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Effect of decreased loading on the metabolic activity of the mandibular condylar cartilage in the rat

Pertti Pirttiniemi*, Tuomo Kantomaa* and Timo Sorsa**
Institutes of Dentistry, *University of Oulu, **University of Helsinki, Finland

SUMMARY The aim of this study was to measure the effect of decreased temporomandibular loading on the proliferative activity and the level of matrix production of the condylar cartilage. The effect of reduced joint loading on the activity of stromelysin-1 (MMP-3), which has been associated with conditions of articular cartilage matrix breakdown, was also examined.

Eighty 14-day-old female rats were assigned to two groups. Following weaning at 20 days, the experimental group was fed a soft diet and the incisors were shortened regularly to keep them out of occlusion. The controls were fed a hard diet. The activity of tritiated thymidine incorporation and the incorporation of radiolabelled sulphur were measured 2, 6, 12, 24 and 48 hours after initiation of the experiment.

The radiolabelled sulphur intake was significantly lower in the condylar cartilage of the experimental group 6–24 hours after initiation of the experiment, and tritiated thymidine activity was lower after 12–24 hours, indicating lower proliferation and matrix production. The cartilage in the experimental group showed marked immunostaining against MMP-3 in all cartilage layers 9 days after initiation of the experiment. In the control group, the staining was clearly seen only in the superficial fibrous layer and in the erosion front.

A marked reduction in proliferative activity and proteoglycan synthesis in mandibular condylar cartilage was found after a continuous soft diet and suppressed incisal mastication in the rat. The results show that sufficient loading is important for condylar cartilage growth, to maintain both ideal proliferation and matrix chondrocyte production.

Introduction

Growth of the condylar cartilage of the temporomandibular joint (TMJ) may be modulated by mechanical loading. It has been shown experimentally that growth of the condylar cartilage can be influenced through environmental stimuli of a mechanical nature and the factors resulting from the functional activity of the TMJ provide the stimulus for the differentiation of proliferative cells into mature chondroblasts (Meikle, 1973; Koski, 1974). Organ culture studies have shown that sufficient intermittent mechanical loading can maintain growth, while static or decreased loading results in less growth (Copray *et al.*, 1985; Kantomaa and Hall, 1988; Pirttiniemi and Kantomaa, 1996). It has also been demonstrated that limited masticatory movements lead to less macroscopic growth of the lower jaw and growth activity of the condylar cartilage decreases (Hinton, 1989; Kantomaa *et al.*, 1994; Kiliaridis *et al.*, 1999). By contrast, overloading of the joint leads to inhibition of growth, and in this respect the TMJ cartilage resembles that of long bones (Copray *et al.*, 1985; Saamanen *et al.*, 1987).

In humans, osteoarthritic changes in the TMJ are common, even in adolescents. These changes can lead to progressive and more degenerative processes in later life. The cause of these degenerative changes is in most cases unknown, although disturbance in occlusion and trauma have been suggested as possible explanations (Seligman and Pullinger, 1991). Increased levels of matrix metalloproteinases (MMPs) are associated with the destructive stage in the human TMJ and in long bone joints (Kapila and Xie, 1998; Tanaka *et al.*, 1998; Towle *et al.*, 1998). Recent cell culture studies have shown that both overloading and decreased loading upregulate the intervertebral disc cartilage stromelysin-1 (MMP-3) and simultaneously decrease proteoglycan and protein synthesis when compared with normal loading (Handa *et al.*, 1997).

The aim of this study was to measure experimentally the effect of decreased loading on the proliferative activity and the level of matrix production of the condylar cartilage in young rats immediately after weaning. In addition, the effect of reduced joint loading on the activity of MMP-3 was examined. The hypothesis tested was that altered masticatory function can alter both the proliferative and degradative activity of the condylar cartilage.

Materials and methods

Eighty 14-day-old female Wistar rats were assigned to two groups: a control group fed on whole pellets

(Hankkija, Finland) and the experimental group on soft, powdery food. Water was available *ad libitum*. At 21 days of age, after the animals had been weaned, the upper and lower incisors in the experimental (soft diet) group were shortened twice a week non-traumatically using a light wire-cutting instrument.

Three experimental and three control rats were killed 2, 6, 12, 24 and 48 hours after initiation of the experiment. The rats were injected with tritiated thymidine, 1 μCi/g of body weight, intraperitoneally 2 hours before they were killed. Correspondingly, three experimental and three control rats were killed 2, 6, 12, 24 and 48 hours after initiation of the experiment, and injected with radiolabelled sulphur (Amersham Biosciences, Amersham, UK), 20 μCi/g of body weight, intraperitoneally 2 hours before they were killed.

