Changes in proteoglycan and collagen content in the mandibular condylar cartilage of the rabbit caused by an altered relationship between the condyle and glenoid fossa

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SUMMARY Twenty 5-day-old New Zealand rabbits underwent surgery to induce premature synostosis of the cranial sutures, resulting in posterior displacement of the glenoid fossa. Twenty sham-operated rabbits served as controls. The animals were killed at age 15 days for histochemical and biochemical analyses. The collagen content of the superior region of the condyle determined biochemically was lower in treated animals than in controls. Biochemical and histochemical analyses revealed the proteoglycan content to be significantly reduced in the superior region of the condyle ($P \leq 0.001$). Low levels of aggregating proteoglycans were seen. Since levels of aggregating proteoglycans decreased, catabolism must have exceeded their synthesis or the monomers must have been unable to aggregate and escaped from the tissue. It is concluded that an experiment in which the location of the mandibular condyle in the glenoid fossa is changed, while causing marked reductions in amounts of both collagen and proteoglycans in the cartilage tissue of the mandibular condyle, will also induce changes resembling those observed in animal models of arthritis. It is possible that the two phenomena have similar mechanisms.

Introduction

Attempts to exploit the dependence of secondary cartilage growth on mechanical factors (Murray and Smiles, 1965; Hall, 1979), for the regulation of mandibular growth raise an intriguing problem. Although histological changes have been observed (Breitner, 1933, 1940, 1941; Buchner, 1982; Kantomaa, 1984) and marked changes occur in the metabolism of the cartilage of the mandibular condyle, which vary both qualitatively (Buchner, 1982; Kantomaa, 1987; Salo and Kantomaa, 1993; Kantomaa *et al.*, 1994a) and quantitatively (Charlier *et al.*, 1969; Tewson *et al.*, 1988; Pirttiniemi *et al.*, 1993) after changing the location of the mandibular condyle in the fossa, macroscopic change is limited.

Synthesis of extracellular matrix has been shown to increase after exertion of intermittent pressure on the condylar cartilage (Copray *et al.*, 1985; Kantomaa *et al.*, 1994b), sometimes depending on the magnitude of manipulation (Takano-Yamamoto *et al.*, 1991). These findings are in line with reports on the behaviour of long-bone articular cartilage in analogous situations (Palmoski *et al.*, 1980; Behrens *et al.*, 1989; Säämänen *et al.*, 1987, 1990; Sah *et al.*, 1991; Helminen *et al.*, 1992). Although extracellular matrix synthesis is considered important for volume growth in the mandibular condyle (Bosshardt-Luehrs and Luder, 1991), no marked increase in condylar growth is observed macroscopically. Tuominen *et al.* (1993) have reported increased condylar growth after diminished masticatory function. It seems clear that there is a missing link between extracellular matrix synthesis and condylar growth at the macroscopic level. It seems logical to look for this link in the amounts and nature of the proteoglycans present in cartilage.

The aims of this study were to investigate and describe a previously unreported effect on rodent condylar cartilage extracellular matrix proteins of an experimental surgical procedure that changes the relationship between the mandibular condyle and glenoid fossa. Glueing of the temporoparietal, interparietal, and lambdoidal sutures of growing rabbits has been shown to result in more posterior location of the glenoid fossa as growth proceeds, and to an altered relationship between the mandibular condyle and the fossa (Kantomaa, 1984). Masticatory function is not actively disturbed.

Materials and methods

Animals

Forty 5-day-old New Zealand rabbits were used. The calvarium was exposed by flap incision in 20 animals, and the periosteum covering the temporoparietal, interparietal, and lambdoidal sutures retracted under local anaesthesia (Citanest®, Astra Pharmaceuticals, Stockholm, Sweden). The adjoining bones were glued together with methylcyanoacrylate, according to the method of Persson *et al*. (1979). Twenty control animals underwent a similar sham-operation except for glueing of the sutures. Protocols had been approved by the Animal Experimentation Committee of the University of Oulu.

