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CHANGES IN CARTILAGE COMPOSITION AND PHYSICAL PROPERTIES DUE TO STROMELYSIN DEGRADATION

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Objective. To determine the effects of stromelysin treatment on biochemical, histologic, and swelling characteristics of intact cartilage explants and to correlate these effects with changes in the functional physical properties of the tissue.

Methods. Bovine articular cartilage explants were cultured for up to 3 days in the presence or absence of recombinant human stromelysin (SLN). Damage to matrix proteoglycans and collagens was assessed and characterized by N-terminal sequencing and Western blot analysis, respectively. Explants were mechanically tested to assess the ability of the tissue to withstand cyclic and static compressive loads.

Results. Treatment with SLN resulted in a time- and dose-dependent loss of proteoglycans from cartilage explants, with significant loss seen after 3 days of exposure to 20 nM SLN. Histology indicated that initial loss of proteoglycans occurred in regions near the tissue surface and proceeded inward with increasing time of SLN exposure. SLN treatment resulted in degradation of matrix collagen types IX and II, and a concomitant increase in tissue swelling. This matrix degradation resulted in severe alterations in functional physical properties of the tissue, including compressive stiffness. The initial, focal loss of proteoglycans that resulted from

SLN treatment was most accurately detected with high-frequency streaming potential measurements.

Conclusion. Exposure of intact cartilage to SLN caused specific, molecular-level degradation of matrix molecules, which resulted in changes in the swelling behavior and marked deterioration of functional physical properties of the tissue.

Articular cartilage is an avascular, aneural, alymphatic, connective tissue which covers the ends of bones in synovial joints. Its primary functions are to distribute loads over bone surfaces and provide a low-friction surface over which bones can move. Articular cartilage is a porous, highly hydrated material, with water content of 70–80% by volume (1). The solid component of cartilage consists of an extracellular matrix and a sparse population of chondrocytes, present in a concentration of approximately 10 to 100 × 10⁶ cells/ml (2). The cartilage matrix is composed primarily of hydrated collagen fibrils, highly charged proteoglycan molecules, and other glycoproteins (3,4). The osmotically swollen matrix and the high water content are mainly responsible for the complex mechanical behavior that characterizes the response of the tissue to physiologic loads (5).

In osteoarthritis (OA), the combination of altered cartilage matrix composition and mechanical wear from joint motion can result in erosion of cartilage down to the bone surface (6). In its degenerated state, OA cartilage exhibits dramatically different biochemical, physicochemical, mechanical, and electro-mechanical properties from normal tissue. OA cartilage shows a distinct loss of proteoglycan (7,8), marked collagen network fibrillation (9,10), increased tissue hydration (11,12), and loss of compressive stiffness (11,13).

The role of enzymatic degradation in OA has been studied, but is not completely understood. It has

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been suggested (14) that families of enzymes, including metalloproteinases (15–18), serine proteases (19), and a novel "aggrecanase" (20) contribute to cartilage degradation in OA. Given that various enzymes have specific expression patterns (21,22) and some act as activators of other enzymes (23–25), it seems likely that OA involves a "cascade" of enzymatic activities that work in tandem to degrade the tissue.

Alterations of biochemical composition and matrix structure that result from enzyme activity are manifest in changes in cartilage material properties and physical behavior. Enzymatic degradation has been shown to result in decreased compressive stiffness (26), changes in tensile stiffness, strength, and fracture strain (19,27), decreased shear modulus (28), changes in creep behavior (19,27), and increased indentation displacement (26). In addition, marked decreases in compression-induced streaming potential have been shown to be particularly sensitive indicators of alterations in matrix proteoglycan composition associated with enzymatic degradation (29).

Stromelysin 1, also known as matrix metalloproteinase 3, is a neutral metalloproteinase produced by chondrocytes and synovial fibroblasts (17,21,22). Prostromelysin has a molecular weight of 55 kd, which can be cleaved by plasmin, trypsin, or organomercurials to produce 45-kd, 22-kd, and 19-kd active forms which cleave several different cartilage matrix components, including aggrecan (16,30), link protein (16), and collagen types II, IX, X, and XI (31).

Stromelysin (SLN) has been shown to be present in arthritic tissue and has been implicated in normal matrix turnover, as well as remodeling and development. It has been found in elevated levels in cartilage and synovial fluid in patients with degenerative OA (17,32), as well as in animal models of OA (15,18). Elevated levels have likewise been detected in cases of knee joint injury (32) and heat shock (33). There is also evidence of SLN gene expression in early stages of soft tissue development (34), as well as immunolocalization of stromelysin in cartilage growth plate (35).

The manner in which stromelysin degrades articular cartilage extracellular matrix constituents has been studied extensively using solutions of isolated matrix molecules in vitro. It has been shown that SLN cleaves the Asn³⁴¹-Phe³⁴² bond in the interglobular domain of purified human aggrecan, generating a large chondroitin sulfate-bearing product with the N-terminal sequence ³⁴²Phe-Phe-Gly-Val, and liberating the G1 domain, with the C-terminal sequence

Ile-Pro-Glu-Asn³⁴¹, from the remainder of the molecule (30,36). Significantly, this form of the G1 domain is also present in human articular cartilage, providing evidence for SLN activity *in situ* (30). Likewise, SLN cleaves the His¹⁶-Ile¹⁷ bond of link protein, and this product has also been detected in human cartilage (37).

In addition to aggrecan and link protein, SLN has been shown to be effective at degrading the 3 major types of collagen (II, IX, and XI) present in articular cartilage. SLN removes short telopeptide sequences from the $\alpha 1$ (II) chain, and cleaves the cross-linking hydroxylysine segments, effectively eliminating this link between $\alpha 1$ (II) chains (31). SLN separates the COL1 domain from the COL2 and COL3 domains of type IX collagen by means of a cleavage of the $\alpha 1$ (IX), $\alpha 2$ (IX), and $\alpha 3$ (IX) chains in the NC2 region (31). In addition, it was noted that SLN could liberate a large globular segment from the $\alpha 1$ (IX) chain of the NC4 domain. These combined actions have the effect of decoupling the type IX-type II collagen complex (31).