The heads of the rats used for autoradiography were first taken for histological sectioning, where five out of the eight most central sagittal sections of the TMJs were chosen. To ensure that consistent sagittal sections of the condyles were obtained, the whole heads of the rats were first carefully sectioned in the midsagittal plane and then embedded. All histological sections were then cut exactly parallel to this plane. The chosen sections were coated with Ilford Nuclear Emulsion K2 (Ilford, Mobberly, Cheshire, UK). These were stored at –20°C for 5 weeks, developed, and stained with toluidine blue. The extent of incorporation of radiolabelled markers was counted using a Leica Q500 MC image analyser connected to a Leica DMR microscope (Leica, Wetzlar, Germany). For analysis, the condyle was divided sagittally into three segments of equal size. The extent of radiolabelled thymidine was measured by counting the total number of marked cells in the proliferative cell layer in the most central segment of the condyle, within an area of 10^5 μm^2. The central segment of the condyle has been found to be most sensitive to changes in loading of the occlusion (Kantomaa *et al.*, 1994; Pirttiniemi *et al.*, 1996).

The extent of radiolabelled sulphur was measured by counting the total number of marked cells in the upper and lower hypertrophied cell layers in the most central segment of the condyle, within an area of 10^5 μm^2.

One-way analysis of variance was used to determine the significance of the differences between groups. The parametric test was used after analysing the skewness and kurtosis of the samples to ascertain the normality of the samples.

The protocols were approved by the animal experimentation committee of the University of Oulu.

Immunohistochemical analysis

Ten experimental and 10 control animals were killed at 30 days of age and the heads were used for immunohistology. The condyles of the right side were fixed in 10 per cent neutral formalin, demineralized for 10 days in 5 per cent formic acid and embedded in glycolmethylacrylate (Historesin, Jung).

Three of the most central sagittal histological sections of the condyles on the right side were analysed immunohistochemically. Sections for immunohistochemistry were digested with 0.4 per cent pepsin (Sigma P-7000, 1780 U/mg, Sigma Chemical Co., St. Louis, Missouri, USA) for 1 hour at 37°C and stained for MMP-3 with a polyclonal antibody, raised in rabbit, specific for MMP-3 (Biogenesis Ltd, Poole, UK). The antibodies were used at ×100 dilution and allowed to react overnight at 4°C. The reaction product was visualized with a Vectastain Elite kit (Vector Laboratories, Burlingame, California, USA) using a peroxidase and diaminobenzine substrate. Negative controls without the primary antibody were prepared. The immunoreactivity in all stained sections and in nine segments of the condyle (Figure 1) was analysed visually with a light microscope (Leica DMR). The immunoreactivity was divided into three grades: no visible staining, light staining or strong staining.

Analysis of measurement error

All the measurements using autoradiography and immunohistology were performed blind on two separate occasions. The intra-examiner measurement error between two sets of measurements of autoradiography was calculated using the formula:

$$s(i) = \sqrt{(\sum d^2/2n)}$$

where d is the difference between repeated measurements and n is the number of double determinations. The intra-examiner error was compared with the total variance of the samples. For immunohistochemical analyses the intra-examiner error was calculated by measuring the frequency of differences between double determinations when counting the stained segments.

Results

The intra-observer methodological error ($s(i)$) showing the difference between repeated cell counts in thymidine autoradiography was 1.2 and the error variance in relation to the total variance of the sample ($s(i)^2/S^2$) was 2.9 per cent. For the measurement of radiolabelled sulphur, the corresponding value for $s(i)$ was 12.5 and the error variance in relation to the total variance of the sample ($s(i)^2/S^2$) was 1.2 per cent. For the immunohistochemical analyses the frequency of differences between double determinations of stained segments was below 5 per cent.

Proliferative activity, measured as radiolabelled thymidine incorporation in the proliferative layer, was

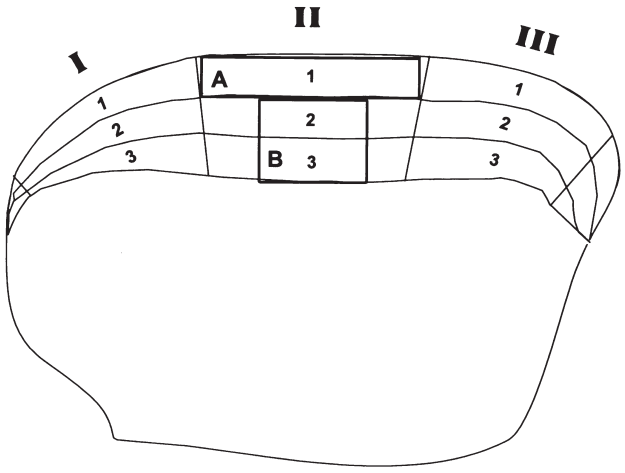

Figure 1 Schematic diagram of the mandibular condyle of the rat. Nine numbered sagittal segments (I–III sagittally and 1–3 vertically) were used in the analyses of immunohistology. Segment A was used for autoradiography using [^{3}H]-thymidine as a marker of proliferative activity and segment B was used for autoradiography using [^{35}S]-sulphur as a marker of proteoglycan production.

