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Original Article

Matrix Metalloproteinase-3 Expression and Structural Changes in Biomechanical Loaded Articular Cartilage

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Background: Cartilage functions as a highly successful load-bearing tissue. However, frequent mechanical loading can affect cartilage metabolism and viability significantly. Intrinsic changes in the metabolism of chondrocytes have also been proposed as an important feature of osteoarthritis. Although many aspects of chondrocyte catabolism have been extensively investigated, the signals that initiate such metabolic changes, and their correlation with structural changes, remain unclear. We hypothesized that matrix metaproteinase-3 (MMP-3) may play a significant role. The purpose of this study was to investigate the activity of MMP-3 in cartilage under load, as well as degeneration-related structural changes of the cartilage matrix under load.

Method: Tissue blocks of porcine cartilages were prepared for tissue culture, and biomechanical forces were applied with a FlexerCell system. Compressive stress of 20 MPa at 0.3 Hz was induced in the experimental cartilage for 240 minutes. The expression and activity of MMP-3 in the experimental cartilage were measured before and at the time points of 4 hours, 24 hours, and 48 hours after start loading. Cartilage samples without loading were used as a control. Structural and ultrastructural changes in the cartilage were investigated under Normarski light microscopy and scanning electron microscopy.

Results: Our results indicated that MMP-3 activity in the loaded cartilages was significantly higher than in the control group. Structural and ultrastructural investigations found evident degradation of the cartilage matrix in loaded cartilages. This study provides information regarding the changes in MMP-3 activity in the loaded cartilages, and the changes in cartilage matrix under load.

Conclusion: This study demonstrated that, under biomechanical force, the matrix of articular cartilage was degraded, with a higher expression of MMP-3. (Tw J Phys Med Rehabil 2011; 39(4): 211 - 217)

Key Words: biomechanical forces, matrix degradation, matrix metalloproteinase-3, articular cartilage

INTRODUCTION

Cartilage functions as a load-bearing tissue because of the coupling of the tension-resisting collagen fibrils with the hydrated proteoglycan component. Neither of these two principal macromolecular components is individually capable of providing a successful compres-

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sion-bearing system. However, the fibrils (and in particular, their interconnected architecture) provide the matrix with an intrinsic strength sufficient to resist rupture under a wide range of loading conditions. Researchers have widely acknowledged that cartilage degeneration involves a breakdown of the fibrillar architecture.[1-3] However, the exact nature of these ultrastructural changes (from normal to abnormal) is not well understood. The correlation between these changes and the intrinsic strength of the cartilage is also unclear.

The widely quoted but largely unproven "wear and tear" hypothesis tends to emphasize that cartilage is a relatively inert tissue-bearing surface, in which loss of thickness results from mechanical wear. The same hypothesis posits that, apart from limiting joint use or loading levels, little can be done to prevent further joint deterioration. The alternative view is vastly more complex but has become more widely accepted. This second hypothesis suggests that the loss of articular cartilage associated with degenerative osteoarthritis results from a biochemically weakened matrix, which is rendered more vulnerable to physiologically normal levels of joint loading.[4] Osteoarthritic changes in pericellular and extracellular matrix properties were also found to significantly alter the mechanical environment of chondrocyte. Such changes lead to higher compressive strain and higher fluid flux near the cell. Some researchers have suggested that changes in the properties of the pericellular matrix associated with osteoarthritis may alter the stress-strain and fluid flow environment of the chondrocytes.[5-7]

Matrix metalloproteinase-3 (MMP-3), also known as stromelysin 1, is a neutral metalloproteinase produced by chondrocytes and synovial fibroblasts.[8-10] 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. These active forms cleave several different cartilage matrix components, including aggrecan,[11,12] link protein,[11] and type II collagen, type IX collagen, type X collagen, and type XI collagen.[13] MMP-3 has been found in arthritic cartilages, 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 osteoarthritis (OA),[8,14-16] and in animal models of OA.[17,18] Elevated levels have also been detected in cases of knee injury[11] and heat shock.[19] Previous studies have demonstrated that MMP-3 increases in osteoblast-like cells under compression.[20] A recent report by Chang et al discussed the role of MMP-3 in response to compressive forces *in vitro* and *in vivo*.[21] In their study, compressive force was applied on human osteoblast-like cells, and MMP-3 was shown to be up-regulated in response. The same study also demonstrated the up-regulation of MMP-3 *in vivo* by orthodontic force.

