Immunolocalization of stromelysin, tumor necrosis factor (TNF) α, and TNF receptors in atrophied canine articular cartilage treated with hyaluronic acid and transforming growth factor β

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Objective—To evaluate the ability of hyaluronic acid (HA), with and without transforming growth factor β (TGF-β), to stabilize the catabolic processes associated with atrophy of articular cartilage.

Animals—20 adult, skeletally normal, hound-type dogs.

Procedure—Dogs (20 to 30 kg) were randomly assigned to 1 of 5 groups. One group served as untreated controls. Bivalve casts were placed on the left hind limbs of the remaining 16 dogs to limit weightbearing and motion of the limb for 92 days. One group served as the cast control. Beginning on day 56, 3 groups received aseptic intra-articular injections in the left stifles of either 5 mg of HA or 5 mg of HA containing either 20 or 50 μg of TGF-β. Intra-articular injections were repeated at 4-day intervals until the end of the study. On day 92, stifles were harvested at necropsy. Medial femoral condyles were histologically processed, and the articular cartilage was stained for the presence of proteoglycans, stromelysin, tumor necrosis factor (TNF) α, and TNF receptors (p55 and p75).

Results—Decreased metachromasia was evident in the cartilage matrix of all cast groups, with the smallest decrease in the HA-treated group. Stromelysin was immunolocalized in articular cartilage of the cast (left) limbs of cast control and both HA/TGF-β-treated groups. TNF-α was localized in articular cartilage of all cast (left) and right limbs, except those of the HA-treated group. Receptors for TNF were observed in both limbs of untreated control and cast control groups and cast limbs of HA/TGF-β-treated groups. The receptors were not localized in the right limbs of the HA with or without TGF-β-treated groups. TGF-β did not decrease stromelysin or TNF-α or receptors at the doses used.

Conclusions—HA may mediate a chondrostabilizing influence on articular cartilage by down-regulating TNF-α. Importantly, HA appeared to exert its inhibitory influence on TNF-α, as well as stromelysin and TNF receptors, on a systemic basis.

Clinical Relevance—Results provide insight into the mode of action of HA as a therapeutic agent for arthritis and its stabilizing influence on cartilage metabolism. (*Am J Vet Res* 1996;57:1488–1496)

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Osteoarthritis (OA) is an age-related, debilitating disease of synovial joints characterized by degradation of articular cartilage and formation of new bone. Although the cause of OA may be multifactorial, destruction of articular cartilage appears to begin in the matrix, with the early alterations of proteoglycans and disorganization and disruption of collagen fibers, which allows an increase in water content.[1,2] Degradation of the matrix is mediated by enzymes (ie, metalloproteinases, serine proteinases, and cysteine proteinases) that may be produced by chondrocytes or cells of the synovial membrane. Stromelysin is a member of the metalloproteinase family of enzymes that degrades proteoglycans.[3]

In OA, chondrocytes synthesize increased amounts of proteoglycans[1]; however, degradation of proteoglycans proceeds at a faster rate than that at which chondrocytes can replace them.[2] The increased amount of catabolic enzymes responsible for degradation of the matrix can be controlled by cytokines, such as interleukin 1 and tumor necrosis factor (TNF).[3] Also, inflamed synovial membrane contains macrophages, monocytes, and lymphocytes, which secrete inflammatory mediators into the synovial fluid that bathes the cartilage.[3]

Tumor necrosis factor is activated by binding to TNF receptors, p55 and p75.[4-6] Although both receptors are present on and expressed by most cells, p55 is considered to be necessary for the biologic activity of TNF-α in many cell lines.[4-6] Activity of TNF has been inhibited by shedding of portions of p55 and p75 receptors from the cell surface.[4-7] These shed cell surface receptors can bind TNF to prevent it from binding to attached cell surface receptors, thus inhibiting activation of the cell.[4,5,7]

Loss of matrical proteoglycans from articular cartilage that accompanies OA has been simulated by numerous animal models. Atrophy of articular cartilage induced by immobilization or decreased loading of a diarthrodial joint, or both for several weeks induces some changes similar to osteoarthritic lesions and has been used to study the catabolic process.[8-10] Proteoglycan aggregation, thickness, metachromatic matrical staining, and glycosaminoglycan content are decreased and water content is increased in immobilized articular cartilage.[8,9] Although the loss of proteoglycans from atrophic articular cartilage is similar to that observed in OA, the mechanisms of depletion are different. In OA, catabolism of existing proteoglycans and synthesis of new proteoglycans are increased.[1] Conversely, during atrophy, degradation of proteoglycans is normal or

slightly accelerated, but synthesis of new proteoglycans is decreased.[8,9] Thus, the secretory activity of chondrocytes of atrophic cartilage is down-regulated, compared with chondrocytes in normal or osteoarthritic cartilage. Importantly, atrophic articular cartilage provides a unique model to study the ability of exogenous mediators of chondrocytic metabolism to up-regulate the synthetic activity and decrease the catabolic actions of metabolically normal, although dormant, chondrocytes. An additional advantage of the atrophic articular cartilage model is the limiting influence of reduced loadbearing/motion on the physical destruction of joint surfaces.[8-10] Thus, the joint immobilization model can be used as an in vivo culture system to evaluate chondrocytic metabolism.

The role of altered chondrocytic nutrition in the pathogenesis of atrophy of articular cartilage in immobilized joints is unclear. Palmoski et al[9] reported that deterioration of articular cartilage resulting from immobilization will develop in a mobile joint if normal loading of the joint is removed. Thus, atrophic changes in immobilized joints could result from altered mechanical loading of the tissue rather than from altered or stagnated nutrition of the resident articular chondrocytes.