Histochemistry

Ten control and 10 experimental animals were used for histological and histochemical studies of differences in proteoglycan content. Animals were killed at age 15 days and left mandibular condyles prefixed in 4 per cent neutral formalin supplemented with 0.7 per cent ruthenium hexamine trichloride (Hunziker *et al*., 1983) for 24 hours, post-fixed in 4 per cent neutral formalin for 24 hours, and decalcified for 1 week in 0.5 M EDTA formalin. Condylar processes were embedded in glycolmethylacrylate (Leica Instruments). Five 4-µm sagittal sections, where the sagittal dimension of the condylar process was largest, were regarded as being the most central, and were stained with Safranin O using the
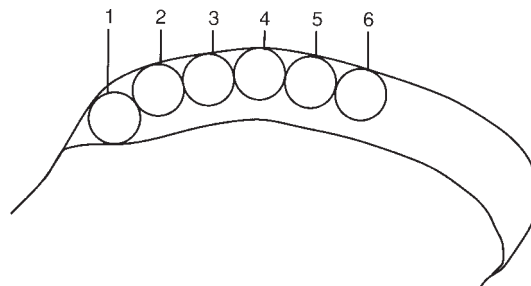
Figure 1 Representation of the six regions of the mandibular condyles where the intensity of Safranin O staining were measured from histological sections. The scanning areas of 0.2 mm² were circular in shape and located tangentially to the surface of the condyle at intervals of 0.5 mm, starting from the anterior edge of the cartilage. The depth of the measurement area was about two-thirds of the thickness of the cartilage, reaching the articular and prechondroblast zones, and also hypertrophying chondrocytes, although the hypertrophied chondrocyte layer lay mainly outside the measurement area.

method of Kiviranta *et al*. (1985), who showed that this marker binds stoichiometrically to proteoglycans. The intensity of Safranin O staining was measured in 6 regions for each condyle (Figure 1) using a digital microcomputer-based image analyser (M2 Imaging, Research Inc., Brock University, St Catherines, Ontario, Canada; Microscope Nikon Optiphot II 40X, CCD camera Dage MTI 72E, Michigan City, Indiana, USA). Significances of differences between control and operated condyles were tested using Student's *t*-test for small samples. Use of a parametric test was based on the assumption that the samples were normally distributed, which was confirmed by calculating skewness and kurtoses. To test the error of the method 10 measurements were repeated after a week, and the method error calculated using the formula

$$s_i = \sqrt{\Sigma d^2 / 2n}$$

where d is the difference between two measurements and n the number of repeated measurements.

Biochemistry

Left and right mandibular condyles of 10 operated and 10 sham-operated 15-day-old animals
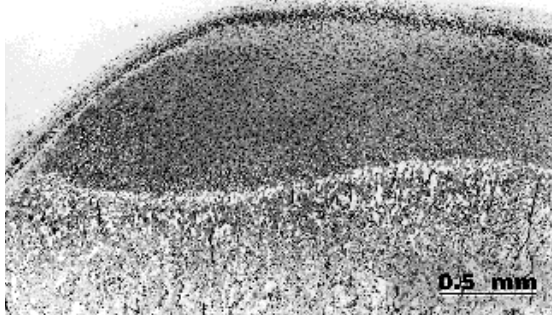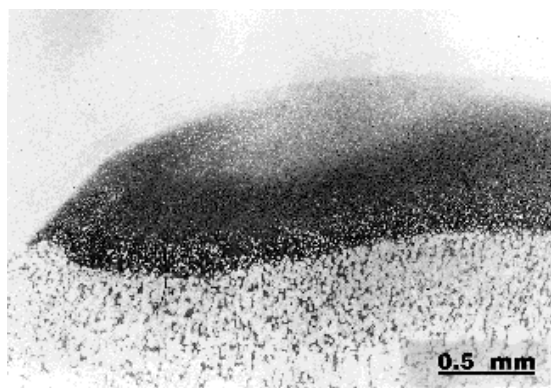
Figure 2 Photograph of sagittal histological section from anterior and superior regions of mandibular condyle of a 15-day-old sham-operated rabbit (Cole haematoxylin and eosin, ×20).
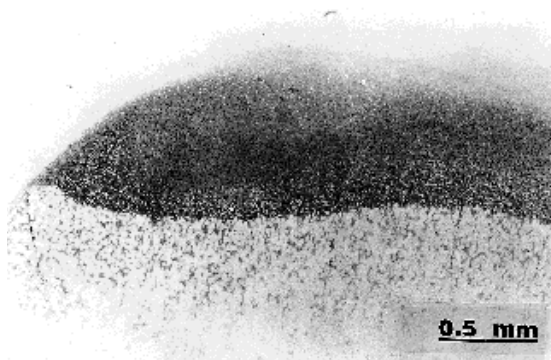
Figure 3 Photograph of sagittal histological section from anterior and superior regions of the mandibular condyle of a 15-day-old sham-operated rabbit (Safranin O staining, ×20).

