

Effects of Interleukin 1(IL1)-Induced Matrix Breakdown on Chondrocyte Morphology in Bovine Nasal Cartilage Explants

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Abstract: The objective of this study was to investigate the influence of matrix loss on regulating chondrocyte phenotype, and the synthesis and activation of collagenolytic enzymes. IL1 was used to enhance matrix breakdown in bovine nasal cartilage explants. Change in matrix composition and its effects on chondrocyte morphology were investigated using histochemical techniques. In bovine nasal explants, chondrocytes responded to IL1 to manifest 2 distinct patterns of cell shape exhibiting either pyknotic or enlarged nuclei. Enzyme activity analysis suggested a positive correlation between collagenolytic activity and the occurrence of proteoglycan loss and cell morphology changes. These data suggest that loss of non-collagenous proteins such as proteoglycans may lead to changes in cell shape but this may not be sufficient on its own to create the threshold stimulus for chondrocytes to commit themselves to produce active collagenolytic enzymes.

Key Words: Interleukin 1, matrix metalloproteinase, cartilage

İnterlökin 1(IL1) ile İndüklenmiş Matriks Yıkımının Sığır Nazal Kıkırdak Kondrosit Morfolojisi Üzerine Etkileri

Özet: Bu çalışmada matriks kaybının kondrosit fenotipinin yeniden düzenlenmesi ve kollajenolitik enzimlerin sentezi ve aktivasyonu üzerine etkileri araştırılmıştır. Matriks yıkımı interlökin1 (IL1) ile sağlandı. Matriks yapısındaki değişiklikler ve kondrosit morfolojisi üzerine etkileri histokimyasal teknikler kullanılarak saptandı. Sığır nazal kıkırdak dokusunun IL1 ile indüklenmesini takiben kondrositlerde iki belirgin, farklı değişim izlendi. Kondrositlerin bazıları piknotik özellikler gösterirken diğer bir grup kondrositte çekirdek boyutlarında artma izlendi. Enzim aktivitesi analizleri proteoglikan yıkımını takiben izlenen hücre morfolojisindeki değişikliklerle kollajenolitik aktivite arasında pozitif bir korelasyon gösterdi. Sonuç olarak oluşturulan doku kültürü modeli proteoglikanlar benzeri non-kollajen proteinlerin kaybının hücre şeklinde değişikliklere yol açabildiğini ama bu değişikliklerin kondrositlerin, doku yıkımına neden olacak düzeyde kollajenolitik enzimler üretmeleri için tek başına yeterli uyarıcı olmadığını sağlamadığına dikkat çekmektedir.

Anahtar Sözcükler: Interlökin 1, matriks metalloproteinaz, kıkırdak

Introduction

Chondrocytes in hyaline cartilage can be identified as having a spherical shape in an abundant extracellular matrix. They are responsible for the synthesis, maintenance and maturation of the matrix within which they are embedded (1). There are multiple interactions between the chondrocyte, its extracellular matrix and its mechanical, hormonal, and biochemical environment. These interactions are considered critical to the biological functions of chondrocytes, such as the synthesis and

degradation of extracellular matrix components and differentiation.

It has been demonstrated that, in the absence of supporting matrix, chondrocytes alter their cell shape (e.g., in monolayer cell cultures) and become fibroblast-like dedifferentiated cells that express type I rather than type II collagen and cease synthesis of aggrecan (2,3). However, recently Kolettas et al. (4) suggested that type I collagen expression and cell shape change may not necessarily correlate with cell phenotype changes.

Shakibaei et al. (5) showed that when grown on type II collagen, chondrocytes do not dedifferentiate to fibroblast-like cells in monolayer cultures. Fell et al. (6) have shown that loss of the collagen network leads to a change in the chondrocytes to a more fibroblastic appearance and presumably to loss of the differentiated phenotype, thereby leading to a potentially irreversible path of destruction of cartilage. Studies by Werb et al. (7) demonstrated that the effects of cell shape change on rabbit synovial fibroblasts might act as a triggering factor on secretion of procollagenase, prostromelysin and related proteolytic enzymes. In monolayer sub-cultures, rabbit articular chondrocytes become dedifferentiated and were found to be more responsive to catabolic agents like IL1 (8). Baici et al. (9,10) also reported similarities between chondrocytes artificially modulated in monolayer cultures and those in advanced stages of osteoarthritis (OA), showing that the chondrocytes in severe cases of OA become phenotypically dedifferentiated, appear more fibroblastic and potentiate the synthesis and secretion of cathepsin B. Recently, Lochter et al. (11) reported that stromelysin 1 can initiate a cascade of events leading to loss of phenotype in mouse mammary cell lines, which upregulates the production of matrix degrading enzymes, such as gelatinase B and collagenase-3 in an irreversible manner.

