Dietary Loading and Aggrecanase-1/TIMP-3 Expression in Rat Mandibular Condylar Cartilage

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Aims: *To examine the expression of aggrecanase-1 and a tissue inhibitor of metalloproteinases (TIMP-3) in the condylar cartilage of young rats and to determine their relationship during altered dietary loading at different time points after weaning.* ***Methods:*** *One hundred Sprague-Dawley rats were randomly assigned to 1 of 2 groups: the soft-diet group, which served as the control group (n = 50), or the hard-diet group, which served as the experimental group (n = 50). Ten soft- and 10 hard-diet rats were killed at 6 hours, 12 hours, 24 hours, 48 hours, and 9 days after weaning (ie, after initiation of diet change for hard-diet rats). The right-side temporomandibular joints (TMJs) were prepared for immunohistochemical staining. The cartilage from the left-side mandibular condyles of all 10 animals in each group was combined for Western blot analysis.* ***Results:*** *Immunohistochemical analysis revealed strong staining for aggrecanase-1 localized mainly in the chondrocytes of proliferative and upper hypertrophic cartilage zones at all time points in both groups. The immunohistological expression of aggrecanase-1 was significantly higher in the hard-diet group at 12 and 24 hours than in the soft-diet group. Strong staining for TIMP-3 was mainly localized in the chondrocytes of proliferative and upper hypertrophic zones at all time points in both groups. The expression of TIMP-3 in the hard-diet group was at a significantly lower level compared to the soft-diet group at 6 hours. Western blot analysis also showed time-related differences in aggrecanase-1 and TIMP-3, but there was no significant difference between the 2 groups.* ***Conclusion:*** *The temporary change in aggrecanase-1 and TIMP-3 expression reflects the complex interaction of these enzymes in the physiologic range and cartilage response to altered dietary loading.* J OROFAC PAIN 2007;21: 232–238

Key words: aggrecanase-1, joint loading, mandibular condylar cartilage, TIMP-3

The condyle of the temporomandibular joint (TMJ) is an important growth site for the mandible. The mandibular ramus is elongated via the tissue-separating capacity of the growth cartilage in the mandibular condyle.[1] The condylar cartilage consists essentially of chondrocytes embedded in a highly hydrated extracellular matrix (ECM), proteoglycans (PGs), and noncollagenous proteins immobilized within a network of mainly type II collagen.[2,3] Normal cartilage metabolism is characterized by a highly regulated balance between the synthesis and degradation of the various components of ECM. In this process, a variety of proteases have been implicated.

Aggrecan, an aggregating PG, provides the cartilage with its mechanical properties of compressibility and resilience during joint loading. One of the suggested causes of cartilage destruction in degenerative joint diseases is the loss of aggrecan. This is considered a critical early event carried out first by cartilage aggrecanases and then by matrix metalloproteinases (MMPs).[4–6] As a consequence, the tissue loses its capacity to resist compression under load, which eventually leads to irreversible mechanical destruction of the cartilage. Aggrecanase-1 (ADAMTS-4), which belongs to the ADAMTS (a metalloproteinase with disintegrin and thrombospondin type-1 motifs) family, plays an important role in aggrecan degradation in ECM.[7,8]

The tissue inhibitors of metalloproteinases (TIMPs) are important regulatory factors in the activity of MMPs. Basically, TIMP-1 and TIMP-2 inhibit the activity of all MMPs, whereas TIMP-3 only inhibits MMP-1, -2, -3, -9, and -13.[9] Aggrecanase-1 is inhibited by TIMP-1, -2, -3, and -4, but it is most efficiently inhibited by TIMP-3.[10]

Growth of the condylar cartilage relies on sufficient biomechanical stimuli.[11] Altered loading delivered by a different diet is a useful method for observation of the possible changes in articular cartilage composition of the condyle. Several studies have shown that a soft diet leads to decreased growth of the mandibular condyle compared with a hard diet.[12–14] A previous study also showed that reduced loading of the TMJ by a soft diet and altered chewing capacity led to a lower number of chrondrocytes and a thinner cartilaginous layer in the articular surface of the rat mandibular condyle.[2,15] Only a few studies have examined the expression of aggrecanase-1 and TIMP-3 in the cartilage.[16,17] The regulation of aggrecanase-1 and TIMP-3 expression during the early growth of the joints remains poorly defined.