Figure 4 Photograph of sagittal histological section from anterior and superior regions of mandibular condyle of a 15-day-old operated rabbit (Safranin O staining). Intensity of the Safranin O staining is markedly lower in the superior region of the condylar cartilage than elsewhere. The difference is greater than in the control section in Figure 3 (×20).

were used for biochemical analysis. The anterior part (1 mm from the anterior edge of the condyle) was dissected out under a dissecting microscope and discarded, and the remaining cartilage divided into two equal halves, a superior and a posterior part. The superior parts were pooled and used for biochemical analysis. Proteoglycans were extracted according to the method of Säämänen *et al.* (1990). The extract was used for proteoglycan analysis and the precipitate was analysed for collagen content by means of a colourimetric reaction (Stegemann, 1958; Woessner, 1961). To exclude the possibility that proteoglycans might not have been fully extracted, the uronic acid contents were measured by the m-hydroxydiphenyl method described by Blumenkrantz and Asboe-Hansen (1973) of both the extract and precipitate after papain digestion.

The extract was subjected to dissociative density gradient ultracentrifugation, and the high density fraction A1 (the bottom two-fifths) was used after re-aggregation for chromatography according to the method of Säämänen *et al.* (1990). Amounts of proteoglycans, measured using Safranin O according to the method of Lammi and Tammi (1988), were compared quantitatively using chrondroitin sulphate C (Sigma, St Louis, USA) standard and computerized digital image analysis, as described above. As a control for the method, the rest of the extract, fraction A2, was dialysed, and its uronic acid

content measured to establish whether appreciable amounts of proteoglycans were present.

Results

Histochemical analysis showed the proteoglycan content to be lower in the superior region of the condyle than in the anterior or posterosuperior regions (Figures 2–4). Proteoglycans were significantly less in amount in the superior regions

Table 1 Amounts of proteoglycans in different regions (Figure 4) of sham-operated ($n = 10$) and operated ($n = 10$) condyles measured as intensity of Safranin O staining.

Region	Sham-operated		Significance	Operated	
	x	SD		x	SD
1	0.53	0.052	NS	0.53	0.058
2	0.40	0.016	***	0.31	0.032
3	0.33	0.049	**	0.26	0.032
4	0.40	0.020	NS	0.35	0.067
5	0.40	0.021	*	0.36	0.027
6	0.40	0.052	NS	0.36	0.041

