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Proteoglycan- and Collagen-Degrading Enzymes from Human Interleukin 1-Stimulated Chondrocytes from Several Species: Proteoglycanase and Collagenase Inhibitors as Potentially New Disease-Modifying Antiarthritic Agents (42416)

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Abstract. Human IL-1-stimulated chondrocytes derived from rabbit, bovine, and human articular cartilage produce proteoglycan- and collagen-degrading enzymes. These studies demonstrate that the biological activity of IL-1 is not species specific. Several thiol, carboxyalkyl, and hydroxamic acid peptide inhibitors showed differential effects. The thiols were equipotent inhibitors of both the collagen- and proteoglycan-degrading enzymes whereas the carboxyalkyls appear to inhibit solely the proteoglycan-degrading enzyme(s). The hydroxamic acid peptides, the most potent inhibitors, appear to be more active against the proteoglycan-degrading enzymes. These synthetic inhibitors of proteoglycan- and/or collagen-degrading enzymes may represent a new class of disease-modifying antiarthritic agents.

Although the underlying mechanisms occurring in osteoarthritis or osteoarthrosis remain obscure, the disease is clinically characterized by pain, inflammation, stiffness, limitation of motion, and deformity (1, 2). Pathophysiologically, one observes a structural breakdown of cartilage macromolecules, namely proteoglycans and collagen, and the development of ulcerations which initially are focal and later involve diffuse areas of the cartilage surface.

The structural integrity of these macromolecules is necessary to maintain the biomechanical properties of cartilage (3, 4). Structural alterations of cartilage in osteoarthritis may result from increased enzymatic breakdown of proteoglycans (5), and articular cartilage may be an active participant in joint destruction (3, 6). The destruction of the extracellular matrix of joint tissue that occurs during the development of arthritides appear to be mediated in part by proteinases synthesized by the tissues themselves (7, 8). Proteoglycan- and collagen-degrading metalloenzymes have been isolated and characterized from human and bovine cartilage (9), rabbit and human chondrocytes (10–12), and explants of rabbit and human synovium (13, 14).

The metabolic activity of chondrocytes can be modified with intercellular messengers such as interleukin 1 (IL-1) or catabolin (15). Recently, it has been reported that rabbit and normal or osteoarthritic human chondrocytes stimulated with IL-1 release into the spent media latent proteoglycan- and collagen-degrading metalloproteinases (11, 12, 16). The human osteoarthritic chondrocytes produced measurable latent proteoglycan- and collagen-degrading enzymes without IL-1 stimulation (12).

Interestingly, IL-1-like activity has been identified in 9 of 11 synovial fluids from patients with various types of joint disease (17, 18). Thus, an understanding of the nature of proteinases and the factors which stimulate or control their synthesis and activity (endogenous inhibitors) is important for developing new types of disease-modifying agents. Additionally, drug development requires information from several species including the human. In this report we compare the proteoglycan- and collagen-degrading enzyme production derived from IL-1-stimulated chondrocytes from several species. In addition, we evaluated several thiol, carboxyalkyl, and hydroxamic acid peptides as inhibitors of the proteoglycan- and collagen-degrading enzymes derived from rabbit chondrocytes.

Materials and Methods. Chondrocytes were obtained from rabbit and bovine articular cartilage; normal and osteoarthritic cartilage from human knee (at arthroplasty) by sequential enzyme treatment as described by Benya *et al.* (19).

The released cells were filtered through a nylon mesh, collected by centrifugation, and

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washed twice with Nutrient Mixture F12 (HAM) medium (GIBCO) supplemented with 10% fetal calf serum (FCS). The chondrocytes were plated at 3×10^4 or 6×10^4 (human cells/cm^2) in Nutrient Mixture F12 containing 10% FCS (GIBCO) and 25 μg/ml of gentamicin (Schering).

The medium was changed every other day, and the cells were allowed to grow to confluency as described by Malemud *et al.* (10). The rabbit and bovine chondrocytes were confluent on Days 7–10 whereas the human chondrocytes required 18–21 days.

The confluent monolayers were washed twice with serum-free DMEM. Human affinity-purified IL-1 (Genzyme, Inc.) was added (30–50 units/ml)[1] to the chondrocyte monolayers maintained in serum-free DMEM at 37°C, 5% CO_2. The cell-free culture supernatant (human) was collected at 3 and 5 days after IL-1 addition. The supernatant was dialyzed against 50 m*M* Tris, 200 m*M* NaCl, 5 m*M* $CaCl_2$, and 0.02% NaN_3, pH 7.4. The collected supernatants were evaluated for collagen- and proteoglycan-degrading enzyme activity as described by DiPasquale *et al.* (12). The collagen- and proteoglycan-degrading enzymes were activated with 0.34 m*M* aminophenylmercuric acetate (APMA). The test agents were evaluated following removal of APMA by exhaustive dialysis (three changes of 100 vol of buffer). The preparation of the proteoglycan subunit (20, 21) as proteoglycan–polyacrylamide beads (22) used to evaluate proteoglycan-degrading enzyme activity and the preparation of the ^{14}C-acetylated collagen (23, 24) used to evaluate the collagenolytic activity of the enzyme preparation were previously described (12). Additionally, we evaluated several thiol, *N*-carboxyalkyl, and hydroxamic acid peptides synthesized by Shaw, Wolanin, and Roberts (Stuart Pharmaceuticals, Wilmington, Del.). These compounds with identifying numbers and nomenclature are listed in Table II.