In this study, the effects of IL1 on chondrocyte morphology in intact cartilage were investigated by histochemical techniques. The commitment of chondrocytes to the synthesis and activation of matrix metalloproteinases (MMP) in response to IL1 was determined in relation to the alterations of the extracellular matrix.

Materials and Methods

Preparation of cartilage explants

Explants from bovine nasal and articular cartilage were prepared and cultured as described previously (12). Bovine nasal and articular cartilage were obtained shortly after slaughter. The nasal septum was dissected out and sectioned into slices of approximately 5 cm x 1.5 cm x 2 mm with a sterile scalpel. The slices were washed once with sterile phosphate buffered saline (PBS) for 20 min. Discs of cartilage (wet weight of 30-50 mg) were punched out using a belt punch under sterile conditions. The outer parts of the cartilage slices were not used to avoid contamination with other tissues.

Culture of cartilage explants

The nasal and articular cartilage explants were cultured in serum free Dulbecco's modification Eagle's medium (DMEM; Gibco Life Technologies Ltd., Renfrew Road, Paisley, UK), containing 2 mM glutamine, 2000 U/ml penicillin G, 0.1 mg/ml streptomycin and 10 mM HEPES (all from Gibco) for 4 weeks in 48-well tissue culture plates (Costar, High Wycombe, UK) at 37 °C in a humidified atmosphere of 5% CO₂/95% air. Cartilage explants were treated with rhIL1 α (0.3 nM, 3 nM) or were cultured in DMEM only. IL1 treatment was given for 2 weeks. At days 7, 14 and 21, medium in each well was replenished. Media from explant cultures were collected weekly. Conditioned media were frozen and stored at -20 °C.

Measurement of type II collagen degradation in culture medium

Type II collagen released into the media was digested as described by Hollander et al. (13) for the detection of type II collagen. Then 1 mg/ml proteinase K (EC 3.4.21.64); (Sigma, Poole, Dorset, UK) in 50 mM Tris HCl, pH 7.6, containing 1 mM EDTA, 10 μ g/ml Pepstatin A and 1 mM iodoacetamide solution, was mixed with medium and incubated at 56 °C for 15 h to digest any soluble (unwound) or intact collagen released into the media. The samples were then boiled at the end of the extraction to inhibit proteinase K.

Measurement of proteoglycan breakdown

Media were digested with proteinase K as described above. Proteoglycan in the digests was measured as sulphated glycosaminoglycan (GAG) using 1, 9-dimethylmethylene blue (Aldrich, Gillingham, UK) by modification of the method of Farndale et al. (14). Chondroitin sulphate (Sigma) was used as a standard.

Light microscopy of bovine nasal cartilage

Bovine nasal cartilage explants were treated with IL1 (3 nM or 0.3 nM) for 2 weeks. At a concentration of 0.3 nM, IL1 has been shown to have a potent effect on proteoglycan breakdown with little or no effect on type II collagen release (15); 3 nM rhIL1 was found to attain the maximal response in preliminary experiments and was therefore used in all subsequent assays. In most cases the effect on collagen was seen after 3 weeks with a 3 nM IL1 dose (data not shown). Controls were cultured in DMEM only. At days 7, 14 and 21, some explants were collected

and fixed in 4% (w/v) paraformaldehyde in PBS, pH 7.2. The others were continued in culture until day 28. Medium in all wells was replenished weekly, and included fresh IL1 α , where indicated. At the end of the culture period all explants were wax-embedded and stained with either haematoxylin and eosin or toluidine blue using standard histological techniques (16,17). Toluidine blue, a metachromatic dye which binds to sulphated glycosaminoglycans, was used to visualise the loss of proteoglycans in response to IL1.