***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; Student's t-test.

Figure 5 Sephacryl S-1000 chromatography of proteoglycans in pooled cartilage samples from 10 sham-operated (☆—☆) and 10 operated (★—★) animals. The quantity of aggregating proteoglycans was markedly reduced and the amounts of non-aggregating proteoglycans slightly increased in the operated animals relative to the sham-operated animals

of operated condyles than in sham-operated condyles (Table 1, Region 2, $P \leq 0.001$ and Region 3, $P \leq 0.01$). The error of method (S_i^2) was 1.2 per cent of the total variance.
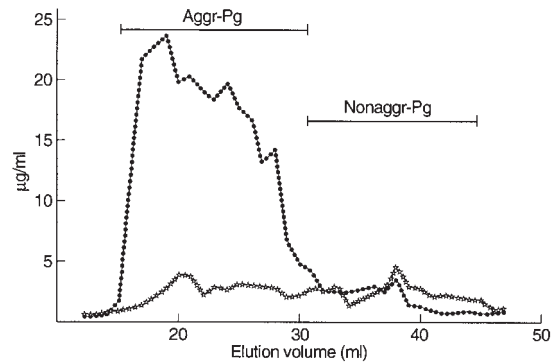
The uronic acid content of the guanidinium chloride extract of the experimental condyles, indicating the amount of proteoglycans, was 20 per cent of the control value, and the quantity of proteoglycans present after density gradient ultracentrifugation (A1 fraction) was 44 per cent of the control value in terms of dry weight. Chromatography of the A1 fraction revealed a marked reduction in quantity of aggregating glycosaminoglycans (Figure 5).

The uronic acid contents of the precipitate used for collagen analysis (41 per cent of the control value) and of the fraction A2 (33 per cent of the control value) from the operated condyles confirmed that proteoglycans had not been lost into these fractions to any greater extent than from control condyles, and had thus been successfully extracted for analysis in fraction A1.

The collagen contents of the operated condyles were 66 per cent of the values from the sham-operated condyles.

Discussion

The purpose of this experiment was to investigate the effects of an altered relationship between mandibular condyle and glenoid fossa on the extracellular matrix of the condylar cartilage.

From the mechanical point of view, it is evident that the altered relationship increased functional loading in the superior region of the condyle. Collagen content was measured in terms of hydroxyproline. Hydroxylation could have altered. With this reservation, the results indicate that the collagen content of the condylar cartilage in the superior region was low in the operated animals. This finding is in line with the immunohistochemical data of Salo and Kantomaa (1993), who noted that the onset of synthesis of collagen type II was retarded, and regarded the phenomenon as an expression of a slowing down in the rate of maturation of the condylar cartilage under increased pressure (Salo and Kantomaa, 1993; Kantomaa *et al.*, 1994a). Collagen synthesis has been found to increase transiently *in vitro* after application of low intermittent pressures to the condylar cartilage, and to decrease after application of higher intermittent pressure or continued forces (Copray *et al.*, 1985). The decreases in the amounts of collagen observed in this study would seem to imply that forces increased, or altered from intermittent to continuous, after backward displacement of the glenoid fossa.

A need for loading to maintain synthesis of glycosaminoglycans in cartilage has been found in several experiments with long-bone articular

cartilage (Palmoski *et al.*, 1980; Behrens *et al.*, 1989; Säämänen *et al.*, 1987, 1990; Sah *et al.*, 1991; Helminen *et al.*, 1992), and a significant proteoglycan reduction has been reported in mandibular condylar cartilage following a decrease in functional loading (Hinton, 1993). Increased synthesis of proteoglycans has been reported in association with increased loading *in vitro* (Copray *et al.*, 1985; Takano-Yamamoto *et al.*, 1991). Glycosaminoglycan synthesis was increased in a similar experiment, when measured with the aid of S^{35} sulphate and computerized image analysis (Kantomaa *et al.*, 1994b). In contrast, a significant reduction in total quantity of proteoglycans was observed in the operated condyles relative to controls used in the study reported here. These results indicate that loss of proteoglycans from the studied tissue must have exceeded increase in its synthesis.

Aggregation of proteoglycans has been regarded as important for inhibition of their escape from tissue (Sah *et al.*, 1991) and thus for ability of cartilage tissue to resist pressure. We found a marked reduction in aggregating proteoglycans, indicating either that catabolism of proteoglycans exceeded any increase in their synthesis, or that proteoglycan monomers synthesized were unable to aggregate. We did not directly measure the catabolism of cartilage matrix.

