated antibody

(Sigma, Poole, Dorset, UK) at a dilution of 1: 50 in PBS for 30 min.

7. Peroxidase activity was demonstrated with 0.5 mg/ml 3, 4, 3', 4'-tetrabiphenyl hydrochloride (DAB; Sigma, Poole, UK) in PBS (pH 7.4) containing 0.3% (v/v) hydrogen peroxide for approximately 5 min, and the Bu20A-positive cells stain brown.

8. The sections were rinsed well with distilled water, then mounted in Aquamount (BDH, Dorset, UK), and analysed under light microscopy.

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