The aim of this study was to examine the expression of aggrecanase-1 and TIMP-3 in the condylar cartilage of young rats and to determine their relationship during altered dietary loading at different time points after weaning.

Materials and Methods

Animals and Tissue Preparation

One hundred female Sprague-Dawley rats were fed soft powdery food from 15 days of age. Water was available ad libitum. After weaning, at 21 days of age, all rats were randomly assigned to 1 of 2 groups (50 animals per group). The soft-diet group, which served as the control group, continued on soft powdery food, and the hard-diet group, which served as the experimental group, was switched to hard pellet food. The hard-diet group was considered the experimental group because the diet was changed in this group. Ten soft- and 10 hard-diet rats were killed at 6 hours, 12 hours, 24 hours, 48 hours, and 9 days after initiation of the experiment. The protocols were approved by the Animal Experiment Committee of University of Oulu.

The cartilage from the left mandibular condyle of all 10 animals in each group was carefully removed with an excavator and pooled together. Cartilage samples were stored in sterile plastic tubes at –80°C. The right-side TMJs were decalcified with 5% formic acid, heated in a microwave oven at 37°C (Micromed T/T MEGA) 6 times for 5 hours each time, and then embedded in paraffin. Serial sections 6 μm wide were cut parallel to the sagittal plane of the mandibular condyle. Deparaffinized sections were stained with toluidine blue, and the most central sections were selected for histologic observation.

Immunohistochemical Analysis

Immunohistochemistry was carried out with a previously described method.[15] The most central sagittal sections of the TMJs were chosen for immunohistochemistry. Deparaffinized sections were pretreated with 0.4% pepsin for 60 minutes at 37°C for TIMP-3 staining or for 30 minutes at 98°C in a microwave oven for aggrecanase-1 staining. Endogenous peroxidase activity was quenched by treatment with 0.2% H_2O_2 for 3 hours. Nonspecific binding of antibodies was blocked by normal goat serum treatments. The sections were incubated with diluted polyclonal anti-goat aggrecanase-1 (0.004 mg/mL) and polyclonal anti-goat TIMP-3 (0.001 mg/mL) antibody (Santa Cruz Biotechnology) overnight at 4°C. For negative controls, polyclonal nonimmune goat serum was used. The immunostaining was visualized with a Vectastain Elite kit (Vector Laboratories) using a peroxidase and diaminobenzine substrate. The sections were counterstained with Mayer's hematoxylin (Histolab Products) and subsequently examined with an image analyzer in a light microscope (Leica Leitz DMRB/E) at a magnification of 150×. The number of positive cells in the entire condylar cartilage area was counted, and the relative number of positively stained cells was calculated. The cell counting was carried out by a single researcher who was blinded to the status of the tissue samples (experimental versus control).

Western Blot Analysis

To investigate the protein expression of aggrecanase-1 and TIMP-3, Western blotting was performed. All 10 left-side mandibular condylar cartilages were pooled together from each group at each time point, snap-frozen in liquid nitrogen, and crushed to powder. Every group was subjected to extraction with 50 mmol/L tris-(hydroxymethyl)aminomethane (TRIS), calcium chloride dihydrate (10 mmol/L $CaCl_2 \cdot 2H_2O$), sodium chloride (150 mmol/L NaCl), and 0.05% Brij-35, pH 7.5, for 2 hours at 4°C, followed by centrifugation for 10 minutes (13,000 rpm). Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories). A sample representing 15 μg of protein was loaded into each lane of a 12% SDS gel and run for 1.5 hours. The proteins were then transferred to nitrocellulose membrane, blocked by 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBST). The blots were incubated in TBST for 1 hour at room temperature at a dilution of 0.0005 mg/mL for aggrecanase-1 and 0.001 mg/mL for TIMP-3 primary antibodies, which were the same antibodies as used for the immunostaining (Santa Cruz Biotechnology). After washing, the blots were incubated with 1:10,000 diluted anti-goat antibodies in TBST for 1 hour at room temperature. After being washed with TBST, the membrane was incubated with electrochemiluminescence (ECL) detection reagents (ECL plus Western blotting detection reagents) for 1 minute at room temperature and immediately exposed to ECL film. Immunoreactivity of protein bands was quantified as optical density using image analysis software (Scion Image for Windows).