Transforming growth factor b (TGF-b) is a multifunctional protein with a molecular weight of 25,000 that is synthesized by chondrocytes and secreted in latent form that requires either acid or alkali activation to become functional.[11-13] Transforming growth factor β has multiple influences on the homeostasis of cartilage that are mediated through its ability to increase synthesis of new proteoglycans and to reduce destruction of existing proteoglycans.[14-18]

Hyaluronic acid (HA) is an integral component of synovial fluid as well as an important component of the articular cartilage matrix where it binds proteoglycan monomers into large aggregates.[19] The movement and potential metabolism of HA is dynamic, as evidenced by its ability to penetrate the synovial membrane within 2 hours and articular cartilage within 6 hours.[20] The half-life of HA in normal synovial joints was determined to be as long as 96 hours in horses[21] and as short as 12 hours in rabbits.[22] A protective and stabilizing influence of HA on articular cartilage has been documented in experimental models of OA and in chondrocyte cultures.[23-29] The palliative influence appears to be mediated through resident cells and inflammatory mediators, such as cytokines.[23,25] In synovial fluid, the mechanism of action of HA may result from an inhibitory influence on inflammatory cell mobility, phagocytosis, and enzyme release.[25]

Because HA and TGF-β are usually present in articular cartilage, their actions to stabilize the integrity of the matrix, to stimulate chondrocytic synthesis of new matrix, and to decrease production of degradative enzymes and inflammatory cytokines could have potential therapeutic value in stimulating maintenance and repair of injured articular cartilage. Evaluation in an immobilized synovial joint is an initial step in determining the efficacy of a composite of HA and TGF-β to maintain the integrity of articular cartilage that is undergoing degradation in the absence of mechanical stimulation. The objective of the study reported here was to evaluate the ability of HA, with and without TGF-β, to maintain chondrocyte matrical production of proteoglycans and to decrease destructive enzyme production in atrophic articular cartilage.

Materials and Methods

Dogs—Twenty skeletally normal, hound-type dogs (10 males and 10 females), 2 to 5 years old and weighing approximately 20 to 30 kg, were studied. They were housed individually in indoor/outdoor runs (2.5 × 4.1 m) for 1 month before and during the study where they were allowed unrestricted activity. Pretrial radiography of both stifles was done to verify skeletal maturity on the basis of complete physeal closure and lack of degenerative disease of the stifles.

The dogs were randomly assigned to 5 groups of 4 dogs each (2 males and 2 females). The 5 groups included: group 1, untreated controls (no casting or injections); group 2, cast controls (no injections); group 3, cast and HA[a] (10 mg/ml) injected; group 4, cast and 20 µg of TGF-β_2[b] in HA (10 mg/ml) injected; and group 5, cast and 50 µg of TGF-β_2 in HA (10 mg/ml) injected. Dogs that received casts were heavily sedated by use of atropine[c] (0.01 mg/kg of body weight), acepromazine[d] (0.1 mg/kg), and tiletamine HCl and zolazepam HCl[e] (8 mg/kg). With the left stifle in 90° flexion, a bivalved full-limb cast,[f] extending from the hip to and encompassing the paw, was applied to all dogs of groups 2–5. The casts were attached to the caudoventral portion of thorax harnesses via metal clips incorporated into the mid-metarsal region of the casts to prevent weightbearing and limit motion of the limb. The dogs wore the casts for 92 days and were monitored daily for comfort. If adjustments or new casts were needed, alterations were made on heavily sedated dogs.

Atrophy of articular cartilage was induced by immobilization/non-weightbearing of the cast stifle for 56 days.[8] On day 56 of immobilization, intra-articular injections in the left femorotibial joints of dogs in treatment groups 3–5 were initiated and repeated at 4-day intervals until day 92. A total of 9 injections were administered. The cast was removed from the dogs under the aforementioned heavy sedation, and the region of the stifle was prepared for an aseptic intra-articular injection. A volume of 0.5 ml was injected into the synovial cavity. The TGF-β was acid activated in 5 m*M* HCl under sterile conditions prior to mixing with the HA. Casts of dogs in group 2 (cast control) also were removed and replaced at 4-day intervals in dogs under heavy sedation, but intra-articular injections were not administered.

Each dog was euthanatized on day 92 by an overdose of barbiturate[g] (39.8 mg/kg). The stifles of both hind limbs were harvested from each dog, and 5-mm, wedge-shape specimens were cut from the proximal medial femoral condyle. Each specimen contained articular cartilage and subchondral bone as well as periarticular synovial membrane. The specimens were placed in neutral-buffered 10% formalin for 48 hours and decalcified in a mixture of 4% concentrated hydrochloric acid and 4% concentrated formic acid in distilled water for 72 hours (verified by radiography). After being rinsed in running tap water, specimens were processed in a vacuum infiltration processor[h] for 24 hours and embedded in a highly purified paraffin-containing low molecular weight polymer.[i] Five-micron serial sections were cut from each specimen, using a rotary microtome.[j]

Sequential sections from both femoral condyles of each dog were stained with H&E and toluidine blue. Additional sections were used for immunolocalization, using antibodies against stromelysin[k] (mouse IgG monoclonal, 1:500), TNF-α[l] (rabbit IgG monoclonal, 1:100), and TNF receptors p55[k] and p75[k] (rabbit IgG polyclonal, 1:200). The TNF-α antibody was specific for TNF-α at a dilution of 1:500 and did not cross-react with TNF-β or bovine serum albumin when immunoassayed by use of western blot. Tumor necrosis factor receptors p55 and p75 were raised against the extracellular portion of recombinant p55 and p75 receptors. Specificity of these antibodies was determined, using western blot analysis. Also, receptors were recognized by these antibodies

Table 1—Summary of the trends of histochemical and immunohistochemical procedures on articular cartilage of right (untreated) medial femoral condyles

Group	MC	Strom	TNF-a	p55	p75
Control	Normal	–	+	+	+
Cast control	Normal	–	+	+	+
HA	Normal	–	–	–	–
HA + 20 µg TGF-β	Normal	–	+	–	–
HA + 50 µg TGF-β	Normal	–	+	–	–

MC = metachromatic staining; strom = stromelysin; TNF-α = tumor necrosis factor α; p55 and p75 = TNF receptors p55 and p75; HA = hyaluronic acid; + = positive reaction; and – = negative reaction.