Whether there are decreases or increases in proteoglycans in long-bone articular cartilage following increases in functional loading will depend on the magnitude of loading. Moderate running, 4 km/day, was followed by increases in proteoglycans in the articular cartilages of beagle dogs (Kiviranta *et al.*, 1988). Strenuous training, 20 km/day, caused a significant decrease in proteoglycans in the condylar summit of the femur, although no changes were observed in other joints. Running 40 km/day significantly decreased all articular cartilages studied. This reduction was considered to cause a risk of subsequent development of osteoarthrosis (Helminen *et al.*, 1992; Arokoski *et al.*, 1993). The findings in our study are in line with those reported in connection with strenuous training.

During orthopaedic treatment, with continuous forwarding jumping of the mandible, the relationship between the mandibular condyle and glenoid fossa is changed as in our study. Our findings are similar to those reported for animal models of osteoarthrosis of the knee. Histological examination after an experiment similar to ours revealed acellular and necrotic areas accompanied by striated and uneven metachromasia in the condylar cartilage of the rat, changes regarded as pathological (Kantomaa, 1987). Buttle *et al.* (1995) have suggested that both normal and pathological breakdown of aggrecan occur via the same cascades. It is possible that early changes in osteoarthrosis and reaction of the mandibular condyle to altered location in the glenoid fossa, for instance, in connection with functional appliances, have similar mechanisms.

Histochemical measurements showed amounts of proteoglycans to be highest in the anterior region of the condyle, with decreases in a superior direction and increases in a posterior direction. The reduction in the superior direction was greater in operated condyles than in sham-operated condyles. The difference can be explained in terms of functional stress. The superior region is obviously subjected to greatest functional stress during mastication (Weijs and Dantuma, 1981). Distal displacement of the glenoid fossa may be assumed to increase this effect.

Reports of the effects of functional appliances on growth of the mandibular condyle vary from marked or moderate change to no effect (McNamara, 1973; Petrovic *et al.*, 1981, Tazumi, 1982; Kantomaa, 1984; McNamara and Bryan, 1987; Tewson *et al.*, 1988). Numerous clinical studies have confirmed the statement of Graber (1985) that 'the total sagittal change is the result of multiple factors, no one of which is responsible for the major change'. This is confusing in relation to the condyle, as both matrix synthesis (Kantomaa, 1994b) and cell proliferation (Charlier *et al.*, 1969) have been reported to increase markedly. Our results provide for the first time an explanation for this in situations where the relationship between the condyle and glenoid fossa is continuously changed, suggesting that matrix synthesized is either catabolized or lost from the tissue at an even higher rate than the synthesis is increased. In the light of our

findings it would seem reasonable to concentrate in future more on inhibition of such loss than on attempts to increase synthesis.

Conclusions

We have shown that when the relationship between the mandibular condyle and glenoid fossa is altered in the rabbit there is a marked reduction in amounts of collagen and proteoglycans in the cartilage tissue of the mandibular condyle. The decrease in amount of proteoglycans is obviously a result of increased catabolism or loss from tissue. The changes resemble those observed in animal models of arthritis. It is possible that mechanisms in the two situations are similar.

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References

Arokoski J, Kiviranta I, Jurvelin J, Tammi M, Helminen H 1993 Long-distance running causes site-dependent decrease of cartilage glycosaminoglycan content in the knee joints of beagle dogs. Arthritis and Rheumatism 36: 1451–1459

Behrens F, Kraft E L, Oegema T R 1989 Biochemical changes in articular cartilage after joint immobilization by casting or external fixation. Journal of Orthopedic Research 7: 335–343

Blumenkrantz N, Asboe-Hansen G 1973 New method for quantitative determination of uronic acids. Analytical Biochemistry 54: 484–489