found to be lower in the experimental group than in the controls 12 ($P < 0.01$), 24 ($P < 0.05$) and 48 ($P < 0.05$) hours after initiation of the experiment (Figure 2). The incorporation of radiolabelled sulphur, an indicator of proteoglycan synthesis, was lower in the experimental group after 6 ($P < 0.05$), 12 ($P < 0.05$) and 24 hours ($P < 0.05$, Figure 3).

The articular cartilage in the control group showed immunostaining against MMP-3 only in the superficial fibrotic layer and in the erosion front where ossification takes place (Figure 4a). In the experimental group, the staining against MMP-3 was seen in all cartilage layers

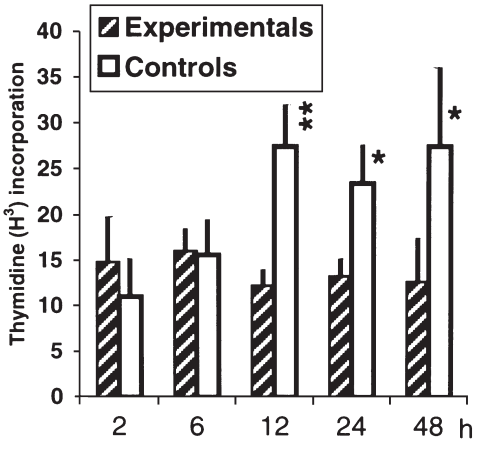

Figure 2 [^{3}H]-Thymidine intake in the proliferative layer of the mandibular condyle measured as the mean active cell count in the experimental and control rats during the 48 hour follow-up period (mean and standard deviation of the total count of marked cells per measured area). *$P < 0.05$; **$P < 0.01$.

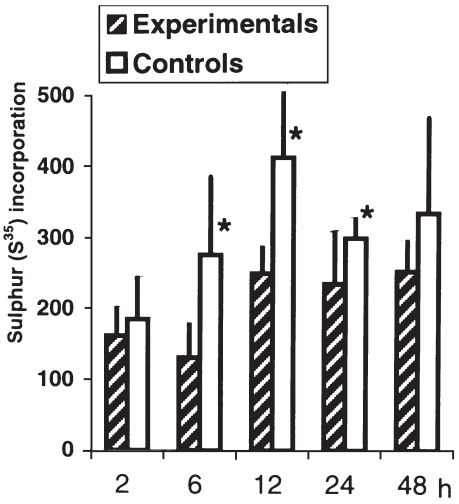

Figure 3 [^{35}S]-Sulphur intake in the upper and lower hypertrophied layer in the experimental and control rats during the 48 hour follow-up period (mean and standard deviation of the total count of [^{35}S]-sulphur activity per measured area). *$P < 0.05$.

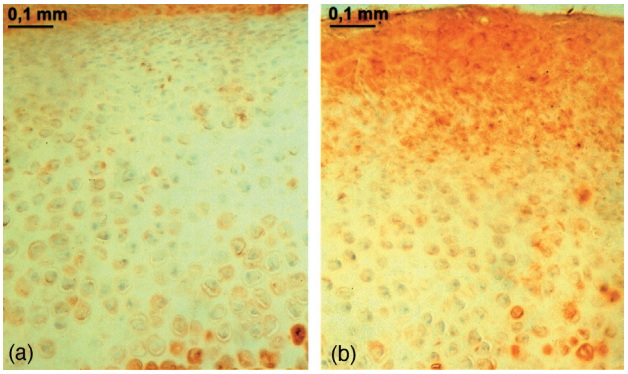

Figure 4 (a) Sagittal central section of the superior condylar head of a control rat at 30 days of age, 9 days after weaning; the articular surface is at the top. The section was stained for stromelysin-1 (MMP-3) antibody. The fibroblastic layer (top) shows intra- and extracellular staining and the lower hypertrophied layer near the erosion front shows intracellular staining (toluidine blue counterstaining). (b) Sagittal central section of the superior condylar head of an experimental rat at 30 days of age, 9 days after the initiation of the experiment, stained for MMP-3 antibody. The articular surface is at the top. There is marked staining in the fibroblasts, the proliferative and upper hypertrophied layer and weaker staining in the lower hypertrophied layer. The normal configuration of the cellular layers cannot be distinguished and the shape and normal polarity in the superior cellular layers are not seen.

at 30 days of age. The staining was most intense intracellularly in the upper hypertrophied layer. There was weak staining in the extracellular matrix. The normal configuration of the cellular layers could not be distinguished in the superior cellular layers. The shape and normal polarity of the cells was lost (Figure 4b).