Table 2—Summary of the trends of histochemical and immunohistochemical procedures on articular cartilage of eft (treated) medial femoral condyles

Group	MC	Strom	TNF-a	p55	p75
Control	Normal	–	+	+	+
Cast control	↓	+	+	+	+
HA	Slight ↓	–	–/↓	–	–
HA + 20 µg TGF-β	↓	+	+	+	+
HA + 50 µg TGF-β	↓	+	+	+	+

↓ = decreased.

in an ELISA format. Antibodies against stromelysin have been documented to react with canine stromelysin.[30]

In the general immunohistochemical procedure, sections were digested for 1 hour in hyaluronidase (1.0 mg/ml), blocked with 3% hydrogen peroxide in methanol for 45 minutes, and serum blocked with either normal goat (10%) or horse (10%) serum for 20 minutes. Sections were incubated with primary antibody overnight at 20 to 22 C (room temperature). Avidin-biotin complex kits[m] were used to label the primary antibodies, and 3,3-diaminobenzidine hydrochloride[n] was used to yield a color reaction. Osteoarthritic articular cartilage lesions were used as a positive control for stromelysin, and rheumatoid arthritic synovial membrane was the control for TNF-α and TNF receptors. In negative controls, phosphate-buffered saline solution was substituted for the primary antibody. All slides (groups 1–5) were stained as block sections for each individual antibody, so no variation in staining intensity attributable to processing would be present. Slides were evaluated, using a binocular research microscope.[o]

Results

Clinical findings—Dogs tolerated immobilization of a hind limb well, and signs of systemic abnormalities or diseases were not observed. Muscular atrophy of the cast limbs of all dogs was progressive during the study and was particularly evident after 2 months of casting. Some alopecia and superficial abrasions developed over pressure points located in the casts. In such instances, additional padding or a new cast was provided to increase comfort of the dog. Dogs of groups 4 and 5, which received intra-articular injections of TGF-β, developed moderate joint effusion in the injected stifle as well as grossly palpable fibrosis of the joint capsules during the course of the study.

Necropsy—Articular surfaces of the femorotibial and femoropatellar joints had a slightly bluish tinge in all cast limbs that was particularly evident in the cast control group. The joint capsules of dogs in the TGF-β-treated groups were fibrotic and thick. Also, TGF-β-treated joints were moderately distended by increased amounts of synovial fluid and fibrosis. Joint distention or fibrosis was not evident in dogs of the cast control or cast/HA-treated group.

Histopathologic findings—Results of histologic examination and histochemical staining, along with immunohistochemical localizations, were compared (Tables 1 and 2).

Untreated control group—Lenticular chondrocytes located in the superficial zone, round chondrocytes in the middle zone, and columnated groups of chondrocytes in the deep zone had normal morphology and morphologic relations. Metachromatic matrical staining extended throughout the depth of the articular cartilage (Fig 1A). Fissures or fibrillations were not observed in the articular cartilage; however, slight erosions of the superficial zone were seen in 2 specimens (1 left and 1 right).

Immunolocalization of stromelysin was negative in all femoral condyles (Fig 2A). Light immunoreactivity in chondrocytes of the superficial zone was present in the left femoral condyle with the superficial erosion. Immunoreactivity for TNF-α was observed in chondrocytes, principally in the middle and deep zones of femoral condyles of both limbs of all dogs (Fig 3A). However, a few superficial chondrocytes contained immunoreactivity in the left femoral condyles. Immunolocalization of receptors p55 and p75 was evident in chondrocytes of the superficial, middle, and deep zones of the articular cartilage of the left and right femoral condyles (Fig 4A), although chondrocytic immunoreactivity was, at times, limited to the middle zone.

Cast control group—Morphology and metachromatic staining of the articular cartilage of the right femoral condyles was similar to those on cartilage from the untreated control group. In the left femoral condyles, increased acidophilia and decreased metachromasia were observed in the matrices of the superficial and middle zones of the articular cartilage (Fig 1B).

Immunolocalization of stromelysin was generally lacking in the right femoral condyles. In left condyles, stromelysin was located in chondrocytes and matrix of the superficial and middle zones of the articular cartilage (Fig 2B). In 1 dog, immunoreactivity was at low intensity in the left condyle.

Tumor necrosis factor α was immunolocalized in chondrocytes of the middle and deep zones of the articular cartilage of the right femoral condyles. In the left femoral condyles, immunoreactivity for TNF-α was present in chondrocytes of the superficial, middle, and deep zones and in the matrices of the superficial and middle zones (Fig 3B). Immunoreactivity for TNF-α was light in 1 left femoral condyle.

Chondrocytic and matrical immunoreactivity of the receptor p55 was present in the superficial zone of the articular cartilage of right femoral condyles. In the left femoral condyles, receptor p55 was immunolocalized in chondrocytes and matrix of the superficial zone (Fig 4B). Chondrocytic and matrical immunoreactivity for receptor p75 was principally located in the superficial zone of the articular cartilage of the right and left femoral condyles, although immunoreactivity was light in the right femoral condyle of 1 dog.