Determination of collagenase activity

The presence of interstitial collagenase in cartilage explant conditioned medium was determined using the quenched fluorescent substrate 7-methoxycoumarin-4-ylAcetyl(Mca)-Pro-Leu-Gly-Leu-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl) (Dpa)-Ala-Arg-NH (from Dr. C. Graham Knight, Strangeways Research Laboratory, Cambridge, UK) (18). The assays were performed at 37 °C using a Perkin Elmer LS50B fluorimeter linked to a computer running the FLUSYS software (19). The substrate and media were diluted in 0.1 M Tris-HCl, pH 7.5, containing 10 mM CaCl₂ and 0.2% v/v Triton X-100. The steady-state rate of cleavage was recorded and expressed as product per minute per cartilage explant.

Results

Histological demonstration of proteoglycan and type II collagen loss from IL1-induced bovine nasal cartilage explants

Bovine nasal cartilage explants cultured in the absence of IL1 stained intensely with toluidine blue (Figure 1a). An effect of IL1 on proteoglycan breakdown was detectable after 1 week of stimulation with the catabolic agent (Figures 1e-f). As shown in Figures 1a, e and f, there was a good correlation between the concentration of rhIL1 α used to induce proteoglycan loss, and the loss of metachromasia with toluidine blue stain. Dark blue staining with toluidine blue disappeared with IL1 treatment, indicating the loss of sulphated proteoglycan. Removal of 0.3 nM but not 3 nM IL1 from the culture system after 2 weeks was followed by increased metachromasia of the pericellular matrix, stained with toluidine blue, indicating considerable recovery from the effects of the lower concentration of cytokine (Figure 1e).

Effects of rhIL1 α on chondrocyte morphology in bovine nasal cartilage explants

The effects of matrix degradation on chondrocyte morphology were assessed histologically using haematoxylin and eosin staining. In the nasal cartilage chondrocytes were round with pale-pink cytoplasm after 1 week of culture in DMEM (Figure 2a). They fully filled their lacunae. In control explants cultured in DMEM only, chondrocytes mostly kept their typical characteristics during the 3-week culture period (data not shown). Histological sections of cartilage explants incubated in the presence of rhIL1 α showed marked morphological changes at the cellular level. After 1 week, chondrocytes started to show heterogeneity in cell shape. One morphological type observed during this period was pyknosis, showing condensed chromatin staining (Figure 2d). A second group of cells, with enlarged nuclei, were also present. Others showed cytoplasmic eosinophilia, suggesting the presence of acidic organelles in the cytoplasm (such as lysosomes, or secretory vesicles). In the third week, an increase in the cell:matrix ratio was observed due to the rapid loss of extracellular matrix (Figure 2f). It has been suggested that most mammalian cells require signals from other cells and/or from the extracellular matrix to survive. To determine whether the cell shape changes were related in any way to cell death, lactate levels in explant conditioned media were measured in the presence and absence of rhIL1 α by the lactate oxidase/oxidase method using a kit supplied by Sigma Chemical Co. As chondrocytes respire largely anaerobically, rates of lactate secretion are reliable measures of the cells' metabolic state. In our study, there was an increase in lactate levels produced by explants treated with IL1 (Table 1). The decrease in lactate levels during the fourth week of IL1 stimulation may be explained by the decreased cell number in explants, following the rapid loss of surrounding matrix and replenishment of the conditioned medium.

MMP activity in rhIL1 α induced nasal cartilage

Bovine nasal cartilage explants were incubated in the presence and absence of both IL1 for 2 weeks and were then transferred into control medium (without IL1) for a further 2 weeks. Under these conditions IL1-induced collagen release and fluorimetric assays also revealed an increase in MMP activity against quenched fluorescent substrate in media from nasal explants cultured with IL1 for 2 weeks (Table 2).

