Cartilage degradation and associated changes in biomechanical and electromechanical properties

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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 [1]. In its degenerated state, OA cartilage exhibits dramatically different biochemical, physicochemical, mechanical, and electromechanical properties from normal tissue, including a distinct loss of proteoglycan [2,3], marked collagen network fibrillation [4], increases in tissue hydration [5], and loss of compressive stiffness [6]. The role of enzymatic degradation in OA has been studied extensively, but is not completely understood. It has been suggested [7] that families of enzymes, including matrix metalloproteinases (MMPs) [8,9], serine proteinases [10], and a novel "aggrecanase" [11] contribute to cartilage degradation in OA. It seems likely that the enzymatic component of OA involves a cascade of activities which work in tandem to degrade the tissue.

MMPs are a family of at least 12 enzymes which are divided into three main classes: collagenases, gelatinases, and stromelysins. The role of the gelatinases in degradation of cartilage matrix is unclear; however, interstitial collagenase (MMP-1) is known to degrade collagen types II and X [12], while stromelysin-1 (MMP-3) is known to degrade aggrecan [13], link protein [8], and collagen types II, IX, X, and XI [14]. Both collagenase and stromelysin (SLN) are produced by chondrocytes and synovial fibroblasts [15]. These MMPs are present in elevated levels in cartilage and synovial fluid of patients with degenerative OA [3,15], as well as in animal models [9]. Elevated levels have likewise been detected in cases of traumatic joint injury [3]. There is also evidence of MMP gene expression in early stages of soft tissue development [16].

Both interleukin-1 and retinoic acid have been commonly used to induce cell-mediated degradation of cartilage explants. Interleukin-1ß (IL-1ß) is an 18 kD cytokine produced by several cell types including endothelial cells, synoviocytes and chondrocytes [17]. Although typically associated with inflammatory diseases such as rheumatoid arthritis [18], both the α and ß forms of IL-1 have been shown to be relevant to degradation in osteoarthritis as well [19]. IL-1 interacts with chondrocytes by means of cell surface receptors [20]. Stimulation of cartilage explants with IL-1 has been shown to result in significant matrix degradation, including loss of tissue PG and collagens [21]. Additionally, IL-1 induces production and mRNA expression of MMPs in chondrocytes [22,23], while modulating production of tissue inhibitors of metalloproteinases (TIMPs) [17]. Although tissue treated with IL-1 contains an excess of MMPs, the PG degradation products which result are not those typically associated with MMP cleavage [24]. These fragments, however, are similar to those found in human OA patients [25]. Despite this apparent discrepancy, MMP inhibitors have been shown to be effective at inhibiting cartilage matrix degradation induced by IL-1 [21,26].

The vitamin A derivative all-trans retinoic acid (RA) has also been shown to induce matrix degradation in cartilage explants [21,27]. RA stimulation involves interaction with nuclear receptors, directly altering DNA transcription [28]. On the tissue levels, RA treatment results in loss of tissue PGs and collagens [21,27] decreased synthesis of PG [27] and collagens [29,30] by chondrocytes. RA has also been shown to induce expression and production of MMPs [21], although degradation products from such systems are not those typically associated with MMP activity (JD Sandy, unpublished results). This matrix degradation is inhibited in the presence of MMP inhibitors, but not in the presence of inhibitors of cathepsin B [21].

Alterations of biochemical composition and matrix structure which 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 [31], changes in tensile stiffness, strength, and fracture strain [10,32], decreased shear modulus [33], changes in creep behavior [10,32], and increased indentation displacement [10,31]. Degradation of matrix collagens due to action of proteinases has been shown to be manifest in increased tissue swelling [34,35]. In addition, a marked decrease in compression-induced streaming potential has been shown to be a particularly sensitive indicator of alterations in matrix proteoglycan composition associated with enzymatic degradation [36], and of the spatial distribution patterns of degradation caused by enzymes such as stromelysin [34].

This study focuses on cartilage matrix degradation which resulted from treatment of calf cartilage explants or rabbit stifle joints with activated stromelysin and on degradation induced by the addition of the MMP-activating agent APMA, IL-1ß, or retinoic acid to calf cartilage explants. Degradation of matrix proteoglycans and collagens was assessed and the resultant changes in mechanical and electromechanical behavior of the tissue were quantified.