Studies on the effects of SLN on cartilage matrix constituents have thus far focused on degradation of isolated matrix constituents in solution. However, the effects of SLN on the composition and physical properties of intact cartilage have received less attention. The primary objectives of this work were to determine the effects of SLN treatment on the biomechanical and electromechanical properties of cartilage explants and to correlate the extent and kinetics of changes in these material properties to changes in certain biochemical, histologic, and physicochemical characteristics of the tissue.

MATERIALS AND METHODS

Cartilage explant and culture. Saddle sections of 1–2-week-old calves were obtained from a local abattoir (A. Arena, Hopkinton, MA) within 4 hours of slaughter. The femoropatellar groove was isolated and 3-mm diameter by 1-mm thick cartilage disks were harvested using a dermal punch (Miltex Instruments, Lake Success, NY) and a sledge microtome (model 860; American Optical, Buffalo, NY), as described previously (38). Recombinant human prostromelysin (200 μ g/ml) in 25 mM Tris HCl, 10 mM CaCl₂, 0.05% Brij 35, and 0.01% NaN₃ (39) was activated with trypsin, as described previously (40). The enzyme had specific activity as native human gingival fibroblast SLN using ³H-transferrin as a substrate (40).

The enzyme was concentrated to ~2 mg/ml using a Centricon 10 ultrafiltration membrane (Amicon, Beverly, MA) and diluted into media for tissue culture experiments. Cartilage disks were incubated in groups of 4 in 24-well culture dishes (Costar, Cambridge, MA) in 1 ml of Dulbec-

co's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) containing 100 units/ml of penicillin G and 100 µg/ml of streptomycin (Gibco) for approximately 16 hours at 37°C in a 5% CO₂ atmosphere. Groups of 4 disks were then placed in 1 ml of DMEM containing graded levels of enzymes, including recombinant human stromelysin 1, trypsin (type III from bovine pancreas; Sigma, St. Louis, MO), or chondroitinase ABC (protease free; Seikagaku America, Rockville, MD), and incubated for various periods (up to 72 hours). Upon removal from culture, groups of plugs were allocated for biochemical, histologic, physicochemical, and biomechanical analyses.

Biochemical analysis. Disks allocated for biochemical analysis were frozen at -20°C, lyophilized, and digested with 1 ml of 125 µg/ml papain digestion solution (Sigma), as described previously (38). Digests and culture media (20-µl portions) were assayed for sulfated glycosaminoglycans (GAG) by reaction with 2 ml of dimethylmethylene blue dye solution in polystyrene cuvettes (VWR, Boston, MA) and spectrophotometry (model λ3B; Perkin Elmer, Norwalk, CT) (41), using whale/shark chondroitin sulfate (Sigma) as the standard.

Aggrecan fragments released into the medium during SLN digestion were purified into the D1 fraction of a CsCl gradient. Core proteins were prepared by enzymic deglycosylation, Superose 12 chromatography, and desalting, and the amino-termini were determined as previously described (20). In addition, portions of medium containing 100 µg of GAG were analyzed by chromatography on Sepharose CL-2B in the presence of 4% (weight/weight) link protein and 4% (w/w) hyaluronan, and fractions were assayed for sulfated GAG.

Degradation products of type IX collagen were detected in the stromelysin-treated disks and culture media by Western blot analysis. Rabbit antiserum against purified bovine type IX collagen containing structurally intact COL1, COL2, COL3, NC2, and NC3 domains was produced as described previously (42). The antiserum reacted specifically with type IX collagen and did not cross-react with any of the noncollagenous matrix proteins of cartilage or with collagen types I, II, III, V, VI, or XI. Tissue slices (14 mg) from SLN-treated bovine plugs were extracted with 200 µl of a 1% (weight/volume) sodium dodecyl sulfate (SDS) solution containing 0.05 M dithiothreitol to cleave disulfide bonds. Extracts were fractionated by SDS-polyacrylamide gel electrophoresis and electroblotted onto a PVDF membrane for Western blot detection of type IX collagen degradation products. The concentration of type IX collagen fragments in culture medium was also quantified by competitive enzyme-linked immunosorbent assay (ELISA) on a microtiter plate using this antiserum.

Histology. Cartilage disks were fixed with ruthenium hexaamine trichloride (RHT) and stained with toluidine blue O as described by Hunziker et al (43). Each disk was fixed in a 2% (v/v) glutaraldehyde (Polysciences, Warrington, PA) solution buffered with 0.05M sodium cacodylate (Bio-Rad, Richmond, CA) and containing 0.15 M NaCl (pH adjusted to 7.4), with 0.7% (w/v) RHT powder (Polysciences) added 10 minutes prior to fixation. Samples were fixed at room temperature for 4 hours and an additional 16 hours at 4°C. Samples were then washed 3 times for 10 minutes at room

temperature in a solution containing 0.075M NaCl and 0.1M sodium cacodylate (pH 7.4) and stored in 1 ml of 70% ethanol solution at 4°C. Tissue was embedded in paraffin, and thick sections ($5 \pm 1 \mu\text{m}$) were stained with toluidine blue O for analysis in the light microscope.

Swelling studies. The protocol for swelling studies was motivated by the technique used by Maroudas (12) to compare the swelling of normal and OA cartilage. Disks removed from culture medium were patted briefly with a paper towel to remove surface water, and wet weights were measured (AE 163 Balance; Mettler Instrument Corp, Hightstown, NJ). Disks were then twice reequilibrated in a hypotonic saline solution (0.01M NaCl at pH 7.0) for 1 hour at room temperature. Surface water was again removed and wet weights were measured. Following wet weight measurements, disks were lyophilized, and dry weights were recorded.

