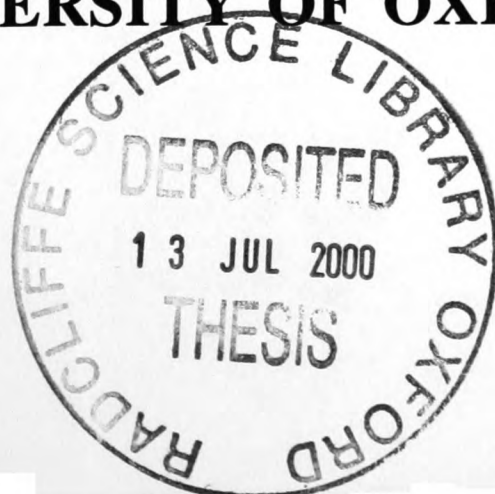


ISCHAEMIA / REPERFUSION INJURY IN RENAL TRANSPLANTATION

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UNIVERSITY OF OXFORD



DEDICATION

In memory of my father **Ngar Long Koo**, and to my mother
Kam Fung Koo, who have encouraged and inspired my
pursuit of knowledge

ISCHAEMIA/REPERFUSION INJURY IN RENAL TRANSPLANTATION

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ABSTRACT

Kidney transplants from both living-related (LRD) and living unrelated (LURD) donors have superior function and survival than transplants from cadaver donors. This may be unsurprising as kidneys from living donors are procured under optimal conditions, from healthy donors with minimal ischaemia times. In contrast, cadaver kidneys are obtained from traumatised donors and may experience extended periods of cold ischaemic storage before transplantation. An immunohistochemical analysis has been performed on biopsies obtained before, and immediately after transplantation, to investigate the potential causes of early inflammatory events associated with cadaver renal transplantation that may influence subsequent graft outcome.

An immunohistochemical analysis of biopsies obtained before transplantation demonstrated upregulated expression of endothelial E-selectin and proximal tubular expression of ICAM-1, VCAM-1 and HLA Class II antigens in cadaver donor kidneys. Analysis of donor parameters demonstrated that traumatic physiological events experienced in intensive care around the time of brain death were significantly associated with the induction of pro-inflammatory antigens. Antigen induction in cadaver donor kidneys before transplantation was significantly associated with early acute rejection. Furthermore, in cadaveric kidneys with long cold ischaemia times, glomerular neutrophil infiltration and deposition of activated platelets expressing P-selectin on intertubular capillaries were detected following reperfusion, in association with impaired short and long term graft function. Expression of inflammatory mediators were absent in all LRD renal allografts before and after reperfusion.

A clinical trial was performed to determine whether ischaemia/reperfusion injury may be ameliorated by reflusing cadaver kidneys after cold storage to remove harmful products that may have accumulated in the vessel lumen. Reflusing did not prevent the inflammatory events observed after reperfusion or improve graft function. Therefore, a novel, oxygen free radical scavenger (lec-SOD) was obtained to assess its potential efficacy in preventing ischaemia/reperfusion injury. Lec-SOD bound with high affinity to macro- and microvascular endothelial cells under cold hypoxic conditions following incorporation into Marshall's preservation solution, significantly inhibiting cold hypoxia induced cell death, adhesion molecule induction and neutrophil adhesion. Furthermore, preservation of kidneys with lec-SOD for 18 hr in an experimental model of chronic renal allograft rejection, significantly attenuated neutrophil infiltration and MHC Class I induction day 1 post-transplant, with improved long term renal function.

The results presented in this Thesis demonstrate that donor factors and cold ischaemia/reperfusion injury elicit an early inflammatory response that may influence graft outcome of cadaver kidneys. Refinements in donor management and organ preservation may limit the deleterious effects of ischaemia/reperfusion injury in cadaver renal allografts, increasing graft survival to that observed in living donor transplantation.

PUBLICATIONS AND PRESENTATIONS RESULTING FROM THIS THESIS

Publications

Roake JA, Fawcett J, **Koo DD**, Fuggle SV, Gray DW, Morris PJ: "Late Reflush in Clinical Renal Transplantation: Protection Against Delayed Graft Function Not Observed." *Transplantation* 62(1): 114-116, **1996**

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Koo DDH, Welsh KI, McLaren AJ, Roake JA, Morris PJ, Fuggle SV: "Cadaver Versus Living Donor Kidneys: Impact of Donor Factors on Antigen Induction Before Transplantation." *Kidney International* (in press) **1999**

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Koo DDH, Welsh KI, Roake JA, Morris PJ, Fuggle SV. "Immunological Differences Between Cadaver and Living Related Donor Renal Allografts." XVII World Congress of the Transplantation Society, Palais des Congres, Montreal, Canada, **1998**

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Koo DDH. “Evidence for Immunohistological Differences Between Cadaver and Living-Related Donor Kidneys.” **Invited Speaker**, Department of Nephrology and Transplantation, Guy’s Hospital, London, UK, **1999**

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Koo DDH, Welsh KI, Roake JA, Morris PJ, Fuggle SV. “A Novel Endothelial-Specific Free Radical Scavenger for Organ Preservation.” International Conference on Superoxide Dismutases: Recent Advances and Clinical Applications, Pasteur Institute, Paris, France, **1998**.

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ABBREVIATIONS

| | |
|-------------------------------|--|
| ATG | Anti-thymocyte globulin |
| ATN | Acute tubular necrosis |
| ATP | Adenosine triphosphate |
| BSA | Bovine serum albumin |
| °C | Celsius |
| CD | Cluster of Differentiation |
| CLA | Cypridina luciferin analog |
| CMV | Cytomegalovirus |
| CO ₂ | Carbon dioxide |
| CPM | Counts per minute |
| ⁵¹ Cr | Chromium |
| CuZn-SOD | Copper,Zinc-superoxide dismutase |
| DDAVP | 1-desamino-8-D-arginine vasopressin |
| DGF | Delayed graft function |
| DMEM | Dulbecco's Modified Eagle's Medium |
| EDTA | Ethylene diamine tetra acetic acid |
| ET | Endothelin |
| FCS | Foetal calf serum |
| FITC | Fluorescein isothiocyanate |
| g | Gravity |
| H ₂ O ₂ | Hydrogen peroxide |
| HBSS | Hank's balanced salt solution |
| HLA | Human Leucocyte Antigen |
| HMEC-1 | Human microvascular endothelial cell-1 |
| HUVEC | Human umbilical vein endothelial cell |
| ICAM | Intercellular adhesion molecule |
| ICU | Intensive care unit |

| | |
|------------------------------|--|
| IFN _γ | Interferon-gamma |
| IL | Interleukin |
| Ig | Immunoglobulin |
| kg | kilogram |
| lec-SOD | Lecithinised superoxide dismutase |
| LFA | Leucocyte function-associated antigen |
| LRD | Living-Related Donor |
| LURD | Living-Unrelated Donor |
| μg | microgram |
| mAb | monoclonal antibody |
| mg | milligram |
| MHC | Major histocompatibility complex |
| min | minutes |
| ml | millilitres |
| mM | millimolar |
| MnSOD | Manganese-superoxide dismutase |
| mRNA | messenger Ribonucleic Acid |
| N ₂ | Nitrogen |
| NHBD | Non-heart-beating donors |
| NO | Nitric oxide |
| ·O ₂ ⁻ | superoxide |
| OH· | hydroxyl |
| PAF | Platelet activating factor |
| PGI ₂ | Prostacyclin |
| PBS | Phosphate buffered saline |
| PMN | Polymorphonuclear |
| PSGL | P-selectin glycoprotein ligand |
| rhSOD | recombinant human superoxide dismutase |
| SD | Standard deviation |

| | |
|------------------|-----------------------------------|
| SEM | Standard error mean |
| secs | seconds |
| sLe ^x | sialyl-Lewis x |
| SOD | Superoxide dismutase |
| TNF | Tumour Necrosis Factor |
| U | Units |
| VCAM-1 | Vascular cell adhesion molecule-1 |
| VLA | Very Late Antigen |
| w/v | weight/volume |
| X/XO | Xanthine/Xanthine Oxidase |

TABLE OF CONTENTS

| | |
|---|-----------|
| CHAPTER 1. INTRODUCTION..... | 1 |
| 1.1 ORGAN TRANSPLANTATION: AN INTRODUCTION..... | 1 |
| 1.2 RENAL TRANSPLANT DONORS | 2 |
| <i>1.2.1 Heart-Beating Cadaver Donors.....</i> | <i>2</i> |
| <i>1.2.2 Non-Heart-Beating Cadaver Donors (NHBD).....</i> | <i>3</i> |
| <i>1.2.3 Living-Related Donors (LRD).....</i> | <i>4</i> |
| <i>1.2.4 Living-Unrelated Donors (LURD).....</i> | <i>5</i> |
| 1.3 LONG TERM GRAFT SURVIVAL IN LIVING AND CADAVERIC RENAL TRANSPLANTATION | 5 |
| 1.4 PHYSIOLOGICAL DIFFERENCES BETWEEN LIVING AND CADAVER DONOR RENAL TRANSPLANTATION | 7 |
| <i>1.4.1 Brain Death and ICU Treatment.....</i> | <i>7</i> |
| <i>1.4.2 Cold Ischaemic Storage.....</i> | <i>9</i> |
| 1.5 DELAYED GRAFT FUNCTION..... | 10 |
| <i>1.5.1 Effects of DGF on Graft Survival.....</i> | <i>10</i> |
| <i>1.5.2 Effects of DGF and Acute Rejection on Chronic Graft Loss.....</i> | <i>11</i> |
| <i>1.5.3 Potential Causative Factors of DGF.....</i> | <i>13</i> |
| 1.6 BIOLOGICAL EFFECTS OF ISCHAEMIA IN TRANSPLANTATION..... | 14 |
| <i>1.6.1 ATP Depletion.....</i> | <i>15</i> |
| <i>1.6.2 Reduction in Intracellular pH.....</i> | <i>15</i> |
| <i>1.6.3 Impaired Regulation of Ionic Environment.....</i> | <i>16</i> |

| | | |
|---------|--|----|
| 1.6.4 | <i>Conversion of Xanthine Dehydrogenase to Xanthine Oxidase</i> | 17 |
| 1.7 | ISCHAEMIA/REPERFUSION INJURY: THE CENTRAL ROLE OF ENDOTHELIAL CELLS | 18 |
| 1.7.1 | <i>Generation of Reactive Oxygen Species</i> | 18 |
| 1.7.2 | <i>Modulation of Vascular Tone</i> | 21 |
| 1.7.2.1 | Nitric Oxide | 22 |
| 1.7.2.2 | Endothelin..... | 23 |
| 1.7.3 | <i>No Reflow Phenomenon</i> | 24 |
| 1.8 | ADHESION MOLECULES INVOLVED IN ENDOTHELIAL- LEUCOCYTE INTERACTIONS | 25 |
| 1.8.1 | <i>Leucocyte Tethering and Rolling</i> | 25 |
| 1.8.1.1 | P-selectin and ligands..... | 26 |
| 1.8.1.2 | E-selectin and ligands..... | 27 |
| 1.8.1.3 | L-selectin and ligands..... | 28 |
| 1.8.1.4 | Leucocyte-leucocyte interactions..... | 29 |
| 1.8.2 | <i>Triggering</i> | 30 |
| 1.8.2.1 | CXC chemokine receptors..... | 31 |
| 1.8.2.2 | CC chemokine receptors..... | 31 |
| 1.8.3 | <i>Firm Adhesion</i> | 32 |
| 1.8.3.1 | Leucocyte integrins..... | 32 |
| 1.8.3.2 | Endothelial immunoglobulin superfamily (IgSF) structures..... | 33 |
| 1.8.4 | <i>Transendothelial Migration</i> | 34 |
| 1.9 | ADHESION MOLECULE EXPRESSION IN NORMAL AND TRANSPLANTED HUMAN KIDNEYS | 35 |
| 1.9.1 | <i>Normal Kidney</i> | 35 |
| 1.9.2 | <i>Transplanted Kidneys</i> | 36 |
| 1.10 | ISCHAEMIA/REPERFUSION INDUCED INFLAMMATORY RESPONSE | 37 |
| 1.10.1 | <i>Endothelial Adhesion Molecules and Neutrophil Infiltration</i> | 37 |
| 1.10.2 | <i>Platelet Interactions</i> | 40 |
| 1.10.3 | <i>Inflammatory Cytokines and Chemokines</i> | 41 |
| 1.11 | ISCHAEMIA/REPERFUSION INJURY IN CLINICAL RENAL TRANSPLANTATION | 43 |

| | |
|--|----|
| 1.12 THERAPEUTIC STRATEGIES FOR ISCHAEMIA/REPERFUSION INJURY | 44 |
| <i>1.12.1 Organ Preservation Solutions</i> | 44 |
| 1.12.1.1 Static cold storage..... | 44 |
| 1.12.1.2 Machine perfusion preservation..... | 46 |
| <i>1.12.2 Post-Storage Reflush</i> | 46 |
| <i>1.12.3 Preconditioning</i> | 46 |
| <i>1.12.4 Inhibition of Endothelial-Leucocyte Adhesion</i> | 47 |
| <i>1.12.5 Antioxidant Therapy</i> | 49 |
| 1.12.5.1 Novel antioxidants..... | 49 |
| 1.12.5.2 Superoxide dismutase..... | 50 |
| 1.13 AIM OF THESIS | 51 |

CHAPTER 2. MATERIALS AND METHODS.....53

| | |
|---|----|
| 2.1 PATIENTS AND BIOPSY MATERIAL | 53 |
| 2.2 IMMUNOHISTOCHEMICAL STAINING | 53 |
| <i>2.2.1 Immunoperoxidase Staining</i> | 54 |
| <i>2.2.2 Double-Immunofluorescent Staining</i> | 54 |
| 2.3 PREPARATION AND MAINTENANCE OF HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVEC). | 55 |
| <i>2.3.1 Isolation of Endothelium from Umbilical Vein</i> | 55 |
| <i>2.3.2 Maintenance and Passaging of HUVEC</i> | 56 |
| 2.4 CULTURE AND MAINTENANCE OF A HUMAN MICROVASCULAR ENDOTHELIAL CELL LINE, HMEC-1..... | 57 |
| <i>2.4.1 Source and Culture Conditions of HMEC-1</i> | 57 |
| <i>2.4.2 Maintenance and Passaging of HMEC-1</i> | 57 |
| 2.5 PHENOTYPIC CHARACTERISATION OF ENDOTHELIAL CELLS..... | 58 |
| <i>2.5.1 Cytokine Stimulation</i> | 58 |

| | |
|---|----|
| 2.5.2 Preparation of Cells for FACS Analysis | 58 |
| 2.6 SOURCE OF LECITHINISED-SOD (LEC-SOD), RECOMBINANT HUMAN CUZn-SOD (RHSOD) AND LECITHIN..... | 59 |
| 2.7 CHEMILUMINESCENT ANALYSIS OF RHSOD AND LEC-SOD ACTIVITY..... | 59 |
| 2.8 INCORPORATION OF LEC-SOD INTO MARSHALL'S PRESERVATION SOLUTION | 60 |
| 2.9 BINDING OF LEC-SOD TO ENDOTHELIAL CELLS..... | 60 |
| 2.9.1 Culture on Glass Coverslips..... | 60 |
| 2.9.2 Confocal Laser Microscopy Analysis of lec-SOD Binding | 61 |
| 2.9.3 Cell Permeabilisation Assay for FACS Analysis..... | 61 |
| 2.10 <i>IN VITRO</i> HYPOXIA/REOXYGENATION MODEL..... | 62 |
| 2.11 CELL VIABILITY DETERMINED BY TRYPAN BLUE EXCLUSION STAINING..... | 63 |
| 2.12 NEUTROPHIL-ENDOTHELIAL CELL ADHESION ASSAY..... | 64 |
| 2.12.1 Purification of Neutrophils | 64 |
| 2.12.2 Hypoxia/Reoxygenation of Endothelial Cells | 65 |
| 2.12.3 Neutrophil-Endothelial Cell Adhesion (NECA) Assay..... | 65 |
| 2.13 PHENOTYPIC STUDY OF ENDOTHELIAL CELLS FOLLOWING HYPOXIA/ REOXYGENATION..... | 66 |
| 2.14 EXPERIMENTAL ALLOGENEIC RENAL TRANSPLANTATION | 67 |
| 2.14.1 Renal Transplant Procedure..... | 67 |
| 2.14.2 Renal Function..... | 68 |
| 2.14.3 Immunohistology | 69 |

| | |
|---|-----------|
| CHAPTER 3. CADAVER VERSUS LIVING RELATED DONOR KIDNEYS: IMPACT OF DONOR FACTORS ON ADHESION MOLECULE AND HLA -DR ANTIGEN EXPRESSION..... | 70 |
| 3.1 INTRODUCTION..... | 70 |
| 3.2 MATERIALS AND METHODS..... | 72 |

| | |
|---|----|
| 3.2.1 Patients and Biopsy Material..... | 72 |
| 3.2.2 Monoclonal Antibodies and Immunohistochemistry | 72 |
| 3.2.3 Assessment of Staining | 73 |
| 3.2.4 Statistical Analyses..... | 73 |
| 3.3 IMMUNOHISTOLOGICAL DIFFERENCES BETWEEN CADAVER AND LIVING-RELATED DONOR KIDNEYS | 74 |
| 3.3.1 Leucocyte Markers..... | 74 |
| 3.3.2 Endothelial Adhesion Molecules..... | 74 |
| 3.3.3 Proximal Tubular Expression of ICAM-1, VCAM-1 and HLA-DR Antigens | 74 |
| 3.3.4 Organ Retrieval Biopsies..... | 75 |
| 3.4 ASSOCIATIONS OF ADHESION MOLECULE AND HLA-DR EXPRESSION WITH CLINICAL PARAMETERS | 76 |
| 3.4.1 Donor Parameters | 76 |
| 3.4.2 Clinical Events in ICU..... | 76 |
| 3.4.3 Drug Administration | 77 |
| 3.4.4 Donor Renal Function..... | 77 |
| 3.5 POST-TRANSPLANTATION EVENTS..... | 77 |
| 3.6 DISCUSSION | 78 |

| | |
|--|-----------|
| CHAPTER 4: ISCHAEMIA/REPERFUSION INJURY IN HUMAN RENAL TRANSPLANTATION..... | 85 |
| 4.1 INTRODUCTION..... | 85 |
| 4.2 MATERIALS AND METHODS..... | 88 |
| 4.2.1 Patients and Biopsy Material..... | 88 |
| 4.2.2 Monoclonal Antibodies and Immunohistochemistry | 88 |
| 4.2.3 Double-Immunofluorescent Staining | 89 |
| 4.2.4 Assessment of Staining | 90 |

| | |
|---|----|
| 4.2.5 <i>Statistical Analyses</i> | 90 |
| 4.3 IMMUNOHISTOCHEMICAL CHANGES FOLLOWING REPERFUSION..... | 90 |
| 4.3.1 <i>Leucocyte Subpopulations</i> | 91 |
| 4.3.2 <i>Adhesion Molecule Expression</i> | 91 |
| 4.4 RELATIONSHIP BETWEEN P-SELECTIN EXPRESSION AND NEUTROPHIL INFILTRATION | 92 |
| 4.5 CHARACTERISATION OF P-SELECTIN EXPRESSION..... | 93 |
| 4.6 ORIGIN OF INFILTRATING CELLS..... | 93 |
| 4.7 CLINICAL SIGNIFICANCE OF IMMUNOHISTOLOGICAL CHANGES DETECTED AFTER REPERFUSION IN CADAVER RENAL ALLOGRAFTS | 94 |
| 4.8 DISCUSSION..... | 95 |

| | |
|--|------------|
| CHAPTER 5. EFFECTS OF LATE REFLUSH ON ISCHAEMIA/ REPERFUSION INJURY IN CLINICAL RENAL TRANSPLANTATION | 102 |
| 5.1 INTRODUCTION..... | 102 |
| 5.2 MATERIALS AND METHODS..... | 104 |
| 5.2.1 <i>Patients and Biopsy Material</i> | 104 |
| 5.2.2 <i>Late Reflush Protocol</i> | 105 |
| 5.2.3 <i>Immunohistochemical Analysis</i> | 106 |
| 5.2.4 <i>Assessment of Staining</i> | 106 |
| 5.3 RESULTS..... | 107 |
| 5.3.1 <i>Clinical Parameters</i> | 107 |
| 5.3.2 <i>Early Graft Function</i> | 107 |
| 5.3.3 <i>Acute Rejection Episodes</i> | 107 |
| 5.3.4 <i>Histological and Immunohistochemical Changes Post-Reperfusion</i> | 108 |
| 5.4 DISCUSSION..... | 109 |

| | |
|---|------------|
| CHAPTER 6. EFFECTS OF LECITHINISED SUPEROXIDE DISMUTASE (LEC-SOD) ON ENDOTHELIUM IN AN IN VITRO MODEL OF ISCHAEMIA/ REPERFUSION INJURY | 114 |
| 6.1 INTRODUCTION..... | 114 |
| 6.2 PHENOTYPIC CHARACTERISATION OF MACROVASCULAR AND MICROVASCULAR ENDOTHELIAL CELLS | 116 |
| 6.2.1 <i>CD31 Expression</i> | 117 |
| 6.2.2 <i>HLA Class I Expression</i> | 117 |
| 6.2.3 <i>HLA Class II Expression</i> | 117 |
| 6.2.4 <i>E-selectin Expression</i> | 118 |
| 6.2.5 <i>ICAM-1 Expression</i> | 118 |
| 6.2.6 <i>VCAM-1 Expression</i> | 118 |
| 6.3 CHEMILUMINESCENT ANALYSIS OF RHO SOD AND LEC-SOD ACTIVITY..... | 119 |
| 6.4 FACS ANALYSIS OF LEC-SOD BINDING TO ENDOTHELIAL CELLS FOLLOWING INCORPORATION INTO MARSHALL'S ORGAN PRESERVATION SOLUTION | 120 |
| 6.5 CONFOCAL LASER MICROSCOPY ANALYSIS OF LEC-SOD BINDING TO ENDOTHELIUM | 122 |
| 6.6 EFFECT OF COLD HYPOXIA ON LEC-SOD BINDING TO ENDOTHELIUM..... | 123 |
| 6.6.1 <i>HMEC-1</i> | 123 |
| 6.6.1.1 Native extracellular SOD..... | 123 |
| 6.6.1.2 Total native SOD following permeabilisation | 124 |
| 6.6.1.3 Binding of lec-SOD to HMEC-1 | 124 |
| 6.6.2 <i>HUVEC</i> | 125 |
| 6.6.2.1 Native extracellular SOD..... | 125 |
| 6.6.2.2 Total native SOD following permeabilisation | 125 |
| 6.6.2.3 Binding of lec-SOD to HUVEC | 125 |
| 6.6.3 <i>Detection of lec-SOD Following Hypoxia and Prolonged Reoxygenation</i> | 125 |
| 6.7 EFFECT OF LEC-SOD ON ENDOTHELIAL CELL VIABILITY FOLLOWING COLD HYPOXIA AND REOXYGENATION | 127 |

| | |
|---|------------|
| 6.7.1 HMEC-1 Viability | 127 |
| 6.7.2 HUVEC Viability | 127 |
| 6.8 EFFECT OF LEC-SOD ON NEUTROPHIL-ENDOTHELIAL CELL ADHERENCE | |
| FOLLOWING HYPOXIA/REOXYGENATION | 128 |
| 6.8.1 <i>Time Course for NECA Assay</i> | 129 |
| 6.8.2 <i>Effect of lec-SOD on NECA Assay</i> | 129 |
| 6.8.3 <i>Effect of Lecithin on NECA Assay</i> | 130 |
| 6.8.4 <i>Comparison of Effects of lec-SOD, rhSOD and Lecithin on NECA Assay</i> | 131 |
| 6.9 FACS ANALYSIS OF ENDOTHELIAL CELL ADHESION MOLECULE EXPRESSION | |
| FOLLOWING HYPOXIA/REOXYGENATION | 131 |
| 6.9.1 <i>Hypoxia/Reoxygenation of HMEC-1</i> | 132 |
| 6.9.2 <i>Hypoxia/Reoxygenation of HUVEC</i> | 132 |
| 6.10 DISCUSSION | 132 |

CHAPTER 7. EFFECTS OF LECITHINISED SUPEROXIDE DISMUTASE ON ISCHAEMIA/REPERFUSION INJURY IN EXPERIMENTAL RENAL TRANSPLANTATION..... 144

| | |
|---|------------|
| 7.1 INTRODUCTION..... | 144 |
| 7.2 MATERIALS AND METHODS..... | 146 |
| 7.2.1 <i>Renal Allograft Model</i> | 146 |
| 7.2.2 <i>Monoclonal Antibodies and Immunohistochemistry</i> | 147 |
| 7.2.3 <i>Assessment of Staining</i> | 148 |
| 7.2.4 <i>Statistical Analyses</i> | 148 |
| 7.3 RESULTS..... | 149 |
| 7.3.1 <i>Renal Function</i> | 149 |
| 7.3.2 <i>Leucocyte Infiltration</i> | 149 |
| 7.3.3 <i>Antigen Induction</i> | 151 |
| 7.4 DISCUSSION | 151 |

CHAPTER 8. SUMMARY AND DISCUSSION..... 159

REFERENCES.....168

CHAPTER 1. INTRODUCTION

1.1 ORGAN TRANSPLANTATION: AN INTRODUCTION

The continuing success of transplantation has resulted in an increase in the number of suitable patients placed on the transplant waiting list, whilst the number of available donor organs has remained stable. The shortfall in conventional cadaver donor organs has renewed interest in transplantation of organs procured from non-heart-beating cadaver donors, living-related (LRD) and living-unrelated (LURD) donors to meet this demand. It is widely recognised that the outcome of organ transplantation from living-related donors is superior to those from cadaver donors, presumably due to the genetic relationship between donor and recipient. Nevertheless, mounting evidence from studies of LURD renal allografts has demonstrated graft survival to be identical to one haplotype-matched LRD allografts, and consistently superior to all cadaver transplants, irrespective of HLA matching [1-8]. The essential differences between cadaveric and LURD renal allografts are physiological, rather than genetic. Thus, the influence of non-immunological events on graft survival may have been underestimated.

In the modern era of renal transplantation, >80% one year graft survival is observed in most transplant centres. Nevertheless, the long term rate of attrition has remained the same regardless of improvements in surgical techniques, organ preservation, HLA-typing and immunosuppression. There is accumulating evidence to suggest that early non-specific inflammatory events may play a major role in determining chronic allograft loss and long term survival following transplantation. The work presented in this Thesis examines the role of ischaemia/reperfusion injury in cadaveric renal transplantation to determine its effects on inflammatory responses following

transplantation, and to investigate the potential therapeutic benefits of a novel free radical scavenging agent for minimising this damage.

1.2 RENAL TRANSPLANT DONORS

1.2.1 Heart-Beating Cadaver Donors

Transplantation of organs from heart-beating, brain-dead cadaver donors is ethically acceptable both religiously and culturally in the western world and is currently the most prevalent organ donor population in these countries. In the UK, the system for cadaveric organ donation has been defined from the publication of the “Diagnosis of Brain Death” by the Conference of the Medical Royal Colleges and Faculties of the United Kingdom in October 1976 and from the publication in 1983 by the Government Working Party on “Cadaveric Organs for Transplantation - A Code of Practice including the diagnosis of Brain Death” derived from the 1961 Human Tissue Act. Brain stem death is determined by clinical diagnostic tests for central neurological tissue damage that results in the permanent loss of the brain’s capacity to maintain vital functions, consciousness and meaningful life, in the absence of intensive care therapy.

Suitable organ donors identified in ICU are maintained on ventilator support and circulatory, fluid and metabolic support provided by intravenous fluid replacement and pharmacological intervention to maintain adequate perfusion through the vasculature of the organs. Immediately prior to organ procurement, the organs are cooled *en-bloc* by *in situ* perfusion through the aorta with chilled balanced electrolyte or preservation solutions to limit the harmful effects of warm ischaemia. Following donor nephrectomy, the kidneys are submerged in iced saline slush and perfused through the renal artery with cold preservation solution until the effluent is clear of blood. The kidneys are then surrounded with preservation solution, placed inside sterile bags and stored in ice until transplantation [9].

The majority of potential cadaveric organ donors in intensive care units (ICU) die as a result of cerebrovascular trauma. However, not all patients who die become cadaveric organ donors. Death that occurs outside ICU (e.g. external medical wards) generally result in organs that are unsuitable for transplantation due to the extensive warm ischaemia times encountered. Several studies have proposed that “elective ventilation” may be beneficial in alleviating the shortfall in potential cadaveric organ donors by identifying the patients on medical wards with rapidly progressing coma due to intracranial haemorrhage and admitting these patients to ICU [10-13]. This procedure increased the number of available donor kidneys in the centre performing elective ventilation but this practice was eventually suspended due to ethical criticisms and ambiguity in the legal consequences of the procedure. The organ shortage has resulted in an expansion in the criteria for cadaver donor acceptability with increasing use of “marginal” (e.g. elderly, non-heart-beating) donors. Although marginal donors have become an important source of kidneys, there is clearly an increased risk of failure and graft loss following transplantation [14, 15].

1.2.2 Non-Heart-Beating Cadaver Donors (NHBD)

The growing divide between supply and demand for heart-beating cadaveric organ transplantation has resulted in renewed interest in the retrieval of organs from non-heart-beating donors (NHBD). NHBD are defined as donors who experience cardiorespiratory arrest immediately before or after admission to hospital, usually as a result of myocardial infarction or severe traumatic injury. In contrast to conventional cadaveric organ retrieval, the rapid onset of cardiac arrest in NHBD may result in a lengthy hypotensive state, prolonged periods of warm ischaemia and inadequate perfusion of the donor organs, possibly resulting in irreversible damage to the kidneys. Therefore, procurement of kidneys from NHBD is limited to specialist centres where a dedicated, well-trained team must be available immediately to assess potential donation,

obtain consent for donation from the family, and initiate *in situ* organ perfusion to limit warm ischaemia times.

Booster and colleagues recommended a standardised criteria for selection of suitable NHBD such that warm ischaemia times should remain below 30 min, donors should be <60 years of age, have no history of kidney disease, hypertension, diabetes mellitus, malignancies or signs of intravenous drug abuse or systemic infection [16]. The implementation of NHBD organ retrieval in this single centre, resulted in an increase of 20% in kidneys available for transplantation. However, many groups have reported a significantly higher incidence of delayed graft function (DGF) in NHBD kidney transplantation compared with conventional cadaveric renal allografts, although surprisingly, no differences were observed in long term graft survival [16-20]. These results suggest that kidneys procured from NHBD may be an important source of organs for transplantation, but with the caveat that this practice should be performed by highly trained retrieval teams with expertise in *in situ* perfusion techniques to minimalise the detrimental effects of warm ischaemia.

1.2.3 Living-Related Donors (LRD)

Living-related donor (LRD) renal allografts are mostly performed between siblings or parent-child combinations and may be better matched for major and minor antigens due to the genetic relationship between donor and recipient. The first successful LRD renal transplants were performed between identical twins in 1954 [21]. However, the potential risks to the living donor of major surgery and long term risks of having only one kidney have made LRD transplantation less appealing to some transplant clinicians. Furthermore, major advances in surgical techniques, organ preservation and immunosuppression have meant that cadaver organ transplantation has become the preferred choice. However, the inadequate supply of cadaver donor kidneys in the face of increasing demand for kidney transplantation and the relatively low risks to the donor (<0.1% mortality) has resulted in a recent rise in the number of LRD transplants

performed. Moreover, the increase in the incidence of LRD renal transplantation has been driven by the superior graft survival rates observed for one, or even two haplotype disparate LRD kidneys over similarly mismatched cadaver kidneys. Indeed, the most successful long term graft survival results have been obtained from HLA-identical sibling transplants compared with transplants performed between any other donor-recipient combination [2, 3, 6, 8, 22].

1.2.4 Living-Unrelated Donors (LURD)

The shortfall in cadaveric organ donors has also been partly alleviated with the growing acceptance of living-unrelated donor (LURD) transplantation. The remarkable long term graft survival rates observed in LURD renal transplantation have resulted in the increased utilisation of this donor pool and has attracted attention to the possible reasons why these allografts have superior survival when faced with similar immunological barriers to cadaveric transplantation. Nevertheless, in light of the controversial issues surrounding payment for organs that resulted in the Human Organ Transplants Act 1989, transplantation of kidneys from genetically unrelated spouses or friends is carefully evaluated to ensure altruistic motives behind donation. The guidelines stipulated in the HOT Act 1989 restricts living donor transplantation to genetically related individuals, whilst non-related living transplants must be approved by the Unrelated Live Transplant Regulatory Authority (ULTRA).

1.3 LONG TERM GRAFT SURVIVAL IN LIVING AND CADAVERIC RENAL TRANSPLANTATION

The genetic relationship between living donors and related transplant recipients increases the probability for sharing one (parent-child or sibling allograft), or two HLA haplotypes (HLA-identical siblings). Therefore, it is unsurprising that HLA-identical sibling renal transplants have superior short and long term graft outcome, whilst one haplotype disparate LRD grafts have only marginally inferior levels of success [2, 3, 6,

8, 22]. Although LRD renal allografts may be better matched for HLA antigens, the immunological barriers encountered in LURD transplantation are similar to those experienced in cadaveric transplantation. However, accumulating evidence from single centre studies and national registry databases have demonstrated that graft survival in LURD renal transplantation is surprisingly similar to one-haplotype disparate LRD renal allografts, and significantly better than similarly matched cadaver renal allografts (Figure 1.1) [1-8].

It may be argued that the unexpected results obtained in LURD transplantation may reflect the use of younger donors. However, analysis of optimal cadaver renal allografts performed from donors under the age of 50 with no HLA-A, -B, or -DR mismatches demonstrated a 5 year graft survival of 77%, similar to LURD allografts with 3 HLA mismatches (74%), but significantly inferior to similarly, zero-mismatched LURD renal allografts (85%) [7]. Furthermore, identical rates of graft survival were observed in LURD spousal transplants from husband-to-wife compared with wife-to-husband, excluding the possibility of potential immunisation of the wife by previous pregnancies [6].

Although 5 year graft survival within the population of LURD transplants decreased proportionally with increasing numbers of mismatched HLA antigens, the differences in graft survival between cadaver and LURD renal allografts could not be explained by HLA-matching alone. Therefore, it appears that non-immunological factors may be involved in the differences observed between cadaver and living donor renal allograft survival.

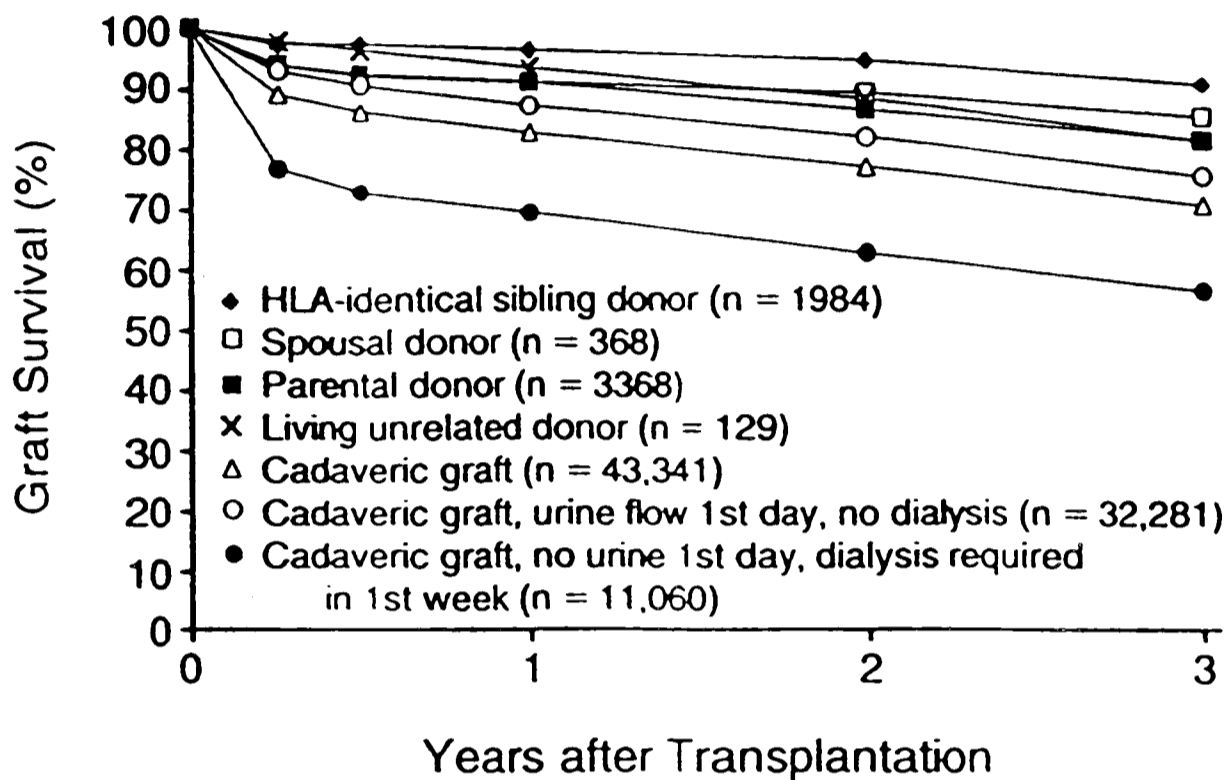


Figure 1.1 Graft Survival Following Clinical Renal Transplantation

Kidney transplant data from the United Network for Organ Sharing Renal Transplant Registry adapted from Terasaki et al. (New Engl J Med 1995; 333: 333-336). Three year survival rates were assessed for renal allografts from HLA-identical sibling donors, living-related donors (parental), living-unrelated donors (spousal and others), and cadaver donors according to the requirement for dialysis within the first week post-transplant

1.4 PHYSIOLOGICAL DIFFERENCES BETWEEN LIVING AND CADAVER DONOR RENAL TRANSPLANTATION

Fundamental physiological differences exist between living and cadaveric renal transplantation. Organs from living donors are procured under optimal conditions from carefully screened healthy individuals with minimal ischaemia times. In marked contrast, selection of cadaver donors is less stringent, and donors may experience abnormal regulation of respiratory, cardiovascular and homeostatic functions associated with cause of death, treatment in intensive care and deleterious events following brain death. In addition, cadaver kidneys are stored for longer cold ischaemic periods prior to transplantation.

1.4.1 Brain Death and ICU Treatment

Cadaveric organ donors may experience a variety of traumatic events leading up to, and following brain death. Irreversible loss of brain activity will inevitably result in progressive deterioration of organ function. Therefore, it is of utmost importance that potential organ donors are carefully managed in ICU to preserve organ quality for successful transplantation. Respiratory function is maintained by ventilator support, cardiovascular support is provided by the administration of inotropes (e.g. dopamine, dobutamine, epinephrine) and homeostatic function is regulated by the administration of vasopressin analogues such as desmopressin and by plasma volume expansion. Nevertheless, the onset of brain death results in severe physiological imbalances of endocrine and haemodynamic parameters that may occur before the actual diagnosis of brain stem death.

In experimental models of brain death induced by a rapid increase in intracranial pressure, dramatic pathophysiological changes have been demonstrated within hours of the onset of brain stem death; commonly referred to as the “autonomic storm” [23, 24]. Organs may be exposed to intense sympathetic stimulation by the rapid release of enormous levels of catecholamines into the circulation by adrenal glands and via direct,

local neural activity. The massive systemic surge in catecholamines results in severe vasoconstriction, creating high perfusion pressure and restricting flow to peripheral organs that may inevitably result in tissue ischaemia [25-27]. Furthermore, the autonomic storm may induce an increase in myocardial activity and oxygen consumption as a result of rising catecholamine levels and peripheral vascular resistance. The abnormal rates of oxygen consumption and delivery to peripheral organs results in accumulating levels of plasma lactate from anaerobic metabolism, further diminishing the availability of oxygen to the organs [26, 28]. Therefore, it is highly likely that circulatory disturbances may lead to severe ischaemic damage of donor organs prior to procurement, diminishing organ quality for transplantation.

Furthermore, significant changes in endocrine hormones may lead to neurogenic diabetes insipidus and impairment of aerobic metabolism in the organs. Diminished production of pituitary hormones such as vasopressin and adrenocorticotrophic hormone following brain death commonly results in diabetes insipidus with loss of homeostatic regulation and excessive urine output [29]. Fluid loss may be replaced with intravenous hypotonic solutions, but infusion of large volumes of hydroxyethylstarch to the donor has been shown to have a detrimental effect on immediate graft function following renal transplantation [30]. Replacement of vasopressin with an analogous compound, desmopressin (DDAVP), has been shown to act as a powerful anti-diuretic in the donor [31], but conflicting evidence has been reported with respect to its effects on renal function and survival following transplantation [32, 33].

In addition, severe fluctuations in thyroid hormones such as triiodothyronine (T_3), thyroxine and thyroid stimulating hormone (TSH) result in progressive depletion of high energy stores due to impaired aerobic metabolism [34, 35]. In cardiac transplantation, treatment of the donor with triiodothyronine significantly reduced serum lactate-pyruvate levels and the need for inotropic support, improving cardiac function in the donor and in the recipient following transplantation [36, 37]. Therefore,

the overall effects of severe hormonal imbalances following brain death appears to greatly impair normal organ function, resulting in abnormal flow of adequately oxygenated blood to the organs.

The functional consequences of the physiological derangements associated with brain death may result in severe histopathological changes to the donor organs. The elevated systemic catecholamine levels produced after brain death have been shown to cause severe morphological changes to the heart, with elongated, hypercontracted sarcomeres, mitochondrial injury and interstitial oedema [38]. In kidneys, systemic hypotension and cardiovascular instability may result in prolonged periods of ischaemia, leading to the development of histopathological changes such as glomerulitis and endothelial proliferation [39]. Furthermore, a high incidence of acute tubular necrosis was detected in post-transplant kidneys procured from haemodynamically unstable donors [40].

It is highly likely that histopathological changes to the organs before procurement, may significantly influence inflammatory responses in the recipient following transplantation. The mounting evidence suggesting that damage occurs to potential donor organs following brain death, indicates that management of cardiovascular, respiratory and homeostatic functions in ICU is of primary importance if the quality of the organs is to be maintained for transplantation.

1.4.2 Cold Ischaemic Storage

A primary physiological difference between living and cadaveric renal transplantation is the significantly longer period of cold ischaemic preservation experienced by cadaveric kidneys. The period of cold storage may be extended beyond 30 hours depending on the time taken for tissue typing, crossmatching, selection of a suitable recipient, transport of the kidney, arranging operating theatre time and preparation of the recipient. In marked contrast, kidneys may be stored for a nominal period of

approximately 2 hours in living donor renal transplantation, as almost all the above procedures are performed before organ procurement.

Removal of the organ from its blood supply will inevitably result in metabolic deterioration, initiating a cascade of events that lead to cell death. The principles of the techniques and solutions used in cold organ preservation are discussed in greater detail below, but the fundamental objective of hypothermia is to limit the progressive damage experienced during ischaemia. Nevertheless, although hypothermia alone will slow down the process of ischaemic damage, it may not prevent injury to the organ if cold storage times are extensive. Prolonged cold ischaemia of cadaveric kidneys, along with other non-specific and immunologically specific injuries may result in impaired graft function in the immediate period following transplantation. It is highly likely that ischaemic damage resulting from cardiovascular instability in the donor and cold preservation injury contribute significantly to the inferior graft survival of cadaver renal allografts compared with living donor transplantation.

1.5 DELAYED GRAFT FUNCTION

1.5.1 Effects of DGF on Graft Survival

In cadaveric renal transplantation, pre-transplant damage to the kidney may result in poor initial function of the graft. Delayed graft function (DGF) is defined in most studies as the requirement for dialysis within a specified period (usually one week) post-operatively. The early effects of DGF lead to complications in the immunosuppressive management of the recipient, additional biopsy sampling to determine intragraft events, extended hospitalisation, added financial costs incurred through dialysis and possible implications for long term graft function and survival [41, 42]. A few studies reported by individual transplant centres suggest that DGF alone has little effect on long term graft outcome [43-47], but other single centre studies, and

reports from national registry databases indicate that DGF is significantly associated with inferior long term graft survival [48-57]. Indeed, recent studies of >25,000 first cadaveric renal transplants from both the U.S. Renal Data System database and the UNOS Scientific Renal Transplant Registry, demonstrated that the occurrence of DGF was a powerful indicator of poor graft survival irrespective of acute rejection and the degree of HLA matching [53, 54].

1.5.2 Effects of DGF and Acute Rejection on Chronic Graft Loss

It is thought that cadaver renal allografts that experience one or more acute rejection episodes provide a poor long term prognosis with respect to chronic rejection and eventual graft loss [58-60].

Acute rejection is characterised by impaired graft function that is responsive to anti-rejection therapy in conjunction with the infiltration of mononuclear leucocytes comprising alloantigen activated CD4 and CD8 T lymphocytes, macrophages and natural killer cells. Histological diagnosis for grades of severity of acute rejection were standardised by the Banff working classification of kidney transplant pathology and recently revised in the Banff 97 classification and defined by tubulitis (number of mononuclear cells/tubular cross section), the level of interstitial infiltration, and severity of intimal arteritis in needle core biopsies of renal cortex [61, 62]. Despite the detrimental effects of acute rejection on long term graft loss, substantial improvements in immunosuppression and clinical management have resulted in a reduction in the number of renal allografts lost to acute rejection [60]. This has focused attention to the rise in grafts lost to chronic rejection.

Chronic rejection has been defined as a slow, progressive decline in renal function (determined by rising serum creatinine levels), in association with hypertension and proteinuria, occurring >90 days following transplantation [63, 64]. Histopathological features of chronic rejection according to Banff criteria includes interstitial fibrosis,

tubular atrophy, glomerular and vascular lesions, and arterial intimal thickening [61, 62]. Chronic rejection may result from specific low level alloimmune responses, but it may also be influenced by a higher frequency of acute rejection or the occurrence of late episodes of acute rejection [65-67]. However, there is mounting evidence that non-immunological factors (e.g. donor organ quality, donor age, nephron mass, DGF, ischaemia/reperfusion injury, recipient diabetes, hypertension) may play a critical role in the progression of chronic rejection and graft loss [60]. Interestingly, the non-immune factors that have been found to predispose grafts to chronic rejection are similar to those which have been identified for DGF (section 1.5.3). There is growing debate as to whether non-immunological factors leading up to and including DGF, or allospecific acute rejection have the most significant impact on long term graft survival.

The most compelling evidence has been provided from large multicentre database studies which indicate that DGF is an important predictive factor for the poor long term survival of cadaveric allografts (Figure 1.2) [49, 50, 53, 54]. It may be argued that multicentre registry studies are limited by the variation between centres in their treatments for DGF and acute rejection, but the large number of cases provides a valuable overview of transplants performed in a relatively recent period of time and enables powerful statistical analyses.

It is evident from these multicentre studies that DGF and acute rejection have an additive adverse effect on long term graft loss (Figure 1.2) [53, 54]. Furthermore, DGF was found to be significantly associated with an increased incidence of acute rejection episodes during the period of hospitalisation and within 6 months of transplantation [54]. It is possible that events which give rise to DGF, or its resulting consequences, may initiate and intensify alloimmune responses, especially in mismatched renal allografts. In support of this hypothesis, Shoskes and colleagues demonstrated that DGF had a detrimental effect on long term survival of 6-HLA-antigen mismatched kidneys but no significant impact on survival of completely matched kidneys.

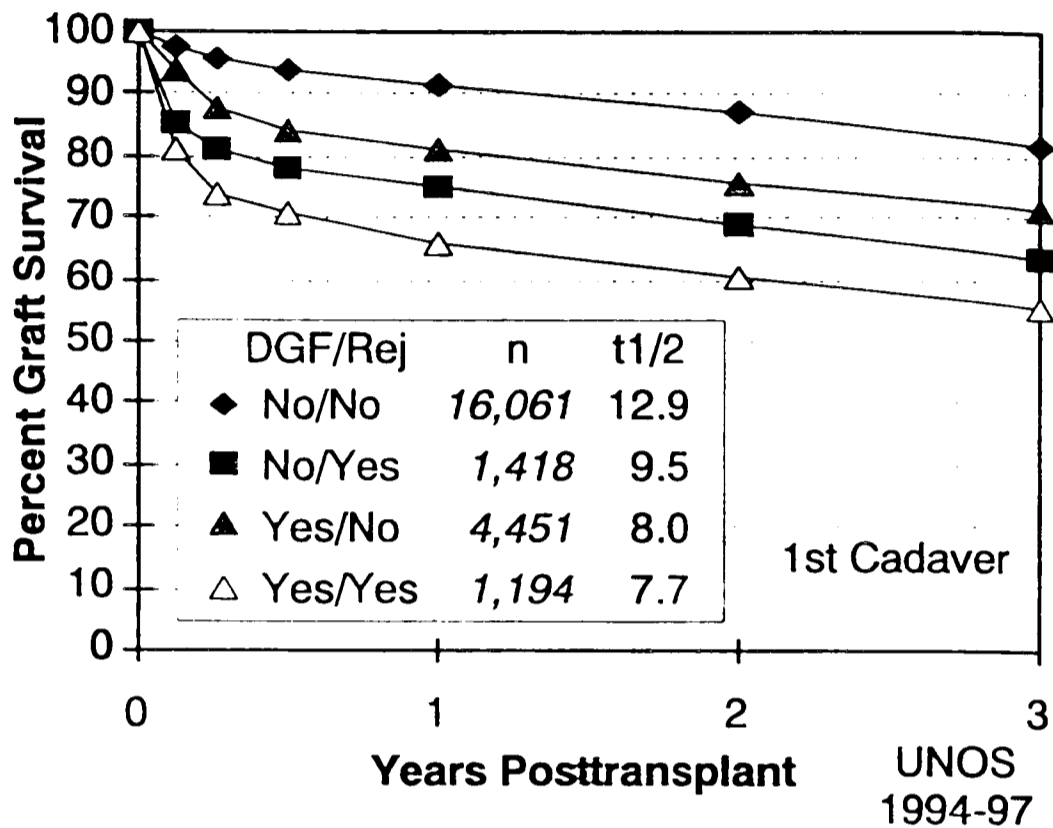


Figure 1.2 Effect of DGF and acute rejection on graft survival following clinical renal transplantation.

Kidney transplant data from the United Network for Organ Sharing Renal Transplant Registry adapted from Shoskes and Cecka (Transplantation 1998; 66: 1697-1701). Both DGF and acute rejection were shown to have an independent, detrimental impact on 3 year graft survival, with the occurrence of both events having an additional adverse effect on graft survival.

Moreover, totally mismatched kidneys with good early function had similar long term graft survival to the 6-antigen matched kidneys [51, 68]. These results strongly suggest that inflammatory events arising from DGF may induce HLA-antigen and accessory molecule expression, thus affecting alloimmune responses in allografts with a greater number of mismatches. Experimental evidence has demonstrated that in renal models of warm ischaemia and reperfusion, induction of MHC antigens and inflammatory cytokine expression were detected in association with ATN [69, 70]. Therefore, it remains an important goal to elucidate the events which cause DGF in clinical renal transplantation, to determine whether or not they influence graft survival, possibly through the initiation and generation of an alloimmune response.

1.5.3 Potential Causative Factors of DGF

DGF may result from a variety of immunological and non-specific factors determined clinically by the requirement for dialysis in the immediate period following transplantation and pathologically by the diagnosis of acute tubular necrosis (ATN). Many of these factors are related to the donor and may result from a reduction in functioning nephron mass. Many studies have demonstrated a higher incidence of DGF in kidneys procured from elderly donors, with progressively poor graft survival and quality of function with increasing donor age [15, 50, 53-55, 57]. The diminished renal function observed in kidneys from older donors may be related to the reduction in the number of active nephrons with age. Similarly, this hypothesis has been supported by studies that demonstrated transplantation of organs from donors with a small body mass into larger recipients experienced a higher incidence of graft loss [71].

Furthermore, pre-transplant ischaemic events in the donor arising from cardiovascular instability, may additionally reduce functioning nephron mass. This is evident from transplants performed from NHBD kidneys that have a significantly higher incidence of DGF as a result of prolonged warm ischaemia times [16-20]. Moreover, the lower incidences of DGF in LURD renal transplantation compared with cadaveric allografts,

demonstrate the importance of donor factors before transplantation. In addition, the effects of prolonged cold ischaemic storage have been demonstrated by both single and multi-centre studies to be the most significant factor influencing DGF following transplantation [45, 48-54, 72]. Ojo and colleagues calculated that a 23% increase in the risk of DGF was observed for every 6 hours of cold ischaemia, following a multicentre analysis of 37,216 primary cadaver renal allografts [53]. Furthermore, prolonged cold ischaemia time additionally decreased graft survival independently of DGF or early acute rejection [54]. The precise mechanisms whereby cold ischaemia causes DGF, and consequently promotes increased acute rejection episodes, chronic rejection and poor graft survival are not fully understood. However, there is accumulating evidence to suggest that ischaemia/ reperfusion injury plays a pivotal role in the aetiology of this process.

1.6 BIOLOGICAL EFFECTS OF ISCHAEMIA IN TRANSPLANTATION

Ischaemia occurs when the blood supply to an area of tissue is interrupted, initiating a sequence of chemical events that lead to cellular dysfunction, oedema and cell death [73-75]. In the context of transplantation, warm ischaemia may arise in the donor as a result of cardiovascular disturbances that affect adequate perfusion of the organs *in situ*, or during procurement and implantation, although warm ischaemia is minimal during these procedures because of modern *in situ* cooling techniques. In addition, all organs experience ischaemic anoxia during the period of preservation, although cooling diminishes metabolic activity and reduces the oxygen demand of the preserved organ. Unfortunately, hypothermia does not arrest all metabolic processes but acts by delaying the breakdown of high energy compounds. A summary of potentially detrimental events experienced during ischaemia is represented in Figure 1.3.

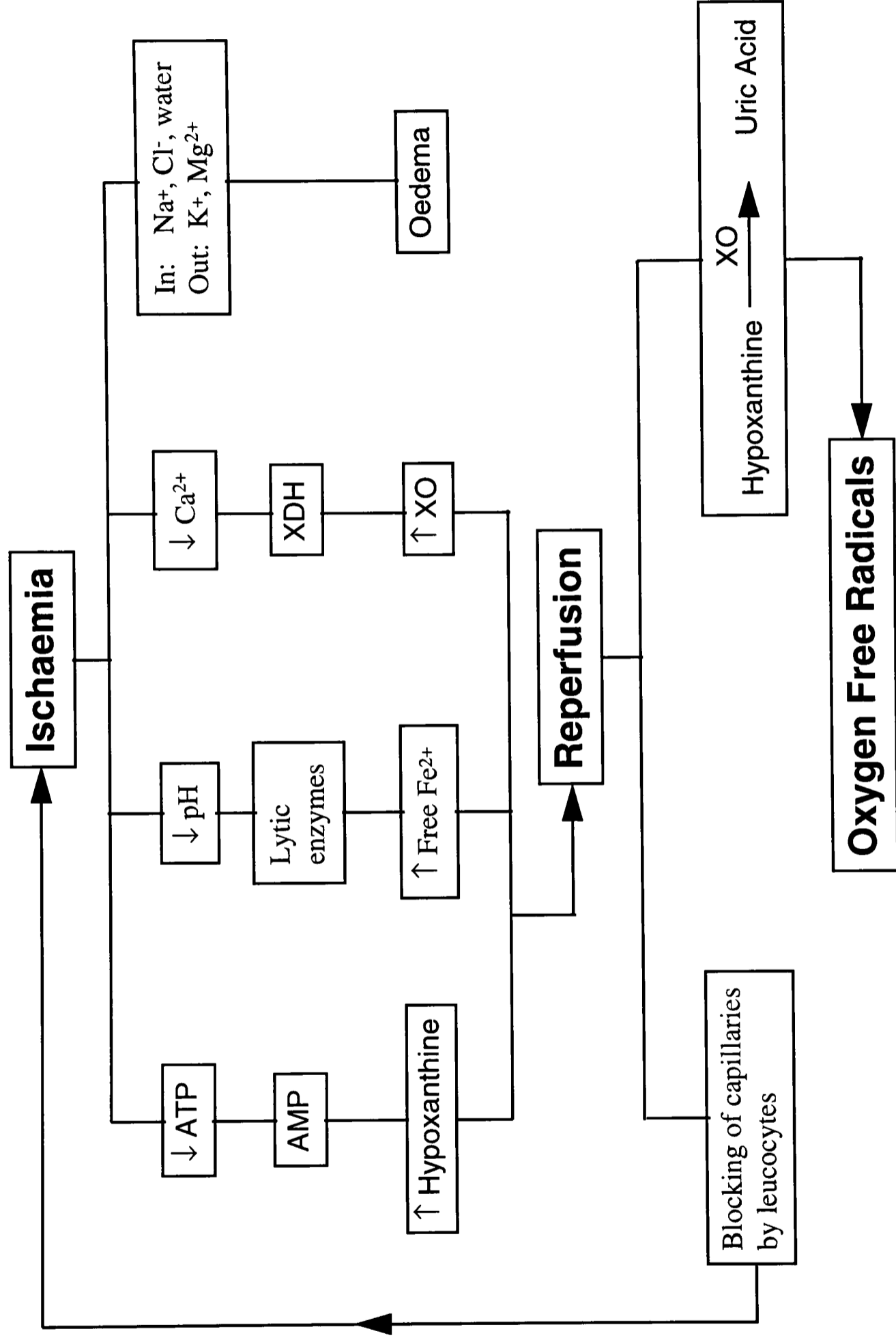


Figure 1.3 Schematic diagram of biological events during ischaemia

1.6.1 ATP Depletion

Cells within normally functioning organs derive their energy through an adequately maintained circulation supplying high energy substrates (glucose, fatty acids, amino acids) and oxygen. Ischaemic tissue is depleted of oxygen and nutrients, with continuing anaerobic metabolism leading to a build up of catabolites and a cascade of ischaemic events, ultimately resulting in irreversible cell damage and death. Anaerobic metabolism generates only two molecules of adenosine triphosphate (ATP) per glucose molecule metabolised, whilst under normal aerobic conditions 38 ATP molecules are produced [9]. ATP is consumed by energy requiring reactions and is further dephosphorylated to adenosine diphosphate (ADP) and adenosine monophosphate (AMP). In the kidney, 95% of ATP is lost within 4 hours of ischaemia, although kidneys may remain viable even after 3 days storage [76]. Accumulation of AMP leads to purine nucleotide catabolism with the formation of inosine, adenosine, xanthine, uric acid and hypoxanthine. Furthermore, purine products of ATP degradation are highly permeable and may diffuse out of the cell at reperfusion, further depleting nucleotides available for regeneration of ATP after reperfusion [77]. The build up of purines such as hypoxanthine may have additional consequences at reperfusion, providing the substrate for the generation of cytotoxic, reactive oxygen free radicals. Oxygen deprivation and subsequent changes in cellular pH directly influence mitochondrial ATP production and can reduce glycolysis [78]. Thus cells with the greatest dependence on mitochondrial ATP production, such as proximal tubule cells, will generally be more susceptible to ischaemic damage [79, 80].

1.6.2 Reduction in Intracellular pH

Another consequence of anaerobic metabolism is the formation of lactic acid leading to the lowering of intracellular pH levels. The acidic environment affects cellular enzyme activity, decreasing the stability of lysosomal membranes, thereby activating lysosomal lytic enzymes [81]. Lysosomal hydrolyses, phospholipases, proteases and endonucleases may disrupt structural components of cells causing permanent damage.

Binding of transition metals (e.g. iron, copper) to carrier proteins (e.g. transferrin, ferritin) is also disrupted such that the free iron (Fe^{2+}) generated can be potentially harmful if it interacts with reactive oxygen species via the Haber-Weiss reaction during reperfusion.

1.6.3 Impaired Regulation of Ionic Environment

Alterations in the intracellular ionic environment has been shown to be important during ischaemia. Under conditions of normal metabolism, high concentrations of potassium and magnesium and low concentrations of sodium and calcium are maintained by sodium-potassium ATPase and calcium-magnesium ATPase membrane-bound pumps which are ATP-dependent [82]. The reduction of ATP levels and hypothermic storage consequently results in the loss of maintenance of the ionic environment, and a tendency toward equilibration of permeable solutes across the cellular membrane. Potassium and magnesium ions diffuse out of the cell whilst sodium, chloride, calcium and water diffuse into the cell causing oedema [83]. Suppression of cellular swelling appears to be one of the most important factors in cold storage and this is currently achieved by the inclusion of impermeable solutes into all modern preservation solutions, providing an extracellular osmotic force opposing the development of oedema [77].

Inactivation of the enzymatic systems responsible for calcium transport during cold ischaemia results in a rapid influx of calcium that may initiate a cascade of events that activates the calcium-calmodulin complex, mediating the breakdown of cell membranes and mitochondrial dysfunction resulting in cellular necrosis [84]. The increase in cytosolic calcium results in the activation of phospholipase A2, an enzyme which lyses cellular membranes [85, 86]. The role of calcium as a toxic factor during ischaemic injury is supported by studies demonstrating that increasing concentrations of calcium in the perfusate were harmful during hypoxia in the isolated perfused kidney [87]. Furthermore, the removal of calcium from the medium ameliorated hypoxic injury to

isolated perfused kidneys [88] and anoxic injury to freshly isolated tubule cells [89]. Addition of calcium channel blockers such as diltiazem and verapamil to existing preservation solutions has been shown to have a protective effect against ischaemic injury in human renal allografts with improved early graft function [90-92]. The protective effect of calcium channel blockers is thought to occur through the inhibition of calcium influxes from the extracellular milieu into the cytoplasm during ischaemia [93, 94]. However, the promising results from these early studies have not been applied routinely in the modern era of organ preservation and indeed, more recent evidence from a large, prospective double-blind trial suggests that addition of calcium channel blockers alone may have no beneficial effect on ischaemic injury of kidneys [95].

1.6.4 Conversion of Xanthine Dehydrogenase to Xanthine Oxidase

Perhaps one of the most significant events associated with the increase in intracellular calcium levels is the activation of a calcium dependent protease which cleaves xanthine dehydrogenase (XDH) to its isomeric form xanthine oxidase (XO) [96]. The conversion of XDH to XO is proportional to the length of ischaemia [97], with the rate of conversion varying between organs, taking about 30 min in the kidney [98]. XDH is constitutively expressed on the surface of endothelium [99], where its normal function is to catalyse the terminal oxidation of purines (hypoxanthine and xanthine) to uric acid, coupled with the reduction of nicotinamide adenine dinucleotide (NAD⁺) to NADH [100]. Although XO also catalyses the oxidation of purines to uric acid, the absence of a binding site for NAD⁺ results in purine oxidation coupled with the reduction of molecular oxygen (O₂) to generate the highly reactive superoxide anion (·O₂⁻) [101]. Therefore, it is apparent that in the absence of oxygen, accumulating levels of XO during the ischaemic period may not be significantly harmful until the reintroduction of oxygen at reperfusion.

1.7 ISCHAEMIA/REPERFUSION INJURY: THE CENTRAL ROLE OF ENDOTHELIAL CELLS

If blood is returned to ischaemic tissue before a critical time point, the tissue is quickly restored to normal function. Kidneys may endure short periods of warm ischaemia (<30 min), but tolerance to ischaemia may be prolonged (12 hours) by hypothermic storage [76]. Beyond this time point, the return of oxygenated blood upon reperfusion may paradoxically lead to a chain of damaging events that result in the production of reactive oxygen species and endothelial damage (Figure 1.4). The endothelial lining of the organ vasculature plays a critical role in transplantation, participating in the initial interactions between donor organ and recipient blood during ischaemia/reperfusion injury, and regulating leucocyte trafficking during subsequent inflammatory and immunological responses.

1.7.1 Generation of Reactive Oxygen Species

The harmful effects of oxygen during reperfusion injury have been demonstrated from elegant studies showing that reperfusion with normoxic blood resulted in microvascular and parenchymal cell injury, whereas hypoxic reperfusion produced minimal damage [102, 103]. Furthermore, 4 hours of ischaemia alone, was less harmful than 3 hours of ischaemia followed by 1 hour of reperfusion [104]. These observations suggest that the reintroduction of oxygenated blood initiates tissue injury.

It is widely recognised that the cytotoxic events resulting from reperfusion of ischaemic tissue involve the formation of reactive oxygen species derived from molecular oxygen. The oxidants formed during reperfusion are members of the "free radical" species; molecules that are capable of independent existence, containing one or more unpaired electrons and are thus chemically highly reactive [105]. Several reactive oxygen species can be produced by reduction or excitation of molecular oxygen [74]. The addition of a single electron to molecular oxygen leads to generation of the superoxide anion (O_2^-) [97]. During normal cellular metabolism, superoxide is a by-product derived from

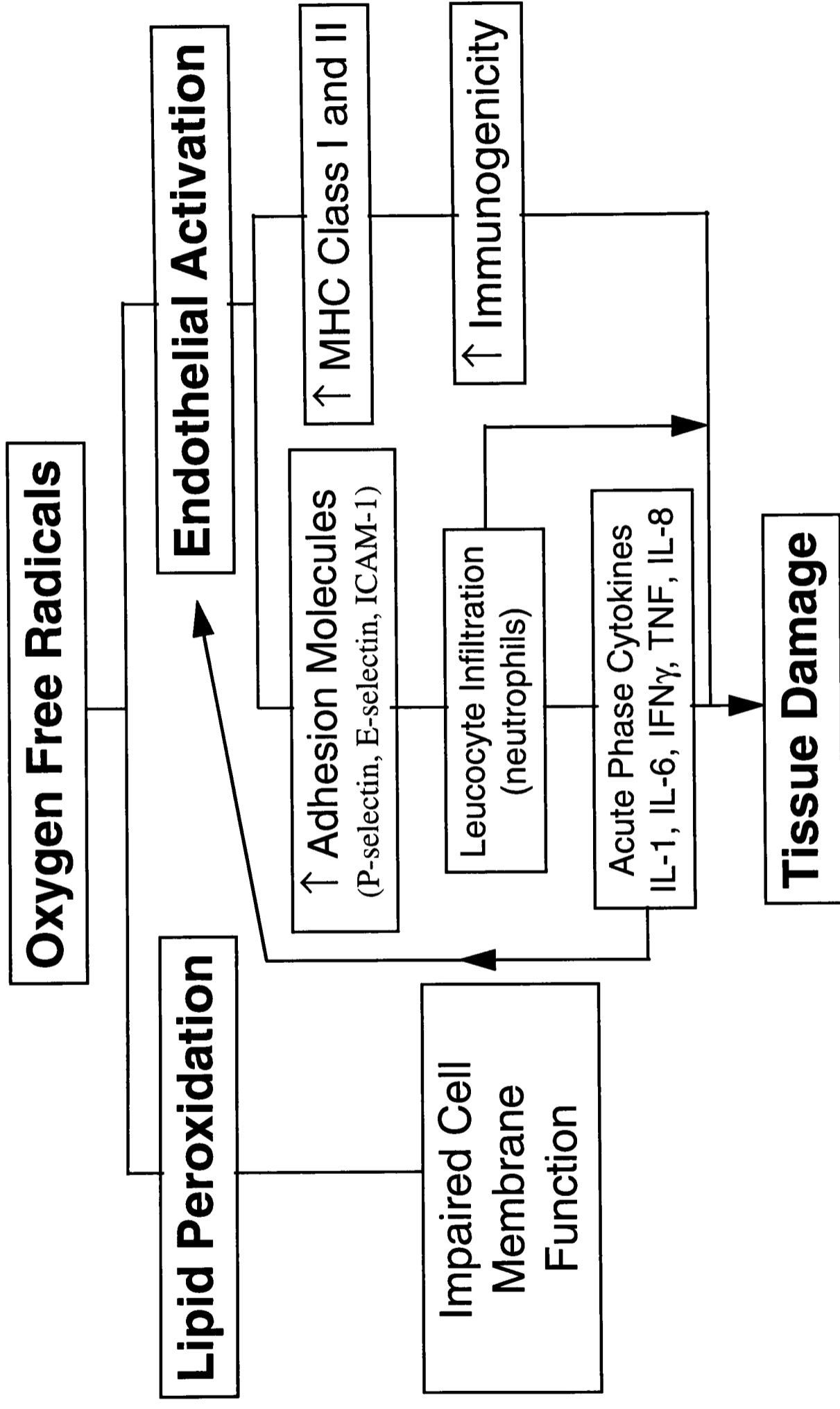


Figure 1.4 Schematic representation of effects of ischaemia/reperfusion injury

mitochondrial, endoplasmic reticular and nuclear membrane electron transport processes [106]. The natural cellular defence against superoxide-mediated damage is provided by superoxide dismutase (SOD), which exists in three different forms. Mn-SOD is restricted to mitochondria, the non-glycosylated dimeric, CuZn-SOD is found in both cytosol and nuclei, and the glycosylated tetrameric CuZn-SOD is bound on the extracellular surface of endothelium via a heparin binding domain [107, 108]. Protection against superoxide is mediated predominantly by the CuZn isoforms of SOD in peripheral vessels, whereas defence against other reactive oxygen species such as H_2O_2 (see below) is mediated by catalase and glutathione peroxidase. The rapid burst of free radical production upon reperfusion of ischaemic tissue is initiated at the endothelial surface by the action of xanthine oxidase, overwhelming the antioxidant capacity of the cell.

