USE OF A TYPE II COLLAGEN NEOEPITOPE ASSAY FOR MEASURING COLLAGEN DEGRADATION IN CARTILAGE EXPLANTS.

Introduction: Osteoarthritis is a disease, which is characterized by the progressive loss of articular cartilage with accompanying loss of joint function. The rate-limiting step in this process is believed to be the degradation of the type II collagen fibrillar network. Four enzymes (collagenases 1 – 3 and MT1-MMP) have been demonstrated to cleave type II collagen at characteristic sites between residues 775 (glycine) and 776 (leucine) resulting in the TC^5 (3/4) and TC^6 (3/4) products. All four enzymes are expressed in human cartilage. However, the primary enzyme responsible for type II collagen degradation has yet to be firmly established. Recent work in cartilage explants with an MMP-1 sparing inhibitor suggests that MMP-1 is unlikely to play a major role in type II collagen degradation (1). In the present work, immunohistochemistry and an ELISA, both based on an antibody to a collagen neoepitope, have been utilized to confirm that an MMP-1 sparing compound (CP-471,474: IC50’s for MMP-1, MMP-8, MMP-13 and MMP-14 are 1200, 4.6, 0.9, and 15nM respectively) is effective at blocking IL-1 induced collagen degradation in IL-1 stimulated bovine nasal cartilage explants. The neoepitope antibody, 9A4, is a single chain monoclonal antibody made to the carboxy-terminal sequence produced after collagenase digestion of type II collagen (EGPGPGPG) (2). In addition to 9A4, the ELISA utilizes a capture monoclonal antibody (5109) to a specific type II collagen epitope (GEPGDDASPS) N-terminal to 9A4 (3). The type II collagen neoepitope ELISA demonstrated that CP-471,474 was effective at blocking IL-1 stimulated release of type II collagen fragments bearing the collagenase-generated neoepitope. The effective concentrations for blocking collagen degradation preclude MMP-1 from playing a significant role in this system. These results were confirmed using traditional hydroxyproline analysis of acid hydrolyzed conditioned media. To directly visualize the production of collagenase-generated cleavage sites in cartilage sections, from IL-1 stimulated cartilage explants stained with 9A4. IL-1 induced a time-dependent increase in staining in cartilage explant sections and this was effectively blocked by treatment with CP-471,474. Our results demonstrate that type II collagen degradation in cartilage explants can be effectively monitored using this new neoepitope ELISA. In addition, our results confirm that MMP-1 plays only a minor role in type II collagen degradation in this explant system.

Methods: Bovine Nasal Cartilage Cultures: Bovine nasal septa were punched into 4 mm cartilage plugs and 3 plugs were placed per well of a 24 well plate cultured in 1ml/well serumless media (20mM HEPES, 50ug/ml ascorbic acid, 0.1mg/ml BSA, 5ug/ml insulin, 50ng/ml sodium selenite and 1% PSF in DMEM). For experiments, the cartilage plugs were cultured in the presence of 10 ng/ml IL-1 alpha and CP-471,474 at 5, 10, 50, 100, and 500nM. Media were replaced every 3-4 days for fresh media, IL-1 and IL-1 + inhibitor. Each condition was assayed in triplicate and cultures were terminated after 14 days of culture. Type II collagen Neoepitope Assay: Antibody 5109 was coated on a 96-well immunosassay plate (1 ug/well). The plate was blocked with 1% non-fat dried milk and pooled conditioned media samples immunabsorbed (for 2 hours). The type II collagen collagenase cleavage site was then immunodecorated with biotinylated 9A4 antibody (1 ug/ml) and visualized with HRP-anti biotin antibody (Jackson ImmunoResearch) and TMB (Amersham). The concentrations of type II collagen neoepitope were calculated using a standard curve generated with a peptide corresponding to the collagenase cleavage fragment. The data were normalized to cartilage wet weight. Data are plotted as concentration of inhibitor vs. % control and IC50’s determined.

Immunohistochemistry: Bovine nasal cartilage explants were prepared and treated with IL-1 and CP-471,474 as above for 13 days. Sectioned cartilage explants were stained with 10 µg/ml 9A4 antibody and anti-mouse IgG alkaline phosphatase (1,200). Hydroxyproline analysis: Cartilage was acid hydrolyzed and hydroxyproline content determined as previously described (4).

Results: CP-471,474 inhibited IL-1 induced collagen degradation in the bovine nasal cartilage assay with an IC50 of 28 nM as determined using the type II collagen neoepitope assay (Figure. 1). Hydroxyproline analysis of the remaining cartilage qualitatively confirmed this result. The collagenase-generated neoepitope was immunolocalized in cartilage sections with staining with 9A4 (Figure 2). Very little staining was observed in unstimulated cartilage explants (Figure 2A). IL-1 treatment induced a time-dependent increase in staining, first evident after 6 days of IL-1 treatment. After 13 days of treatment (Figure 2B), intense 9A4 staining was observed, and this was almost completely abrogated in cultures incubated with IL-1 in the presence of CP-471,474 (Figure 2C).

Discussion: The explant model is a convenient assay for both studying the mechanism of type II collagen degradation in vitro and for measuring the relative efficacy of various collagenase inhibitors. We have demonstrated that the cleavage of type II collagen in bovine nasal cartilage can be measured by following the release of collagen fragments in the conditioned media using the type II collagen neoepitope assay and that the data generated is comparable to the traditional hydroxyproline analysis. The IC50’s of CP-471,474 (approximately 25 nM) using both the type II collagen neoepitope assay and hydroxyproline analysis clearly demonstrate that MMP-1 does not play a major role in type II collagen degradation in this explant system. Immunolocalization of the collagenase-generated neoepitope in cartilage explants demonstrated that CP-471,474 inhibited generation of the cleavage site and not just the release of the epitope into the conditioned medium. Collagenase inhibitors such as CP-471,474 may be therapeutically useful in blocking type II collagen degradation in diseases such as osteoarthritis.

Figure 1: CP-471,474 dose response curve.

Figure 2: Bovine nasal cartilage staining with 9A4 antibody. Explants were cultured for 13 days prior to staining. A. Control, B. IL-1, C. IL-1 + 1µM CP-471,474.

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