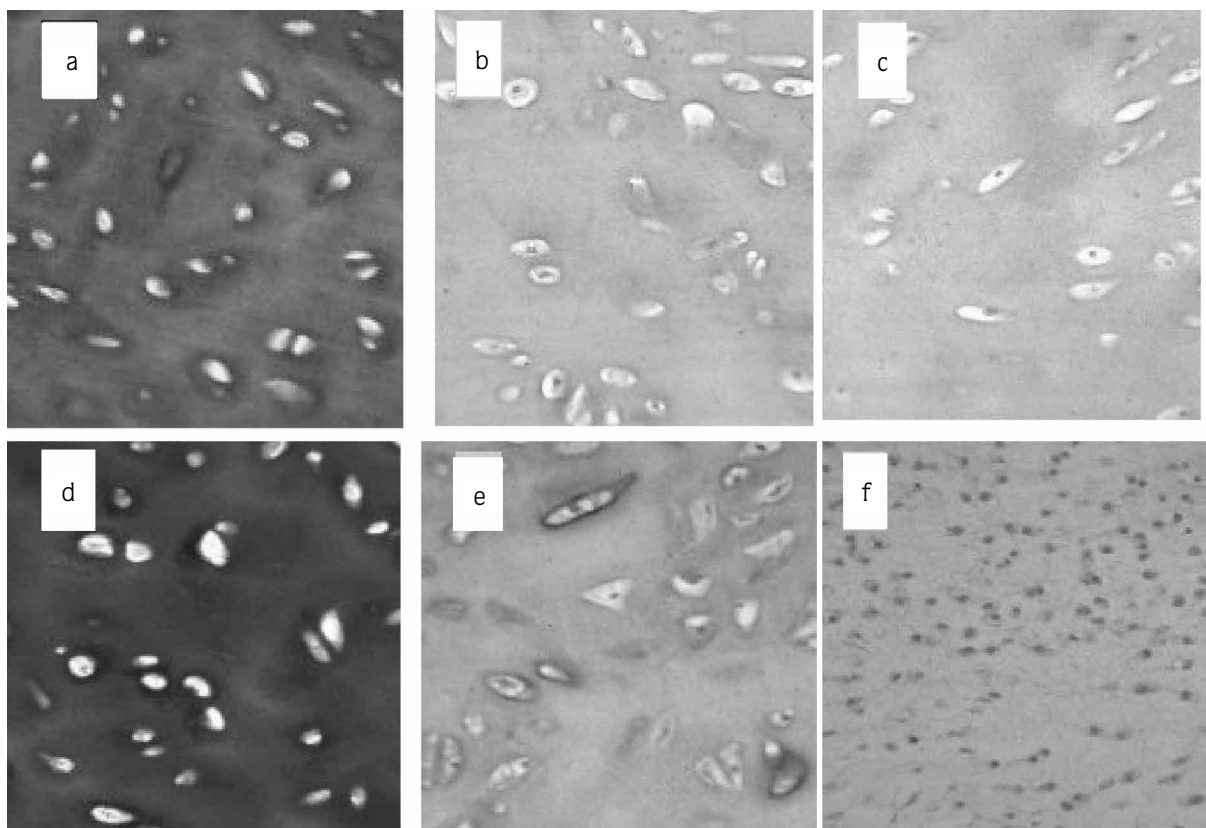


Figure 1 Metachromasia in control and IL1-treated bovine nasal cartilage explants.

Explants were cultured with or without IL1 for 4 weeks. Medium was replenished on days 7, 14 and 21. Explants were fixed in 4% (w/v) paraformaldehyde in PBS, pH 7.2, processed for histology and stained with toluidine blue (x 160).

- a) Section from a cartilage explant cultured for 1 week in DMEM alone.
- b) Section from a cartilage explant cultured for 1 week in the presence of IL1 (0.3 nM).
- c) Section from a cartilage explant cultured for 1 week in the presence of IL1 (3 nM).
- d) Section from a cartilage explant cultured for 4 weeks in DMEM alone.
- e) Section from a cartilage explant cultured for 2 weeks in the presence of IL1 (0.3 nM) and 2 weeks in DMEM.
- f) Section from a cartilage explant cultured for 2 weeks in the presence of IL1 (3 nM) and 2 weeks in DMEM.

Discussion

The experiments described here suggest that IL1 may drive chondrocytes towards a morphological change. Such a change must be long-term, as removing IL1 after 2 weeks does not lead to reversion to the original morphology. Chondrocytes in bovine nasal explants cultured with IL1 seem to reveal 2 distinct patterns of cell shape, pyknotic or enlarged chondrocytes. Kouri et al. (20), in a case study, described such phenotypic variations in OA chondrocytes. They reported large, round cells forming “clones”. Large secretory chondrocytes and degenerating cells with pyknotic nuclei were also present in the same OA patient. Interaction between these morphologically different

chondrocytes may be important in inducing MMP synthesis and activation, since chondrocytes have been reported to be dependent on autocrine signals from other chondrocytes for their survival (21). Previous studies on OA chondrocytes have shown increased synthesis of MMPs (22). Baici et al. (10) also reported that chondrocytes in OA cartilage contain more intracellular organelles, indicating a potentiation of their synthetic and secretory activities, and increase secretion of cathepsin B. In agreement with reports by Baici et al. (9), in the present study, IL1 caused increased cytoplasmic eosinophilia in some chondrocytes in bovine nasal cartilage explants (Figure 2d), suggesting increased lysosomal activity.