Histomorphometric Analysis

For histomorphometric analysis, the total thickness of the mandibular condylar cartilage was measured at the central area of the condylar surface. The central area was defined as the highest point of the condylar surface opposite the mandibular fossa. The soft- and hard-diet groups at 9 days were selected for analysis.

Statistical Analysis

The normality of the samples was tested before the analyses, and the distribution was found to be normal. Therefore, a parametric test was chosen. The Student *t* test was used to examine the statistical significance of differences. *P* values less than .05 were considered significant.

Results

Immunohistochemical Analysis

Immunohistochemical analysis revealed a clear staining for aggrecanase-1 localizing mainly in the chondrocytes of the proliferative and upper hypertrophic cartilage zones at all time points in both groups. There was only weak or no staining in chondrocytes and ECM of the lower hypertrophic zone (Fig 1a). The number of positively stained aggrecanase-1 cells was significantly higher in the hard-diet group compared to the soft-diet group at 12 ($P = .044$) and 24 ($P = .042$) hours after initiation of the experiment (Fig 1b). Clear staining for TIMP-3 was mainly localized in the chondrocytes of proliferative and upper hypertrophic zones at all time points in both groups. There was only weak or no staining in the chondrocytes and ECM of the lower hypertrophic zone (Fig 2a). The number of TIMP-3 positive chondrocytes was significantly lower in the hard-diet group compared to the soft-diet group at 6 hours ($P = .009$; Fig 2b).

Western Blot Analysis

Using an antibody against aggrecanase-1, Western blot analysis was utilized to recognize a band about 100 kDa corresponding to the latent form of aggrecanase-1 and 2 very weak bands about 60 and 70 kDa corresponding to the active forms of aggrecanase-1 in the soft- and hard-diet groups, respectively, at all time points (Fig 3a). The 100 kDa band of immunoreactivity was quantified (Fig 3b).

Aggrecanase-1 immunoreactivity showed a weak band at 12 and 24 hours in the soft-diet group, but a stronger band at 6 and 48 hours, after which a weaker band was seen at the 9-day time point. In the hard-diet group there was a weak band at 6 and 12 hours, after which there was a stronger band at 24 and 48 hours and a weaker band at 9 days (Fig 3b). Statistical analysis of immunoreactivities between the groups revealed no significant differences at any time point ($P > .05$).

In the Western blot analysis using antibody against TIMP-3, 2 clear bands about 30 kDa and 50 kDa were visible, 30 kDa corresponding to the active form of TIMP-3 and 50 kDa best corresponding to the TIMP-3 dimer. The active form of 30 kDa was further quantified (Fig 4a). In the soft-diet group TIMP-3 immunoreactivity showed weak bands at 6 to 24 hours and at 9 days and a stronger band at 48 hours. In the hard-diet group the immunoreactivity of the 30 kDa band was also

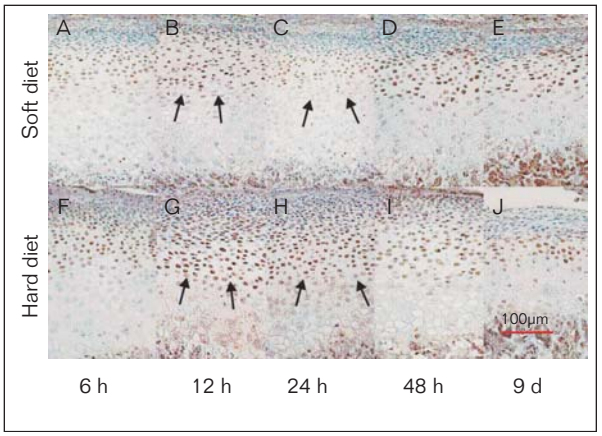

Fig 1a Sagittal central sections of mandibular condylar cartilage 6 μm thick. The soft- (A, B, C, D, E) and hard- (F, G, H, I, J) diet groups with aggrecanase-1 immunostaining at 6 hours (A, F), 12 hours (B, G), 24 hours (C, H), 48 hours (D, I), and 9 days (E, J) after initiation of the experiment. More staining against aggrecanase-1 was seen in the hard-diet groups compared to the soft-diet groups at 12 and 24 hours *(arrows)*. The articular surface is shown at the top of each image (original magnification ×200).