Hyaluronic acid group—Routine staining of articular cartilage from the right femoral condyles was similar to that of cartilage from untreated controls. In the left femoral condyles, increased acidophilia and de-

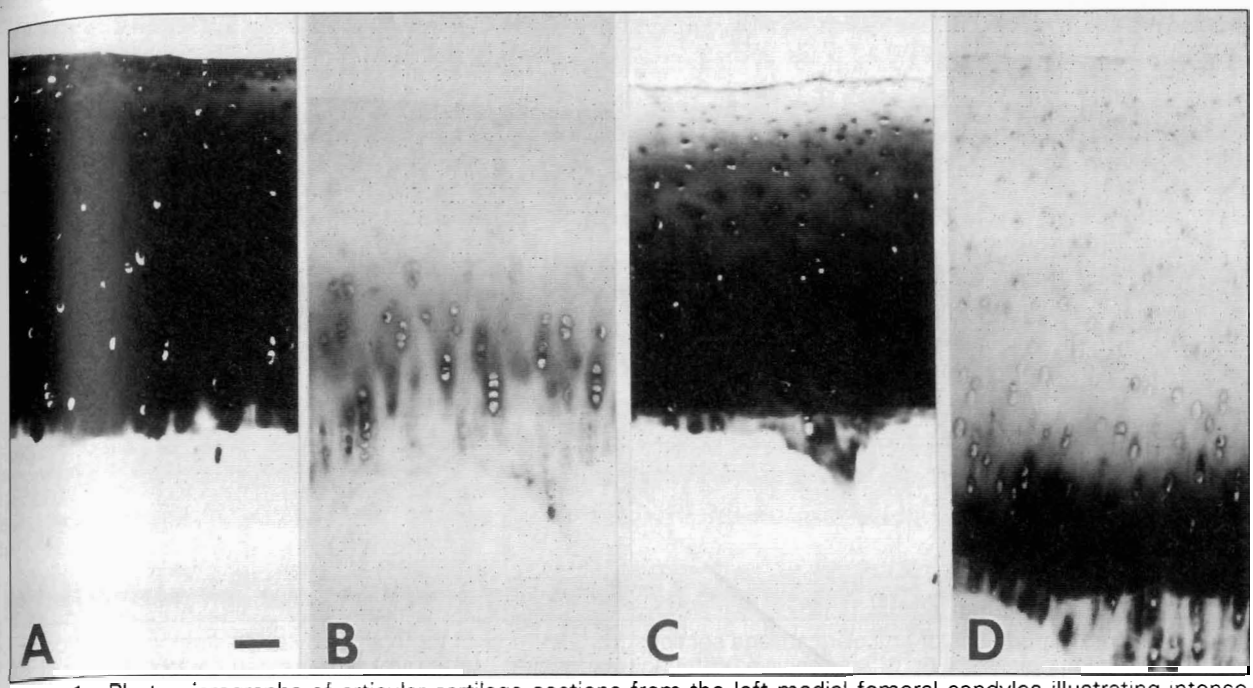

Figure 1—Photomicrographs of articular cartilage sections from the left medial femoral condyles illustrating intense matrical metachromatic staining of an untreated control dog (A), compared with extensive loss of metachromasia in cast control dog (B). Notice only mild loss of matrical staining in the superficial layer of cast/hyaluronic acid (HA)-treated dogs (C), whereas almost complete loss of staining was present in cast/HA/transforming growth factor β (TGF-β)-treated dogs (D). Toluidine blue stain; bar = 50 μm.

creased metachromasia were present in the matrix of the superficial zone (Fig 1C).

Immunoreactivity for stromelysin was lacking in the right or left femoral condyles of all dogs (Fig 2C), other than slight chondrocytic immunolocalization in the superficial zone of 1 left femoral condyle. Similarly, TNF-α was not immunolocalized in the right femoral condyles (Fig 3E), other than light immunoreactivity in a few chondrocytes of the middle zone of 1 specimen. In the left condyle, immunolocalization of TNF-α was lacking in 2 dogs (Fig 3C) and was light in a few deep chondrocytes of 2 others. The only immunoreactivity for receptors p55 and p75 was light localization of p55 in the matrix of the middle zone of the articular cartilage of 1 right and 1 left condyle. Immunoreactivity was absent in the right and left femoral condyles of all other dogs (Fig 4C).

Dosage of 20 mg of TGF-b in HA—Articular cartilage of the right femoral condyles had routine staining patterns that were similar to that in untreated controls. However, articular cartilage of the left femoral condyles had increased matrical acidophilia and decreased metachromasia of the superficial, middle, and deep zones (Fig 1D). Blood vessel ingrowth from the subchondral bone into the articular cartilage and a few fibrous tags attached to the articular surface were observed. The fibrous tags originated from synovial membrane at the margin of the articular cartilage. Hyperplasia of the periarticular synovial membrane was accompanied by infiltration of mononuclear inflammatory cells (Fig 5).

Immunoreactivity for stromelysin was lacking in the right femoral condyles. In the left femoral condyles, stromelysin was immunolocalized in chondrocytes and matrix of the superficial, middle, and deep zones of the articular cartilage (Fig 2D).

Tumor necrosis factor α was immunolocalized in chondrocytes of the middle and deep zones of the articular cartilage of the right femoral condyles. In the left femoral condyles, TNF-α was immunolocalized in chondrocytes and matrix in the superficial, middle, and deep zones of the articular cartilage (Fig 3D).

Other than a focal site of light chondrocytic immunoreactivity in the superficial zone of 1 specimen, immunoreactivity for receptors p55 and p75 was absent in the right femoral condyles. In the left femoral condyles, both receptors were immunolocalized principally in the matrix of the middle zone of all specimens (Fig 4D), although immunoreactivity in chondrocytes of the superficial and middle zones was observed. In some specimens, the matrix appeared to contain a thick line of immunoreactivity oriented parallel to the surface at the junction of the middle and deep zones. The intensity of immunoreactivity was greatest in this linear region.

Dosage of 50 mg of TGF-b in HA—The microscopic appearance and patterns of routine staining and immunoreactivity of articular cartilage from joints treated with 50 μg of TGF-β were similar to those described for specimens treated with 20 μg of TGF-β.