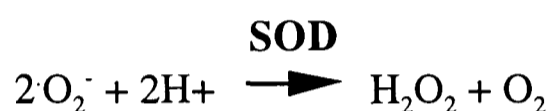
The role of reactive oxygen species in ischaemia/reperfusion injury has been investigated through *in vitro* studies of endothelial cells exposed to periods of hypoxia and reoxygenation. Electron paramagnetic resonance studies of endothelial cells exposed to hypoxia/reoxygenation demonstrated that XO was the primary source of superoxide generation [109, 110]. In mammalian cells, XO is converted from constitutively expressed xanthine dehydrogenase (XDH) as a result of proteolytic cleavage [100, 101, 111]. XDH and XO catalyse the oxidation of hypoxanthine and xanthine to xanthine and uric acid, respectively. However, XO has been shown to generate reactive oxygen species as part of this process and is involved in the endothelial injury that occurs in ischaemia/reperfusion injury [98].

Generation of $\cdot O_2^-$ by Xanthine Oxidase



Exposure of endothelial cells to hypoxia and reoxygenation results in an increase in the enzymatic activities of XDH/XO but with no induction of mRNA and protein synthesis. Furthermore, exogenous administration of SOD or allopurinol (a xanthine oxidase inhibitor) prevented free radical induced endothelial cell injury and neutrophil adhesion in this model [112, 113]. Superoxide inactivates specific cellular enzymes, is a precursor for the formation of hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical (OH^\cdot), and has been demonstrated to be involved in inflammatory processes of a variety of pathological disorders.

Dismutation of $\cdot O_2^-$ by SOD



H_2O_2 is a less powerful oxidant than $\cdot O_2^-$, but can inactivate nucleic acids as a result of its ability to traverse cellular membranes [74]. Although H_2O_2 has low reactivity with biological molecules, it may interact with free intracellular iron accumulated during ischaemia to produce the highly reactive hydroxyl radical (OH^\cdot) via the Haber-Weiss reaction.

Generation of OH^\cdot radicals (Haber-Weiss Reaction)



The hydroxyl radical is far more reactive than either superoxide or hydrogen peroxide and can attack all biologic molecules, initiating a cascade of free radical reactions. OH^\cdot radical may be responsible for oxidised sulphhydryl groups, inactivated cytochrome enzymes and altered membrane associated transport proteins [74]. The dependence of OH^\cdot radical formation on the availability of free iron has been demonstrated with the powerful iron-chelating agent deferoxamine, which abolished OH^\cdot mediated damage following ischaemia/reperfusion [114-116]. However, the universal reactivity of OH^\cdot

with an abundance of cellular reactants and its short half-life, means that it may be unable to survive to attack more critical macromolecules. In contrast, O_2^- is a more discriminating reagent, thus enabling molecular damage at critical sites. Many studies have demonstrated that specific scavenging of O_2^- with SOD has been shown to have a beneficial effect on ischaemia/reperfusion injury [117-126].

One of the most damaging effects of free radical mediated damage during reperfusion is lipid peroxidation. Reactive oxygen species peroxidise phospholipid and polyunsaturated fatty acids in cell membranes resulting in structural and functional cell damage [127]. Lipid peroxidation is a complex event that may result in the formation of lipid peroxides or lipid hydroperoxides, that are further broken down to form malondialdehyde or hydroxynonenal; stable end-products of the process which can be used as markers of lipid peroxidation and free radical damage as reactive oxygen species only have very short half-lives [128].

The initial burst of superoxide production by xanthine/xanthine oxidase reactions at the endothelial surface following reperfusion may initiate a cascade of biochemical events, producing a variety of reactive oxygen species, thus accentuating the pathological process of ischaemia/reperfusion injury.

1.7.2 Modulation of Vascular Tone

It is widely recognised that ischaemia/reperfusion leads to microvascular injury that exhibits similar characteristics to an acute inflammatory response, primarily through alterations in normal endothelial function. Endothelial cells may release and express a variety of mediators that result in endothelial dysfunction and recruitment of leucocytes to the tissue, thereby furthering the degradative process.

The endothelial barrier between the circulation and surrounding tissues secretes vasoconstrictors (e.g. endothelin), and vasodilatory molecules (e.g. nitric oxide (NO)),

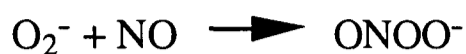
to regulate blood pressure and flow [129]. NO is constitutively expressed by the endothelium, whereas endothelin is synthesised in response to external stimuli such as hypoxia or ischaemia/reperfusion, thus disrupting the balance in vasoregulation resulting in a net vasoconstrictory effect .

1.7.2.1 Nitric Oxide

Nitric oxide (NO) was first identified as the vasodilator molecule, endothelium-derived relaxing factor (EDRF), involved in the regulation of vascular tone [130]. NO is constitutively synthesised and expressed at basal levels on the surface of endothelium by the calcium/calmodulin dependent enzyme nitric oxide synthase (cNOS), and also by an inducible isoform (iNOS) [122, 131]. cNOS is expressed in the renal vasculature and tubular epithelium, whereas iNOS may be expressed by the proximal tubules, glomeruli and inner medullary collecting duct [132-134]. L-arginine is converted to L-citrulline by NOS with the subsequent release of nitric oxide on endothelial cells [135]. Thus under normal conditions, nitric oxide is produced at basal levels, having a vasodilatory effect by relaxing vascular smooth muscle cells through the binding of NO to guanyl cyclase [136]. Furthermore, NO has been shown to participate in normal endothelial anti-coagulative functions through the inhibition of platelet adhesion, activation and aggregation in part, via suppression of the calcium-sensitive conformational change of the platelet GP IIb-IIIa integrin molecule [137, 138]. In addition, NO has been shown to impair neutrophil adherence [139-141] and leucocyte chemotaxis across the endothelium [142].

NO carries an unpaired electron and will rapidly react with other oxygen derived free radical species. Production of superoxide at reperfusion may expose the endothelium to damage by its reaction with NO to form peroxynitrite (ONOO⁻) [105, 143], reducing NO bioavailability, increasing vasoconstriction and thus its protective effect on the endothelial surface.

Generation of peroxynitrite



Peroxynitrite and its decomposition products have been shown to initiate lipid peroxidation without the requirement for iron [144]. Hence, this reaction may have a further detrimental effect on endothelial cell function. In experimental models of renal ischaemia/reperfusion injury, conflicting evidence on the contribution of NO has been reported. Garcia-Criado and colleagues demonstrated a protective effect on renal function following intravenous administration of a NO donor before reperfusion [145]. A more detailed study performed by Linas and coworkers demonstrated that NO had a detrimental effect on ischaemic renal injury in the absence of neutrophils, but prevented neutrophil-mediated ischaemic injury [146]. Shoskes and colleagues demonstrated a significant increase in NOS activity that was cytotoxic over the first 24 hr following reperfusion of ischaemic kidneys, but subsequently dropped below baseline levels until day 21 [147]. Similarly, Weight and colleagues demonstrated a cytotoxic effect of increased NOS activity in the initial period following reperfusion that was attenuated by the NOS inhibitor, L-NAME [148]. The evidence from these studies suggest that increased NO production may paradoxically promote ischaemia/reperfusion injury. It has been proposed that the initial rate of superoxide and NO production following reperfusion may be critical in determining whether NO has a protective or cytotoxic effect through peroxynitrite formation, although no conclusive evidence has yet been provided [141].

1.7.2.2 Endothelin

The vasoconstriction resulting from reduced levels of available NO may be further exacerbated by the action of endothelin. Endothelin-1 (ET-1) is synthesised and expressed by endothelial cells and is the most potent vasoconstrictor discovered to date [149]. ET-1 has been shown to be predominantly expressed in endothelial cells, but

may also be expressed by cardiac myocytes, vascular smooth muscle cells and in renal tissue [150]. Under normal physiological conditions, endothelin and NO act in concert to regulate vascular tone. Similar to NO, endothelin has also been shown to play a role in the pathogenesis of ischaemia/reperfusion injury [151].

ET-1 mRNA levels have been shown to be upregulated in post-ischaemic kidneys persisting for several days following reperfusion [152]. Wilhelm and colleagues recently demonstrated that ET-1 peptide was detected at high levels on intertubular capillaries following 60 min of warm ischaemia in association with elevated mRNA levels [153]. There is evidence to suggest that the upregulated expression of ET-1 following ischaemia/reperfusion is mediated by reactive oxygen species [154]. Furthermore, two specific receptors for endothelin have been identified in human kidneys, ET_A and ET_B [155]. The detrimental role of endothelin in ischaemia/reperfusion injury of the kidney has been demonstrated by the improved renal function and histology observed following administration of antibodies to ET or ET receptor antagonists [156-159].

It appears that loss of vasoregulation in the kidney following ischaemia/reperfusion may play a critical role in the pathogenesis of this event. Endothelial dysfunction may result in impaired NO release during ischaemia, resulting in inhibition of vasodilation. The effects of ischaemia, endothelial dysfunction and reduced NO levels may all stimulate endothelin release, which in turn accentuates vasoconstriction and exacerbates the effects of ischaemia/reperfusion injury. The critical balance between NO and endothelin may be a major determinant in the regulation of regional and systemic vasoregulation.

1.7.3 No Reflow Phenomenon

Net vasoconstriction and cellular oedema in the microvasculature may result in impaired reperfusion through the organ, creating ischaemic areas following the return of blood to the organ [74]. This phenomenon is known as the “reflow paradox.” In addition, the no

reflow phenomenon may result in trapping of leucocytes within vessels, causing capillary plugging [160].

Loss of normal endothelial function following ischaemia/reperfusion, exposure of endothelial cells to reactive oxygen species and production of inflammatory mediators may cause the rapid upregulation and expression of adhesion molecule structures on the endothelial surface that are critically involved in the process of leucocyte capture, activation and transmigration. There is direct evidence from many studies to indicate that neutrophils are implicated in the pathophysiology of numerous models of ischaemia/reperfusion injury [74, 97, 141, 161]. The endothelium plays a critical role in regulating this response and the leucocyte sub-populations recruited into the inflammatory area.

1.8 ADHESION MOLECULES INVOLVED IN ENDOTHELIAL-LEUCOCYTE INTERACTIONS

Transplantation of a renal allograft leads to immediate and sustained contact between the circulation of the recipient and the endothelium of the donor allograft. The process of allograft damage can begin immediately through reperfusion injury and subsequently via rejection episodes, all of which involve leucocyte infiltration. The mechanism by which leucocytes transmigrate from the circulation at a site of an inflammatory response involves a cascade of interactions which can be divided into four major steps: (i) tethering; (ii) triggering; (iii) firm adhesion; (iv) transmigration (Figure 1.5 and Table 1.1) [162-166]. Many of the molecules involved in the cascade may be targets for immune intervention therapy following transplantation.

1.8.1 Leucocyte Tethering and Rolling

The initial event by which leucocytes interact with endothelium is mediated by lectin-like carbohydrate molecules called selectins. The role of the selectins and their cognate

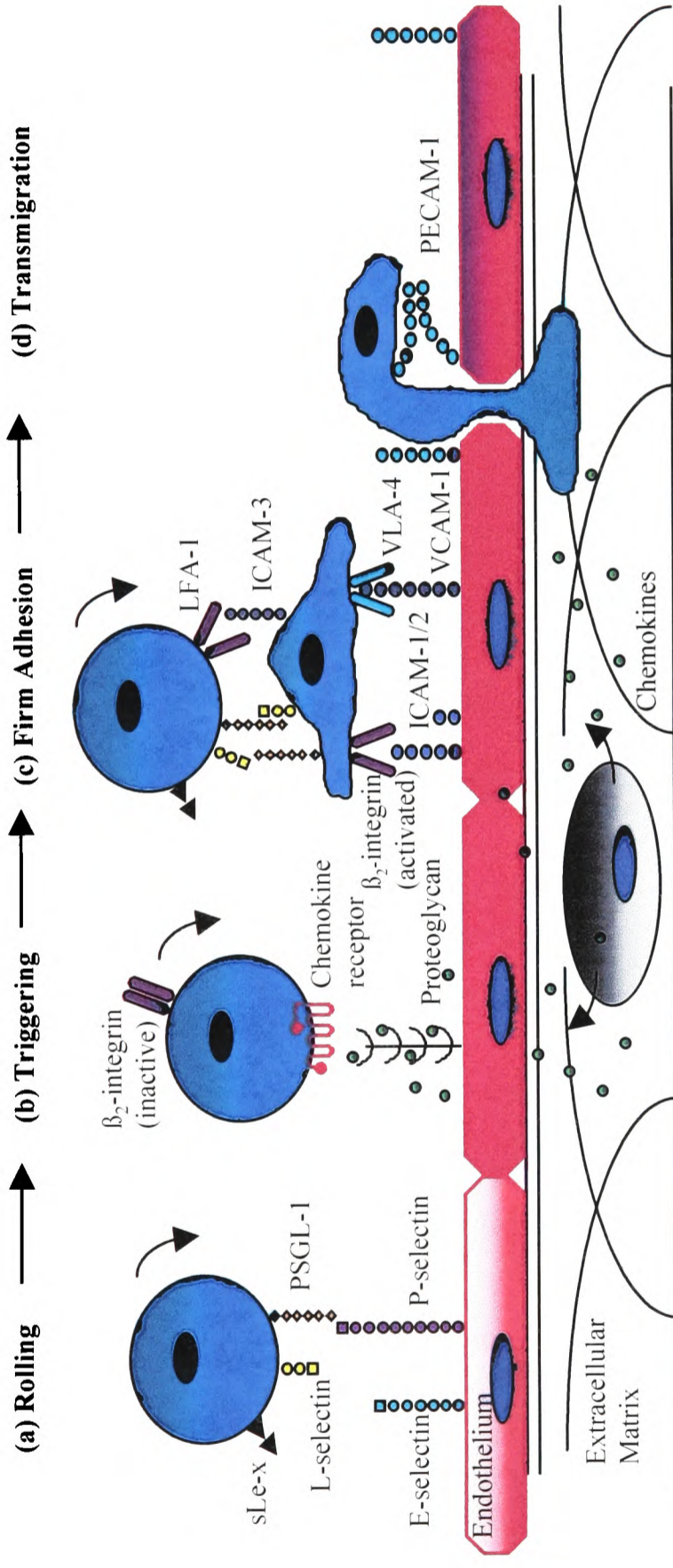


Figure 1.5 A schematic diagram representing the molecules involved in the four distinct steps of the adhesion cascade.

Leucocytes are initially attracted from the circulation to the luminal surface of the endothelium by selectin-mediated tethering and subsequent rolling as a consequence of the weak interactions with their ligands in flowing conditions. Chemokines produced from the site of inflammation can become attached to proteoglycans on the endothelial surface where they may be presented to rolling leucocytes. Interaction between chemokines and the seven-membrane spanning G-protein receptors present on leucocytes results in the triggering of intracellular signalling events that lead to leucocyte activation and firm adhesion. Activated leucocytes express $\beta 2$ -integrins that have undergone a conformational change resulting in higher avidity binding with its ligands ICAM-1, ICAM-2 and ICAM-3. VCAM-1 is also upregulated on activated endothelium and this molecule participates in leucocyte firm adhesion through its interaction with VLA-4. Neutrophils do not express VLA-4 and are not thought to require VCAM-1 for firm adhesion. Leucocytes which have firmly adhered are also capable of supporting rolling of other leucocytes from the circulation via interactions between ICAM-3 and LFA-1, and L-selectin and PSGL-1. Firmly adhered leucocytes extend specialised structures known as "uropods" into the vessel lumen that are highly enriched in adhesion molecules such as ICAM-3. Once the leucocytes have firmly attached they migrate towards endothelial cell junctions via homotypic PECAM-1 interactions between leucocyte and endothelium. The precise mechanism for leucocyte transmigration is unknown but there is evidence to show that leucocytes may cause structural rearrangements of endothelial cells at the cellular junctions and transmigrate along a chemotactic gradient.

| Inflammatory Adhesion Molecules | Function | Distribution | Major ligands during inflammation |
|--|---------------------------|---|--|
| Selectins | | | |
| P-selectin (CD62P) | Rolling | Endothelium Platelets | PSGL-1 (CD162) SLe ^x (CD15s) Sialyl-Lewis a L-selectin (CD62L) |
| E-selectin (CD62E) | Rolling | Activated endothelium | PSGL-1 (CD162) SLe ^x (CD15s) Sialyl-Lewis a L-selectin (CD62L) Cutaneous lymphocyte antigen (CLA) |
| L-selectin (CD62L) | Rolling | Most resting leukocytes | E- and P-selectin SLe ^x (CD15s) PSGL-1 (CD162) CLA |
| Immunoglobulin Supergene Family | | | |
| ICAM-1 (CD54) | Firm Adhesion | Endothelium T and B cells Monocytes Dendritic cells Tubular epithelia | LFA-1 (CD11a/CD18) Mac-1 (CD11b/CD18) Fibrinogen |
| ICAM-2 (CD102) | Firm Adhesion | Endothelium T cells Dendritic cells | LFA-1 (CD11a/CD18) |
| ICAM-3 (CD50) | Rolling | Most resting leucocytes Dendritic cells | LFA-1 (CD11a/CD18) |
| PECAM-1 (CD31) | Transmigration | Endothelium Platelets Leukocytes | PECAM-1 (CD31) $\alpha_v\beta_3$ (CD51/CD61) |
| Integrins | | | |
| LFA-1 (CD11a/CD18) | Firm Adhesion | Most leukocytes | ICAM-1 ICAM-2 ICAM-3 |
| Mac-1 (CD11b/CD18) | Firm Adhesion | Most leukocytes | ICAM-1 Fibrinogen iC3b |
| p150,95 (CD11c/CD18) | Firm Adhesion | Most leukocytes | Fibrinogen iC3b |
| VLA-4 (CD49d/CD29) | Firm Adhesion, Rolling | Lymphocytes Monocytes | VCAM-1 |

Table 1.1 Inflammatory adhesion molecules, their function, distribution and the major ligands involved in leucocyte recruitment.

ICAM, intercellular adhesion molecule; LFA, leukocyte function-associated antigen; PECAM, platelet-endothelial cell adhesion molecule; PSGL, P-selectin glycoprotein ligand; VCAM, vascular cell adhesion molecule; VLA, very late antigen.

ligands is to capture circulating leucocytes to the blood vessel wall, where the combination of weak binding and direction of blood flow lead to leucocyte rolling along the luminal surface of the endothelium.

1.8.1.1 P-selectin and ligands

P-selectin (previously called GMP-140, PADGEM) is a protein molecule which was first identified in α -granules of platelets, but has subsequently been shown to be present in Weibel-Palade bodies (secretory storage granules) of endothelial cells [167]. The fusion of these granules with the plasma membrane leads to the rapid expression of P-selectin at the cell surface along with the release of other storage granule products (e.g. von Willebrand factor). Therefore, upon stimulation with histamine, thrombin or reactive oxygen species, pre-stored P-selectin may be rapidly mobilised within minutes to the luminal endothelial cell surface without *de novo* protein synthesis. The expression of P-selectin on the cell surface is transient, such that if the molecule is not engaged with a ligand then it will be reinternalised, either returning to storage granules, to lysosomes where they will be degraded, or shed extracellularly into the circulation [168-170]. P-selectin may be regulated at the transcriptional level by inflammatory mediators such as IL-1 β , TNF and LPS as determined by *in vitro* and *in vivo* experiments measuring mRNA [171].

The ligands for P-selectin that have been identified on leucocytes appear to be sialylated, fucosylated and/or sulphated carbohydrate structures (reviewed in [171-174]). Sialyl Lewis^x (sLe^x) and related tetrasaccharides such as sLe^a, have been proposed as ligands for P- and E-selectin based on blocking monoclonal antibody studies [175-178]. Although sLe^x has been shown to be expressed at high density on neutrophils and at lower density on monocytes, the binding affinity of this molecule with the selectins is weak.

A glycoprotein ligand which was originally identified by Moore and coworkers, has been extensively characterised (P-selectin glycoprotein ligand-1 (PSGL-1)) [179]. PSGL-1 is the major, high affinity ligand for P-selectin which is expressed on all leucocytes but at a lower density than sLe^x [180-183]. Although PSGL-1 is expressed on a wide range of leucocytes, the molecule can not function as a ligand for P-selectin unless it is correctly glycosylated and tyrosine sulphated [171, 174, 184-186]. PSGL-1 has been shown to be localised on microvilli of leucocytes in a similar manner to L-selectin, thereby providing greater accessibility for contact with endothelial structures [187]. The topographical advantage of PSGL-1 is further consolidated by a study showing that PSGL-1 is a highly extended molecule with the P-selectin binding domain at the N-terminus of the molecule. Furthermore, P-selectin itself has been shown to mediate neutrophil rolling only when P-selectin extends above the plasma membrane [188, 189].

1.8.1.2 E-selectin and ligands

E-selectin (ELAM-1) was first identified as a molecule that was expressed on cultured endothelial cells after stimulation with IL-1 β and TNF [190]. Unlike P-selectin, E-selectin expression requires *de novo* synthesis of mRNA and protein before expression on the surface of the endothelium. *In vitro* data shows that E-selectin expression is observed 1 hour post-stimulation, peaking at between 4-6 hours, with a return to basal levels after 24 hours [190]. The return to basal levels after 24 hours stimulation has been shown to result from downregulation of transcription [191], and internalisation or shedding of E-selectin [192]. E-selectin has been shown *in vitro* to support neutrophil rolling [193, 194].

As previously described, a major ligand for E-selectin is thought to be sLe^x. PSGL-1 is another ligand common to E- and P-selectin that binds to E-selectin with lower affinity and does not require tyrosine sulfation for binding [184-186, 195]. There is mounting evidence to suggest that PSGL-1 may be important in mediating T helper cell

recruitment to inflamed tissues expressing E- and P-selectin. PSGL-1 expressed on T helper 1 (Th1), but not T helper 2 (Th2) cells has been shown to be functionally active in supporting migration of Th1 cells into inflamed tissue where they produce cytokines involved in activating cytotoxic and inflammatory functions [196, 197]. Furthermore, activation of naive CD4+ T cells with the Th1 polarising cytokine IL-12, but not IL-4 (Th2 polarising), augmented binding to E- and P-selectin through increased expression of α 1,3-fucosyltransferase VII, an enzyme that is crucial for correct fucosylation of functional PSGL-1 [198, 199]. Similarly, an E-selectin specific ligand identified on murine neutrophils, ESL-1, is recognised by E-selectin only after correct fucosylation [200].

These ligands provide valuable evidence for the requirement for tight regulation of post-translational modification before recognition by the selectins. It has been proposed that sLe^x may be responsible for the initial interaction between leucocytes and endothelium, thus enabling subsequent interactions with higher affinity ligands such as PSGL-1 on the same cell [173].

1.8.1.3 L-selectin and ligands

L-selectin is the smallest of the three selectin molecules and is constitutively expressed on leucocytes. It was originally identified as the molecule responsible for lymphocyte homing, but has subsequently been shown to be expressed on neutrophils, monocytes, circulating T and B cells, and a subset of NK cells [201-204]. L-selectin has been localised to leucocyte microvilli, thus enabling optimal endothelial contact and efficient tethering to the vessel wall [205-207]. Upon leucocyte activation, L-selectin is actively shed from the plasma membrane at a cleavage site external to the membrane [208]. There is evidence to suggest that the enzyme responsible for cleavage of L-selectin is a matrix metalloproteinase termed "L-selectin sheddase" [209]. If L-selectin shedding is inhibited on activated neutrophils, rolling velocity is reduced and leucocyte

accumulation results [210, 211]. The shedding of L-selectin after leucocyte activation may act to control the number of leucocytes present at an inflammatory site.

The ligands recognised as the molecules involved in lymphocyte homing, were classified as the mucosal addressins, GlyCAM-1, CD34 and MadCAM-1 which are found in high endothelial venules of lymph nodes (reviewed in [164, 166]). Specific L-selectin ligands on non-lymphoid inflammatory endothelium is less clearly defined. *In vivo and in vitro* experiments have shown that monoclonal antibodies directed against L-selectin prevent neutrophil and monocyte rolling [194, 212-214] but not T cells [215, 216] on cytokine activated endothelium. However, the identification of specific ligands for L-selectin on cytokine stimulated, non-lymphoid endothelium remains to be determined. It has been proposed that sLe^x present on L-selectin, may function as a ligand for E- and P-selectin on activated endothelium [205]. Indeed there is recent evidence to suggest that a subset of sLe^x antigen, cutaneous lymphocyte antigen (CLA), can be induced on cytokine activated endothelium *in vitro*, acting as a functional ligand for L-selectin [217].

1.8.1.4 Leucocyte-leucocyte interactions

Another mechanism by which L-selectin may be involved in leucocyte recruitment to an inflammatory site involves leucocytes that roll on adherent leucocytes via L-selectin [218, 219]. This would enable leucocyte accumulation at sites of inflammation so that aggregates may form along the vessel wall. The counter structure for L-selectin-mediated leucocyte aggregation has been identified as PSGL-1 [220-222]. The elongated structure of PSGL-1 and its location on the microvilli, provides an ideal location to attract passing leucocytes via L-selectin, which itself is located on microvilli. Furthermore, adherent leucocytes have also been shown to rearrange their morphology such that a specialised uropod structure, extends into the vessel lumen. Uropods are highly enriched in ICAM-3, (a member of the Ig superfamily), and other leucocyte adhesion molecules, such that passing leucocytes may become attached and activated at

the site of inflammation without prior contact with the endothelium [223]. Therefore, there is evidence to suggest that in conjunction with leucocyte-endothelial interactions, leucocyte-leucocyte contact has a significant role in leucocyte recruitment to an inflammatory site.

1.8.2 Triggering

Once tethered at the endothelial surface, leucocytes require activation before the cascade can proceed. This signal is provided by chemoattractants and chemokines which are synthesised at an inflammatory site by both leucocytes and endothelial cells. They may have a direct effect in the circulation, but there is evidence to show that they are retained on proteoglycans such as heparan sulphate, or CD44 on the endothelial surface, thereby enhancing the concentration gradient at the inflammatory site [224-226]. Integrin-mediated adhesion occurs by means of intracellular signalling events resulting from chemokine interaction with specific chemokine receptors that are seven-membrane spanning G-proteins expressed on the surface of leucocytes [227]. The currently identified chemoattractants, chemokines, and their receptors are listed in Table 1.2.

The chemokines may be classified into families according to the amino acid sequence within the N terminal region. The CXC chemokines have two cysteine residues separated by another amino acid, the CC chemokines two cysteines in direct contact, whereas the C chemokines contain only one cysteine residue in the region. In the most recently identified CX₃C chemokines, fractalkine and neurotactin, the two cysteine residues are separated by three amino acids [228, 229]. Many chemokine receptors have the capacity to interact with more than one chemokine, but the precise reactivity profile of all receptors has yet to be determined (Table 1.2). Nevertheless, expression of chemokine receptors is restricted to specific leucocyte subsets.

| Chemokine Receptor | Major Cellular Distribution | Chemokines |
|---|--|--|
| <i>Classical Chemoattractant Receptors</i> | | |
| FPR | Neutrophils | N-formyl peptide |
| PAFR | Most leukocytes | Platelet Activating Factor |
| C5aR | Granulocytes, monocytes | C5a |
| <i>CXC Chemokine Receptors</i> | | |
| CXCR1 | Neutrophils, monocytes | IL-8, GRO- α , GCP-2 |
| CXCR2 | Neutrophils, monocytes, subsets of NK cells and CD8+ T cells, not CD4+ T cells | IL-8, GRO- α , NAP-2, MIP-2 |
| CXCR3 | Activated CD4+ and CD8+ T cells | IP10, MIG |
| CXCR4 | Wide variety of cell types | SDF-1 |
| CXCR5 | B cells | BCA-1 |
| <i>CC Chemokine Receptors</i> | | |
| CCR1 | Monocytes, memory T cells, granulocytes, NK cells | RANTES, MIP-1 α,β , MCP-1-3 |
| CCR2 | Monocytes, memory T cells | MCP-1-4 |
| CCR3 | Eosinophils, monocytes | eotaxin, RANTES |
| CCR4 | Peripheral T and B cells, monocytes, basophils | RANTES, MIP-1 α , MCP-1 |
| CCR5 | Monocytes, T cells | MIP-1 α, β , RANTES |
| CCR6 | CD4+ and CD8+ T cells B cells | MIP-3 α |
| CCR7 | Activated T and B cells | MIP-3 β |
| CCR8 | T cells, monocytes | TARC, 1309 |
| <i>C Chemokine Receptor</i> | | |
| Lymphotactin receptor | T lymphocytes | Lymphotactin |
| <i>CX₃C Chemokine Receptor</i> | | |
| CX ₃ CR1 | NK cells, T cells, monocytes | Fractalkine, neurotactin |

Table 1.2 Summary of currently identified classes of chemokine receptors, their distribution and reactivity profiles.

The chemokines may be classified into families according to the sequence surrounding cysteine residues in the N terminal region. In CXC chemokines the cysteine residues are separated by a single amino acid, while the CC chemokines have two cysteine residues adjacent to each other and the C chemokines only have a single cysteine residue in the region. In the recently identified CX₃C chemokines, fractalkine and neurotactin, the cysteine residues are separated by three amino acids. The chemokines can be further divided according to their reactivity with specific receptors which mediate the recruitment of particular leucocyte populations at a site of inflammation.

FPR, N-formyl peptide receptor; GRO, growth related gene; IP-10, gamma interferon inducible protein-10; MCP, monocyte chemoattractant protein; MIG, monokine induced by interferon gamma; MIP, macrophage inflammatory protein; NAP, neutrophil activating protein; PAFR, platelet activating factor receptor; RANTES, regulated activation, normal T cell expressed and secreted; SDF, stromal cell derived factor; TARC, thymus and activation-regulated chemokine.

1.8.2.1 CXC chemokine receptors

Neutrophils predominantly express CXCR1 and CXCR2, mediating neutrophil activation and potent chemotaxis to IL-8 [230], whereas other leucocytes expressing these receptors such as monocytes, basophils and eosinophils, have a weaker response to IL-8 mediated chemotaxis [230]. CXCR1 has been identified as having high affinity for IL-8 alone, whereas CXCR2 has high affinity for all other CXC chemokines that are chemotactic for neutrophils, and is found at higher density on other leucocytes [231]. IL-2 activated T lymphocytes upregulate expression of CXCR3 receptor, whereas resting T lymphocytes, B lymphocytes, monocytes and granulocytes were found to be negative for CXCR3 [227, 232]. CXCR4, the SDF-1 receptor, is widely distributed amongst a variety of cell types, making it unique from other CXC chemokine receptors, and indeed has been shown to function as a coreceptor for HIV infection of CD4+ T cells [233]. CXCR5 was found to be expressed Burkitt's lymphoma cells and B lymphocytes [234].

1.8.2.2 CC chemokine receptors

The CC chemokine receptors have a more promiscuous specificity, expressed on a wide variety of resting and activated T cell subsets, monocytes, dendritic cells and granulocytes (see Table 1.2). CCR1 was originally identified as the ligand for the CC chemokines RANTES and MIP-1 α , and subsequently found to bind to MCP-2 and MCP-3, and possibly MCP-4. CCR2, which has two RNA splice-variants, CCR2a and CCR2b, recognises MCP-1-4 [227]. CCR1 and CCR2 have been shown to be expressed on CD45RO+ peripheral blood lymphocytes only after pre-treatment with IL-2, suggesting that T cells may only be responsive to chemokines after the initial events of antigen-dependent activation [235]. CCR3 is the high affinity receptor for eotaxin, RANTES, MCP-3 and MCP-4, and is expressed primarily on eosinophils, but also on monocytes. CCR4 and CCR5, bind mainly RANTES and MIP-1 α . CCR4 mRNA has been detected in basophils treated with IL-5, peripheral T and B cells, and monocytes [236]. CCR5 has also been shown to bind to MIP-1 β in stably transfected CHO cells

and is preferentially expressed on Th1 cells, whereas CCR3 and CCR4 are mainly expressed on Th2 cells [237]. CCR6 and CCR7 are expressed on similar cell types, predominantly expressed on CD4+ and CD8+ T cells and B cells, but may differentiate between immature and mature dendritic cells, respectively [237]. CCR8 has been shown to mediate monocyte chemotaxis to the cytokine I-309 [238].

It is clear from the evidence above, that the production of specific chemokines at a site of inflammation will attract a particular leucocyte subpopulation expressing the appropriate chemokine receptors. This interaction enables intracellular signalling events to occur via the chemokine receptors, resulting in activation of integrin-mediated firm adhesion to the endothelium. Recent evidence suggests that there may be an alternative route by which integrins can be activated. Crosslinking and ligation of L-selectin on neutrophils has been shown to cause increased intracellular signalling events which result in activation of the β_2 integrins [239-242]. Taken together, these results suggest that the early events of selectin-mediated rolling and chemokine interactions with leucocyte receptors may act in concert to ensure that activation of integrin-mediated firm adhesion occurs on the appropriate leucocyte population.

1.8.3 Firm Adhesion

1.8.3.1 Leucocyte integrins

Firm adhesion is mediated by leucocyte integrin interactions with immunoglobulin superfamily (IgSF) structures on endothelium. The β_2 integrins share a common β -chain (CD18) and are comprised of three different non-covalently associated α -chains: CD11a (lymphocyte function-associated antigen-1, LFA-1), CD11b (Mac-1) and CD11c (p150,95). Upon leucocyte activation, LFA-1 rapidly undergoes a conformational change increasing its avidity for ligand, without increased surface expression [163, 164, 166]. The binding of LFA-1 with its ligands, ICAM-1, ICAM-2 and ICAM-3 (see below), is cation dependent [243-245], and may cause

multimerisation of LFA-1, further enhancing its ligand avidity [246]. Furthermore, activation of neutrophil phagocytic activity has been shown to be mediated through intracellular signalling events from ICAM-1 interactions with LFA-1 [247].

Mac-1 may be upregulated on the surface of neutrophils with a conformational change required to augment ligand avidity [166]. Mac-1 can also bind to ICAM-1 on the endothelium, but in addition, may function as a receptor for fibrinogen [248]. Furthermore, Mac-1 has been found to bind and internalise oligodeoxynucleotide, making it a possible target for antisense therapy [249]. The β 1-integrin VLA-4 ($\alpha_4\beta_1$) is involved primarily in lymphocyte adhesion, but is also expressed on monocytes, NK cells, basophils and eosinophils, but not neutrophils [163]. The activation of VLA-4 leads to an increase in avidity for its cytokine-induced endothelial ligand VCAM-1 [250]. VLA-4 has also been shown to bind to extracellular matrix proteins fibronectin and thrombospondin suggesting that it may play a role in transendothelial migration [163, 164].

1.8.3.2 Endothelial immunoglobulin superfamily (IgSF) structures

IgSF molecules expressed on endothelium are the counter ligand structures for leucocyte integrins. ICAM-1, ICAM-2 and ICAM-3 share distinct but homologous genes with different numbers of Ig domains and were identified by their ability to bind to the LFA-1 I domain. ICAM-1 is constitutively expressed on endothelial cells but may be upregulated upon cytokine stimulation [251]. ICAM-1 can also bind to Mac-1 via a site in the third Ig domain [252]. In contrast, ICAM-2 only has two Ig domains and appears to bind to LFA-1 with lower affinity than ICAM-1 [253, 254], and is not upregulated by cytokine stimulation [255]. The crystal structure for ICAM-2 has been elucidated and demonstrates the importance of the I domain of LFA-1 in ligand recognition [256]. ICAM-3 is the most recently identified member of this family of molecules expressed on resting and activated leucocytes [253, 254, 257]. ICAM-3 mediates leucocyte-leucocyte interactions by binding with the I domain of LFA-1 [258-

260]. The precise function of this interaction has yet to be determined, but it is possible that it has a central role in the recruitment of leucocytes to an inflammatory site via its expression on uropod structures, that extend into the vessel lumen from firmly adhered leucocytes [223].

Another member of the IgSF involved in leucocyte adhesion is VCAM-1, which is upregulated on stimulated endothelium with the peak of expression observed at 12-18 hours [261, 262]. Interaction with its ligand VLA-4, mediates lymphocyte arrest on endothelium, but it has also been shown to participate in lymphocyte rolling [263]. Although VLA-4 is not expressed on neutrophils [163], recent evidence suggests that the integrin $\alpha_9\beta_1$ expressed on neutrophils may mediate adhesion and transmigration through interactions with VCAM-1 [264].

1.8.4 Transendothelial Migration

Integrin-mediated firm adhesion results in morphological changes to the leucocyte causing it to flatten along the surface of the endothelium. The transitory nature of integrin interactions provides a mechanism for leucocytes to break their contact with the endothelium at their trailing edge as they migrate towards endothelial junctions. PECAM-1 (CD31), a member of the Ig superfamily, has been shown to be a critical molecule for leucocyte transmigration both *in vivo* and *in vitro* [265-267]. It is constitutively expressed on leucocytes and at tenfold higher levels on the surface of endothelial cells, where they form homotypic (e.g. PECAM-1 to PECAM-1) interactions [268, 269]. PECAM-1 has also been shown to bind heterotypically to heparan sulphate and glycosaminoglycans. Leucocyte migration is thought to occur via a haptotactic gradient of PECAM-1 at endothelial cell junctions [270]. The precise mechanism by which leucocytes migrate between endothelial cells has yet to be completely defined, but there is evidence to suggest that polymorphonuclear leucocyte adhesion causes the rearrangement of endothelial cell adherens junctions and that this is not mediated by proteases or oxygen reactive metabolites [271].

The adherence of neutrophils to endothelium has been shown to cause intracellular signalling events that results in structural rearrangements of cytoskeletal proteins and disruption of endothelial cell monolayers [272-274]. Lorenzon and colleagues demonstrated that, in addition to signalling events within adherent leucocytes, signalling was reciprocated to endothelial cells via neutrophil binding to endothelial E- and P-selectin and VCAM-1, resulting in structural rearrangements of endothelial cells [275]. These results suggested that following neutrophil adhesion, transmigration may be facilitated by intracellular signalling events within endothelial cells, causing rearrangement of actin filaments and disruption of endothelial junctions. Migration assays performed *in vitro* have demonstrated through selective inhibition with blocking antibodies that VLA-4, VLA-5, LFA-1 and CD44 may also be involved in migration through endothelial monolayers [164]. Following transmigration leucocytes traverse through the extracellular matrix using β_1 integrin-ligand interactions and are thus retained at the site of inflammation [276].

1.9 ADHESION MOLECULE EXPRESSION IN NORMAL AND TRANSPLANTED HUMAN KIDNEYS

1.9.1 Normal Kidney

In a normal kidney P- and E-selectin may be expressed at low levels on the endothelium of an occasional intertubular capillary and large vessel. ICAM-1 is constitutively expressed on endothelium whereas VCAM-1 is found on the parietal epithelial cells of the Bowman's capsule, weakly on the glomerular mesangium and may be detected on an occasional intertubular structure (either capillary endothelium or leucocyte). PECAM-1, as may be anticipated, is strongly expressed on the endothelium throughout normal kidneys and thus may be used as a marker to detect endothelial destruction in a transplanted kidney [277, 278].

1.9.2 Transplanted Kidneys

There have been many studies on the expression of adhesion molecules in biopsies from renal transplants with particular emphasis on the cytokine inducible molecules which may become upregulated during rejection.

In transplanted kidneys significant upregulation of E- and P-selectin may be detected, with strong expression on intertubular capillaries and larger vessels, often associated with areas of leucocyte infiltration [277, 278].

Upregulation of ICAM-1 can be clearly demonstrated following cytokine stimulation *in vitro*, but in the kidney, the basal level of endothelial expression is very high making it difficult to detect increased levels by normal histochemical methods. Nevertheless, ICAM-1 upregulation may be detected in proximal tubules following transplantation, where it is most strongly expressed at the brush border [277-280]. In addition, VCAM-1 expression may be upregulated on the endothelium of intertubular capillaries and large vessels, and on proximal tubular epithelium [277-281]. In contrast to ICAM-1, VCAM-1 expression occurs at the basolateral surface of proximal tubules, usually surrounding a nucleus.

Upregulation of all the cytokine inducible molecules is frequently focal, perivascular and associated with areas of interstitial infiltration rather than being a generalised phenomenon extending throughout the biopsy. Results from *in vitro* analyses have demonstrated that increased levels of adhesion molecules on the renal tubular epithelial cells may enhance the binding of alloreactive lymphocytes and augment cell mediated damage [281-284]. Furthermore, T cells cultured from renal allograft rejection biopsies have been shown to be tissue specific for donor-derived tubular epithelial cells, but not donor splenocytes [285, 286]. There is evidence to suggest that an antigen expressed by renal epithelial cells, E-cadherin, may be recognised by specific cytotoxic T cells that express the cognate $\beta 7$ -integrin ligand, ($\alpha^E\beta 7$) [287].

There have been fewer studies of adhesion molecule expression in renal allografts undergoing chronic rejection. A spectrum of staining patterns have been reported, but E-selectin was consistently absent from the renal endothelium and a more uniform expression of VCAM-1 was found on the intertubular capillaries than has been generally found in biopsies from acutely rejecting kidneys. In addition, induction of tubular VCAM-1 and ICAM-1 has been detected in chronically rejecting renal allografts [280, 288].

Analysis of adhesion molecule expression following ischaemia/reperfusion injury has not been reported in clinical renal transplantation. In human liver allografts, ICAM-1 expression has been detected in biopsies obtained before transplantation with upregulated expression in biopsies taken 2 hours after reperfusion in association with the induction of platelet and neutrophil inflammatory mediators [289]. It is of interest that in wedge biopsies obtained from cadaver kidneys before implantation, there is considerable variation between kidneys in the level of expression of endothelial E-selectin, P-selectin and VCAM-1 and tubular ICAM-1 and VCAM-1 [278], although induction of these molecules following reperfusion of renal allografts is unknown.

1.10 ISCHAEMIA/REPERFUSION INDUCED INFLAMMATORY RESPONSE

1.10.1 Endothelial Adhesion Molecules and Neutrophil Infiltration

Neutrophils are the most abundant leucocyte population in the bloodstream and are the first to appear at inflammatory sites. They are produced in the bone marrow at a rate of 10^9 cells/kg body wt/day, where they are stored before being released into the circulation in response to inflammatory stimuli, with a half-life of 7 hours in the circulation and only a few hours following extravasation [166]. The normal function of

neutrophils is to respond against invading microbes by the release of a complex assortment of agents, but may destroy normal cells and connective tissue as a consequence of this action. The inability of neutrophils to distinguish between foreign and host antigens may result in inappropriate damage to surrounding tissue. Activated neutrophils generate reactive oxygen species ($\cdot\text{O}_2^-$, OH^- , H_2O_2) via the NADPH oxidase enzyme system located on the cell membrane and release proteolytic enzymes (e.g. elastase, collagenase, gelatinase, myeloperoxidase) from intracellular granules that fuse to the membranes, releasing their products into the extracellular milieu. Therefore, neutrophils have the potential to act as mediators of tissue destruction in ischaemic injury [290, 291].

Many studies have shown that ischaemia/reperfusion injury leads to increased adhesion molecule expression and leucocyte infiltration. Results from animal models have demonstrated that neutrophils are a major component of ischaemia/reperfusion induced damage in whole organs [141, 292-297]. Investigations of ischaemia/reperfusion injury have implicated a major role for endothelial induction of E- and P-selectin and ICAM-1 for neutrophil recruitment. Animal studies have shown that the addition of blocking antibodies to selectins or sLe^x analogues either immediately before or during reperfusion, significantly prevented reperfusion injury [295, 298-303]. In addition, mice deficient in selectin and ICAM-1 expression have been shown to be protected against neutrophil-mediated damage following renal ischaemia and reperfusion [304-306]. However, the synergistic effect of all three selectins for leucocyte recruitment to inflammatory sites has been demonstrated in transgenic mice deficient for E-, P- and L-selectin in all combinations [307, 308]. In addition to neutrophil recruitment, L-selectin deficiency may also be protective through a second pathway, as binding of L-selectin has been shown to be necessary for potentiation of the neutrophil oxidative burst [240].

Upregulated endothelial selectin expression may preferentially recruit neutrophils which express high levels of sLe^x and the high affinity ligand PSGL-1 on their surface [171-

174]. Activation of neutrophils in the microvasculature and underlying tissue via molecules such as IL-8 and PAF may result in generation of oxygen free radicals and granular release of proteolytic enzymes [290]. Furthermore, the expression of endothelial selectins following ischaemia/reperfusion may initiate more severe inflammatory responses, with the selective recruitment of Th1 cells into the inflamed area [196, 197].

Many studies have been performed to examine the effects of hypoxia/reoxygenation on cultured endothelial cells, to simulate similar conditions to those experienced during ischaemia/reperfusion. Arnould and colleagues performed several studies to determine the effects of hypoxia on HUVEC by exposure to 100% nitrogen for periods between 30-120 min, followed by reoxygenation for 5 min in the presence of neutrophils. Their results demonstrated a time-dependent increase in unstimulated neutrophil adherence compared with normoxic controls, and this was abrogated by the administration of antibodies against ICAM-1, CD11b/CD18 and PAF receptor antagonists. Furthermore, neutrophil activation and free radical release were also observed when in contact with hypoxic HUVEC but not by hypoxia conditioned medium [309-311]. A similar study performed by Yoshida and colleagues demonstrated that in HUVEC exposed to 30 min hypoxia (93% N₂, 5% CO₂, 2% H₂) and reoxygenated for 30 min in the presence of neutrophils, increased neutrophil adherence was observed that was inhibited by blockade of β_2 integrin-ICAM-1 interactions. In contrast to the studies performed by Arnould and colleagues, incubation of neutrophils with conditioned medium from hypoxic HUVEC resulted in increased expression of CD11b, CD18 but not CD11a, and adherence to resting HUVEC [312].

There are additional disparities in the source of endothelium and the length of hypoxic exposure. Studies performed by Terada and colleagues, demonstrated that bovine pulmonary arterial endothelial cells exposed to 48 hours hypoxia (95% N₂, 5% CO₂) and reoxygenation showed increased XO/XDH activity resulting in generation of $\cdot\text{O}_2^-$,

endothelial damage and increased neutrophil adherence [112, 313]. Other investigators have demonstrated that exposure of endothelial cells to 5 hours of hypoxia and reoxygenation for periods up to 24 hours exhibited transient neutrophil adhesion at 30 min and a second adhesion response 4 hours after reoxygenation [314].

Many *in vitro* studies have been performed on endothelial cells exposed to exogenous reactive oxygen species (e.g. hydrogen peroxide, X/XO), which resulted in elevated expression of ICAM-1, PAF and P-selectin with increased binding of neutrophils [315-318]. It is likely that the recruitment and activation of neutrophils by endothelial cells following ischaemia/reperfusion may further aggravate the injury resulting from reactive oxygen species and interact with other inflammatory mediators.

1.10.2 Platelet Interactions

The endothelial damage resulting from ischaemia/reperfusion and neutrophil-mediated injury may initiate a chain of inflammatory events that further exacerbates this damage. Denudation of damaged endothelial cells may expose sub-endothelial matrix proteins of the vessel wall to the circulation, resulting in platelet attachment [319]. The physiological function of platelets is to limit blood loss at sites of vascular injury by formation of a mechanical plug. Platelets interact with matrix proteins such as collagen via the CD41/CD61 (α IIB β 3) integrin molecule, resulting in platelet activation. This is characterised by morphological alterations from discoid to spherical forms with the formation of extended, spiky pseudopods and the extrusion of secretory organelles [319]. Adhesion and activation facilitates the aggregation of platelets and neutrophils to the damaged area through the expression of CD41/CD61 and β 2-integrins [320]. Platelet aggregation may be mediated by either the release of endoperoxides/thromboxane A₂ or gelatinase A [321]. Furthermore, platelets may bind to activated, non-denuded endothelium via bridging interactions between CD41/CD61 and fibrinogen with endothelial ICAM-1 [322], where they may induce endothelial expression of chemokines and adhesion molecules [323, 324]. Moreover, adherent

platelets have been shown to function in a similar manner to endothelial monolayers, mediating neutrophil rolling, attachment and transmigration [325-329].

Although platelets may be involved in the process of vascular repair, it is possible that they may contribute to the pathophysiology of ischaemia/reperfusion injury by the recruitment of neutrophils. Platelets may be activated by exposure to reactive oxygen species resulting from ischaemia/reperfusion [330, 331]. Activated platelets express P-selectin enabling binding to neutrophils via PSGL-1 [332], resulting in activation of Mac-1, aggregation and potentiation of inflammatory mediators [333-335]. There is evidence to suggest that platelet P-selectin expression may be critical in neutrophils interactions, providing an additive adverse effect on ischaemia/reperfusion injury [336-338]. Accumulation and aggregation of platelet and neutrophil complexes is likely to contribute to the no-reflow phenomenon as a result of capillary plugging [339, 340].

1.10.3 Inflammatory Cytokines and Chemokines

The generation of an acute inflammatory state within post-ischaemically reperfused organs may be facilitated by the expression of acute phase cytokines such as IFN γ , IL-1 β , TNF and IL-6, and chemotactic factors, IL-8 and PAF. IL-1 β and TNF are produced by activated monocytes and macrophages and play a major role in coordinating the acute phase inflammatory response, inducing expression of adhesion molecules and other inflammatory mediators on endothelial cells [341]. In a syngeneic rat renal transplant model, IL-1 β and TNF were expressed within 15 min of reperfusion after periods of warm and cold ischaemia [342]. Daemen and colleagues demonstrated that administration of neutralising antibodies against TNF significantly attenuated reperfusion injury of warm ischaemic kidneys [343].

IL-6 is synthesised by monocytes and macrophages, but may be induced in endothelial cells following IL-1 β and TNF stimulation [344]. IL-6 is involved in the regulation of acute phase responses [345] and also induces adhesion molecule expression on

endothelial cells. There is evidence to suggest that IL-6 may be upregulated following endothelial hypoxia with subsequent alterations in barrier function [346].

IL-8 is produced by a variety of cell types including monocytes, macrophages and endothelium and is a potent neutrophil chemotactic and activating factor [224, 347, 348]. IL-8 expression may be induced by a variety of stimuli but has been shown to be synthesised by endothelial cells in response to reactive oxygen species [349, 350]. It is attached onto the surface of endothelial cells via proteoglycans and presented to rolling leucocytes where it may interact with specific receptors expressed on neutrophils, CXCR1 and CXCR2, resulting in activation of the β 2-integrins necessary for neutrophil adhesion and chemotaxis [348].

Platelet activating factor (PAF) is synthesised by a variety of cells including platelets, monocytes and endothelium. Reactive oxygen species have been shown to induce the expression of PAF on endothelial cells [315, 351]. Furthermore, PAF levels rise following reperfusion of ischaemic tissue and leucocyte adherence to endothelium is significantly reduced with a PAF receptor antagonist [312, 352, 353]. Similar to IL-8, interaction of PAF with its leucocyte receptor initiates the upregulation and conformational change of the integrin molecules, enabling firm adhesion to endothelial adhesion molecules [351].

It is evident from these studies that the generation of reactive oxygen species and an inflammatory response following ischaemia/reperfusion injury, is critically controlled by the endothelial expression of cytokines, chemokines and adhesion molecules. Therefore, it is likely that the expression of these molecules following transplantation may contribute to inflammatory and alloimmune responses within the graft.

1.11 ISCHAEMIA/REPERFUSION INJURY IN CLINICAL RENAL TRANSPLANTATION

Investigation of ischaemia/reperfusion injury in clinical renal transplantation is limited. Several studies have demonstrated elevated levels of malondialdehyde (a marker of lipid peroxidation and free radical damage) in plasma obtained from patients following reperfusion of the graft, but no correlations with subsequent graft function were examined [354-356].

During the early period of transplantation, analysis of renal allograft biopsies was frequently performed to determine histological changes associated with hyperacute rejection. In biopsies obtained approximately one hour after reperfusion of the graft, neutrophils were detected in the glomerular capillaries and diagnosis of hyperacute rejection presumed, but it may be likely that in some of these biopsies, the presence of neutrophils represented ischaemia/reperfusion injury [357-361]. Gaber and colleagues examined one hour post-revascularisation renal biopsies that demonstrated the presence of polymorphonuclear leucocytes to be significantly associated with prolonged cold storage times [362]. However, comparison with biopsies obtained before reperfusion was not performed, and thus were unable to determine whether the neutrophils had infiltrated upon reperfusion or were donor neutrophils present as a result of previous inflammatory events.

1.12 THERAPEUTIC STRATEGIES FOR ISCHAEMIA/ REPERFUSION INJURY

1.12.1 Organ Preservation Solutions

1.12.1.1 Static cold storage

It is evident from experimental and clinical studies that prolonged cold ischaemic storage of organs has detrimental effects on initial graft function. Thus, organ preservation solutions have been developed to minimise the harmful effects of cold ischaemia.

During the early periods of organ transplantation, the method of preservation relied mainly on simple surface cooling of the organ in ice [363]. Cooling was further enhanced by flushing blood from the organ with a cold preserving solution containing solutes that mimicked plasma constituents. However, modern preservation solutions were developed from initial studies by Collins and colleagues, demonstrating that a crystalloid preserving solution containing high concentrations of potassium and phosphate to mimic the intracellular milieu, markedly improved organ preservation [364]. Since the development of Collins' solution, a wide variety of preservation solutions with modifications in their content have been employed with similar levels of success. Belzer's University of Wisconsin solution enabled extended storage times for pancreas, liver, kidney, heart, lung and small bowel transplantation [365].

The principle aims of organ preservation are to cool the organ rapidly and uniformly by an intravascular flush containing solutes that may limit the effects of cellular oedema and improve preservation of the vasculature, parenchyma and interstitium. Modern preservation solutions contain impermeable molecules to provide an extracellular osmotic force to oppose the development of oedema, which would otherwise result

from loss of cellular membrane ionic pumps. Therefore molecules such as lactobionate, gluconate, sucrose, citrate and mannitol are included into preservation solutions to prevent oedema [77]. Furthermore, effective buffers (e.g. citrate, phosphate, bicarbonate, histidine) are necessary for countering intracellular acidosis. Addition of electrolytes to preservation solutions (e.g. sodium, potassium, chloride) may be less important, with no significant differences between solutions containing diverse ranges of electrolyte concentrations [77].

The constituents of the four major preservation solutions employed in transplantation are presented in Table 1.3. Euro-Collins' solution contains high concentrations of potassium and phosphate with sucrose as the main impermeant. Hyperosmolar Citrate (HOC) solutions differ marginally from Euro-Collins', with citrate acting as the buffer and mannitol as the impermeant. Results for kidney preservation were comparable to those obtained for Euro-Collins' solution [366, 367]. Bretschneider's HTK solution contains large and relatively impermeable solutes such as histidine and mannitol, with low concentrations of electrolytes, buffering by histidine and free radical scavenging properties of tryptophan, histidine and mannitol. Results from a randomised multicentre trial demonstrated that HTK solution was more effective than Euro-Collins' solution in reducing the incidence of delayed graft function in renal transplantation [368]. By comparison, the University of Wisconsin (UW) solution is more complex than other solutions, with lactobionate as the impermeant, a phosphate buffer, free radical inhibitors (allopurinol and glutathione), vasoactive agents and hormones (steroids, insulin) and a colloid (hydroxyethylstarch). The benefits of preservation with UW solution have been demonstrated in liver transplantation, enabling extended periods of cold storage [369]. In clinical renal transplantation, it has been shown that preservation in UW solution significantly improved primary function and one year graft survival compared with Euro-Collins' solution [370]. Similar results were obtained when compared with HTK solution [371]. Despite the beneficial effects of UW solution for prolonged cold storage of cadaveric kidneys, the high cost of preservation with UW

solution has resulted in many transplant centres preserving kidneys with conventional solutions, with a greater effort on minimising long cold ischaemia times.

1.12.1.2 Machine perfusion preservation

Continuous, pulsatile machine perfusion has also been employed for organ preservation. This method originally described by Belzer and colleagues [372], has been employed in some preservation protocols as a replacement for normal static preservation. Beneficial effects have been described for the continuous hypothermic perfusion of kidneys obtained from marginal donors (e.g. non-heart-beating donors, elderly, long cold ischaemia times), with significant reductions in the incidence of delayed graft function and abnormal histological changes [373-376]. However, despite the beneficial effects observed with pulsatile perfusion of marginal donor organs, the increased cost, practical difficulties of transporting perfusion machines and lack of a significant advantageous effect on conventional donor organs has meant that machine perfusion is not routinely practised.

1.12.2 Post-Storage Reflush

Several studies have been performed to analyse the effects of perfusing kidneys after the period of cold storage but before transplantation, with conventional preservation solutions to remove toxic ischaemic products from the kidneys. Early studies demonstrated a significant reduction in the incidence of delayed graft function [377, 378], but a subsequent larger, prospective randomised study demonstrated no beneficial effect of reflushing the kidneys [379].

1.12.3 Preconditioning

It has been well established that brief periods of ischaemia followed by reperfusion increases the tolerance of a variety of tissues to a subsequent ischaemic challenge [74, 380-383]. The mechanism of ischaemic preconditioning is poorly understood but may involve adenosine A1 receptors, ATP-sensitive K⁺ channels, heat shock protein

induction, or induction of free radical scavengers. Ischaemic preconditioning of rat kidneys for four periods of 4 min warm ischaemia and 11 min reperfusion, prior to the main ischaemic insult of 30 min followed by reperfusion, demonstrated no beneficial effects on renal function or tissue integrity [384].

Preconditioning of organs may also be achieved by thermotolerance whereby organs are exposed to increased temperature with the aim of inducing expression of cytoprotective heat shock proteins to protect the organ against subsequent ischaemia/reperfusion injury. [385]. Exposure of rat kidneys to a temperature of 41°C for 15 min, 18 hours before ischaemia/reperfusion injury significantly improved renal function, inhibited neutrophil infiltration and attenuated upregulation of ICAM-1 and MHC Class II antigens [386]. Nevertheless, it is unlikely that exposure of human kidneys to repeated periods of warm ischaemia or increased organ temperature would become acceptable in clinical practice.

1.12.4 Inhibition of Endothelial-Leucocyte Adhesion

A major focus of research in prevention of reperfusion injury has been the inhibition of endothelial-leucocyte interactions. Antibodies against endothelial-leucocyte adhesion molecules that potentially disrupt stages of the adhesion cascade (e.g. P- and E-selectin ligand interactions, ICAM-1 binding to β 2-integrins) have been shown to be effective in preventing neutrophil infiltration following reperfusion of occluded ischemic tissue [295, 298-300, 302, 303, 387, 388].

In models of renal warm ischaemia created by *in situ* occlusion of the renal vessels, inhibition with antibodies against ICAM-1-ligand interactions has been shown to attenuate reperfusion injury [389-391]. In addition, models of *in situ* cold ischaemia and reperfusion have been studied to simulate the physiological conditions encountered in renal transplantation. Takada and colleagues demonstrated elevated expression of E-selectin with an accompanying neutrophil infiltration were detected within 6 hours of

reperfusion, with increased expression of MHC Class II antigens, B7 costimulatory molecules, Th1 and macrophage-associated cytokines, 2 to 5 days post-reperfusion, and long term induction of MCP-1, endothelin and iNOS expression with progressive deteriorating renal function [392-395]. Treatment with either soluble PSGL-1 [392, 393] or CTLA4Ig (which blocks B7 costimulation) [394, 395] significantly attenuated expression of all inflammatory and immunological molecules, with reduced leucocyte infiltration and improved renal function.

In experimental renal transplant models where kidneys were exposed to prolonged periods of cold ischaemia, treatment with PAF antagonists significantly reduced neutrophil infiltration and improved renal function and survival [396, 397]. Furthermore, novel antisense oligodeoxynucleotide therapy for ICAM-1 significantly reduced ICAM-1 expression, neutrophil infiltration, with improved primary graft function and prolonged graft survival in rat renal autografts [398-400].

There are many possible adhesion molecule interactions which, if disrupted, may modulate the immune response to an allograft, but in renal transplantation the interaction between LFA-1 and ICAM-1 has been most extensively targeted. In cynomologous monkeys an anti-ICAM-1 antibody has been used as the sole immunosuppressive therapy for renal allografts and was found to delay allograft rejection significantly when compared to untreated controls [401]. The distribution of ICAM-1 in monkey is similar to that found in human and there was no evidence of vascular damage in the antibody treated animals. The same antibody gave encouraging results when used in a Phase I clinical trial in combination with conventional immunosuppression for patients with a high risk of delayed graft function (recipients of allografts with long cold ischaemia times and highly sensitised patients). The incidence of delayed graft function and rejection was significantly decreased in those patients with adequate circulating antibody levels [402]. Despite the promising results from these initial trials, a large, randomised

multicentre trial has been performed with an anti-ICAM-1 monoclonal antibody that demonstrated no beneficial effects on DGF or acute rejection [403].

In addition, clinical studies have been performed using antibody therapy directed towards LFA-1. The antibody was ineffective in reversing rejection [404], but was used prophylactically for the first 10 days following transplantation in conjunction with azathioprine and corticosteroids with more encouraging results. Cyclosporine was withheld for the first 9 days and the results showed a decreased incidence of rejection in the first month after transplantation in patients receiving antibody therapy [405]. The prophylactic use of anti-LFA-1 has been compared to ATG in a multicentre randomised study where the antibody appeared to decrease the incidence of delayed graft function. While there were no significant differences in the number of rejection episodes between therapies, more of the rejection episodes occurred in the early post transplant course in LFA-1 treated patients.

1.12.5 Antioxidant Therapy

Exposure of tissue to extended periods of ischaemia results in a gradual decline in cellular antioxidant defences against reactive oxygen species generated at reperfusion [74, 141, 406]. Free radical scavengers that are present in preservation solutions (e.g. allopurinol, glutathione, ascorbate) have not been shown to be specifically effective in preventing reperfusion injury and reducing the incidence of DGF. Therefore, development of more effective antioxidant therapies has been investigated.

1.12.5.1 Novel antioxidants

A number of novel antioxidants have been developed to inhibit the detrimental effects of reactive oxygen species generated following ischaemia/reperfusion injury in experimental models. Infusion of multivitamins containing antioxidants such as Vitamin E was shown to inhibit lipid peroxidation with beneficial effects on renal graft function [407]. Cold storage of rat kidneys for 24 hours with lazaroids (a lipid peroxidation

inhibitor) significantly reduced the level of lipid peroxidation and expression of inflammatory cytokines at day 7 post-transplant [408]. Novel bioflavonoid compounds such as quercetin and curcumin have also been shown to inhibit the effects of renal warm ischaemia resulting in preservation of histological integrity, improved renal function and reduction in the expression of inflammatory mediators [409].

1.12.5.2 Superoxide dismutase

Many studies have demonstrated a beneficial effect of administering superoxide dismutase (SOD) against ischaemia/reperfusion injury [117-120, 122-126]. In renal transplantation, early experimental studies demonstrated that intravenous infusion of SOD at reperfusion significantly improved graft function [117, 119]. Furthermore, transfection of endothelial cells with human CuZn-SOD cDNA significantly improved cell viability following exposure to 24 hours cold preservation and 4 hr rewarming [410] and attenuated ICAM-1 upregulation by TNF [411]. In animal models of organ ischaemia, transgenic mice overexpressing the human CuZn-SOD gene demonstrated a reduction in intracellular superoxide generation and infarct size of ischaemic hearts [412], whilst direct gene therapy with a recombinant adenoviral vector expressing human mitochondrial superoxide dismutase significantly attenuated ischaemia/reperfusion injury of livers in the mouse [413].

In clinical renal transplantation, treatment with recombinant human superoxide dismutase (rhSOD) has been performed in two separate trials. Pollak and coworkers administered half of a 20mg/kg rhSOD solution immediately before reperfusion and the remainder one hour post-reperfusion intravenously [121]. No significant differences were observed between the treatment (n=58) and placebo (n=58) groups with respect to glomerular filtration rate and creatinine clearance up to day 6 post-transplantation, although long term analysis was not performed. Land and colleagues administered a single, higher dose (200mg rhSOD) intravenously just prior to reperfusion and demonstrated that although no immediate benefits were observed in the incidence of

DGF in patients given superoxide dismutase (n=81), a significant reduction in first acute rejection episodes (35% vs 18%) and improved 1 year (86% vs 78%) and 4 year (74% vs 52%) graft survival were observed compared with the placebo (n=96) group [123, 414].