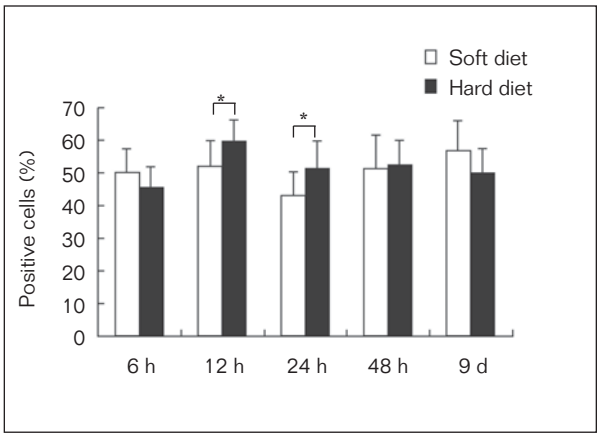

Fig 1b Percentage of cells labeled by immunostaining for aggrecanase-1 in the total condylar cartilage area (the number of positive cells divided by the total number of cells). Values are presented as means ± SD. Statistically significant differences between soft- and hard-diet groups are marked with asterisks ($P < .05$).

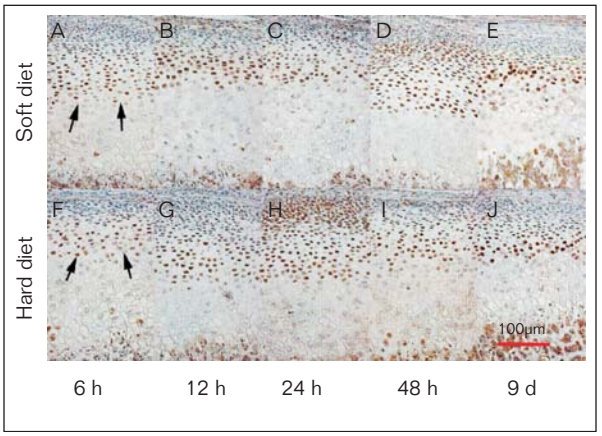

Fig 2a Sagittal central sections 6 μm thick of mandibular condylar cartilage. Soft- (A, B, C, D, E) and hard- (F, G, H, I, J) diet groups with immunostaining for TIMP-3 at 6 hours (A, F), 12 hours (B, G), 24 hours (C, H), 48 hours (D, I), and 9 days (E, J) after initiation of the experiment. Stronger staining against TIMP-3 was seen in the hard-diet groups compared to the soft-diet groups at 6 hours *(arrows)*. The articular surface is shown at the top of each image (original magnification ×200).

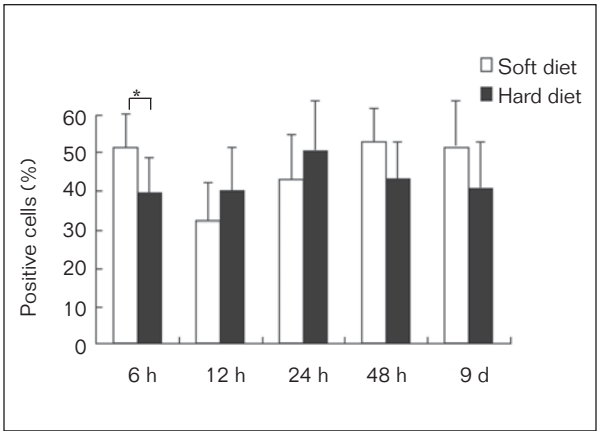

Fig 2b Percentage of cells labeled by immunostaining for TIMP-3 in the total condylar cartilage area (the number of positive cells divided by the total number of cells). Values are presented as means ± SD. A significant difference between the soft- and hard-diet groups is marked with an asterisk ($P < .05$).