Discussion

Homeostasis of matrical proteoglycans in articular cartilage results from a balance between degradation of existing proteoglycans and synthesis of new proteoglycans. In this study, immobilization/non-weightbearing of the stifle resulted in loss of staining of proteoglycans in articular cartilage as described by other investigators,[31] which suggests an imbalance in metabolism toward catabolism. Degradation of proteoglycans is also a characteristic lesion of OA that results from the action of enzymes, such as the metalloproteinase stro-

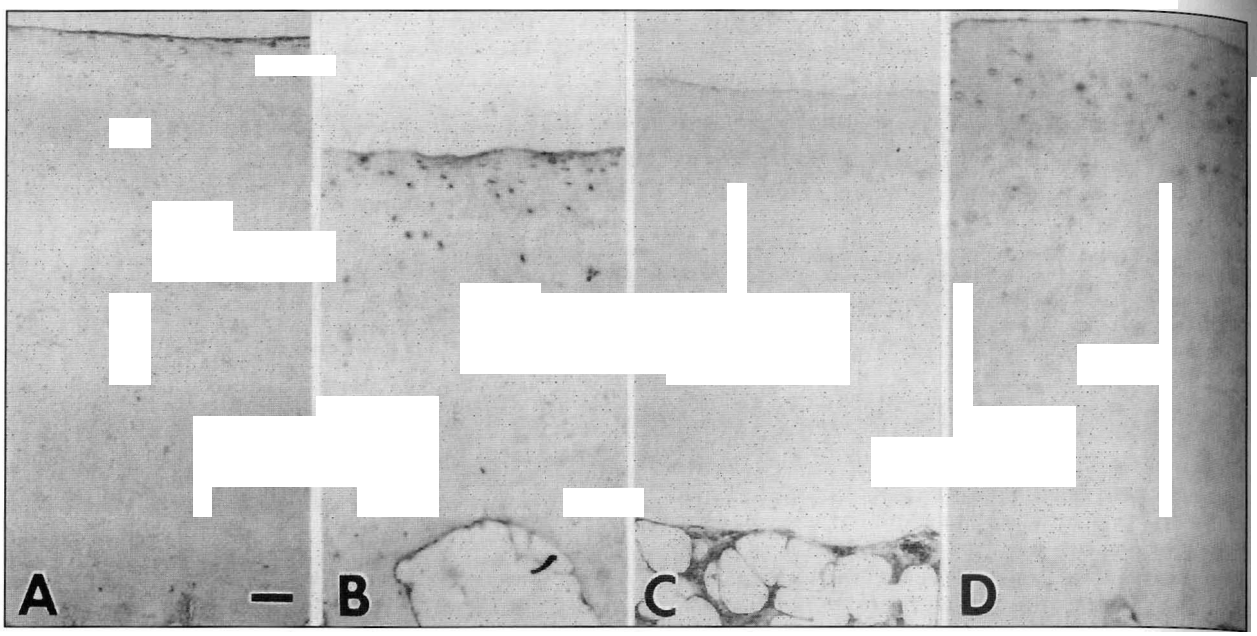

Figure 2—Photomicrographs of articular cartilage sections from left medial femoral condyles illustrating chondrocytic and matrical immunolocalization of stromelysin. Immunolocalization is lacking in the untreated control (A) and cast/HA-treated (C) dogs. However, immunolocalization was present in chondrocytes and matrix of the superficial and middle zones of the cast control (B) and cast/HA/TGF-β-treated (D) dogs, as well as those in the deep zone of the latter. Antistromelysin stain; bar = 50 μm.

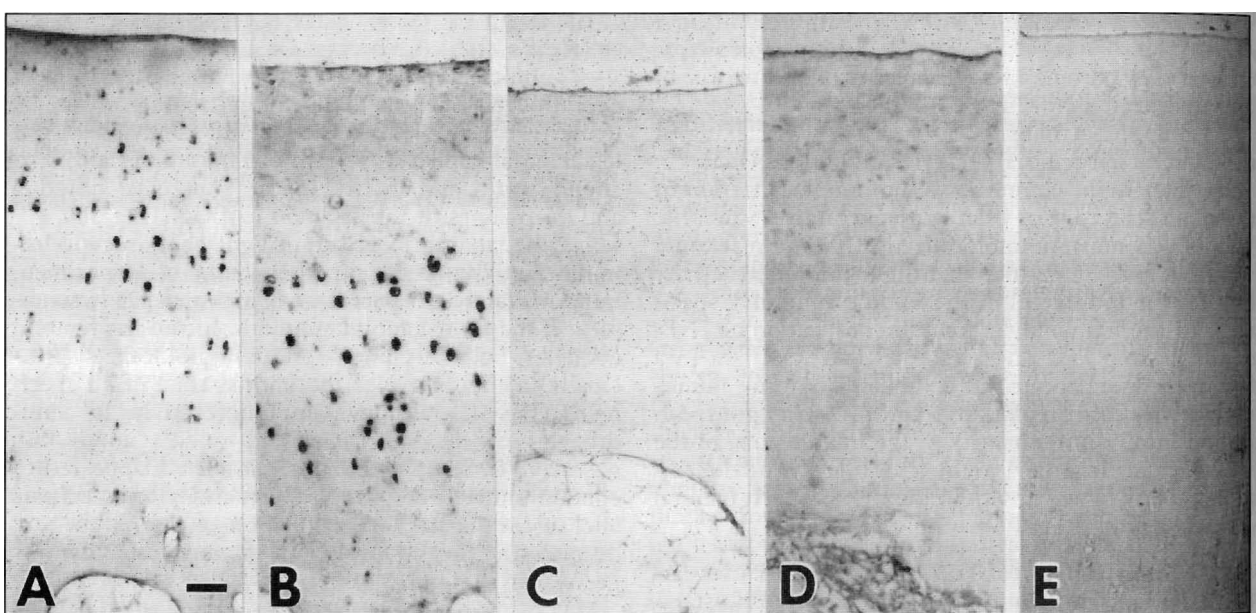

Figure 3—Photomicrographs of sections of articular chondrocytes of left medial femoral condyles illustrating tumor necrosis factor (TNF) α immunolocalized in untreated control (A), cast control (B), and cast/HA/TGF-β-treated (D) dogs, as well as in the matrix of cast control and cast/HA/TGF-β-treated dogs. The TNF-α was not immunolocalized in articular cartilage from the left (C) or the right (E) femoral condyles of this cast/HA-treated dog. Anti-TNF-α stain; bar = 50 μm.