Results. Human IL-1-stimulated chondrocytes from several species to release proteoglycan- and collagen-degrading enzymes (Table I). The rabbit chondrocyte appeared to be the most responsive. Human osteoarthritic chondrocytes released more proteoglycanase but less collagenase than human chondrocytes from normal-appearing cartilage (from cadaver with no clinically diagnosed OA). Additionally, human osteoarthritic chondrocytes released significant proteoglycan-degrading enzyme(s) without IL-1 stimulation. Preliminary studies show that chondrocytes obtained from rat or dog articular cartilage also release detectable proteoglycan- and collagen-degrading enzymes in the media when stimulated with IL-1.

Rabbit-derived proteoglycan- and collagen-degrading enzymes are inhibited with EDTA, phenanthroline, and α2-macroglobulin but not with pepstatin, PMSF, TLCK, or α1-antitrypsin (11, 12, 25, 26). These studies suggested that the enzymes inhibited are metalloenzymes and not thiol or serine proteinases.

Evaluations with several thiol, carboxyalkyl, and hydroxamic acid peptides show differential effects on the proteoglycan- and collagen-degrading enzymes (Table II). The thiols appear to be equipotent inhibitors of both enzymes, whereas the carboxyalkyls inhibit solely the proteoglycan-degrading enzyme. The hydroxamic acids are more potent inhibitors of the proteoglycan- than the collagen-degrading enzymes.

U24522 and U24531 (hydroxamic acids) were the most potent proteoglycanase inhibitors with IC_{50} of approximately 4.2×10^{-8} and 3.4×10^{-8} *M*, respectively. These agents were approximately three times more potent than U24278 and U24279 (IC_{50} of 1×10^{-7} and 1.2×10^{-7} *M*) and approximately 100 times more potent than the carboxyalkyl and thiol peptides. U24522 and U24631 were also the most potent collagenase inhibitors with IC_{50} of approximately 4.1×10^{-7} and 4.5×10^{-7} *M*, respectively.

As previously indicated, the carboxyalkyl peptides did not inhibit the collagen-degrading enzyme(s). The hydroxamic acid peptide inhibitors were approximately 10 times more potent against the proteoglycan- than the col-

[1] All material received from Genzyme was retitered. The IL-1 activity was assessed by the augmentation of PHA-induced C3H/HeJ mouse thymocyte proliferation as described by Mizel *et al.* (1978, *J. Immunol.* **120,** 1497). One unit of IL-1 induced a doubling of [^{125}I]iododeoxyuridine incorporation in phytohemagglutinin-stimulated thymocytes when compared to cultures without IL-1. [(CPM with IL-1 + PHA) − (CPM with PHA alone)].

The various thiol, carboxyalkyl, and hydroxamic peptides demonstrated differential effects on the proteoglycan- and collagen-degrading enzymes. The hydroxamic acid peptides were the most potent, inhibiting proteoglycanase and collagenase at concentrations ranging from 3×10^{-8} to 1×10^{-7} *M* and 4×10^{-7} to 1×10^{-6} *M*, respectively. A comparison of the inhibitory capacity of the hydroxamic acid peptides shows that they are approximately 10 times more potent as proteoglycanase inhibitors than collagenase inhibitors. U24522 and U24631, the most potent inhibitors evaluated, are approximately 100 times more active than the thiols or carboxyalkyl peptides as proteoglycanase inhibitors and approximately 10 times more active than the thiol agents as collagenase inhibitors. The thiols appear to be equipotent inhibitors of either enzyme, whereas the carboxyalkyl peptides appear to inhibit only the proteoglycan-degrading enzyme(s). These differential activities can be useful biochemical tools to separate and further characterize the enzymes and develop more potent inhibitors.

As previously indicated, the degradative neutral metalloenzymes, which are derived from cartilage and/or chondrocytes and found in osteoarthritic cartilage, appear to be responsible for the loss of matrix observed in osteoarthritis.

It has also been reported that synovial fluid obtained from joints with arthritis contain cartilage debris (34). Additionally, the intraarticular injections of cartilage particles induce an experimental arthritis in animals (35–37). From these studies the authors speculated that cartilage particles or cartilage degradation products may induce secondary symptom-producing changes in osteoarthritis. The addition of a "chemical" inflammation may be responsible for increased cartilage degradation and the expansion and potentiation of clinical symptoms. This appears to be emphasized by Katona (2) who suggested, after evaluating 160 patients with osteoarthritis, that osteoarthritis may be regarded as an inflammatory condition and that the use of nonsteroidal anti-inflammatory agents (NSAIFA) rather than pure analgesics should be encouraged. However, it should also be kept in mind that many presently available NSAIFA appear to inhibit cartilage matrix synthesis (38–42) but do not inhibit the degradative mechanisms which are operative during the osteoarthritic process. These actions may be detrimental although it appears that the NSAIFA may suppress the "chemical" inflammation and pain induced by cartilage degradation products.

The advantages of a proteoglycan/collagen-degrading enzyme inhibitor in arthritic conditions appear unique since these agents would (a) inhibit the "spreading" cartilage degradation evident in moderate to severe osteoarthritis and (b) reduce and/or inhibit the "chemical" inflammation induced with the cartilage degradation products accumulating in synovial fluid.

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