Methods

In vitro culture

Cartilage disks were incubated in groups of 4 in 1 ml DMEM containing 100 U/ml penicillin G and 100 µg/ml streptomycin for approximately 16 hours at 37 °C in a 5% CO_2 atmosphere. Groups of 4 disks were then placed in 1ml DMEM alone or containing 100µg/ml recombinant human stromelysin (SLN), 1mM aminophenylmercuric acetate (AMPA), 100ng/ml recombinant human interleukin-1ß (IL-1ß) or 1µM all-*trans* retinoic acid (RA) in 0.1% dimethylsulfoxide. Tissue incubated in the presence of SLN or APMA was removed from culture at select times up to 3 days. Cartilage treated with IL-1ß or RA was cultured for up to 8 days, in media which also contained 0.1% bovine serum albumin and was changed every two days. After culture, selected cartilage disks were assayed for type II collagen denaturation by analysis with the CB11B antibody [37]. Media from SLN cultures was assayed for type IX collagen fragments by ELISA using a type IX collagen antibody [38].

Tissue swelling behavior was measured as an indicator of damage to matrix collagens [5]. After culture, cartilage disks were removed from media and patted briefly with a paper towel to remove surface water, and wet weights were measured. Disks were then twice reequilibrated in 1 ml of hypotonic saline solution (0.01M NaCl at pH 7.0) for 1 hour at room temperature [34]. Surface water was again removed and wet weights were measured. Following wet weight measurements, disks were lyophilized, and dry weights were recorded.

After swelling measurements were completed, lyophilized samples were digested with 1 ml of 125 µg/ml papain digestion solution and assayed for sulfated GAG by reaction with 2 ml of dimethylmethylene blue dye solution [39] using whale/shark chondroitin sulfate as the standard.

In vivo model

At Merck Research Laboratories, 6–8 week old NZW female rabbits received intraarticular injections of 100 µg of trypsin-activated rhSLN in buffer into one hind stifle joint, with the contralateral control joint receiving only buffer. After one hour the animals were sacrificed and joints were lavaged. Stifle joints were dissected and stored on dry ice. Subsequently, 2–4 cartilage/bone cylinders were cut from each femoropatellar groove with a 3 mm dermal punch. Immediately prior to testing, the cartilage was separated from the bone, and each plug was tested in confined compression (see below), then weighed, digested with papain and analyzed for GAG content [40].

Physical properties

Cartilage disks were placed in a cylindrical confining chamber, similar to that used by Frank et al. [36]. The chamber was mounted in a servo-controlled Dynastat mechanical spectrometer which was interfaced to a computer and frequency generator. Samples were equilibrated in 0.15 M phosphate buffered saline at pH 7.4 containing 100 U/ml penicillin G and 100 µg/ml streptomycin at room temperature. Disks were subjected to confined compression between a porous polyethylene platen and a 6.35 mm Ag/AgCl pellet electrode mounted at the base of the chamber. An identical electrode was mounted in the surrounding PBS bath.

Disks were subjected to sequential increments of ~1% strain, up to a maximum of 20% total strain and the resultant equilibrium stresses were recorded. 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, and the resultant oscillatory load and streaming potential were recorded. Equilibrium modulus, dynamic stiffness, and streaming potential data were used in com-

	Calf explants [a]						Rabbit in vivo [b]			
	Control		24 hr		72 hr		Control		1 hr	
GAG content (µg GAG/mg wet weight)	61.8	*16.9*	37.2	*7.3*	6.3	*0.5* *	43.0	*9.6*	36.4	*17.2*
Type II collagen (% denaturation)	2.53	*0.94*			2.45	*0.68*				
Type IX collagen release to media (nmol)	48				156					
Streaming potential @ 1 Hz (µV/%)	234	*38*	120	*15* *	33	*16* *	22.7	*9.8*	5.4	*2.8* *
Dynamic stiffness @ 1 Hz (MPa)	11.5	*1.7*	8.9	*3.0*	1.4	*0.2* *	1.36	*0.67*	1.23	*0.64*
Equilibrium modulus (MPa)	0.89	*0.25*	0.65	*0.24*	0.06	*0.03* *	0.25	*0.11*	0.18	*0.09*
Hydraulic permeability ($m^2/Pa \cdot s \times 10^{-15}$)	1.08	*0.19*	2.25	*1.26*	15.7	*4.65* *	15.3	*12.7*	23.6	*15.4*
Electrokinetic coupling coefficient ($V/Pa \times 10^{-9}$)	1.92	*0.28*	1.33	*0.26* *	0.91	*0.15* *	1.17	*0.32*	0.70	*0.27* *
Swelling ratio (0.01 M NaCl/DMEM)	1.06	*0.03*	1.17	*0.06* *	1.25	*0.07* *				

[a] Cartilage explants (n = 4) were cultured in the presence of 100 µg/ml SLN for 24 or 72 hours.
[b] Rabbits (n = 6) received intraarticular injection of 100 µg SLN in one stifle joint and were sacrificed after 1 hour.
* Results which varied significantly ($p < 0.05$) from controls

bination with the method of Frank and Grodzinsky [41] to calculate the effective hydraulic permeability and electrokinetic coupling coefficient of the tissue samples.