melysin. The degradative process appears to be regulated at least in part by TNF-α, which is known to increase the production of metalloproteinase enzymes.[32-35] This coordinated degradative action results in the release of proteoglycan fragments into synovial fluid.[p] In this study, the immunolocalization of stromelysin in regions where metachromatic staining was decreased and TNF-α was immunolocalized supports the interrelation of the 2 molecules. The absence of stromelysin in the control group and uncast (right) limbs of all groups coincided with reports by other investigators.[30,34,q] Concomitantly, immunolocalization of stromelysin in chondrocytes[30,34,q] and matrix in atrophic cartilage in this study is similar to osteoarthritic articular cartilage.[30,34,36,37,q]

Hyaluronic acid is considered to have a stabilizing influence on degenerating articular cartilage, although its mechanism of action has not been clearly defined.[23-29] Restoration of rheologic properties of synovial fluid by viscosupplementation with high molecular weight HA has been suggested.[38] This concept is consistent with degradation of HA from phospholipid

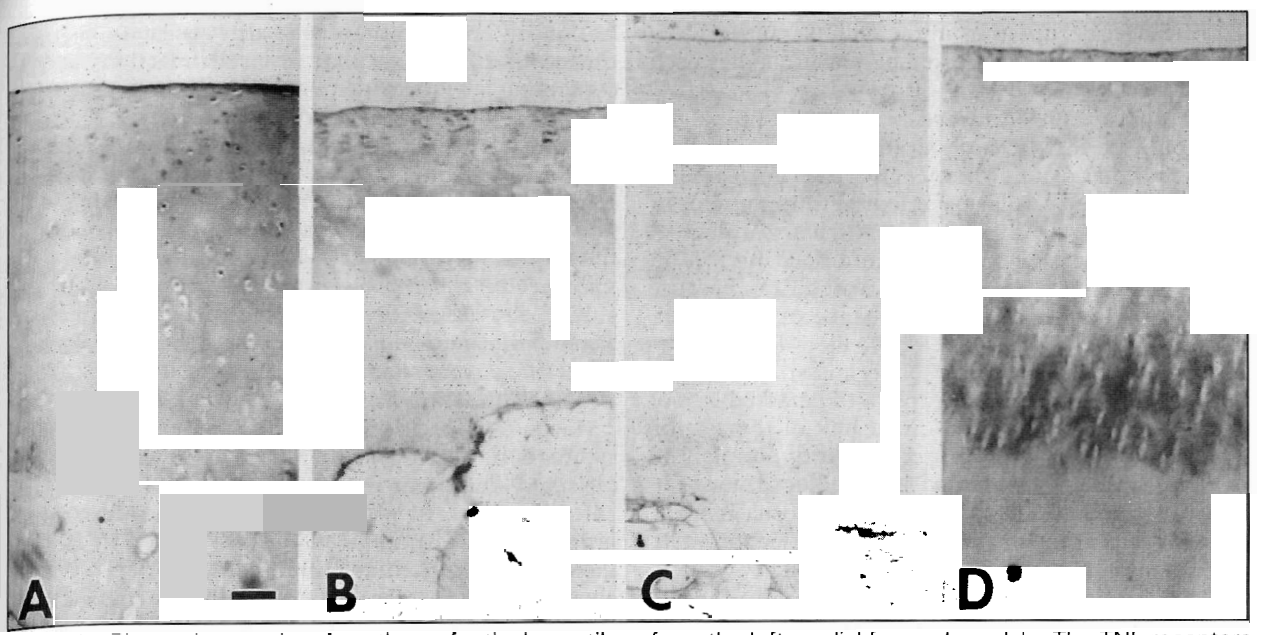

Figure 4—Photomicrographs of sections of articular cartilage from the left medial femoral condyle. The TNF receptors were immunolocalized in chondrocytes of an untreated control dog (A). Immunolocalization in cast control dogs (B) was principally concentrated in chondrocytes and matrix of the superficial zone. Immunoreactivity was not observed in the articular cartilage of cast/HA-treated dogs (C); however, dense immunoreactivity was located in the middle and deep zones of cast/HA/TGF-β-treated dogs (D). Anti-TNF receptors stain; bar = 50 μm.

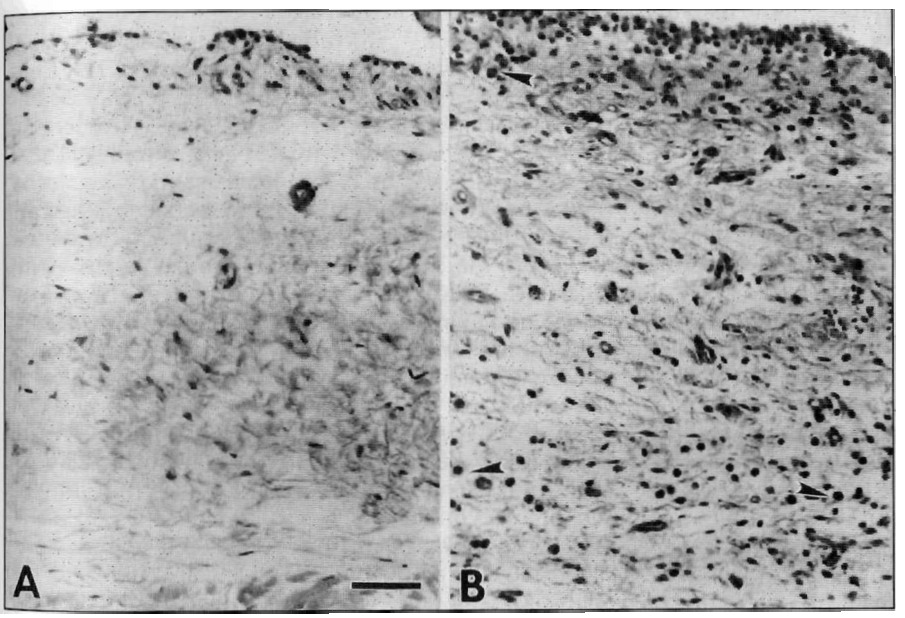

Figure 5—Photomicrograph of a section of periarticular synovial membrane from the left stifle that is representative of the cast control group (A). Hyperplasia of the synovial membrane and mononuclear inflammatory cells (arrowheads) are visible in this photomicrograph of the periarticular synovial membrane from dogs of the cast/HA/TGF-β-treated group (B). H&E stain; bar = 50 μm.