Biomechanical and electromechanical evaluation. Cartilage disks were placed in an electrically insulating polymethylmethacrylate cylindrical confining chamber, similar to that used by Frank et al (29). The chamber was mounted in a servo-controlled Dynastat mechanical spectrometer (IMASS, Hingham, MA) which was interfaced to a computer and frequency generator (model 5100; Rockland Systems, West Nyack, NY). Samples were equilibrated at room temperature in 0.15M phosphate buffered saline (PBS; pH 7.4) containing 100 units/ml of penicillin G and 100 µg/ml of streptomycin. Disks were subjected to confined compression between a porous polyethylene platen and a 6.35-mm Ag/AgCl pellet electrode (Annex Research, Costa Mesa, CA) mounted at the base of the chamber. An identical electrode was mounted in the surrounding PBS bath.

Disks were first compressed by sequential increments of 0.5–1.5% strain, up to a maximum of 20% total strain. After stress relaxation, the equilibrium stress corresponding to each increment of static strain was detected with the load cell of the Dynastat and recorded on the computer. The resultant equilibrium stress was plotted against applied strain, and the slope of the best-fit linear regression curve gave the equilibrium confined-compression modulus. At a given static offset strain, sinusoidal strains of <1% amplitude were superimposed on the static strain at frequencies ranging from 0.01 Hz to 1 Hz. The resultant oscillatory load was detected by the load cell, and the oscillatory streaming potential was detected by the chamber electrodes, which were connected to a high-impedance universal amplifier (model 11-4113-02; Gould, Cleveland, OH). Load and streaming potential were simultaneously recorded by the computer. The amplitude of the dynamic load was normalized to the disk area and to the amplitude of the applied strain to give dynamic stiffness, while the amplitude of the streaming potential was normalized to the amplitude of the applied strain (44,45).

Equilibrium modulus and dynamic stiffness data were used in combination with the method of Frank and Grodzinsky (45) to calculate the effective hydraulic permeability of the tissue specimen. In all tests the amplitude of the applied strain was chosen to be small enough to elicit a linear mechanical response from the sample (44,45) (i.e., the total harmonic distortion in the measured load was <10%). The

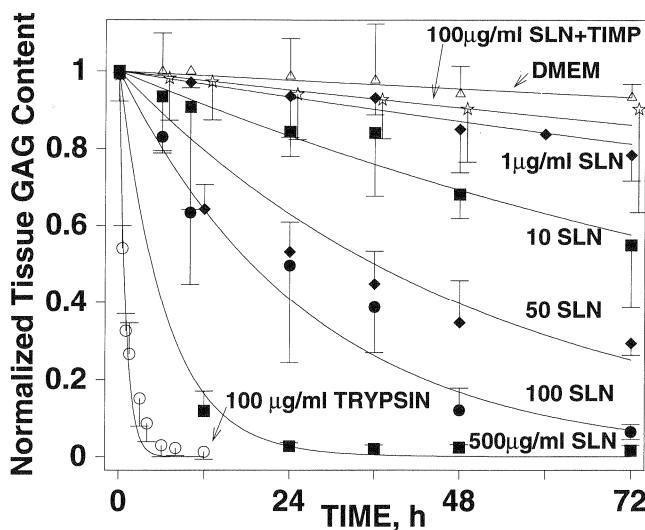


Figure 1. Normalized glycosaminoglycan (GAG) loss from cartilage disks incubated in Dulbecco's modified Eagle's medium (DMEM) with graded levels of recombinant human stromelysin 1 (SLN), 100 µg/ml SLN + 100 µg/ml tissue inhibitor of metalloproteinase (TIMP), or 100 µg/ml trypsin. Values are the mean ± SD ($n = 4$) GAG remaining in the tissue normalized to total GAG (tissue + media GAG) at each time point. Solid lines are best-fit singles exponential decay curves of the form $le^{-t/\tau}$. The best-fit decay times are $\tau = 344$, 130, 52, 26, and 6.6 hours for respective SLN concentrations of 1, 10, 50, 100, and 500 µg/ml; $\tau = 2.8$ hours for 100 µg/ml trypsin, $\tau = 624$ hours for 100 µg/ml SLN + 100 µg/ml TIMP, and $\tau = 1,019$ hours for DMEM alone.

porous platen remained in contact with the sample at all times during the testing cycle.

RESULTS

Findings of biochemical analysis. Treatment with SLN induced both a time- and dose-dependent loss of proteoglycan constituents from bovine cartilage explants (Figure 1). The data are reported as the amount of GAG remaining in the tissue normalized to the total GAG (tissue + media GAG) at that time point, versus time in culture for disks incubated in media containing SLN (concentration range 1–500 µg/ml; 20 nM to 10 µM). Control tissue at time zero contained 270 ± 31 µg of GAG/mg dry weight (mean ± SD). For comparison between SLN concentrations, the time course of GAG loss was fit to an exponential of the form $e^{-t/\tau}$, giving decay times ranging from 344 hours at 1 µg/ml to 6.6 hours at 500 µg/ml. GAG loss clearly increased with the SLN dose, with maximal loss achieved in 72 hours for concentrations exceeding 100 µg/ml.

Control disks incubated in DMEM alone showed little GAG loss. By comparison, disks incubated in media containing 100 µg/ml trypsin demonstrated maximal GAG loss in ~3 hours, while disks incubated with 100 µg/ml SLN and a 2-fold molar excess of tissue inhibitor of metalloproteinases (TIMP) showed GAG loss that was indistinguishable from that of the controls.