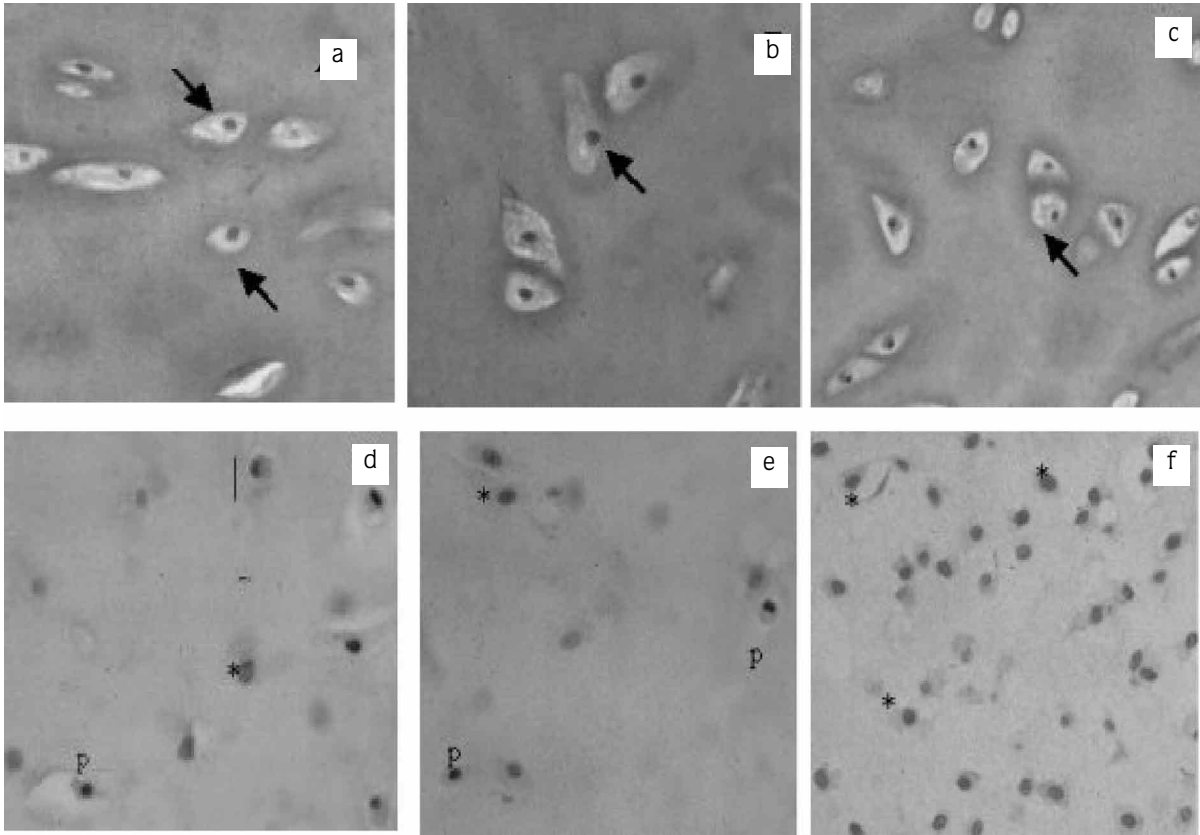


Figure 2. Effects of IL1 treatment on chondrocytes in bovine nasal explants.

Explants were cultured with or without rhIL1 α (3 nM) for 3 weeks. IL1 treatment was given for up to 2 weeks. Explants were then cultured for a further week in DMEM, sectioned and stained with haematoxylin and eosin (x 400). Chondrocytes in control explants fully filled their lacunae, keeping the characteristic appearance during the cultures (arrows). In explants cultured with IL1 some cells contained pyknotic nuclei (p), others with enlarged nuclei (*). Some cells showed cytoplasmic eosinophilia †

a-c) Sections from cartilage explants cultured for 1-3 weeks, respectively, in DMEM alone.

d) Section from cartilage explant cultured in the presence of IL1 for 1 week.

e) Section from cartilage explant cultured in the presence of IL1 for 2 weeks.

f) Section from cartilage explant cultured in the presence of IL1 for 2 weeks and then 1 week in DMEM alone.