In addition to the cleavage of aggrecan and link protein, MMP-3 has been shown to be effective at degrading the three major types of collagen (II, IX, and XI) present in articular cartilage. MMP-3 had the effect of decoupling the type II-type IX collagen complex.[13]

The extracellular matrix turnover rate is extremely low; aggrecan half-life has been calculated at two years, while that of type II collagen is over 100 years.[22] A recent study demonstrated that, from the instance of loading, the degenerated matrix generated a higher peak hydrostatic excess pore pressure in a shorter period of time than the normal matrix.[23]

The purpose of the current study was to detect structural changes and the response of MMP-3 to biomechanical forces in the cartilage matrix.

METHODS

Tissue Culture

All animal experiments were performed according to a protocol approved by the Review Committee of Kaohsiung Veterans General Hospital. Ten freshly slaughtered immature pigs (aged 3-5 months) were used to harvest 120 samples of cartilage, which were excised as thin blocks (approximately 2 mm x 2 mm x 0.5 mm) from the front foot knee joints. The cartilage blocks were cultured in serum-free Dulbeco's modified Eagle's medium, supplied with 1 % penicillin and streptomycin plus essential vitamins. Sixty samples were used for the biomechanical experimental group, and the remaining 60 samples were used for the control group.

Biomechanical Experiments

All specimens were prepared for the biomechanical

experiment using the FlexerCell system (McKeesport, PA, USA). In this system, specially designed plates with six wells are used for tests. We placed each specimen in its own well, with the articular surface facing upward, and added 1500 µl of medium to each well. Stress was then applied to the tissue to determine the effects of mechanical compression on the cartilage. Cartilage explants were subjected to a cycle of repeated compressive loading to a stress of 20 MPa at 0.3 Hz for 240 minutes. Control samples were not subjected to loading stress.

Prior to loading and at the time points of 4 hours, 24 hours, and 48 hours after the start of mechanical loading, the culture media of the 60 normal (control) samples and the 60 loaded cartilage explant cultures were collected. These were analyzed for MMP-3 activity. To examine the most extreme changes in structure, the 48-hour loaded samples were fixed with 4% paraformaldehyde and preserved at -70 ^{o}C. These samples would undergo further cryosection for structural and ultrastructural study and would be compared with the control samples.

Evaluation of MMP-3 Activity

The activities of MMP-3 in the compressed and normal samples were compared using MMP-3/Stromelysin Activity Assay Kit (Chemicon International Co., USA). The fluorescence intensity of the resulting product was measured at 520 nm (Em)/495 nm (Ex) and correlated with MMP-3 activity.

Structural and Ultrastructural Studies

To investigate how loading influences structural change, 60 cryosections (60 µm in thickness) from the normal and the fixed loaded cartilage samples were examined separately. We used both Nomarski light microscopy and scanning electron microscopy 48 hours after loading. No further preparation is necessary for hydrated fixed slices to be observed with Nomarski light microscope.

After the Nomarski light microscopy investigation, the same slices were prepared for scanning electron microscopy. The fixed sections were digested with hyaluronidase and trypsin (1 mg/1 ml 0.1M PBS) for three days, with the PBS rinse and digestion solution being changed daily. After being fixed in 2 % OsO_4 for 2 h at 4 ^{o}C, the sections were treated with 2 % tannic acid for 4 h at 4^{o} C. We then applied a degraded ethanol dehydration procedure to dehydrate the specimens, with critical point drying, and they were subsequently coated with gold.

STATISTICAL ANALYSIS

Results are presented as the mean ± standard deviation (SD) of 15 independent cultures. Statistical significance was calculated using one-way analysis of variance (ANOVA), followed by a post hoc procedure (Bonferoni analysis. The significance level was set at $p<0.005$).

RESULTS

Our results indicated a higher expression of MMP-3 in loaded cartilage than in the control. Figure 1 shows the analysis of MMP-3 activities as measured before loading and at 4 hours, 24 hours, and 48 hours after loading began. The results for the experimental group and the control