To examine the effect of SLN treatment on the structure of the released aggrecan, media from the disks exposed to 100 µg/ml SLN for 72 hours was first evaluated for aggregability with hyaluronan on associative Sepharose CL-2B. The profile (Figure 2A) showed that the fragments eluted as a broad peak over K_{av} range of 0.3–0.7, and >95% were nonaggregating. This indicated that stromelysin had cleaved the aggrecan to separate the chondroitin sulfate-rich region from the hyaluronan-binding region and, additionally, had cleaved the chondroitin sulfate-rich region in several places.

This interpretation was supported by N-terminal sequence analysis of the aggrecan fragments which gave evidence for at least 2 cleavage products (Figure 2B). One sequence, detected at 5 pmoles, was FFGVXGX, and this is consistent with the known interglobular cleavage of the Asn³⁴¹-Phe³⁴² bond in human aggrecan (30,36). Another sequence, detected at about 10 pmoles, was IXGLP, and this is consistent with cleavage between aspartic acid and isoleucine residues in the sequence GVEDISGLP, which is present in the chondroitin sulfate-rich region of the bovine aggrecan core protein (46). N-terminal sequencing showed no evidence of cleavage products which could not be attributed to SLN.

In addition to aggrecan degradation, SLN treatment resulted in degradation of type IX collagen, as evidenced by the presence of type IX collagen fragments in the culture media of disks treated with 100 µg/ml of SLN for 72 hours (Figure 3A). The ELISA showed that the level of immunoreactive type IX collagen fragments in the culture media of treated tissue was 3 times higher than that of controls. Western blot analysis (Figure 3B) indicated that more of the immunoreactive type IX collagen fragments were extracted from SLN-treated cartilage disks than from untreated controls.

The identity of each type IX fragment and the sites of SLN cleavage remain to be determined. However, the fragments are large and do not appear to be equivalent to those produced in vitro from monomeric type IX collagen molecules by SLN (31). Similar

analyses using anti-type II collagen antibodies also revealed the presence of elevated levels of type II collagen fragments in extracts of treated explants and in the incubation media (data not shown).

Findings of histologic analysis. The loss of proteoglycan constituents from SLN-treated disks as determined by biochemical analysis was reflected in the absence of toluidine blue staining of SLN-treated disks (Figure 4B) compared with untreated disks (Figure 4A). While control disks showed toluidine blue

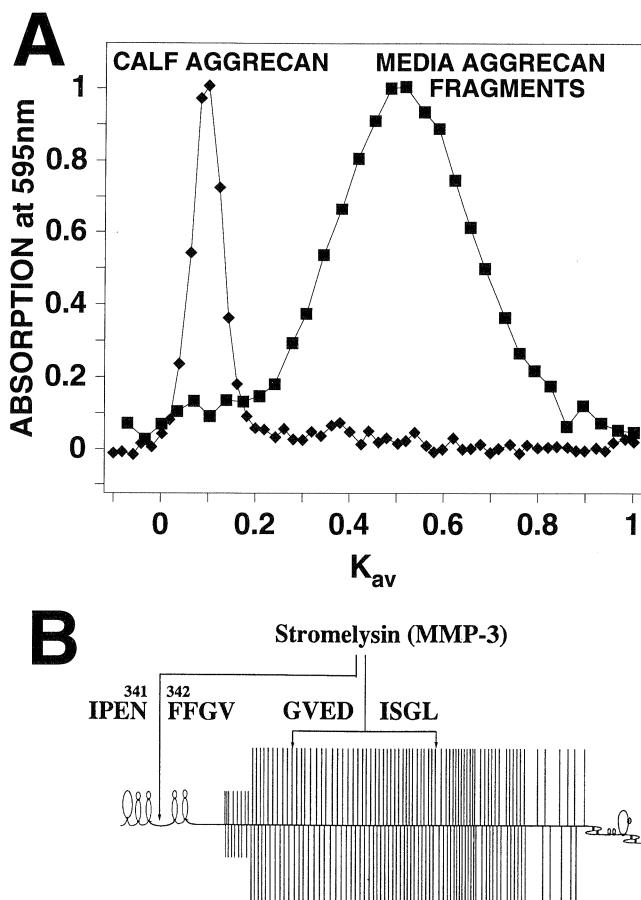


Figure 2. A, Sepharose CL-2B fractionation of culture media. A portion of medium from disks treated with 100 $\mu\text{g}/\text{ml}$ SLN for 72 hours containing 100 μg GAG was mixed with 4% (w/w) hyaluronan and 4% link protein and fractionated under associative conditions. Also shown is the elution profile of control calf aggrecan A1D1 fractionated under the same conditions. Control A1D1 eluted in the void volume of $K_{av} = 0.1$, while aggrecan fragments in the media from SLN-treated disks eluted with a broad profile of $K_{av} = 0.5$. B, Schematic diagram showing suggested sites of stromelysin cleavage of bovine aggrecan. The sequences and residue numbers shown are based on bovine data obtained in the present studies and the original human complementary DNA sequence (56). See Figure 1 for definitions.