1.13 AIM OF THESIS

The superior outcome of living unrelated donor renal allografts compared to cadaveric donors that undergo similar immunological challenges, suggests that detrimental physiological events in cadaveric transplantation, such as cold ischaemic damage and traumatic events surrounding brain death, may significantly influence long term graft survival. Furthermore, modern preservation techniques have not been effective in reducing the incidence of DGF in kidneys with prolonged periods of cold ischaemia. Moreover, DGF has been shown in many studies to have an additive adverse effect with acute rejection on long term graft survival, suggesting that the inflammatory events of ischaemia/reperfusion injury may play a major role in this process. The results of the rhSOD trial performed by Land and colleagues supports the hypothesis that ischaemia/reperfusion injury may contribute towards early inflammatory and immunological events within the graft, with subsequent effects on long term graft survival. However, the precise inflammatory events that occur in the donor before transplantation and subsequent ischaemia/reperfusion injury in clinical renal transplantation remains to be determined.

The aims of this Thesis were to characterise by immunohistology, the inflammatory events that arise in cadaver donor kidneys prior to transplantation and changes resulting from reperfusion, in comparison with healthy LRD kidneys with minimal ischaemia times. Furthermore, refushing cadaveric kidneys after cold storage was examined to determine whether or not this procedure afforded a beneficial effect on reperfusion injury and delayed graft function. In addition, a novel free radical scavenging agent was

obtained to determine whether incorporation of this agent into preservation solutions protected endothelial cells from hypoxic injury and whether in a rat renal allograft model of prolonged cold ischaemia, preservation with the agent afforded protection against inflammatory events and graft dysfunction in both the short and long term.

CHAPTER 2. MATERIALS AND METHODS

2.1 PATIENTS AND BIOPSY MATERIAL

Renal wedge biopsies from cadaver and living-related donor (LRD) kidney allografts performed at the Oxford Transplant Centre were obtained at three time points before and after transplantation. (i) Organ retrieval: after nephrectomy, perfusion with cold Marshall's hypertonic citrate solution (Baxter Healthcare Ltd, Berkshire, UK), but before cold storage; (ii) pre-reperfusion: after cold storage but before implantation of the kidney; (iii) post-reperfusion: approximately 20-40 min after anastomosis and clamp release, but before wound closure. Biopsies were placed in labelled cryovials (Greiner Labortechnik Ltd, Gloucestershire, UK) and snap frozen in liquid nitrogen before storage at -80°C.

Following transplantation all patients received standard triple therapy immunosuppression (cyclosporine, azathioprine and steroids) [415]. Donor information and clinical parameters were obtained from the Oxford Transplant Centre database for statistical analyses.

2.2 IMMUNOHISTOCHEMICAL STAINING

Snap frozen wedge biopsies were embedded in O.C.T. (Miles Inc. USA), and 7µm sections were cut using a Bright cryostat microtome. The sections were air-dried before fixation with 100% acetone (BDH, Lutterworth, UK) for 10 min. Fixed sections were

stored at -30°C until required for staining. All staining procedures were performed in an humidified chamber at room temperature.

2.2.1 Immunoperoxidase Staining

Optimal working dilutions of primary monoclonal antibodies were determined by doubling dilutions of the antibodies on positive control sections (e.g. normal kidney, acute rejection biopsy), to obtain maximal signal with minimal background staining. The sections were incubated with primary monoclonal antibody (see Table 2.1) diluted in phosphate buffered saline (PBS) and 0.5% bovine serum albumin (BSA; Sigma Ltd, Gillingham, Dorset, UK) for 30 min. After washing in PBS, monoclonal antibody (mAb) bound to the sections was detected by incubating with a peroxidase-conjugated rabbit anti-mouse Ig (1:100 dilution) (DAKO Ltd, Ely, Cambridgeshire, UK) for 30 min. in which anti-human antibody activity was blocked by pre-incubation with 20% human AB serum. The signal for E- and P-selectin was enhanced by incubating with a peroxidase-conjugated swine anti-rabbit Ig (1:50 dilution) (DAKO Ltd) which was also pre-blocked using 20% human AB serum. The reaction was developed using 600µg/ml 3,3'-diaminobenzidine tetrachloride (DAB; Sigma, UK) and 0.009% H₂O₂ (Thornton and Ross Ltd, Huddersfield, UK) for 7 min, counterstained with Harris' Haematoxylin (Merck Ltd., Atherstone, UK) for 15 secs and rinsed with water. The sections were then dehydrated via a series of graded ethanol baths (100%, 90% and 70% for 1 min in each), cleared with xylene and mounted in DPX mountant (Merck Ltd, UK).

2.2.2 Double-Immunofluorescent Staining

Double immunofluorescent staining was performed to clarify the origin of the increased expression of P-selectin detected in the post-reperfusion biopsies. All incubations were performed for 30 minutes at room temperature in the dark. Acetone fixed cryosections of post-reperfusion biopsies were first incubated with antibodies to either P-selectin (IgG₁) or ICAM-1 (IgG_{2a}) (see Table 2.1) and following washing, bound antibody was detected with the appropriate Texas Red-conjugated, isotype-specific, goat anti-mouse

| Antigen Specificity | Antibody Clone | Experiment | Source (Reference) |
|---------------------|----------------|--------------|---|
| CD3 | UCHT-1 | IP | [416] |
| CD14 | UCHM-1 | IP | [417] |
| CD31 | BBA7 | IP, FACS | British Biotech. Ltd, UK |
| CD31 (IgG2a) | 7E4 | IF | Walter Knapp, Institute of Immunology, University of Vienna, Austria |
| CD41 | 5B12 | IP | Dako Ltd, Bucks, UK |
| CD45 | F10.89.4 | IP | [418] |
| CD68 | EBM/11 | IP | [419] |
| CuZnSOD | SD-6 | IF, FACS | Sigma Ltd, UK |
| Dog Thy-1 | F3.20.7 | IP, IF, FAC | [420] |
| E-selectin (CD62E) | 5D11 | IP, FACS | British Biotech. Ltd, UK |
| HLA-A2/B17 | MA 2.1 | IP | [421] |
| HLA Class I | PA2.6 | IP, FACS | [422] |
| HLA-DR | Cyto-DR | IP, FACS | Coulter Immunol, USA |
| ICAM-1 (CD54) | 14C11 | IP, FACS | British Biotech. Ltd, UK |
| ICAM-1 (IgG2a) | MEM111 | IF | Vaclav Horejsi, Institute of Molecular Genetics, Prague, Czech Republic |
| Neutrophil elastase | NP57 | IP | DAKO Ltd, UK |
| PAF (CD109) | 8A3 | FACS | Robert Sutherland, Oncology Research Lab, Toronto Hospital, Canada |
| P-selectin (CD62P) | G1 | IP, IF, FACS | [423] |
| VCAM-1 (CD106) | 4B2 | IP, FACS | British Biotech. Ltd, UK |

Table 2.1. List of primary monoclonal antibodies against human antigens used in this Thesis.

The isotype of the monoclonal antibodies was IgG1 unless otherwise stated. Abbreviations: IP= immunoperoxidase staining, IF= immunfluorescent staining, FACS= flow cytometry analysis.

Ig antibodies (1:50 dilution) (Southern Biotechnology Associates, Birmingham, Alabama, USA) pre-incubated with 20% human AB serum. Sections were then incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-CD41 (platelet-specific) antibody (5B12, DAKO Ltd). After final washing, slides were mounted in Vectashield™ (Vector Labs, Peterborough, UK) to preserve fluorescence and analysed by fluorescent microscopy. The specificity of the staining procedure was confirmed by including isotype control antibodies to ensure that there was no non-specific binding of the secondary antibodies. All antibodies were used in isolation to check that the binding was in no way altered by the double staining protocol.

2.3 PREPARATION AND MAINTENANCE OF HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVEC).

2.3.1 Isolation of Endothelium from Umbilical Vein

Umbilical cords were obtained from the Delivery Suite, Department of Obstetrics and Gynaecology, John Radcliffe Hospital, Oxford, UK, and stored for a maximum period of 3 hours at 4°C prior to endothelial cell isolation in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, UK) supplemented with 100U/ml penicillin and 100µg/ml streptomycin (PAA Laboratories Ltd, Kingston-upon-Thames, UK). All procedures were performed in a sterile flow cabinet with autoclaved instruments. Isolation of umbilical vein endothelial cells was adapted from the method of Jaffe *et al* [424].

Umbilical cords were washed with PBS and external surfaces of the cord cleaned with 70% ethanol (BDH, UK). Once the vein was located, 50 ml of DMEM medium was perfused through the vein to remove blood and related constituents from the vessel lumen. The cord was clamped at one end, and 10ml of 0.01% (w/v) collagenase solution (Type IA-S; Sigma, UK) was carefully administered until the vein was

distended, and clamped to seal the collagenase solution within the vein before being suspended in a 37°C water bath for 12 min. Following this period, the collagenase solution containing the endothelial cells was flushed from the cord by perfusion with 40 ml DMEM using a Jencons Powerpette Plus pipette to remove endothelial cells from the vessel lumen. The effluent was collected in a sterile 50 ml centrifuge tube (Greiner Labortechnik Ltd, Gloucestershire, UK) and denuded endothelial cells were pelleted by centrifugation at 300 g for 5 min, and washed three times with DMEM to remove any residual collagenase. Finally, the endothelial cell pellet was resuspended in 5 ml of M199 medium (Sigma, UK) supplemented with 20% heat inactivated foetal calf serum (FCS; PAA Labs, UK) (heat inactivated at 56°C for 30 min to destroy potential complement activity), 2mM L-glutamine (PAA Labs, UK), 1x endothelial cell growth factor (Sigma, UK), 100U/ml penicillin and 100µg/ml streptomycin (PAA Labs, UK) and cultured in 50ml tissue culture flasks (Greiner, UK) pre-coated with 1% (w/v) gelatine (Sigma, UK) for 1 hour at 37°C. Endothelial cells were incubated at 37°C in an humidified incubator in 95% air and 5% CO₂.

2.3.2 Maintenance and Passaging of HUVEC

Growth of HUVEC were carefully monitored and supplemented M199 medium was changed daily in each flask until HUVEC monolayers were confluent. Passaging was performed by washing once with 15 ml PBS, incubation with 15 ml PBS/1mM EDTA at 4°C for 10 min, followed by 10 min incubation at 37°C. The flasks were gently shaken to detach the endothelial cells from the flasks and placed into 15 ml centrifuge tubes (Greiner, UK) for centrifugation at 300 g for 5 min. Passage was performed at 1:3 dilution in supplemented M199 medium and cells were never used beyond passage 4 for all experiments. HUVEC cells were identified by their characteristic cobblestone morphology in confluent monolayers following culturing under selective conditions for endothelial cell growth, and for their expression of CD31 determined by FACS analysis.

2.4 CULTURE AND MAINTENANCE OF A HUMAN MICROVASCULAR ENDOTHELIAL CELL LINE, HMEC-1.

2.4.1 Source and Culture Conditions of HMEC-1

Frozen stocks of a human microvascular endothelial cell line (HMEC-1) were obtained from the Centre for Disease Control (Emory University, Atlanta, GA, USA) [425]. HMEC-1 is an immortalised human microvascular endothelial cell line derived from the transformation of endothelial cells (isolated from neonatal foreskin) with a pBR322 construct containing the SV40 large T antigen [425]. The cells were maintained in culture in MCDB-131 medium (Sigma, UK) supplemented with 15% heat inactivated FCS (PAA Labs, UK), 10ng/ml epidermal growth factor (Sigma, UK), 1µg/ml hydrocortisone (Sigma, UK), 100U/ml penicillin and 100µg/ml streptomycin (PAA Labs, UK) and incubated at 37°C in 5% CO₂ and 95% room air under humidified conditions.

2.4.2 Maintenance and Passaging of HMEC-1

Once the cells had reached 80% confluence (~Day 4-5), they were washed once with PBS and passaged by incubation with PBS/1mM EDTA at 4°C for 10 min, followed by 10 min incubation at 37°C. Passage was performed at 1:3 dilution in supplemented MCDB-131 and cells were never used when retarded growth and senility were noticed, usually observed beyond passage 18.

2.5 PHENOTYPIC CHARACTERISATION OF ENDOTHELIAL CELLS

2.5.1 Cytokine Stimulation

Confluent monolayers of HMEC-1 and HUVEC were stimulated with the following cytokines according to previously published concentrations [190, 425, 426]: 1000U/ml recombinant human Interferon- γ (IFN γ) (Cambridge Biosciences, Cambridge, UK) for 72 hours, 200U/ml recombinant human Tumour Necrosis Factor (TNF) (Cambridge Biosciences, UK) for 6 hours, and 4U/ml recombinant human Interleukin-1 β (IL-1 β) (Cambridge Biosciences, UK) for 6 hours. Cytokine stimulation was performed in 10 ml of the appropriate supplemented medium at 37°C in 5% CO₂ and 95% room air under humidified conditions.

2.5.2 Preparation of Cells for FACS Analysis

Following the period of cytokine stimulation, cells were washed twice with PBS and removed from the flasks using the passaging methods described above to preserve surface antigens (as opposed to trypsinisation). Endothelial cells were aliquotted equally into 5ml FACS tubes (Becton-Dickinson Immunocytometry Systems, Oxford, UK) (approximately 1.0 - 2.0 x 10⁵ cells/tube), and pelleted at 300 g for 5 min at 4°C. 50 μ l of the appropriate mAb (see Table 2.1) were added to the tubes, cells resuspended and incubated on ice for 45 min. Following this period cells were washed three times with PBS/1% FCS at 4°C and then incubated with 50 μ l of secondary, FITC-conjugated goat anti-mouse Ig (1:50 dilution) (Sigma, UK) for a further 45 min in the dark on ice. After three washes in cold PBS/1% FCS, cells were fixed with FACS fixative (1.0 % formalin solution in PBS/1% FCS) and stored at 4°C in the dark until analysis. Data acquisition was performed on a FACSort flow cytometer (Becton-Dickinson, Immunocytometry System, CA, USA) and analysed using CellQuest™ software.

2.6 SOURCE OF LECITHINISED-SOD (LEC-SOD), RECOMBINANT HUMAN CUZn-SOD (RHSOD) AND LECITHIN

Lec-SOD, rhSOD and lecithin were kindly provided by the Seikagaku Corporation, Tokyo, Japan. Lec-SOD was synthesised by the covalent linkage of 4 molecules of an active ester form of lecithin to rhSOD (Figure 2.1) [427]. Stock solutions of lec-SOD and rhSOD were supplied in 5% mannitol at concentrations of 42 mg/ml and 100 mg/ml, respectively, and stored at -30°C until required. The pharmacological activity of lec-SOD and rhSOD per 100µg protein was reported as 300U and 510U, respectively (as determined by the xanthine/xanthine oxidase system [428]). Lecithin was reconstituted to a concentration of 100mg/ml in sterile water.

2.7 CHEMILUMINESCENT ANALYSIS OF RHSOD AND LEC-SOD ACTIVITY

The pharmacological activity of rhSOD and lec-SOD was determined using a xanthine/xanthine oxidase system based on a method by Skatchkov et al [429]. 2.0 ml of Kreb's HEPES Buffer pre-heated to 37°C (99mM NaCl, 4.7mM KCl, 1.2mM MgSO₄, 1.9mM CaCl₂, 25mM NaHCO₃, 11.1mM glucose, 20mM HEPES and 1.0mM KH₂PO₄) was added to a cuvette with 1.0µM Cypridina luciferin analog (CLA) (a kind gift from Dr. Keith Channon, Department of Cardiovascular Medicine, University of Oxford, Oxford, UK) and 200µl of the test sample (\pm SOD). The background level of luminescence was measured over a 2 min period in the luminometer (Turner Design TD20-20, UK). Chemically induced superoxide generation was performed by the addition of 10µl xanthine oxidase (0.002U; Sigma, UK) and 80µl xanthine (400nM; Sigma, UK) and the level of luminescence produced from superoxide reactivity with CLA recorded for 5 min. The data was analysed using AcqKnowledge™ software and the level of background luminescence produced over 2 min subtracted from the level of

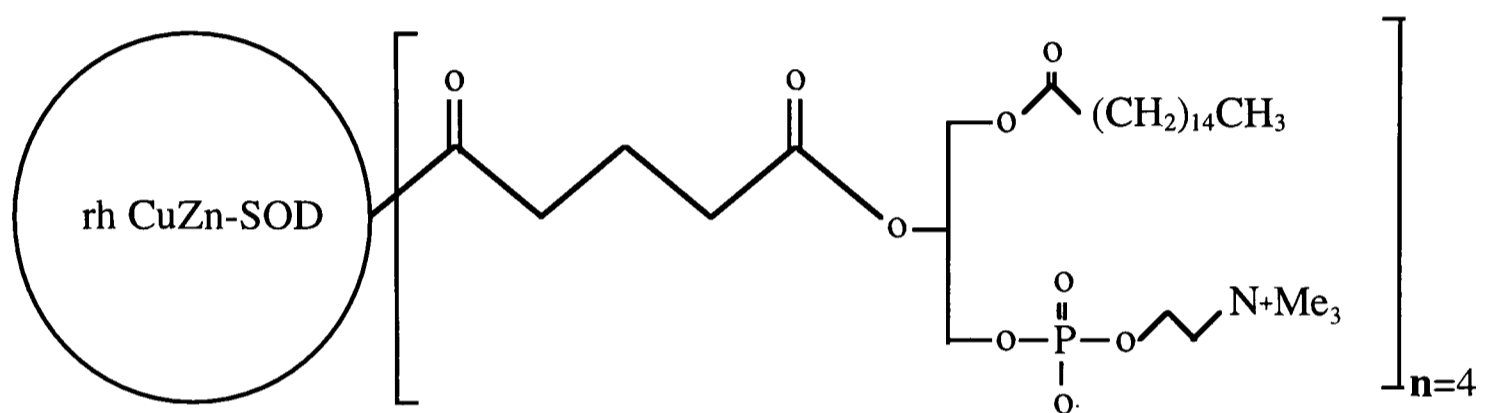


Figure 2.1. Chemical structure of lecithinised superoxide dismutase (lec-SOD).

Lec-SOD was formulated by the covalent linkage of recombinant human CuZn-SOD with an average of 4 molecules of lecithin.

luminescence produced in 5 min following addition of X/XO and expressed as the rate of luminescence/min.

2.8 INCORPORATION OF LEC-SOD INTO MARSHALL'S PRESERVATION SOLUTION

Endothelial cells were cultured on 6-well plates (Greiner, UK) until confluence. Cells were incubated with varying concentrations of lec-SOD and rhSOD incorporated into Marshall's preservation solution (Baxter Healthcare Ltd, UK) for 1, 3, 12 and 24 hours at 4°C. Following this period, the wells were washed twice with PBS, detached (see above) and transferred to 5ml FACS tubes (Becton-Dickinson, UK) for centrifugation at 300 g for 5 min. The cell pellet was resuspended in 50µl of anti-CuZn-SOD mAb (Sigma, UK) and incubated on ice for 45 min. Following two washes in PBS, anti-SOD mAb was detected with a FITC-conjugated goat anti-mouse Ig (1:50) for 45 min in the dark, on ice. Cells were washed twice with PBS and fixed with FACS fixative before data analysis by flow cytometry.

2.9 BINDING OF LEC-SOD TO ENDOTHELIAL CELLS

2.9.1 Culture on Glass Coverslips

Sterile glass coverslips were coated with 10% (w/v) collagen solution Type I (Sigma, UK) diluted with PBS for 2 hours at 37°C. Washing was performed with PBS throughout. Unbound collagen was washed off and 1×10^5 endothelial cells in 200µl of supplemented medium were spread carefully onto the surface of the coverslips and left overnight in an humidified 37°C incubator to adhere. Once cells had firmly adhered, 2ml/well (6-well plate, Greiner, UK) of supplemented medium was added and incubated until confluent.

2.9.2 Confocal Laser Microscopy Analysis of lec-SOD Binding

Endothelial cells were cultured to confluence on collagen-coated glass coverslips as described above. Analysis for the internalisation of lec-SOD was performed by a double-immunofluorescent staining procedure using mAb against the endothelial surface marker, CD31 (an IgG2a mAb) (Table 2.1), and an anti-SOD mAb (IgG1 mAb). Confluent monolayers of HUVEC were incubated with 50µg/ml lec-SOD for 3 hours, washed twice with PBS and fixed with Reagent A (Cytoperm™ kit, Serotec Ltd, UK) for 15 min. The anti-SOD mAb was diluted 1:10 with the Permeabilisation solution (Reagent B) and incubated with the cells for 30 min. Cells were washed twice with PBS before incubation with a FITC-conjugated secondary goat anti-mouse Ig (Sigma, UK) for 30 min in the dark. All subsequent procedures were performed in the dark. Following two further washes in PBS, the cells were incubated with the anti-CD31 mAb for 30 min, washed twice in PBS, and stained with a Texas Red-conjugated goat anti-mouse IgG2a antibody (1:50 dilution) (Southern Biotechnology Associates Inc., Birmingham, Alabama, USA) for a further 30 min. The cells were washed twice with PBS, fixed with FACS fixative and mounted with Vectashield™ to preserve fluorescence (Vector Labs, UK). Cells were stored at 4°C in the dark until confocal microscope analysis. Cellular staining was analysed on a Zeiss LSM410 confocal laser microscope performed in the School of Biological and Molecular Sciences, Oxford Brookes University, Oxford, UK.

2.9.3 Cell Permeabilisation Assay for FACS Analysis

HMEC-1 and HUVEC were prepared for incubation with primary antibody as described above. Intracellular levels of SOD were detected using the Cytoperm™ kit (Serotec Ltd, Oxford, UK) according to the manufacturer's protocol. Briefly, the cells were resuspended after removal from the coverslips, fixed with a formaldehyde-based reagent (Reagent A) for 15 min, washed twice with PBS and incubated for 30 min at room temperature with primary antibody diluted 1:10 in a Permeabilisation solution (Reagent B). The cells were then washed twice with PBS and incubated on ice with a

secondary FITC-conjugated goat anti-mouse Ig (Sigma, UK) for 45 min in the dark. After two further washes, the cells were fixed with FACS fixative before analysis by flow cytometry.

2.10 *IN VITRO* HYPOXIA/ REOXYGENATION MODEL

A perspex hypoxia chamber has been designed and was constructed by the Department of Anaesthetics, John Radcliffe Hospital, Oxford, UK (Figure 2.2). Confluent endothelial monolayers in culture flasks or plates were placed inside the chamber with the lids removed to expose the medium to the hypoxic environment, and the chamber sealed with high vacuum grease (Philip Harris Ltd, London, UK). Hypoxic conditions were created by perfusion through the chamber with 95% nitrogen and 5% CO₂ until 0% oxygen was detected via a Class T-7 Teledyne Oxygen Sensor (Viamed, Keighley, UK) analysed with a TED 60T oxygen meter (Viamed, UK). Perfusion was continued for a further 15 min before the chamber was completely sealed via 3-way taps. The chamber was then placed in a 37°C incubator or at 4°C depending on the required conditions. Following the period of hypoxia, cells were washed once with PBS and fresh supplemented medium added before reoxygenation at 37°C in an humidified incubator with 95% air and 5% CO₂.

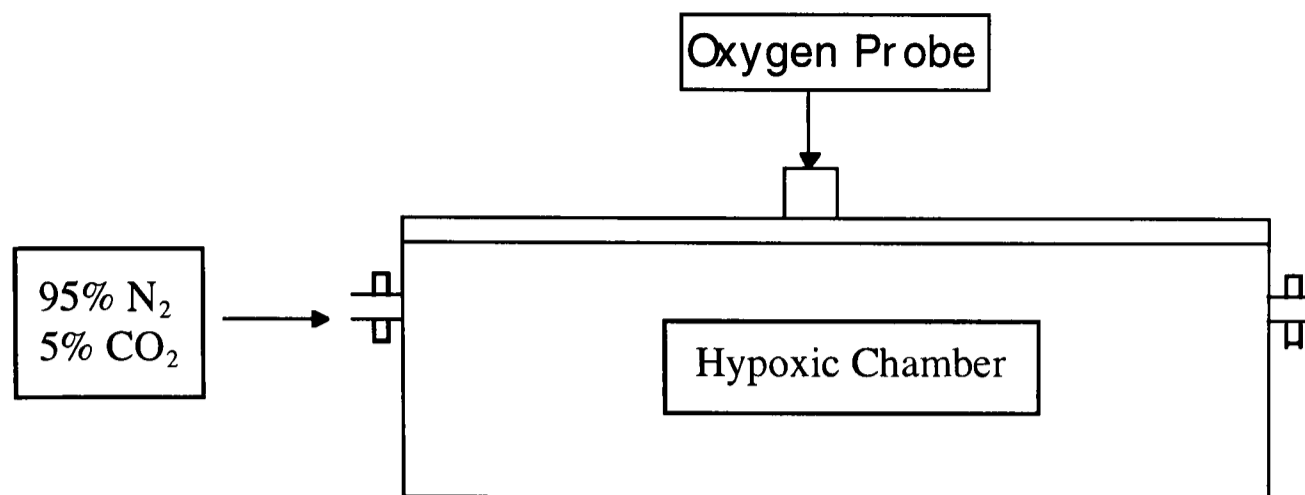


Figure 2.2. Perspex hypoxic chamber for *in vitro* hypoxia/reoxygenation experiments.

2.11 CELL VIABILITY DETERMINED BY TRYPAN BLUE EXCLUSION STAINING

Endothelial cells were cultured in 6-well plates (Greiner, UK) until they had attained confluence. Wells were washed twice with PBS and then incubated under cold hypoxic conditions (95% N₂ and 5% CO₂) (see 2.10) for 18, 24 and 27 hours in the presence of 1ml of lec-SOD, rhSOD, lecithin or lecithin and rhSOD unconjugated in Marshall's solution. Following cold hypoxia, the supernatant (Supernatant A) was transferred to 15ml centrifuge tubes (Greiner, UK), stored at 4°C, and 2ml of supplemented medium added to each well for reoxygenation at 37°C in a humidified 5% CO₂ incubator for 24 hours. Following reoxygenation, supernatant (Supernatant B) was removed from each well and transferred to the same 15ml centrifuge tubes containing the post-cold hypoxia supernatant (Supernatant A + B). Remaining adherent cells were carefully detached by gentle pipetting following incubation with 2ml PBS/1mM EDTA at 4°C for 10 min, and 37°C for 10 min and transferred to the 15ml centrifuge tubes (Supernatant A + Supernatant B + Adherent Cells = Total Cells). Total viable and dead cells were spun at 300 g for 5 min and then resuspended in 200µl supplemented medium. Percentage cell viability was calculated following staining of cell suspension with 0.6% Trypan Blue,

and the number of viable (unstained) and dead (blue) cells counted in a Neubauer haemocytometer.

2.12 NEUTROPHIL-ENDOTHELIAL CELL ADHESION ASSAY

2.12.1 Purification of Neutrophils

50 ml of fresh peripheral blood were obtained from healthy volunteers and anticoagulated with 15% EDTA in 4.5ml Vacutainer™ tubes (Becton Dickinson, UK). The blood was transferred to 50ml Falcon tubes and centrifuged at 210 g for 10 min, following which the platelet-rich plasma was carefully aspirated and discarded. Separation of red blood cells from the remaining blood constituents was performed by dextran sedimentation. 5ml of sterile 10% dextran solution (200-300,000 MW, ICN, Ohio, USA) was added to the blood and the total volume brought up to 40ml with 0.9% saline. The tube was inverted four times and red blood cells were allowed to settle at room temperature for 30 min. The supernatant containing leucocytes was carefully transferred to a fresh 50 ml centrifuge tube (Greiner, UK) and centrifuged for 10 min at 210 g. Following centrifugation, the supernatant was removed and the leucocyte pellet resuspended in 12ml 0.9% saline. 3ml of Lymphoprep™ 1077 (Gibco, UK) was layered underneath the cell suspension and then centrifuged at 470 g for 15 min. The supernatant and lymphocyte layer were carefully removed and discarded, and the remaining pellet resuspended in 10 ml 0.2% saline for exactly 40 sec at 4°C to lyse any remaining red blood cells. Immediately after this period, 10 ml of 1.6% saline at 37°C was added to the suspension to return the solution to physiological conditions. The cell suspension was then centrifuged at 470 g for 5 min and the cell pellet resuspended in sterile PBS (Gibco, UK) to a concentration of 1×10^7 cells/ml. Cell viability was assessed by Trypan Blue exclusion staining and purity of the preparation determined by staining with Rapid Romanowski Stain (HD Supplies, UK) to identify

polymorphonuclear (PMN) leucocytes (Figure 2.3). 96-99% viability of neutrophils was observed and cell purity of >99% was obtained for each experiment.

Purified neutrophils were labelled with 50 μ Ci of sodium chromate (^{51}Cr) (Amersham International plc, Bucks, UK) at 37°C for 1 hour. Neutrophils were then washed three times with ice-cold Hank's balanced salt solution (HBSS; PAA Labs, UK) by resuspending in 50 ml HBSS and centrifugation at 300 g for 5 min at 4°C, to remove unincorporated ^{51}Cr . The neutrophils were then resuspended in HBSS to a concentration of 1×10^7 cells/ml and stored at 4°C until the assay was performed. The maximum period between the end of the ^{51}Cr -labelling and the final assay did not exceed 4 hours.

2.12.2 Hypoxia/Reoxygenation of Endothelial Cells

96-well plates (Greiner, UK) were pre-coated with 40 μ l/well of 50 μ g/ml fibronectin solution (Boehringer Mannheim, Lewes, UK) for 45 min at room temperature. The wells were washed once with PBS and 1×10^4 endothelial cells in 180 μ l supplemented medium were seeded into each well. Upon confluence, the monolayers were washed once in PBS, and 50 μ l of fresh supplemented medium (\pm SOD) added to each well. The plates were placed inside the hypoxic chamber and exposed to 1 hour hypoxia (95% N_2 and 5% CO_2) at 37°C (see section 2.10). Following this period, the monolayers were washed once with PBS, and 180 μ l fresh supplemented medium added to each well for reoxygenation at 37°C in a humidified 5% CO_2 incubator for specified periods of time (see Chapter 6).

2.12.3 Neutrophil-Endothelial Cell Adhesion (NECA) Assay

After hypoxia/reoxygenation of endothelial cells, 5×10^5 neutrophils were added to each well in a volume of 50 μ l to achieve a neutrophil:endothelial cell ratio of 10:1. Hypoxia/reoxygenation stimulated endothelial cells were incubated with neutrophils for 30 min at 37°C after the period of reoxygenation. After 30 min, the supernatant was

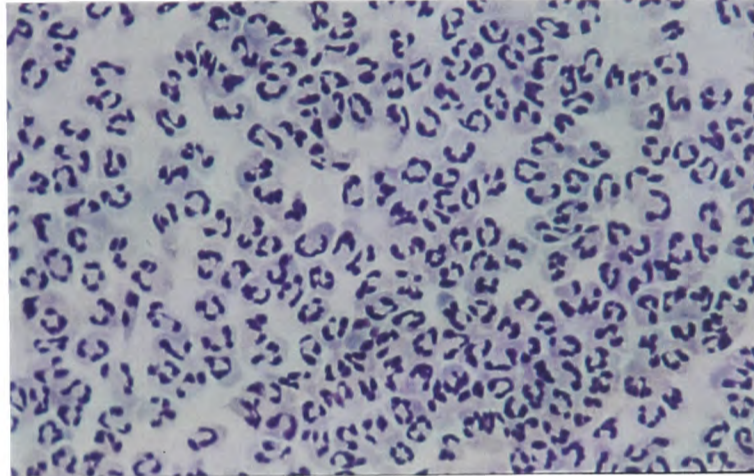


Figure 2.3. Purified human neutrophils from fresh whole blood.

Neutrophils were isolated from peripheral blood obtained from healthy volunteers by a Dextran sedimentation technique. Neutrophil purity was assessed following Rapid Romanowski staining and observations by light microscopy. The purity for each neutrophil preparation was >99%, with very occasional eosinophils detected. (magnification x400).

removed, transferred onto a Spot-On filtermat (Wallac, Milton Keynes, UK) and 50µl of HBSS added to each well using a multichannel pipette. This first washing step was carefully performed by tilting the plate to an angle of 45° and gently pipetting 50µl of HBSS down the inside of the wells with the multichannel pipette. The wash was then removed from the wells maintaining minimal disturbance to the monolayers to ensure uniformity for each washing step. This was transferred to the filtermats and the washing process to remove non-adherent neutrophils was repeated a total of three times. After the final wash, the adherent neutrophils and endothelial cells were lysed with 50µl 0.1M sodium hydroxide and transferred to filtermats. Filtermats were dried overnight and placed inside Sample bags (Wallac, UK) filled with 8ml of scintillant fluid (Wallac, UK), heat-sealed and gamma-emission quantified on a Wallac 1205 Betaplate Counter (Wallac, UK). The percentage neutrophil adherence was calculated by the following formula:

$$\% \text{ PMN adherence} = \frac{\text{Cell lysate (CPM)} (x100)}{(\text{supernatant (CPM)} + 3x \text{ wash (CPM)} + \text{lysate (CPM)})}$$

2.13 PHENOTYPIC STUDY OF ENDOTHELIAL CELLS FOLLOWING HYPOXIA/REOXYGENATION

Endothelial cells were cultured in 250ml tissue culture flasks (Greiner, UK) till confluence. The medium was removed and replaced with 5ml of fresh supplemented medium (\pm SOD). Flasks were then placed inside the hypoxic chamber and perfused with 95% N₂ and 5% CO₂ through a sterile glass pipette for 15 min after 0% oxygen was detected to ensure thorough exposure of the medium to hypoxia. Hypoxic conditions were maintained by taping the perimeter of the hypoxic chamber lid for this

period. After 15 min, the pipette and tape were removed, and the hypoxic chamber sealed with vacuum grease and gassed with 95% N₂ and 5% CO₂ for a further 5 min before placing the sealed chamber into a 37°C incubator for 1 hour. Following hypoxia, the flasks were removed from the chamber, the medium removed, washed once with PBS, and 10ml of fresh supplemented medium added to the flasks prior to 4 hour reoxygenation in the humidified 37°C incubator (95% air and 5% CO₂).

After the period of reoxygenation, the medium was removed and washed once with PBS, before detachment of the endothelial cell monolayers from the flasks with 15 ml PBS/1mM EDTA for 10 min at 4°C, and 10 min at 37°C. The cells were aliquotted into 5 ml FACS tubes (approximately 1.0 - 2.0 x 10⁵ cells/tube), and pelleted at 300 g for 5 min at 4°C. The cell pellet was resuspended in 50µl of the appropriate mAb (Table 2.1) and incubated on ice for 45 min. Cells were washed twice in PBS/1% FCS before incubation with a FITC-conjugated goat anti-mouse Ig for 45 min in the dark and on ice. After two further washes the cells were fixed in FACS fixative and analysed by flow cytometry on the FACSort cytometer with CellQuest™ software.

2.14 EXPERIMENTAL ALLOGENEIC RENAL TRANSPLANTATION

2.14.1 Renal Transplant Procedure

The rat renal transplantation procedure was performed by Dr. Ken Nakagawa. Inbred male rats (250-300g) (Harlan Olac Ltd, Bicester, UK) were used for allogeneic renal transplants performed from donor Fischer rats (F344, RT1^{lv1}) to recipient Lewis rats (LEW, RT1^l). The microsurgical procedure was performed using an operating microscope (Carl Zeiss, West Germany). All animals were fasted overnight. A xifopubic incision was performed on anaesthetised donor rats and the renal vessels and ureter mobilised after ligation of the adrenal and gonadal veins. The animals were

heparinised and the main vessels clamped before both kidneys were flushed through the aorta with 5 ml of ice-cold Marshall's hypertonic citrate solution (Baxter Healthcare Ltd, Berkshire, UK). Kidneys were removed and cold stored for either short periods of cold storage (1 hour), or long cold ischaemia (18 hours) in Marshall's solution alone, or with Marshall's containing 50µg/ml lec-SOD (a kind gift of Seikagaku Corp., Japan). 18 hours cold ischaemia was determined as optimal for the experimental protocol from Lewis rat renal isografts transplanted into bilaterally nephrectomised recipients, resulting in delayed graft function immediately following transplantation, but with 100% graft survival 2 weeks post-transplant.

Recipient rats were fasted overnight, anaesthetised, the aorta and vena cava dissected and clamped, the left native kidney removed, and an end to side anastomosis of the graft renal vessels to the recipient aorta performed with a running 10-0 nylon suture. After the release of the clamps an end to end ureteric anastomosis was performed by an interrupted 10-0 suture. Recipients received low-dose cyclosporine A (5mg/kg/day) for 10 days to prevent early rejection. Groups of animals were sacrificed at day 1, day 3 and 24 weeks post-transplantation, and renal tissue snap frozen in liquid nitrogen for immunohistology. In the recipients maintained for 24 weeks, the right native kidney was removed 10 days post-transplantation.

2.14.2 Renal Function

Renal function was determined by measurement of proteinuria. Urine samples were collected at 4 week intervals from rats placed in metabolic cages for 24 hours, up to and including 24 weeks post-transplant. Protein excretion was determined by measuring precipitation after interaction with 3% sulfosalicylic acid (Sigma, UK). Turbidity was assessed by absorbance measurements at a wavelength of 595nm.

2.14.3 Immunohistology

Snap frozen rat renal tissue was prepared for immunohistochemistry in an identical manner to that described in Section 2.2 for human renal biopsy material and stained using an identical indirect immunoperoxidase technique. Briefly, cryosections were stained with monoclonal antibodies (Table 2.2) diluted with 0.5% BSA in PBS for 30 min, washed with PBS and incubated with a peroxidase-conjugated rabbit anti-mouse IgG (1:50 dilution) (Serotec, UK) for 30 min, pre-blocked with 20% rat serum (DAKO Ltd, UK). The signals for MHC Class I, ICAM-1, CD45 and ED1 were amplified with a further incubation step for 30 min using a peroxidase-conjugated swine anti-rabbit Ig (1:50 dilution) (Serotec, UK) pre-incubated with 20% rat serum. Monoclonal antibodies against CD3 and HIS48 were of the IgM isotype and thus were developed with a peroxidase-conjugated goat anti-mouse IgM (1:50 dilution) (Serotec, UK) and enhanced with a rabbit anti-goat Ig (1:200 dilution) (Sigma, UK), both pre-incubated with 20% rat serum.

| Antigen Specificity | Antibody Clone | Source |
|----------------------------|-----------------------|---|
| CD3 (IgM) | IF4 | Serotec, UK |
| CD45 | OX1 | Professor K.J. Wood Nuffield Department of Surgery Oxford |
| Granulocytes (IgM) | HIS48 | Serotec, UK |
| Macrophages | ED1 | Serotec, UK |
| ICAM-1 | TLD-4C9 | Serotec, UK |
| MHC Class I | F16-4.4 | Professor K.J. Wood Nuffield Department of Surgery Oxford |
| MHC Class II | OX3 | Professor K.J. Wood Nuffield Department of Surgery Oxford |

Table 2.2. List of primary monoclonal antibodies against rat antigens for immunohistochemical analysis.

The isotype of the monoclonal antibodies was IgG1 unless otherwise stated.

**CHAPTER 3. CADAVER VERSUS LIVING RELATED
DONOR KIDNEYS: IMPACT OF DONOR FACTORS ON
ADHESION MOLECULE AND HLA -DR ANTIGEN
EXPRESSION**

3.1 INTRODUCTION

In clinical renal transplantation, allografts from living-related donors (LRD) have superior graft function and survival when compared to cadaver allografts [1, 430, 431]. LRD kidneys are obtained from carefully screened, healthy individuals who are genetically related to the recipient, whereas cadaver donor kidneys are transplanted into unrelated recipients and may have experienced physiological changes associated with brain death and undergone prolonged cold storage times. In living-unrelated donor (LURD) transplantation, the immunological barriers are similar to those encountered with cadaveric allografts, but the clinical outcome of LURD allografts mismatched for two HLA haplotypes, is significantly better than cadaveric transplantation, and similar to one haplotype disparate LRD transplants [1-7].

The interpretation of these studies resulted in several groups suggesting controversially that HLA matching could be omitted from transplantation protocols in an effort to reduce the detrimental effects of longer cold ischaemia times [432, 433]. However, the additional time required for shipping of organs to better matched recipients within an organ allocation scheme was not found to have a significant impact on graft survival, with a minimal increased risk of delayed graft function in the face of significantly improved outcome of zero-mismatched allografts [53, 434] Furthermore, recent evidence has demonstrated that when graft survival for LURD renal allografts were

stratified according to the degree of HLA matching, 5 year graft survival declined proportionally with the number of HLA mismatches [7]. Nevertheless, graft survival of cadaver renal allografts from donors under 50 years of age with zero-HLA mismatches was inferior to zero-mismatched LURD allografts, and identical to LURD allografts with 3 mismatches [7]. Thus, the differences between graft survival of LURD and cadaver renal allografts must partly be attributable to factors other than histocompatibility.

The high success rates of LURD transplantation probably reflects the use of organs obtained under optimal conditions. Living donors are carefully selected following examination for conditions such as diabetes and hypertension, whereas cadaver kidney donors do not undergo such close scrutiny. In addition, cadaver donors experience traumatic physiological abnormalities associated with brain death which may result in pathophysiological damage to the donor organs. It is possible that early damage to cadaveric organs may render them more susceptible to harmful physiological and immunological events following transplantation.

The effects of the host immune response against the allograft have been studied by analysing post-transplant biopsies. During episodes of acute rejection, leucocyte infiltration is detected in association with upregulated expression of adhesion molecules and HLA Class II antigens (reviewed in [435]). Interestingly, several studies have shown that varying levels of expression of these molecules may be detected in biopsies from cadaver donor kidneys before transplantation [278-280, 436-439]. However, in many of these studies only a few pre-transplant biopsies were analysed, thus precluding the meaningful analysis of possible donor-derived factors that may be involved in the induction of antigen expression. The presence of high levels of HLA Class II antigens and adhesion molecules in donor kidneys may be indicative of inflammatory events before transplantation and provide a mechanism for subsequent leucocyte recruitment into the graft and initiation of an alloimmune response.

In this chapter, the expression of adhesion molecules and HLA-DR antigens has been analysed in LRD and cadaver donor kidneys prior to transplantation, and the levels of expression related to donor parameters. Furthermore, high levels of antigen expression in kidneys before transplantation were examined for significant associations with subsequent post-transplant rejection or impaired graft function.

3.2 MATERIALS AND METHODS

3.2.1 Patients and Biopsy Material

An immunohistochemical analysis was performed on renal biopsies obtained from two groups of renal transplant donors: (i) cadaveric donors (n=65); and (ii) living-related donors (n=29). As controls, 5 biopsies were obtained from cadaver donor kidneys at the time of organ retrieval, prior to cold perfusion and storage.

Relevant donor and clinical parameters for cadaver and living-related donors are listed in Table 3.1. Donor data was obtained from Kidney Donor Information Forms completed by the Donating Centres and recipient surgeons and supplied by the United Kingdom Transplant Support Service Authority (UKTSSA).

3.2.2 Monoclonal Antibodies and Immunohistochemistry

7 μ m cryostat sections from wedge biopsies were stained using an indirect immunoperoxidase technique (see Chapter 2). The sections were stained with monoclonal antibodies against adhesion molecules E-selectin, P-selectin, ICAM-1, VCAM-1 and HLA-DR, and anti-leucocyte markers CD45, CD3, CD14, CD68 and neutrophil elastase (see Table 2.1). Detection of E- and P-selectin was enhanced by an additional incubation step with a peroxidase-conjugated swine anti-rabbit Ig (DAKO Ltd) which was pre-blocked with human AB serum. The isotype control anti-dog Thy-1 antibody resulted in completely negative staining on all biopsies.

| Clinical Parameters | Cadaver (n=65) | LRD (n=29) |
|---|-----------------|---------------|
| Donor Sex (M:F) | 33:32 | 7:22 |
| Donor Age (years) \pm SD | 40 \pm 14.7 | 43 \pm 10 |
| ^a Trauma at Death | 22/65 | N/A |
| Ventilator Support (Days) \pm SD | 2.17 \pm 1.45 | N/A |
| Episode of Cardiac Arrest | 12/65 | N/A |
| Inotropic Support | 42/65 | N/A |
| Desmopressin (DDAVP) Treatment | 18/65 | N/A |
| Donor Infection | 17/65 | N/A |
| Urine Output (final hour) (ml) \pm SD | 194 \pm 197 | N/A |
| Rate of Urine Output (ml/hour) \pm SD | 183 \pm 110 | N/A |
| Blood Urea (mmol/L) \pm SD | 5.9 \pm 2.7 | N/A |
| Donor Serum Creatinine (μ mol/L) \pm SD | 94 \pm 38 | N/A |
| Donor Blood Transfusion | 16/65 | N/A |
| Local vs Shipped Kidney (L:S) | 48:17 | N/A |
| Multi/Single Organ Donor (M:S) | 56:9 | N/A |
| ^b Cold Ischaemia Time (hours) \pm SD | 25.1 \pm 10.0 | 1.9 \pm 0.5 |
| ^c Delayed Graft Function | 10/65 | 6/29 |
| Serum Creatinine (3 months) (μ mol/l) \pm SD | 153 \pm 51 | 141 \pm 37 |
| Serum Creatinine (6 months) (μ mol/l) \pm SD | 154 \pm 48 | 140 \pm 29 |
| ^d No. of Rejection Episodes (0:1:2:3) | 30:22:6:7 | 12:12:5:0 |
| Rejection by Day 7 Post-Transplantation | 11/65 | 5/29 |

Table 3.1 Demographics of donor and clinical parameters following renal transplantation.

^a Traumatic death was defined by the Donating Centre as death resulting from road traffic accidents or other forms of severe physical injury

^b Significant difference between LRD and cadaver donors ($p < 0.01$)

^c Delayed graft function was defined as the requirement for dialysis in the first week after transplantation.

^d Acute rejection was defined as an elevation of serum creatinine ($>15\%$) above baseline and a response to anti-rejection therapy (98% biopsy proven)

3.2.3 Assessment of Staining

Semi-quantitative staining was scored by two independent observers without prior knowledge of the clinical information. Leucocyte infiltration was analysed with respect to localisation in (i) the glomeruli; (mean number of positive cells/glomerulus per section) (ii) intertubular areas; mean leucocyte number per field of view (x10 objective). The entire biopsy was analysed in each case. The semi-quantitative grades given for endothelial expression of E- and P-selectin were scored as:

Grade 0 - negative

Grade 1 - negative with an isolated positive vessel

Grade 2 - focus of positive vessels or occasional positive vessels throughout the biopsy

Grade 3 - multiple foci or a single focus with positive vessels distributed throughout the biopsy.

The semi-quantitative grading for ICAM-1, VCAM-1 and HLA-DR antigens was based on proximal tubular expression and was scored as, Grade 0 - negative or weakly positive; Grade 1 - strongly positive.

3.2.4 Statistical Analyses

Student's T-test, Fisher's exact and χ^2 tests were performed to analyse the results of the immunohistological staining with respect to clinical parameters. Furthermore, multiple logistic regression using the SPSS statistical program (version 8.0) was performed to confirm or refute univariate comparisons between elevated antigen expression in cadaver kidneys and possible predictive donor parameters. Hosmer-Lemeshow χ^2 test in this model determined whether the elevated antigen expression provided a reasonable fit with donor parameters.

3.3 IMMUNOHISTOLOGICAL DIFFERENCES BETWEEN CADAVER AND LIVING-RELATED DONOR KIDNEYS

3.3.1 Leucocyte Markers

No significant differences were observed in the level of leucocyte infiltration between cadaveric and LRD kidneys. CD3+ T cells were absent in the majority of donor kidneys, but in occasional biopsies, isolated cells were detected in the interstitium. Both cadaveric and LRD kidneys had high numbers of resident macrophages, with no differences between the two donor groups. In 7/65 (11%) cadaver donor kidneys, a large number of neutrophils were detected in the glomeruli and intertubular areas, but there was no statistically significant difference compared to LRD kidneys.

3.3.2 Endothelial Adhesion Molecules

Low levels of P-selectin expression were detected on isolated intertubular capillaries in wedge biopsies obtained from both cadaveric and LRD kidneys, with no significant differences in the percentage of kidneys with high levels (\geq Grade 2) of P-selectin expression (Figure 3.1a). In marked contrast, significantly higher levels of intertubular capillary E-selectin expression (\geq Grade 2) were detected in 35/65 (54%) cadaver donor kidneys compared with LRD kidneys, none of which expressed E-selectin ($p < 0.00001$) (Figure 3.1b, and Figure 3.2a and 3.2b).

3.3.3 Proximal Tubular Expression of ICAM-1, VCAM-1 and HLA-DR Antigens

Biopsies from cadaveric and LRD kidneys were examined for differences in expression of ICAM-1, VCAM-1 and HLA-DR antigens on the proximal tubules. Constitutive expression of HLA-DR antigens was detected on glomerular endothelium and mesangium, intertubular capillaries and interstitial leucocytes, as previously reported [437]. However, variable levels of HLA-DR antigens were detected in the cytoplasm and on the membranes of proximal tubules, with strong HLA-DR antigen expression (Grade 1) in 43/65 (66%) cadaver donor kidneys whereas only 2/29 (7%) LRD kidneys

were positive for HLA-DR antigens ($p < 0.00001$) (Figure 3.2c and 3.2d, and Figure 3.3).

ICAM-1 expression was detected constitutively at high levels on all vascular endothelium in both the LRD and cadaver donor kidneys. High levels of proximal tubular ICAM-1 expression, predominantly on the brush borders, (Grade 1) were detected in 40/65 (62%) cadaver donor kidneys, whereas none of the 29 LRD kidneys expressed tubular ICAM-1 ($p < 0.00001$) (Figure 3.2e and 3.2f, and Figure 3.3). Similarly, constitutive expression of VCAM-1 was detected on the Bowman's capsule of all the biopsies analysed, but in 30/65 (46%) cadaver donor kidneys high levels of proximal tubular VCAM-1 were detected, mainly at the basolateral surface, whereas all 29 LRD kidneys were negative for tubular VCAM-1 ($p < 0.00001$) (Figure 3.2g and 3.2h, and Figure 3.3).

Analysis of associations between pairs of adhesion molecule markers (using a Chi-squared test with Yates' correction) showed significant associations between high levels of expression of the three tubular markers studied, with the strongest associations between HLA-DR and other markers ($p < 0.0001$). Elevated tubular antigen expression, (defined as elevated levels of ICAM-1, VCAM-1 or HLA-DR antigens either alone or in combination), was detected in 50/65 (77%) cadaver kidneys (Figure 3.3). No significant associations were observed between elevated tubular antigen expression and high levels of E-selectin expression on the endothelium.

3.3.4 Organ Retrieval Biopsies

No differences in adhesion molecule and HLA-DR antigen expression were detected between biopsies obtained at the time of organ retrieval and subsequent pre-transplant biopsies from the same kidneys taken after cold storage. That is to say that in cases where expression of these molecules was upregulated in kidneys at the time of organ retrieval, the appearance was subsequently unchanged following cold storage.

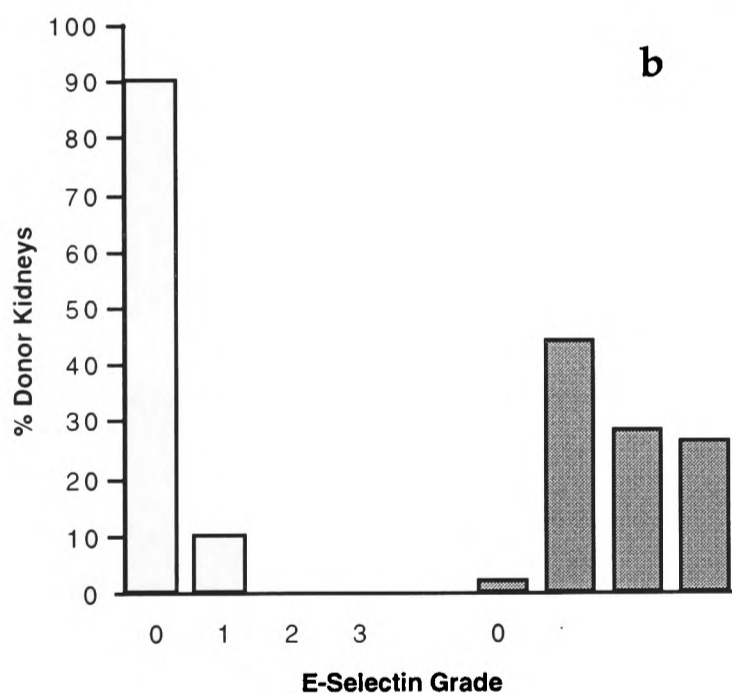
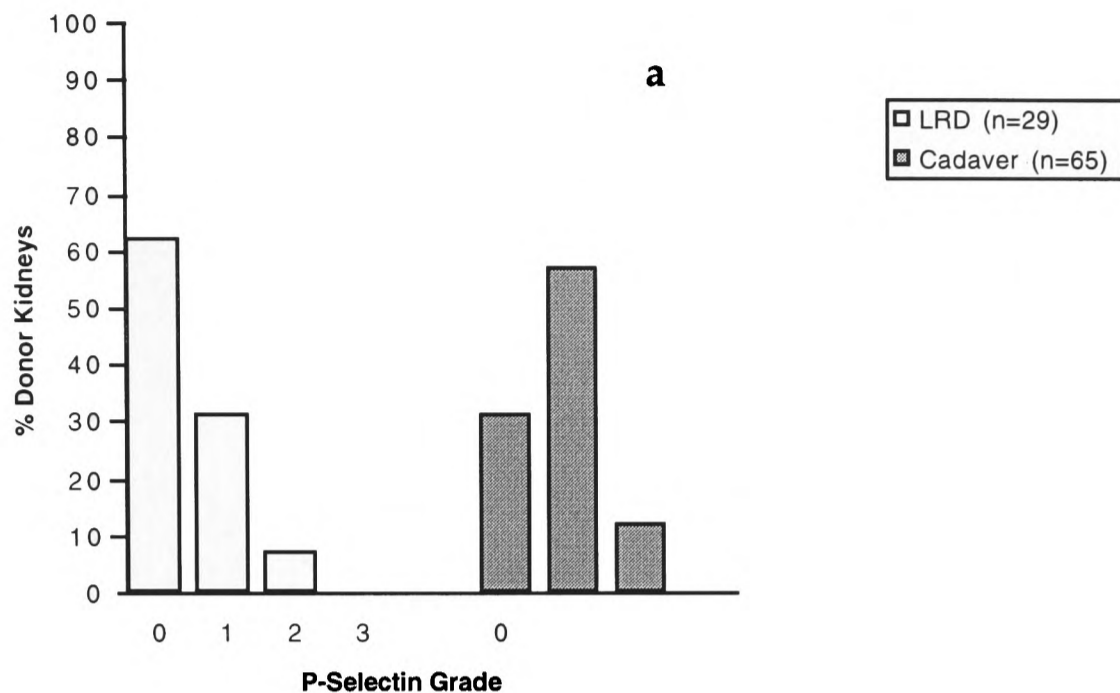


Figure 3.1. Levels of P- and E-selectin expression in cadaveric and living-related donor (LRD) kidneys.

(a) Similar patterns of low level P-selectin expression were detected in cadaveric and LRD kidneys. (b) High levels of E-selectin expression (\geq Grade 2) were detected in 54% of cadaveric kidneys, whereas minimal expression was observed in LRD kidneys.

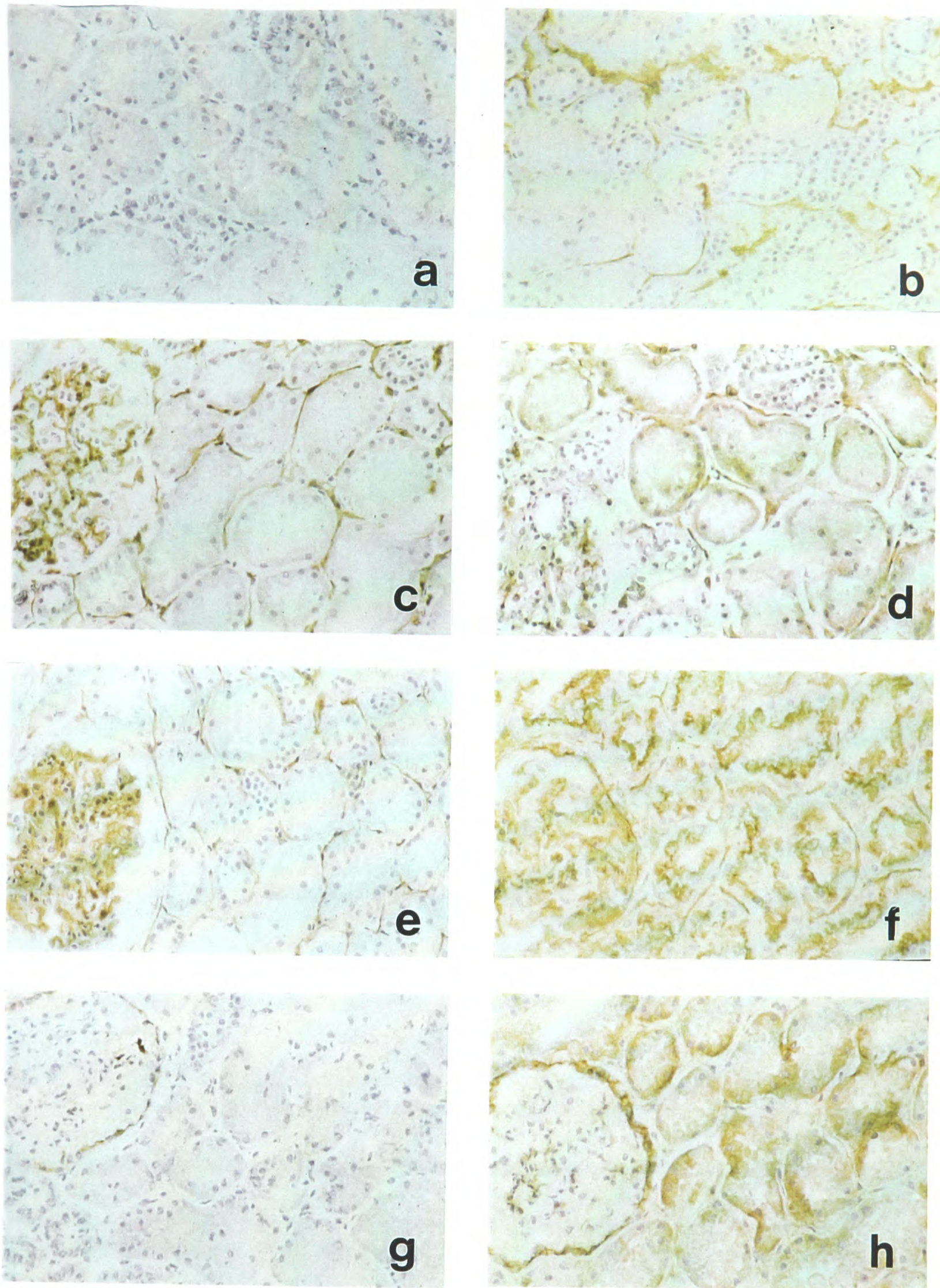


Figure 3.2 Immunohistological differences in adhesion molecule and HLA-DR antigen expression between cadaveric and LRD kidneys.

All biopsies were stained using monoclonal antibodies and developed by an indirect immunoperoxidase method. (a) LRD kidney stained with an anti-E-selectin antibody demonstrating negative expression and (b) a cadaveric kidney with high levels of E-selectin on the intertubular capillaries. (c) LRD kidney with negative tubular expression of HLA-DR antigens and (d) a cadaveric kidney with strong expression of HLA-DR antigens on the proximal tubules. (e) ICAM-1 was absent on the proximal tubules of all LRD kidneys, whereas (f) tubular ICAM-1 was detected at high levels in a proportion of cadaveric kidneys. (g) Similarly, all LRD kidneys were negative for expression of tubular VCAM-1, whereas (h) a proportion of cadaveric kidneys had high levels of tubular VCAM-1. (Magnification x200)

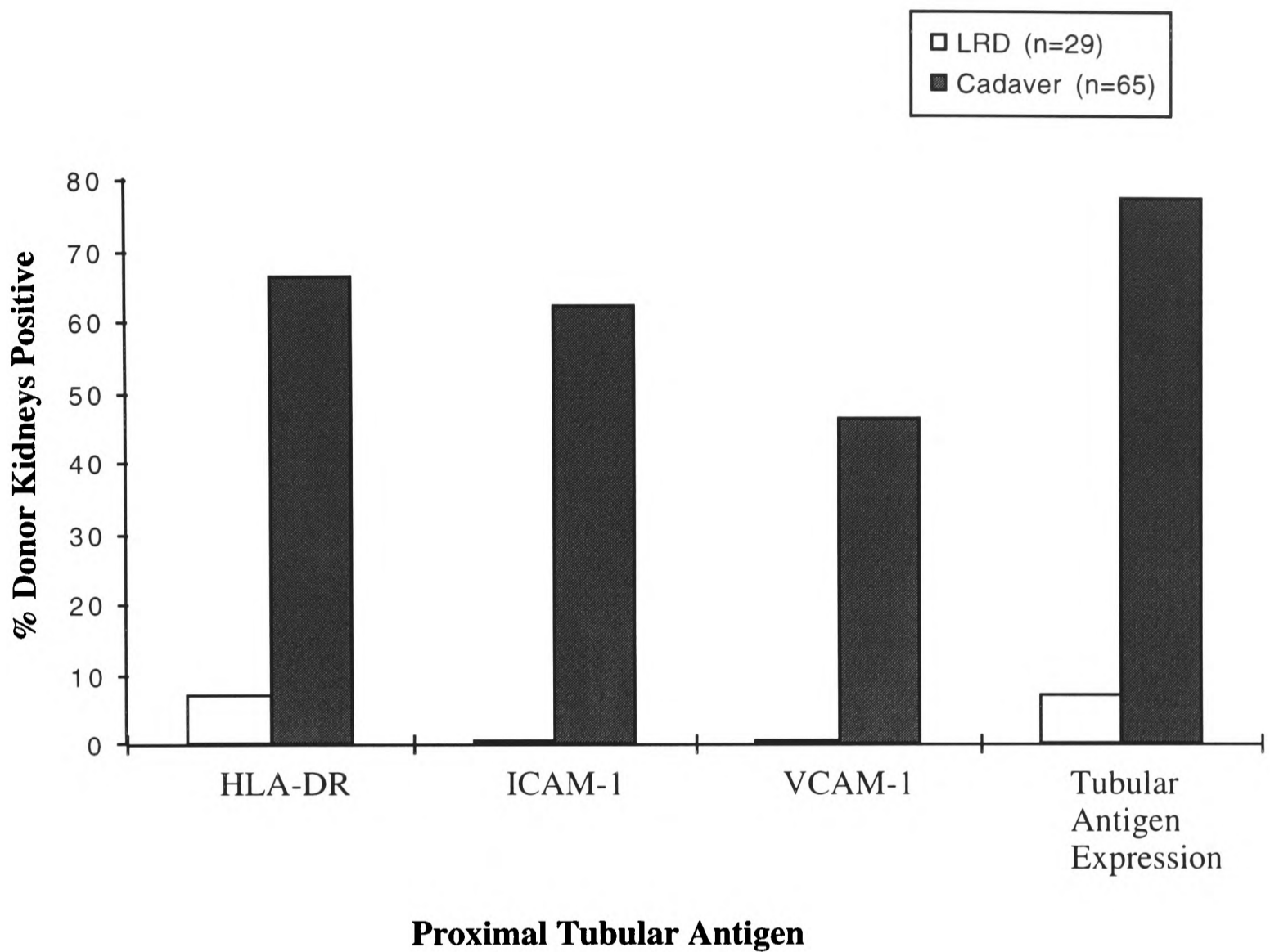


Figure 3.3. Expression of HLA-DR antigens, ICAM-1 and VCAM-1 on the proximal tubules of cadaveric (n=65) and LRD (n=29) kidneys.

High levels of HLA-DR antigens were detected on the proximal tubules of 66% of cadaveric kidneys whereas only 7% of LRD kidneys had elevated HLA-DR antigen expression ($p < 0.00001$). Elevated ICAM-1 and VCAM-1 expression were detected in 62% and 48% of cadaveric kidneys, respectively, whereas all 29 LRD kidneys were negative for tubular ICAM-1 and VCAM-1 expression ($p < 0.00001$). Elevated tubular antigen expression, defined as expression of either HLA-DR antigens, ICAM-1 or VCAM-1 either alone or in combination, were detected in 50/65 (77%) cadaver kidneys whereas only 2/29 (7%) LRD kidneys had induced tubular antigen expression ($p < 0.00001$).

3.4 ASSOCIATIONS OF ADHESION MOLECULE AND HLA-DR EXPRESSION WITH CLINICAL PARAMETERS

The expression of high levels of endothelial and tubular antigens in a large proportion of cadaver donor kidneys was analysed with respect to relevant donor and clinical parameters (Table 3.1). Statistical analysis between clinical parameters listed in Table 3.1 and elevated expression of endothelial E-selectin or tubular antigens are presented in Tables 3.2 and 3.3, respectively. Multiple logistic regression analysis was performed in view of the large number of univariate comparisons. Analysis by the Hosmer-Lemeshow χ^2 test established that the donor parameters identified by univariate analysis were significantly associated with E-selectin expression ($\chi^2 = 32.46$, $p=0.0087$) and induced tubular antigen expression ($\chi^2 = 27.37$, $p=0.0375$).

3.4.1 Donor Parameters

No significant associations were observed between high levels of adhesion molecule and HLA-DR antigen expression with donor age, sex, HLA type, local or shipped kidneys, multi/single organ donors and cold ischaemia time. However, elevated expression of proximal tubular antigens was detected in 20/22 (91%) kidneys from cadaver donors who suffered a traumatic death (death resulting from road traffic accidents or severe physical injury), whereas it was present in only 30/43 (69%) of cadaver donors who experienced non-traumatic death ($p<0.05$).

3.4.2 Clinical Events in ICU

In all 15 cadaver donor kidneys with no tubular antigen induction, the period of ventilator support was ≤ 3 days, whereas in 11/50 cadaver kidneys with induced tubular antigen expression, the period of ventilator support was > 3 days ($p=0.041$). Furthermore, in 16/17 (94%) of donors with a recorded incidence of infection, there were elevated levels of proximal tubular antigens, although in 34/58 (59%) of donors with no infection, tubular antigens were also detected ($p<0.05$). The mean period of ventilation for donors with infection episodes (3.18 ± 2.0 days) was significantly

| Clinical Parameters | E-selectin+ | E-selectin- |
|---|--------------------|--------------------|
| Donor Sex (M:F) | 19:16 | 14:16 |
| Donor Age (years) \pm SD | 44 \pm 14.6 | 37 \pm 14.5 |
| Trauma at Death | 10/35 | 12/30 |
| Ventilator Support (Days) \pm SD | 1.97 \pm 1.09 | 2.40 \pm 1.77 |
| Episode of Cardiac Arrest | 6/35 | 6/30 |
| Inotropic Support | 21/35 | 21/30 |
| ^aDesmopressin (DDAVP) Treatment | 14/35 | 4/30 |
| Donor Infection | 6/35 | 11/30 |
| Urine Output (final hour) (ml) \pm SD | 209 \pm 228 | 178 \pm 156 |
| Rate of Urine Output (ml/hour) \pm SD | 197 \pm 134 | 307 \pm 776 |
| Blood Urea (mmol/L) \pm SD | 6.0 \pm 2.3 | 5.8 \pm 3.3 |
| Donor Serum Creatinine (μ mol/L) \pm SD | 97 \pm 36 | 91 \pm 40 |
| Donor Blood Transfusion | 6/35 | 10/30 |
| Local vs Shipped Kidney (L:S) | 29:6 | 19:11 |
| Multi/Single Organ Donor (M:S) | 31:4 | 25:5 |
| Cold Ischaemia Time (hours) \pm SD | 25.9 \pm 10.3 | 24.3 \pm 9.9 |
| Delayed Graft Function | 5/35 | 5/30 |
| Serum Creatinine (3 months) (μ mol/l) \pm SD | 148 \pm 46 | 157 \pm 56 |
| Serum Creatinine (6 months) (μ mol/l) \pm SD | 154 \pm 49 | 150 \pm 52 |
| No. of Rejection Episodes (0:1:2:3) | 15:10:1:4 | 15:12:5:3 |
| Rejection by Day 7 Post-Transplantation | 8/35 | 3/30 |

Table 3.2 Demographics of donor and clinical parameters in cadaver donor kidneys with negative or induced endothelial E-selectin expression.