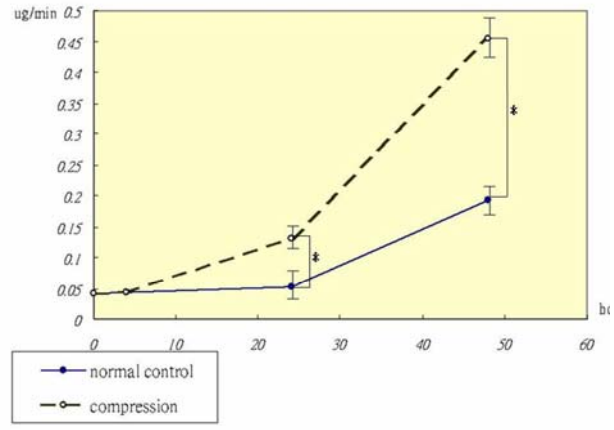
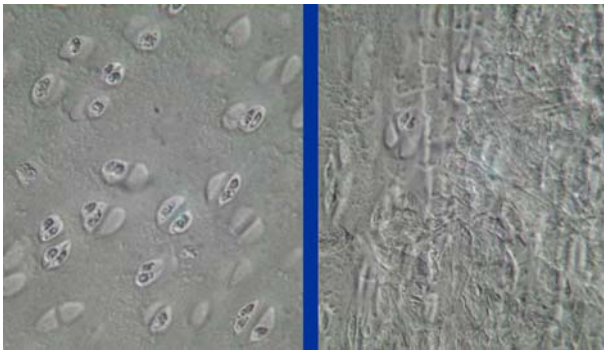


Figure 1. Comparison of MMP-3 activities of articular cartilage in the experimental group before loading and at the time points of 4 hours, 24 hours, and 48 hours after start loading, and the MMP-3 activities in the control group without loading in the same time schedule (Horizontal axis: time (hour), Vertical axis: MMP-3 activity (µg/min). The activities of MMP-3 in the articular cartilages were increased 24 hours and 48 hours after start loading in the experimental group. The asterisk denotes a significant difference ($p<0.005$) compared with the control group, determined by the post hoc procedure (Bonferoni analysis).

group are shown according to the same time schedule. The activity of MMP-3 in the experimental group was shown to be the same as that in the control group before loading and 4 hours after loading. By contrast, 24 hours and 48 hours after loading, respectively, the activity of MMP-3 was elevated in the experimental group relative to the control. This difference in activity level was statistically significant.

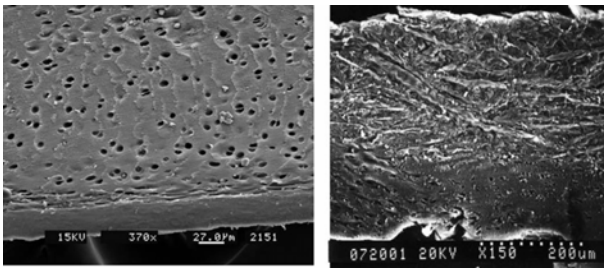
At the light microscope level of resolution, the structural consequences of loading were observed. The control tissue (Figure 2a) exhibited an entirely normal-appearing matrix, whereas the loaded matrix was extensively modified and disrupted (Figure 2b).

The ultrastructural studies supported the microscopic findings, that the articular surface was intact in the control group, with the underlying matrix finely textured (Figure 3a). By contrast, the loaded matrix exhibited severe fragmentation of its articular surface, with the underlying general matrix characterized by the presence of degradation (Figure 3b).



(a) (b)

Figure 2. Images of articular cartilage observed under Normarski light microscopy: (a) Normal cartilage without loading was shown with normal matrix; (b) Cartilage samples 48 hours after start loading was shown with disrupted matrix (Magnification=200 x).



(a) (b)

Figure 3. Ultrastructural investigation of articular cartilage under scanning electron microscopy: (a) Normal cartilage was shown with finely textured matrix; (b) Loaded cartilage sample was shown with degraded matrix 48 hours after start loading.

DISCUSSION

This study investigated the expression of MMP-3 in loaded cartilages with matrix degradation. Previous studies had examined the expression of MMP-3 in osteoarthritic patients.[12,13] Researchers have proposed that cartilage in human joints is continuously subjected to normal mechanical loads *in vivo*, which can cause peak contact stresses as high as 15-20 MPa.[22] A previous study also found that stress of approximately 20 to 30 MPa, and strains of 20 % or more, were required to produce significant cell death.[24] The same report suggested that actual tissue damage was restricted by the design of the researchers' drop tower, which was intended to produce a constant strain rate during sample compression. A separate report showed that stresses and strains of such magnitude are comparable to those that are known to fracture the patella or femur in humans.[25]