Bosshardt-Luehrs C P B, Luder H U 1991 Cartilage matrix production and chrondrocyte enlargement as contributors to mandibular condylar growth in monkeys (*Macaca fascicularis*). American Journal of Orthodontics and Dentofacial Orthopedics 100: 362–369

Breitner C 1933 Experimental change of the mesio-distal relations of the upper and lower dental arches. Angle Orthodontist 3: 67–76

Breitner C 1940 Bone changes resulting from experimental orthodontic treatment. American Journal of Orthodontics and Oral Surgery 6: 155–174

Breitner C 1941 Further investigations of bone changes resulting from experimental orthodontic treatment. American Journal of Orthodontics and Oral Surgery 27: 605–632

Buchner R 1982 Induced growth of the mandibular condyle in the rat. Journal of Oral Rehabilitation 9: 7–22

Buttle D J, Bramwell H, Hollander A P 1995 Proteolytic mechanisms of cartilage breakdown: a target for arthritis therapy. Journal of Clinical Pathology and Molecular Pathology 48: M167–M177

Charlier J-P, Petrovic A, Hermann-Stutzmann J 1969 Effects of mandibular hyperpropulsion on the prechondroblastic zone of young rat condyle. American Journal of Orthodontics 55: 71–74

Copray J C V M, Jansen H W B, Duterloo H S 1985 Effects of compressive forces on proliferation and matrix synthesis in mandibular condylar cartilage of the rat *in vitro*. Archives of Oral Biology 30: 299–304

Graber T M 1985 Functional appliances. In: Graber T M, Swain B F (eds) Orthodontics: current principles and techniques. C V Mosby Company, St Louis, pp. 369–404

Hall B K 1979 Selective proliferation and accumulation of chrondroprogenitor cells as the mode of action of biochemical factors during secondary chondrogenesis. Teratology 20: 81–92

Helminen H J *et al.* 1992 Effect of motion and load on articular cartilage in animal models. In: Kuettner K E, Schleyerbach R, Peyron J G, Hascall V C (eds) Articular cartilage and osteoarthritis. Raven Press, New York, pp. 501–509

Hinton R J 1993 Effect of dietary consistency on matrix synthesis and composition in the rat condylar cartilage. Acta Anatomica 147: 97–104

Hunziker E B, Hermann W, Schenk R K 1983 Ruthenium hexamine trichloride (RHT) mediated interaction between plasmalemmal components and pericellular matrix proteoglycans is responsible for the preservation of chrondrocytic plasma membranes *in situ* during cartilage fixation. Journal of Histochemistry and Cytochemistry 31: 717–727

Kantomaa T 1984 Effect of increased posterior displacement of the glenoid fossa on mandibular growth: a methodological study on the rabbit. European Journal of Orthodontics 6: 15–24

Kantomaa T 1987 Reactions of the condylar tissues to attempts to increase mandibular growth. Scandinavian Journal of Dental Research 95: 335–339

Kantomaa T, Tuominen M, Pirttiniemi P 1994a Effect of mechanical forces on chondrocyte maturation and differentiation in the mandibular condyle of the rat. Journal of Dental Research 73: 1150–1156

Kantomaa T, Pirttiniemi P, Tuominen M, Poikela A 1994b Glycosaminoglycan synthesis in the mandibular condyle during growth adaptation. Acta Anatomica 151: 88–96

Kiviranta I, Jurvelin J, Tammi M, Säämänen A-M, Helminen H J 1985 Microspectrophotometric quantitation of glycosaminoglycans in articular cartilage sections stained with Safranin O. Histochemistry 82: 249–255

Kiviranta I, Tammi M, Jurvelin J, Säämänen A-M, Helminen H J 1988 Moderate running exercise augments glycosaminoglycans and thickness of articular cartilage in the knee joint of young beagle dogs. Journal of Orthopedic Research 6: 188–195

Lammi M, Tammi M 1988 Densitometric assay of nanogram quantities of proteogycans precipitated on nitrocellulose membrane with Safranin O. Analytical Biochemistry 168: 352–357