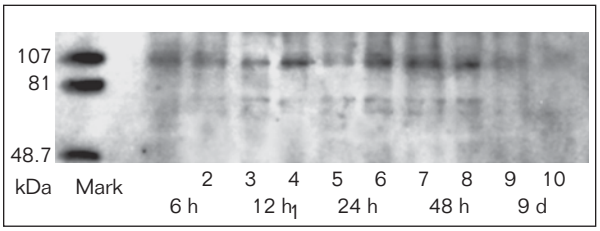

Fig 3a Expression of aggrecanase-1 in cartilage extracts at different time points, analyzed by Western blot analysis. Lanes 1, 3, 5, 7, and 9 show soft-diet groups, and lanes 2, 4, 6, 8, and 10 show hard-diet groups.

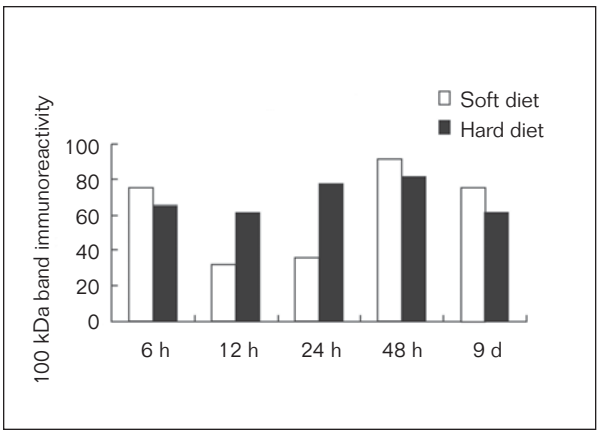

Fig 3b The 100 kDa band immunoreactivity representing a latent form of aggrecanase-1. In the soft-diet group there was a weak band at 12 and 24 hours but a stronger band at 6 and 48 hours, after which a weaker band was seen at the 9-day time point. In the hard-diet group there was a weak band at 6 and 12 hours, after which there was a stronger band at 24 and 48 hours and a weaker band at 9 days.

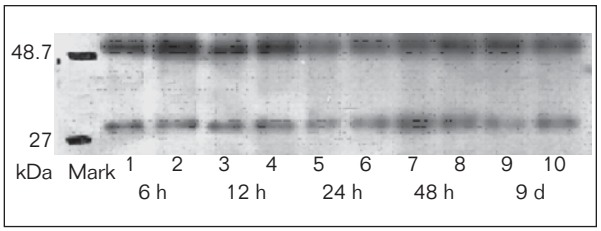

Fig 4a Expression of TIMP-3 in mandibular cartilage extracts at different time points, analyzed by Western blotting. Lanes 1, 3, 5, 7, and 9 show soft-diet groups, and lanes 2, 4, 6, 8, and 10 show hard-diet groups.

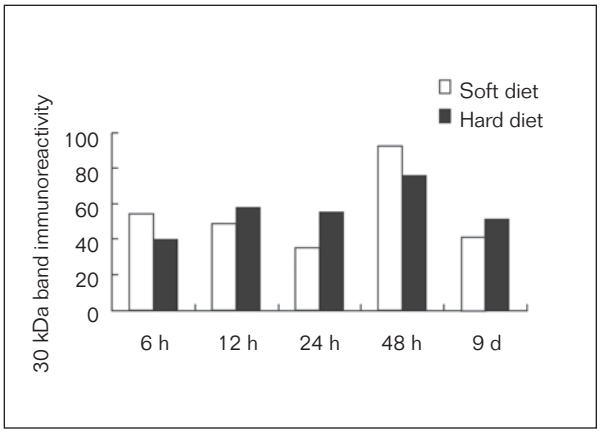

Fig 4b The 30 kDa band immunoreactivity representing an active form of TIMP-3. In the soft-diet group there was a weaker band at 6, 12, and 24 hours as well as at 9 days. There was a stronger band at 48 hours. In the hard-diet group the immunoreactivity of the 30 kDa band was also stronger at 48 hours and weaker at the other time points.

stronger at 48 hours and weaker at the other time points (Fig 4b).

Statistical analysis of immunoreactivities between the groups revealed no significant differences between the 2 groups at any time point ($P > .05$).