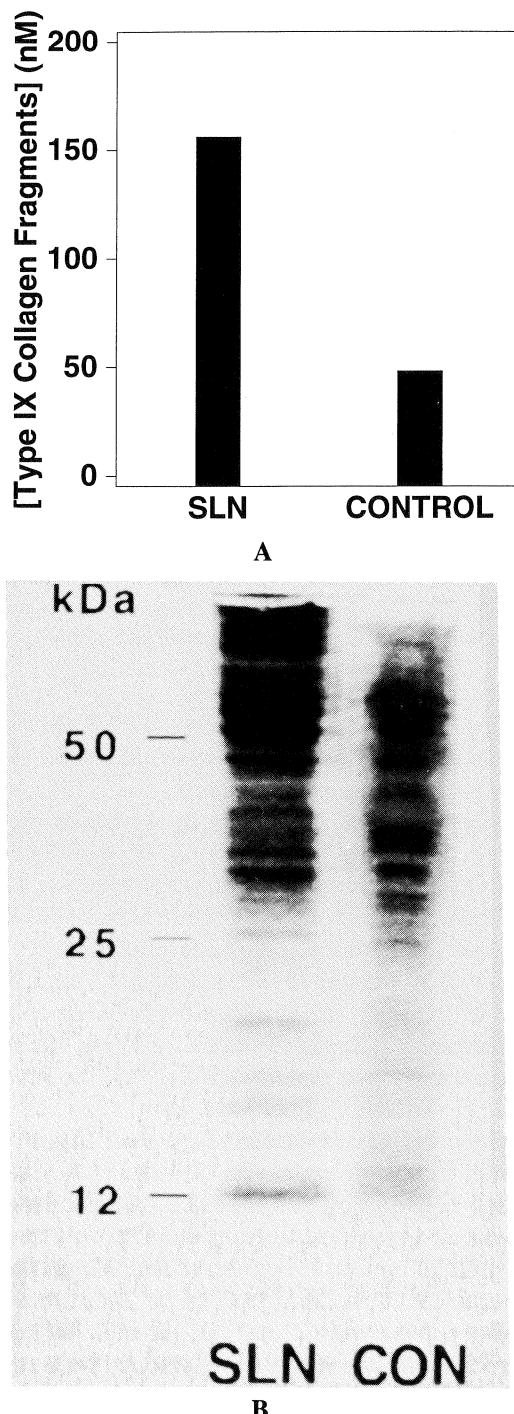
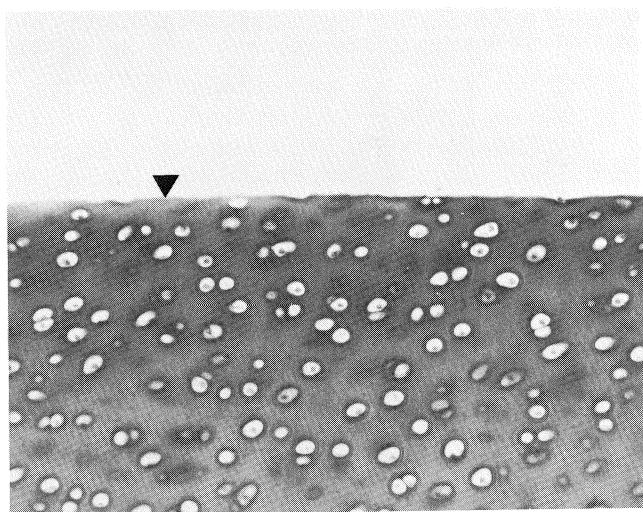
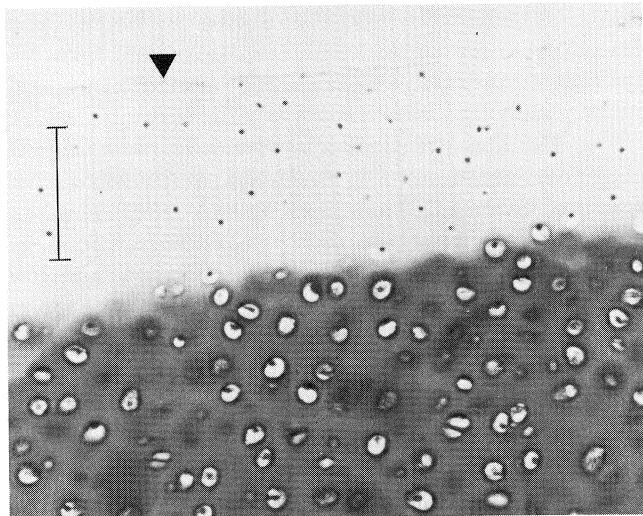


Figure 3. A, Enzyme-linked immunosorbent assay of immunoreactive type IX collagen fragments in the culture media from disks treated with 100 $\mu\text{g}/\text{ml}$ stromelysin (SLN) for 72 hours, using anti-bovine type IX collagen antibodies (40). B, Western blot analysis of immunoreactive type IX collagen fragments from the extracts of tissue slices (1% sodium dodecyl sulfate, 0.05M dithiothreitol) taken from disks treated with 100 $\mu\text{g}/\text{ml}$ SLN for 72 hours. CON = control.



A



B

Figure 4. Light micrographs of 5μ sections of cartilage incubated in **A**, DMEM alone for 72 hours and **B**, DMEM with $100\mu\text{g}/\text{ml}$ SLN for 72 hours. SLN treatment resulted in an apparent front of enzymatic digestion moving inward from all exposed specimen surfaces (arrowhead), shown by the absence of toluidine blue staining for GAG. See Figure 1 for definitions. (Bar = 100μ ; original magnification $\times 100$.)

throughout the tissue, those treated with SLN showed an absence of stain, most pronounced in the surface region, at early times in culture, consistent with a substantial loss of GAG. Thus by 24 hours of treatment with $100\mu\text{g}/\text{ml}$ SLN, the loss of toluidine blue-stained GAG had progressed into the upper $\sim 100\mu\text{m}$ of the tissue, with a marked boundary between degraded and undegraded regions. The section shown in Figure 4 is a magnified view which shows only a portion of the disk; full cross-section views at 24, 48, and 72 hours (47) indicate that GAG loss had started at the periphery and progressed into the plug from all surfaces exposed to SLN.

Findings of swelling studies. Swelling of tissue incubated in the presence of $100\mu\text{g}/\text{ml}$ of SLN, $50\mu\text{g}/\text{ml}$ of trypsin, or 0.1 units/ml of chondroitinase ABC was quantified as the ratio of plug wet weight in $0.01M$ NaCl to wet weight in DMEM (Figure 5). Untreated plugs swelled by 6–7% in hypotonic saline at all time points, while those treated with $100\mu\text{g}/\text{ml}$ of SLN showed increased swelling with time, up to a maximum of 25% after 72 hours in culture. Plugs treated with $50\mu\text{g}/\text{ml}$ of trypsin or 0.1 units/ml of chondroitinase ABC showed swelling that was indistinguishable from controls.