^a Fisher's exact test (p<0.05)

| Clinical Parameters | Tubules+ | Tubules- |
|--|----------------------------------|----------------------------------|
| Donor Sex (M:F) | 26:24 | 7:8 |
| Donor Age (years) \pm SD | 40 \pm 14.6 | 43 \pm 16.1 |
| ^aTrauma at Death | 20/50 | 2/15 |
| ^bVentilator Support (Days) \pm SD | 2.38 \pm 1.5 | 1.60 \pm 0.9 |
| Episode of Cardiac Arrest | 7/50 | 5/15 |
| Inotropic Support | 34/50 | 8/15 |
| Desmopressin (DDAVP) Treatment | 13/50 | 5/15 |
| ^aDonor Infection | 16/50 | 1/15 |
| Urine Output (final hour) (ml) \pm SD | 183 \pm 198 | 236 \pm 198 |
| Rate of Urine Output (ml/hour) \pm SD | 271 \pm 607 | 169 \pm 79 |
| Blood Urea (mmol/L) \pm SD | 6.1 \pm 2.8 | 5.1 \pm 2.6 |
| Donor Serum Creatinine (μ mol/L) \pm SD | 96 \pm 36 | 91 \pm 43 |
| Donor Blood Transfusion | 12/50 | 4/15 |
| Local vs Shipped Kidney (L:S) | 40:10 | 8:7 |
| Multi/Single Organ Donor (M:S) | 44:6 | 12:3 |
| Cold Ischaemia Time (hours) \pm SD | 25.3 \pm 10.5 | 24.7 \pm 9.0 |
| Delayed Graft Function | 7/50 | 3/15 |
| Serum Creatinine (3 months) (μ mol/l) \pm SD | 153 \pm 46 | 147 \pm 64 |
| Serum Creatinine (6 months) (μ mol/l) \pm SD | 155 \pm 46 | 143 \pm 64 |
| No. of Rejection Episodes (0:1:2:3) | 21:20:5:4 | 9:2:1:3 |
| ^aRejection by Day 7 Post-Transplantation | 11/50 | 0/15 |

Table 3.3 Demographics of donor and clinical parameters in cadaver donor kidneys with negative or induced tubular antigen expression.

^a Fisher's Exact Test ($p < 0.05$)

^b Students T-test (one-tailed assuming unequal variance?????) ($p = 0.015$)

greater than those with no infection (1.83 ± 1.0 days) ($p < 0.01$). The relationship between prolonged ventilation, tubular antigen expression and donor infection is illustrated in Figure 3.4. There were no significant associations between high levels of adhesion molecules or HLA-DR antigen expression and donor blood transfusions or episodes of cardiac arrest in the donors.

3.4.3 Drug Administration

There was a significant association between high levels of E-selectin expression and the administration of desmopressin (DDAVP) to the donor prior to organ retrieval. In 14/18 (78%) donors that received DDAVP, there were high levels of E-selectin expression, whereas E-selectin expression was only detected in 21/47 (44%) donors that did not receive DDAVP ($p = 0.015$). No significant associations were observed between the administration of inotropes (e.g. adrenaline, noradrenaline) and elevated levels of endothelial or tubular adhesion molecule or HLA-DR antigen expression.

3.4.4 Donor Renal Function

No significant associations were observed with the rate of urine output, volume of urine produced in the final hour, serum creatinine or urea levels.

3.5 POST-TRANSPLANTATION EVENTS

Elevated tubular antigen expression was evident before transplantation in all 11 cadaver renal allografts with a biopsy confirmed acute rejection episode within 7 days of transplantation. In contrast, tubular antigens were absent in 15/54 (28%) donor kidneys with no rejection in the first 7 days ($p < 0.05$). The degree of HLA-A, -B and -DR mismatching in the 11 cadaver renal allografts with early acute rejection episodes was not significantly different from cadaver allografts with no rejection. There were no significant associations with delayed graft function, number of rejection episodes and 3-month and 6-month serum creatinine levels.

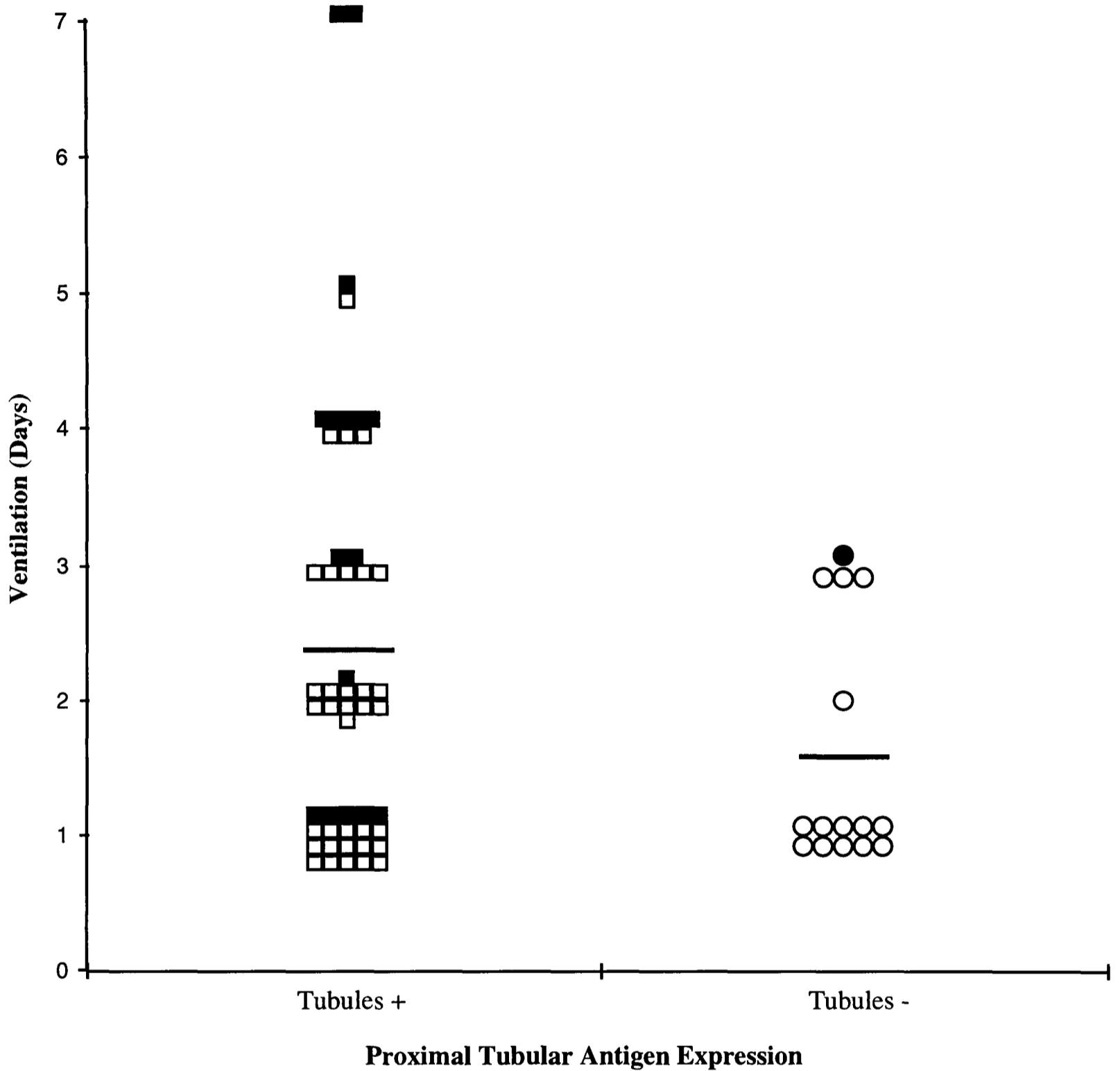


Figure 3.4. Relationship between ventilation time, induced tubular antigen expression and episodes of infection in cadaver donors.

The mean period of ventilator support for cadaver donors with induced tubular antigen expression (2.4 ± 1.5 days) (squares) was higher than that of donors with no tubular antigen induction (1.6 ± 0.9 days) (circles). Donors with an episode of infection were also identified with respect to induced tubular antigen expression (black squares) or no tubular antigen induction (black circles).

3.6 DISCUSSION

It is widely recognised that graft function and survival of living-related donor (LRD) renal allografts is superior to that of cadaver renal allografts [1, 430, 431]. The differences observed between cadaveric and LRD allografts have been attributed to the fact that in LRD transplantation, there is a genetic relationship between donor and recipient, and kidneys are procured from healthy donors with short cold ischaemia times. We have performed an immunohistological analysis to determine whether or not pre-existing factors in cadaver donors promote the expression of adhesion molecules and HLA-DR antigens which in turn, may predispose cadaveric kidneys to subsequent immune-mediated events following transplantation.

A striking difference in adhesion molecule and HLA-DR antigen expression was detected between cadaveric and LRD kidneys. High levels of E-selectin expression were detected on the intertubular capillaries of a large proportion of cadaver donor kidneys, whereas E-selectin expression was not detected in any of the 29 LRD kidneys ($p < 0.00001$). E-selectin is expressed on the surface of activated endothelial cells following cytokine stimulation, with a peak of expression observed between 4-6 hours following stimulation and a return to basal levels after 24 hours [190]. E-selectin is involved in the initial recruitment of leucocytes from the circulation to an inflammatory site [193, 194, 197], and has been shown to be upregulated during allograft rejection [277, 278, 440]. The expression of E-selectin in cadaver donor kidneys may facilitate the recruitment of leucocytes following transplantation, thus rendering the graft susceptible to cell mediated damage.

Elevated levels of proximal tubular ICAM-1 and VCAM-1 were detected in a proportion of cadaver donor kidneys, whereas all LRD kidneys were negative for tubular adhesion molecule expression ($p < 0.00001$). ICAM-1 and VCAM-1 have been shown to be upregulated on proximal tubules during renal allograft rejection [277-280, 441]. *In vitro* experiments on cultured renal tubular epithelial cells have demonstrated that the

peak of ICAM-1 and VCAM-1 expression occurs between 1-2 days following cytokine stimulation [281, 283]; a greater period than that required for endothelial E-selectin expression. The difference between the kinetics of expression may explain why no significant associations were found between elevated E-selectin expression and the tubular adhesion molecules, whereas a highly significant association was observed in cadaver donor kidneys expressing both tubular ICAM-1 and VCAM-1.

The recruitment of leucocytes by adhesion molecules expressed on vascular endothelium at a site of inflammation has been well characterised [166, 435], but the role of tubular adhesion molecule expression is less clearly defined. *In vitro* data has demonstrated that renal tubular epithelial cells with induced ICAM-1 and VCAM-1 expression are capable of binding lymphocytes [281-284]. The expression of VCAM-1 at the basolateral surface of proximal tubules provides a possible mechanism for lymphocyte migration into the tubules following extravasation from the circulation. Furthermore, ICAM-1 expression on the proximal tubular brush borders may enable leucocytes to migrate into the tubule lumen. Thus, the induction of tubular adhesion molecule expression may facilitate the infiltration of T lymphocytes into the tubules resulting in tubulitis, a pathophysiological event associated with allograft rejection. Indeed, there is evidence to demonstrate that in biopsies from renal allografts with severe acute rejection, CD8+ cytotoxic cells expressing perforin were detected within tubules in association with tubular epithelial cell proliferation [442, 443].

In addition to tubular adhesion molecule expression, HLA-DR antigens were also detected on the proximal tubules of 43/65 (66%) cadaver donor kidneys, whereas only 2/29 (7%) of LRD kidneys were positive for HLA-DR antigens ($p < 0.00001$). Tubular HLA-DR antigen expression was significantly associated with elevated tubular ICAM-1 and VCAM-1 expression ($p < 0.0001$). This supports *in vitro* studies which have shown that tubular HLA Class II expression has similar kinetics to tubular ICAM-1 and VCAM-1 expression [284, 444]. It may be surprising that high levels of adhesion molecule expression were detected in the absence of increased levels of leucocyte

infiltration, but little is known with respect to immune regulation following brain death. It is probable that following traumatic events in the donor (e.g. severe physical injury, infection, brain death) there may be systemic release of cytokines into the circulation, resulting in elevated adhesion molecule expression in peripheral organs but no specific site-directed leucocyte infiltration as detected during allograft rejection or infection. The expression of tubular VCAM-1, ICAM-1 and HLA Class II antigens in cadaver donor kidneys may render renal allografts more susceptible to cell-mediated damage following transplantation.

Several studies have demonstrated that T cells cultured from renal allograft rejection biopsies were tissue specific for donor-derived tubular epithelial cells, but not donor splenocytes [285, 286]. Furthermore, kidney tubule cells isolated from a murine model of lupus interstitial nephritis were found to be capable of CD4+ T cell activation, mediated by ICAM-1 and a novel costimulatory signal in the absence of B7 T cell costimulatory molecule expression [445]. The constitutive expression of MHC Class I and the costimulatory molecule CD40 have been demonstrated on cultured tubular epithelia [446], together with inducible expression of MHC Class II, ICAM-1 and VCAM-1, provide potential mechanisms for lymphocyte attachment, antigen presentation and costimulation to recipient lymphocytes during allograft rejection. Indeed, in this study, all 11 cadaver renal allografts with a rejection episode by Day 7 post-transplant, had high levels of induced tubular antigen expression pre-transplant ($p < 0.05$). Analysis of HLA mismatching in allografts with or without an early rejection episode demonstrated no significant difference for HLA-A, -B, or -DR, although in all 11 allografts with rejection by day 7, one or more mismatches were detected for HLA-A and -B, with 8/11 having ≥ 1 mismatch for HLA-DR.

Comparison of biopsies obtained from 5 cadaver kidneys before and after cold storage, demonstrated that antigen expression was detected at the time of organ procurement, and was not elevated following cold ischaemia. These results suggest that inflammatory responses occur in cadaver donors, probably several hours or days before organ

procurement, enabling a sufficient period for transcription and expression of adhesion molecule and HLA-DR antigens. In experimental models of transplantation, induced antigen expression is not detected in donor organs because they are procured from healthy, anaesthetised animals which do not experience brain death; a situation similar to that in human LRD transplantation.

One of the major differences between living and cadaveric donors is that physiological abnormalities may occur in the procurement of cadaver donor organs which are not encountered in living donors. Upon brain death in cadaveric donors, a variety of traumatic events ensues as a result of loss of brain activity leading to deteriorating organ function. In the immediate period following brain death, there is a massive release of circulating and endogenous catecholamines from the adrenal glands and nerve endings, referred to as the “autonomic storm.” This has a direct effect on the cardiovascular system creating an increase in systemic vascular resistance and hypertension, with potential repercussions to the donor organs as a result of transient periods of ischaemia and reperfusion [23]. In addition there are significant alterations in endocrine hormones with significant reductions in free triiodothyronine (T_3) and thyroxine, and rises in reverse triiodothyronine (rT_3) and thyroid stimulating hormone (TSH), all of which result in impaired cellular aerobic metabolism [34, 35]. Indeed, donor hormonal therapy with triiodothyronine demonstrated significant reductions in serum lactate-pyruvate and requirement for inotropic support, and improved cardiac function in the donor and following transplantation [36, 37]. Brain death also results in a rapid decrease in the production of pituitary gland hormones vasopressin and adrenocorticotrophic hormone leading to diabetes insipidus and loss of homeostatic regulation [29]. Thus, potential cadaveric donors require careful management of cardiovascular, pulmonary and homeostatic functions in intensive care, but if stability of these vital functions is not maintained, adverse alterations in renal blood flow may result.

Vascular tone is modulated with the administration of inotropes (e.g. dopamine, dobutamine, epinephrine), but prolonged inotropic support has been shown to cause

cellular and functional changes to peripheral organs [39]. Furthermore, there is evidence to suggest that donor inotropic support may have a detrimental effect on human renal allograft function and has also been shown to be associated with severe ultrastructural changes in donor hearts [38, 447]. Animal studies have demonstrated that reduction of blood flow to peripheral organs as a result of hypotension or blood flow redistribution following brain death may result in ischemic damage [25, 27]. The problems of ischemic damage prior to organ retrieval may be further exacerbated by the abnormal rates of oxygen consumption and delivery following brain death, leading to an accumulation of plasma lactate as a result of anaerobic metabolism and thus, reduced oxygen availability to the organs [26, 28]. In this current study, no correlation was observed between donor inotropic support and poor allograft function. Furthermore, we were unable to detect any correlations between elevated adhesion molecule and HLA Class II antigen expression with the administration of inotropes. This may reflect efficient monitoring and treatment of fluctuations in vascular stability during intensive care treatment.

The secondary effects of brain damage may result in markedly reduced production of vasopressin, with ensuing diabetes insipidus and loss of homeostatic regulation. Excessive diuresis may result in dehydration and is controlled by either plasma-volume expansion or the administration of the L-arginine vasopressin analogue, DDAVP (1-desamino-8-D-arginine vasopressin), a powerful anti-diuretic [31]. Maintenance of plasma volume with hydroxyethylstarch in brain dead kidney donors has been demonstrated to impair early renal function following transplantation [30]. There are also conflicting reports regarding the effects of donor treatment with DDAVP upon subsequent renal function following transplantation. In a retrospective study, Hirschl and colleagues demonstrated that administration of DDAVP to the donor resulted in a two-fold increase in the rate of delayed graft function following renal transplantation [32]. In contrast, a recent study reported that there were no adverse effects of DDAVP administration on subsequent renal graft function and survival [33]. Our results demonstrated that a significant number of cadaveric donors receiving DDAVP treatment

had elevated levels of E-selectin expression on the intertubular capillaries. DDAVP has been used in the treatment of bleeding disorders [448, 449], and studies have shown that DDAVP stimulates platelet and endothelial P-selectin expression and increased platelet-activating factor expression on monocytes [450-452]. Therefore, it is possible that administration of DDAVP to potential donors to control excessive diuresis may result in secondary effects on haemostasis and endothelial activation.

The potential organ donor requires ventilatory support and remains on ventilation until organ donation. In a proportion of cadaver donor kidneys, elevated proximal tubular expression of inflammatory antigens were detected in donors who received prolonged ventilator support. It is widely recognised that patients on prolonged ventilation are susceptible to respiratory tract infections [453], therefore, unsurprisingly, significant associations were detected between elevated tubular antigen expression and recorded incidences of infection. Our results suggest that the increased risk of infection as a result of prolonged ventilation in the potential organ donor may increase the likelihood of systemic inflammatory events affecting peripheral organs.

Another possible explanation may be that patients requiring ventilatory support are close to brain death. The physiological effects of brain death on neurological and hormonal functions are well defined, but little is known about the immunological events associated with brain death [24]. However, there is recent evidence from a rat model of acutely induced brain death demonstrating increased expression of inflammatory cytokines in association with upregulated levels of adhesion molecules, MHC antigens and the costimulatory molecule, B7, in peripheral organs up to 6 hours following brain death [454]. Furthermore, dramatic increases in IL-6 and severe hormonal imbalances were detected in blood samples obtained from patients after brain death diagnosis [455]. The results from these studies may in part, explain the high levels of adhesion molecule and HLA-DR antigen expression in cadaver donor kidneys, and not in LRD kidneys. The prolonged period of ventilatory support observed in a proportion of cadaveric kidneys with induced tubular antigen expression may represent the time taken for the

expression of these molecules following brain death. In addition, we have shown that cadaveric donors who suffered a traumatic death as a result of road traffic accidents or severe physical injury were found to have a significantly higher incidence of tubular antigen expression. Victims of traumatic death may have experienced systemic release of inflammatory mediators as a response to injury, but in addition, it is possible that this particular group of donors may have suffered acutely induced brain death.

In summary, our results demonstrate significantly higher levels of adhesion molecule and HLA-DR antigen expression prior to transplantation in a proportion of cadaver donor kidneys, whereas almost all LRD kidneys were negative. Moreover, we have demonstrated a significant association between elevated pre-transplant levels of these proinflammatory molecules with early rejection episodes following transplantation of cadaver kidneys. Nevertheless, in this current study, the incidence of acute rejection was similar in both the cadaver and LRD renal allografts despite the higher incidence of pre-transplant antigen expression in the cadaver kidneys. The precise effects of pre-transplant antigen expression in cadaver kidneys are uncertain, but may be involved in ischaemia/reperfusion injury and subsequent alloimmune responses. Further improvements in donor management and organ retrieval may benefit renal allograft outcome.

**CHAPTER 4: ISCHAEMIA/REPERFUSION INJURY IN
HUMAN RENAL TRANSPLANTATION**

4.1 INTRODUCTION

Organs that are used for transplantation require effective *ex vivo* preservation from the moment the organ is retrieved to the time of transplantation. Hypothermic preservation solutions have been developed to maintain tissue viability by reducing metabolic activity and the accumulation of toxic substances during the cold ischaemic period. Organs used for transplantation can undergo lengthy periods of cold ischaemic storage following devascularisation and cold perfusion, resulting in an increased susceptibility to damage upon reperfusion.

In clinical renal transplantation, prolonged cold storage has been demonstrated in many studies to be strongly associated with delayed graft function (DGF) [45, 48-54, 72]. DGF is broadly defined as the requirement for dialysis within the first week following transplantation and results in complications in the immunosuppressive management of the transplant patient, prolonged hospitalisation and potentially detrimental effects to subsequent graft function and survival [41, 42]. Some studies have suggested that DGF has little or no effect on graft survival, especially when the compounding effects of acute rejection are taken into account [43-46]. In marked contrast, other studies show a profound effect of DGF on subsequent short and long term graft survival [48-53]. This effect has recently been highlighted in a multivariate analysis of 37,216 primary cadaver renal allografts from the U.S. Renal Data System, where DGF was shown to be an independent factor in determining poor short and long term graft

survival, regardless of both the incidence of early rejection episodes and the degree of HLA matching [53].

Although it is widely accepted that prolonged cold storage has a detrimental effect upon graft function, the precise mechanisms by which this occurs are not completely understood. During cold ischaemic storage of organs prior to transplantation, biochemical events occur within the tissue leading to free radical mediated damage upon reperfusion of the vascularised graft (see Chapter 1). Free radicals appear to mediate tissue injury through lipid peroxidation and the activation of endothelial cells resulting in functional and structural cell damage.

In vitro experiments on human umbilical vein endothelial cells (HUVEC), have shown that reactive oxygen species induce adhesion molecule expression resulting in activation and increased binding of neutrophils [310, 316, 317, 456-458]. Furthermore, in experimental animal models, *in situ* cold ischaemia followed by reperfusion of the kidney, has been shown to lead to increased expression of adhesion molecules and neutrophil infiltration within hours of reperfusion, followed by mononuclear cell infiltration and upregulation of MHC Class II expression several days later [392, 394]. This increase in immunogenicity resulting from the early non-specific inflammatory events may intensify subsequent alloimmune responses and play a major role in determining the quality of graft function in the long term [67, 69, 70, 395].

Studies investigating the effects of ischaemia/reperfusion injury in clinical renal transplantation are limited, and have relied mainly on the measurement of a marker of lipid peroxidation, malondialdehyde [354-356]. While these studies showed elevated levels of malondialdehyde in plasma following reperfusion of the graft, potential correlations with subsequent graft function were not examined. A more informative method of investigating reperfusion injury of renal allografts would be to analyse biopsies obtained immediately following transplantation.

During the early era of transplantation, biopsies were frequently obtained an hour after revascularisation when hyperacute rejection was suspected. A neutrophil infiltration in the glomeruli was an indicator of hyperacute rejection of the allograft [357, 358], but subsequent reports did not show a direct correlation between neutrophil infiltration and either hyperacute rejection or acute rejection episodes [359-361]. In the modern era of transplantation, hyperacute rejection has been virtually eliminated because of improvements in antibody screening, crossmatching and immunosuppression, and thus there have been few studies of post-reperfusion biopsies. In one recent study of post-reperfusion biopsies, polymorphonuclear leucocytes were detected in biopsies from cadaver renal allografts and this was found to be associated with long cold storage times [362]. Interpretation of these results was complicated by the presence of hyperacute rejection in 4/57 allografts studied and by the fact that pre-reperfusion biopsies were not available for comparison, thus it is not clear whether these cells entered upon reperfusion or were already present within the donor kidney.

In order to investigate the potential effects of cold ischaemic damage and reperfusion injury in renal transplantation, we have performed an immunohistochemical study on renal allograft biopsies obtained immediately after transplantation, and for comparison, on biopsies from the same kidney prior to transplantation. This has enabled the analysis of changes resulting from reperfusion of the allograft, whilst excluding pre-existing factors associated with the donor kidneys. Furthermore, biopsies from living-related donor renal allografts with minimal cold ischaemia times have been obtained for comparison. The results from the analysis have been related to relevant donor parameters and factors relating to graft function and rejection.

4.2 MATERIALS AND METHODS

4.2.1 Patients and Biopsy Material

Biopsy material was obtained from transplants of cadaveric (n=55) and living-related (LRD) (n=11) kidney allografts performed at the Oxford Transplant Centre. Wedge biopsies were obtained from all transplanted kidneys at two time points: (i) **pre-reperfusion**: after nephrectomy, flushing with ice-cold hypertonic citrate (Marshall's solution) and storage, but before implantation and (ii) **post-reperfusion**: approximately 20-40 min. after reperfusion of the kidney, immediately before wound closure. In 5 of the cadaver kidneys, additional biopsies were obtained at the time of nephrectomy, immediately after flushing and before the period of cold storage. All biopsies were snap frozen in liquid nitrogen and stored at -80°C.

Following transplantation all patients received standard triple therapy immunosuppression (cyclosporine, azathioprine and steroids) [415]. Details relating to important clinical parameters and outcome indicators are given in Table 4.1. There were no significant differences observed between cadaver and LRD renal allografts in donor age, HLA-A, -B, and -DR mismatches, number of retransplants, anastomosis time, and recipient sex.

4.2.2 Monoclonal Antibodies and Immunohistochemistry

7µm cryostat tissue sections from wedge biopsies were stained with monoclonal antibodies using an indirect immunoperoxidase technique described in Chapter 2. The sections were stained with the following monoclonal antibodies; 5D11 (anti-E-selectin [CD62E]), 4B2 (anti-VCAM-1 [CD106]), 14C11 (anti-ICAM-1 [CD54]) all obtained from British Biotechnology Ltd, Oxford, UK, F10.89.4 (anti-CD45 leucocyte common marker [418]), UCHT-1 (anti-CD3 T cell marker [416]), UCHM-1 (anti-CD14 macrophage/monocyte marker [417]), EBM/11 (anti-CD68 macrophage/monocyte

| Clinical Details | Cadaver (n=55) | LRD (n=11) |
|---|-----------------------|-------------------|
| Donor age (\pm SD) | 41 \pm 14.8 | 41 \pm 11.3 |
| ^a Cold Ischaemia Time (hours) \pm SD | 24.7 \pm 9 | 1.8 \pm 0.5 |
| ^b Positive crossmatch | 7/55 | 0/11 |
| ^c Delayed Graft Function | 8/55 | 1/11 |
| ^a Recipient age (\pm SD) | 47.8 \pm 12 | 29.4 \pm 12 |
| Serum Creatinine μ mol/l (3 mth) \pm SD | 154.7 \pm 52 | 142.3 \pm 37 |
| Serum Creatinine μ mol/l (6 mth) \pm SD | 154.2 \pm 51 | 143.7 \pm 31 |
| No. of rejection episodes (0:1:2:3) | 29:16:5:5 | 4:5:2:0 |

Table 4.1. Clinical factors and graft outcome indicators following transplantation.

^a Significant difference between cadaver and LRD groups of $p < 0.01$.

^b Positive crossmatch resulting from non-HLA, autoreactive IgM antibodies

^c Delayed graft function is defined as the requirement for dialysis in the first week after transplantation.

marker [419]), G1 (anti-P-selectin [CD62P] [423]), 1G10 (anti-CD15s neutrophil marker [459]), 5B12 (anti-CD41 platelet-specific marker) and anti-neutrophil elastase (DAKO Ltd., High Wycombe, Bucks., UK.). An anti-dog Thy-1 (F3.20.7 [420]) monoclonal antibody was used as a negative isotype control.

Identification of recipient cells within the allograft was performed by staining pre- and post-reperfusion biopsies from transplants mismatched for HLA-A2 or B17 with an anti-HLA-A2/B17 antibody (MA2.1 [421]) and a monomorphic anti-HLA-A,B,C antibody, (PA2.6 [422]), as a control for the presence of HLA class I antigens.

4.2.3 Double-Immunofluorescent Staining

Double immunofluorescent staining was performed to clarify the origin of the increased expression of P-selectin detected in the post-reperfusion biopsies. All incubations were performed for 30 minutes at room temperature in the dark. Acetone fixed cryosections of pre- and post-reperfusion biopsies were first incubated with antibodies to either P-selectin (IgG₁) or ICAM-1 (IgG_{2a}) and following washing, bound antibody was detected with the appropriate Texas Red-conjugated isotype-specific goat anti-mouse Ig antibodies (Southern Biotechnology Associates, Birmingham, Alabama, USA) pre-incubated with human AB serum. Sections were then incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-CD41 (platelet-specific) antibody (5B12, DAKO Ltd). After final washing, slides were mounted in Vectashield™ (Vector Labs, Peterborough, UK) and analysed by fluorescent microscopy. The specificity of the staining procedure was confirmed by including isotype control antibodies, to ensure that there was no non-specific binding of the secondary antibodies. All antibodies were used in isolation to check that the binding was in no way altered by the double staining protocol.

4.2.4 Assessment of Staining

Staining of endothelial and leucocyte markers was scored by two independent observers without knowledge of the clinical status of the patients. Minor differences in the scoring were resolved by conference. The semi-quantitative grades given for E-selectin, P-selectin and CD41 detected on endothelium were scored as follows: 0 - negative; 1- predominantly negative- an isolated positive vessel; 2- focus of positive vessels/ occasional positive vessels; 3- multiple foci/ positive vessels throughout biopsy. A significant increase in the level of expression of adhesion molecules after reperfusion was considered as an increase in Grades of ≥ 1 . Changes between Grades 0 and 1 were not considered significant.

Leucocytes were quantified and expressed as; (i) mean number of positive cells/glomerulus per section, with a minimum of 3 glomeruli required for inclusion in the analysis and (ii) mean number of positive cells in the intertubular areas per field of view (x10 objective). The entire biopsy was analysed in each case. A significant increase in glomerular infiltration after reperfusion was taken as an increase in mean glomerular count of ≥ 1.5 and an increase in intertubular infiltration was scored as an increase of ≥ 10 positive cells.

4.2.5 Statistical Analyses

Statistical analyses of the immunohistochemical results and the clinical data were performed using the Student's T-test, Fisher's exact and χ^2 tests.

4.3 IMMUNOHISTOCHEMICAL CHANGES FOLLOWING REPERFUSION

Biopsies obtained from donor kidneys before and after reperfusion were stained with monoclonal antibodies to leucocyte subpopulations and endothelial adhesion molecules, to provide information about the changes which may occur immediately following

reperfusion in cadaveric and LRD transplants. In addition, five biopsies were obtained at the time of donor nephrectomy and compared with pre-reperfusion biopsies from the same kidney to determine changes arising from cold storage.

4.3.1 Leucocyte Subpopulations

The major leucocyte population in the pre-reperfusion biopsies was CD14/CD68+ macrophages localised in the interstitial areas, but very occasionally, CD3+ T lymphocytes were detected. There were no quantifiable differences in either of these cell populations immediately following reperfusion. In contrast, an increase in neutrophil infiltration, as determined by staining with antibodies to CD15s and neutrophil elastase, was observed in 29/55 (53%) of cadaver renal allografts following reperfusion (Figure 4.1). In the 29 cadaver allografts where a neutrophil infiltration was detected, the infiltration was localised in the glomeruli of 16/29 (55%) (Figure 4.2a and 4.2b) and in the intertubular regions of 25/29 (86%) of cadaver allografts (Table 4.2). The increase in neutrophil infiltration following reperfusion was restricted to cadaver allografts; there was no neutrophil infiltration detected in the post-reperfusion biopsies of LRD renal allografts (Table 4.2). Furthermore, no changes occurred during the period of cold storage as determined by comparison between the 5 biopsies obtained at donor nephrectomy and the corresponding pre-reperfusion biopsies taken after cold storage.

4.3.2 Adhesion Molecule Expression

There was no difference in the levels of ICAM-1, VCAM-1 and E-selectin between pre- and post-reperfusion biopsies from any of the transplants studied, as may be anticipated since all of these molecules require protein synthesis for surface expression. Nevertheless there was considerable variation between kidneys in the extent of endothelial E-selectin expression and tubular ICAM-1 and VCAM-1. In marked contrast, P-selectin, which is expressed on the surface of endothelium and platelets within minutes of stimulation, was noticeably increased after reperfusion in 24/55

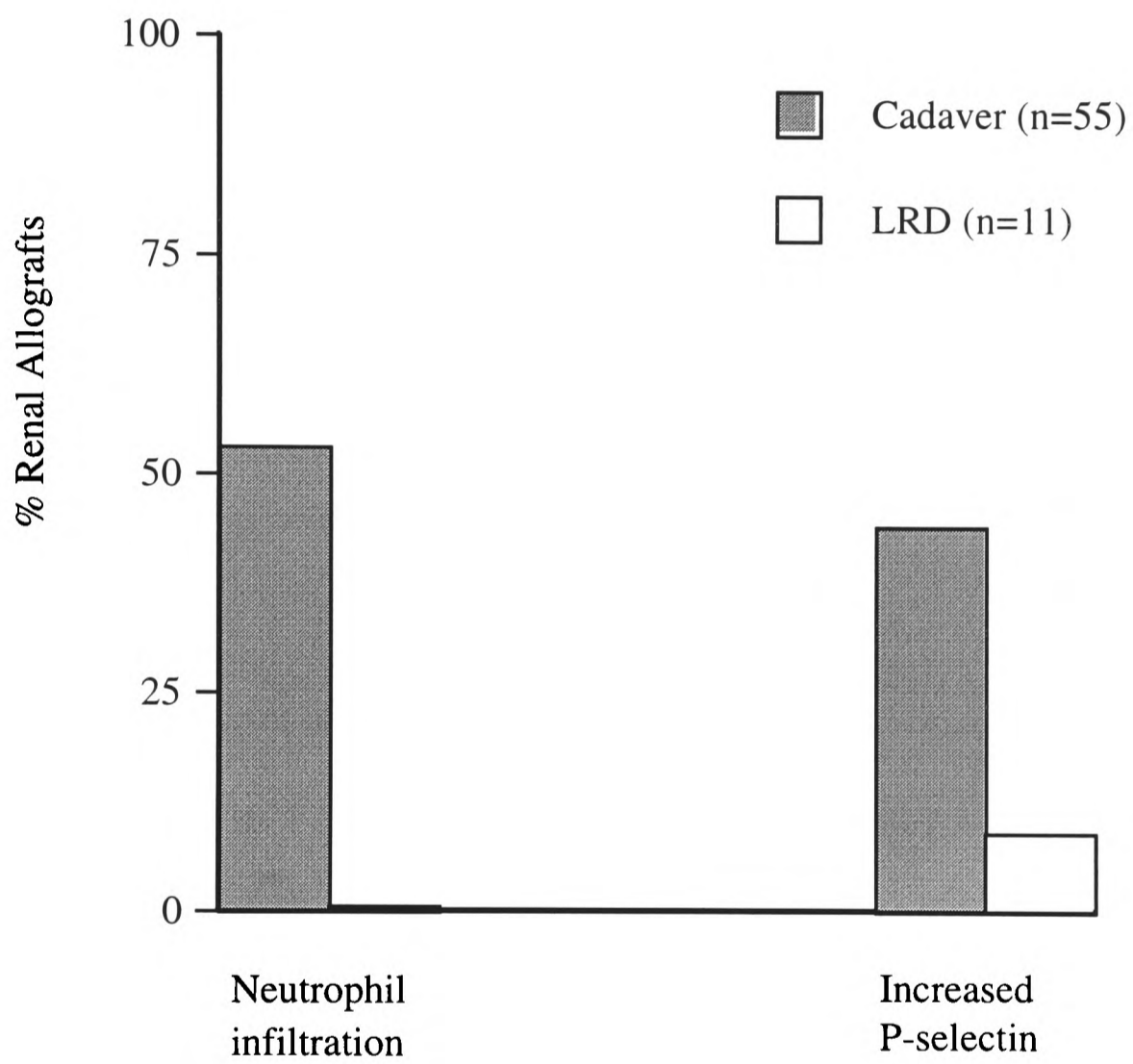


Figure 4.1. Percentage of cadaver (n=55) and LRD (n=11) renal allografts with an increase in neutrophil infiltration and P-selectin expression after reperfusion.

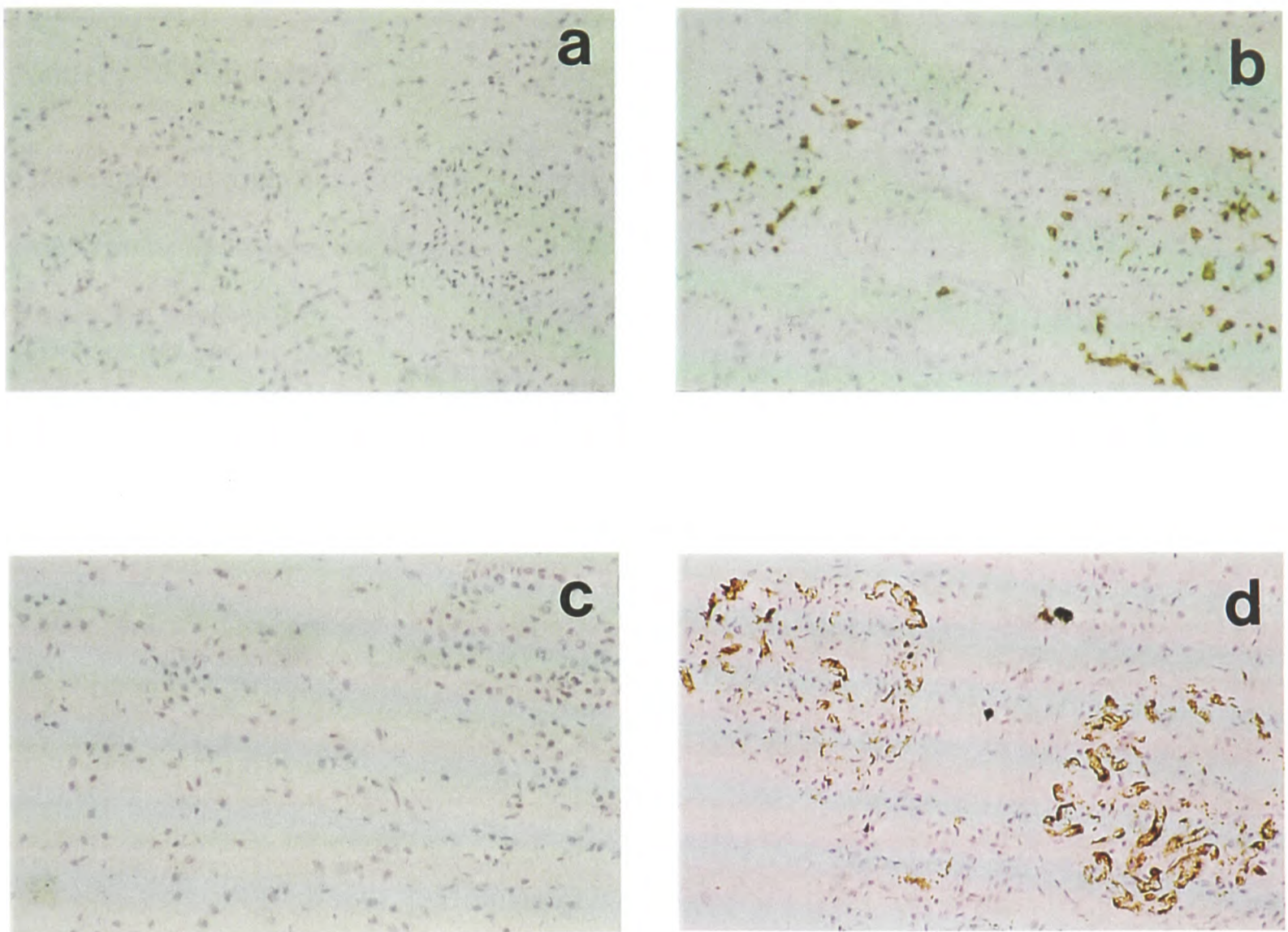


Figure 4.2 Immunohistological changes observed after reperfusion in cadaver renal allografts.

(a) Indirect immunoperoxidase staining of a pre-reperfusion biopsy with an anti-neutrophil elastase monoclonal antibody demonstrating the absence of neutrophils and (b) the subsequent post-reperfusion biopsy stained with the same antibody showing neutrophils infiltrating into the glomeruli (magnification, x200). (c) Pre-reperfusion biopsy demonstrating negative staining for P-selectin and (d) increased P-selectin expression detected in the glomeruli of the subsequent post-reperfusion biopsy. (Magnification x200)

| Changes detected between pre-post reperfusion biopsies | Cadaver (n=55) | LRD (n=11) | p-value |
|---|-----------------------|-------------------|----------------|
| Neutrophil infiltration (Glomerular and/or ITC) | 29/55 (53%) | 0/11 (0%) | <0.01 |
| Glomerular neutrophil infiltration | 16/29 (55%) | 0/11 (0%) | <0.01 |
| Intertubular neutrophil infiltration | 25/29 (86%) | 0/11 (0%) | <0.01 |
| P-selectin expression (Glomerular and/or ITC) | 24/55 (44%) | 1/11 (9%) | <0.03 |
| Glomerular P-selectin expression | 13/24 (54%) | 0/11 (0%) | <0.01 |
| ITC P-selectin expression | 20/24 (83%) | 1/1 (100%) | n/s |

Table 4.2. Comparison of immunohistochemical changes detected following reperfusion of cadaver and living-related donor (LRD) renal allografts. (ITC=intertubular capillaries)

(44%) cadaver renal allografts (Figure 4.1). In 13/24 (54%) of these transplants, P-selectin localised to the glomeruli (Figure 4.2c and 4.2d) and in 20/24 (83%) it was detected within the intertubular areas (Table 4.2). An increase in P-selectin following reperfusion occurred in only 1/11 LRD renal allografts and was localised in the intertubular areas. Comparison of 5 biopsies obtained at donor nephrectomy with pre-reperfusion biopsies taken after cold storage showed identical staining patterns, suggesting that changes in adhesion molecule expression did not occur during the period of cold storage.

4.4 RELATIONSHIP BETWEEN P-SELECTIN EXPRESSION AND NEUTROPHIL INFILTRATION

An analysis of cadaver renal allografts with either a neutrophil infiltration and/or increased P-selectin expression after reperfusion was performed to assess whether there were significant correlations between these post-reperfusion changes. Of the 24 cadaver renal allografts with an increase in P-selectin expression following reperfusion, 14 had a corresponding increase in neutrophil infiltration. When considering changes detected within the glomeruli, 5 of the 16 cadaver allografts with a glomerular neutrophil infiltration, had increased P-selectin expression in the glomeruli. With respect to the intertubular capillaries, in 12 of the 25 cadaver renal allografts with a neutrophil infiltration, an increase in P-selectin expression was also detected. Therefore, although a proportion of cadaver renal allografts had corresponding post-reperfusion changes in the glomeruli and intertubular capillaries, there were no statistically significant correlations observed between neutrophil infiltration and P-selectin expression.

4.5 CHARACTERISATION OF P-SELECTIN EXPRESSION

To investigate whether the P-selectin expression resulted from endothelial stimulation and thus release of P-selectin from Weibel-Palade bodies or from deposition of activated platelets on the endothelium, consecutive sections were stained with an antibody to P-selectin and with the platelet-specific marker, CD41. The patterns of staining with the two antibodies appeared to be identical, indicating that P-selectin may, at least in part, be of platelet origin (Figure 4.3a and 4.3b).

To confirm that the P-selectin was attributable to platelets, double staining was performed. The specificity of the staining protocol was confirmed with negative, isotype control antibodies. Biopsy sections were double-stained with a FITC-conjugated anti-CD41 (platelet-specific) antibody in combination with an antibody against ICAM-1 (expressed constitutively on endothelium). The results showed that platelets detected after reperfusion were localised on the endothelium of the graft following reperfusion. FITC-labelled platelets were clearly identified on Texas Red stained endothelium (Figure 4.4a-c). Double staining was then performed using antibodies against P-selectin and CD41. All stained structures were double-positive for CD41 and P-selectin, indicating that the P-selectin detected in the post-reperfusion biopsies was present as a result of activated platelets attached onto the microvascular endothelium (Figure 4.4d-f). Furthermore, P-selectin was not observed on vessels in the absence of CD41 signal, suggesting that endothelial P-selectin was not expressed in these biopsies.

4.6 ORIGIN OF INFILTRATING CELLS

To determine whether or not the post-reperfusion infiltration was attributable to recipient cells infiltrating into the allograft, selected transplants, where donor and

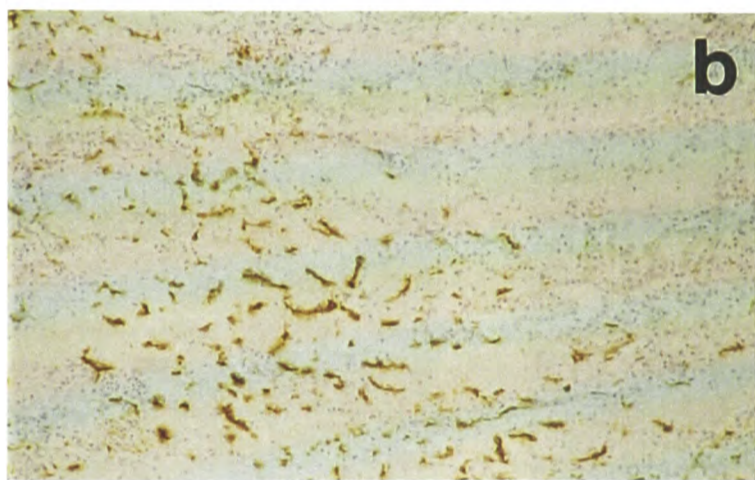
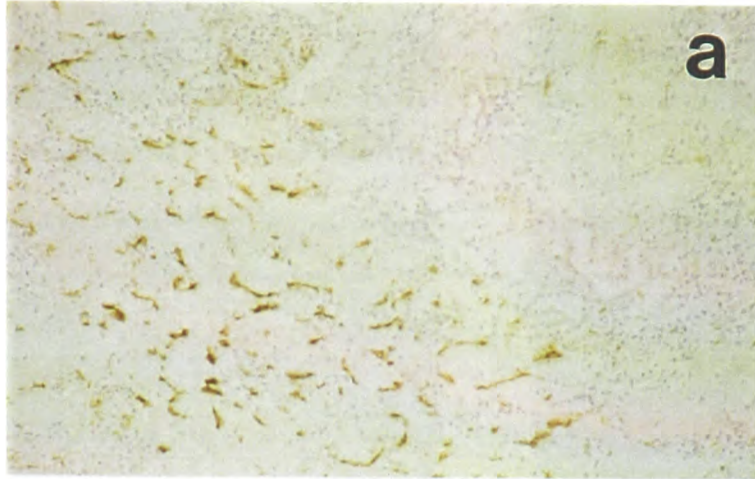


Figure 4.3 P-selectin and CD41 expression in post-reperfusion biopsies.

- (a) Post-reperfusion biopsy stained by the indirect immunoperoxidase method using an anti-P-selectin antibody, showing a positive signal on the intertubular capillaries.
- (b) A consecutive section from the same post-reperfusion biopsy stained with the anti-CD41 platelet-specific antibody showing a similar pattern of staining to that detected for P-selectin (magnification, x100).

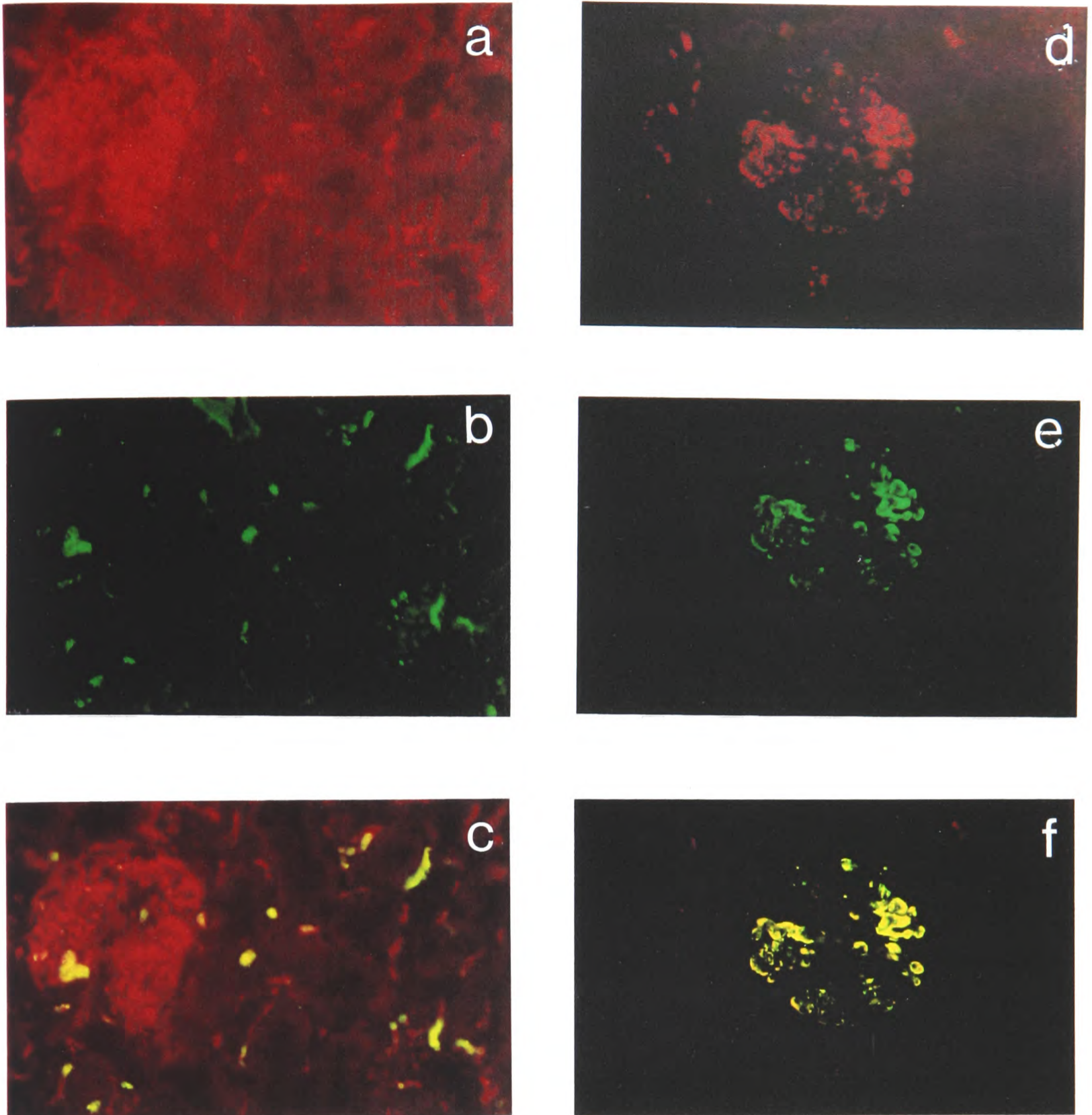


Figure 4.4 Double-immunofluorescent staining to identify the origin of the P-selectin expression in post-reperfusion biopsies.

(a-c) Post-reperfusion biopsy double-stained with a directly conjugated anti-CD41FITC mAb and an anti-ICAM-1 antibody detected with a Texas-Red conjugated secondary antibody. (a): ICAM-1 staining (red) of glomerular and intertubular capillaries on the kidney. (b): CD41+ platelets (green) detected following reperfusion. (c): CD41 positive staining of platelets overlaid on ICAM-1+ endothelial structures, suggesting that platelet deposition on the microvascular endothelium is detectable following reperfusion (magnification, x200). (d-f): Double immunofluorescent staining of a post-reperfusion biopsy with an anti-CD41 FITC mAb and an anti-P-selectin mAb developed with a Texas-Red conjugated secondary antibody. (d): P-selectin expression (red) detected in glomeruli following reperfusion. (e): CD41+ platelets (green) detected on identical structures in the glomerulus. (f): Double-positive (yellow) structures within the glomerulus for CD41 and P-selectin positive staining indicating that P-selectin expression occurs on CD41+ platelets (magnification, x200).

recipient were mismatched for HLA-A2 or B17, were stained with a polymorphic antibody specific for these antigens (MA2.1). The results demonstrated that recipient cells were present following reperfusion (Figure 4.5). Furthermore, in biopsies where platelet deposition and neutrophil infiltration were detected, it was evident that these cells were positive for recipient antigen.

4.7 CLINICAL SIGNIFICANCE OF IMMUNOHISTOLOGICAL CHANGES DETECTED AFTER REPERFUSION IN CADAVER RENAL ALLOGRAFTS

In order to determine the possible factors which may be associated with the changes detected after reperfusion, relevant parameters were examined for the cadaver renal allografts. No significant associations were observed between intertubular neutrophil infiltration and any of the clinical parameters analysed. However in kidneys with a glomerular neutrophil infiltration following reperfusion, the mean cold ischaemia time was 29.9 hours; significantly higher than in cadaver allografts without a neutrophil infiltration in which the mean cold ischaemia time was 22.7 hours ($p=0.0067$) (Table 4.3). This strongly suggests that a long cold ischaemia time plays an important role in causing neutrophil infiltration immediately following transplantation.

The effect of glomerular neutrophil infiltration upon graft function after transplantation was also analysed. Interestingly, delayed graft function was observed in 5/16 (31%) kidneys with a glomerular neutrophil infiltration, but in only 3/38 (8%) of cadaver allografts with no infiltration ($p=0.041$). Similarly, the mean serum creatinine levels at both 3 months and 6 months post-transplant for kidneys with a glomerular neutrophil infiltrate were significantly higher than in those kidneys without an increase in neutrophils post-reperfusion ($p=0.002$ and $p=0.024$, respectively); (Table 4.3). There was no significant association between allografts with neutrophil infiltration post-

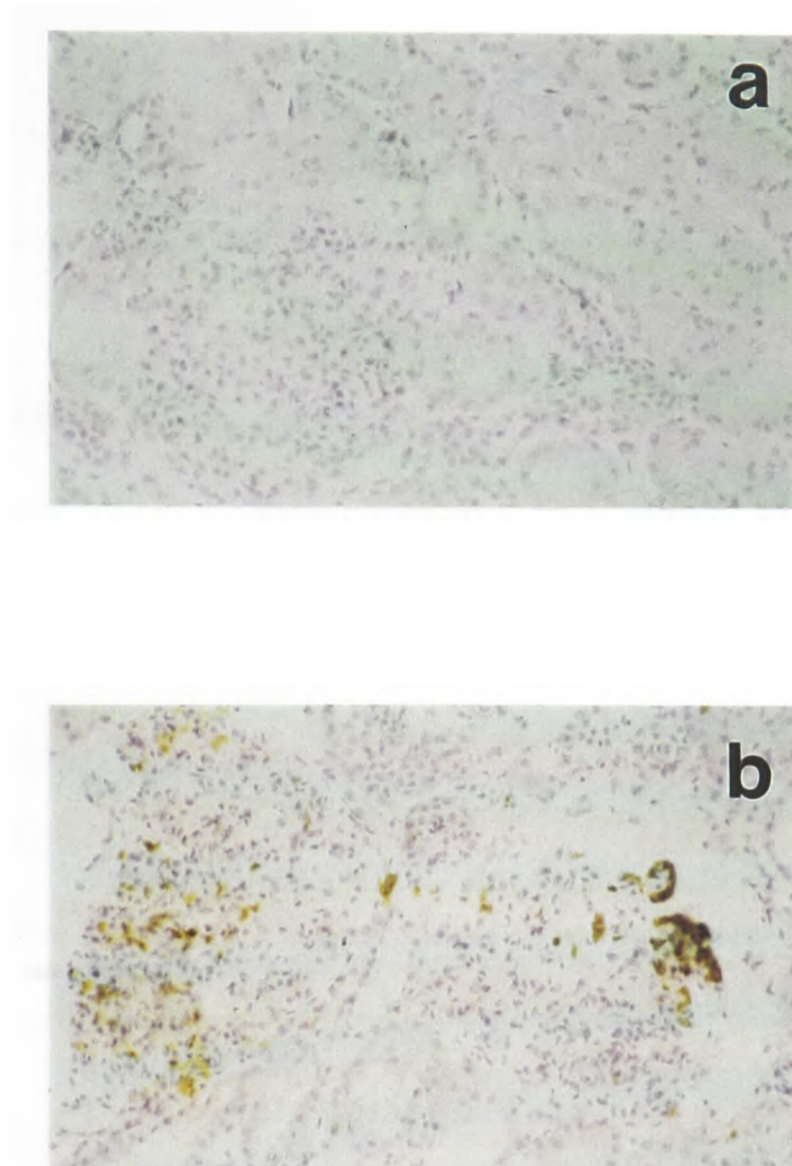


Figure 4.5 Detection of recipient-derived cells in cadaver renal allografts following reperfusion.

Indirect immunoperoxidase staining of (a) pre-reperfusion biopsy from an HLA-A2/B17 negative donor kidney showing the absence of positive staining with MA2.1, an anti-HLA-A2/B17 mAb. (b) Post-reperfusion biopsy stained with the same antibody showing the presence of recipient-derived HLA-A2/B17 positive cells within the graft following reperfusion (magnification, x200).

| Clinical Parameter | Neutrophil infiltration | No infiltration | P-value |
|-------------------------------------|-------------------------|-----------------|---------|
| Donor age (years) | 41.9 ± 12 | 41.3 ± 14 | n/s |
| Mean cold ischaemia time (hr) | 29.9 ± 13.7 | 22.7 ± 7.0 | 0.0067 |
| CIT > 30hr | 8/16 (50%) | 5/39 (13%) | 0.011 |
| Glomerular P-selectin | 5/16 (31%) | 8/39 (21%) | n/s |
| Delayed graft function | 5/16 (31%) | 3/39 (8%) | 0.041 |
| 3 mth serum creatinine (µmol/l) | 188.6 ± 63 | 141.1 ± 40 | 0.0015 |
| 6 mth serum creatinine (µmol/l) | 177.1 ± 59 | 144.6 ± 45 | 0.024 |
| No. of rejection episodes (0:1:2:3) | 10:4:0:2 | 19:12:5:3 | n/s |

Table 4.3. Clinical significance of glomerular neutrophil infiltration after reperfusion in cadaver renal allografts (n=55).

| Clinical Parameter | P-selectin expression | No P-selectin | P-value |
|-------------------------------------|------------------------------|----------------------|----------------|
| Donor age | 44.9 ± 15 | 39.8 ± 14 | n/s |
| Mean cold ischaemia time (hr) | 27.7 ± 15 | 23.7 ± 8 | n/s |
| CIT > 30hr | 4/13 (31%) | 9/42 (25%) | n/s |
| Glomerular neutrophil influx | 5/13 (38%) | 11/42 (26%) | n/s |
| Delayed graft function | 3/13 (23%) | 5/42 (12%) | n/s |
| 3 mth serum creatinine (μmol/l) | 185.6 ± 52 | 144.6 ± 48 | 0.008 |
| 6 mth serum creatinine (μmol/l) | 178.5 ± 58 | 145.9 ± 46 | 0.023 |
| No. of rejection episodes (0:1:2:3) | 5:5:2:1 | 24:11:3:4 | n/s |

Table 4.4. Clinical significance of glomerular P-selectin expression after reperfusion in cadaver renal allografts (n=55).

reperfusion and increases in the number of acute rejection episodes or early rejection episodes.

The increase in platelet deposition detected after reperfusion was also assessed for associations with pre- and post-transplant factors. No significant associations were observed between platelet deposition on the intertubular capillaries and any clinical parameters analysed. In cadaver allografts with glomerular platelet deposition following reperfusion, a significantly higher serum creatinine level was observed than in allografts without an increase in platelets at both 3 months and 6 months following transplantation ($p=0.002$ and 0.023 , respectively); (Table 4.4). Platelet deposition was only observed in 1/11 LRD allografts post-reperfusion. It is possible that the presence of both neutrophils and platelets immediately following reperfusion is indicative of an antibody-mediated event but all patients were transplanted in the absence of a positive crossmatch resulting from clinically relevant antibodies. There were no significant associations in the 7 patients transplanted with autoreactive antibodies.

4.8 DISCUSSION

All solid organs used for transplantation undergo varying degrees of ischaemic damage and reperfusion injury following retrieval, storage and transplantation into the recipient. Hence, the immune response against a transplanted organ may not solely involve an MHC-specific alloimmune response, but in addition, an immediate non-specific inflammatory response caused by ischaemia/reperfusion injury.

In this study we have investigated the events which occur following reperfusion of living-related and cadaver renal allografts, by comparing biopsies obtained from the same kidneys at two time points: pre-reperfusion, (after cold storage but before transplantation), and post-reperfusion biopsies (20-40 min. after revascularisation).

The most striking observations of this study were the clear differences between living-related and cadaver renal allografts in the immediate post-transplant period.

Our results demonstrated that a neutrophil infiltration was observed following reperfusion in 29/55 (53%) cadaver allografts whereas no increase in neutrophil infiltration was detected in any of the living donor renal allografts analysed. An increase in the level of P-selectin expression following reperfusion was also detected in 24/55 (44%) cadaver renal allografts but in only 1/11 (9%) of living-related donor allografts. No direct correlations were observed between P-selectin expression and neutrophil infiltration in the intertubular and/or glomerular regions of cadaver renal allografts following reperfusion. The absence of any significant association between neutrophil infiltration and P-selectin expression following reperfusion may in part, result from the time frame in which the post-reperfusion biopsies were taken.

Another pathway by which neutrophils can attach to endothelium and infiltrate into the graft is through interactions with E-selectin [193, 194]. Although the level of E-selectin expression on the endothelium did not increase after reperfusion, there were high levels of E-selectin detected on the intertubular capillaries of 28/55 (51%) of cadaver renal allografts, but there was no significant association between high levels of E-selectin and neutrophil infiltration. In addition to E-selectin expression, high levels of tubular ICAM-1 and VCAM-1 were detected in cadaver kidneys, but not in LRD kidneys. The expression of high levels of adhesion molecules in cadaver but not LRD kidneys, suggests that injury to the organs as a result of trauma, donor management or brain death may be partly responsible for these detectable differences (see Chapter 3).

Neutrophil infiltration in cadaver renal allografts was significantly associated with prolonged cold storage times suggesting that cold ischaemia and reperfusion may, in part, be responsible for initiating the early inflammatory response against the graft. The presence of a significant neutrophil infiltrate within the microvasculature of renal

allografts suggests that these cells may be involved in active damage to the glomeruli as a result of the granular release of proteolytic enzymes and generation of reactive oxygen metabolites [290, 291, 460]. Activation of oxidative production by neutrophils has been shown *in vitro* to be mediated by cross-linking of L-selectin [239, 240]. Furthermore, interactions between β 2-integrin molecules and endothelial ICAM-1 result in the activation of cytotoxic and phagocytic functions in neutrophils [247, 461]. The release of neutrophil elastase from activated neutrophils may also further enhance endothelial expression of ICAM-1 [462]. In this study, the effects of neutrophil-mediated damage may have been manifest by the presence of neutrophils in the glomeruli of cadaver allografts which was found to be significantly associated with delayed graft function (DGF). This suggests a possible effect of neutrophil-mediated damage upon early graft function.

A recent study of the UNOS Scientific Renal Transplant Registry demonstrated that in 27,096 first cadaveric renal transplants, DGF was a predictor of poor graft survival independent of early acute rejection and HLA matching [54]. Indeed, DGF increased the incidence of acute rejection during the period of hospital stay following transplantation and any acute rejections within six months post-transplant. Furthermore, prolonged cold ischaemia time additionally decreased graft survival independently of DGF or early acute rejection. Our results suggest that neutrophil infiltration resulting from prolonged cold ischaemia and reperfusion injury of cadaver renal allografts may contribute to DGF, initiating an ongoing inflammatory response, which may become manifest in poor long term graft function (e.g. elevated serum creatinine levels at 3 months and 6 months post-transplant). This study provides corroborative immunohistological evidence to support the clinical results from the multicentre studies described above.

The presence of neutrophils after reperfusion was detected along with increased P-selectin expression although no significant associations were detected. However, P-

selectin expression may also be a contributing factor to the initial inflammatory response. P-selectin has been shown to be mobilised from intracellular Weibel-Palade bodies in endothelial cells, or from α -granules in activated platelets and expressed on the cell surface within minutes of initial stimulation [319, 463-465]. Therefore, it was necessary to determine the source of P-selectin expression. Double-immunofluorescent staining using an anti-CD41 platelet-specific antibody, a non-platelet endothelial antibody against ICAM-1, and an anti-P-selectin mAb, enabled us to conclude that P-selectin detected was of platelet, and not endothelial origin. It is surprising that endothelial P-selectin was not detected since there is evidence showing that endothelial P-selectin is expressed rapidly following oxygen free-radical mediated damage *in vitro* [310, 456-458, 466]. The transient nature by which P-selectin is expressed and reinternalised on the endothelium could provide a possible explanation for the lack of endothelial P-selectin observed, if this event occurred prior to the time at which the post-reperfusion biopsies were obtained. The results from *in vivo* studies of ischaemia/reperfusion injury also demonstrate a significant role for P-selectin by the administration of mAb against P-selectin or selectin ligands which attenuated the detrimental effects of neutrophil infiltration [140, 295, 298, 301-303, 387, 392, 393, 467]. However, many of these studies did not provide evidence to differentiate between endothelial or platelet-derived P-selectin expression as the cause of reperfusion injury. It appears that in a variety of experimental models in *in vivo* studies of ischaemia/reperfusion, the role of platelet-derived P-selectin expression may have been underestimated and mistaken for endothelial P-selectin.