A comparison of biochemical analyses and physical property measurements between in vitro SLN treatment of calf cartilage explants and in vivo SLN treatment of cartilage in rabbit stifle joints is shown in Table 1. Data are reported as mean *SD* with n=4 disks for explant studies and n=6 animals for in vivo studies. Data on culture media from explants studies indicates the cumulative release of macromolecules from 4 cartilage disks. Tissue GAG content of calf explants treated with 100 µg/ml SLN decreased with time in culture and was significantly less than that of controls ($p < 0.05$, denoted by *) by 24 hours, while the GAG content of cartilage disks removed from the rabbit stifle joints which received 100 µg SLN did not differ significantly from controls after only 1 hour of treatment. The amount of type II collagen denaturation in calf explants was not affected by SLN treatment; however, explants treated with SLN showed a greater than 3-fold increase in loss of type IX collagen fragments to culture media.

After 24 hour treatment with SLN, cartilage explants showed significant decreases in streaming potential at 1 Hz and electrokinetic coupling coefficient and a significant increase in swelling. However, there were no significant changes in dynamic stiffness at 1 Hz, equilibrium modulus, and hydraulic permeability. After 72 hours, streaming potential at 1 Hz, electrokinetic coupling coefficient, dynamic stiffness at 1 Hz, and equilibrium modulus all decreased significantly while hydraulic permeability and tissue swelling both increased significantly. A decrease in high frequency streaming potential was the only change observed in the rabbit cartilage after the 1 hour treatment.

The frequency response of dynamic stiffness and streaming potential for calf cartilage explants treated with 1mM APMA, 100ng/ml IL-1ß, and 1 µM retinoic acid are shown in Figure 1 (A, B, and C, respectively). Explants treated with APMA showed significant decreases in streaming potential and dynamic stiffness at all frequencies after 1 day in culture and continued to decrease through the course of the experiment. Streaming potential and dynamic stiffness of explants cultured with IL-1ß also decreased with time in culture. However, by day 2 of culture, changes in dynamic stiffness were not statistically significant at any frequency tested, while the streaming potential differed significantly only at testing frequencies >0.5 Hz. By day 4, dynamic stiffness had decreased significantly at all testing frequencies and streaming potential measurements significantly different at frequencies >0.03 Hz. By day 8, both streaming potential and dynamic stiffness had decreased significantly at all frequencies tested. Similar to APMA studies, explants treated with retinoic acid showed significant decreases in streaming potential and dynamic stiffness at all frequencies after 2 days in culture and continued to decrease through the course of the experiment.

Data from Figure 1 and independent measurements of equilibrium modulus were used to calculate material properties of explants treated with APMA, IL-1ß, and retinoic acid. These data, along with biochemical analyses of these disks, are shown in Table 2. After 3