Trypsin treatment resulted in nearly complete GAG loss by 4 hours, while chondroitinase ABC treatment resulted in 50% GAG loss by 72 hours (data not shown), with GAG loss proceeding from the

surfaces into the tissue in a front-like manner (similar to that in Figure 4), as described previously (29). Given that both trypsin and chondroitinase ABC treatment induced significant GAG loss, it seems likely that the increased swelling of SLN-treated disks is not related to loss of proteoglycan constituents.

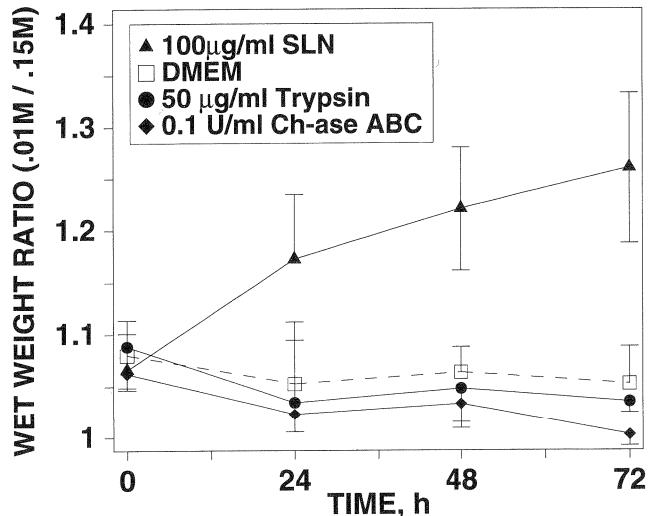


Figure 5. Ratio of wet weight in $0.01M$ NaCl to wet weight in DMEM for samples incubated in DMEM alone, DMEM with $100\mu\text{g}/\text{ml}$ SLN, DMEM with $50\mu\text{g}/\text{ml}$ trypsin, or DMEM with 0.1 units/ml chondroitinase ABC (Ch-ase ABC). Data are the mean \pm SD. See Figure 1 for other definitions.

Biomechanical and electromechanical properties.

Treatment with 100 $\mu\text{g}/\text{ml}$ of SLN resulted in a dramatic decrease in the equilibrium confined-compression modulus, H_A (Figure 6A). The modulus of control disks was $0.89 \pm 0.25 \text{ MPa}$ (mean \pm SD) at time zero and remained essentially constant over 72 hours in culture. In contrast, the modulus of disks incubated in the presence of 100 $\mu\text{g}/\text{ml}$ of SLN decreased to 10% of the initial value by 72 hours (Figure 6A), with kinetics similar to those of GAG loss for an SLN dose of 100 $\mu\text{g}/\text{ml}$ (see Figure 1).

The hydraulic permeability, k , of these same disks (Figure 6B) was calculated from the measured equilibrium modulus (Figure 6A) and the measured dynamic stiffness (Figure 7A), as previously described (45). The hydraulic permeability of control disks was $1.08 \pm 0.19 \times 10^{-15} \text{ m}^2 (\text{Pa} \cdot \text{s})^{-1}$ at time zero and remained constant over 3 days in culture. However,

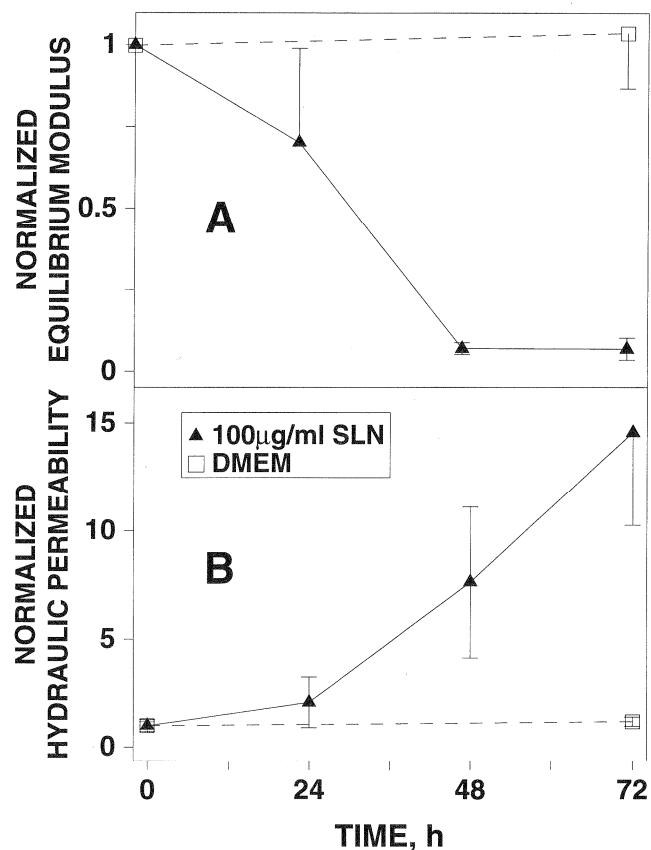


Figure 6. A, Equilibrium modulus and B, hydraulic permeability of cartilage disks as a function of time in culture. Disks were incubated in DMEM alone (broken line) or in DMEM with 100 $\mu\text{g}/\text{ml}$ SLN (solid line). Values are the mean \pm SD ($n = 4$); mean values are normalized to the value at time zero. See Figure 1 for definitions.