Platelets have been shown to attach to damaged blood vessels by binding directly to components of the sub-endothelial matrix, such as collagen, via the CD41/CD61 integrin complex (reviewed in [319]). Platelets may be activated after exposure to free radicals resulting from ischaemia/reperfusion [330, 331]. Activation of platelets leads to the upregulation and increased binding avidity of the CD41/CD61 integrin, and also of the β 2-integrins, thus resulting in localised platelet accumulation [319, 320]. Activated

platelets have been shown to adhere to intact endothelium via CD41/CD61 interactions with fibrinogen and endothelial ICAM-1 [322]. This interaction may facilitate endothelial expression of chemokines such as MCP-1 and adhesion molecules (e.g. ICAM-1) via activation of the transcription factor NF κ B and thus may result in further recruitment of leucocytes to inflammatory regions [323]. The increased expression of endothelial adhesion molecules as a result of platelet interactions have been shown to be mediated by CD40 ligand expressed on platelets [324]. Furthermore, there is evidence to suggest that adherent platelets support neutrophil rolling, arrest and transmigration in a similar manner to activated endothelium [325-329].

The expression of P-selectin by activated platelets enables them to bind to neutrophils, causing aggregation and modifications in the activity of both cell types [333-335]. The deleterious effects of neutrophil-platelet interactions have been shown to act in synergy following ischaemia/reperfusion injury of rat hearts [338]. Moreover, recent evidence suggests that platelet P-selectin interaction with neutrophils is mediated by the selectin ligand PSGL-1, resulting in intracellular signalling events within neutrophils and activation of the β 2-integrin CD11b/CD18 [332]. In transplantation, platelets have been implicated in ischaemia/reperfusion injury of human liver allografts [468] and rat syngeneic lung transplants, where the level of platelet accumulation was shown to be proportional to the preservation time [469]. To our knowledge, the presence of platelets following reperfusion in clinical renal transplantation has not been previously reported.

The interaction of platelets and neutrophils can result in fibrin deposition and thrombus formation [333]. Indeed, in the early era of clinical renal transplantation, the formation of microthrombi in the glomeruli in association with neutrophil accumulation were found during hyperacute rejection of renal allografts, a result of preformed humoral antibodies in the recipient against donor antigens [357, 358, 470]. However, no incidences of hyperacute rejection were observed in this study. Nevertheless, platelet-neutrophil interactions may have a significant contributing factor to the no-reflow

phenomenon where cellular aggregates block capillaries causing further ischaemic damage in the microvasculature [339, 340]. Therefore, although no direct association was found between platelet deposition and neutrophil infiltration in the time frame in which the biopsies were obtained for this study, it is possible that platelet-neutrophil interactions at subsequent time points in the microvasculature of the kidney, may result in further recruitment and accumulation of both cell types at the site of damage and production of pro-inflammatory mediators.

Our results indicate that the presence of platelets and/or neutrophils in the glomeruli post-reperfusion is significantly associated with higher serum creatinine levels at three and six months following transplantation. It is possible that reperfusion injury to the glomerular capillaries may result in partial endothelial denudation and thus platelet attachment to the underlying extracellular matrix. We have demonstrated that neutrophils and platelets detected following reperfusion are of recipient origin and are not pre-existing cells within the donor kidney. Their presence immediately following transplantation of a renal allograft is indicative of an early non-specific, inflammatory event which is potentially detrimental to long term graft function. Results from experimental models of renal transplantation suggest that inhibition of the early events of cold ischaemia and reperfusion injury with immunomodulatory therapy significantly reduces subsequent increases in graft immunogenicity and improves long term graft function and survival [392-395].

The immunohistological results from our study may in part, explain the success of clinical trials where treatment with anti-ICAM-1 and anti-LFA-1 antibodies resulted in a reduction in the incidence of delayed graft function following renal transplantation [402, 471]. Moreover, ICAM-1 antisense oligodesoxynucleotides therapy in a rat renal transplant model attenuated ICAM-1 expression, leucocyte infiltration and improved initial graft function [399]. It is possible that anti-adhesion molecule therapy may limit the damage resulting from ischaemia/reperfusion injury by inhibiting the infiltration of neutrophils into the kidney. Further evidence of the significant effect of ischaemia/

reperfusion injury in clinical renal transplantation has been demonstrated by the increased 1 year and 4 year graft survival observed in patients treated with superoxide dismutase, (a free radical scavenger) given intravenously just prior to reperfusion [123]. While investigations were not performed to analyse the biological effects of administering superoxide dismutase at reperfusion, the results from the clinical outcome were promising and indicate that reperfusion injury may have a significant impact on chronic changes to the graft.

We have demonstrated that immunohistological changes following reperfusion of renal allografts are significantly associated with short and long term graft function. Improved methods of preservation and therapeutic strategies directed at reducing the detrimental effects of ischaemia/reperfusion injury require further investigation. Furthermore, immunohistological analyses performed in conjunction with clinical studies would provide a more effective approach to understanding the mechanisms involved in this process.

**CHAPTER 5. EFFECTS OF LATE REFLUSH ON
ISCHAEMIA/ REPERFUSION INJURY IN CLINICAL
RENAL TRANSPLANTATION**

5.1 INTRODUCTION

Delayed graft function (DGF) occurs in approximately one third of all cadaveric renal allografts performed in Europe and is defined by the requirement for dialysis in the first post-operative week [370]. This results in prolonged hospitalisation of the recipient, complications in the management of immunosuppressive regimens and may adversely affect graft outcome and survival [41, 42]. Studies performed on large multicentre registry databases suggest that DGF, independent of acute rejection or HLA mismatching, may be an important indicator of poor long term survival in cadaveric allografts [49, 50, 53, 54].

DGF may be influenced by a number of factors including donor age, cause of death, and treatment of the recipient with nephrotoxic drugs (e.g. cyclosporine). However, prolonged cold ischaemic storage has been demonstrated by both single and multi-centre studies to be the most significant factor influencing DGF following transplantation [45, 48-54, 72]. The precise mechanisms by which cold ischaemia causes DGF in renal transplantation are not fully understood, but there may be a number of significant events that contribute to this process.

Ischaemia to the organ results in anaerobic metabolism within the tissues, leading to reduction in the levels of ATP and accumulation of cytotoxic products [77].

Hypothermic storage reduces the rate at which these events occur during ischaemia. However, the loss of ATP and further dephosphorylation increases cellular levels of adenosine, inosine and hypoxanthine, depleting the total nucleotide pool required for rephosphorylation once blood flow is restored. Loss of function of membrane ion channel regulators results in cellular oedema, with diffusion of water, sodium and chloride into the cell and extracellular diffusion of potassium [83]. In addition, the influx of calcium into the cytosol results in the activation of phospholipase, a membrane lytic enzyme [85, 86]. The processes involved in ischaemic damage are further exacerbated by the generation of lactic acid leading to progressive intracellular acidosis and activation of lysosomal enzymes [81].

The cellular deterioration that occurs during ischaemia may be limited by flushing the organs before storage with cold preservation solutions designed to prevent specific degradative pathways experienced during ischaemia. A major requirement for preservation solutions is inclusion of an impermeable molecule (e.g. lactobionate, gluconate, sucrose, mannitol) to provide an extracellular osmotic force [77]. Moreover, inclusion of effective buffers (e.g. phosphate, citrate, bicarbonate) to counter intracellular acidosis and addition of electrolytes may also provide protection against ischaemic damage during storage. Addition of other agents with biochemical, pharmacological and vascular effects to preservation solutions have resulted in varying degrees of success.

Upon reperfusion of the kidney, accumulated end-products of anaerobic conditions such as hypoxanthine and xanthine oxidase, may generate oxygen free radicals, resulting in cellular damage and microvascular injury. Reperfusion injury is characterised by the generation of oxygen free radicals and infiltration of neutrophils into the tissue, further exacerbating the damaging events encountered during ischaemia [74, 97, 161, 292]. Treatment with antioxidants or mAb directed against leucocyte-

endothelial interactions have demonstrated beneficial effects on graft outcome following reperfusion of renal allografts [117, 119, 123, 402, 471].

Two studies have been performed to determine whether simple reflushing of the kidneys with preservation solution after the period of cold storage may remove the harmful products that have accumulated in the vasculature, and prevent the detrimental effects of ischaemia/reperfusion injury on DGF. Parrott and colleagues demonstrated a significant reduction in the incidence of DGF in 39 consecutive reflushed kidneys compared with the previous 106 renal transplants performed without reflushing [377]. Lodge and coworkers performed a prospective, randomised trial, but with only 10 patients in each arm of the study, also demonstrating a significant reduction in the incidence of DGF [378]. However, histological analysis was not presented in either study. Therefore, a randomised, prospective pilot study of reflushed and non-reflushed cadaveric kidneys was undertaken to confirm or refute the published observations and in addition, to evaluate histological and immunohistochemical changes to understand the process that reflushing kidneys may have on ischaemia/reperfusion injury. Unfortunately, the trial presented in this Thesis was stopped before the desired number of patients were recruited to each arm of the trial because of abnormal histological findings in the reflushed kidneys.

5.2 MATERIALS AND METHODS

5.2.1 Patients and Biopsy Material

A prospective randomised control study was performed in which 18 consecutive kidneys from cadaveric donors were randomised to “reflush” or “control” arms of the study. All kidneys were initially flushed with and stored in Marshall’s hypertonic citrate solution. Immediately before transplantation, kidneys were prepared in routine fashion

and pre-transplant wedge biopsies taken for histology and immunohistochemical analysis. Control and reflush kidneys were biopsied again just before wound closure (approximately 30-60 min after reperfusion).

Patients were managed with a standard triple immunosuppression therapy protocol. Cyclosporine was initially given at a dosage of 10mg/kg/day and adjusted according to trough blood levels. Azathioprine was given at 1.5 mg/kg/day and prednisolone at 20mg/day. All patients received standard post-operative monitoring and management. In cases of delayed graft function (DGF) where there was no rejection, cyclosporine dose was reduced by 50% on about day 7 until graft function recovered. Highly sensitised patients (panel reactive antibody >85%) at risk of rejection or DGF received antithymocyte globulin (ATG) (Fresenius; 10-14 days), azathioprine, and prednisolone, with delayed introduction of cyclosporine 3 days before completion of the administration of ATG.

DGF was defined as the need for dialysis in the first week after transplantation and the date of resolution defined as the first day on which there was a spontaneous fall in plasma creatinine. Acute rejection was diagnosed as the presence of acute graft dysfunction that was responsive to anti-rejection treatment in association with histological evidence of rejection from a needle core biopsy. In cases of DGF, kidneys were monitored by daily fine needle aspiration cytology (from day 4) and suspected rejection was confirmed by needle core biopsy. Acute rejection was treated with methylprednisolone (500 mg, intravenously) on 3 consecutive days. Biopsy proven steroid resistant rejection was treated with ATG or OKT3 (Ortho-Cilag, High Wycombe, UK).

5.2.2 Late Reflush Protocol

Reflush kidneys were perfused via the renal artery with 500ml of ice-cold Marshall's solution from a height of 1 meter as described by Lodge and coworkers after the period

of cold storage, immediately prior to implantation [378]. Control kidneys were transplanted without further intervention.

5.2.3 Immunohistochemical Analysis

7 μ m cryostat tissue sections from wedge biopsies were stained with monoclonal antibodies using an indirect immunoperoxidase technique described in Chapter 2. The results from Chapter 4 demonstrated that changes in neutrophil infiltration and P-selectin expression were evident following reperfusion. Therefore sections were stained with monoclonal antibodies; G1 (anti-P-selectin [CD62P] [423]), and 1G10 (anti-CD15s neutrophil marker [459]). A positive control was provided by staining of sections with an anti-CD45 mAb (F10.89.4 [418]) and an anti-dog Thy-1 (F3.20.7 [420]) monoclonal antibody was used as a negative isotype control.

5.2.4 Assessment of Staining

Staining for P-selectin and neutrophil infiltration were scored "blind" by two independent observers. The semi-quantitative grades for P-selectin expression were scored as follows: 0 - negative; 1- predominantly negative- an isolated positive vessel; 2- focus of positive vessels/ occasional positive vessels; 3- multiple foci/ positive vessels throughout biopsy. A significant increase in the level of expression of P-selectin after reperfusion was considered as an increase in Grades of ≥ 1 . Changes between Grades 0 and 1 were not considered significant.

Leucocytes were quantified and expressed as; (i) mean number of positive cells/glomerulus per section, with a minimum of 3 glomeruli required for inclusion in the analysis and (ii) mean number of positive cells in the intertubular areas per field of view (x10 objective). A significant increase in glomerular infiltration after reperfusion was taken as an increase in mean glomerular count of ≥ 1.5 and an increase in intertubular infiltration was scored as an increase of ≥ 10 positive cells.

5.3 RESULTS

5.3.1 Clinical Parameters

The reflush and control groups were well matched with respect to recipient age and sex, number of previous transplantations, degree of sensitisation, donor sex, cold ischaemia time, and HLA matching, but the mean donor age was significantly lower in the reflush group (Table 5.1). Warm ischaemia time was less than 5 min in all cases and the kidneys were bathed in ice-cold saline during implantation to prevent rewarming. ATG induction therapy was used for one of the highly sensitised patients in the control group but none of the reflush group.

5.3.2 Early Graft Function

In the control group, only 3/9 (33%) kidneys had poor initial graft function requiring dialysis in the immediate period following transplantation. A higher incidence of delayed graft function (DGF) was observed in the reflush group 6/9 (66%) (Table 5.2). The duration of DGF in each of the grafts in the reflush group was 5, 6, 11, 13 and 32 days, with one of the reflushed kidneys lost secondary to renal vein thrombosis on day 0. The corresponding figures for the 3 grafts with DGF in the control group were 10, 14 and 16 days. At 1 week, this was reflected in the median plasma creatinine (217 vs 610 $\mu\text{mol/L}$) and creatinine clearance (30 vs 7 ml/min) of surviving grafts in the control and reflush groups, respectively, but by 1 month there were no significant differences in graft function (Table 5.2).

5.3.3 Acute Rejection Episodes

Only two of the controls experienced acute rejection episodes within the first year and there were no cases of steroid-resistant rejection. In contrast, acute rejection was diagnosed in 7/8 patients at risk of rejection in the reflush group. Four of these patients experienced >1 rejection episode and two required ATG therapy for biopsy-proven steroid-resistant rejection. Two control kidneys failed within the first 6 months: one at 2

| Clinical Parameter | Control (n=9) | Reflush (n=9) |
|--|-----------------|-----------------|
| Recipient age (mean \pm SD) | 50.7 \pm 15.6 | 38.2 \pm 11.6 |
| Recipient gender (M:F) | 5:4 | 5:4 |
| First graft : Retransplant | 8:1 | 8:1 |
| Highly sensitised (PRA >85%) | 2:9 | 0:9 |
| ^a Donor age (mean \pm SD) | 52.3 \pm 12.0 | 37.6 \pm 8.9 |
| Donor gender (M:F) | 5:4 | 5:4 |
| Local : Shipped Kidney | 6:3 | 6:3 |
| CIT (hours \pm SD) | 28.1 \pm 8.8 | 27.7 \pm 7.6 |
| CIT (\leq 18hrs : 18-30hrs : > 30hrs) | 1:4:4 | 2:2:5 |
| HLA-DR mismatches (0:1:2) | 7:2:0 | 5:4:0 |
| HLA-A mismatches (0:1:2) | 3:3:3 | 1:6:2 |
| HLA-B mismatches (0:1:2) | 0:6:2 | 2:4:3 |

Table 5.1 Demographics and risk factors for cadaveric renal allografts.

^ap<0.05, Student's T-test.

CIT = cold ischaemia time

| Outcome measurements | Control | Reflush | p-value |
|---|----------------|----------------|----------------|
| Delayed graft function (DGF) | 3/9 | 6/9 | n/s |
| Abnormal post-reperfusion biopsy | 0/8 | 6/9 | 0.007 |
| Increased P-selectin expression | 5/7 | 5/7 | n/s |
| Glomerular neutrophil infiltration | 5/7 | 5/7 | n/s |
| Histological acute tubular necrosis (1 week) | 4/9 | 6/8 | n/s |
| 1 week serum creatinine ($\mu\text{mol/L}$, median) | 217 | 610 | n/s |
| 1 week creatinine clearance (ml/min, median) | 30 | 7 | n/s |
| 1 week creatinine clearance ($>10\text{ml/min}$) | 6/9 | 3/9 | n/s |
| 1 mth serum creatinine ($\mu\text{mol/L}$, median) | 185 | 201 | n/s |
| 1 mth creatinine clearance (ml/min, median) | 32 | 36 | n/s |
| 1 mth creatinine clearance ($>10\text{ml/min}$) | 9/9 | 7/9 | n/s |
| Acute rejection in first year | 2/9 | 7/8 | 0.012 |

Table 5.2 Graft outcome indicators in refushed and control renal allografts.

P-values were calculated by Fisher's exact test or Mann-Whitney test.

n/s = not significant

months and the other at 6 months as a result of death from complications from tissue-invasive CMV disease. At 1 year, there was no apparent difference in the function of the surviving grafts.

5.3.4 Histological and Immunohistochemical Changes Post-Reperfusion

Histological analysis of post-reperfusion biopsies showed abnormal cell debris within the tubules or eosinophilic proteinaceous material within Bowman's capsule in 6/9 reflushed kidneys. These features were not evident in any of the control kidneys. In addition, the changes were not present in the pre-transplant biopsies, did not correlate with the length of the cold ischaemia time and had largely resolved by one week. Histological acute tubular necrosis was reported more frequently in the 1 week biopsies of reflushed kidneys (6/8 vs 4/9). All cases of clinical acute tubular necrosis were confirmed histologically.

An increase in neutrophil infiltration and P-selectin expression was detected predominantly in the glomeruli of both control (5/7) and reflush (5/7) post-reperfusion biopsies (Table 5.3). However, in this small number of biopsies, no significant associations were observed between renal allografts with increased P-selectin expression or neutrophil infiltration and the length of cold ischaemia time or incidence of delayed graft function, irrespective of the treatment group. This may be a reflection of the small sample number and the relatively high mean cold ischaemia times of both the reflushed and control kidneys. These results suggest that the effect of late reflush does not prevent these post-reperfusion changes associated with ischaemia/reperfusion injury of cadaveric renal allografts.

| | | Reflush Group (n=7) | | | | Control Group (n=7) | | | |
|-----|--------------------------------|----------------------------|-------------------|----------------------|--------------------------------|----------------------------|-------------------|----------------------|--|
| | Neutrophil Infiltration | No Infiltration | P-selectin | No P-selectin | Neutrophil Infiltration | No Infiltration | P-selectin | No P-selectin | |
| n | 5/7 | 2/7 | 5/7 | 2/7 | 5/7 | 2/7 | 5/7 | 2/7 | |
| DGF | 3/5 | 1/2 | 4/5 | 0/2 | 2/5 | 1/2 | 2/5 | 1/2 | |
| CIT | 28.5 ± 7.6 | 20.2 ± 3.9 | 26.4 ± 7.1 | 25.5 ± 9.2 | 30.1 ± 7.2 | 30.5 ± 10.2 | 29.1 ± 8.0 | 32.8 ± 8.0 | |

Table 5.3 Immunohistochemical analysis for neutrophil infiltration and P-selectin expression following reperfusion of reflushed and control renal allografts.

Pre- and post-reperfusion biopsies from reflushed kidneys (n=7) and control kidneys (n=7) were assessed semi-quantitatively for changes in neutrophil infiltration and increased P-selectin expression following reperfusion. No significant differences were observed between the two treatment groups in P-selectin expression, neutrophil infiltration, delayed graft function (DGF) and cold ischaemia time (CIT). No significant differences were observed between allografts with immunohistochemical changes and those without post-reperfusion changes within each treatment group.

5.4 DISCUSSION

In renal transplantation, prolonged cold storage times have been found to be significantly associated with delayed graft function [45, 48-54, 72]. In an attempt to alleviate this problem, development of new preservation solutions and therapeutic strategies to prevent ischaemic damage have been employed with varying degrees of success. The potentially beneficial effects of reflushing kidneys after the period of cold storage and before transplantation to remove harmful products that may have accumulated over the ischaemic period have been shown to significantly improve graft outcome following transplantation. The results from an initial non-randomised, prospective study of 145 renal transplants performed by Parrott and colleagues demonstrated that reflushing the kidneys immediately prior to transplantation significantly reduced the incidence of DGF by approximately 50% [377]. Furthermore, a prospective randomised trial using a similar reflushing protocol but on a smaller cohort of renal allografts, demonstrated similar reductions in the incidence of DGF [378]. In both these studies, kidneys were stored in hyperosmolar citrate (HOC) solution following organ procurement, currently the preservation solution most widely used in the UK [76] and the solution used for the study reported in this Thesis.

The initial aim of the clinical trial presented in this Chapter was to conduct a large, prospective randomised study to determine the effects of reflushing kidneys on reperfusion injury, subsequent graft function and acute rejection. However, the study was abandoned earlier than anticipated with only 18 patients recruited into the trial due to the histological abnormalities detected in post-reperfusion biopsies from reflushed renal allografts and the high incidence of DGF observed. Abnormal cellular debris within the tubules and eosinophilic proteinaceous material deposited within Bowman's capsules were detected in 6/9 reflushed kidneys following reperfusion that were not present in any of the control kidneys. No histological data was presented in the studies previously published by Parrot et al. and Lodge et al.

Contrary to the results from the published reflush studies described above, the incidence of DGF was higher in the reflushed kidneys (6/9) than in controls (3/9) despite the fact that the reflush procedure performed in this study was similar to that performed by Lodge et al. and Parrott et al. Parrott and colleagues found that the period of warm ischaemia experienced during anastomosis significantly influenced the incidence of DGF [377]. It is unlikely that a warm ischaemic insult was experienced during implantation of the kidneys in the current study, as all kidneys are routinely bathed in ice-cold saline during this period.

Lodge and coworkers examined the effects of reflushing with either HOC or phosphate buffered sucrose (PBS140) solutions compared with non-reflushed kidney controls [378]. Although histological biopsies were not obtained, 20ml effluent samples from each of the reflushed kidneys (n=10 per group) were analysed for the presence of sodium, potassium, calcium, creatine phosphokinase and pH changes. The results demonstrated that reflushing with PBS140 resulted in an overall loss of both sodium and potassium from the kidney, and venous effluent from both reflush solutions contained significant levels of calcium, lactate dehydrogenase and creatine phosphokinase with a reduction in pH. Comparison with effluent samples obtained at the end of the reflush demonstrated that reflushing the kidneys prior to transplantation removed calcium and ATP breakdown products released into the venous effluent. The depletion of potential oxygen free radical precursors from the vasculature of reflushed kidneys may prevent ischaemia/reperfusion injury of the grafts and thus explain the significant reduction in the incidence of DGF. Furthermore, the reduction in DGF was achieved despite the increased use of high dose cyclosporine monotherapy in the reflushed group.

The absence of a reduction in the incidence of DGF in the reflushed kidneys presented in this Chapter may be difficult to interpret considering the similarities in the conditions

employed between the studies. However, it is possible that the long cold ischaemia times in the current study were responsible for the high incidences of DGF observed. Although both the control (28.1 ± 8.8 hrs) and reflushed (27.7 ± 7.6 hrs) kidneys had similar mean cold storage times, the length of cold ischaemia was unusually high compared with the studies performed by Parrott et al (21 hrs) and Lodge et al. (23 hrs), and from the cohort of patients studied in Chapter 4 of this Thesis (24 hrs). Furthermore, immunohistochemical analysis demonstrated significant increases in neutrophil infiltration and P-selectin expression in 5/7 (71%) kidneys from both the reflushed and control kidneys following reperfusion. These results suggest that reflushing of cadaver kidneys prior to transplantation did not reduce the immunohistological changes associated with ischaemia/reperfusion injury that were reported in Chapter 4.

Corroborative evidence to the results presented in this Chapter have been published in a prospective, randomised trial of 99 first cadaveric renal allografts [379]. The reflushed (n=50) and non-reflushed (n=49) groups were well-matched with respect to HLA matching, cold ischaemia time, anastomosis time and cyclosporine monotherapy, with no significant differences in DGF, acute rejection episodes or serum creatinine levels at day 7 and 3 months post-transplant. Although no histological or biochemical data was available in this larger study, the results indicate that simple reflushing of kidneys after cold storage has no beneficial effects with respect to DGF and acute rejection episodes.

Therefore, despite the detection of ischaemic breakdown products in the venous effluent of reflushed kidneys in the trial performed by Lodge et al, it is likely that the removal of these products alone is not sufficient to maintain good renal function after transplantation. Other pathways by which ischaemic injury exerts a pathological effect on graft function may have a greater impact than simple reflushing of kidneys. The development of a variety of preservation solutions to limit the effects of cold ischaemic damage have improved the length of time an organ may be stored, but the incidence of

DGF in the modern era of renal transplantation remains relatively high at approximately 30% in cadaveric renal allografts. Therefore, modifications to current preservation solutions have been investigated in an attempt to reduce DGF.

Protection of kidneys against ischaemia/reperfusion injury has been studied with the use of antioxidants to inhibit the effects of oxygen free radical damage. Development of University of Wisconsin (UW) solution containing the xanthine oxidase inhibitor, allopurinol, has been shown to significantly improve primary graft function in experimental and human renal transplantation [370, 472], and prolonged viable cold storage times in liver transplantation [369]. Experimental transplant studies to determine the effects of allopurinol alone, or in combination with superoxide dismutase (SOD), demonstrated protective effects in kidneys exposed to prolonged periods of ischaemia [117, 119]. Clinical trials performed with SOD demonstrated no immediate benefit following transplantation, but significantly improved long term graft survival compared with placebo controls [123].

It is widely recognised that acute tubular necrosis (ATN) diagnosed histologically by the characteristic flattening and denucleation of tubule cells is strongly associated with ischaemic injury and DGF in kidney transplantation. The precise mechanisms by which ATN occurs over the cold ischaemic period is unclear, but it is likely that a combination of events involving cell membrane and mitochondrial dysfunction and activation of cytolytic enzymes contribute to this process. Experimental evidence suggests that ATN induced by warm ischaemia may induce an inflammatory response with upregulation of cytokines and MHC antigens [69, 70]. Furthermore, *in vitro* data from cultured human renal tubular cells exposed to hypoxia demonstrated an increase in ICAM-1 expression [473]. The effects of cold ischaemia and reperfusion induced inflammatory responses in a rat kidney model demonstrated increased expression of cytokines, adhesion molecules and MHC antigens, in association with leucocyte infiltration [392-394]. The upregulation of these molecules was abrogated by treatment with anti-leucocyte

adhesion molecule therapy and costimulatory signal inhibitors. Furthermore, clinical trials have been performed in renal transplantation with antibodies directed against either ICAM-1 or LFA-1 to inhibit leucocyte-endothelial interactions, resulting in a reduction in the incidence of DGF in both trials [402, 471]. More recently, a multicentre trial using an anti-ICAM-1 mAb demonstrated no beneficial effects with respect to graft function, rejection episodes or 1 year graft survival, although the authors suggested that a higher dose may have had greater therapeutic efficacy [403]. It is possible that anti-adhesion molecule therapy may limit the damage caused by ischaemia/reperfusion injury by inhibiting the infiltration of neutrophils into the kidney, but the optimal conditions for antibody therapy requires further investigation.

The results from these studies, and those presented in Chapter 4, demonstrate that ischaemia/reperfusion injury may be dependent on a combination of events that occur over the period of cold ischaemic storage and that simple reflusing of the kidneys to remove ischaemic by-products from the vasculature are inadequate for the prevention of this damage. It is likely that interventional therapies designed specifically to target oxygen free radical production and neutrophil infiltration may be more effective in limiting the harmful effects of ischaemia/reperfusion injury. Currently, an ongoing Phase I trial is being performed in the Oxford Transplant Centre to look at the efficacy of administering an anti-CD18 mAb on graft outcome following renal transplantation. In addition, a novel form of superoxide dismutase has been obtained to determine whether this may be successfully incorporated into preservation solutions to enable direct protection of endothelial cells against oxygen free radical damage following reperfusion.

**CHAPTER 6. EFFECTS OF LECITHINISED SUPEROXIDE
DISMUTASE (LEC-SOD) ON ENDOTHELIUM IN AN IN
VITRO MODEL OF ISCHAEMIA/ REPERFUSION INJURY**

6.1 INTRODUCTION

Ischaemia of an organ graft results from the transient period of warm ischaemia experienced during removal from the donor, cold ischaemia associated with preservation and storage, and a second period of warm ischaemia during revascularisation of the graft. During the period of ischaemia, natural sources of free radical scavengers (e.g. glutathione, ascorbic acid, tryptophane, superoxide dismutase) are depleted, such that upon reperfusion, the burst of free radical production overwhelms the cellular capacity for protection against free radical-mediated damage. Studies in experimental models have demonstrated that reperfusion injury is characterised by oxygen free radical production, induced adhesion molecule expression and neutrophil infiltration [74, 75, 97]. Indeed, we have demonstrated that in cadaver renal allografts with prolonged cold ischaemia, neutrophil infiltration was detected in the microvasculature of the graft approximately 30 minutes after reperfusion, and this was not observed in living-related allografts with minimal ischaemia times (see Chapter 4). Leucocyte homing to lymphoid and non-lymphoid tissues during acute inflammatory responses occurs almost exclusively in the microvasculature, and it is in these regions where ischaemia/reperfusion injury may exert its pathophysiological effects. Furthermore, the endothelial barrier provides the initial point of contact between the graft and the recipient circulation carrying oxygen and leucocytes. Results from *in vitro* studies have shown that endothelial cells exposed to hypoxia/reoxygenation

increase their expression of adhesion molecules and bind neutrophils as a result of oxygen free radical production [309-311, 456, 458].

In transplantation, alleviation of the effects of ischaemia/reperfusion injury have been attempted with the development of organ preservation solutions such as Euro-Collins, HTK (histidine, tryptophan, α -ketoglutarate), UW (University of Wisconsin) and Marshall's (hypertonic citrate) solution [9]. These solutions contain a variety of buffers, slowly permeable solutes to prevent oedema, glucose metabolites and free radical scavengers. However, despite improvements in organ preservation solutions, the effects of prolonged cold ischaemia have been shown to be significantly associated with delayed graft function and poor long term graft survival [53].

Free radical scavengers that are present in preservation solutions (e.g. allopurinol, glutathione, ascorbate) have not been shown to be effective in preventing reperfusion injury or improving graft outcome. However, two trials have been performed with superoxide dismutase (SOD) (an enzyme that converts the highly reactive $\cdot\text{O}_2^-$ molecule to molecular oxygen and hydrogen peroxide) in renal transplantation. Pollak and coworkers administered SOD intravenously before and 1 hour post-reperfusion, but no beneficial effects were observed with respect to early graft function [121]. In a separate trial, Land and colleagues administered a single, higher dose of SOD intravenously immediately prior to reperfusion. The results demonstrated a significant reduction in first acute rejection episodes and improved 1- and 4-year graft survival compared with placebo controls [123].

A novel form of superoxide dismutase (lec-SOD) has been obtained for this study which has been shown to have a higher affinity for endothelial cells, a longer half-life and is thus a more effective free-radical scavenger than unmodified, recombinant human CuZn-SOD (rhSOD) [474]. The increased affinity of lec-SOD for cell membranes is attributed to the covalent linkage of 4 molecules of lecithin, (a phospholipid molecule

found ubiquitously in cellular membranes and found to be highly cytotoxic [475, 476]) to one molecule of recombinant human CuZn-SOD [427]. In renal transplantation, it would be desirable to incorporate lec-SOD into the preservation solution during cold storage and localise its protective capacity to the endothelium where the initial burst of superoxide generation is thought to occur. Therefore, we have obtained a microvascular endothelial cell line, HMEC-1 (created from transfection with pBR322 plasmid containing SV40 gene of microvascular endothelial cells isolated from neonatal foreskin) and freshly isolated human umbilical vein endothelial cells (HUVEC), to determine the efficacy of lec-SOD binding after incorporation into Marshall's preservation solution. Furthermore, to simulate the physiological conditions experienced in the clinical setting, we have developed a cold hypoxia/reoxygenation model to investigate the effect of lec-SOD on hypoxia/reoxygenation induced cell death, neutrophil adhesion and adhesion molecule expression.

6.2 PHENOTYPIC CHARACTERISATION OF MACROVASCULAR AND MICROVASCULAR ENDOTHELIAL CELLS

A phenotypic analysis of HUVEC and HMEC-1 was performed to confirm characteristic endothelial responses to cytokine stimulation reported in previously published studies. The results from this study form the basis for subsequent experiments in which comparisons are made between the responses of microvascular and large vessel endothelial cells to hypoxia/reoxygenation in the presence of lec-SOD.

Confluent monolayers of HUVEC and HMEC-1 displayed characteristic cobblestone morphology in culture flasks prior to stimulation with the following cytokines using doses previously reported [190, 425, 426]: 1000U/ml IFN γ for 72 hours; 200U/ml TNF for 6 hours; 4U/ml IL-1 β for 6 hours. Following cytokine stimulation, HUVEC were stained with mAb against CD31, HLA Class I, HLA Class II, E-selectin, ICAM-1

and VCAM-1 (See Table 2.1) and analysed by FACS following negative gating against cells stained with an irrelevant isotype control antibody. Each experiment was repeated at least three times.

6.2.1 CD31 Expression

Unstimulated HUVEC and HMEC-1 both constitutively expressed CD31 as has been previously reported for endothelial cells *in vivo* and *in vitro*, with higher baseline levels of CD31 expression on HUVEC (Figure 6.1a and 6.2a). Stimulation with IL-1 β and TNF did not increase the expression of CD31 on either cell type, whereas stimulation with IFN γ resulted in upregulated expression on both HUVEC and HMEC-1 cells (Figure 6.1a and 6.2a).

6.2.2 HLA Class I Expression

Higher levels of constitutively expressed HLA Class I were detected on HMEC-1 (mean fluorescence intensity = 204) compared with HUVEC (mean fluorescence intensity = 99) (Table 6.1 and 6.2). Stimulation with IFN γ significantly upregulated HLA Class I expression on both HMEC-1 and HUVEC, whereas HLA Class I expression was unchanged following IL-1 β and TNF stimulation on HMEC-1 cells (Figure 6.1b and 6.2b). In contrast, a small subpopulation of HUVEC upregulated HLA Class I expression in response to TNF stimulation.

6.2.3 HLA Class II Expression

In accordance with results obtained from previously published studies, HLA Class II expression was absent on resting HUVEC and HMEC-1 cells, with HLA Class II induction detected following IFN γ stimulation of both cell types, but not by IL-1 β or TNF stimulation (Figure 6.1c and 6.2c).

6.2.4 E-selectin Expression

The expression of E-selectin was absent on both unstimulated HUVEC and HMEC-1 cells as has been previously reported (Figure 6.1d and 6.2d). *De novo* E-selectin expression was detected on 63% and 57% HUVEC cells stimulated with TNF and IL-1 β respectively, but not by IFN γ (1%) (Table 6.1). In contrast, the magnitude of E-selectin induction in response to cytokine stimulation was markedly reduced in HMEC-1 cells, with a small positive shift in total cell populations stimulated with either TNF, IL-1 β or IFN γ (Figure 6.2d).

6.2.5 ICAM-1 Expression

Higher levels of constitutive ICAM-1 expression were detected on HMEC-1 cells (mean channel fluorescence = 141) than HUVEC (mean channel fluorescence = 27) (Tables 6.1 and 6.2). Following stimulation with IL-1 β and TNF, the magnitude of ICAM-1 upregulation was greater in HUVEC than observed for HMEC-1 cells (Figure 6.1e and 6.2e). The response to stimulation with IFN γ resulted in the greatest level of ICAM-1 upregulation in HMEC-1 cells compared with stimulation by IL-1 β and TNF (Figure 6.2e). In contrast, upregulation of ICAM-1 expression in HUVEC cells stimulated with IFN γ was less pronounced than in HUVEC stimulated with IL-1 β or TNF (Figure 6.1e).

6.2.6 VCAM-1 Expression

Low levels of VCAM-1 were detected on resting HMEC-1 (11%) and HUVEC (20%) (Figure 6.1e and 6.2e). Following stimulation of HUVEC with TNF, IL-1 β and IFN γ , upregulated VCAM-1 expression was detected on 45%, 34% and 32% of cells, respectively (Table 6.1). The response of HMEC-1 to TNF, IL-1 β and IFN γ stimulation was less pronounced, with 24%, 18% and 22% of cells being positive for VCAM-1 expression, respectively (Table 6.2).

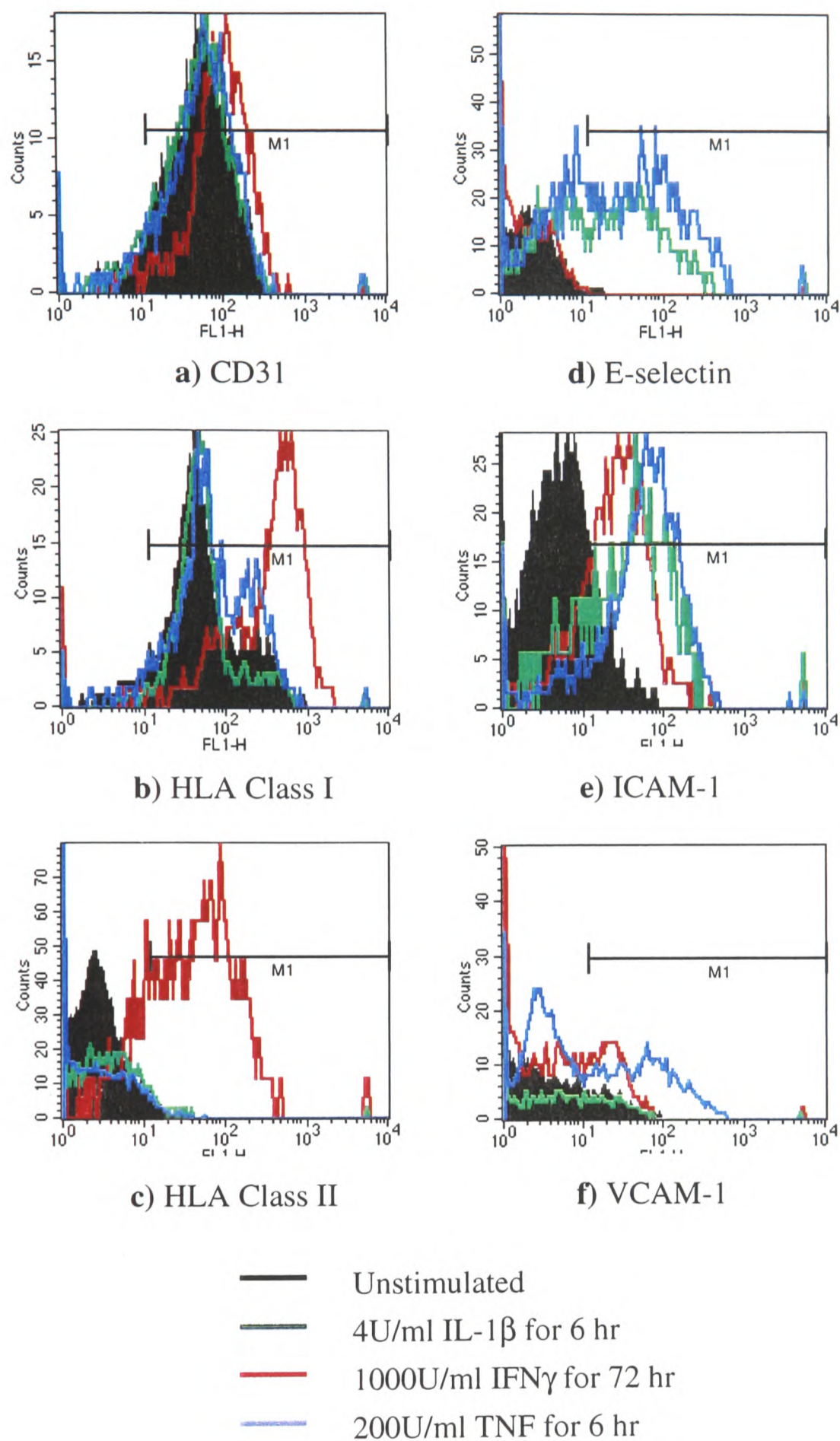


Figure 6.1 Phenotypic characterisation of HUVEC by FACS analysis following cytokine stimulation.

HUVEC were stimulated with the inflammatory cytokines IL-1β, IFNγ and TNF to assess the endothelial expression of adhesion molecules and HLA antigens. The cells were stained with mAb against (a) CD31; (b) HLA Class I; (c) HLA Class II; (d) E-selectin; (e) ICAM-1; (f) VCAM-1 and detected with a secondary FITC-labelled goat anti-mouse Ig. Mean fluorescence intensity and percentage of positive cells are represented in Table 6.1.

| Endothelial Antigen | Cytokine stimulation of HUVEC (mean fluorescence values) | | | |
|---------------------|--|----------------------|----------------------|----------------------|
| | Unstimulated (% +ve) | IFN γ (% +ve) | TNF α (% +ve) | IL-1 β (% +ve) |
| CD31 | 74 (97%) | 121 (96%) | 95 (96%) | 86 (96%) |
| HLA Class I | 99 (96%) | 447 (98%) | 126 (97%) | 91 (98%) |
| HLA Class II | 13 (4%) | 116 (81%) | 14 (6%) | 28 (7%) |
| E-selectin | 18 (2%) | 17 (1%) | 135 (63%) | 124 (57%) |
| ICAM-1 | 27 (19%) | 60 (79%) | 108 (90%) | 109 (78%) |
| VCAM-1 | 28 (20%) | 30 (32%) | 81 (45%) | 51 (34%) |

Table 6.1 Mean fluorescence intensity values for phenotypic characterisation of HUVEC by FACS analysis following cytokine stimulation (see Figure 6.1).

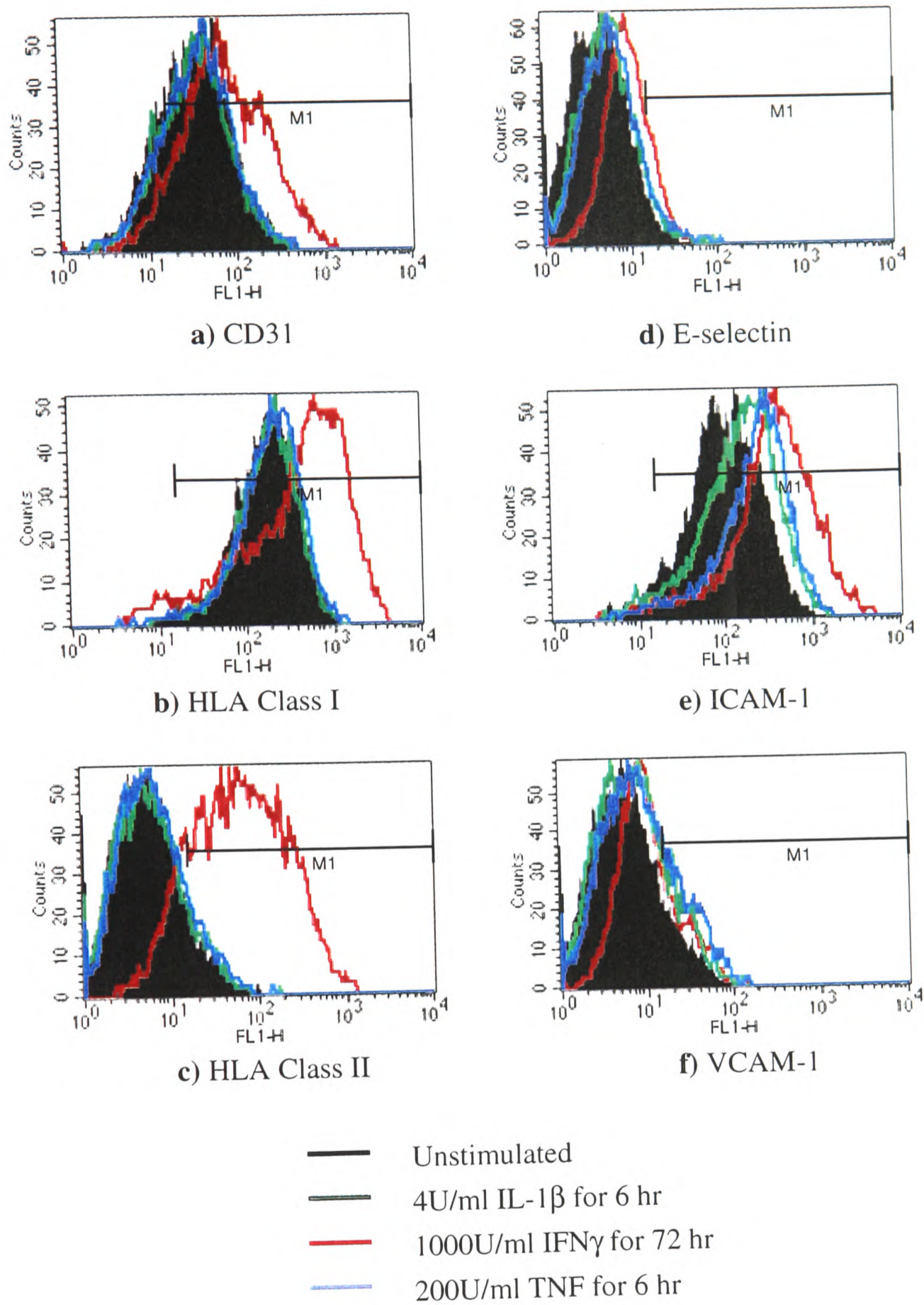


Figure 6.2 Phenotypic characterisation of HMEC-1 by FACS analysis following cytokine stimulation.

HMEC-1 were stimulated with the inflammatory cytokines IL-1 β , IFN γ and TNF to assess the endothelial expression of adhesion molecules and HLA antigens. The cells were stained with mAb against (a) CD31; (b) HLA Class I; (c) HLA Class II; (d) E-selectin; (e) ICAM-1; (f) VCAM-1 and detected with a secondary FITC-labelled goat anti-mouse Ig. Mean fluorescence intensity and percentage of positive cells are represented in Table 6.2.

| Endothelial Antigen | Cytokine stimulation of HMEC-1 (mean fluorescence values) | | | |
|---------------------|---|----------------------|----------------------|----------------------|
| | Unstimulated (% +ve) | IFN γ (% +ve) | TNF α (% +ve) | IL-1 β (% +ve) |
| CD31 | 58 (77%) | 122 (92%) | 57 (80%) | 55 (78%) |
| HLA Class I | 204 (98%) | 647 (95%) | 226 (98%) | 206 (98%) |
| HLA Class II | 10 (9%) | 126 (82%) | 10 (12%) | 8 (11%) |
| E-selectin | 7 (3%) | 31 (14%) | 28 (7%) | 26 (6%) |
| ICAM-1 | 141 (95%) | 513 (97%) | 279 (97%) | 204 (96%) |
| VCAM-1 | 28 (11%) | 35 (22%) | 34 (24%) | 30 (18%) |

Table 6.2 Mean fluorescence intensity values for phenotypic characterisation of HMEC-1 by FACS analysis following cytokine stimulation (see Figure 6.2).

The results from this analysis demonstrate that HUVEC and HMEC-1 have similar endothelial characteristics with cobblestone morphology observed in confluent monolayers and expression of similar adhesion molecules in their resting state. However, phenotypic differences exist in the level of constitutive adhesion molecule expression between HUVEC and HMEC-1. Unstimulated HMEC-1 cells constitutively expressed higher levels of ICAM-1 and HLA Class I than HUVEC, but lower levels of CD31. Furthermore, the magnitude of cytokine induced expression of E-selectin and VCAM-1 was greater in HUVEC than HMEC-1 cells. These results confirm previously published reports on adhesion molecule induction in HMEC-1 and HUVEC and provides the basis for the interpretation of future leucocyte adherence assays.

6.3 CHEMILUMINESCENT ANALYSIS OF RhsOD AND LEC-SOD ACTIVITY

The aim of the experiments in this Chapter were to assess the potential efficacy of lecithinised superoxide dismutase (lec-SOD) following its incorporation into Marshall's organ preservation solution. To verify the enzymatic activities of both unmodified rhSOD (rhSOD) and lec-SOD when incorporated into Marshall's solution, a chemiluminescent technique for the detection of superoxide production was developed using a xanthine/ xanthine oxidase (X/XO) system to generate superoxide ($\cdot\text{O}_2^-$). $\cdot\text{O}_2^-$ production was detected by reaction with a chemiluminescent probe, Cypridina luciferin analog (CLA) [429], resulting in a signal that could be quantified by luminometer analysis. To determine the superoxide scavenging activities of rhSOD and lec-SOD, 30U/ml of lec-SOD (10 $\mu\text{g/ml}$), 30U/ml rhSOD (6 $\mu\text{g/ml}$) or Marshall's solution alone were equilibrated for 2 min with CLA, before the addition of X/XO to assess the generation of $\cdot\text{O}_2^-$ for a further 5 min.

Addition of Marshall's solution alone had no effect on peak $\cdot\text{O}_2^-$ production over the 5 min period in the presence of X/XO (Figure 6.3). In marked contrast, $\cdot\text{O}_2^-$ -induced chemiluminescence was completely abrogated in the presence of 30U/ml of either rhSOD or lec-SOD. Furthermore, the capacity of lec-SOD or rhSOD to scavenge $\cdot\text{O}_2^-$ was not affected following cold storage in Marshall's preservation solution for 48 hours prior to the assay (Figure 6.3). Quantification of the rate of superoxide production (calculated by subtraction of the background level of luminescence in the 2 min equilibrium phase from the 5 min X/XO phase and expressed as the level of luminescence per minute) demonstrated that addition of Marshall's solution alone resulted in a rate of 45.1 luminescence units per minute, but following addition of rhSOD or lec-SOD, the level of luminescence was reduced to 0.21 and 0.16 luminescence units per minute, respectively. Furthermore, after 48 hours cold storage in Marshall's solution (a period widely considered to be towards the upper limit for storage of kidneys prior to transplantation), rhSOD and lec-SOD remained active resulting in a 100-fold reduction in the rate of $\cdot\text{O}_2^-$ induced luminescence (0.46 and 0.43 luminescence units per minute, respectively) (Figure 6.4). Therefore, the results demonstrated that incorporation of either rhSOD or lec-SOD into Marshall's preservation solution did not affect their pharmacological potency, even after storage for 48 hours.

6.4 FACS ANALYSIS OF LEC-SOD BINDING TO ENDOTHELIAL CELLS FOLLOWING INCORPORATION INTO MARSHALL'S ORGAN PRESERVATION SOLUTION

During the period of organ preservation, an organ may be stored on ice for over 24 hours before transplantation. To recreate similar physiological conditions, HMEC-1 cells were incubated at 4°C for 1, 3, 12 and 24 hours with 100µg/ml (300U/ml), 200µg/ml (600U/ml) and 400µg/ml (1200U/ml) of lec-SOD diluted in Marshall's

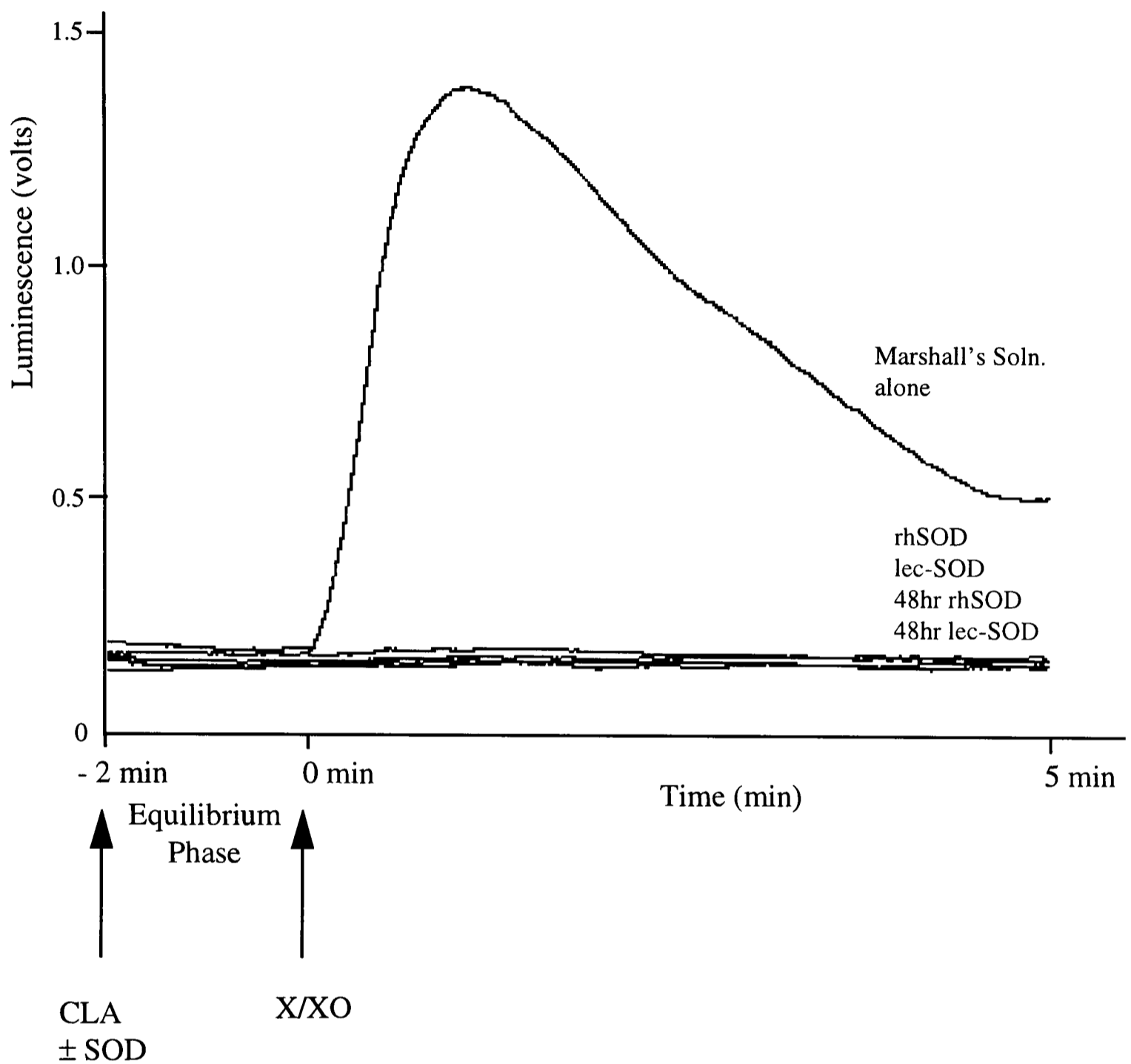


Figure 6.3. Effect of rhSOD and lec-SOD on CLA chemiluminescence profiles induced by xanthine/xanthine oxidase.

Background levels of CLA chemiluminescence in the presence of Marshall's solution alone, 30U/ml rhSOD or lec-SOD were evaluated for 2 min prior to the addition of xanthine/xanthine oxidase (X/XO). Chemiluminescence resulting from X/XO generated superoxide reaction with CLA was assessed for a further 5 min. Addition of 30U/ml of rhSOD or lec-SOD, before and after 48hr cold storage completely abrogated the superoxide induced chemiluminescent signal.

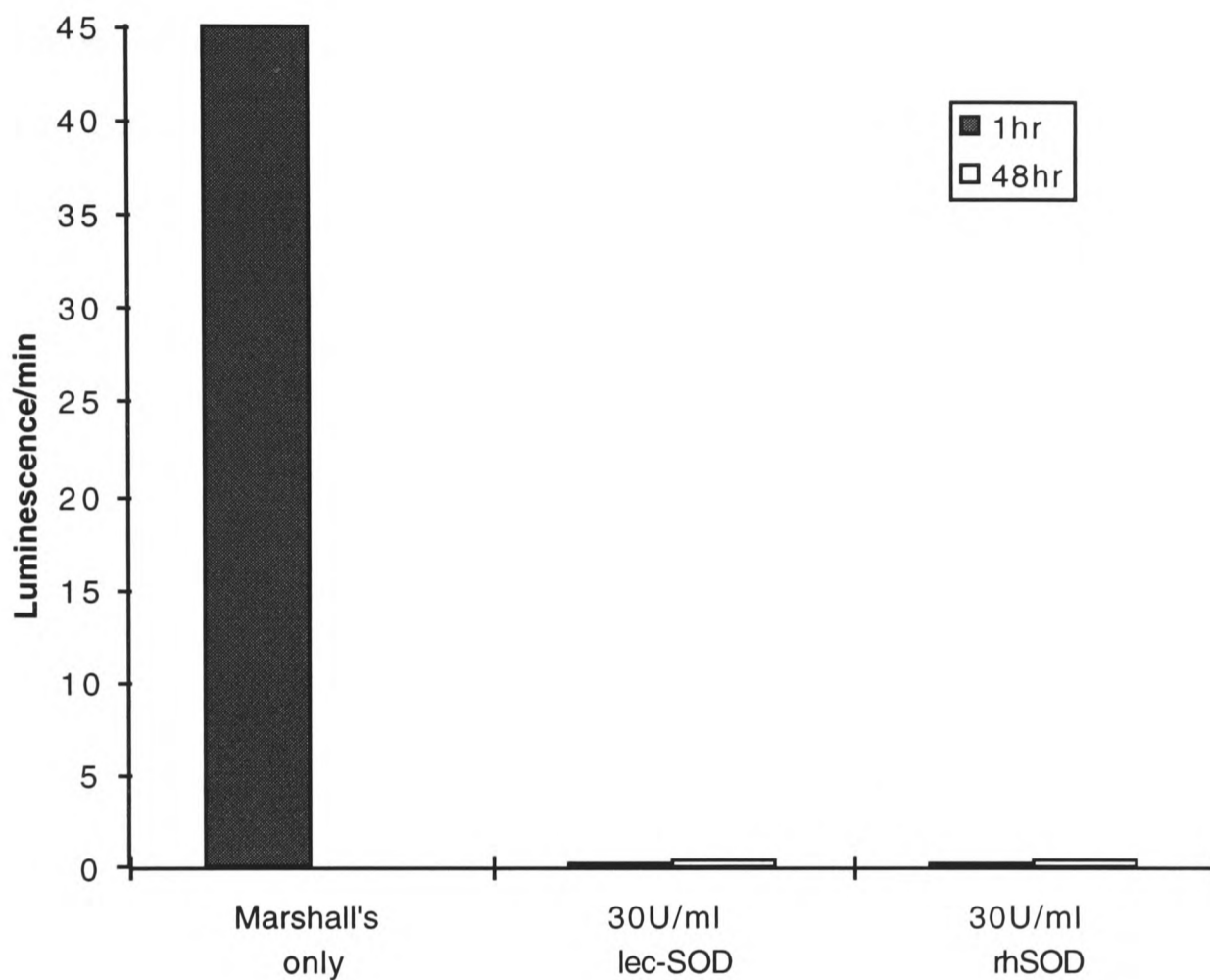


Figure 6.4. Effect of SOD on rate of CLA chemiluminescence induced by xanthine/xanthine oxidase.

The rate of CLA chemiluminescence was calculated following subtraction of background levels determined prior to the addition of xanthine/xanthine oxidase. Marshall's solution alone had no effect on $\cdot\text{O}_2^-$ production, whereas lec-SOD and rhSOD completely abrogated chemiluminescence signal in response to X/XO, even after 48 hours cold storage in Marshall's solution.

(hypertonic citrate) preservation solution. These concentrations of lec-SOD were selected as a result of studies performed by Igarashi and colleagues who demonstrated that 100µg/ml lec-SOD bound with high affinity to endothelial cells after incubation at 37°C for 3 hours [474]. The degree of lec-SOD binding at 4°C was compared to similar concentrations of rhSOD and the results analysed by FACS following staining with an anti-human CuZn-SOD mAb and detection with a secondary FITC-conjugated goat anti-mouse Ig.

Binding of lec-SOD to HMEC-1 was detected for all concentrations and at all time points up to 24 hours post-incubation, above the basal levels of native SOD produced by the endothelial cells (Figure 6.5). Levels of lec-SOD appeared to decrease with increasing concentrations of lec-SOD following incubation for 1 hour and 3 hours (as shown by decrease in mean fluorescence intensity). Direct observation of the wells in which HMEC-1 was incubated with lec-SOD demonstrated formation of a white precipitate when 200 and 400µg/ml of lec-SOD was incorporated into Marshall's solution.

Incubation of HMEC-1 with identical concentrations of rhSOD demonstrated no rhSOD binding above the basal levels of native SOD at all time points examined, whereas high levels of the positive control were detected using 100µg/ml lec-SOD which demonstrated that lec-SOD binds preferentially to HMEC-1 in this experiment (Figure 6.6).

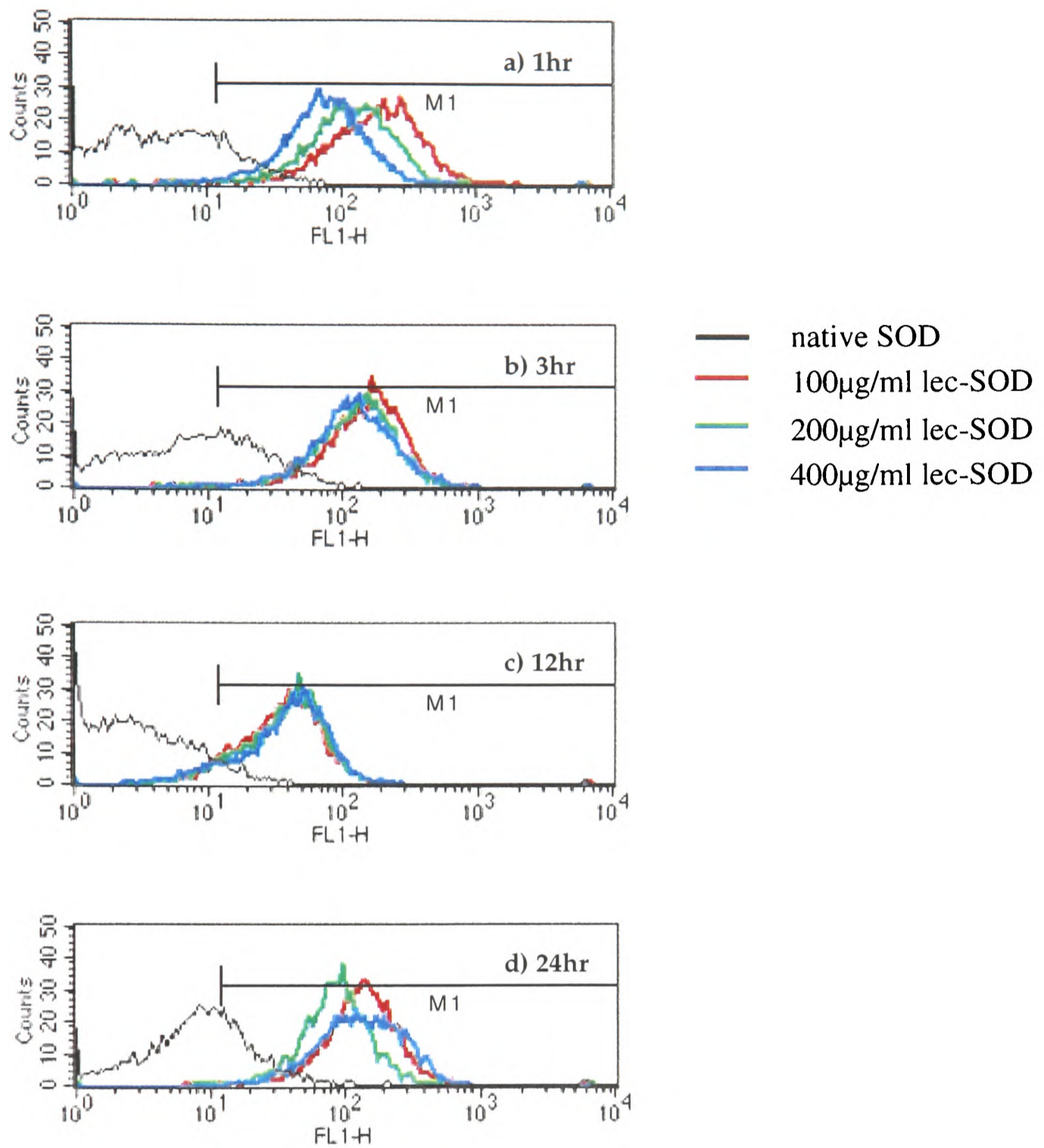
The precipitation of lec-SOD in the preservation solution from the preliminary analysis suggested that a lower concentration of lec-SOD may be more effective. Therefore, a range of doses, 25, 50 and 100 µg/ml of lec-SOD was tested to determine the optimum range for binding to endothelial cells. Both HMEC-1 and HUVEC were examined to compare the binding efficiency of lec-SOD under the same conditions as previously described. Our results demonstrated that for both endothelial cell types, lec-SOD bound

with high affinity at all concentrations and time points analysed above the levels of native SOD detected produced by the cells (Figure 6.7 and Figure 6.8). Therefore, an optimum range of 25-100µg/ml lec-SOD with no detectable precipitation had been established for binding to HMEC-1 and HUVEC following incorporation into Marshall's solution.

6.5 CONFOCAL LASER MICROSCOPY ANALYSIS OF LEC-SOD BINDING TO ENDOTHELIUM

To determine the precise localisation of lec-SOD binding to endothelial cells, a double-immunofluorescent staining technique was employed using mAb directed against CD31, an endothelial surface antigen, and to SOD, following cell permeabilisation. HMEC-1 grown to confluence on collagen-coated glass coverslips were incubated with 50µg/ml of lec-SOD for 3 hours at 4°C in Marshall's solution and analysed by confocal laser microscopy. Native levels of SOD were determined by staining cells with an anti-SOD mAb in the absence of lec-SOD. Staining specificity was determined with an irrelevant isotype control antibody which demonstrated no cellular autofluorescence or non-specific binding of the secondary isotype-specific, fluorescein-labelled antibodies. Incubation of collagen-coated coverslips alone with lec-SOD demonstrated no binding to collagen, and incubation of cells with lec-SOD followed by staining with an isotype control mAb showed no lec-SOD autofluorescence.