bonding or depolymerization induced by oxygen-derived free radicals.[39] Alternatively, analgesic or anti-inflammatory actions of HA, or both could be central to the therapeutic efficacy of HA.[39,40-42]

Hyaluronic acid decreases the release of matrical proteoglycans.[23-25,27-29,43-45] In this study, staining for proteoglycans was retained and concurrent immunolocalization of stromelysin was absent in the atrophic articular cartilage of dogs in the HA-treated group. These observations support the suggested local chondroprotective influence of HA on immobilized articular cartilage[24,31,46] and articular cartilage predisposed to OA.[26-28] Thus, this study provides in vivo evidence of down-regulation of stromelysin and/or its regulatory pathway through TNF-α as a mechanism of action of HA.[47]

Tumor necrosis factor α was localized in chondrocytes of the middle and deep zones of the articular cartilage of the untreated control group. This observation disagreed with that of Pickvance et al,[48] who observed chondrocytic and matrical immunolocalization of TNF-α in early osteoarthritic lesions but not in control articular cartilage. However, the observation agreed with the report by Kammermann et al,[9] who suggested that TNF-α had a role in regulating the metabolism of proteoglycans in normal articular cartilage. The reason for the discrepancy among studies is not apparent, but may result from differences in processing of the artic-

ular cartilage specimens or in the epitope on the TNF-α molecule that was recognized by the antibodies used in the respective studies.

During atrophy of articular cartilage, proteoglycans could be lost by normal attrition of the matrix because synthesis of new proteoglycans is decreased in atrophic articular cartilage.[9,49] However, immunolocalization of TNF-α in the atrophic articular cartilage of this study suggests that TNF-α continued its role in the regulation of proteoglycan catabolism. Because TNF-α mediates its catabolic influence on proteoglycans through the action of enzymes, such as stromelysin,[27,34,50] localization of TNF-α and stromelysin in the atrophic cartilage could explain the marked loss of proteoglycans. Although no direct link of the regulatory action of TNF-α to up-regulation of stromelysin was found other than mutual immunolocalization, in vitro treatment of slices of normal and osteoarthritic articular cartilage with TNF-α has been reported to increase immunolocalization of stromelysin.[34]

Tumor necrosis factor α also was observed in the articular cartilage of the untreated (right) limbs of all groups of the present study, except dogs that were treated with HA alone. This observation suggests that HA had a systemic influence on TNF-α expression that has hitherto not been reported, to the authors' knowledge. Additional support for this systemic effect is provided by the ability of HA to inhibit the secretion of TNF-α by cultured monocytic cell lines.[51] The inability of HA to prevent the expression of TNF-α in the TGF-α/HA-treated group may have resulted from accelerated catabolism of the exogenous HA by hyaluronidase that is present in synovial fluid[52,53] or oxygen-derived free radicals originating from mononuclear inflammatory cells, such as those observed in the synovial membrane.[54] Alternatively, excess production of TNF-α by the latter cells may have resulted from the stimulatory influence of the large amount of TGF-β injected into the joint.[55]

The origin of TNF-α was not determined by us. However, immunolocalization of TNF-α in chondrocytes in control articular cartilage suggests that they produced the molecule. The uniform localization of TNF-α in chondrocytes and matrix of the superficial zone of atrophic articular cartilage of cast control limbs unlike untreated control cartilage suggests that, as atrophy of the cartilage progressed, the chondrocytes may have released TNF-α into the surrounding matrix. This hypothesis is supported by other investigators who have reported that chondrocytes are capable of production of TNF-α.[35,56] Alternatively, TNF-α may have originated from mononuclear cells, such as activated monocytes and macrophages, that are a major source of TNF-α and can be located in the synovial membrane.[57] Regardless of its origin, the expanded immunolocalization of TNF-α into chondrocytes and matrix of the superficial zone of atrophic cartilage, compared with untreated controls, suggests that the expression of TNF-α was increased in atrophic cartilage.

The immunolocalization of TNF receptors of the treated and contralateral untreated limbs also was influenced by HA. Although TNF receptors were immunolocalized in the articular cartilage of both limbs of the untreated control and the cast limb of the cast control groups, immunolocalization was not observed in either limb of the HA-treated group. Immunolocalization of TNF receptors was also absent in the untreated limbs of dogs that received injections of HA containing TGF-β in the contralateral cast stifle. This result was unexpected because chondrocytic localization of TNF-α was present in the same limbs. The reason for this finding is not known but could be a result of differential action of HA or its subunits on the expression of TNF-α and TNF receptors. Collectively, the results suggest that HA had a local and a systemic influence on the expression of TNF receptors. The resulting absence of TNF-α, its receptors, and, in turn, stromelysin may be a basis for the chondroprotective effect of HA on articular cartilage.

Results of in vitro studies of TGF-β and articular cartilage indicated decreased destruction and enhanced synthesis of proteoglycans.[14-16] However, Elford et al[58] observed that intra-articular injection of TGF-β resulted in loss of proteoglycans. In this study, intra-articular administration of TGF-β resulted in loss of metachromatic staining throughout the depth of the atrophic articular cartilage, indicative of loss of proteoglycans. The pathogenesis of the degradation of proteoglycans after intra-articular injection of TGF-β has not been clarified, but may have been mediated by metalloproteinase enzymes. The immunolocalization of stromelysin in all zones of the articular cartilage of all dogs treated with TGF-β, compared with that in only the superficial and middle zones in cast control dogs, supports this hypothesis.

