Introduction
Aggrecan degradation by one or more aggrecanases appears to be responsible for much of the cartilage pathology seen in human arthritis (1). The aggrecanase group of the ADAMTS family (ADAMTS1, 4, 5, 8, 9, 15) appear to be controlled by complex interactions at the cell surface which activate the proteases and facilitate access to the substrate (2). A major important question is the identification of the family members responsible for human joint tissue destruction at each stage of the disease. With this in mind we have obtained human biopsies from notchplasty surgery for ruptured ACL (a model of early human disease) and also from knee joints at knee replacement surgery (end-stage disease). Site-matched normal tissues have been obtained from tissue donors. Immunolocalization analysis for each aggrecanase family member with monospecific antibodies and confocal colocalization studies with aggrecanase products, provides a novel approach to this question.

Methods
Human cartilage biopsies were obtained from the lateral femoral condylar notch expansion as part of routine ACL repair surgery (Figure 1). Age-matched and site-matched samples were obtained at tissue harvest under the Michigan Gift of Life donation program. Samples removed at total knee surgery included punch biopsies from the interface of cartilage, bone and soft tissues on lateral, medial, superior and inferior aspects of the patella. Samples were fixed in neutral-buffered formalin, processed for paraffin embedding and sectioning by published methods (3). Affinity purified IgGs used for immunohistochemistry were JSCNIT (anti-NITEGE392), JSCTAS (anti-TASELE1539), JSCVMN (anti-ADAMTS4), JSCKNG (anti-ADAMTS5), JSCHE (anti-ADAMTS1), JSCYNA (anti-ADAMTS8) JSCFIDG (anti-ADAMTS9), JSCSTH (anti-ADAMTS15). Specificity of antibodies was established by comparison with irrelevant IgGs, titration to minimal staining and application to knock-out mouse tissues where available (3). For confocal localization, sections were incubated with primary Ab diluted in blocking serum washed in PBS and incubated with AlexaFluorStreptavidin 488 (1:500) goat anti-rabbit IgG (1:250) for 1h at room temperature and nuclei were stained with DRAQ5 (Alexis). Stained sections were examined using a Leica TCS SP2 Confocal scanning Laser Microscope at a resolution of 1024x1024 pixels and images generated with Leica Confocal Software.

Results
Immunohistochemical analysis of notch cartilage from a normal 33 y male, 41 y female and 49 y male (data from 49 y male shown in Figure 2) revealed that in each case the cartilage had an apparent “fibrous” surface layer (about 20 uM deep) which was GAG-negative and which stained clearly positive for all antigens except ADAMTS4 and ADAMTS5. Below this surface layer the chondrocytes (and some intercellular matrix) also exhibited this general staining preference. The same analysis of ACL repair patients (18 y male, 3 months post-injury; 30 y male, 5 months post-injury; 41 y female, 6 months post-injury, see Fig. 1 bottom panel) showed a profound change in tissue morphology and epitope abundances. The “fibrous” surface layer now accounted for up to one-third of the tissue depth and it was very strongly stained for aggrecan fragments (NIT and TAS), ADAMTS1, 9 and 15. ADAMTS 4, 5 and 8 were less evident. In addition, the midzone of the cartilage showed corresponding relative staining intensities and even the deep zones of the cartilage were strongly positive for NIT and ADAMTS 1 and 9. To determine which of the aggrecanase(s) are responsible for aggrecanolysis in post-injury human cartilage degradation, we have now established high resolution confocal co-localization analyses of enzymes and cleavage products. In preliminary work with bovine cartilage explants treated with retinoic acid, to induce aggrecanolysis, cartilage stained with JSCNIT, anti-ADAMTS4 (Figure 3) showed both antigens localized in the immediate pericellular space, consistent with a role for ADAMTS 4 in this model system of aggrecan catabolism.

Discussion
The data shown is typical of results from both normal and post-injury tissue samples. Most notably, the injury induced cellular activation and matrix remodeling both at the surface and the subchondral zones of the cartilage at this site. However, it is possible that this pronounced change at the cell and matrix level is confined to cartilage which closely interfaces with other soft tissues, and in this location would include the ruptured ACL. The effect of ACLT on aggrecanolysis in cartilages located near the centre of the condyles and plateaus remains to be investigated. It is interesting that in the areas of major aggrecan depletion in the superficial zone (decreased methyl green staining intensity), ADAMTS 1, 9 and 15 appeared most abundant in the matrix, whereas ADAMTS 4 and 5 signals were not markedly elevated compared to the normal controls. Delineation of aggrecanase(s) responsible for post-injury aggrecanolysis and tissue destruction in end-stage disease in the human knee joint, will require more sophisticated techniques to localize the interaction of each protease with aggrecan in situ. Possible experimental approaches include confocal immunolocalization (Figure 3), atomic force microscopy (4) and fluorescence based digital video imaging (5).


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