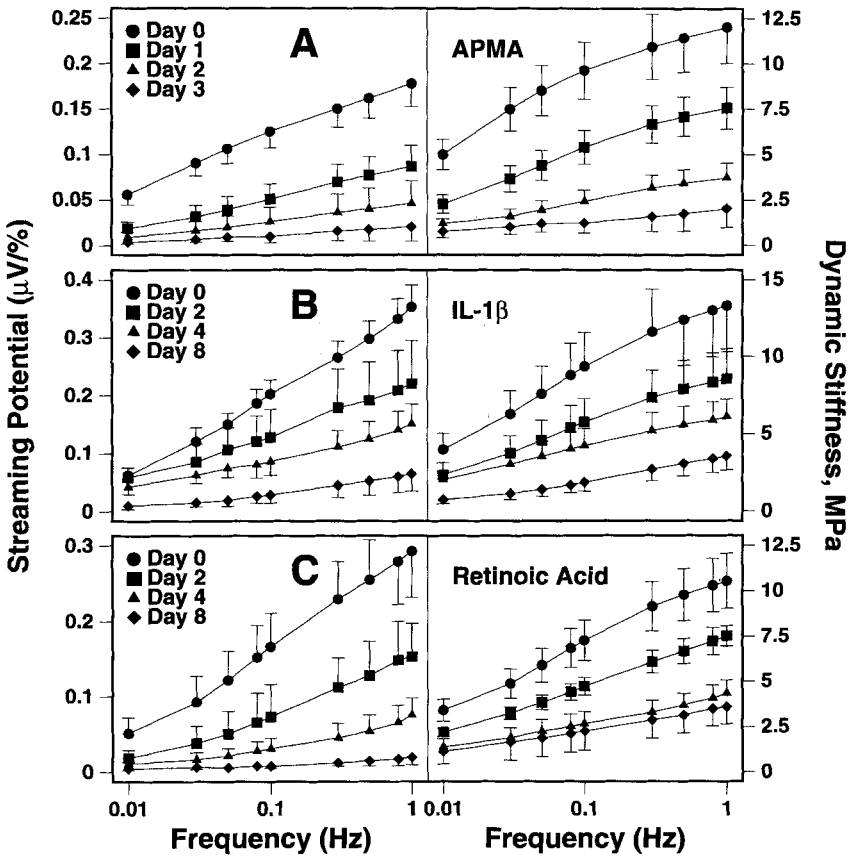

Figure 1. Frequency profiles of streaming potential and dynamic stiffness of calf cartilage explants cultured in the presence of 1mM APMA on days 0, 1, 2, and 3 of culture (A) and 100 ng/ml interleukin-1ß (B), or 1 µM retinoic acid (C) on days 0, 2, 4, and 8 of culture. All data are reported as mean SD with n=4.

Table 2. Results of biochemical analyses and physical properties measurements from calf cartilage explants (N=4) treated with 1mM APMA, 100 ng/ml IL-1ß, or 1 µM retinoic acid and cultured up to 8 days. All data are reported as mean *SD*

	APMA		IL-1ß			Retinoic acid		
	Day 0	Day 3	Day 0	Day 2	Day 8	Day 0	Day 2	Day 8
GAG content [a]	95 *3.8*	34 *6.5* *	97 *1.5*	90 *7.5*	6.8 *4.2* *	97 *1.9*	41 3.5 *	4.8 2.3 *
Type II collagen [b]	2.5 *0.94*	6.9 *0.54* *						
Swelling ratio [c]	1.0 *0.02*	1.2 *0.09* *	1.0 *0.01*	1.0 *0.03*	1.1 *0.02* *	1.0 *0.02*	1.0 *0.02*	1.0 *0.02*
Equilibrium modulus [d]	1.3 *0.35*	0.29 *0.12* *	1.2 *0.43*	0.79 *0.19*	0.20 *0.05* *	1.1 *0.27*	0.49 *0.26* *	0.24 *0.20* *
Hydraulic permeability [e]	0.59 *0.22*	1.9 *0.98* *	1.4 *1.2*	1.8 *1.1*	9.8 *4.8* *	1.0 *1.0*	1.8 *0.45* *	10 *3.7* *
Electrokin. coupl. coeff. [f]	1.9 *0.28*	1.2 *0.26* *	3.9 *0.27*	3.1 *0.99*	1.4 *0.34* *	3.3 *0.52*	2.0 *0.84* *	0.75 *0.42* *

[a] (% GAG in tissue), [b] (% denaturation), [c] (0.01 M NaCl/DMEM), [d] (MPa), [e] ($m^2/Pa \cdot s \times 10^{-15}$), [f] ($V/Pa \times 10^{-9}$).
* Results which varied significantly ($p<0.05$) from measurements Day 0.

days in culture, APMA-treated disks showed significant decreases in tissue GAG content, equilibrium modulus, and electrokinetic coupling coefficient, as well as significant increases in hydraulic permeability, tissue swelling, and type II collagen denaturation. Explants treated with IL-1ß showed no significant changes in these parameters after two days in culture, but showed significant decreases in tissue GAG content, equilibrium modulus, and electrokinetic coupling coefficient, as well as significant increases

in hydraulic permeability, and tissue swelling after 8 days. Explants treated with retinoic acid showed significant decreases in GAG content, equilibrium modulus, and electrokinetic coupling coefficient as well as an increase in hydraulic permeability after only 2 days in culture; however, there was no change in tissue swelling even after 8 days in culture.