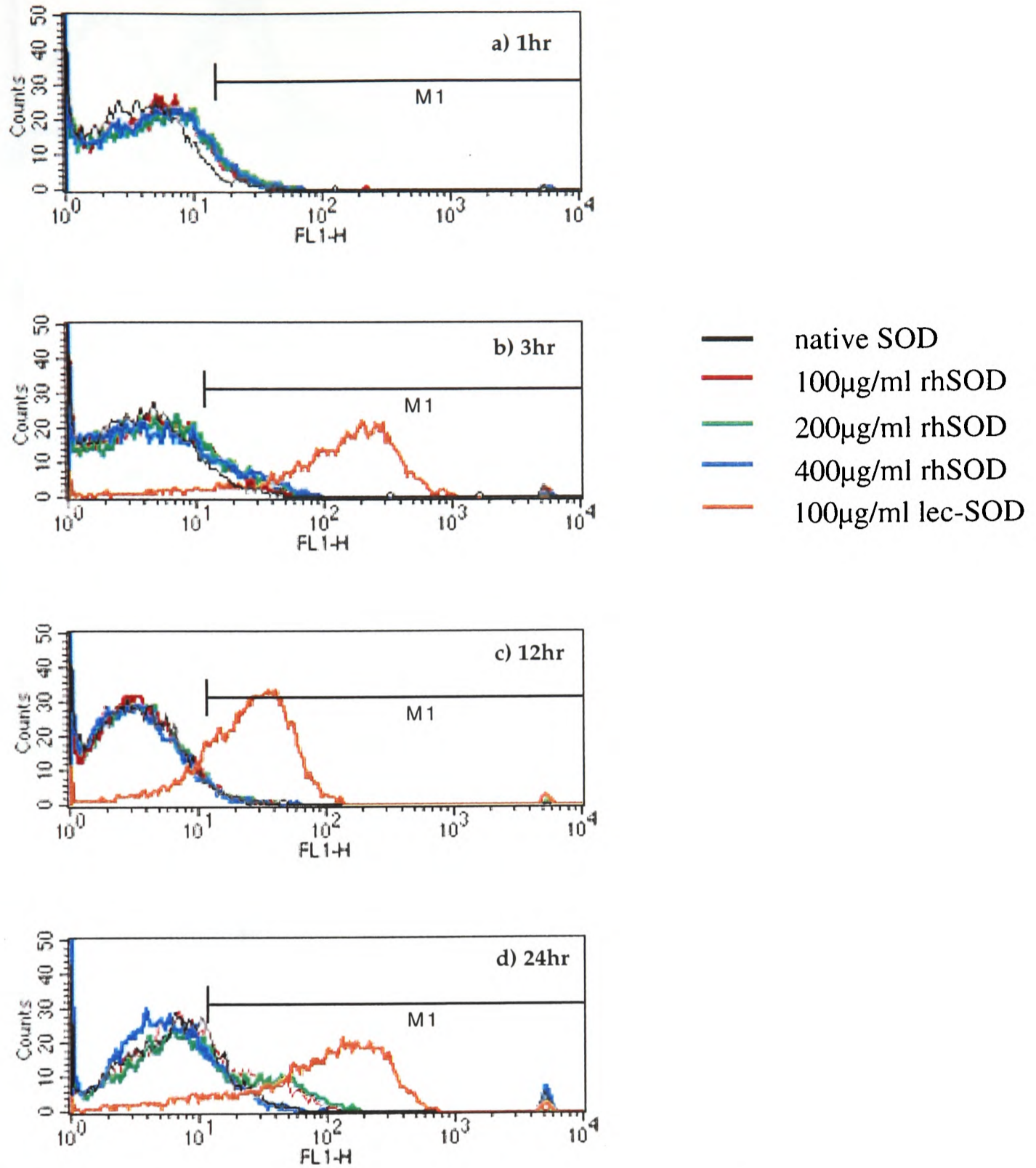
Laser confocal microscopy revealed that following cell permeabilisation, lec-SOD was detectable only on the surface of endothelial cells with no intracellular SOD observed in 3.0µm serial planar laser images captured through the endothelial cells (Figure 6.9). This was confirmed by double-immunofluorescent staining of endothelial cells with mAb specific for CD31 and SOD under identical conditions to that described above,



| lec-SOD (µg/ml) | 1 hour (% +ve) | 3 hour (% +ve) | 12 hour (% +ve) | 24 hour (% +ve) |
|--------------------|-------------------|-------------------|--------------------|--------------------|
| 100 | 233 (98%) | 183 (99%) | 62 (92%) | 153 (99%) |
| 200 | 153 (98%) | 159 (99%) | 51 (91%) | 105 (99%) |
| 400 | 104 (98%) | 157 (99%) | 57 (89%) | 160 (99%) |
| native SOD | 53 (25%) | 38 (39%) | 12 (6%) | 63 (32%) |
| negative | 19 (5%) | 15 (6%) | 11 (5%) | 20 (4%) |

Figure 6.5. Incubation of HMEC-1 with lec-SOD in Marshall's solution at 4°C.

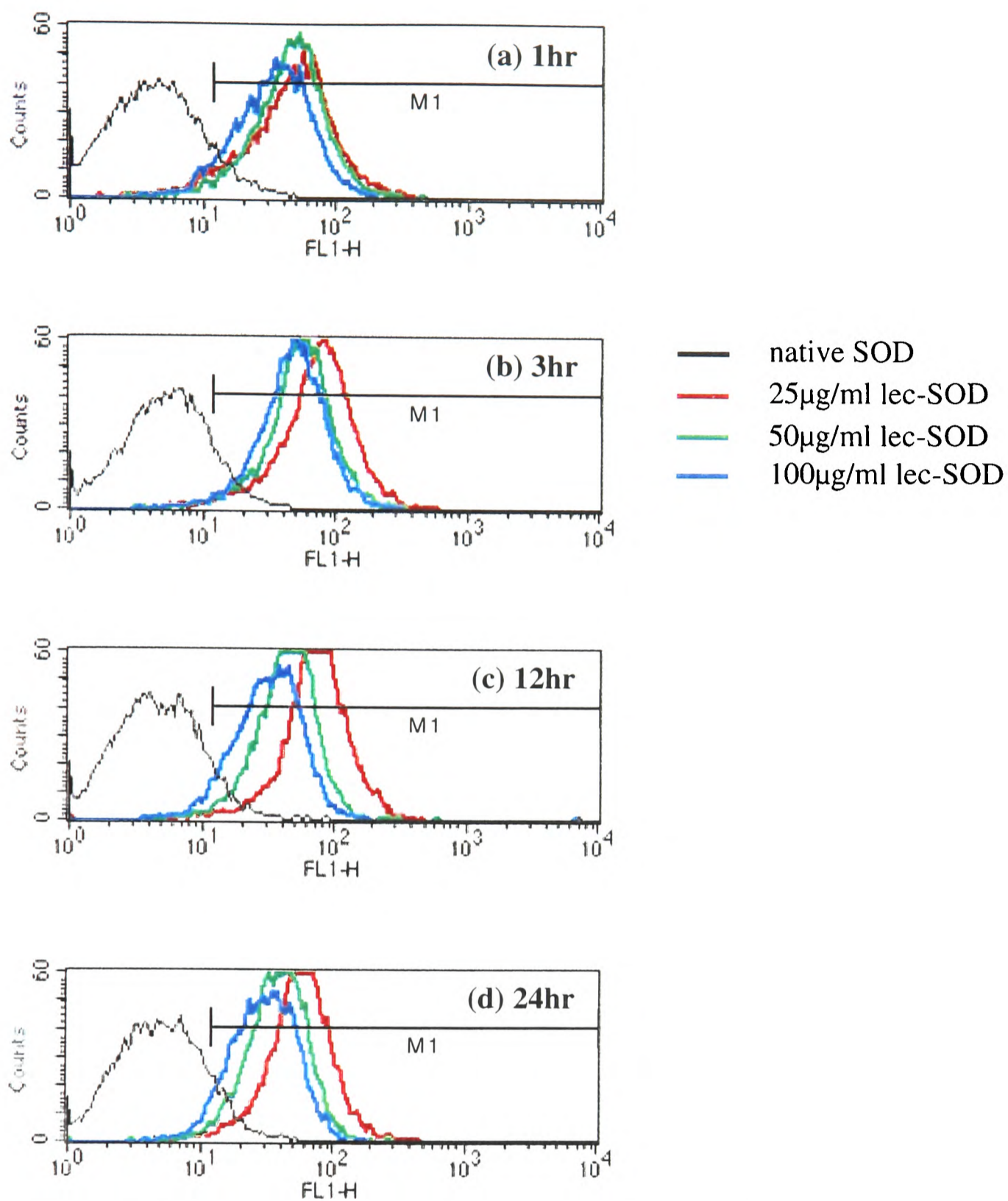
HMEC-1 cells were incubated with 100, 200 and 400µg/ml of lec-SOD at 4°C for (a) 1 hour; (b) 3 hours; (c) 12 hours; and (d) 24 hours. SOD binding was analysed by FACS with an anti-SOD mAb detected with a FITC-conjugated goat anti-mouse Ig.



| rhSOD (µg/ml) | 1 hour (% +ve) | 3 hour (% +ve) | 12 hour (% +ve) | 24 hour (% +ve) |
|---------------|-------------------|-------------------|--------------------|--------------------|
| 100 | 21 (7%) | 35 (16%) | 5 (4%) | 30 (31%) |
| 200 | 18 (10%) | 18 (19%) | 13 (5%) | 38 (34%) |
| 400 | 23 (9%) | 19 (19%) | 5 (3%) | 88 (18%) |
| native SOD | 14 (3%) | 33 (9%) | 6 (4%) | 73 (21%) |
| lec-SOD | - | 193 (94%) | 73 (81%) | 176 (88%) |

Figure 6.6. Incubation of HMEC-1 with rhSOD in Marshall's solution at 4°C.

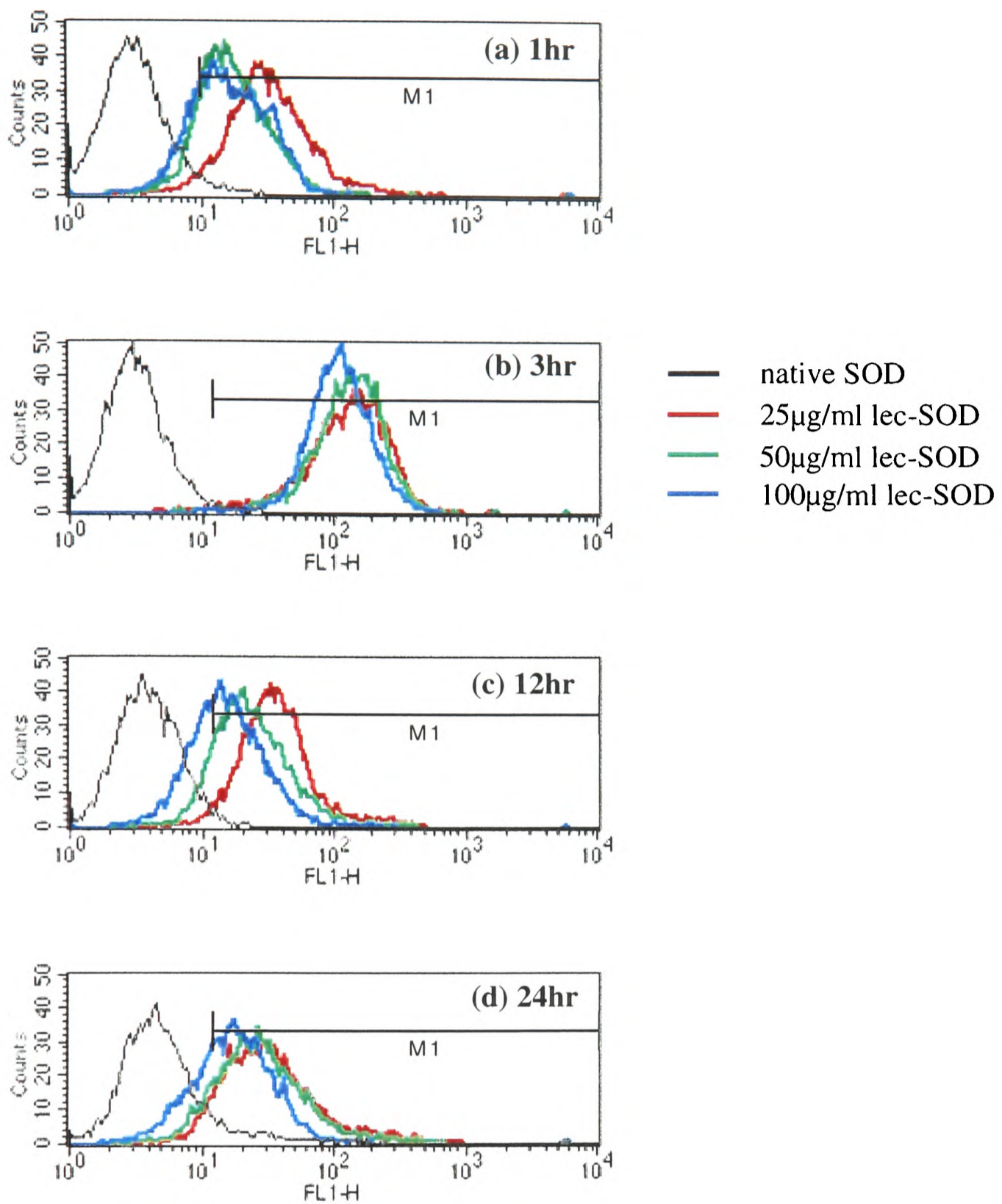
HMEC-1 was incubated with 100, 200 and 400µg/ml of rhSOD at 4°C for (a) 1 hour; (b) 3 hours; (c) 12 hours; and (d) 24 hours. Exposure of cells to 100µg/ml of lec-SOD was analysed for comparison. SOD binding was determined by FACS with an anti-SOD mAb detected with a FITC-conjugated goat anti-mouse Ig.



| lec-SOD (µg/ml) | 1 hour (% +ve) | 3 hour (% +ve) | 12 hour (% +ve) | 24 hour (% +ve) |
|--------------------|-------------------|-------------------|--------------------|--------------------|
| 25 | 63 (94%) | 86 (98%) | 88 (99%) | 66 (99%) |
| 50 | 55 (97%) | 62 (98%) | 50 (98%) | 43 (97%) |
| 100 | 47 (93%) | 57 (98%) | 40 (94%) | 36 (94%) |
| native SOD | 60 (9%) | 42 (12%) | 9 (9%) | 11 (11%) |
| negative | 7 (3%) | 7 (3%) | 7 (3%) | 6 (4%) |

Figure 6.7. Incubation of HMEC-1 with lower concentrations of lec-SOD at 4°C.

HMEC-1 were incubated with 25, 50 and 100µg/ml of lec-SOD at 4°C for (a) 1 hour; (b) 3 hours; (c) 12 hours; and (d) 24 hours. SOD binding was analysed by FACS with an anti-SOD mAb detected with a FITC-conjugated goat anti-mouse Ig.



| lec-SOD (µg/ml) | 1 hour (% +ve) | 3 hour (% +ve) | 12 hour (% +ve) | 24 hour (% +ve) |
|--------------------|-------------------|-------------------|--------------------|--------------------|
| 25 | 61 (96%) | 157 (98%) | 55 (96%) | 64 (91%) |
| 50 | 25 (84%) | 147 (99%) | 44 (86%) | 57 (86%) |
| 100 | 33 (76%) | 127 (99%) | 33 (62%) | 43 (69%) |
| native SOD | 4 (10%) | 11 (2%) | 9 (2%) | 18 (10%) |
| negative | 13 (3%) | 15 (5%) | 10 (3%) | 12 (4%) |

Figure 6.8. Incubation of HUVEC with lower concentrations of lec-SOD at 4°C.

HUVEC were incubated with 25, 50 and 100µg/ml of lec-SOD at 4°C for (a) 1 hour; (b) 3 hours; (c) 12 hours; and (d) 24 hours. SOD binding was analysed by FACS with an anti-SOD mAb detected with a FITC-conjugated goat anti-mouse Ig.

demonstrating that lec-SOD was localised to surface structures positive for CD31 (Figure 6.10).

6.6 EFFECT OF COLD HYPOXIA ON LEC-SOD BINDING TO ENDOTHELIUM

During the period of cold organ storage, the tissue and endothelium is deprived of oxygen, resulting in anaerobic metabolism and impaired cellular function. Therefore, it was necessary to investigate whether binding of lec-SOD to HUVEC and HMEC-1 was affected by exposure to cold hypoxic conditions. HUVEC and HMEC-1 cells were incubated with 50µg/ml lec-SOD under cold normoxic (room air) or cold hypoxic (95% N₂, 5% CO₂) conditions for 3 and 24 hours. Staining for SOD binding was assessed by FACS analysis following either cell permeabilisation (Cytoperm™) or non-permeabilisation (fixation with 10% neutral buffered formalin (NBF) alone). Fixation of endothelial cells following permeabilisation allowed the evaluation of total SOD present both extracellularly and intracellularly, whereas fixation with 10% NBF enabled the detection of extracellular SOD alone.

6.6.1 HMEC-1

6.6.1.1 Native extracellular SOD

Before determining the level of lec-SOD binding to HMEC-1, it was necessary to assess the levels of native SOD produced under cold normoxic and hypoxic conditions.

After 3 hours of cold storage in Marshall's solution, the levels of extracellular SOD were minimal in a small percentage of cells incubated under either normoxic (15%) or hypoxic conditions (23%) (Figures 6.11a and 6.12a). After 24 hours cold storage, a marginal increase in native extracellular SOD was detected under both normoxic (32%)

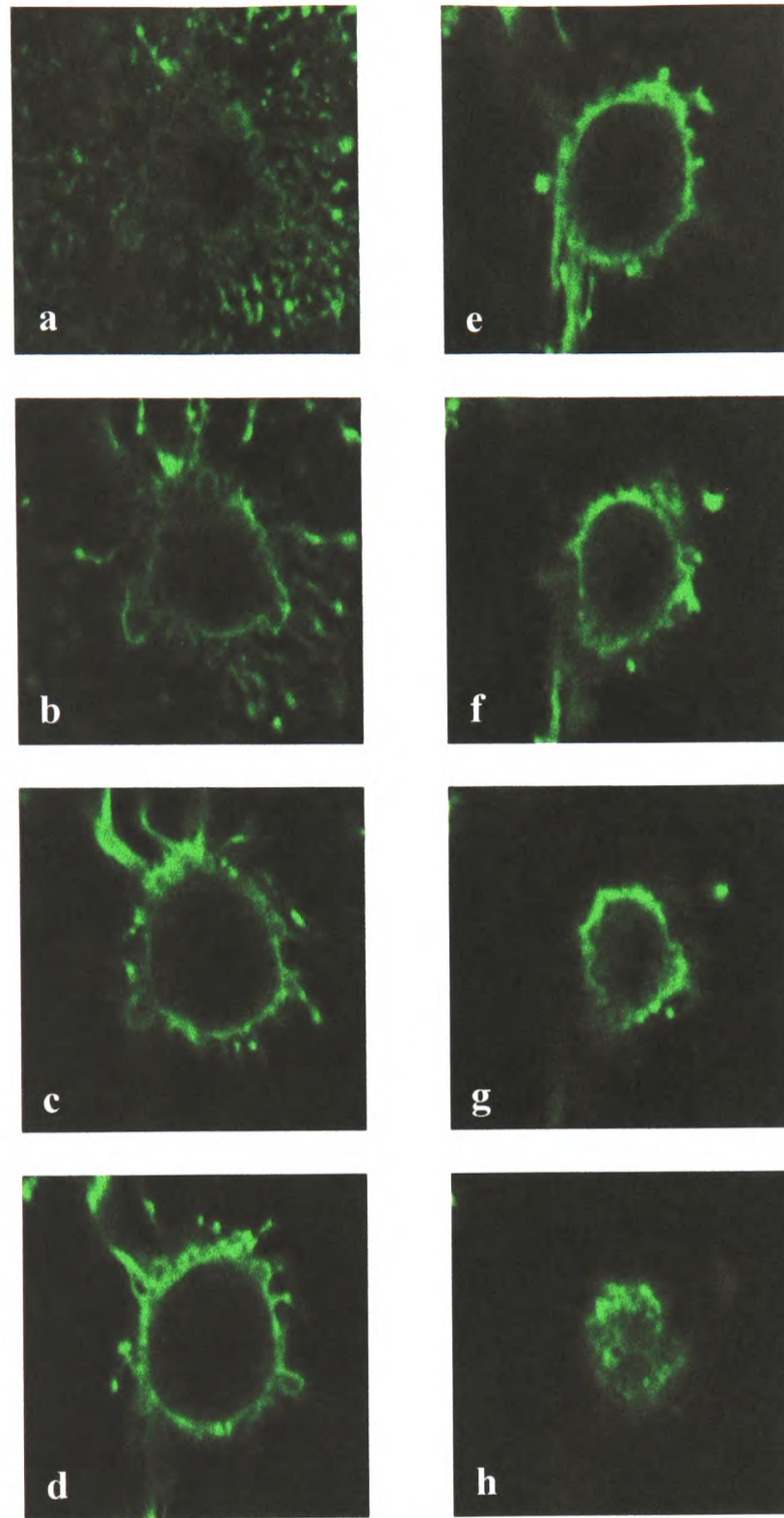


Figure 6.9. Serial planar confocal laser microscopy images of endothelial cell incubated with lec-SOD

HUVEC cells grown on collagen-coated glass coverslips were incubated with 50 μ g/ml of lec-SOD for 3 hours. Cells were stained with an anti-SOD mAb following permeabilisation and staining detected with a FITC-conjugated goat anti-mouse Ig. Figures 6.6 a-h represent serial laser confocal 3.0 μ m sections through a single endothelial cell, demonstrating no detectable SOD within the cell.

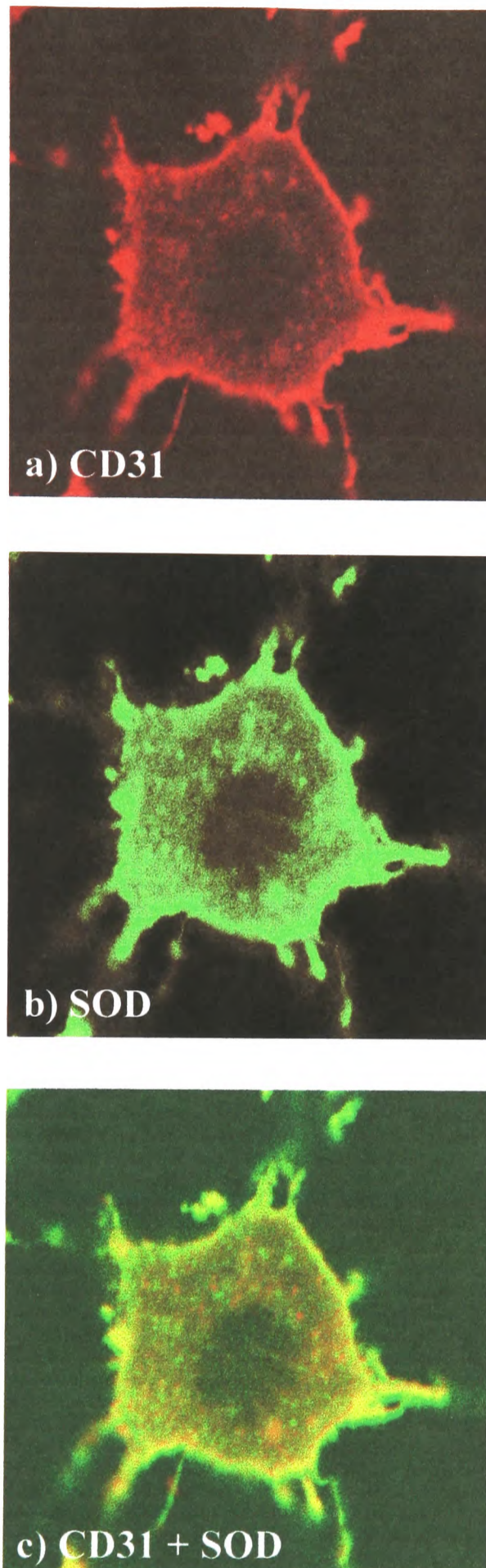


Figure 6.10. Double-immunofluorescent staining to determine the localisation of lec-SOD binding to endothelial surface.

HUVEC cells grown on collagen-coated glass coverslips were incubated with 50µg/ml lec-SOD for 3 hours. Cells were permeabilised and stained with mAbs specific for the surface antigen (a) CD31, and (b) SOD, and detected with isotype-specific Texas-Red- and FITC-conjugated secondary antibodies, respectively. Images were captured by confocal laser microscopy. (c) Images (a) and (b) were superimposed, localising lec-SOD to the endothelial surface expressing CD31.

and hypoxic (33%) conditions compared with levels detected after 3 hours (Figures 6.11c and 6.12c).

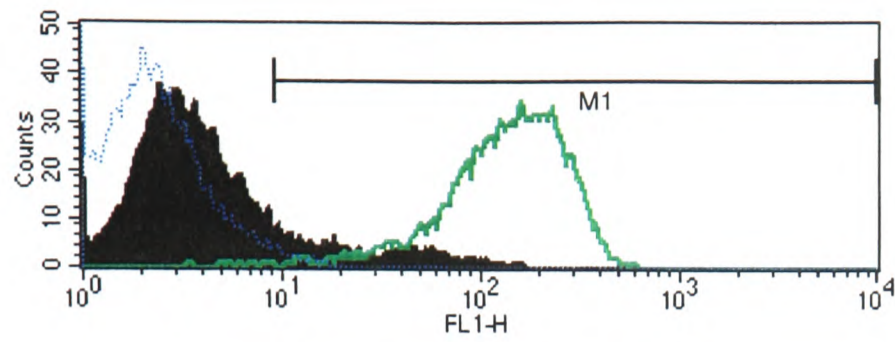
6.6.1.2 Total native SOD following permeabilisation

After permeabilisation, the percentage of cells positive for total extracellular and intracellular SOD increased to 98% in cells incubated under both normoxic and hypoxic conditions, showing that a high level of intracellular SOD was present in HMEC-1 cells (Figures 6.11b and 6.12b). Intracellular SOD detected following permeabilisation remained relatively stable after 24 hours cold hypoxia (96%), whereas in cells incubated under cold normoxia, intracellular levels of native SOD were detected in 86% of cells, with two distinct subpopulations containing high and low levels of intracellular SOD (Figure 6.11d).

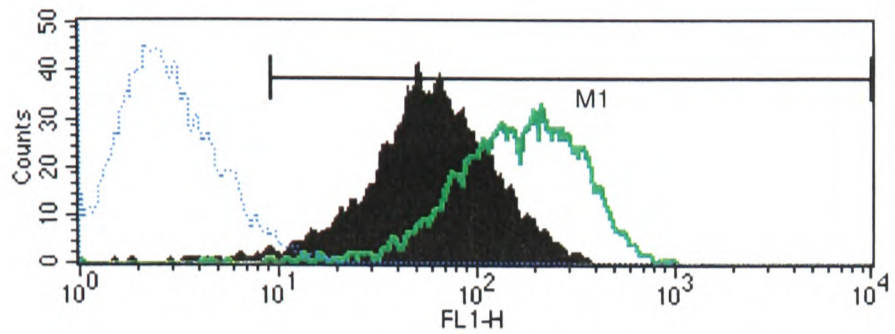
These results suggest that levels of intracellular and extracellular SOD may fluctuate in HMEC-1 cells during cold storage, but higher levels of intracellular SOD may be present following prolonged hypoxia.

6.6.1.3 Binding of lec-SOD to HMEC-1

Incubation of HMEC-1 with 50µg/ml lec-SOD under cold normoxia or hypoxia resulted in a significantly higher level of lec-SOD binding over and above the level of native SOD produced by the cells (e.g. 99% of HMEC-1 cells were positive for high levels of extracellular lec-SOD at all time points). Furthermore, the level of lec-SOD remained relatively constant at all time points, irrespective of incubation under cold hypoxic conditions (Figure 6.11 and 6.12).

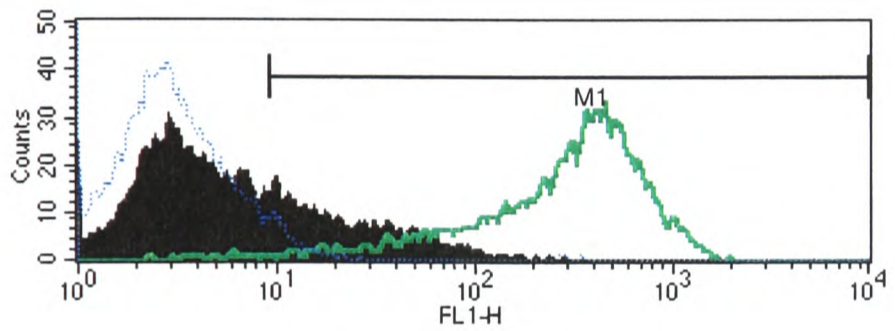


a) 3hr - Non-permeabilised

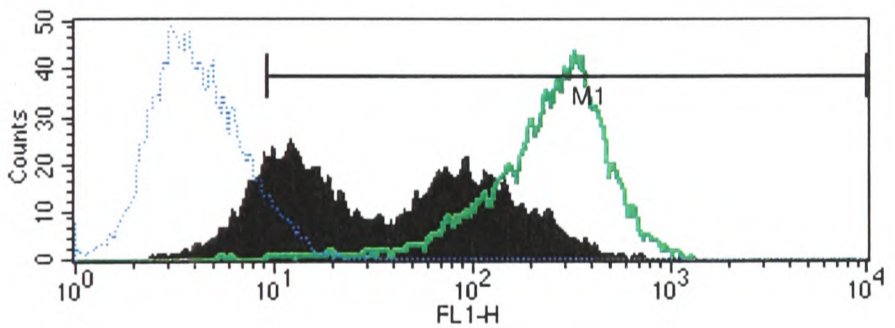


b) 3hr - Permeabilised

— Negative isotype control
 — Native SOD
 — lec-SOD



c) 24hr - Non-permeabilised

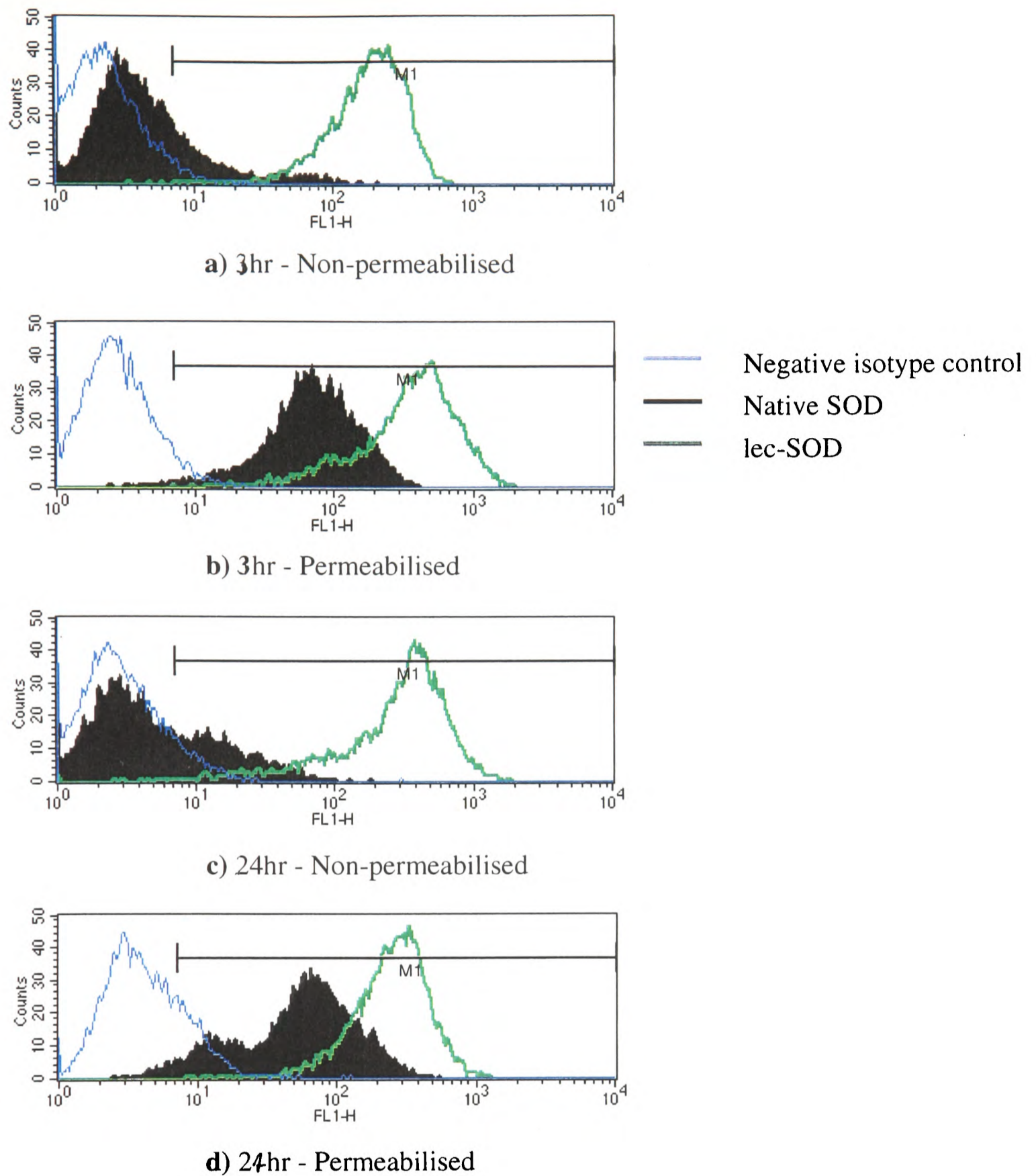


d) 24hr - Permeabilised

| Treatment | -ve Control (% +ve) | NativeSOD (% +ve) | lec-SOD (% +ve) |
|----------------|------------------------|----------------------|--------------------|
| 3hr - No Perm | 3 (2%) | 36 (15%) | 158 (99%) |
| 3hr - Perm | 5 (3%) | 73 (98%) | 195 (99%) |
| 24hr - No Perm | 8 (6%) | 33 (32%) | 371 (98%) |
| 24hr - Perm | 9 (8%) | 80 (86%) | 280 (99%) |

Figure 6.11. Detection of intracellular and extracellular SOD on HMEC-1 following incubation under cold normoxic conditions.

HMEC-1 were incubated with 50µg/ml of lec-SOD at 4°C for (a,b) 3 hours; and (c,d) 24 hours. Extracellular (a,c) and intracellular (b,d) levels of SOD were analysed by FACS with an anti-SOD mAb detected with a FITC-conjugated goat anti-mouse Ig following NBF fixation or permeabilisation, respectively.



| Treatment | -ve Control (% +ve) | Native SOD (% +ve) | lec-SOD (% +ve) |
|----------------|------------------------|-----------------------|--------------------|
| 3hr - No Perm | 15 (4%) | 26 (23%) | 194 (99%) |
| 3hr - Perm | 11 (5%) | 87 (98%) | 409 (99%) |
| 24hr - No Perm | 16 (10%) | 23 (33%) | 342 (99%) |
| 24hr - Perm | 14 (16%) | 77 (96%) | 270 (99%) |

Figure 6.12. Detection of intracellular and extracellular SOD on HMEC-1 following incubation under cold hypoxia.

HMEC-1 were incubated with 50µg/ml of lec-SOD at 4°C for (a,b) 3 hours; and (c,d) 24 hours under hypoxic conditions. Extracellular (a,c) and intracellular (b,d) levels of SOD were analysed by FACS with an anti-SOD mAb detected with a FITC-conjugated goat anti-mouse Ig following NBF fixation or permeabilisation, respectively.

6.6.2 HUVEC

6.6.2.1 Native extracellular SOD

The expression of native levels of SOD in HUVEC were examined following incubation under the same conditions as described above for HMEC-1. A subpopulation of HUVEC were positive for extracellular SOD exposed to normoxia (47%) or hypoxia (43%) following 3 hours cold storage (Figure 6.13a and 6.14a). The level of extracellular SOD was reduced following 24 hours cold normoxia (24%), whereas in cells exposed to 24 hours cold hypoxia, the extracellular levels of native SOD increased in 63% of the cell population.

6.6.2.2 Total native SOD following permeabilisation

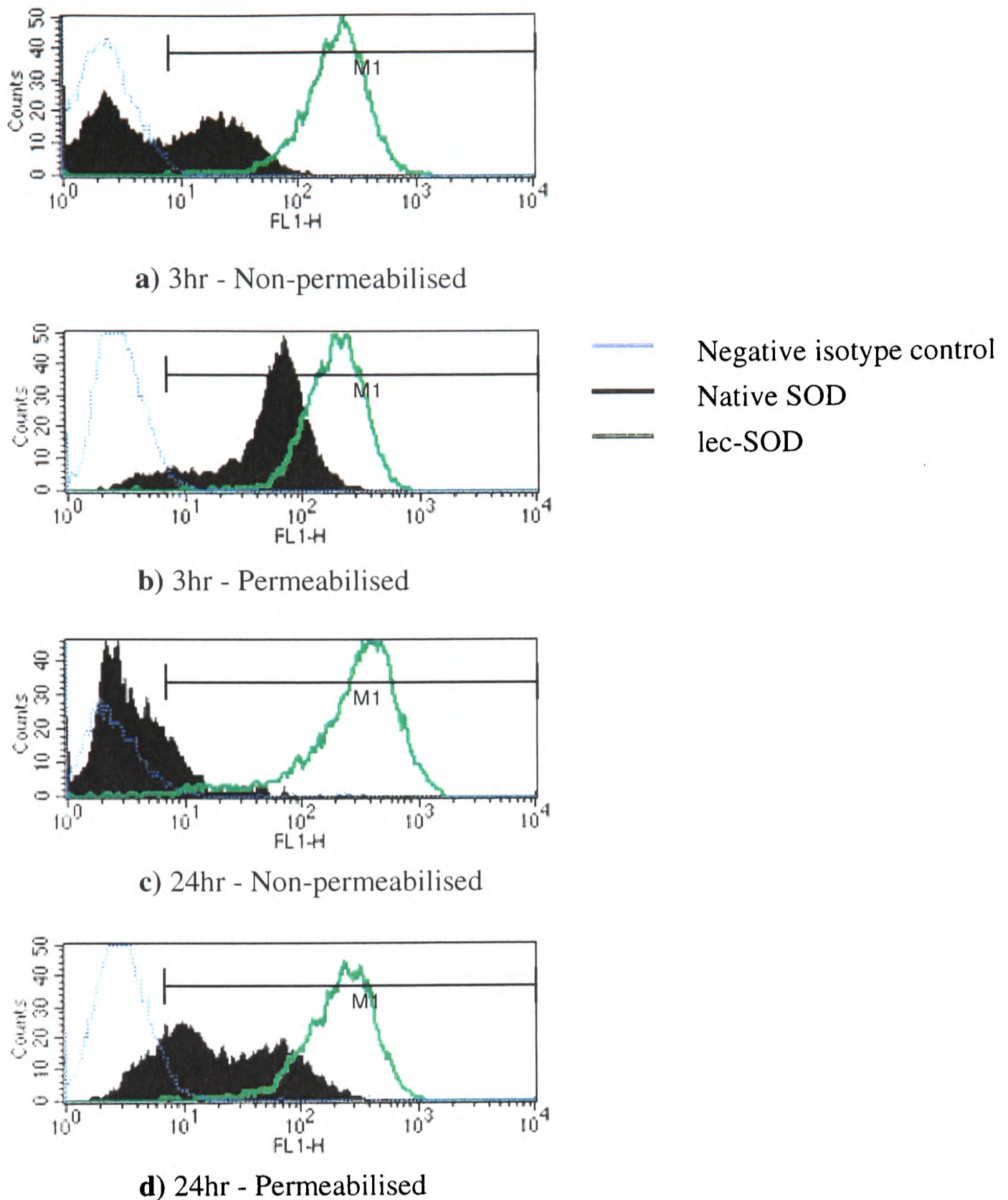
No significant differences were observed between HUVEC exposed to 3 hours cold normoxia and 3 hours cold hypoxia with respect to the levels of extracellular and intracellular SOD (Figure 6.13 and 6.14). Similarly, no significant differences in native intracellular SOD levels were detected after 24 hours cold normoxia (81%) or hypoxia (72%).

6.6.2.3 Binding of lec-SOD to HUVEC

Similar to the results obtained for HMEC-1, lec-SOD binding was detected on HUVEC above the levels of native SOD produced by the cells at all time points analysed. Furthermore, constant levels of lec-SOD were detected irrespective of the fluctuations in native SOD produced by the endothelial cells.

6.6.3 Detection of lec-SOD Following Hypoxia and Prolonged Reoxygenation

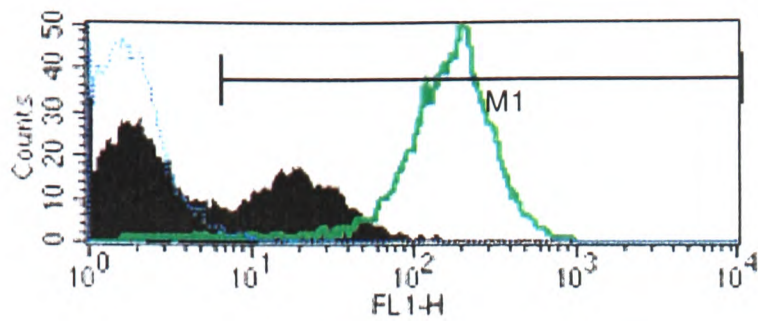
Binding of lec-SOD to the surface of HUVEC and HMEC-1 following incorporation in Marshall's preservation solution at 4°C under hypoxic conditions has been established, but the level of lec-SOD remaining after prolonged periods of reoxygenation once the



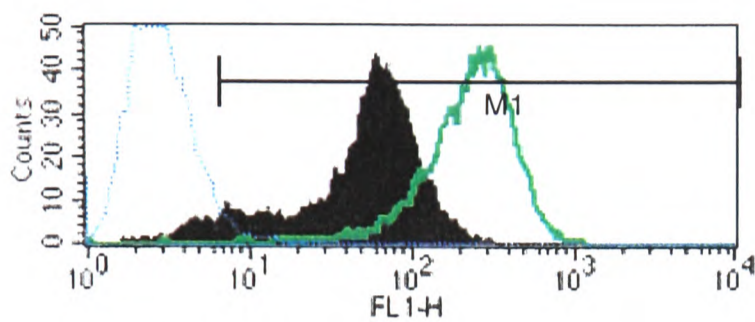
| Treatment | -ve Control (% +ve) | Native SOD (% +ve) | lec-SOD (% +ve) |
|----------------|------------------------|-----------------------|--------------------|
| 3hr - No Perm | 3 (2%) | 31 (47%) | 229 (99%) |
| 3hr - Perm | 9 (2%) | 66 (93%) | 203 (99%) |
| 24hr - No Perm | 3 (2%) | 29 (24%) | 344 (99%) |
| 24hr - Perm | 4 (5%) | 53 (81%) | 243 (99%) |

Figure 6.13. Detection of intracellular and extracellular SOD on HUVEC following incubation under cold normoxic conditions.

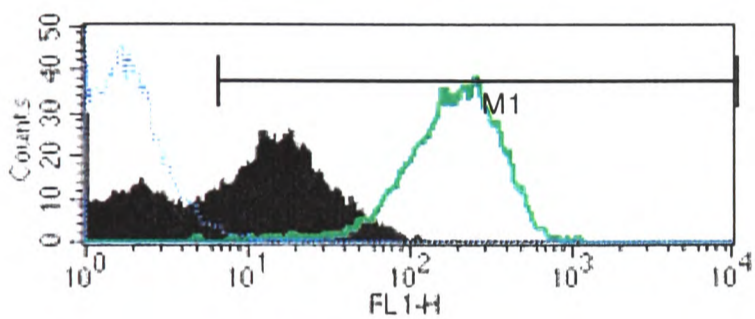
HUVEC were incubated with 50µg/ml of lec-SOD at 4°C for (a,b) 3 hours; and (c,d) 24 hours. Extracellular (a,c) and intracellular (b,d) levels of SOD were analysed by FACS with an anti-SOD mAb detected with a FITC-conjugated goat anti-mouse Ig following NBF fixation or permeabilisation, respectively.



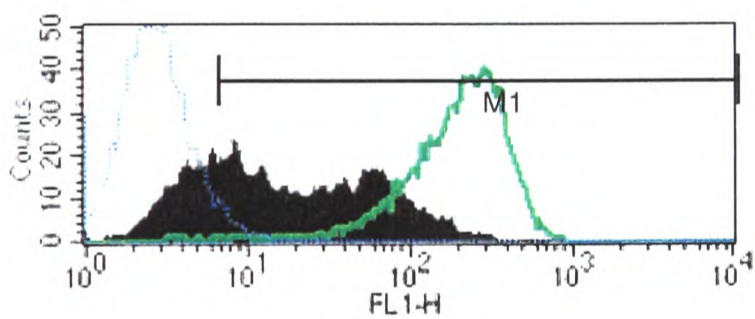
a) 3hr - Non-permeabilised



b) 3hr - Permeabilised



c) 24hr - Non-permeabilised



d) 24hr - Permeabilised

— Negative isotype control
 — Native SOD
 — lec-SOD

| Treatment | -ve Control (% +ve) | Native SOD (% +ve) | lec-SOD (% +ve) |
|----------------|------------------------|-----------------------|--------------------|
| 3hr - No Perm | 2 (1%) | 23 (43%) | 184 (98%) |
| 3hr - Perm | 3 (3%) | 66 (94%) | 253 (99%) |
| 24hr - No Perm | 3 (1%) | 23 (63%) | 209 (99%) |
| 24hr - Perm | 3 (4%) | 45 (72%) | 223 (99%) |

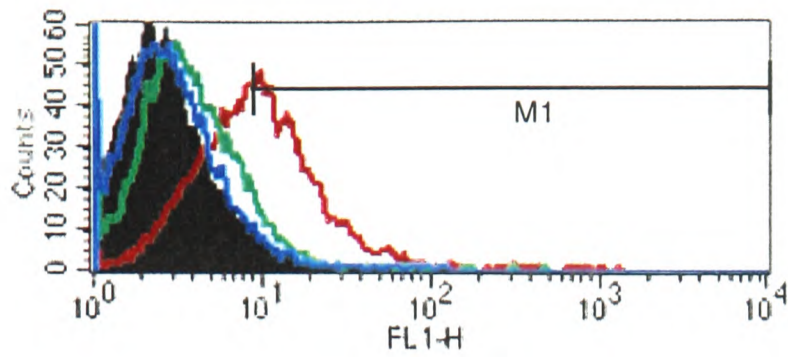
Figure 6.14. Detection of intracellular and extracellular SOD on HUVEC following incubation under cold hypoxia.

HUVEC were incubated with 50µg/ml of lec-SOD at 4°C for (a,b) 3 hours; and (c,d) 24 hours under hypoxic conditions. Extracellular (a,c) and intracellular (b,d) levels of SOD were analysed by FACS with an anti-SOD mAb detected with a FITC-conjugated goat anti-mouse Ig following NBF fixation or permeabilisation, respectively.

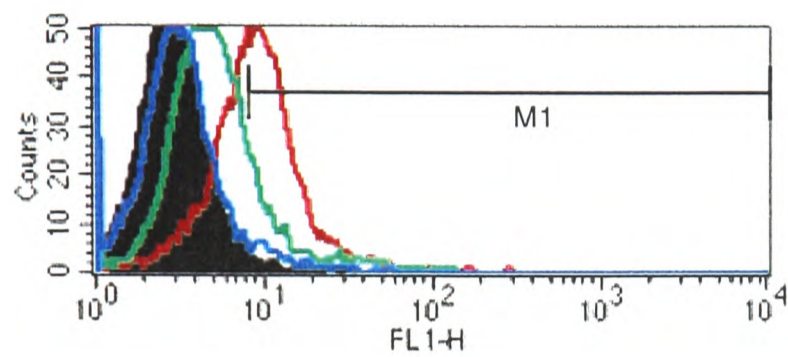
cells resume metabolic activity remained to be determined. HUVEC and HMEC-1 cells were incubated with 50µg/ml lec-SOD for 1 hour hypoxia at 37°C (95% N₂, 5% CO₂) followed by reoxygenation (95% air, 5% CO₂) for 4 hours, 1 day and 3 days at 37°C. In addition HMEC-1 cells were incubated for 18 hours cold hypoxia in the presence of 50µg/ml lec-SOD and also reoxygenated at 37°C for the same periods of time.

Incubation of HMEC-1 and HUVEC with lec-SOD for 1 hour warm hypoxia demonstrated that lec-SOD was detectable 4 hours after reoxygenation, declining proportionately with prolonged reoxygenation, such that after 3 days, the level of extracellular lec-SOD was no greater than in cells with no SOD treatment (Figure 6.15a and 6.15b). In HMEC-1 cells incubated with lec-SOD under cold hypoxic conditions for 18 hours, maximal levels of lec-SOD were detected after 4 hours of warm reoxygenation, declining in a time dependent fashion after 1 day and 3 days reoxygenation (Figure 6.15c). Furthermore, the level of lec-SOD after 18 hours cold hypoxia and 3 days reoxygenation (17%) was higher than basal levels of native SOD in HMEC-1 cells (2%).

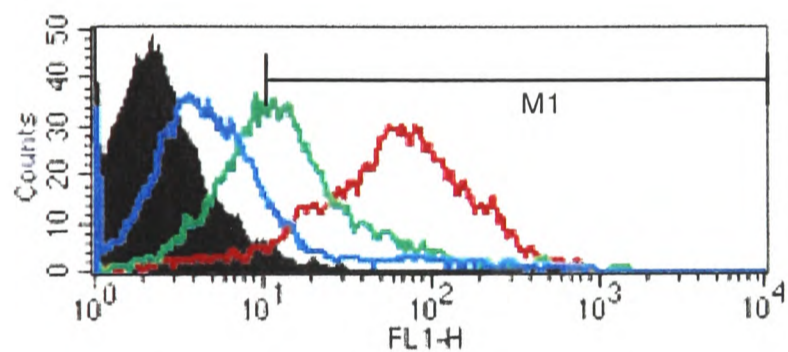
The combined results from this study demonstrate that lec-SOD can be successfully incorporated into Marshall's solution whilst retaining its pharmacological potency under prolonged cold storage. Incubation of HMEC-1 and HUVEC with lec-SOD under cold normoxic and hypoxic conditions for periods up to 24 hours demonstrated increased and constantly high levels of binding above the fluctuating basal levels of native SOD produced by endothelial cells. Furthermore, surface levels of lec-SOD were detected after prolonged reoxygenation of hypoxia-conditioned endothelial cells. To examine whether the surface binding of lec-SOD exerted a beneficial pharmacological and physiological effect following hypoxia/reoxygenation, cell viability and neutrophil-endothelial cell adhesion was assessed.



a) HMEC-1: 1hr hypoxia and reoxygenation



b) HUVEC: 1hr hypoxia and reoxygenation



c) HMEC-1: 18hr cold hypoxia and reoxygenation

— Native SOD
 — 4hr reoxygenation
 — 1 day reoxygenation
 — 3 day reoxygenation

| Hypoxia | Native SOD | 4 Hour Reoxygen. | 1 Day Reoxygen. | 3 Day Reoxygen. |
|-----------------------|------------|------------------|-----------------|-----------------|
| HMEC-1 1 hr @ 37°C | 3 (5%) | 36 (50%) | 7 (9%) | 5 (5%) |
| HUVEC 1 hr @ 37°C | 3 (2%) | 20 (54%) | 11 (16%) | 4 (6%) |
| HMEC-1 18 hr @ 4°C | 3 (2%) | 96 (93%) | 54 (58%) | 23 (17%) |

Figure 6.15. Analysis of endothelial lec-SOD binding following hypoxia and prolonged reoxygenation.

HUVEC and HMEC-1 cells were incubated with lec-SOD for either 1hr warm hypoxia (a,b) or 18hr cold hypoxia (c), then reoxygenated for 4hr, 1 day and 3 days to determine the period in which lec-SOD remains bound to the surface. Cells were stained with an anti-SOD mAb and developed with a secondary FITC-conjugated goat anti-mouse Ig for FACS analysis.

6.7 EFFECT OF LEC-SOD ON ENDOTHELIAL CELL VIABILITY FOLLOWING COLD HYPOXIA AND REOXYGENATION

To determine whether lec-SOD had a beneficial effect on cold hypoxia/reoxygenation induced cell death, HMEC-1 and HUVEC were incubated for 18, 24 and 27 hours under cold hypoxic conditions (95% N₂, 5% CO₂) in the presence of 25µg/ml lec-SOD, 50µg/ml lec-SOD, 50µg/ml lecithin alone and 50µg/ml lecithin and rhSOD unconjugated, followed by reoxygenation for 24 hours. Cell viability was assessed by Trypan Blue exclusion staining and each experiment performed in duplicate in three experiments. Statistically significant differences were determined by paired T-test analysis.

6.7.1 HMEC-1 Viability

Incubation of HMEC-1 cells for 18 and 24 hours of cold hypoxia and reoxygenation did not result in a significant reduction in cell viability compared with normoxic endothelium (82 ± 7% cells viable) (Figure 6.16a). In marked contrast, following 27 hours cold hypoxia, a significant reduction in cell viability was detected in HMEC-1 cells (27 ± 7%), and this remained significant regardless of treatment (p<0.01). However, in HMEC-1 treated with 50µg/ml lec-SOD, the reduction in cell viability was less marked (53 ± 7%) and significantly better than treatment with either 25µg/ml lec-SOD (31 ± 8%), 50µg/ml lecithin (25 ± 6%) or 50µg/ml lecithin and rhSOD (24 ± 7%) (p<0.01). Furthermore, incubation of HMEC-1 with lecithin, resulted in a noticeable degree of cell detachment at all hypoxia times, but with no effect on cell viability as determined by Trypan Blue staining. In contrast, incubation with lec-SOD did not result in endothelial cell detachment.

6.7.2 HUVEC Viability

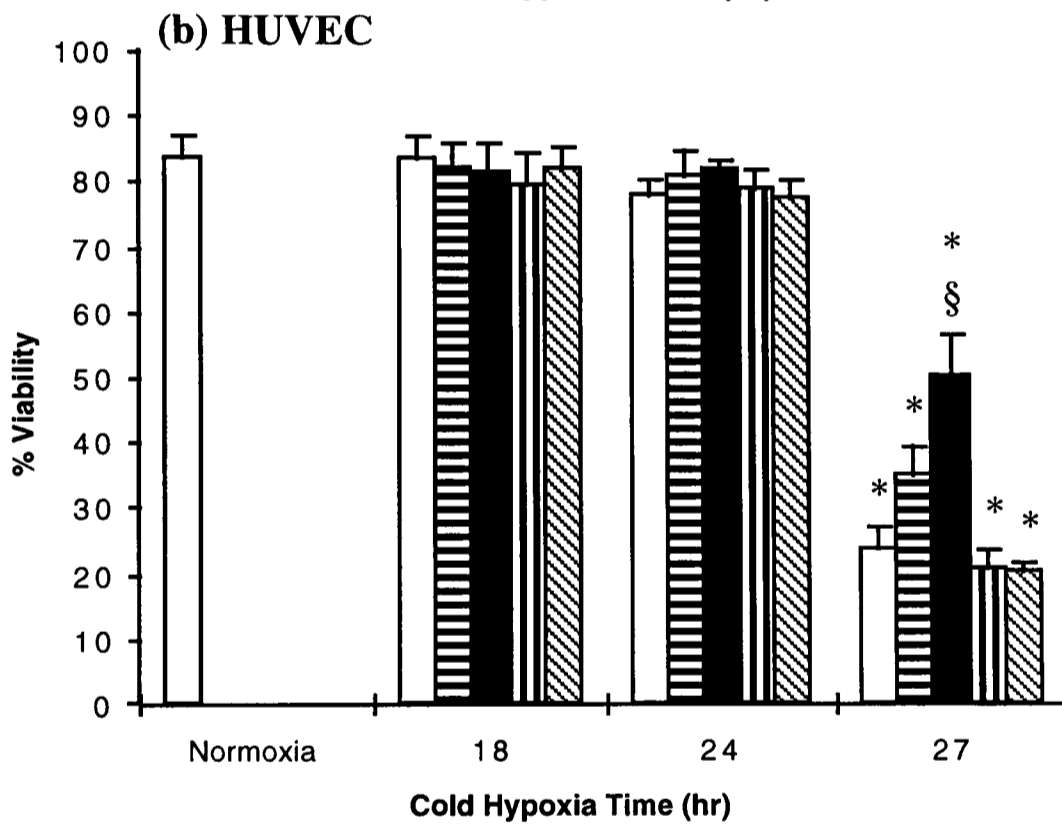
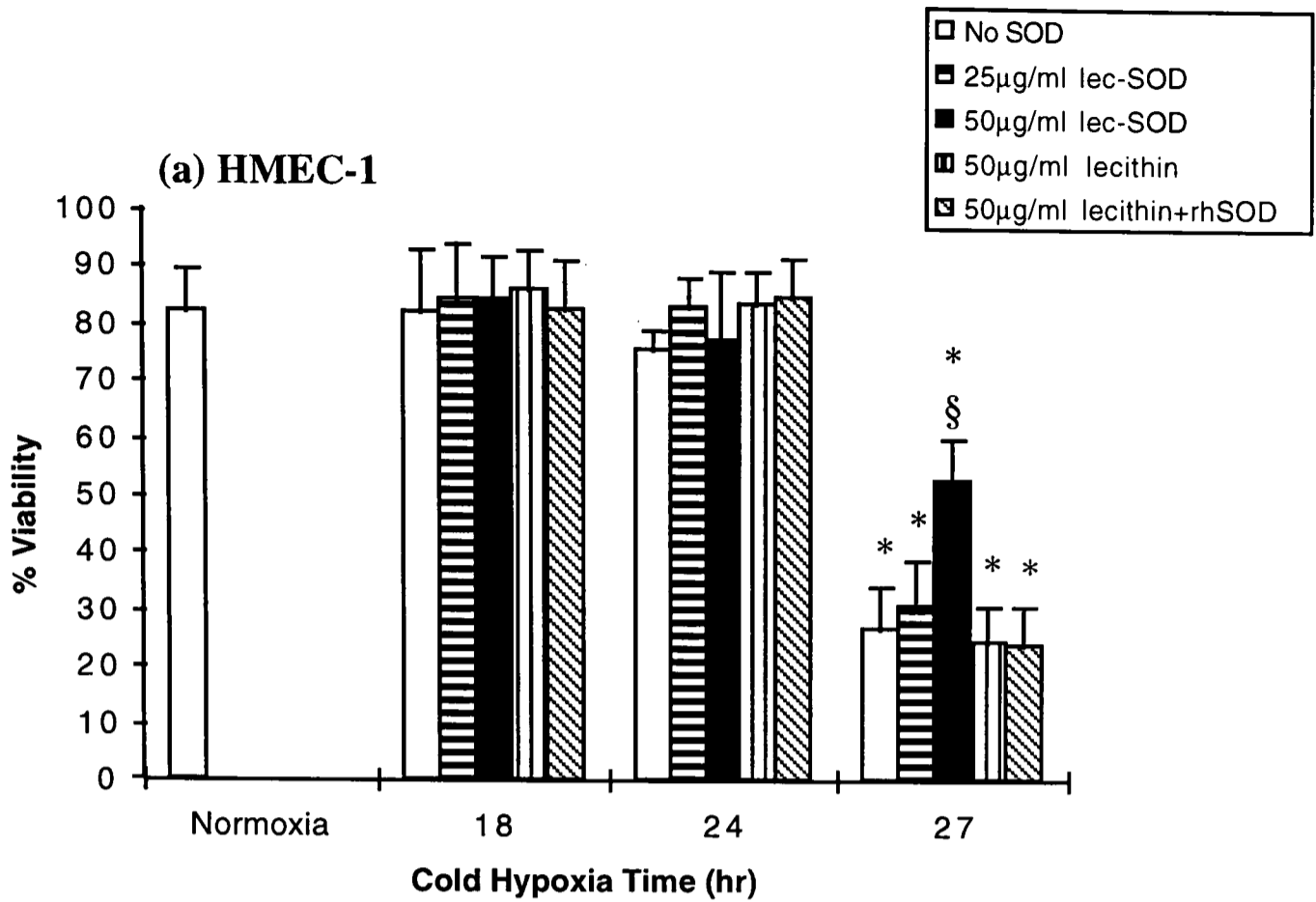
The effects of cold hypoxia/reoxygenation on HUVEC viability were similar to those observed for HMEC-1. No significant reduction in cell viability was detected following

24 hours of cold hypoxia ($78 \pm 3\%$) compared with normoxic controls ($84 \pm 3\%$) (Figure 6.16b). In contrast, a significant reduction in HUVEC viability was observed following 27 hours cold hypoxia/reoxygenation ($24 \pm 4\%$) ($p < 0.01$). Similar to results for HMEC-1, 50 $\mu\text{g/ml}$ lec-SOD afforded a significant improvement in cell viability following 27 hours cold hypoxia ($50 \pm 7\%$) compared with HUVEC treated with 25 $\mu\text{g/ml}$ lec-SOD ($35 \pm 5\%$), 50 $\mu\text{g/ml}$ lecithin ($21 \pm 3\%$) and 50 $\mu\text{g/ml}$ lecithin plus rhSOD unconjugated ($20 \pm 2\%$) (all $p < 0.01$). A marginal level of protection was provided by 25 $\mu\text{g/ml}$ lec-SOD, although this did not attain statistical significance. The effects of lecithin on HUVEC were similar to HMEC-1 with respect to the level of cell detachment observed.

6.8 EFFECT OF LEC-SOD ON NEUTROPHIL-ENDOTHELIAL CELL ADHERENCE FOLLOWING HYPOXIA/REOXYGENATION

Ischaemia/reperfusion injury is characterised by the production of oxygen free radicals resulting in a neutrophil-mediated inflammatory response in the reperfused tissue (see Chapter 1). In Chapter 4 the possible detrimental effects of neutrophil infiltration following ischaemia/reperfusion injury of cadaver renal allografts have been described. Therefore, it would be highly desirable to prevent neutrophil-mediated damage following ischaemia/reperfusion. The initial contact between neutrophils and the reperfused tissue occurs at the endothelial surface as a result of adhesion molecule interactions.

A neutrophil-endothelial cell adhesion (NECA) assay reported by Ichikawa and colleagues has been adapted [458], to investigate the effects of lec-SOD on neutrophil interactions with HUVEC and HMEC-1 cells exposed to conditions of hypoxia and reoxygenation. Briefly, endothelial cell monolayers cultured on fibronectin-coated plates were incubated for 1 hour hypoxia (95% N_2 , 5% CO_2) at 37°C, washed once and



* $p < 0.01$ (vs normoxia and 18 + 24hr CHT)

§ $p < 0.01$ (vs other treatments at 27hr CHT)

Figure 6.16. Viability of HMEC-1 and HUVEC following cold hypoxia and reoxygenation for 24 hr.

(a) HMEC-1 and (b) HUVEC cells were incubated for 18, 24 and 27 hours under cold hypoxic conditions with 25µg/ml of lec-SOD, or 50µg/ml of lec-SOD, lecithin or lecithin and rhSOD then reoxygenated under normoxic conditions for 24 hours. Cell viability was assessed by Trypan Blue exclusion staining after reoxygenation. Each value represents mean \pm SD for three experiments performed in duplicate. Incubation of endothelial cells with 50µg/ml lec-SOD had a significantly protective effect against 27 hours cold hypoxia and 24 hours warm reoxygenation.

then reoxygenated. Following reoxygenation, ^{51}Cr -labelled neutrophils were incubated with endothelial cells for 30 min and the percentage neutrophil adherence calculated. Each experiment was performed at least three times, with ten measurements per experiment. Statistically significant differences were determined by paired T-test analysis.

6.8.1 Time Course for NECA Assay

HUVEC and HMEC-1 cells were exposed for 1 hr hypoxia and then reoxygenated for 30, 60, 120 and 240 min to determine the optimal period of reoxygenation for maximum neutrophil adherence. A time-dependent increase in NECA was observed for HMEC-1, with a significant increase in neutrophil adhesion following 120 min reoxygenation ($9.2 \pm 3.2\%$) compared with normoxic HMEC-1 ($5.3 \pm 1.8\%$) ($p < 0.01$), rising to a maximum after 240 min reoxygenation ($12.3 \pm 2.6\%$) ($p < 0.01$) (Figure 6.17a). A significant increase in neutrophil adherence was also observed in HUVEC reoxygenated for 120 min ($11.2 \pm 1.7\%$) compared with normoxic controls ($5.7 \pm 0.4\%$) ($p < 0.01$), with maximal adherence detected after 240 min reoxygenation ($12.7 \pm 1.9\%$) ($p < 0.01$) (Figure 6.17b). As controls for the experiments, non-specific binding of neutrophils to normoxic endothelial cells, fibronectin alone and blank wells containing medium alone were assessed, and detectable with only a baseline level of adherence of approximately 5%, significantly less than exposure of endothelial cells to 1hr hypoxia and 240 min reoxygenation ($12.3 \pm 2.6\%$) ($p < 0.01$) (Figure 6.18). Therefore, for all subsequent experiments, endothelial cells were exposed to 1 hour hypoxia and reoxygenated for 240 min.

6.8.2 Effect of lec-SOD on NECA Assay

HMEC-1 and HUVEC were incubated with 5, 10, 25, 50 and 100 $\mu\text{g}/\text{ml}$ lec-SOD during the 1 hour hypoxia period and then reoxygenated for 240 min prior to NECA assay. Incubation of hypoxia/reoxygenation conditioned HMEC-1 with 5-100 $\mu\text{g}/\text{ml}$ lec-SOD significantly diminished neutrophil adherence for all concentrations of lec-SOD

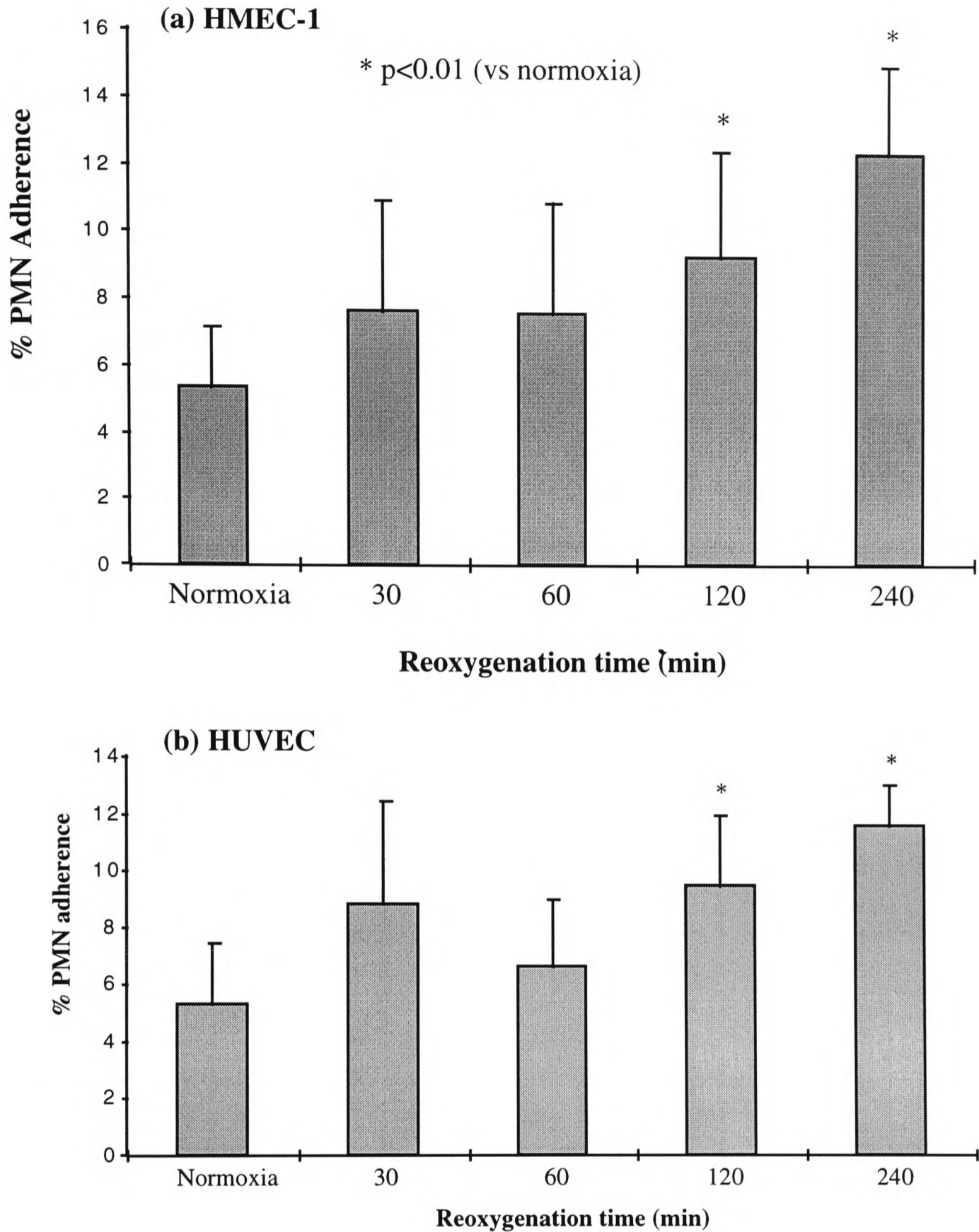


Figure 6.17. Time course of neutrophil adherence to HMEC-1 and HUVEC after hypoxia/reoxygenation.