McNamara J A 1973 Neuromuscular and skeletal adaptations to altered function in the orofacial regions. American Journal of Orthodontics 64: 578–606

McNamara J A, Bryan A 1987 Long term mandibular adaptations to protrusive function: an experimental study in *Macaca mulatta*. American Journal of Orthodontics and Dentofacial Orthopedics 92: 98–108

Murray P D F, Smiles M 1965 Factors in the evocation of adventitious (secondary) cartilage in the chick embryo. Australian Journal of Zoology 13: 351–381

Palmoski M J, Colyer R A, Brandt K D 1980 Joint motion in the absence of normal loading does not maintain normal articular cartilage. Arthritis and Rheumatism 23: 325–334

Persson K M, Roy W A, Persing J A, Rodeheaver G T, Winn H R 1979 Craniofacial growth following experimental craniosynostosis and craniectomy in rabbits. Journal of Neurosurgery 50: 187–197

Petrovic A, Stutzmann J J, Gasson N 1981 The final length of the mandible: Is it genetically predetermined? In: Carlson D (ed.) Craniofacial biology. Monograph 10, Craniofacial Growth Series. Center for Human Growth and Development, University of Michigan, Ann Arbor, pp. 105–126

Pirttiniemi P, Kantomaa T, Tuominen M 1993 Increased condylar growth after experimental relocation of the glenoid fossa. Journal of Dental Research 72: 1356–1359

Säämänen A-M, Tammi M, Kiviranta I, Jurvelin J, Helminen H J 1987 Maturation of proteoglycan matrix in articular cartilage under increased and decreased joint loading. A study in young rabbits. Connective Tissue Research 16: 163–175

Säämänen A-M, Tammi M, Jurvelin J, Kiviranta I, Helminen H J 1990 Proteoglycan alterations following immobilization and remobilization in the articular cartilage of young canine knee (Stifle) joint. Journal of Orthopaedic Research 8: 863–873

Sah R L-Y, Doong J-Y H, Grodzinsky A J, Plass A H, Sandy J D 1991 Effects of compression on the loss of newly synthesized proteoglycans and proteins of cartilage explants. Archives of Biochemistry and Biophysics 286: 20–29

Salo L, Kantomaa T 1993 Type II collagen expression in the mandibular condyle during growth adaptation: an experimental study on the rabbit. Calcified Tissue International 52: 465–469

Stegemann H 1958 Mikrobestimmung von hydroxyprolin mit chloramin-T und p-dimethylaminobezaldehyd. Zeitschrift für Physiologie und Chemie 311: 41–45

Takano-Yamamoto T, Soma S, Nakagawa K, Kobayashi Y, Kawakami M, Sakuda M 1991 Comparison of the effects of hydrostatic compressive force on glycosaminoglycan synthesis and proliferation in the rabbit chondrocytes from mandibular condylar cartilage, nasal septum, and spheno-occipital synchondrosis *in vitro*. American Journal of Orthodontics and Dentofacial Orthopedics 99: 448–455

Tazumi T 1982 Dentofacial changes produced by extraoral anterior traction of the mandible in *Macaca irius*. Journal of Japan Orthodontic Society 41: 466–492

Tewson D H T K, Heath J I, Meikle C 1988 Biochemical and autoradiographical evidence that anterior mandibular displacement in the young growing rat does not stimulate cell proliferation or matrix formation at the mandibular condyle. Archives of Oral Biology 33: 99–107

Tuominen M, Kantomaa T, Pirttiniemi P 1993 Effect of food consistency on the shape of the articular eminence and the mandible: an experimental study on the rabbit. Acta Odontologica Scandinavica 51: 65–72

Weijs W A, Dantuma R 1981 Functional anatomy of the masticatory apparatus in the rabbit (*Oryctolagus cuniculus L*). Netherlands Journal of Zoology 31: 99–147

Woessner J F Jr 1961 The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. Archives of Biochemistry and Biophysics 93: 440–447