Discussion

Cartilage explants treated with SLN and cartilage from lapine stifle joints exposed to SLN in vivo showed similar patterns of proteoglycan degradation (Table 1). By 24 hours, calf cartilage explants exhibited moderate loss in tissue GAG, and significant decreases in electromechanical properties, streaming potential measured at 1 Hz and electrokinetic coupling coefficient, but did not exhibit significant changes in purely mechanical properties, dynamic stiffness, equilibrium modulus, and hydraulic permeability. Similarly, cartilage disks from lapine stifle joints exposed to SLN for 1 hour did not have significantly different GAG content, showed significant changes in measured streaming potential at 1Hz and electrokinetic coupling coefficient, but did not exhibit significant changes in dynamic stiffness, equilibrium modulus, and hydraulic permeability. These data highlight the sensitivity of electromechanical measurements to the fixed charge density, and therefore the proteoglycan content of the tissue [36]. High frequency streaming potential measurements are particularly sensitive to degradation at the tissue surface [34,40]. This is particularly relevant to these systems given that transport of SLN (45 kDa) is restricted by the cartilage matrix. Thus at early times during these experiments, the surface regions of the tissue are degraded while deeper regions remain relatively intact [34,42,43].

By 3 days in culture, tissue GAG content decreased by ~90% and all measured physical properties changed dramatically. The loss of proteoglycans reduces the tissue fixed charge density and consequently results in decreased streaming potential and electrokinetic coupling coefficient. This degradation, combined with damage to the collagen network impairs the ability of the tissue to withstand compressive loads, as reflected in decreases in dynamic stiffness and equilibrium modulus. Additionally, removal of proteoglycans allows fluid to flow through the matrix more easily, as indicated by the large increase in hydraulic permeability.

The addition of SLN to culture media caused an increase in swelling with time in culture up to 17% after 1 day and 25% after 3 days. As described by Maroudas [5], 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 [5], it is less able to counteract the osmotic swelling forces, and the tissue swells. Given that other enzymes which remove proteoglycans but do not damage collagen do not cause this type of swelling response [34], it seems apparent that SLN has degraded the collagen network. It is interesting that SLN did not cause an increase in type II collagen unwinding, but did cause type IX collagen release along with tissue swelling. This suggests that type IX collagen may play an important role in maintaining the integrity of the collagen network [44]. The lack of type II collagen unwinding is curious, since it is known to be a result of collagenase activity and SLN is known to activate collagenase in solution [45]. This activation does not appear to be taking place in the tissue, despite the large amount of SLN to which the explants were exposed.

The frequency profiles of streaming potential and dynamic stiffness (Figure 1) and the corresponding changes in material properties and biochemical composition (Table 2) again point out the relationship between matrix degradation and changes in physical properties and highlight the sensitivity of electromechanical measurements to tissue proteoglycan content. The 65% loss of tissue GAG due to 3 day APMA treatment is consistent with the decreases in streaming potential, electrokinetic coupling coefficient, dynamic stiffness, and equilibrium modulus, as well as the increase in hydraulic permeability. In contrast to studies using SLN to degrade the cartilage matrix, the decrease in streaming potential is similar across all frequencies tested. This is due to the fact that APMA is small (0.5 kDa) and can move through the cartilage matrix more easily, thus the resulting degradation is more spatially uniform. The degradation of the collagen network indicated by type II collagen denaturation is also consistent with the increase in tissue swelling. Given that tissue treated with SLN showed no increase in type II collagen denaturation, it seems likely that the type II collagen degradation observed here is due to collagenase activated by APMA.

Cartilage matrix degradation induced by treatment with IL-1ß was slower than that induced by retinoic acid (Table 2). After 2 days in culture retinoic acid treated disk showed significant decreases in GAG

content, equilibrium modulus, and electrokinetic coupling coefficient and an increase in hydraulic permeability, while these measurements in disks treated with IL-1ß did not differ significantly from disks at the start of the experiment. This is also likely due to transport restrictions, given that retinoic acid is relatively small (0.4 kDa) and can stimulate cells to degrade the cartilage matrix more quickly and more uniformly than the larger IL-1ß (18 kDa).

As with SLN, the restricted transport of IL-1ß results in initial degradation at the tissue surface. This is reflected in streaming potential measurements at high compression frequencies, where fluid flow occurs only near the tissue surface. After 2 days, IL-1ß treated disks showed significant decreases in streaming potential at frequencies >0.5 Hz; after 4 days, streaming potential was significantly less at frequencies >0.03Hz; and after 8 days streaming potential decreased significantly at all frequencies tested. This is consistent with a pattern of degradation starting at the surface and moving into the tissue with time. This is in contrast to retinoic acid treatment which showed significant decreases in streaming potential across all frequencies tested after only two days in culture.

Another interesting difference between the IL-1ß and retinoic acid culture systems was the observation that swelling was induced by IL-1ß but not by retinoic acid. This suggests that different pathways of collagen catabolism are initiated by these agents.

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