(a) HMEC-1 and (b) HUVEC monolayers were exposed to 1 hour of hypoxia (or normoxia) and then reoxygenated for 30-240 min. ^{51}Cr -labelled neutrophils were added to endothelial monolayers following the period of reoxygenation and percentage neutrophil adherence determined 30 minutes later. Each value represents mean \pm SD for 10 measurements, replicated in three experiments.

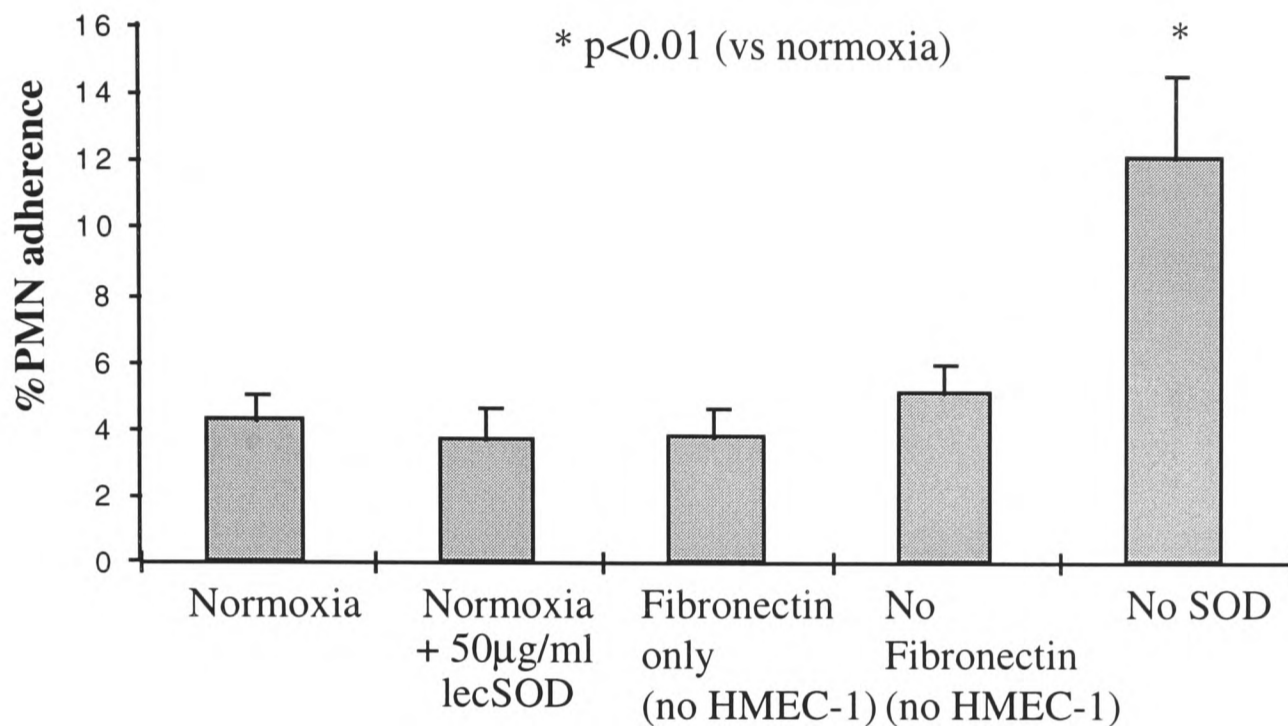


Figure 6.18. Assessment of specificity of neutrophil endothelial cell adherence assay.

HMEC-1 monolayers were incubated under normoxic conditions in the presence or absence of 50µg/ml lec-SOD. Furthermore, wells coated with fibronectin alone, and no HMEC-1 or fibronectin were incubated with ⁵¹Cr-labelled neutrophils for 30 min and neutrophil adherence determined. As a positive control, HMEC-1 were exposed to 1hr hypoxia and 240 min reoxygenation containing no SOD before determining neutrophil adherence. Each value represents mean ± SD for ten replicates.

compared with HMEC-1 cells with no SOD treatment ($12.4 \pm 3.0\%$) ($p < 0.01$) (Figure 6.19a). A significant reduction in neutrophil adherence to HUVEC treated with 5-50 $\mu\text{g/ml}$ lec-SOD during the hypoxic period was also observed ($p < 0.01$) (Figure 6.19b). However, incubation with 100 $\mu\text{g/ml}$ lec-SOD ($9.0 \pm 1.9\%$) did not result in a significant decline in neutrophil adherence, but was significantly higher than normoxic HUVEC ($5.0 \pm 1.1\%$) ($p < 0.01$).

6.8.3 Effect of Lecithin on NECA Assay

The results from section 6.4 demonstrated that rhSOD does not bind to endothelial cells, whereas the effects of lecithin incorporation on endothelial cells required further investigation, especially with regards to the cell detachment observed in section 6.7. Therefore, HMEC-1 and HUVEC exposed to hypoxia/reoxygenation were incubated with a range of concentrations of lecithin (20, 40, 80 and 160 $\mu\text{g/ml}$) to determine its effect on NECA.

Exposure of HMEC-1 to hypoxia/reoxygenation resulted in a significant increase in NECA from $6.9 \pm 2.3\%$ to $12.8 \pm 3.1\%$ ($p < 0.01$). Incorporation of lecithin did not significantly reduce NECA at concentrations of 20 $\mu\text{g/ml}$ ($11.3 \pm 2.2\%$), 40 $\mu\text{g/ml}$ ($10.1 \pm 2.1\%$), 80 $\mu\text{g/ml}$ ($10.1 \pm 3.6\%$) and 160 $\mu\text{g/ml}$ ($10.8 \pm 3.1\%$) (Figure 6.20a). Similarly, following exposure of HUVEC to hypoxia/reoxygenation, no significant reduction in NECA was observed for cells treated with 20 $\mu\text{g/ml}$ ($12.3 \pm 2.8\%$), 40 $\mu\text{g/ml}$ ($11.2 \pm 2.7\%$), 80 $\mu\text{g/ml}$ ($10.5 \pm 2.2\%$) and 160 $\mu\text{g/ml}$ ($10.2 \pm 3.4\%$) compared with hypoxia/reoxygenation conditioned HUVEC with no treatment ($13.0 \pm 1.8\%$) (Figure 6.20b). The marginal reduction in neutrophil adhesion may have resulted from cell detachment and thus disruption of the endothelial cell monolayer.

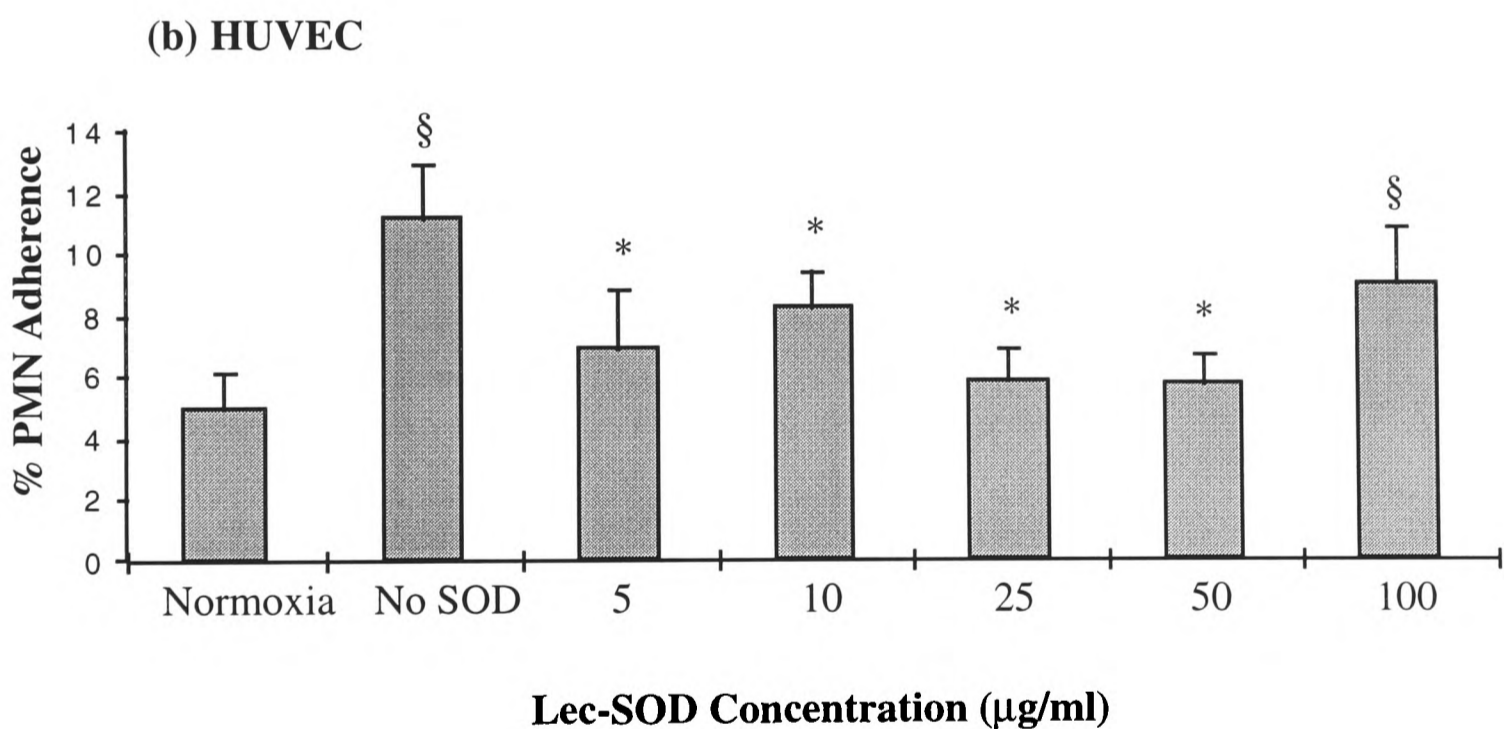
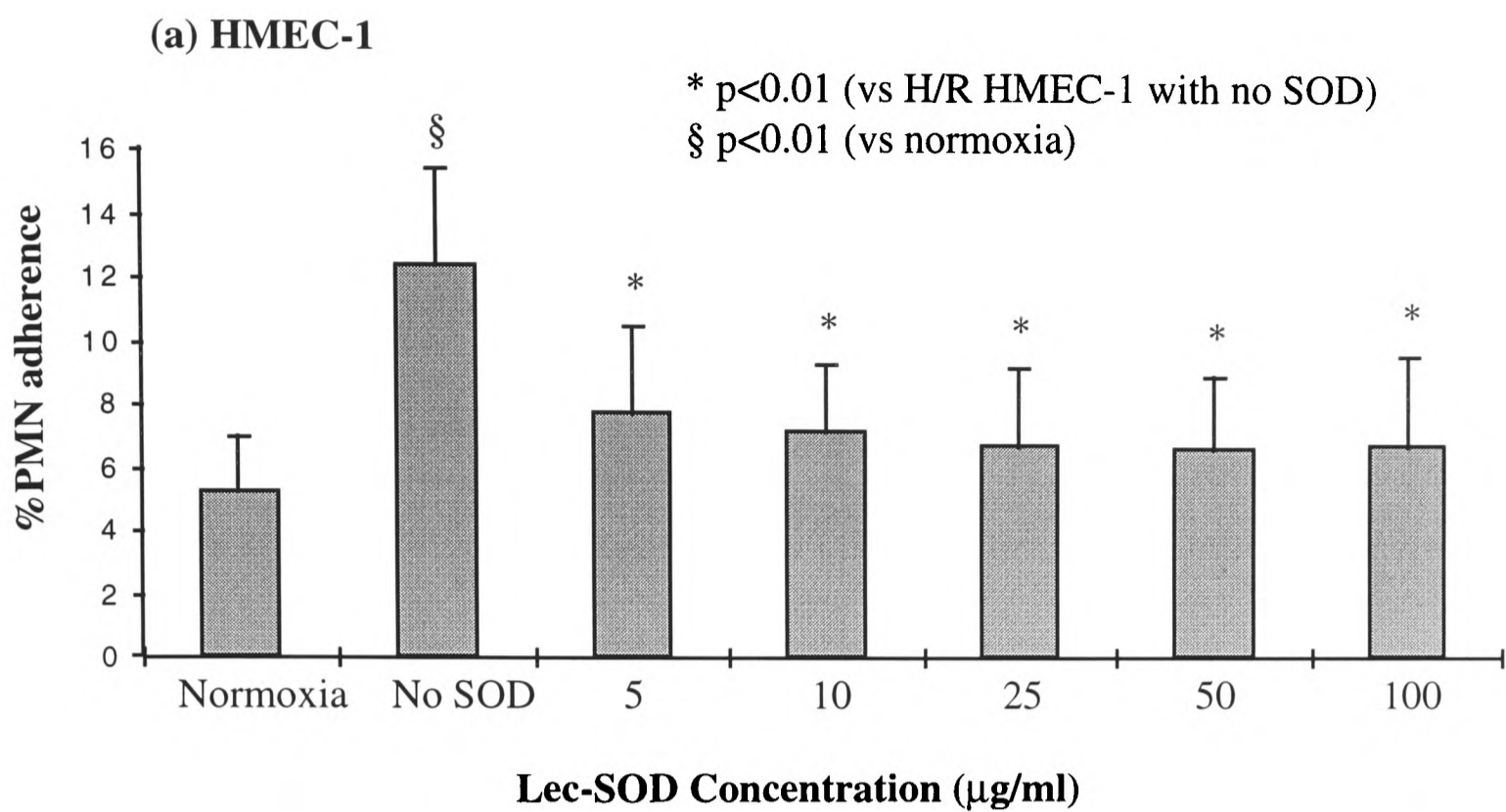


Figure 6.19. Titration of lec-SOD on HMEC-1 and HUVEC exposed to 1hr hypoxia and 4hr reoxygenation.

(a) HMEC-1 and (b) HUVEC monolayers were exposed to 1 hour hypoxia in the presence of 5-100 µg/ml lec-SOD and reoxygenated for 4 hours. ⁵¹Cr-labelled neutrophils were added to endothelial monolayers for 30 min following reoxygenation and percentage neutrophil adherence determined. Each value represents mean ± SD for 10 measurements, replicated in three experiments.

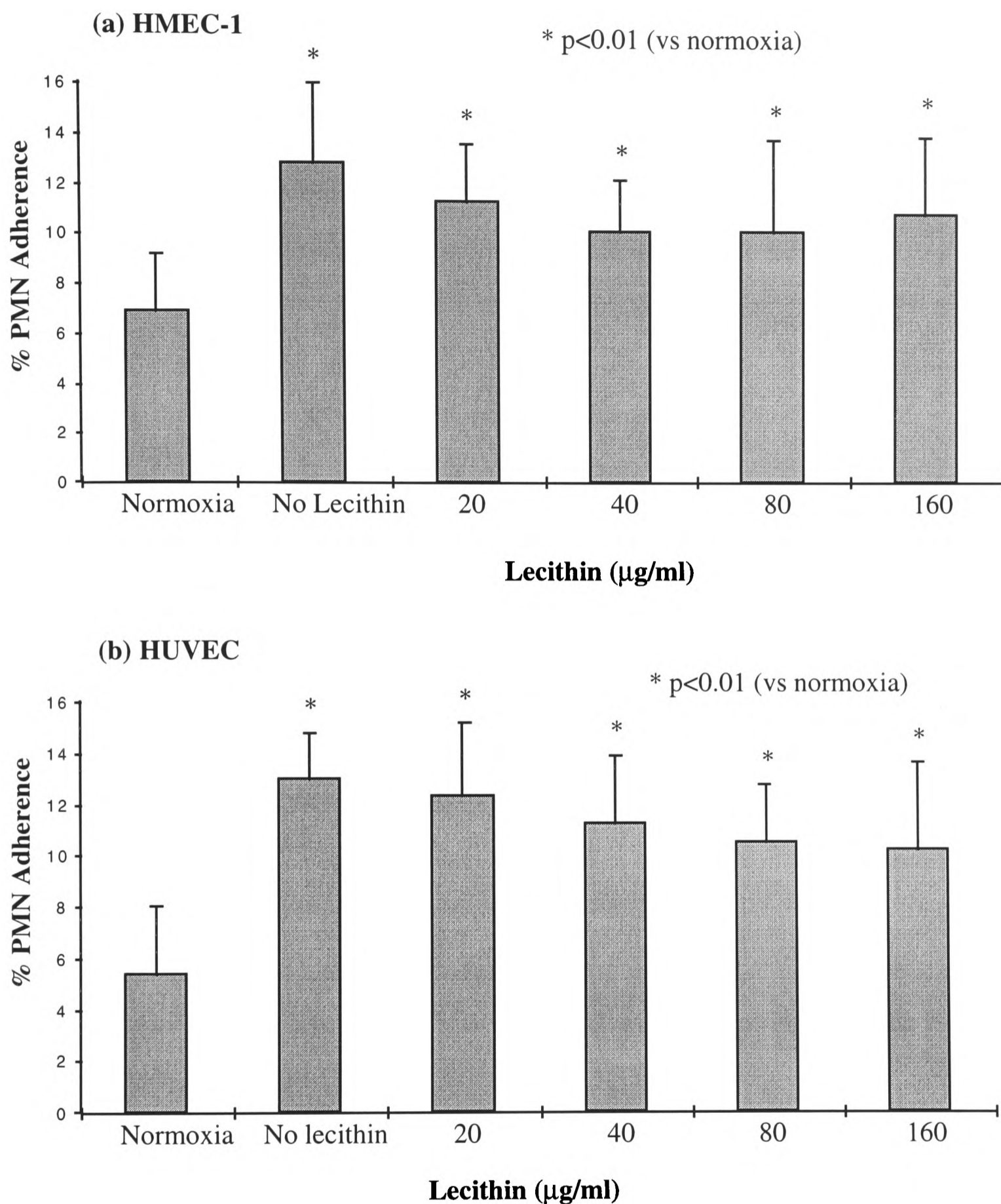


Figure 6.20. Titration of lecithin on HMEC-1 and HUVEC exposed to 1hr hypoxia and 4hr reoxygenation.

(a) HMEC-1 and (b) HUVEC monolayers were exposed to 1 hour hypoxia in the presence of 20-160µg/ml lecithin and reoxygenated for 4 hours. ⁵¹Cr-labelled neutrophils were added to endothelial monolayers for 30 min following reoxygenation and percentage neutrophil adherence determined. Each value represents mean ± SD for 10 measurements, replicated in three experiments.

6.8.4 Comparison of Effects of lec-SOD, rhSOD and Lecithin on NECA Assay

Confluent monolayers of HMEC-1 and HUVEC were exposed to hypoxia/reoxygenation and incubated with 50µg/ml of lec-SOD, 50µg/ml lecithin, 50µg/ml rhSOD and 50µg/ml lecithin plus rhSOD unconjugated, prior to the NECA assay. Statistical analyses using the paired T-test were performed to examine whether or not there were significant differences between all the treatment groups, unstimulated endothelial cells and hypoxia/reoxygenation conditioned endothelium.

HMEC-1 cells exposed to hypoxia/reoxygenation exhibited > 2-fold increase in NECA ($5.6 \pm 1.6\%$ to $12.8 \pm 2.7\%$; $p < 0.01$) which was not significantly reduced in the presence of 50µg/ml rhSOD ($10.7 \pm 3.3\%$), 50µg/ml lecithin ($10.4 \pm 3.7\%$), or 50µg/ml of unconjugated lecithin and rhSOD ($10.6 \pm 3.2\%$) (Figure 6.21a). In contrast, treatment of HMEC-1 cells with 50µg/ml lec-SOD significantly reduced NECA ($6.2 \pm 1.7\%$) ($p < 0.01$) to similar levels obtained under normoxic conditions (Figure 6.21a). Exposure of HUVEC to hypoxia/reoxygenation resulted in a > 2-fold increase in NECA ($5.6 \pm 0.8\%$ to $12.0 \pm 2.6\%$; $p < 0.01$) which was not significantly inhibited with 50µg/ml rhSOD ($10.1 \pm 0.9\%$), 50µg/ml lecithin ($8.4 \pm 1.6\%$), or 50µg/ml lecithin and rhSOD ($8.9 \pm 1.9\%$) (Figure 6.21b), but treatment with 50µg/ml lec-SOD significantly abrogated increased NECA ($6.3 \pm 1.4\%$; $p < 0.01$) (Figure 6.21b).

6.9 FACS ANALYSIS OF ENDOTHELIAL CELL ADHESION MOLECULE EXPRESSION FOLLOWING HYPOXIA/REOXYGENATION

FACS analysis was performed to determine the endothelial adhesion molecules and antigens which may be involved in the increased neutrophil adherence to hypoxia/reoxygenation conditioned HUVEC and HMEC-1 monolayers. HUVEC and HMEC-1

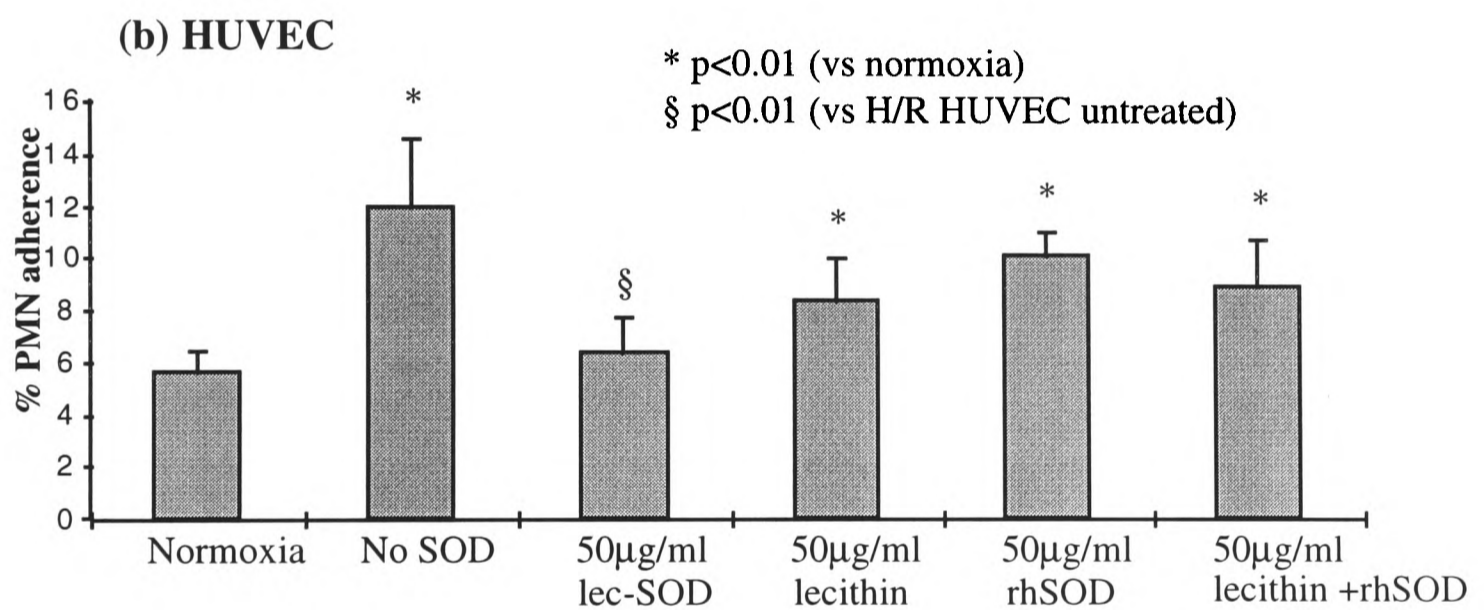
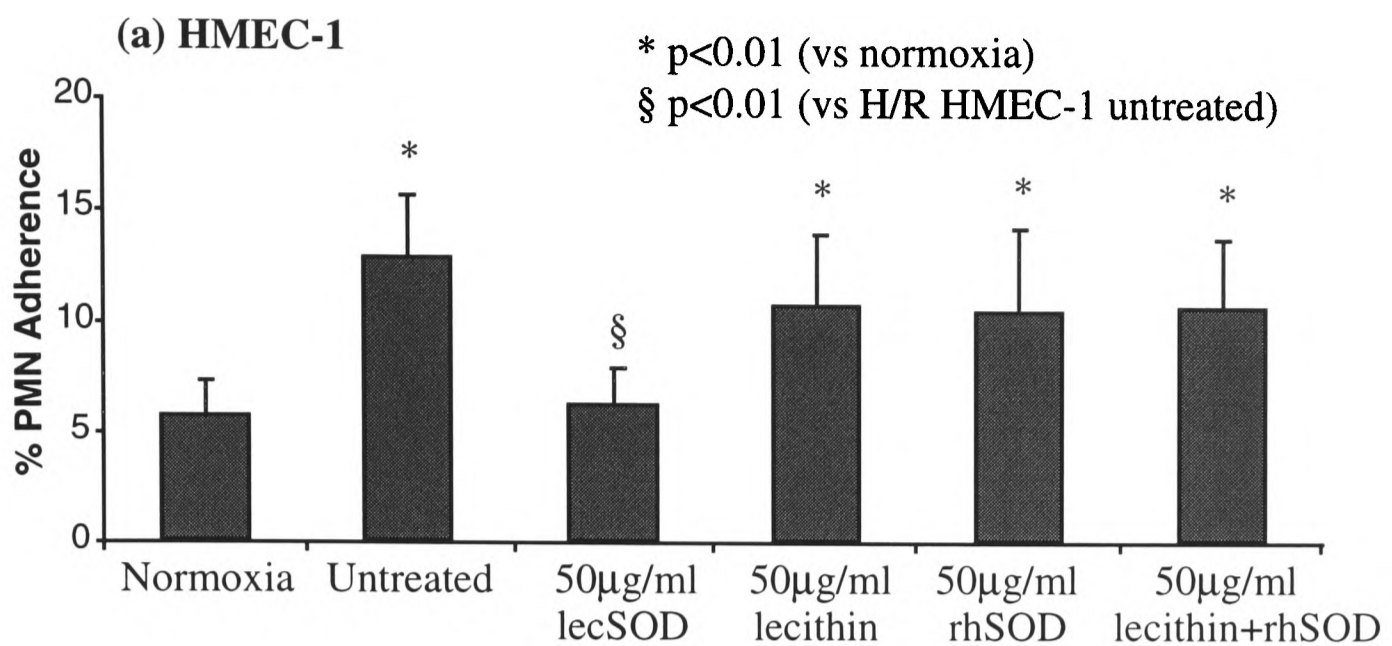


Figure 6.21. Comparison of effects of lec-SOD, rhSOD and lecithin on neutrophil adhesion to HMEC-1 and HUVEC following 1hr hypoxia and 4hr reoxygenation.

Confluent monolayers of (a) HMEC-1 and (b) HUVEC were incubated for 1 hour under hypoxic conditions either untreated or with 50µg/ml of lec-SOD, rhSOD, lecithin alone or lecithin and rhSOD, together but unconjugated, then reoxygenated for 4 hours. ⁵¹Cr-labelled neutrophils were incubated with HMEC-1 after this period for 30 min before determining percentage neutrophil adherence. Each value represents mean ± SD for 10 measurements, replicated in three experiments.

were exposed to 1 hour hypoxia in the presence or absence of 50µg/ml lec-SOD and reoxygenated for 4 hours prior to FACS analysis for expression of CD31, P-selectin, E-selectin, ICAM-1, VCAM-1, HLA Class I, HLA Class II and platelet activating factor (PAF). This analysis was repeated at least three times.

6.9.1 Hypoxia/Reoxygenation of HMEC-1

Constitutive expression of CD31, ICAM-1, HLA Class I and PAF were detected on unstimulated HMEC-1, with no expression of P-selectin, E-selectin, VCAM-1 and HLA Class II (Figure 6.22 and Table 6.3). Following hypoxia/reoxygenation, percentage of HMEC-1 cells positive for ICAM-1 increased from 86% to 95%, and this was inhibited by 50µg/ml lec-SOD (85%). A marginal increase in E-selectin expression (4% to 12%) following hypoxia/reoxygenation was also abrogated by 50µg/ml lec-SOD (5%). Induction of other antigens analysed was not observed on hypoxia/reoxygenation conditioned HMEC-1.

6.9.2 Hypoxia/Reoxygenation of HUVEC

Similar to HMEC-1, CD31, ICAM-1, HLA Class I and PAF were constitutively expressed on HUVEC, whilst P-selectin, E-selectin, VCAM-1 and HLA Class II were absent (Figure 6.23 and Table 6.4). Upregulated expression of ICAM-1 was detected on HUVEC exposed to hypoxia/reoxygenation (38% to 65%) and this was inhibited with 50µg/ml lec-SOD (37%). E-selectin expression was also induced on HUVEC following hypoxia/reoxygenation (4% to 22%) but was only marginally inhibited by 50µg/ml lec-SOD with 16% of cells being positive. Similar to HMEC-1, induction of other antigens analysed in this study was not observed.

6.10 DISCUSSION

Ischaemia/reperfusion injury exists in transplantation following removal of the organ from the donor circulation, extracorporeal cold ischaemic storage and reperfusion with

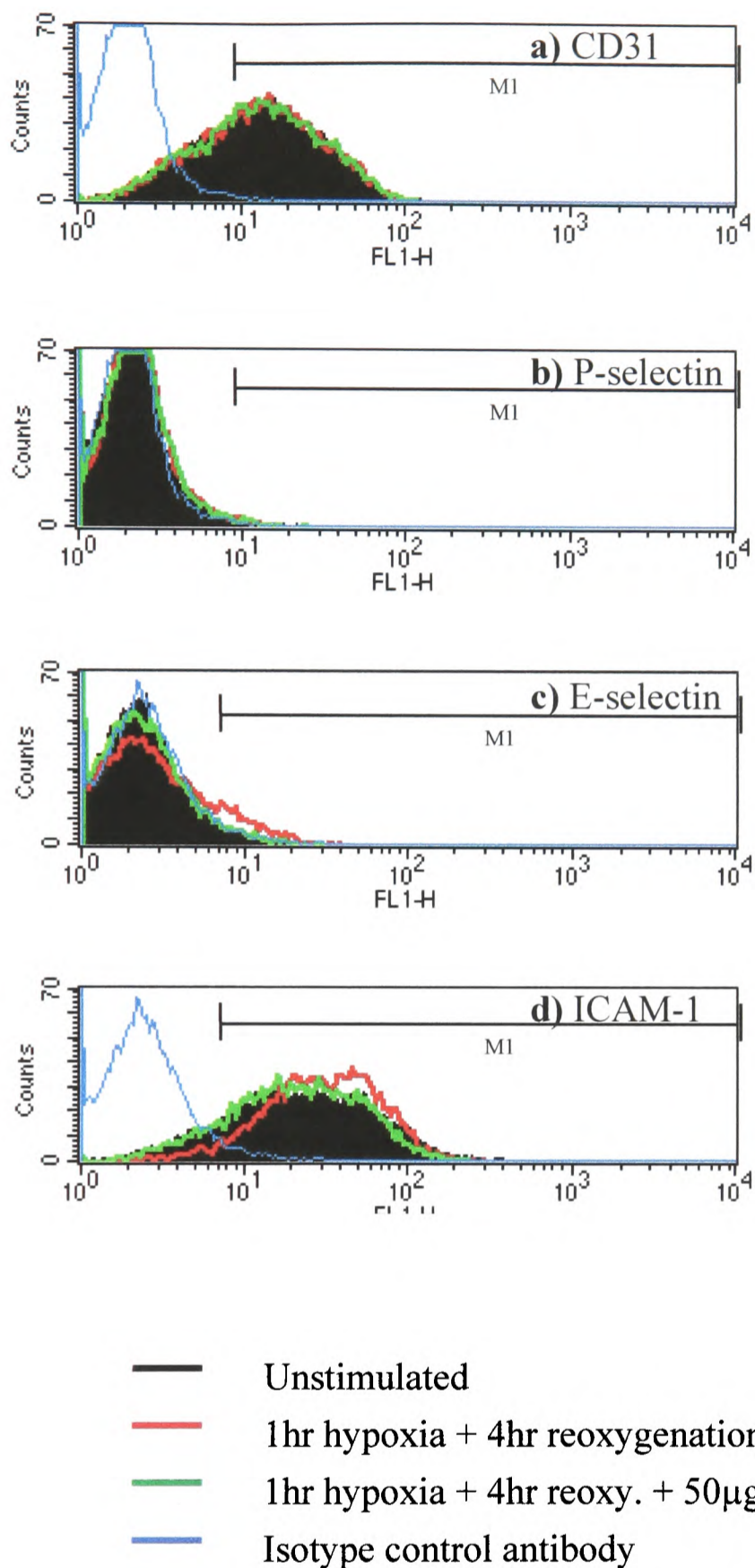


Figure 6.22a-d Phenotypic analysis of HMEC-1 following 1 hr hypoxia and 4 hr reoxygenation.

Confluent monolayers of HMEC-1 were incubated for 1 hr hypoxia and 4 hr reoxygenation either untreated or in the presence of 50 μ g/ml lec-SOD. HMEC-1 were stained with mAb against (a) CD31; (b) P-selectin; (c) E-selectin; (d) ICAM-1; (e) VCAM-1; (f) HLA Class I; (g) HLA Class II; (h) PAF, and surface antigen expression detected with a secondary FITC-conjugated goat anti-mouse Ig for FACS analysis.

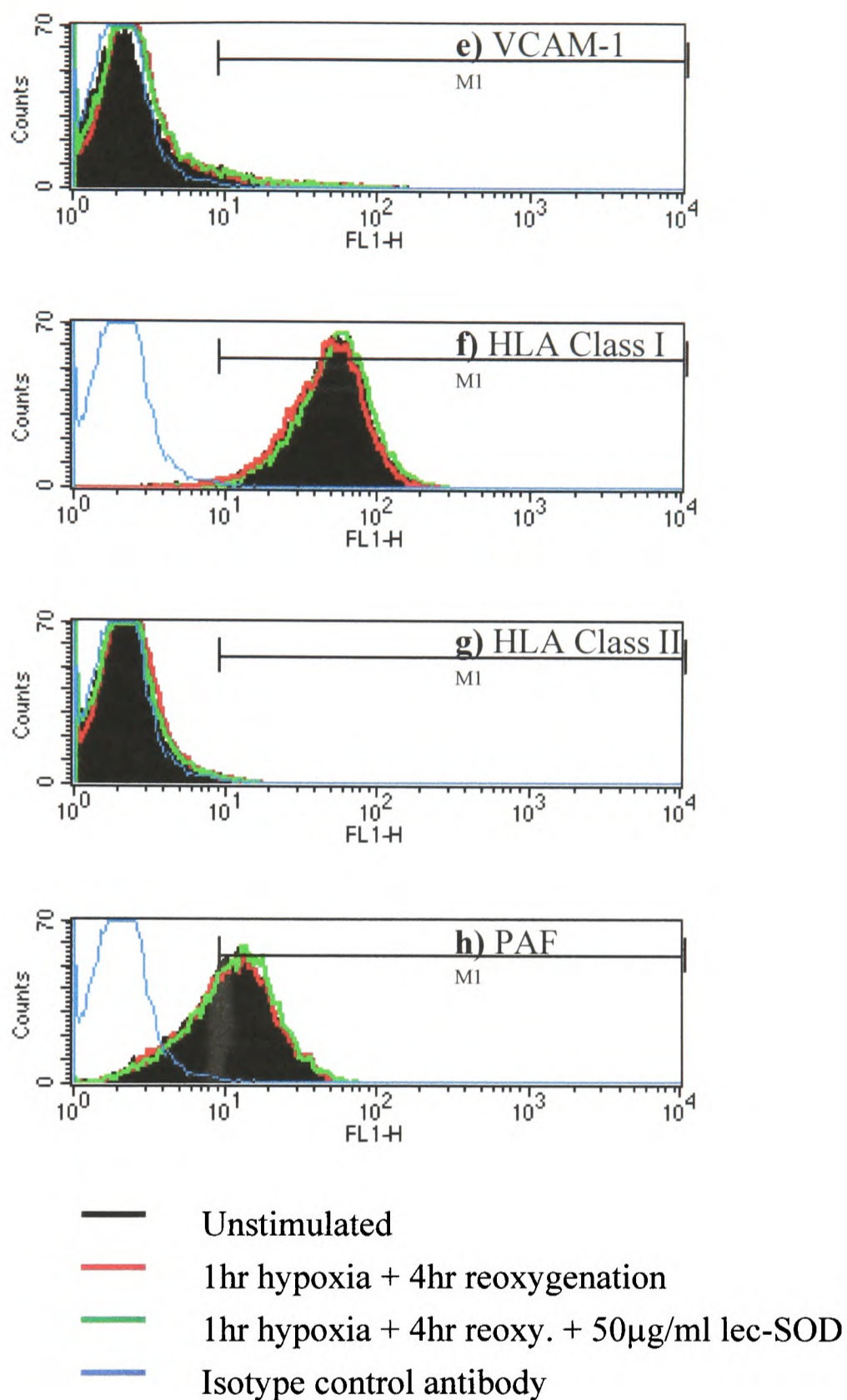


Figure 6.22e-h Phenotypic analysis of HMEC-1 following 1 hr hypoxia and 4 hr reoxygenation.

Confluent monolayers of HMEC-1 were incubated for 1 hr hypoxia and 4 hr reoxygenation either untreated or in the presence of 50 μ g/ml lec-SOD. HMEC-1 were stained with mAb against (a) CD31; (b) P-selectin; (c) E-selectin; (d) ICAM-1; (e) VCAM-1; (f) HLA Class I; (g) HLA Class II; (h) PAF, and surface antigen expression detected with a secondary FITC-conjugated goat anti-mouse Ig for FACS analysis.

| Endothelial marker | Isotype Control | Normoxia | Hypoxia | Hypoxia + lec-SOD |
|--------------------|-----------------|----------|----------|-------------------|
| CD31 | 3 (1%) | 39 (99%) | 43 (99%) | 40 (98%) |
| P-selectin | 3 (1%) | 3 (2%) | 3 (2%) | 2 (1%) |
| E-selectin | 3 (1%) | 3 (4%) | 28 (22%) | 20 (16%) |
| ICAM-1 | 2 (1%) | 21 (38%) | 28 (65%) | 18 (37%) |
| VCAM-1 | 2 (1%) | 4 (8%) | 3 (6%) | 3 (6%) |
| HLA Class I | 3 (1%) | 49 (99%) | 52 (99%) | 48 (98%) |
| HLA Class II | 3 (1%) | 3 (2%) | 3 (2%) | 3 (1%) |
| PAF | 3 (1%) | 48 (99%) | 51 (99%) | 53 (99%) |

Table 6.3. Phenotypic FACS analysis of HUVEC following 1hr hypoxia and 4hr reoxygenation. Values represent mean channel fluorescence intensity and percentage positive cells for Figure 6.22.

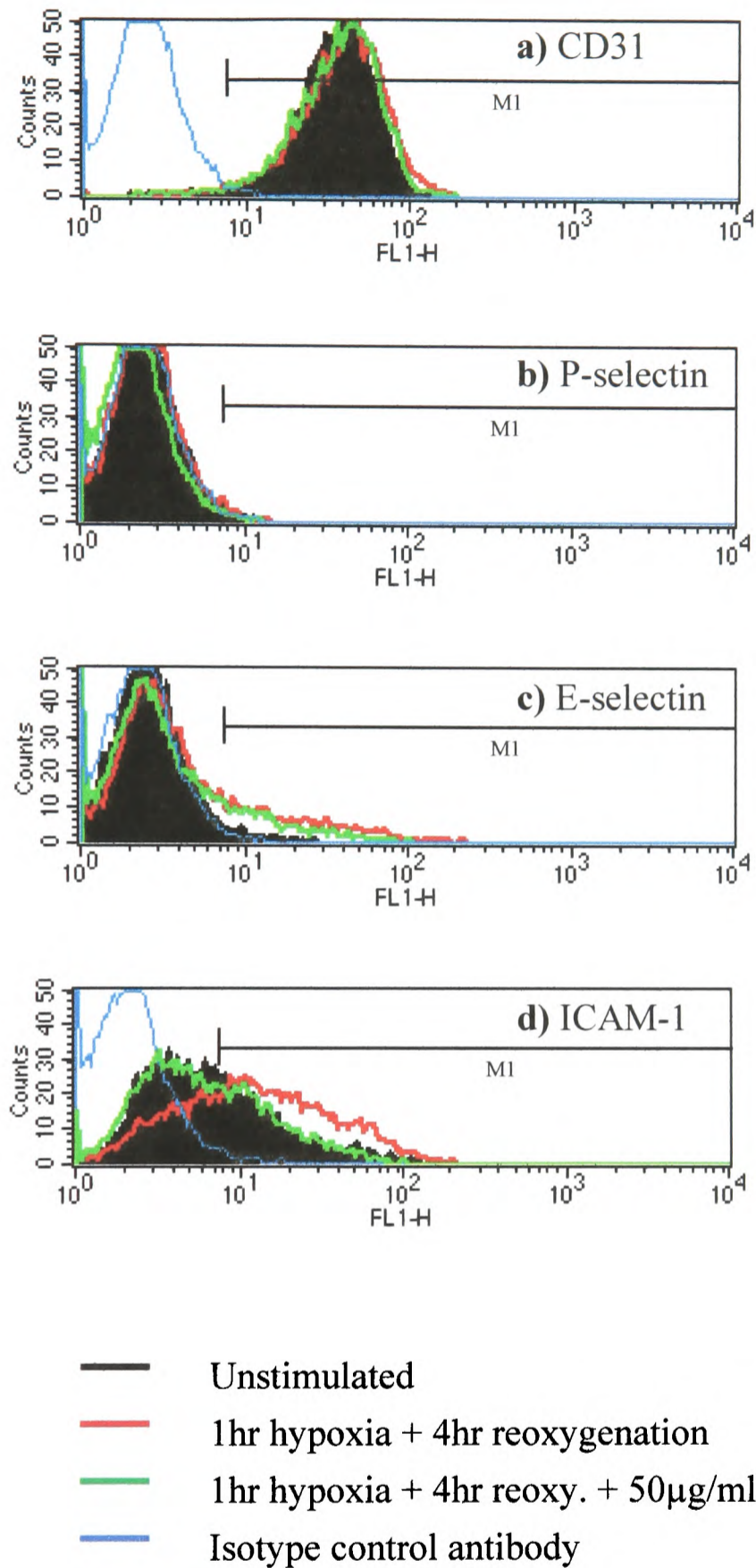


Figure 6.23a-d Phenotypic analysis of HUVEC following 1hr hypoxia and 4hr reoxygenation.

Confluent monolayers of HUVEC were incubated for 1 hr hypoxia and 4 hr reoxygenation either untreated or in the presence of 50µg/ml lec-SOD. HUVEC were stained with mAb against (a) CD31; (b) P-selectin; (c) E-selectin; (d) ICAM-1; (e) VCAM-1; (f) HLA Class I; (g) HLA Class II; (h) PAF, and surface antigen expression detected with a secondary FITC-conjugated goat anti-mouse Ig for FACS analysis.

| Endothelial marker | Isotype Control | Normoxia | Hypoxia | Hypoxia + lec-SOD |
|--------------------|-----------------|----------|----------|-------------------|
| CD31 | 3 (4%) | 24 (69%) | 25 (70%) | 25 (69%) |
| P-selectin | 2 (1%) | 2 (1%) | 3 (1%) | 3 (1%) |
| E-selectin | 3 (4%) | 3 (4%) | 12 (12%) | 3 (5%) |
| ICAM-1 | 3 (4%) | 39 (86%) | 40 (95%) | 32 (85%) |
| VCAM-1 | 2 (1%) | 28 (8%) | 28 (7%) | 27 (7%) |
| HLA Class I | 2 (1%) | 53 (99%) | 50 (99%) | 56 (99%) |
| HLA Class II | 2 (1%) | 3 (1%) | 2 (1%) | 2 (1%) |
| PAF | 2 (1%) | 16 (60%) | 17 (61%) | 17 (64%) |

Table 6.4. Phenotypic FACS analysis of HMEC-1 following 1hr hypoxia and 4hr reoxygenation. Values represent mean channel fluorescence intensity and percentage positive cells for Figure 6.23.

oxygenated blood carrying recipient leucocytes to the graft. The initial interaction between recipient leucocytes and the graft occurs at the endothelial surface where a range of pathophysiological events may take place. Reperfusion injury becomes manifest in the microcirculation of the graft with the generation of endothelial oxygen free radicals and neutrophil-mediated damage. In Chapter 4 the effects of prolonged cold ischaemia and reperfusion injury in cadaveric renal transplantation have been described, which were characterised by a neutrophil infiltration in the microvasculature of the kidney. The aim of the work performed in this Chapter was to simulate the events that occur during ischaemia/reperfusion injury in renal transplantation using an *in vitro* endothelial cell model to examine whether lecithinised superoxide dismutase (lec-SOD), would be beneficial in preventing oxygen free-radical derived damage.

The first human endothelial cell type to be successfully cultured was derived from umbilical vein (HUVEC), and is currently the most commonly studied primary endothelial cell type from which the foundations of endothelial cell biology have been based. HUVEC have been widely used because umbilical cords are easily obtainable on a frequent basis, (a necessity for primary cell cultures), with the additional advantage of a large, single vein enabling simple enzymatic removal of cells from the internal lining of the blood vessel. However there are disadvantages in studying HUVEC due to the potentially unphysiologic *in vitro* conditions, the rare incidences of inflammatory events in umbilical veins, inter-individual variability and the absence of HLA Class II antigens that are expressed on the microvascular endothelium.

Both *in vivo* and *in vitro* studies have demonstrated that there is functional, physiological and phenotypic heterogeneity between macrovascular and microvascular endothelial cells, with additional variation dependent on the organ from which the cells have been isolated [129]. For example, capillary endothelium in the retina and brain are connected by tight junctions forming a continuous layer to maintain the blood brain barrier, whereas discontinuous, fenestrated endothelial cells are found in intestinal villi,

endocrine glands and kidneys to facilitate selective permeability [477]. Furthermore, there are phenotypic differences in constitutive surface protein expression and activation responses to inflammatory mediators and cytokines. The expression of inducible endothelial adhesion molecules is critical for the selective recruitment of leucocyte subpopulations into an area of inflammation. Cultured HUVEC have been shown to constitutively express PECAM-1, ICAM-1 and ICAM-2, with induced expression of ICAM-1, E-selectin and VCAM-1 following stimulation with the inflammatory cytokines TNF and IL-1 β [190, 191, 261, 262, 465, 478, 479].

The expression of adhesion molecules in microvascular endothelial cells has been shown to differ from that of HUVEC. Human intestinal microvascular endothelial cells (HIMEC) have been shown to upregulate identical adhesion molecules to HUVEC, but the kinetic profiles for E-selectin and VCAM-1 induction demonstrated that there were regulatory differences compared with HUVEC [480]. Microvascular endothelial cells isolated from human dermal tissue (HDMEC) also demonstrated sustained E-selectin expression following TNF stimulation as a result of slower internalisation and degradation of E-selectin protein, a characteristic also observed in microvascular endothelial cells isolated from lung and subcutaneous fat, but not large vessel endothelium from saphenous vein, aorta or HUVEC' [481]. The biological and functional differences between microvascular and large vessel endothelial cells, in particular growth characteristics and adhesion molecule expression have been well documented [425, 426, 482-492]. However, despite the functional and physiologically relevant characteristics that make investigation of microvascular endothelial cells attractive, fewer studies have been performed than might be expected due to their fastidious growth requirements and practical difficulties in isolating pure microvascular endothelial cells compared with HUVEC.

This problem has largely been overcome with the creation of an immortalised human microvascular endothelial cell line, HMEC-1. HMEC-1 was created by transfection of

human dermal microvascular endothelial cells (isolated from neonatal foreskin tissue) with a plasmid containing simian virus SV40 gene and has been shown to display a characteristic endothelial phenotype with cobblestone morphology in confluent monolayers and tube formation in matrigel culture similar to HDMEC, but with the advantage of less fastidious growth requirements and the capacity for prolonged passaging through many cell cycles [425, 426]. Furthermore, inter-individual variation experienced in primary HUVEC and microvascular endothelial cell preparations is not encountered in HMEC-1 cells.

In this Chapter, experiments comparing the effects of lec-SOD binding on both HMEC-1 and HUVEC under normoxic conditions and following hypoxia/reoxygenation have been performed. A phenotypic characterisation of HUVEC and HMEC-1 demonstrated that adhesion molecule expression before and after cytokine stimulation were similar to results which have been previously reported. Both cell types constitutively expressed PECAM-1, ICAM-1 and HLA Class I, but with variations in the level of expression. HMEC-1 constitutively expressed higher levels of ICAM-1 and HLA Class I, but with lower levels of CD31 compared with HUVEC. Following stimulation of HMEC-1 and HUVEC with IFN γ , ICAM-1 and HLA Class I expression were upregulated, with *de novo* synthesis of HLA Class II. Incubation of HUVEC with TNF or IL-1 β elicited upregulated expression of ICAM-1 and induction of E-selectin and VCAM-1 expression, whereas in HMEC-1 cells, the magnitude of adhesion molecule upregulation was less pronounced. The results from this study demonstrated that HMEC-1 had similar growth characteristics to HUVEC but variations were detected in the level of constitutive adhesion molecule expression and the magnitude of the response following cytokine stimulation. Our results and those from other studies, suggest that HMEC-1 is a suitable endothelial cell line for *in vitro* investigation of microvascular cell function [318, 493-498].

An *in vitro* model of ischaemia/reperfusion injury has been developed to determine the effects of lec-SOD following periods of hypoxia and reoxygenation of large vessel and microvascular endothelial cells. During the period of ischaemia, endothelial levels of antioxidants become depleted and are rapidly overwhelmed by the burst of free radical production at reperfusion. A number of studies have utilised the scavenging effects of SOD for the protection of ischaemic tissue from reperfusion injury [117-126]. However, therapeutic efficacy could be further improved by increasing the half-life of exogenous SOD and localising SOD activity to the reperfused tissue. Previous studies have demonstrated that following lecithinisation of rhSOD, lec-SOD bound with higher affinity to HUVEC than rhSOD alone or polyethylene glycol-bound SOD, and in addition, was pharmacologically more potent with respect to the enzymatic half-life and protection afforded to HUVEC from neutrophil-mediated damage [474]. In this current study, we determined the binding capacity of lec-SOD to HMEC-1 under similar conditions to those previously described for HUVEC, with additional analysis for the detection of intracellular SOD.

The long term aim of this study would be to incorporate lec-SOD into organ preservation solution to enable targeting to the endothelium. It was our hypothesis that following flushing through the vasculature of the organ with preservation solution, lec-SOD may be directly targeted to the endothelium during the period of cold storage and exert its protective effect upon reperfusion. We have determined the optimum range (10-50µg/ml) in which lec-SOD may be incorporated effectively on HUVEC and HMEC-1 following 24 hours cold storage in Marshall's solution. In contrast, rhSOD was not detectable on endothelial cells incubated with similar concentrations and under identical conditions. Furthermore, binding of lec-SOD to HMEC-1 and HUVEC was not affected by incubation under cold hypoxic conditions, as would be experienced in the clinical setting.

Analysis by confocal laser microscopy demonstrated that lec-SOD was localised to the endothelial surface with PECAM-1, but was not detectable by planar laser imaging within the cells following permeabilisation. Although intracellular uptake of lec-SOD was not proven, the increased surface levels of lec-SOD may be more effective in reducing subsequent ischaemia/reperfusion insults. Exogenous SOD administered immediately prior to reperfusion has been shown to significantly reduce the impact of oxygen free radical derived damage at the endothelial surface in renal transplantation [117, 119, 123].

Interestingly, differences in native intracellular and extracellular SOD levels were detected between HUVEC and HMEC-1 following cold hypoxia. In eukaryotic cells, SOD is characterised by the identity of the transition metal in the catalytic site and their cellular localisation. Mn-SOD is located in the mitochondria, whereas the dimeric, non-glycosylated CuZn-SOD is cytosolic and nuclear. The tetrameric, glycosylated CuZn-SOD has a C-terminal heparin binding domain enabling anchoring of the molecule to heparan sulphate proteoglycans on the extracellular surface (EC-SOD) [107, 108]. These studies also demonstrated that the predominant activity of SOD in peripheral vessels was attributable to the CuZn isoforms. Furthermore, in a study performed by Terada, hypoxia/reoxygenation conditioned endothelial cells were shown to generate $\cdot\text{O}_2^-$ intracellularly and exported to the extracellular compartment where it exerted its deleterious effects [313]. This was abolished following administration of exogenous SOD which prevented hypoxia/reoxygenation induced cellular leakage without affecting intracellular levels of $\cdot\text{O}_2^-$. Furthermore, treatment with an anion channel blocker reduced the levels of extracellular SOD transported from the cytosol. The transport of intracellular SOD to the cell surface may be important for explaining the differences observed between intracellular and extracellular SOD levels produced by endothelial cells following hypoxia/reoxygenation.

Our results suggest that intracellular levels of CuZn-SOD produced by HMEC-1 were higher in cells exposed to 24 hours cold hypoxia compared with normoxic controls, but without a change in extracellular (EC-SOD) levels. In marked contrast, higher levels of EC-SOD were detected in HUVEC exposed to cold hypoxia for 24 hours, whereas intracellular CuZn-SOD was unchanged. This is the first demonstration of cold hypoxia-induced EC-SOD upregulation on the surface of large vessel (HUVEC) but not microvascular (HMEC-1) endothelium *in vitro*. The effects of ischaemia/reperfusion injury in transplantation is manifest in the microvasculature of the organ, where the antioxidant capacity of the endothelium is overwhelmed by the generation of oxygen free radicals [74, 161, 340, 499]. Therefore, it is possible that the inability of microvascular endothelial cells to respond to $\cdot\text{O}_2^-$ mediated damage at the cell surface following hypoxia [313], may compromise their response to ischaemia/reperfusion injury, and thus targeting of lec-SOD to microvascular endothelium may compensate for this deficiency.

After hypoxia and warm reoxygenation, lec-SOD remained on the cell surface and was detectable maximally after 4 hours of reoxygenation, declining in a time dependent manner after 3 days. Daily fine needle aspirate biopsy analysis of cadaver renal allografts have demonstrated that levels of CuZn-SOD were minimal in the initial period following transplantation, but a delayed increase in CuZn-SOD production was detected, peaking at day 2 post-transplant [500]. These results suggest that grafts would be unable to respond to $\cdot\text{O}_2^-$ mediated damage in the critical period immediately following reperfusion. Our results suggest that storage of organs with lec-SOD may provide protection in the initial phase of reperfusion injury when the levels of native SOD within the graft are low. Despite the decline in lec-SOD after 3 days of reoxygenation in our *in vitro* model, the native levels of SOD produced by the graft may have increased sufficiently to provide protection from further damage.

Indirect evidence for the role of free radicals in ischaemia/reperfusion injury have been provided from early studies which demonstrated a protective effect of antioxidants (e.g. SOD, allopurinol) in renal transplantation [117, 119]. The effects of ischaemia/reperfusion injury have been simulated in many *in vitro* studies by exposure of endothelial cells to various periods of hypoxia/reoxygenation. However, direct evidence for the role of oxygen free radicals was provided in 1994 from electron paramagnetic resonance spectroscopy studies which demonstrated that in human aortic endothelial cells subjected to a period of hypoxia and reoxygenation, superoxide and hydroxyl radicals were detected [109, 110]. These studies demonstrated that the enzyme xanthine oxidase (XO) was the primary source of free radical production.

To determine whether lec-SOD had a protective role in hypoxia induced endothelial cell death, HMEC-1 and HUVEC were exposed to 18, 24 and 27 hours of cold hypoxia in Marshall's solution and reoxygenated for 24 hours before assessment of cell viability by Trypan Blue exclusion staining. Our results demonstrated that following 24 hours of cold hypoxia and reoxygenation, no reduction in cell death was detected compared with normoxic controls. In contrast, following 27 hours cold hypoxia and reoxygenation, a significant reduction in HUVEC and HMEC-1 viability with increased cell detachment was observed, that was not prevented by the addition of rhSOD, lecithin or lecithin and rhSOD in combination. However, incubation of HUVEC or HMEC-1 with 50µg/ml lec-SOD significantly improved cell viability and endothelial attachment compared with cells incubated with Marshall's solution alone. The protective effects of lec-SOD, but not rhSOD or lecithin, suggest that lec-SOD may be effective in preventing cold hypoxia/reoxygenation induced cell death as a result of oxygen free radical production.

The effects of various organ preservation solutions on endothelial cell viability and alterations in morphology have been studied. Incubation of cultured human saphenous vein endothelial cells with HTK and UW solutions afforded a significantly greater level of protection for cell viability compared with Euro-Collins and St. Thomas' solutions

following 24 and 36 hours of cold storage [501]. Killinger and colleagues also demonstrated that modified Belzer UW solution and addition of lazaroïd (an inhibitor of lipid peroxidation) to Euro-Collins solution significantly improved endothelial cell viability following cold storage [502, 503]. The involvement of free radicals in the reduction of cell viability following cold preservation of hepatocytes and liver endothelial cells was reported by Rauen and colleagues, but contrary to the results in this Chapter, they demonstrated that apoptosis was prevented by incubation under cold hypoxic conditions [504, 505]. In contrast, evidence from other studies demonstrated that prolonged cold hypoxic storage of HUVEC reduced cell viability and adversely affected cell morphology and integrity [506, 507].

The mechanisms by which hypoxia induces cell death or apoptosis are not fully understood and it is likely that there are several pathways by which this occurs. Incubation of bovine aortic and pulmonary artery endothelial cells under hypoxic conditions results in the expression of a specific set of hypoxia-associated proteins which were found to be distinct from heat shock proteins [508]. Recent evidence suggests that hypoxia induced endothelial cell apoptosis may be mediated by upregulated expression of the tumour suppressor protein p53, independent of nuclear factor- κ B and Bcl-2 [509].

To determine the potential efficacy of lec-SOD in preventing neutrophil-mediated damage upon ischaemia/reperfusion injury in transplantation, an *in vitro* model of hypoxia/reoxygenation was developed to simulate this response. Numerous studies have been performed to examine effects of hypoxia/reoxygenation on endothelial cells, but many discrepancies exist in the percentage of oxygen for hypoxia, period of hypoxia and reoxygenation, and source of endothelia. These discrepancies were investigated in a comprehensive study by Ichikawa and colleagues, to determine the periods of reoxygenation that elicited increased neutrophil adherence, the effects of various oxygen free radicals, adhesion molecules and transcription factors [458]. The

results from this study demonstrated that maximal neutrophil adherence was observed following 1 hour hypoxia (95% N₂, 5% CO₂) and 4 hours reoxygenation, and this was dependent on endothelial expression of PAF, ICAM-1 and E-selectin, which was inhibited by catalase, oxypurinol and SOD.

Therefore, we have adapted the hypoxia/reoxygenation model from the study of Ichikawa and coworkers to examine the effect of lec-SOD on neutrophil adherence and adhesion molecule expression on HUVEC and HMEC-1. Both HUVEC and HMEC-1 exhibited a time-dependent increase in neutrophil endothelial cell adhesion (NECA) following 1 hour hypoxia (95% N₂, 5% CO₂) and reoxygenation for 30, 60, 120 and 240 min, with maximal NECA detected after 240 min reoxygenation. Incubation of HUVEC and HMEC-1 with 50µg/ml lec-SOD during the 1 hour hypoxic period significantly attenuated NECA to similar levels obtained for normoxic endothelium, whereas treatment with lecithin alone, rhSOD or lecithin and rhSOD unconjugated did not reduce NECA. Following hypoxia/reoxygenation of HUVEC and HMEC-1, upregulated expression of E-selectin and ICAM-1 were detected by FACS analysis, whereas expression of CD31, P-selectin, HLA Class I, HLA Class II, VCAM-1 and PAF were unchanged. Incubation of HMEC-1 with 50µg/ml lec-SOD completely inhibited induction of E-selectin and ICAM-1, whereas similar treatment of HUVEC prevented ICAM-1 upregulation and partially inhibited E-selectin induction.

Hypoxia/reoxygenation induced NECA has been demonstrated on HUVEC in this study, but also to the microvascular endothelial cell line, HMEC-1. Our results suggest that upregulation of endothelial E-selectin and ICAM-1 may be important for this process, and inhibition by lec-SOD may indicate a role for $\cdot\text{O}_2^-$ mediated induction. Indeed, the participation of ICAM-1 and E-selectin in NECA to hypoxia stimulated endothelial cells has been demonstrated by blocking mAb treatment [314, 458]. Inhibition of NECA by administration of free radical scavengers also implicated a role for $\cdot\text{O}_2^-$, H₂O₂ in the induction of ICAM-1 and E-selectin. Furthermore, reagents that

interfered with the activation of the transcription factors NF κ B and AP-1 attenuated the upregulated expression of ICAM-1, and especially E-selectin [458]. The transcription factor NF κ B is a pleiotropic regulator of many genes involved in immune and inflammatory responses, including endothelial adhesion molecules [510]. In resting cells, NF κ B occurs in an inactive cytosolic form which is complexed to the inhibitory protein I κ B [511]. Upon activation, I κ B is phosphorylated and targeted for degradation enabling the active NF κ B to translocate to the nucleus, bind to its recognition DNA element and participate in transcriptional activation. Creation of a proteolysis-resistant mutation in I κ B in human endothelial cells prevented TNF induced expression of ICAM-1 and E-selectin [512]. These studies suggest that following hypoxic treatment of endothelial cells, increased expression of adhesion molecules such as ICAM-1 and E-selectin may be upregulated by oxygen free radicals, which activate NF κ B mediated transcription.

In addition to hypoxic stimulation, many *in vitro* studies have been performed on endothelial cells exposed to exogenous oxygen free radicals (e.g. hydrogen peroxide, X/XO), which resulted in elevated expression of ICAM-1, PAF and P-selectin and increased binding of neutrophils [315-318]. P-selectin expression has been demonstrated in hypoxia/reoxygenation of endothelial cells [456-458, 466], but in the results presented in this Chapter, P-selectin expression was not detected in HUVEC or HMEC-1, which may reflect the time point that adhesion molecule expression was analysed (4 hours post-reoxygenation).

The recruitment and activation of neutrophils by endothelial cells following ischaemia/reperfusion may further aggravate the inflammatory processes that have been initiated. Upregulated endothelial selectin expression may preferentially recruit neutrophils which express high levels of sialyl-Lewis x and the high affinity ligand PSGL-1 on their surface [171-174]. Activation of neutrophils in the microvasculature via molecules such as IL-8 and PAF result in generation of oxygen free radicals and granular release of

proteolytic enzymes [290]. Furthermore, the expression of selectins following ischaemia/reperfusion may initiate more severe inflammatory responses, with the selective recruitment of Th1 cells into the inflamed area [197].

In summary, we have determined an optimum concentration of 50µg/ml of lec-SOD for incorporation into Marshall's preservation solution, that binds with high affinity to both large vessel and microvascular endothelial cells. Our results suggest that lec-SOD may be effective in protecting endothelial cells against cold hypoxia induced cell death and upregulated adhesion molecule expression following hypoxia/reoxygenation. The reduction in neutrophil adhesion following incubation of endothelial cells with lec-SOD may be mediated partly by the small reduction in E-selectin and ICAM-1 expression, but is likely to involve the expression of other molecules that mediate neutrophil adhesion, chemotaxis and activation. This *in vitro* study supports animal and clinical studies which demonstrate that the effects of ischaemia/reperfusion injury may be attenuated by therapeutic strategies directed against oxygen free radical damage and neutrophil-endothelial cell adhesion molecules in renal transplantation [123, 399, 402, 410, 471]. Direct targeting of lec-SOD to the endothelium of the organ prior to transplantation and reperfusion would be a very attractive strategy but further *in vivo* studies are required before clinical trials can be commenced.

**CHAPTER 7. EFFECTS OF LECITHINISED SUPEROXIDE
DISMUTASE ON ISCHAEMIA/REPERFUSION INJURY IN
EXPERIMENTAL RENAL TRANSPLANTATION**

7.1 INTRODUCTION

Despite progressive improvements in the short-term results of first cadaver renal allografts over the past few decades, the problems of long term graft loss remain unresolved. Although chronic rejection may not be the sole reason for late graft loss, there is evidence to indicate that it is the major cause of late graft failure [60, 430, 513]. Chronic rejection has been defined as the progressive deterioration in graft function occurring over 90 days post-transplantation in association with histopathological diagnosis characterised by interstitial fibrosis, arterial intimal thickening, vascular lesions and tubular atrophy [61-64]. Experimental models of chronic renal allograft rejection have been developed between Fischer F344 and Lewis rat strains where there are minor genetic disparities which result in weak alloimmune responses, leading to the development of long term histological features associated with chronic rejection [514]. Immunohistochemical analysis of renal allografts from this model demonstrated a progressive increase in cytokine and ICAM-1 expression up to 16 weeks post-transplant [515]. Furthermore, retransplantation of renal allografts back into the donor strain resulted in reversibility of immunohistological changes of chronic rejection if performed within 12 weeks of initial transplantation. Nevertheless, pathological changes eventually progressed in the retransplanted kidneys suggesting that alloantigen independent factors may be involved in pathogenesis of chronic rejection [516].