Researchers have also suggested that higher impact loads may cause tissue injury[26] and initiate cartilage degradation.[27] Previous *in vitro* models of mechanical injury to cartilage explants have demonstrated increased turnover of matrix proteoglycans and proteins.[28,29] Previous research has also established that articular chondrocytes can respond to mechanical stimuli and to alterations in their physicochemical environment. In addition, previous *in vitro* studies of chondrocyte-mediated matrix turnover following mechanical injury showed that mechanical load can produce nonviable cell populations exhibiting condensed nuclei.[29]

Studies of mechanical injury to cartilage explants have demonstrated that tissue swelling is consistent with damage to the collagen network.[30,31] The experiments described in this paper provide a means of producing physical disruption of the original fibrillar network. This structural damage involved the formation of clefts predominantly at the articular surface and in the adjacent

superficial zone, and at approximately 45 degrees to the surface. This suggests that cleft formation involves a type of shear mechanism because any uniaxial compression can be resolved into a deviatoric and a dilatational component. The dilatational strain is a bulk volume change; the deviatoric comprises two perpendicular shear strains at 45 degrees to the applied load. Similar observations of this type of oblique rupture of cartilage have also been made by other researchers.[30,32] Full thickness clefts extending from the bone to the articular surface are rarely found in animal experiments.[26] These findings suggest that the cartilage matrix itself might resist crack propagation, as previous researchers have noted.[30]

Structural differences between normal and abnormally softened cartilage were previously reported with a fibrillar transformation model.[1,2] The general matrix of human cartilage with osteoarthritis was characterized as having a predominantly radial configuration of collagen fibril bundles.[3] Though not properly tested, the structural changes in articular cartilage were proposed to possibly be related to MMP-3.[1] Previous studies had found a higher expression of MMP-3 in osteoarthritic patients than in normal patients.[12,13] Our study indicated that under biomechanical force, cartilage has a higher expression of MMP-3, with matrix degradation; this finding may explain the pathological change in cartilage in osteoarthritic patients.

We utilized the FlexerCell to apply compressive loading. The FlexerCell provides a controllable means of producing physical disruption to articular cartilage, without immediately altering the macromolecular composition. Our study found that the increased levels of MMP-3 activity occurred after one day loading.

CONCLUSION

This study demonstrated that, under biomechanical force, the matrix of articular cartilage became degraded with a higher expression of MMP-3 than in normal cartilage. This finding is important for furthering the understanding of the pathological changes of cartilage in osteoarthritic patients.

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關節軟骨生物機械受力後細胞外基質金屬蛋白酶與結構表現變化

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高雄榮民總醫院復健科

前言：軟骨是承受壓力很好的組織，常給予反覆的機械力會影響軟骨的代謝及活性。軟骨細胞代謝的內部改變可能是導致退化性關節炎的重要因素。雖然軟骨代謝的現象曾被廣泛探討，而啓動其中代謝反應的變化及相關的結構改變中的訊息傳導機制仍不清楚。本研究的目的乃是探討軟骨在受力之後的微結構變化，與受力後細胞外基質金屬蛋白酶(matrix metalloproteinase-3, MMP-3)的變化。

方法：本研究分析軟骨在組織培養下接受機械力之後，MMP-3 的表現並探討細胞外基質的降解性變化與結構和微結構的改變。軟骨組織切片經由組織培養環境利用 FlexorCell 系統給予壓力，在 0.3Hz，20MPa compressive stress 之下作用 4 小時，將受力前及於受力開始後經 4 小時、24 小時及 48 小時軟骨之 MMP-3 表現與控制組進行比較，並將軟骨結構及微結構變化利用 Nomarski 光學顯微鏡及掃瞄式電子顯微鏡進行觀察。經由此方法，我們探討軟骨在受力之後 MMP-3 之表現，並探討軟骨在受力之後的微結構變化。

結果：本研究結果顯示在受力的軟骨中，相較於控制組會有較高的 MMP-3 的表現。在受力的關節軟骨，由 Nomarski 光學顯微鏡可看到破壞性的結構改變，而在電子顯微鏡下可看到受力的軟骨會有軟骨細胞外基質的降解性變化。

結論：由本研究，我們發現在生物機械力作用下，關節軟骨之細胞外基質會有降解變化並表現較多的 MMP-3。（台灣復健醫誌 2011；39(4)：211 - 217）

關鍵詞：生物機械力(biomechanical forces)，細胞外基質降解性變化(matrix degradation)，細胞外基質金屬蛋白酶(matrix metalloproteinase-3)，關節軟骨(articular cartilage)

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