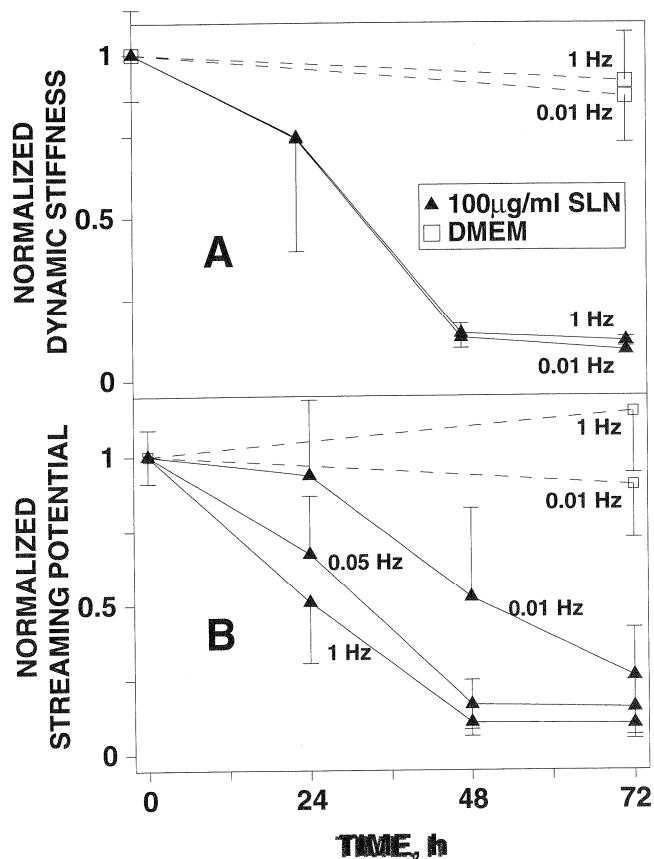


Figure 7. A, Dynamic stiffness and B, streaming potential of cartilage disks as a function of time in culture. Values are the mean \pm SD ($n = 4$); mean values are normalized at each frequency to the value at time zero. See Figure 1 for definitions.

the permeability of disks incubated in the presence of 100 $\mu\text{g}/\text{ml}$ of SLN markedly increased with time, to 15 times that of control disks at 72 hours.

Similar to the equilibrium modulus, the magnitude of dynamic stiffness decreased dramatically after treatment with 100 $\mu\text{g}/\text{ml}$ of SLN (Figure 7A). Applying constant-amplitude sinusoidal strains in displacement control, the measured dynamic stiffness and streaming potential (Figure 7B) increased monotonically with frequency, as described previously (44,45). In the present experiments, the dynamic stiffness of control disks at time zero was $4.40 \pm 0.84 \text{ MPa}$ at 0.01 Hz and $11.56 \pm 1.67 \text{ MPa}$ at 1 Hz, and did not change significantly over 72 hours. In contrast, the dynamic stiffness of disks incubated in 100 $\mu\text{g}/\text{ml}$ of SLN decreased to $\sim 10\%$ of initial values after 3 days in culture. As with the equilibrium modulus, the kinetics of changes in dynamic stiffness were similar to those of GAG loss for a 100- $\mu\text{g}/\text{ml}$ SLN dose (see Figure 1).

The relative decrease in dynamic stiffness with time in culture was independent of frequency in the 0.01–1 Hz range.

Electromechanical measurements revealed that streaming potential magnitude also decreased dramatically for disks treated with 100 µg/ml of SLN (Figure 7B). Initial values of streaming potential ranged from 0.10 ± 0.02 mV/% at 0.01 Hz to 0.33 ± 0.05 mV/% at 1 Hz. The streaming potential of control disks did not vary significantly during time in culture at any frequency, while that of treated plugs decreased to 30% and 10% of initial values at 0.01 Hz and 1 Hz, respectively, after 3 days. In marked contrast to dynamic stiffness, the amount by which the streaming potential of disks treated with 100 µg/ml of SLN decreased with time showed a marked frequency dependence. At high frequency (1 Hz), the streaming potential decreased quickly with time in culture, while at low frequency (0.01 Hz), the decrease in streaming potential was less rapid.

DISCUSSION

The combined results shown in Figures 1 and 2 strongly suggest that SLN is responsible for the proteoglycan loss seen in our studies. The dose dependence of GAG loss and the absence of loss seen when TIMP was added simultaneously with SLN demonstrate that the GAG loss was induced by the addition of SLN. Moreover, the presence of specific SLN-generated aggrecan cleavage products in culture media and the lack of detectable cleavage products generated by other proteinases further suggests that direct SLN cleavage of aggrecan occurred within the tissue during culture. This is of special interest, given that recent work has shown that total levels of SLN (active plus inactive) in synovial fluid from patients with OA are as high as 300 nM (15 µg/ml) (32), which is within the concentration range tested here. Given that matrix degradation in OA may progress gradually over many years, it is interesting that the concentrations of active SLN in the 15 µg/ml range appear quite capable of causing significant cartilage matrix damage in a much shorter time frame (Figure 1). A likely explanation for this is that a majority of the SLN in OA synovial fluid is most probably inactive, being either in the proenzyme form or bound to endogenous inhibitors (32,48).

Histologic examination (Figure 4) showed that initial loss of GAG occurred primarily in the surface regions of the disks, leaving interior regions relatively undegraded. This observation of a moving front of

enzymatic degradation progressing inward from exposed tissue surfaces has been documented previously for the cases of degradation of proteoglycan constituents by chondroitinase ABC (29) and *Streptomyces* hyaluronidase (49). Together, these results suggest that such high molecular weight enzymes ($M_r \sim 20\text{--}150$ kd), having limited numbers of spatially accessible cleavage sites within a dense matrix, do not simply diffuse rapidly into the tissue and degrade it uniformly; rather, they slowly digest their way into the tissue from the surface with diffusion-reaction, substrate-limited kinetics. In vivo, it is conceivable that the degradative enzymes in synovial fluid or those produced by chondrocytes in the superficial zone must first degrade the matrix in the superficial zone before they can gain access to the interior regions of the tissue. Indeed, Okada et al (50) showed immunolocalization of SLN in chondrocytes mainly in the superficial and transition zones of 90% of the human OA samples analyzed, in regions where there was depletion of proteoglycans, as revealed in toluidine blue-stained sections.