A number of alloantigen-dependent and independent factors have been proposed to contribute to chronic rejection. Ischaemia/reperfusion injury is a putative factor that may play a major role in this process. In renal transplantation, prolonged cold ischaemic storage of kidneys has been shown to be significantly associated with a higher incidence of delayed graft function (DGF) [45, 48-54, 72]. DGF has been shown to have a significant impact on poor long term graft survival, especially in association with acute rejection [53, 54]. There is evidence to suggest that the initial inflammatory events arising from ischaemia/ reperfusion injury may upregulate alloantigens and thus increase the immunogenicity of the graft [161, 340, 499].

Experimental evidence from studies examining exposure of kidneys to periods of warm ischaemia demonstrated upregulated expression of MHC antigens, adhesion molecules and inflammatory cytokines resulting in pathophysiological changes associated with chronic rejection [69, 70, 517]. Furthermore, in renal models of *in situ* cold ischaemia and reperfusion, progressive long term deterioration in renal function was detected in association with upregulated expression of MHC antigens, costimulatory molecules, cytokines, chemokines, adhesion molecules and leucocyte infiltration [393, 395].

The mechanisms involved in the generation of an inflammatory response following ischaemia/reperfusion are complexed, but production of reactive oxygen species may be a critical event. Many studies have shown that ischaemia/reperfusion injury may be significantly attenuated by administration of an oxygen free radical scavenger, superoxide dismutase (SOD) [117-120, 122-126]. Indeed, Land and colleagues demonstrated that administration of SOD at reperfusion significantly reduced the incidence of first acute rejection episodes and improved long term graft survival in cadaver renal allografts [123]. The results from this study suggest that the production of reactive oxygen species during the initial period of transplantation may accelerate subsequent immune mediated events and affect long term graft survival.

In Chapter 4 of this Thesis, prolonged cold ischaemia times were found to be significantly associated with an initial inflammatory response characterised by neutrophil infiltration and platelet deposition in the renal microvasculature following reperfusion. A novel form of SOD (lec-SOD) has been obtained to determine whether it may be potentially effective in limiting the development of ischaemia/reperfusion injury in renal transplantation. Lec-SOD has been shown to be more stable than unmodified rhSOD and bind with higher affinity to HUVEC providing protection against neutrophil damage [474]. In Chapter 6, lec-SOD was shown to bind with high affinity to microvascular and large vessel endothelial cells incubated in Marshall's solution under cold hypoxic conditions, inhibiting hypoxia induced cell death, endothelial adhesion molecule expression and neutrophil adhesion. The aim of this Chapter was to examine immunohistologically the effects of prolonged cold ischaemia in an *in vivo* renal allograft model of chronic rejection, to determine whether or not preservation with lec-SOD ameliorated the initial inflammatory response associated with ischaemia/reperfusion injury and late changes in graft function.

7.2 MATERIALS AND METHODS

7.2.1 Renal Allograft Model

Kidneys were harvested from inbred Fischer F344 rats, perfused with ice-cold Marshall's preservation solution either containing 50µg/ml lec-SOD or without SOD and stored at 4°C for 1 hr or 18 hr.

- Group A: 1 hr cold storage with Marshall's solution alone
- Group B: 1 hr cold storage with Marshall's solution and 50µg/ml lec-SOD
- Group C: 18 hr cold storage with Marshall's solution alone
- Group D: 18 hr cold storage with Marshall's solution and 50µg/ml lec-SOD

| Group | No. of Animals per Time Point Post-Transplant | | |
|-------|---|-------|----------|
| | Day 1 | Day 3 | 24 Weeks |
| A | 4 | 4 | 6 |
| B | 4 | 4 | 6 |
| C | 6 | 5 | 6 |
| D | 6 | 7 | 6 |

Table 7.1 Number of animals examined for each transplant group at Day 1, Day 3 and 24 weeks following transplantation.

After the period of cold ischaemia, kidneys were transplanted into uninephrectomised Lewis rat recipients that received 5mg/kg/day cyclosporine A up to 10 days following transplantation.

Rats were sacrificed 1 day, 3 days and 24 weeks post-transplant and portions of renal allograft snap frozen in liquid nitrogen before storage at -80°C. In animals that were sacrificed at week 24, the remaining native kidney was removed 10 days after transplantation. Renal function was assessed by measurement of proteinuria collected over a 24 hour period at 4-weekly intervals, up to and including 24 weeks. Measurement of proteinuria has been shown to be an accurate and convenient method for determining renal function [393].

7.2.2 Monoclonal Antibodies and Immunohistochemistry

7µm cryosections were obtained from frozen kidney portions and stained with mouse anti-rat monoclonal antibodies using an indirect immunoperoxidase technique described in Section 2.2. The sections were stained with the following monoclonal antibodies:

OX1 (CD45 leucocyte common antigen), IF4 (CD3 T lymphocytes), ED1 (macrophages), HIS48 (granulocytes), F16-4.4 (MHC Class I), OX3 (MHC Class II) and TLD-4C9 (ICAM-1) (see Table 2.2). An anti-dog Thy-1 (F3.20.7 [420]) mouse monoclonal antibody was used as an irrelevant antibody control.

7.2.3 Assessment of Staining

All sections were examined blind to treatment groups. Leucocyte infiltration was quantitated by determining the area occupied by a particular cell type using an established morphometric point counting technique [436]. The percentage area of renal tissue occupied by cells of each antigenic specificity was calculated following counting of 10 random fields (x400 magnification) assessed using a graticuled eyepiece (441 points). % infiltration was calculated from: (number of positive points x 100) / (total number of points counted).

Semi-quantitative grading for expression of ICAM-1, MHC Class I and MHC Class II antigens were assessed by two independent observers without prior knowledge of the experimental details. The semi-quantitative grades for ICAM-1 and MHC Class I were; Grade 1 - occasional weakly positive tubule; Grade 2 - isolated foci of positive tubules; Grade 3 - multiple foci of more extensive positive tubular staining. MHC Class II expression was graded as; Grade 1 - Negative; Grade 2 - foci of weakly positive tubules.

7.2.4 Statistical Analyses

Statistical analyses were performed to determine significant differences between treatment groups for each time point using the Student's T-test and F-test.

7.3 RESULTS

7.3.1 Renal Function

A progressive rise in proteinuria level was detected in renal allografts exposed to 18 hours cold ischaemia (Figure 7.1). In contrast, kidneys preserved for 18 hr with lec-SOD demonstrated relatively stable renal function, with significantly lower proteinuria at 16, 20 and 24 weeks post-transplantation ($p < 0.05$). Furthermore, organ preservation for a minimal 1 hr period in the presence or absence of lec-SOD had no detrimental effects on graft function, with stable proteinuria levels over the 24 week period (Figure 7.1).

7.3.2 Leucocyte Infiltration

Renal allografts were evaluated immunohistologically for the presence of CD45+ leucocytes, CD3+ lymphocytes, ED1+ macrophages and HIS48+ granulocytes at 1 day, 3 days and 24 weeks after transplantation. In kidneys stored for 1 hour, low levels of CD45+ leucocytes were detected following transplantation, irrespective of treatment with lec-SOD. In contrast, the CD45+ leucocyte infiltration in kidneys stored for 18 hr was significantly elevated at day 1 compared with kidneys that had been stored for 1 hr ($3.9 \pm 1.1\%$ vs $2.2 \pm 0.3\%$; $p < 0.01$), but was attenuated by preservation with lec-SOD for 18 hr ($2.7 \pm 0.3\%$; $p = 0.013$) (Figure 7.2). Total CD45+ leucocyte infiltration continued to increase at day 3 in kidneys stored for 18 hr ($6.1 \pm 0.7\%$), but declined by 24 weeks ($4.5 \pm 2.9\%$). Preservation with lec-SOD did not significantly reduce the total CD45+ leucocyte infiltration at day 3 or 24 weeks after transplantation.

Occasional CD3+ T lymphocytes were detected at day 1 or day 3 post-transplant, irrespective of the period of cold storage or preservation with lec-SOD (Figure 7.3). However, by 24 weeks post-transplant, a significant increase in CD3+ T lymphocytes was detected in kidneys stored for 18 hr ($1.4 \pm 1.0\%$) compared with T cells present at

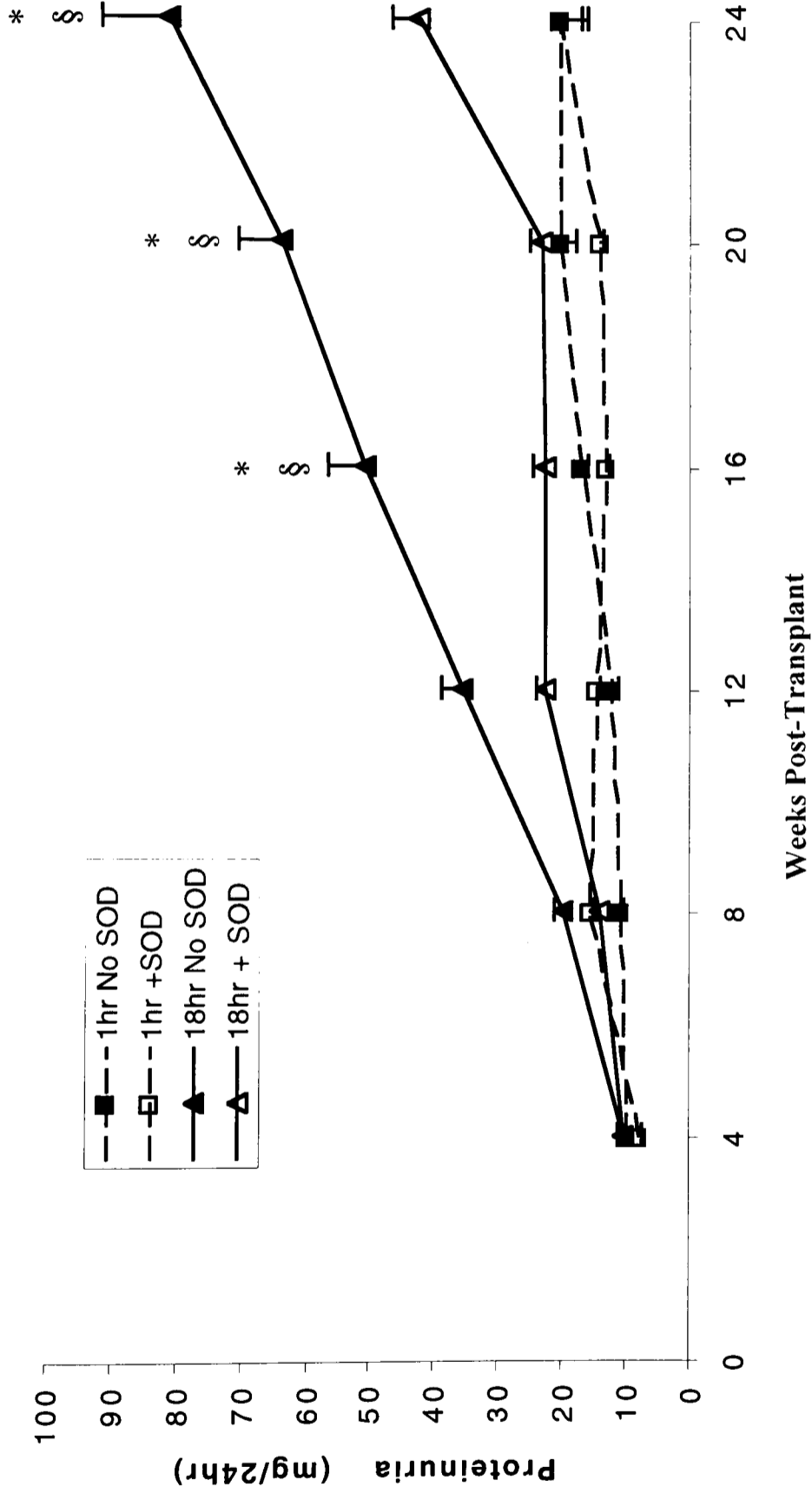


Figure 7.1 Effect of cold ischaemia and lec-SOD on renal function.

Progressive increase in proteinuria was observed in renal allografts with 18 hours cold ischaemic storage. A significant increase in proteinuria was detected at 16, 20 and 24 weeks post-transplant in renal allografts stored for 18 hours compared with kidneys that were stored for only 1 hour ($p<0.05$). Impaired renal function following 18 hours cold storage was significantly abrogated by preservation with lec-SOD beyond 16 weeks post- transplantation ($p<0.05$). Each time point represents the mean \pm SEM for 6 animals.

* ($p<0.05$) 18 hr CIT vs 1 hr CIT

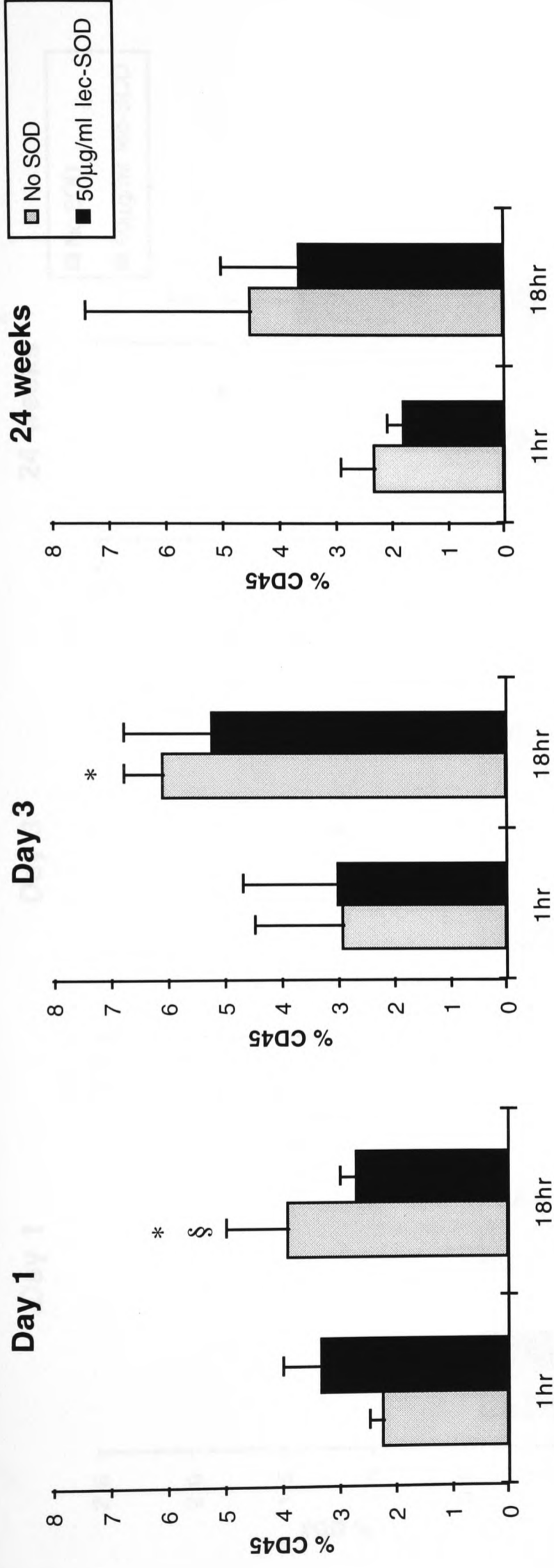
§ ($p<0.05$) 18 hr CIT No SOD vs 18 hr CIT + lec-SOD

day 1 ($0.4 \pm 0.2\%$; $p < 0.01$) and day 3 ($0.3 \pm 0.1\%$; $p < 0.01$) (Figure 7.3). The rise in T lymphocytes at 24 weeks was not inhibited by renal preservation with lec-SOD.

A significant increase in the percentage of ED1+ macrophages was evident in kidneys stored for 18 hours ($2.3 \pm 0.5\%$) compared with kidneys with minimal 1 hr cold storage ($1.0 \pm 0.1\%$; $p < 0.01$) at day 1 post-transplantation (Figure 7.4). Furthermore, at 3 days post-transplant, an additional increase in the percentage of macrophages was detected ($4.1 \pm 1.5\%$), but this decreased after 24 weeks ($1.2 \pm 0.3\%$) in kidneys stored for 18 hr. Although preservation with lec-SOD for 18 hours appeared to inhibit the level of ED1+ macrophage infiltration at day 1 and day 3, this did not attain statistical significance (Figure 7.4). Interestingly, the pattern of ED1+ macrophage infiltration observed at all time points was similar to that detected for total CD45+ leucocyte infiltration.

The pattern of HIS48+ granulocyte infiltration was similar to that observed for ED1+ macrophages and total CD45+ leucocytes, suggesting that neutrophils and macrophages form the major cellular component of infiltration. Significantly higher levels of HIS48+ granulocytes were present in kidneys stored for 18 hr at day 1 ($2.8 \pm 0.4\%$), remaining at elevated levels day 3 post-transplant ($2.7 \pm 0.9\%$) compared with renal allografts stored for only 1 hr ($0.8 \pm 0.6\%$; $p < 0.01$) (Figure 7.5). The increase in granulocyte infiltration at day 1, but not day 3, was significantly attenuated by 18 hr preservation with lec-SOD ($1.9 \pm 0.7\%$; $p = 0.017$).

The results from this study suggest that following 18 hr cold ischaemia, an initial inflammatory response mediated by macrophages and neutrophils was evident within the first few days post-transplantation, but after 24 weeks, T lymphocytes were the predominant leucocyte population.



Cold Ischaemia Time (hours)

Figure 7.2 CD45+ leucocyte infiltration in renal allografts exposed to short and long periods of cold ischaemia.

Total CD45+ leucocytes were assessed by morphometric point counting following indirect immunoperoxidase staining of kidneys harvested 1 day, 3 days and 24 weeks post-transplant. A constant low level of CD45+ leucocytes were observed in kidneys stored for 1 hr, at all time points sampled. In contrast, significant leucocyte infiltration was detected in kidneys stored for 18 hr after day 1 and day 3 ($p < 0.01$). Preservation with lec-SOD for 18 hr significantly attenuated leucocyte infiltration at day 1 post-transplant ($p = 0.013$). Each time point is representative of the mean \pm SD for 4-7 animals.

* $p < 0.01$ versus preservation for 1hr with no SOD at the same time point

§ $p = 0.013$ versus 18 hr preservation with lec-SOD at the same time point

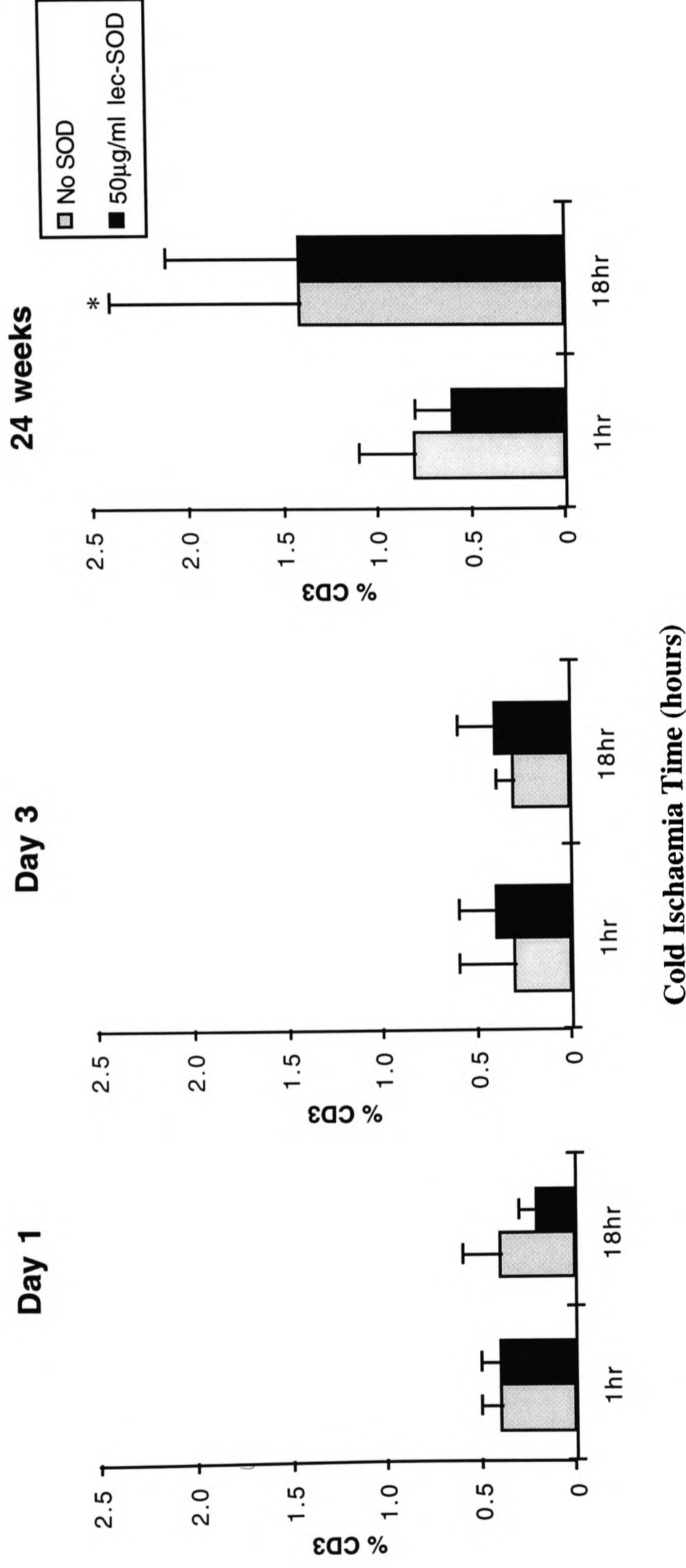


Figure 7.3 CD3+ T lymphocyte infiltration in renal allografts exposed to short and long periods of cold ischaemia.

CD3+ lymphocytes were assessed by morphometric point counting following indirect immunoperoxidase staining of kidneys harvested 1 day, 3 days and 24 weeks post-transplant. Only occasional T cells were detected at day 1 or day 3 post-transplant irrespective of the cold ischaemia time or treatment with lec-SOD. A significantly higher number of T lymphocytes were detected 24 weeks post-transplant in kidneys stored for 18 hr that was not prevented by preservation with lec-SOD ($p < 0.01$). Each time point is representative of the mean \pm SD for 4-7 animals.

* $p < 0.01$ versus preservation for 18 hr with no SOD at day 1 and day 3.

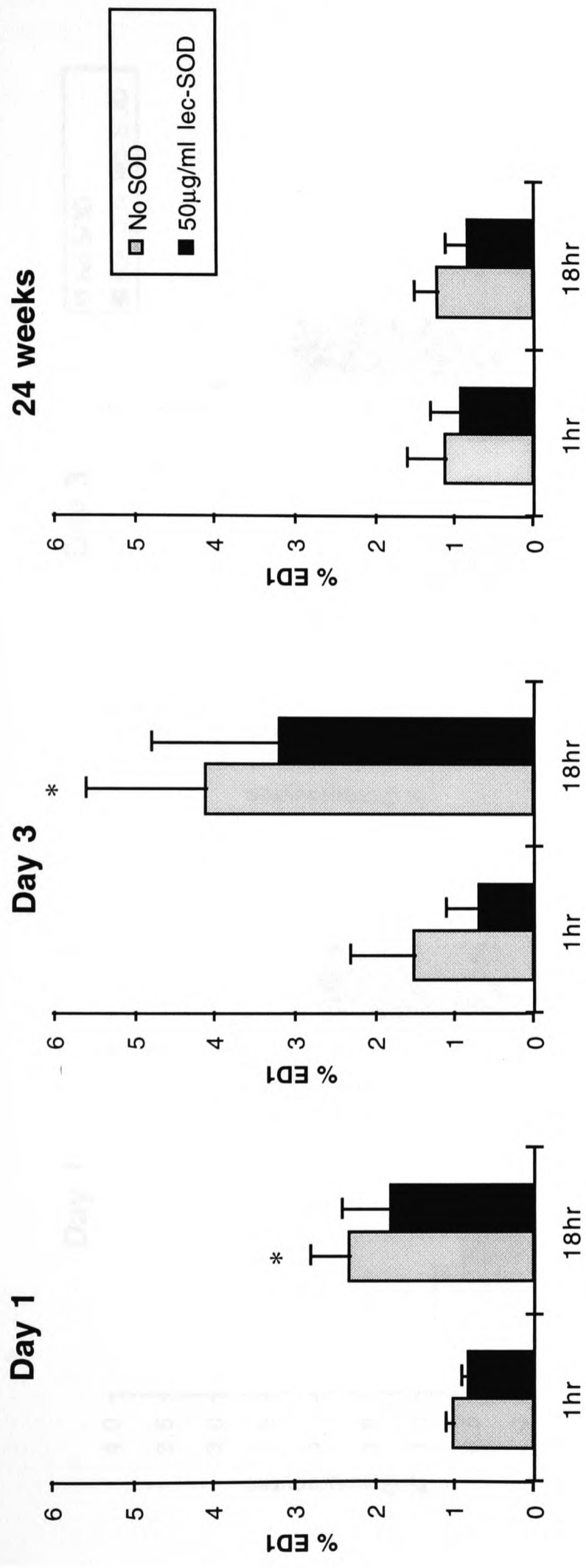


Figure 7.4 ED1+ macrophages in renal allografts exposed to short and long periods of cold ischaemia.

ED1+ macrophages were assessed by morphometric point counting following indirect immunoperoxidase staining of kidneys harvested 1 day, 3 days and 24 weeks post-transplant. A significant increase in ED1+ macrophages was detected in kidneys stored for 18 hr at day 1 post-transplant, with maximal infiltration observed at day 3 (p<0.01). Preservation with lec-SOD did not significantly inhibit ED1+ macrophage infiltration. The presence of ED1+ macrophages returned to baseline levels by 24 weeks post-transplant. Each time point is representative of the mean ± SD for 4-7 animals.

* p<0.01 versus preservation for 1hr with no SOD at the same time point

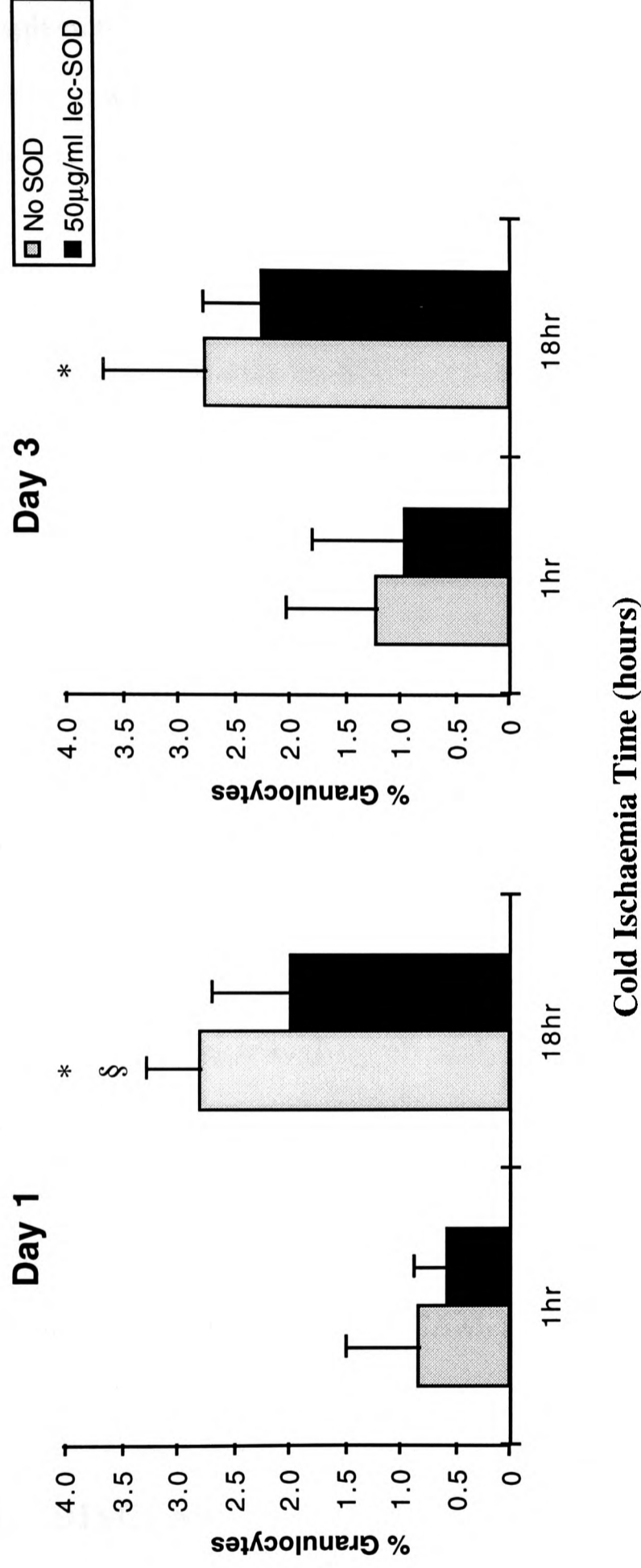


Figure 7.5 HIS48+ granulocytes in renal allografts exposed to short and long periods of cold ischaemia.

Granulocytes were assessed by morphometric point counting following indirect immunoperoxidase staining of kidneys harvested 1 day, 3 days and 24 weeks post-transplant. A significant increase in granulocytes was detected in kidneys stored for 18 hr at day 1, remaining at high levels day 3 post-transplant ($p < 0.01$). Preservation with lec-SOD for 18 hr significantly inhibited granulocyte infiltration 1 day after transplantation ($p = 0.017$). Each time point is representative of the mean \pm SD for 4-7 animals.

* $p < 0.01$ versus preservation for 1hr with no SOD at the same time point

§ $p = 0.017$ versus preservation for 18 hr with lec-SOD at the same time point

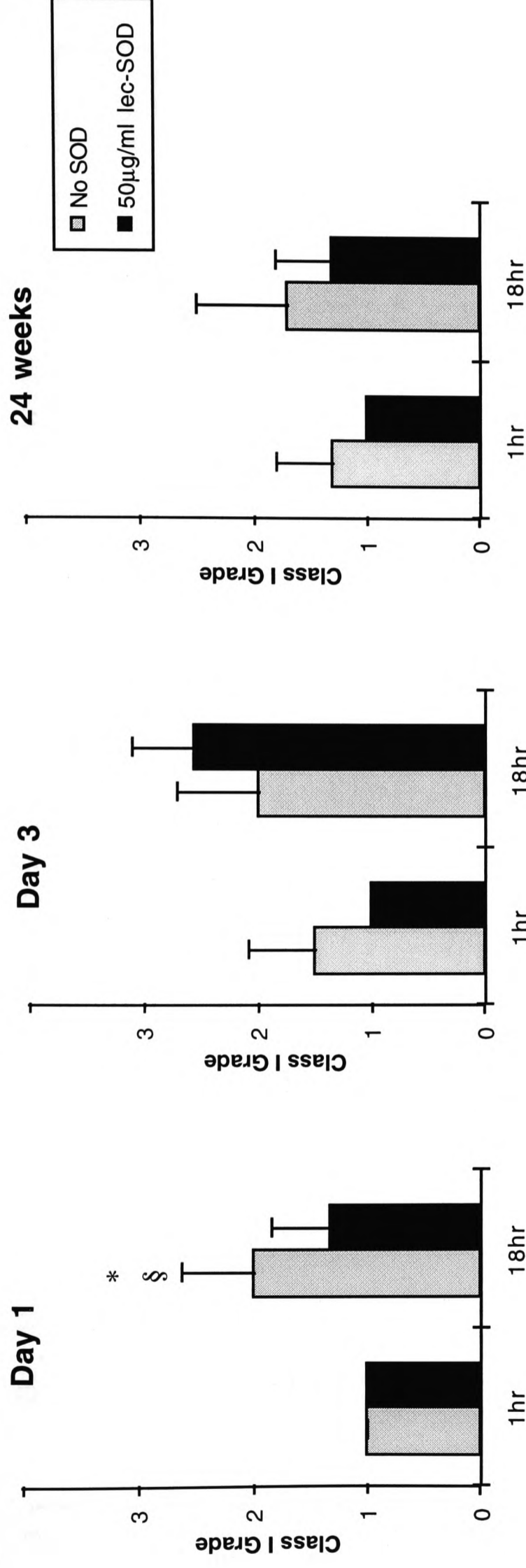
7.3.3 Antigen Induction

Expression of MHC Class I, Class II and ICAM-1 were assessed semi-quantitatively to determine the level of antigen induction at day 1, day 3 and 24 weeks post-transplantation. In renal allografts stored for 1 hour, no increase in MHC Class I expression was detected over the 24 week period, irrespective of preservation with lec-SOD (Figure 7.6). In contrast, kidneys stored for 18 hr had significantly upregulated MHC Class I expression by day 1 (Grade 2.0 ± 0.6) remaining elevated at day 3 (Grade 2.0 ± 0.7) and declining gradually by 24 weeks post-transplant (Grade 1.7 ± 0.8). Preservation with lec-SOD significantly attenuated induction of MHC Class I antigens at day 1 post-transplant (Grade 1.3 ± 0.5 ; $p=0.037$) (Figure 7.6). Expression of MHC Class II antigens were not upregulated by storage for 18 hr, although a low level of tubular Class II antigens were detected in all renal allografts 24 weeks post-transplant (Figure 7.7).

In kidneys stored for 18 hr, low levels of ICAM-1 expression were detected at day 1 post-transplant (1.5 ± 0.6), increasing significantly by day 3 (2.6 ± 0.6 ; $p<0.01$), with high levels remaining at 24 weeks (2.5 ± 0.6 ; $p<0.01$) (Figure 7.8). In contrast, kidneys stored for 1hr demonstrated a marginal increase in ICAM-1 expression at day 3 (1.5 ± 0.6), but increased significantly by 24 weeks post-transplant compared with levels detected at day 1 (1.8 ± 0.4 vs 1.0 ± 0 ; $p<0.01$). Nevertheless, increased ICAM-1 expression in kidneys stored for 18 hr was significantly greater than kidneys stored for 1 hr at day 3 and 24 weeks ($p<0.01$) (Figure 7.8). Preservation of kidneys with lec-SOD did not inhibit induction of ICAM-1 expression.

7.4 DISCUSSION

There is mounting evidence to suggest that ischaemia/reperfusion injury may play a critical role in the pathogenesis of chronic allograft rejection. In clinical renal



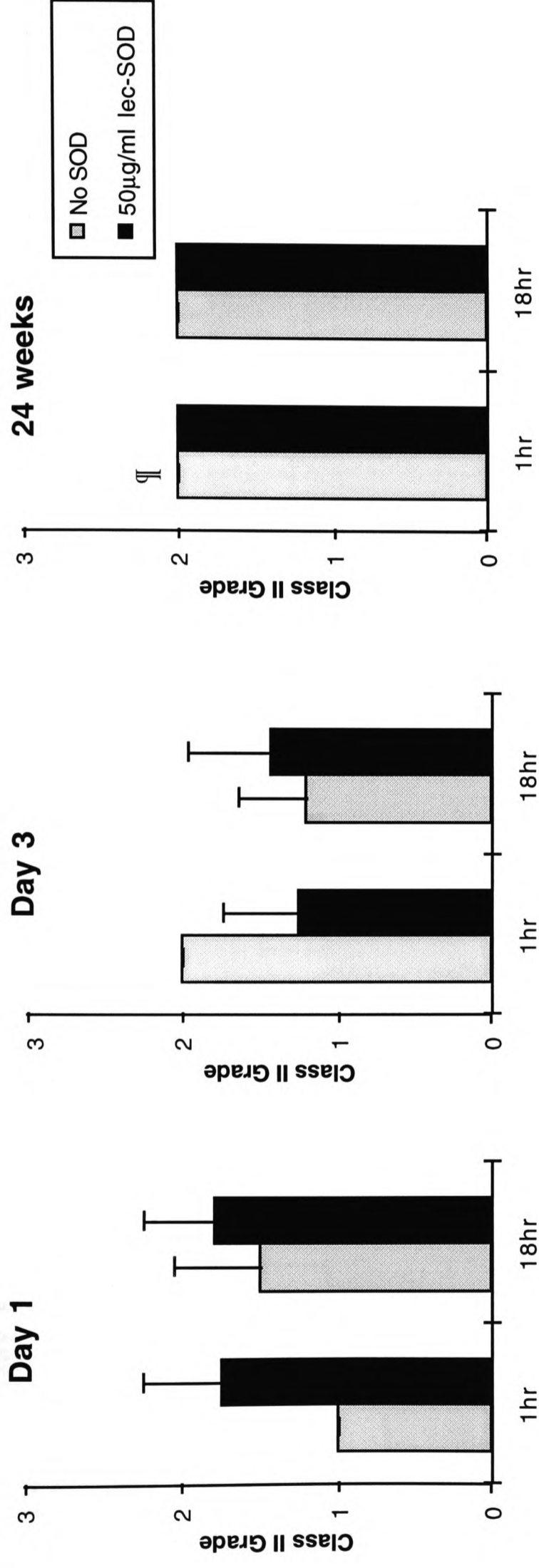
Cold Ischaemia Time (hours)

Figure 7.6 Expression of MHC Class I in renal allografts exposed to short and long periods of cold ischaemia.

MHC Class I expression was assessed semi-quantitatively following indirect immunoperoxidase staining of kidneys harvested 1 day, 3 days and 24 weeks post-transplant. A significant increase in Class I expression was detected in kidneys stored for 18 hr at day 1 ($p < 0.01$), that was significantly attenuated after preservation with lec-SOD for 18 hr ($p = 0.037$). Each time point is representative of the mean \pm SD for 4-7 animals.

* $p < 0.01$ versus preservation for 1hr with no SOD at the same time point

§ $p = 0.037$ versus preservation for 18 hr with lec-SOD at the same time point



Cold Ischaemia Time (hours)

Figure 7.7 Expression of MHC Class II in renal allografts exposed to short and long periods of cold ischaemia.

MHC Class II expression was assessed semi-quantitatively following indirect immunoperoxidase staining of kidneys harvested 1 day, 3 days and 24 weeks post-transplant. No significant increase in Class II expression was detected in kidneys stored for 18 hr compared with kidneys stored for 1 hr. However, all renal allografts at 24 weeks post-transplant expressed weakly elevated levels of MHC Class II antigens, irrespective of cold storage time. Each time point is representative of the mean \pm SD for 4-7 animals.

‡ p<0.01 versus preservation for 1hr with no SOD at day 1

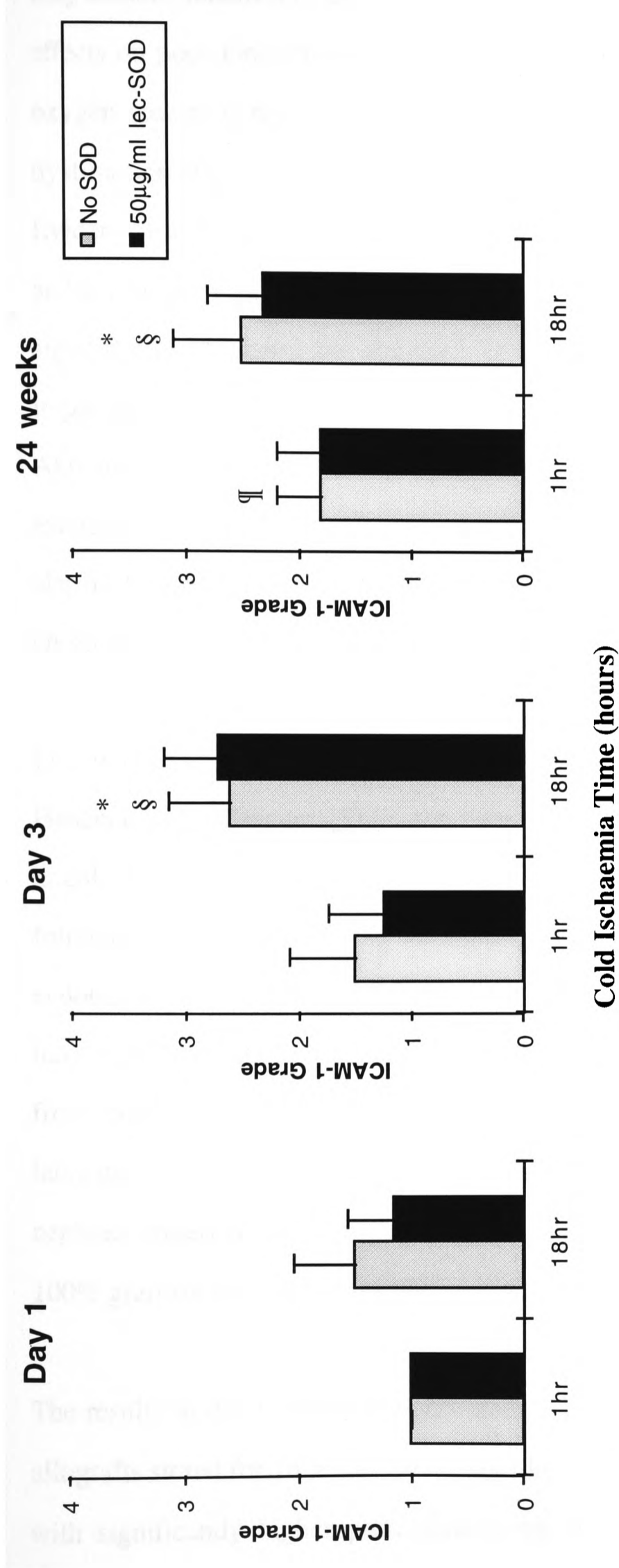


Figure 7.8 Expression of ICAM-1 in renal allografts exposed to short and long periods of cold ischaemia.

Proximal tubular ICAM-1 expression was assessed semi-quantitatively following indirect immunoperoxidase staining of kidneys harvested 1 day, 3 days and 24 weeks post-transplant. A significant increase in ICAM-1 expression was detected at day 3 post-transplant that remained elevated after 24 weeks in kidneys stored for 18 hr ($p < 0.01$). Furthermore, induction of ICAM-1 in kidneys stored for 18 hr was greater than those stored for 1 hr at day 3 and 24 weeks post-transplant ($p < 0.01$). In addition, a significant increase in ICAM-1 expression was detected in kidneys stored for 1 hr after 24 weeks post-transplant ($p < 0.01$). Induction of ICAM-1 expression was not inhibited by preservation with lec-SOD. Each time point is representative of the mean \pm SD for 4-7 animals.

* $p < 0.01$ versus preservation for 1hr with no SOD at the same time point

§ $p < 0.01$ versus preservation for 18hr with no SOD at day 1

¶ $p < 0.01$ versus preservation for 1hr with no SOD at day 1

transplantation, the effects of prolonged cold ischaemic storage of cadaveric kidneys may become manifest as delayed graft function following transplantation, with resulting effects on poor long term graft survival [49, 50, 53, 54]. The production of reactive oxygen species at reperfusion may be a primary event in the development of allograft dysfunction, but in addition, may significantly influence chronic allograft dysfunction. Evidence for this hypothesis has been provided from a clinical trial performed by Land and colleagues who demonstrated that intravenous administration of SOD at reperfusion significantly attenuated the incidence of first acute rejection episodes, in addition to improving long term graft survival up to 10 years post-transplant [123, 161, 499]. Although the beneficial effects of treatment with SOD were demonstrated clinically, examination of the intragraft events by biopsy analysis were not performed to enable clarification of the underlying mechanisms involved in limiting the progression of chronic graft loss.

In this current study, a rat renal allograft model for chronic rejection developed by Hancock and colleagues [515], has been adapted to examine the additive effects of 18 hr cold ischaemic storage on renal allograft function and on immunohistological events following transplantation. In addition, the effects of preservation with a potent, endothelial-specific free radical scavenger (lec-SOD) on ischaemia/reperfusion injury have been assessed in this model. A cold preservation time of 18 hr has been derived from experiments in a syngeneic Lewis rat renal transplant model performed in our laboratory, whereby delayed graft function was demonstrated in bilaterally nephrectomised recipients up to day 3 post-transplant, with recovering function and 100% graft survival after 2 weeks.

The results in this Chapter demonstrated progressive deterioration in renal function in allografts stored for 18 hr compared with kidneys stored for a minimal period of 1 hr, with significantly higher proteinuria levels by 16 weeks post-transplant, rising to maximal levels at 24 weeks. Preservation of kidneys with lec-SOD for 18 hr

significantly attenuated the rise in proteinuria levels at 16, 20 and 24 weeks post-transplant. The progressive elevation in proteinuria arising from cold ischaemic damage is indicative of chronic graft dysfunction and the finding is supported by evidence from a Lewis rat model of *in situ* cold ischaemia, where proteinuria also developed 16 weeks after the initial ischaemia/reperfusion injury [393, 395]. In contrast to the study performed by Hancock and colleagues, deteriorating long term graft function was not observed in the renal allografts with minimal periods of ischaemic storage, in the time period studied. Hancock and colleagues demonstrated that transplantation of kidneys with minimal ischaemic damage from Fischer to Lewis rats (differing only at weak histocompatibility loci) resulted in deteriorating renal function and histopathological changes between 12 to 16 weeks post-transplant similar to those associated with clinical chronic rejection, in addition to an early neutrophil infiltration with the presence of large numbers of macrophage and T cells at 16 weeks [515]. The lack of a significant increase in proteinuria in the chronic renal allograft model with minimal ischaemia employed in this Chapter, may be attributable to the brief period of preservation of kidneys under optimal conditions with Marshall's preservation solution. In contrast, in the study performed by Hancock and colleagues, sufficient preservation may not have been achieved between donor harvesting and transplantation, resulting in a period of warm ischaemia during the transplantation procedure.

To determine the possible factors that may be involved in cold ischaemia/reperfusion induced chronic renal dysfunction, an immunohistochemical analysis was performed to investigate the level of leucocyte infiltration and antigen induction in renal allografts with prolonged cold ischaemia. The results from this Chapter demonstrated a significant impact of 18 hr cold ischaemia on immunohistological events both in the short and long term following transplantation, over any additional weak alloimmune responses in this model. At day 1 post-transplantation, a significant leucocyte infiltration of neutrophils and macrophages were detected, with induction of MHC Class I antigens in renal allografts stored for 18 hr compared with kidneys exposed to only 1 hr cold ischaemia.

Only occasional CD3+ T lymphocytes and minimal expression of ICAM-1 and MHC Class II antigens were detected at day 1. Neutrophil and macrophage infiltration remained elevated at day 3 post-transplant, with significantly increased expression of ICAM-1 in kidneys stored for 18 hr.

The neutrophil infiltration detected at day 1 and day 3, is characteristic of an early inflammatory response generated as a result of oxygen free radical damage following ischaemia/reperfusion [141, 292-297]. Reactive oxygen species have been shown to be generated by endothelial cells following hypoxia/reoxygenation [109, 110], resulting in increased expression of adhesion molecules, facilitating binding and activation of neutrophils [309-311, 456-458, 466]. Experimental models of ischaemia and reperfusion have demonstrated that organ function was markedly improved and neutrophil infiltration significantly inhibited following treatment with exogenously administered SOD or transfection with human SOD cDNA [117-120, 122-126, 410, 412].

The results in this current study demonstrated that preservation of kidneys with lec-SOD under similar physiological conditions as that experienced in the clinical setting, significantly inhibited the infiltration of neutrophils at day 1 post-transplant, presumably through its localised free radical scavenging activity at the endothelial surface. These results provide corroborative experimental evidence to support the results obtained in Chapter 4, where an increase in neutrophils was found 1 hr post-reperfusion in cadaver renal allografts with prolonged cold ischaemia times. Taken together, these results suggest that preservation of organs with lec-SOD incorporated into Marshall's solution, may be an attractive method for the prevention of reperfusion injury in clinical renal transplantation.

In addition, the induction of MHC Class I antigens at day 1 post-transplantation was also inhibited by preservation with lec-SOD. Induction of MHC Class I antigens on

renal tubules has been demonstrated in a murine model of renal warm ischaemia in association with acute tubular necrosis [69, 70]. MHC antigens play a major role in alloimmune responses, thus upregulated expression may increase the immunogenicity of the graft. Therefore, the correlation between ischaemia induced delayed graft function and acute rejection in clinical renal transplantation, may be partially explained by the early induction of MHC antigens in the kidney. The beneficial effects of preservation with lec-SOD on MHC Class I antigen expression in this study and results from the SOD trial performed by Land and colleagues, who demonstrated a reduction in first acute rejection episodes in cadaver renal allografts treated with SOD at reperfusion [123], suggests that induction of MHC antigens and generation of an alloimmune response may result from deleterious pathways initiated by the generation of reactive oxygen species at reperfusion.

A marginal increase in ICAM-1 expression was detected at day 1 post-transplant, but significant upregulation of ICAM-1 expression was detected 3 days after transplantation in kidneys stored for 18 hr. These results are similar to those obtained by Dragun and colleagues who demonstrated that ICAM-1 expression was upregulated 24 hours after autotransplantation of Lewis rat kidneys exposed to 30 min cold and 60 min warm ischaemia [399]. Induction of ICAM-1 expression and neutrophil infiltration were significantly inhibited by intravenous administration of ICAM-1 antisense oligodesoxynucleotides. In clinical renal transplantation, a Phase I trial has demonstrated a protective effect of an anti-ICAM-1 monoclonal antibody against DGF and rejection [402]. Nevertheless a larger, randomised multicentre study using anti-ICAM-1 mAb therapy demonstrated no beneficial effects on DGF or acute rejection [403]. In this current study, ICAM-1 induction was not significantly inhibited by preservation with lec-SOD.

Takada and colleagues demonstrated similar results with elevated ICAM-1 mRNA levels following 45 min of *in situ* cold ischaemia and reperfusion, in addition to

neutrophil infiltration and elevated E-selectin mRNA levels observed after 6 hours. Increased MHC Class II expression, infiltration of ED1+ macrophages and CD4+ T cells in association with cytokine gene expression were detected up to 5 days post-transplantation [392, 394]. Furthermore, this initial inflammatory response was ameliorated by administration of either sPSGL or CTLA4Ig, strongly suggesting that ischaemia/reperfusion injury elicited a mononuclear cell-mediated immunological response in the absence of alloantigens. Although the studies performed by Takada and colleagues demonstrated the potentially harmful effects of *in situ* cold ischaemia/reperfusion injury, it would be more pertinent to examine the effects of cold ischaemic preservation and transplantation, to mimic the events encountered in clinical renal transplantation. Recently, a rat renal allograft model of acute rejection was analysed following 45 min cold ischaemia, where treatment with sPSGL in combination with low-dose cyclosporine, significantly inhibited infiltration of T-lymphocytes and ED1+ macrophages, with absence of elevated proteinuria, and protection against development of chronic rejection [518].

Early infiltration of ED1+ macrophages was evident at day 1 and day 3 post-transplant in renal allografts stored for 18 hr in the present study. Although preservation with lec-SOD reduced the number of macrophages present during this period, this did not attain statistical significance. The apparent difference between preservation with lec-SOD in the current study and treatment with sPSGL or CTLA4Ig in the studies performed by Takada and colleagues, probably reflects the inhibition of different stages towards the progression of ischaemia/reperfusion injury. Preservation with lec-SOD may prevent endothelial damage via scavenging of the initial burst of superoxide anion production, whereas administration of sPSGL or CTLA4Ig may prevent endothelial- and leucocyte-leucocyte interactions that may occur at a later stage in the process.

In addition to the analysis of immunohistological changes in the short term, examination of intragraft events was performed at 24 weeks. By 24 weeks post-transplant, MHC

Class I expression and the number of macrophages had returned to baseline levels, whereas high levels of ICAM-1 were detected in addition to a significant increase in CD3+ T cell infiltration in kidneys exposed to 18 hr cold storage. Although all the kidneys studied demonstrated increased MHC Class II antigen expression at 24 weeks post-transplant, the intensity and extent of tubular Class II expression was low. It was surprising that the presence of macrophages after 24 weeks was diminished in kidneys stored for 18 hr, as other studies of renal ischaemia have demonstrated a marked increase in macrophages at similar time points after the initial ischaemia/ reperfusion injury [393, 400]. Nevertheless, upregulated expression of MHC Class II antigens and ICAM-1, with significant T lymphocyte infiltration was also evident in these studies, in association with histopathological and functional changes associated with chronic rejection. Furthermore, inhibition of adhesion molecule interactions via antisense therapy or treatment with CTLA4Ig significantly attenuated these immunological events [393, 395, 400]. The induction of ICAM-1 in association with mononuclear cell infiltration in kidneys following an initial damaging ischaemia/ reperfusion injury event, would significantly accentuate alloimmune responses in the context of transplantation.

In summary, the results from this study strongly suggest that prolonged cold ischaemia has an additional adverse effect on chronic rejection, significantly inducing an initial inflammatory response mediated by neutrophils and macrophages, with subsequent elevation in long term expression of ICAM-1, in association with a significant T cell infiltration. Preservation with lec-SOD for 18 hr significantly inhibited neutrophil infiltration and MHC Class I expression at day 1, with significant improvements in renal function after 16 weeks post-transplant, but marked protection was not observed on chronic immunological events. The absence of an effect by preservation with lec-SOD on long term immunohistological changes, despite beneficial effects on graft function, may reflect the complexed pathogenesis of chronic rejection. Lec-SOD may be a suitable agent for protection of renal allografts against initial ischaemia/reperfusion injury with potentially beneficial effects on graft function, but additional treatment

directed against leucocyte adhesion may be required for the prevention of chronic rejection.

CHAPTER 8. SUMMARY AND DISCUSSION

There is mounting evidence from both single centre and national registry database studies to demonstrate that renal allografts from both living-related (LRD) and living-unrelated (LURD) donors have superior rates of long term graft survival compared with cadaveric renal transplantation [1-8]. Although the genetic relationship between donor and recipient of cadaveric and LURD renal allografts is similar, graft survival of LURD transplants is significantly better both in the short and long term, even compared with better HLA-matched cadaver renal allografts [7]. These results indicate that physiological and non-specific inflammatory events may be critically involved in the process of graft failure.

Two key physiological factors distinguish LURD transplantation from cadaveric renal allografts. Cadaver donors may experience a number of traumatic physiological events before transplantation as a result of physical injury to the donor, treatment in intensive care and severe haemodynamic instability and hormonal imbalances resulting from brain death. In contrast, LURD kidneys are procured under optimal conditions from carefully screened, healthy individuals and thus do not undergo pre-transplant damage. In addition, cadaveric kidneys experience longer periods of cold ischaemic storage. Evidence that cold ischaemia may affect graft outcome can be derived from studies that demonstrate cold ischaemia to be the most significant factor influencing the incidence of DGF in cadaver renal transplantation [45, 48-54, 72]. The fact that living donor renal allografts with minimal ischaemia times have significantly lower incidences of DGF lend further support to these observations. Furthermore, long term graft survival is significantly impaired in cadaver renal allografts with DGF, independently of acute rejection, but when allografts experience both DGF and acute rejection, a synergistic

adverse effect on graft survival is observed [53, 54]. It is likely that the mechanisms involved in ischaemia/ reperfusion injury, DGF and acute rejection may be inter-related, and that progression towards chronic graft dysfunction involves a combination of these events.

The specific factors involved in traumatic injury to the donor organ and cold ischaemic damage leading to the inferior outcome of cadaver renal allografts remain to be resolved. In this Thesis investigations into the early pathophysiological events associated with cadaver renal transplantation have been performed to determine whether induction of antigen expression is observed in kidneys before transplantation, and to analyse the effects of cold ischaemia/reperfusion injury on renal allograft outcome.

In Chapter 3, an immunohistochemical analysis demonstrated that upregulation of adhesion molecules and MHC Class II antigens in cadaver donor kidneys before transplantation may play a significant role in the pathogenesis of cadaver renal allografts. The results demonstrated that high levels of E-selectin, ICAM-1, VCAM-1 and MHC Class II antigens were detected in approximately half of the 65 cadaver donor kidneys examined, whereas expression of these antigens was absent in all 29 living donor kidneys. Furthermore, the expression of these molecules remained elevated 1 hr after reperfusion of the graft as would be expected, and with no further increase in expression following reperfusion due to insufficient time for gene transcription and protein expression.

The upregulated adhesion molecule expression in cadaver donor kidneys provides a mechanism for the recruitment of leucocytes into the graft following transplantation. Furthermore, induction of donor MHC Class II antigens may increase the immunogenicity of the kidney and enhance host alloimmune responses. It is possible that the development of rejection in kidneys with upregulated donor MHC Class II antigen expression may be enhanced in HLA-DR mismatched allografts. Indeed, in all

11 cadaver renal allografts with acute rejection diagnosed within 7 days of transplantation, elevated tubular expression of adhesion molecules and MHC Class II antigens were detected in the donor kidney before transplantation, with 8/11 having ≥ 1 mismatch for HLA-DR.

To determine the donor factors that may be involved in the induction of adhesion molecule and MHC class II antigen expression, a statistical analysis was performed on donor parameters, clinical events in intensive care and drug administration. Administration of desmopressin used for the regulation of excessive diuresis, was significantly associated with upregulated E-selectin expression on the intertubular capillaries. Induction of tubular adhesion molecule and MHC Class II antigen expression was significantly associated with donors who had experienced severe physical injury, episodes of infection and prolonged ventilator support. It is possible that these traumatic events in the donor may elicit systemic cytokine release that result in activation within peripheral organs. In addition, clinical and experimental studies have demonstrated that following brain death, normal regulation of vital functions are lost with haemodynamic instability and periods of ischaemia in the organ [25-28], that may result in histopathological changes and activation of inflammatory mediators [24, 454, 455]. Furthermore, induction of adhesion molecules and MHC antigens have been demonstrated in animal models of renal warm ischaemia and reperfusion [69, 70]. Thus, it is possible that the induction of these molecules in cadaver donor kidneys may have resulted from episodes of warm ischaemia in the donor prior to organ retrieval.

The results presented in Chapter 3 demonstrated that the induction of inflammatory molecules in cadaver donor kidneys was significantly associated with pathophysiological events in the donor before and during treatment in ICU, presumably through the systemic release of cytokines or localised ischaemic damage to the organs *in situ*. It is evident that careful management of cadaver donors in ICU is critical for preserving the integrity of organs for transplantation. Development of therapeutic

strategies to prevent the induction of adhesion molecules and MHC antigens may limit subsequent attack by the host immune system following transplantation. A large, retrospective study is currently being undertaken to assess the effects of donor parameters on the expression of adhesion molecules, cytokines, chemokines and MHC class II antigens in cadaver donor kidneys compared with living donor kidneys. Multivariate analyses will be used to determine the effects of both donor parameters and antigen expression on post-transplant outcome.

In Chapter 4 further immunohistochemical analysis was performed on cadaver and LRD renal allograft biopsies obtained immediately after reperfusion to determine the effects of donor parameters and cold ischaemia times on cellular infiltration and adhesion molecule expression. In cadaver kidneys that had experienced prolonged periods of cold ischaemic storage, a significant neutrophil infiltration of recipient origin was detected within the renal microvasculature, predominantly in the glomeruli, that was absent in all LRD renal allografts. Moreover, neutrophil infiltration in the glomeruli was significantly associated with DGF and impaired graft function at 3 and 6 months post-transplantation. In addition, deposition of activated platelets expressing P-selectin were detected on the glomerular and intertubular capillaries of certain cadaver renal allografts following reperfusion, that were also significantly associated with poor graft function at 3 and 6 months post-transplant. These results suggest that prolonged cold ischaemia of cadaver renal allografts may generate an inflammatory response that is characteristic of ischaemia/reperfusion injury, influencing short and long-term graft function.

A possible mechanism by which neutrophils mediate impaired graft function following ischaemia/reperfusion injury, may be through glomerular damage resulting from the release of proteolytic enzymes and reactive oxygen species by activated neutrophils infiltrating the glomeruli. Substitution of damaged glomerular tissue with fibrous tissue as part of the repair process, may result in reduction of total glomerular filtration

efficiency. Furthermore, the deposition of platelets in the microvasculature of the allograft following reperfusion, may lead to platelet-neutrophil interactions and the formation of cellular aggregates, resulting in capillary plugging and progression of the no reflow phenomenon [160, 499]. This in turn may further exacerbate cold ischaemic damage, causing global ischaemia of the allograft at reperfusion, and thus, contribute to impaired graft function through the development of acute tubular necrosis, a histological indicator of DGF. Damage to glomeruli and tubules before transplantation and immediately after reperfusion might be expected to reduce the functioning nephron mass of the kidney, a factor that has been implicated in the inferior outcome of renal allografts from elderly and size-mismatched donors [15, 50, 53-55, 57, 71].

It has been proposed that the accumulation of toxic products during the period of cold ischaemia may have a detrimental effect on ischaemia/reperfusion injury and subsequent graft function [377, 378]. A clinical trial presented in Chapter 5 demonstrated that simple reflusing of cadaver kidneys after the period of cold storage, but before transplantation, did not inhibit the infiltration of neutrophils or platelets, and had no significant impact on the incidence of DGF. These results, and those from a similar study [379], suggest that ischaemia/reperfusion injury does not result merely from substances accumulated in the vessel lumen during cold storage.

Many studies have demonstrated that the generation of reactive oxygen species following reperfusion of ischaemic tissue plays a major role in the pathogenesis of reperfusion injury [75, 141, 340, 499]. Therefore, a novel form of superoxide dismutase, lec-SOD, was obtained to evaluate its potential efficacy for clinical transplantation. An *in vitro* endothelial cell model of ischaemia/reperfusion was developed in Chapter 6 which demonstrated that lec-SOD bound with high affinity to the surface of macrovascular and microvascular endothelium under cold hypoxic conditions, inhibiting hypoxia-induced cell death, adhesion molecule induction and neutrophil adhesion. The results from this study and those published by Ichikawa and

colleagues [458], demonstrate that endothelial activation and injury resulting from hypoxia/reoxygenation may be ameliorated by treatment with oxygen free radical scavengers.

Although the inflammatory events associated with ischaemia/reperfusion injury may take place within a short time frame, there is accumulating evidence to suggest that these initial events contribute to chronic allograft dysfunction. Many studies have demonstrated that DGF resulting from prolonged cold ischaemia, may significantly diminish long term renal allograft survival [49, 50, 53, 54]. Furthermore, inhibition of the initial burst of oxygen free radical production at reperfusion by a single intravenous dose of SOD, has been shown to significantly improve long term renal allograft survival [123, 161, 499].

To investigate whether preservation of kidneys with lec-SOD would be effective in limiting the effects of reperfusion injury following transplantation, a rat renal allograft model of chronic rejection was examined in Chapter 7. Fischer donor rat kidneys were transplanted into Lewis recipient rats generating a weak alloreactive immune response that was suppressed by low dose cyclosporine in the initial 10 day post-operative period. To determine the additional impact of cold ischaemia on chronic renal allograft dysfunction, donor kidneys were subjected to 18 hr cold ischaemic storage either in the presence or absence of lec-SOD prior to transplantation.

Preservation with lec-SOD following 18 hr cold storage significantly inhibited neutrophil infiltration and MHC Class I antigen induction in transplanted kidneys at day 1 post-transplant, and significantly prevented the development of proteinuria after 16 weeks post-transplant. Inhibition of reactive oxygen species at reperfusion may reduce the damaging effects of neutrophil damage on subsequent graft function, but induction of ICAM-1 and T lymphocyte infiltration were detected at 24 weeks post-transplant in kidneys stored with lec-SOD, despite stable graft function. These results suggest that

neutrophil infiltration following ischaemia/reperfusion injury may occur as a result of pathways initiated by reactive oxygen species, causing allo-independent inflammatory events that may reduce nephron mass leading to chronic graft dysfunction, whereas progression of chronic alloimmune responses may be generated by an allospecific pathway mediated by T lymphocyte-MHC antigen interactions.

Experimental evidence from renal models of *in situ* cold ischaemia and reperfusion have also demonstrated neutrophil infiltration within hours of reperfusion, with increased expression of MHC antigens and costimulatory molecules in association with macrophage and T cell infiltration 5 days after reperfusion injury [392, 394]. Administration of soluble PSGL or CTLA4Ig to inhibit leucocyte-endothelial adhesion and T cell costimulation, respectively, significantly ameliorated the progression of all immunological events following ischaemia/reperfusion injury. Studies performed by Takada and colleagues demonstrated that administration of sPSGL or CTLA4Ig were also effective in limiting the development of chronic immunological changes following *in situ* cold ischaemia/reperfusion injury [393, 395]. However, when this model was challenged by alloimmune responses following transplantation into MHC-disparate recipient rats, sPSGL was only effective in preventing chronic renal allograft changes in combination with low-dose cyclosporine therapy [518]. These results suggest that the initiation and development of chronic allograft dysfunction is facilitated by both alloantigen-dependent and independent pathways.

Taken together, the results in this Thesis suggest that ischaemia/reperfusion injury in renal transplantation may lead to endothelial activation and damage, neutrophil infiltration and attachment of platelets to damaged vessels, partly mediated by the action of reactive oxygen species. The effect of glomerular neutrophil infiltration and platelet deposition in the capillaries, may in part, provide a rational explanation for the mechanisms by which prolonged cold ischaemia is significantly associated with DGF in clinical observations [45, 48-54, 72].

Therapeutic strategies aimed at inhibiting the effects of ischaemia/reperfusion injury have been developed for clinical renal transplantation. Preliminary Phase I trials have been performed to examine the effects of antibodies directed against adhesion molecules, ICAM-1 or LFA-1, in the initial period following transplantation. The results demonstrated that anti-adhesion molecule therapy was effective in reducing the incidence of DGF and acute rejection in cadaver renal allografts [402, 405]. However, subsequent multicentre trials showed no beneficial effects following antibody therapy against ICAM-1 or LFA-1 [403, 471]. More promising results were obtained from the trial performed by Land and colleagues, demonstrating that administration of SOD at reperfusion significantly reduced the incidence of acute rejection episodes and improved long term graft survival [123].

In summary, the results in this Thesis provides immunohistological evidence to suggest that pathophysiological events around the time of brain death may result in the induction of pro-inflammatory molecules in cadaver donor kidneys before transplantation. Furthermore, following cold ischaemia and reperfusion, neutrophil infiltration and platelet deposition may significantly influence subsequent renal function in cadaver allografts. These events were not observed in LRD renal allografts before or after reperfusion, suggesting that both these non-specific inflammatory events may in part, be involved in the inferior outcome of cadaver renal allografts. Simple reflusing of cadaver kidneys after the period of cold storage did not prevent the immunohistological changes following reperfusion, but experimental studies using a novel oxygen free radical scavenger, suggest that this may be effective in limiting the harmful effects of cold ischaemia/reperfusion injury. The results from this Thesis, and those from the experimental and clinical studies on ischaemia/reperfusion injury detailed above, suggest that a combination of antioxidant therapy and inhibition of leucocyte adhesion may be required to attenuate the effects of ischaemia/reperfusion injury and acute rejection. Further investigations into methods of improving intensive care therapy

during brain death of potential donors, optimising organ preservation and limiting reperfusion injury in cadaveric renal transplantation, may improve the quality of cadaveric organs and increase long-term graft survival towards that observed in living donor transplantation.

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