Table 1. Lactate levels in media conditioned by cartilage explants.

Week of Culture	Control	IL1- α
1	285	339
2	289	335
3	251	318
4	304	198

The conditioned medium from control and rhIL1 α (3nM) stimulated cultures of bovine cartilage explants were assayed by the lactate oxidase/peroxidase method. Results are expressed as μ g of lactate per explant.

Table 2. MMP activity in the conditioned medium.

Week of Culture	Control	IL1-a
1	2.7	2.7
2	4	2.7
3	4	4189
4	5.3	2833

The conditioned medium of control and rhIL1 α stimulated cultures of bovine cartilage explants. Results are expressed as pmol of product produced per explant per min.

In this work enzyme activity analysis suggested a correlation between collagenolytic activity and the occurrence of pyknotic and enlarged cells. It is possible that IL1 may have an effect on cell shape change secondary to the direct molecular or biochemical effects it has on chondrocytes, such as breakdown of non-collagenous extracellular matrix components. The cell shape change may then lead to synthesis of proteolytic enzymes via changes in the cytoskeleton (7). Interactions between the extracellular matrix and cells via surface receptors such as integrins can be critical for the functions of living cells. Such receptors are the sites at which extracellular matrix molecules, such as fibronectin, and type II and VI collagens bind to the cell surface to regulate the behaviour of the chondrocytes via signalling pathways. Integrins are one class of these multiple cell membrane receptors. Adams and Watt (23) demonstrated that integrin-mediated events regulate differentiation via ligation of common cell surface receptors, leading to cell-type-specific responses. A study by Homandberg and Hui (24) reported that fibronectin fragments might cause enhanced release of stromelysin-1 and catabolic agents, such as IL1, TNF- α and IL6, from articular cartilage. It has also been shown that fibronectin acts via an integrin receptor, $\alpha 5 \beta 1$, which is present on the chondrocyte surface. Together with the data by Loeser et al. (25) reporting the increased expression of $\alpha 5 \beta 1$ heterodimer in a non-human primate model of OA, it is likely that integrins play an important role in cartilage remodelling. Wang et al. (26) suggested such roles for other transmembrane molecules such as cadherins or cell surface proteoglycans, for instance CD44. Indeed, IL1-treated chondrocytes in bovine nasal cartilage explants seemed to be retracted from the edges of their lacunae and started to show nuclear polarity during the first weeks of cultures, at a time point which corresponded with massive proteoglycan loss, which may indicate a relation between the cell surface receptors and changes in cell behaviour.

In bovine nasal cartilage, chondrocytes degraded proteoglycan in their surrounding matrix, in response to

IL1 and only after the loss of proteoglycan morphological changes (both pyknotic and enlarged) became more prominent, suggesting that the morphological change might be related to the alteration in the matrix surrounding the chondrocytes. Increased MMP activity occurred in the presence of 2 distinct types of cell morphology. High levels of MMP activity could only be detected following treatment with 3 nM IL1 but not with 0.3 nM. At lower doses of IL1 treatment, breakdown of proteoglycan and other non-collagenous matrix proteins seemed to be reversible and there was no significant increase in MMP activity. One possibility for the variability in responses to different doses of IL1 may be that a critical threshold of IL1 concentration is required for the cell shape change and to commit the cells to secrete MMPs as described by Werb et al. (7). It is also possible that degradation of some non-collagenous proteins may act as an anabolic factor in cartilage turnover and promote protein synthesis at low doses of IL1 stimulation and removal of the stimulus allows the chondrocytes to recover, whereas high doses of IL1 exposure may further increase degradation of such proteins and their breakdown products (e.g., fibronectin fragments), which may then irreversibly lead to catabolic changes. A study by Homandberg and Hui (24) reported such differential responses in the release of proteoglycans in bovine articular explants treated with different concentrations of fibronectin fragments.

To conclude, this study suggests that loss of proteoglycans may not be sufficient on its own to induce the synthesis of collagenolytic enzymes. Presence of a catabolic agent, such as IL1, the duration and the amount of the stimulation and the extent of the initial matrix loss may be the other determinants for the cells to commit themselves to produce collagenolytic enzymes and for the irreversible collagen loss.

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