Histomorphometric Analysis

Histomorphometric analysis of the thickness of the condylar cartilage in the soft- and hard-diet groups at 9 days revealed a statistically significant difference ($P < .05$) between the 2 study groups. The total thickness of the condylar cartilage was smaller in the soft-diet group (mean, 424.8 μm; SD, 25.5) compared to the hard-diet group (mean, 582.3 μm, SD 69.2).

Discussion

The results of the present study show that dietary loading seems to be an important factor affecting the expression of aggrecanase-1 and TIMP-3 in the mandibular condylar cartilage. There is a limited amount of information available about the effects of mechanical stress on aggrecanase-1 expression. Dynamic compression in the intervertebral disc cells has been shown to elevate the expression of aggrecanase-1 in 2 hours in vivo.[18,19] Static compressive loading has also been shown to increase aggrecanase-1 expression in bovine cartilage explants.[20] The results of the present study show that the aggrecanase-1 expression in the hard-diet group was significantly higher compared to the

soft-diet group at 12 and 24 hours after weaning. At later time points no significant difference was seen. There was a similar change in the intensity of the latent form of 100 kDa band in the Western blot analysis. This finding indicates that there is a temporary change of aggrecanase-1 expression in the physiologic range due to a change of diet and thus dietary loading.

TIMP-3 is considered an inhibitor of aggrecanase-1.[10,21,22] TIMP-3 expression in rodent knee menisci has been shown to increase significantly in tissues cultured by reduced loading. Subjecting menisci of the rabbit knee to compressive hydrostatic pressure significantly prevents the increases in TIMP-3 mRNA levels.[23] Le Maitre et al[24] reported an increase in the number of immunoreactive cells for aggrecanase-1 with increasing degradation of the human intervertebral disc. This was not paralleled by a rise in its inhibitor TIMP-3.[24] The possible explanation is that the range of the physiologic process may represent a positive correlation between aggrecanase-1 and TIMP-3, analogous to the relation between MMPs and TIMPs, in order to maintain the physiologic balance.[25] In pathologic processes, the balance would be altered if expression of aggrecanase-1 were increased and the level of its inhibitor were decreased.

There are no previous in vivo reports concerning the changes in aggrecanase-1 and TIMP-3 expression or immunolocalization in mandibular condylar cartilage during dietary loading. In the present study, a similar expression of aggrecanase-1 and TIMP-3 at different time points was seen in both the Western blot and immunohistochemical analysis. This finding confirms the reliability of the results and suggests that cartilage is very sensitive to loading.

In the immunohistochemical analysis, TIMP-3 expression showed a statistically significant difference between the soft- and hard-diet groups at 6 hours. TIMP-3 reaction to altered dietary loading seemed to precede aggrecanase-1 reaction; significant difference of expression was observed at the 12-hour and 24-hour time points. Aggrecanase-1 and TIMP-3 expression are both mainly located in the upper cell layer of the condylar cartilage from 21 to 30 days of age. The results were also supported by the Western blot analysis, which showed changes in TIMP-3 (molecular form) and aggrecanase-1 (latent form) and expression at all time points. This finding suggests that TIMP-3 and especially aggrecanase-1 may take part in normal cartilage metabolism in the early stages of growth.

The physiologic metabolism of cartilage includes the upper layer of the cartilage proliferation and lower layer degeneration. The growth of the mandibular condylar cartilage requires chondrocyte differentiation, apoptosis, and degradation of the cartilage matrix, followed by vascular invasion and the formation of trabecular bone in the lower layer.[3,26] Aggrecanase-1 and TIMP-3 expression in the upper layer of the condylar cartilage indicates that they are already active in the area of undifferentiated chondrocytes. However, as described earlier, the loss of aggrecan is a critical early event mediated by cartilage aggrecanases. It may indicate that there is also a very early breakdown of matrix by aggrecanase-1 in the upper cartilage region, and the process is further regulated by TIMP-3.

In conclusion, this study demonstrates different changes in the expression of aggrecanase-1 and TIMP-3 between soft- and hard-diet groups in the early stages of growth and altered dietary loading in young rats. The results suggest that aggrecanase-1 and TIMP-3 may take part in cartilage metabolism in the early growth stage. The results may reflect a temporary adaptive change of aggrecanase-1 and TIMP-3 expression in the physiologic range. Thus, mechanical loading is an important factor affecting the adaptive responses of mandibular condylar cartilage.

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