Transforming growth factor β has also been documented to down-regulate interleukin 1 and TNF-α in animal models of OA.[59,60] However, the prominent immunolocalization of TNF-α, in addition to stromelysin, in dogs of the TGF-β/HA-treated groups indicates that the composite was unable to decrease the presence of this catabolic pathway in atrophic cartilage. Likewise, the TNF receptors predominately located in the matrix of dogs in the TGF-β/HA-treated groups may have indicated that these receptors were up-regulated and released into the matrix once they bound with the increased amounts of TNF-α. The up-regulation of stromelysin and TNF-α in the cartilage of the TGF-β/HA-treated groups may have been mediated by cytokines from the synovial fluid. Transforming growth factor β has been documented to chemotactically recruit and activate circulating monocytes, resulting in release of cytokines such as interleukin 1 and TNF-α.[61,62] The high amount of TGF-β used in this study may have elicited the mononuclear cell response in the synovial membrane, which could have resulted in the release of cytokines into synovial fluid that subsequently diffused into the articular cartilage or mediated degradation of the exogenous HA.

The potential for TGF-β to elicit undesirable secondary effects, such as local connective tissue proliferation at sites of administration,[55,63] was recognized before the study was initiated. To counteract these effects, TGF-β was administered in HA as a carrier vehicle for injection. In addition to being a normal constituent of synovial fluid and having a stabilizing influence on matrical proteoglycans, HA has been documented to decrease scar formation and angiogenesis,[29] inhibit proliferation and chemotaxis of lymphocytes,[40,41] and reduce mobility of macrophages.[42] Exogenous HA can diffuse into intact articular cartilage within hours[20] and has a half-life of approximately 12 to 96 hours in

synovial joints that is species dependent.[21,22] Because of these characteristics, HA was considered to be a useful carrier of TGF-β to potentially moderate the side effects of TGF-β, retain it in the articular cartilage for an extended period, and slow exposure of the synovial membrane to TGF-β.

Unfortunately, the clinical response of stifles injected with the composite of TGF-β and HA was accentuated by secondary effects of joint capsule thickening and angiogenesis that were attributable to TGF-β.[58,64] Thus, HA was unable to counteract the secondary effects that were attributable to the high doses of TGF-β. Transforming growth factor β has been documented to have angiogenic or antiangiogenic properties.[63,65,66] In this study, invasion of blood vessel ingrowth from the subchondral bone developed only in dogs of the TGF-β-treated groups. Alternatively, the angiogenesis may have been indirectly mediated by TGF-β through the up-regulation of TNF-α from activated synovial monocytes, as described earlier. The inability of HA to ameliorate the secondary effects of TGF-β probably resulted because of the aggressive destruction of HA or excess production of TNF-α as previously discussed. It remains to be determined whether HA could have prevented the secondary effects of TGF-β at lower doses of the latter.

An alternative explanation of the inability of TGF-β to maintain proteoglycans in the matrix may be based on the potential influence of the HA administered with the TGF-β on proteoglycan synthesis. Hyaluronic acid is regulated through hyaluronan-binding proteins.[67] When excess HA is in the milieu, there may not be enough binding proteins to effectively bind the HA. High concentrations of HA have been found to have a negative effect on chondrogenesis.[67] Thus, TGF-β may have been unable to maintain or increase proteoglycan synthesis because of the excess HA in the joint.[67] The concomitant increased inflammatory response mediated by TGF-β and decreased chondrogenesis resulting from exogenous HA may have negated the local and systemic chondroprotective effects of HA.

Although the TGF-β/HA composite used in this study was unable to provide a positive effect on atrophic cartilage, the ability of HA to decrease the presence of inflammatory mediators on a systemic as well as a local basis was important. This observation provides a potential basis for the chondroprotective action of HA in synovial joints and for the continued clinical use of HA for treatment of joint diseases. Further studies are needed to determine how HA exerts its systemic influence on articular cartilage, albeit the variable molecular size of HA, binding affinities with the HA-binding protein receptors on the different cells, or potentially different mechanisms.[67] However, HA has been found to be chondroprotective, and results of this study explain that down-regulation of TNF-α and stromelysin may be one of the possible mechanisms for HA's clinical efficacy.

[a]Hyalovet, Fort Dodge Laboratories, Fort Dodge, Iowa.
[b]TGF-β2, Celtrix Pharmaceuticals Inc, Santa Clara, Calif.
[c]Atropine, Vedco Inc, St Louis, Mo.
[d]Acepromazine, Fort Dodge Laboratories, Fort Dodge, Iowa.
[e]Tiletamine HCl and Zolazepam HCl, Fort Dodge Laboratories, Fort Dodge, Iowa.
[f]Vet Cast Plus, 3M Animal Care Products, St Paul, Minn.
[g]Socumb, The Butler Co, Columbus, Ohio.
[h]VIP 3000, Miles Inc, Elkhart, Ind.
[i]Paraplast X-tra, Fisher Scientific, Pittsburgh, Pa.
[j]Reichert-Jung 2050 microtome, Leica Inc, Deerfield, Ill.
[k]Courtesy of Bayer Inc, Westhaven, Conn.
[l]Sigma Chemical Co, St Louis, Mo.
[m]ABC Elite Kit, Vector Labs Inc, Burlingame, Calif.
[n]DAB, Sigma Chemical Co, St Louis, Mo.
[o]American Optical, Leica Inc, Deerfield, Ill.
[p]Lohmander LS, Walakovitz LA, Lark MW. Metalloproteinases, tissue inhibitor and proteoglycan fragments in knee synovial fluid in human osteoarthritis (abstr), in *Proceedings*. 38th Annu Meet Orthop Res Soc 1992;273.
[q]Kammermann JR, Kincaid SA, Rumph PF, et al. Immunolocalization of TNF-α and TNF receptors in iatrogenically-induced model of canine osteoarthritis (abstr), in *Proceedings*. 41st Annu Meet Orthop Res Soc 1995;20:317.

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