The results shown in Figures 3, 5, and 6 also suggest that SLN has caused significant damage to other constituents of the cartilage matrix besides the proteoglycans. Data in Figure 3 show that the addition of SLN to the cartilage culture media causes an increase in the release of type IX collagen fragments into the culture media, as well as an increase in residual type IX collagen fragments within the tissue. It can be seen (Figure 3B) that the extracts show a broad spectrum of immunoreactive bands, with a greater proportion of higher molecular weight components in the SLN-treated disk extract. In interpreting this finding it is necessary to consider that type IX collagen molecules are heavily cross-linked to type II collagen and to each other (51). Thus, because SLN cleaves the type IX molecule only at very specific sites, it is expected that a ladder of polymeric fragments will result from the degradation of the cross-linked matrix protein. Since type IX collagen has been implicated as a possible crosslinking agent between type II fibrils (52), studies on cartilage swelling were specifically designed to assess the integrity of the collagen network during and after SLN treatment. As described by Maroudas (12), the swelling state of cartilage is determined by a balance between the osmotic pressure of the proteoglycans, which tends to swell the tissue, and the restraining tensile forces of the collagen network, which tend to limit tissue swelling. If the collagen network is compromised, as in OA

(12), it is less able to counteract the osmotic pressure, and the tissue swells.

The addition of SLN to culture media caused an increase in the swelling induced by equilibration in 0.01M NaCl, up to 25% after 3 days. The addition of trypsin or chondroitinase ABC, enzymes known to cause loss of GAG from cartilage, did not alter swelling compared with that of untreated controls (Figure 5). Taken together these results strongly suggest that SLN treatment reduced the ability of the collagen network to restrain the residual swelling pressure of the remaining proteoglycan constituents. The data shown in Figure 3 motivate the hypothesis that degradation of type IX collagen and damage to the type II-type IX complex contribute to this swelling. Although there is no direct evidence of cleavage by proteinases other than SLN, it should be noted that the possibility of some degradation by endogenous proteinases, in addition to and perhaps activated by the exogenous SLN, cannot be ruled out when interpreting the results.

Additional evidence of damage to the cartilage matrix beyond removal of proteoglycans is seen in the results of mechanical compression experiments (Figure 6A). Previous studies have shown that neutralization or shielding of proteoglycan-fixed charge groups (by pH or ionic strength alteration) or removal of charge-bearing GAG chains by enzymatic treatment could decrease the modulus or dynamic stiffness of cartilage disks by ~50% (29,53,54). The results in Figure 6A show that exposure to SLN resulted in a 90% loss in equilibrium modulus, a significantly greater loss than that associated with enzymes which degrade or remove only proteoglycan constituents. The substantial decrease in equilibrium modulus suggests that degradation of the cartilage matrix occurred beyond loss of proteoglycans, possibly including damage to the collagen network.

Dynamic compression studies also showed a large increase in hydraulic permeability (Figure 6B) with time in culture after treatment with 100 µg/ml SLN; at the same time the GAG content had decreased to 10% of its original value (Figure 1). This increase in hydraulic permeability which accompanied GAG loss is consistent with the previous work of Maroudas (55), who demonstrated that proteoglycans are the primary source of resistance to fluid flow in the cartilage matrix.

The streaming potential and its dependence on frequency (Figure 7B) are of particular interest regarding the sensitivity of electromechanical measurements

to the spatial distribution of enzymatic degradation (Figure 4). The fluid flow that occurs within the cartilage disk during sinusoidal compression not only contributes to dynamic stiffness (Figure 7A), but also tends to separate counterions in the interstitial fluid from negative fixed-charge groups of the proteoglycans (44,45). The voltage which results from this charge separation is known as the streaming potential. The measured streaming potential shown in Figure 7B is the total potential drop across the disk. This potential can be viewed as the sum of the local potential drops across incremental layers of the tissue.

At each depth, the local potential drop is proportional to the proteoglycan charge density and the local fluid velocity relative to the matrix (54). At high frequency, the fluid velocity profile is steep, and fluid flow occurs predominantly in a thin region near the surface of the disk. At lower frequencies, the velocity profile is less steep and extends further into the tissue (45). Thus, most of the streaming potential drop at 1 Hz will occur within the topmost 50–100 µm of the 1 mm-thick disks and the potential is therefore most sensitive to the proteoglycan content in the topmost layer. At frequencies of ≤0.01 Hz, however, the potential drops across a much thicker portion of the disk, and therefore reflects the proteoglycan density of deeper as well as surface regions.

Interpreted in this context, the significant decrease in dynamic stiffness and streaming potential with increasing incubation time in SLN is an expected consequence of the loss of GAG (Figure 1). These results are consistent with findings of previous studies of the effects of enzymatic degradation on cartilage electromechanical behavior (29). However, the time dependence of the decrease in potential is highly dependent on frequency, while the decrease in stiffness is independent of frequency. At 24 hours, the tissue has experienced ~50% loss of GAG content (Figure 1), primarily confined to the outer 100–150-µm tissue layer (Figure 4B). The mean stiffness at 24 hours decreased by 25% at all frequencies, but was not significantly different from that of controls. The streaming potential frequency response was quite different, with negligible loss by 24 hours at 0.01 Hz, but a significant loss at 1 Hz. This decrease in potential at 1 Hz but not at 0.01 Hz is consistent with the sensitivity of the streaming potential at higher frequencies to the focal loss of GAG from the surface region of the disk. Given that the dynamic stiffness had no such frequency dependence, it is apparent that streaming potential is more sensitive to the initial, focal loss of

proteoglycans from cartilage that results from SLN treatment.

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