When the number of stained sections (Figure 1) was counted, there were statistically more positively stained

sections in the experimental group than in the controls (chi-squared test, $P < 0.05$).

Discussion

The intra-observer methodological error, measured as the difference between double determinations in autoradiography, was found to be relatively low when compared with the total variance of the samples. This finding shows the usefulness of computerized image analysis in autoradiographic measurements.

There have been various experimental designs that have shown condylar cartilage growth to be dependent on sufficient and appropriate loading. A common finding of all these studies has been that a reduction in growth can be achieved by various means, but overgrowth is difficult to produce experimentally (Copray *et al.*, 1985; Pirttiniemi *et al.*, 1996; Kiliaridis *et al.*, 1999). The rats used in the present experiment were relatively young. Functional occlusion in the rat, when considering molar occlusion, is achieved by the first molar at the 25th day, the second molar at the 28th day, and the third molar at the 40th day (Hunt *et al.*, 1970). Thus, the timing of the whole experiment was during active development of molar occlusion. In previous experimental studies it has been found that the rat condylar cartilage is very sensitive to changes in occlusal loading, especially during the time after weaning and before the 35th day (Kantomaa *et al.*, 1992). For this reason, the present age group of rats, before complete occlusal development, was chosen.

It has been previously shown in an analogous cross-sectional experiment that reduced loading leads to a lower number of chondrocytes and a thinner cartilaginous layer in the articular surface of the condyle (Pirttiniemi *et al.*, 1996). The fact that in the present experiment proliferative activity was reduced may explain, at least in part, the earlier finding of a reduced number of chondrocytes in the condyle. The reduced matrix production, as indicated by the lower level of radioactive sulphur intake in the soft diet group in the present experiment, supports this view. It is obvious that the effect of reduced loading, after a change in diet and occlusion, was slow in the experimental animals. Rats are basically most active during the night, which may explain the relatively high proliferative activity measured at 12 hours after initiation of the experiment in daytime.

The present results show that continuing with soft diet with reduced occlusion induced marked changes which may be considered adverse. Late effects on total growth until maturity or macroscopic defects late in growth were not monitored.

It is important that altered loading *in vivo* is also able to regulate MMP-3 activity. The increased expression of MMP-3 in the TMJ articular cartilage in the experimental group may be an early marker of a degradative process in the joint. Increased levels of active MMP-3 have been detected in osteoarthritic cartilage explants (Kozaci *et al.*, 1998; Towle *et al.*, 1998; Ishiguro *et al.*, 1999). If the increase in the activity of MMP-3 in the experimental group was a sign of a pathological rather than physiological change, it may have been from the traumatic occlusion, resulting from incisive cutting. The only occlusal contacts in the present experiment were shifted to the posterior teeth, and this altered the loading of the condyles, in spite of the soft diet. Thus, the altered incisal occlusion could increase such masticatory movements that are not normal and would in this way lead to the pathological destructive processes observed at the cellular and molecular levels.

Another possible explanation for the increased MMP-3 levels and activation could be the physiological breakdown of excess cartilage matrix, because of reduced occlusal function. This is supported by the finding that less matrix was produced, as indicated by a lower level of sulphur intake. The reduced matrix was reflected in a narrower cartilage layer. There is a wide range of normal variation during growth and the changes found in the present experiment in the soft diet group do not necessarily cause pathology in the condylar cartilage, but may be part of an adaptation process. Additional data could have been obtained if a third group had been included in which only the incisal edges had been cut and they had been fed a normal diet.

It is of note that a short-term decrease in mechanical stimulation of human intervertebral disc cartilage in organ culture results in a marked increase in MMP-3 activity (Handa *et al.*, 1977). It is apparent that normal regulation of cartilage metabolism is highly balanced during the most active period of growth and can respond to changes in loading by various mechanisms, including tissue degradation. On the other hand, it is possible that the thinner articular cartilage layer, which will develop after reduced occlusal function, is not equally resistant as thicker cartilage to possible later increases in loading forces.

Conclusion

A marked reduction in proliferative activity and proteoglycan synthesis in mandibular condylar cartilage was found after a continuous soft diet and suppressed incisal mastication in the rat. These changes were associated with an increase in MMP-3 expression and activation. This enzyme is associated with the cartilage matrix breakdown process. Loading is important for condylar cartilage growth to maintain both ideal proliferation and matrix production of the chondrocytes.

Address for correspondence

Pertti Pirttiniemi
Department of Orthodontics and Oral Development
Institute of Dentistry
University of Oulu
PL 5281
90401 Oulu
Finland

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