

Screening for selective aggrecanase substrates to detect early proteolytic events in cartilage degradation

Milan M. Fowkes, Morten P. Meldal, Tonia L. Vincent and Ngee H. Lim

Purpose

The two major structural proteins in articular cartilage are type-II collagen and aggrecan. The degradation of these components is primarily mediated by the matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin type-I motifs (ADAMTS) class of enzymes. We have previously reported the *in vivo* optical imaging of type-II collagen-degrading MMP-13 activity using an activity-based probe (*MMP13ap*) in a destabilised medial meniscus (DMM) model of osteoarthritis. *MMP13ap* distinguished between DMM and sham operated knees eight weeks post-surgery, where type-II collagen degradation is histologically evident. Given that aggrecan degradation can be observed four weeks after DMM surgery by histology, we hypothesise that an activity-based probe selective for the aggrecanases ADAMTS-4 or ADAMTS-5 would not only enable earlier disease detection, but would also indicate the time-point for targeting these proteinases during the progression of osteoarthritis.

Methods

An activity-based probe is a proteinase-selective peptide that contains a cleavage site flanked by a near-infrared fluorophore and quencher. The increase in fluorescence upon substrate proteolysis can be detected *in vivo* using an optical imaging system. The substrate is reverse-designed from a selective phosphinic peptide inhibitor, which in turn is obtained by screening against a solid-phase combinatorial library of approximately 165,000 unique phosphinic peptide inhibitors. The library consists of beads that each contain two compounds: a general peptide substrate containing a fluorophore and quencher, and a unique phosphinic peptide inhibitor. Screening is performed by incubation of the proteinase with the beads. In the presence of a poor inhibitor, substrate cleavage occurs leading to a fluorescent bead. However, in the presence of a potent inhibitor, cleavage is abrogated and the resultant dark beads are sorted and the sequence of the peptide inhibitor determined by mass spectrometry. The beads limit the size of the proteinase to < 50 kDa. We therefore expressed and purified the catalytic and disintegrin (Cat-Dis) domains of ADAMTS-4 and ADAMTS-5 from transfected human embryonic kidney 293 cells. Purification was carried out by affinity chromatography and analysis by western blotting, coomassie and silver staining. The activity of the Cat-Dis domains of ADAMTS-4 and ADAMTS-5 was validated with the fluorescent substrates 5-carboxyfluorescein-AELQGRPISIAK-N,N,N',N'-tetramethyl-6-carboxyrhodamine and ortho-aminobenzoyl-TESESRGAIY-(N-3-[2,4-dinitrophenyl]-2,3-diaminopropionyl)-KK-NH₂, respectively. Active site titrations with the N-terminal inhibitory domain of tissue inhibitor of matrix metalloproteinases-3 were used to determine the concentration of active proteinase.

Results

A total of 8.2 mg of ADAMTS-4 and 7.5 mg of ADAMTS-5 Cat-Dis proteinases were obtained with purification efficiencies of 3.3 mg/l and 15 mg/l, respectively. Concentration dependent

cleavage of the fluorescent substrates was observed after active site titrations with N-TIMP-3 confirmed the concentration of active proteinase.

Conclusions

ADAMTS-4 and ADAMTS-5 Cat-Dis proteinases were purified and characterised for library screening. We are currently proceeding with the synthesis and assembly of the peptide inhibitor library by combinatorial chemistry. This will be screened against the purified proteinases and potent lead inhibitors will be re-synthesised as substrates and tested for selectivity against a panel of MMPs. Imaging the *in vivo* activity of ADAMTS-4 and ADAMTS-5 could enable earlier osteoarthritis diagnosis, as well as determining the *in vivo* efficacy of their respective inhibitors.