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Forward mandibular positioning enhances condylar adaptation in adult rats

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SUMMARY The aim of this investigation was to assess quantitatively the adaptive changes in the condyles of adult rats to forward mandibular positioning. The level of types II and X collagen expressed in the condyles of adult rats was compared with that formed in response to forward mandibular positioning and the levels of expression were correlated to the amount of bone formed in response to mandibular advancement. Seventy-eight 120-day-old female Sprague-Dawley rats were included in this study. The rats were randomly allocated to six groups. Each group consisted of nine rats with bite-jumping devices and four untreated controls. The animals in each group were sacrificed on days 3, 7, 14, 21, 30, and 60. Immunostaining was used for the detection of types II and X collagen, while Alcian blue–PAS was used to observe the extracellular matrix and new bone formation.

The results showed that new cartilage was formed in the posterior condyle. The highest level of expression of types II and X collagen were present on day 21, the amount of increase was 247.99 and 540.08 per cent, respectively. The highest level of new bone formation was measured at day 30 of advancement when the amount of increase in new bone formation was 318.91 per cent. These findings indicate that forward mandibular positioning causes changes in the biophysical environment of the temporomandibular joint (TMJ) of adult rats that leads to condylar adaptation.

Introduction

Mandibular forward positioning provokes cellular and molecular responses in the temporomandibular joint (TMJ) of growing animals, leading to condylar and glenoid fossa growth (Rabie *et al.*, 2001, 2002a, b). Similar tissue changes have been stated to occur at the condyle and glenoid fossa in non-growing animals (McNamara *et al.*, 1982; Woodside *et al.*, 1983; Hinton and McNamara, 1984). Recently, McNamara *et al.* (2003) reported histological changes associated with mandibular advancement in adult Rhesus monkeys. In these adult monkeys, adaptive changes with condylar cartilage were evident after 3 weeks of advancement. Furthermore, the dimensions of the condylar cartilage showed a gradual increase throughout the experimental period, whereas the untreated controls had a bony cap. In contrast, other reports demonstrated that such adaptive responses to forward mandibular positioning are negligible or non-existent (Hiniker and Ramfjord, 1966; Ramfjord and Enlow, 1971; Ramfjord *et al.*, 1971; Adams *et al.*, 1972; McNamara, 1973; McNamara *et al.*, 1975; Ramfjord and Blankenship, 1981). Clinical reports (Ruf and Pancherz, 1999a, b) demonstrated that functional appliance therapy results in an increase in mandibular prognathism in young adult patients. The use of magnetic resonance imaging in that study provided an opportunity to visualize TMJ responses to forward mandibular positioning (Ruf and Pancherz, 1999a). However, the mechanism by which these changes are triggered in adult patients is not known and the tissue responses to such treatment in adults remain unclear. It is of importance that our clinical treatment is based on sound scientific understanding of the tissue responses to different treatment modalities.

Recently, it has been reported that growth of the condyle is regulated by a series of factors that are endogenously expressed by cells in the condyles (Rabie and Hägg, 2002). Forward mandibular positioning precipitates a series of changes in the level of expression of these factors that leads to condylar growth in growing rats (Rabie and Hägg, 2002; Rabie *et al.*, 2002a, 2003a), particularly the level of expression of type II collagen (Rabie *et al.*, 2003a), the major collagenous framework of cartilage, as well as the level of expression of type X collagen (Rabie *et al.*, 2000), the major component of the hypertrophic cartilage matrix. A close correlation exists between the amount of these collagens and the amount of bone formed in the condyles in response to forward mandibular positioning in growing rats. As the amounts of cartilage, as well as the hypertrophic matrix, are critical to the potential amount of bone formed in the condyle, it is of importance to determine the level of expression of types II and X collagen expressed in the condyles of adult rats and then compare it with the level of expression during forward mandibular positioning.

Therefore, the aims of this study were:

1. To quantify the level of types II and X collagens expressed in the condyles of adult rats.

2. To compare their level of expression with that formed in response to forward mandibular positioning.
3. To correlate their levels of expression to the amount of bone formed in response to mandibular advancement.

Materials and methods

This experiment was approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong (CULATER 586-01).

Experimental groups

Seventy-eight female Sprague-Dawley rats were bred and kept under standard conditions and provided with water *ad libitum*, artificial light and normal rat pellets (Laboratory Rodent Chow 5010, PMI Feeds Inc., St. Louis, Missouri, USA) in the Laboratory Animal Unit of the University of Hong Kong. All the rats were fed with grounded normal pellets instead of normal pellets from 90 days of age. The rats were randomly allocated to six groups. Each group consisted of nine rats with bite-jumping devices and four untreated controls. The experimental animals were fitted with bite-jumping appliances to result in a continuous anterior advancement of 4 mm at the age of 120 days (Xiong *et al.*, 2004). The appliance fitting was conducted under anaesthesia (10 per cent ketamine and 2 per cent xylazine, 2:1, 0.1 ml/100 g). Light-curing Panavia F (Kuraray Medical Inc., Okayama, Japan) was used as the bonding material to provide sufficient retention for the appliance. The animals in each experimental group and matched controls were killed on days 3, 7, 14, 21, 30, and 60.

Tissue preparation

The rats were sacrificed by intraperitoneal injection of 20 per cent Dorminal (200 mg/ml pentobarbital sodium; Alfasan, Woerden, The Netherlands) with a dosage of 100 mg/kg body weight. Immediately after death, the heads were fixed in 4 per cent paraformaldehyde for 2 days. The heads were then carefully dissected along the middle sagittal plane into two halves. The left TMJs were harvested and decalcified according to the method of Rabie *et al.* (2002a). After decalcification, the samples were embedded in paraffin. Serial sections of 4 μm were cut through the TMJ in the sagittal plane using a rotary microtome (Leica RM 2155; Wetzlar, Germany) and mounted on TESPA-coated glass slides.

Immunohistochemistry

Immunohistochemical staining of types II and X collagen was carried out according to the avidin-biotin complex method and counterstained with haematoxylin. Goat polyclonal IgG (C-19, Santa Cruz Biotechnology, California, USA) was used for immunolocalization of type II collagen and mouse monoclonal IgG (Quartett, 211406, Berlin, Germany) for the detection of type X collagen. The staining procedure was followed according to the method described by Rabie *et al.* (2002a).

Alcian blue–PAS staining

The combined Alcian blue–PAS staining was used to identify acid and neutral mucins. The acid mucins, which stained blue in cartilage, and neutral mucins, which stained distinct magenta in cartilage, represent the calcifying cartilage matrix and new bone. The staining protocol followed the method described by Rabie *et al.* (2003b).

Quantitative analysis

The amount of type II collagen, type X collagen and new bone formation was quantified at a magnification of ×180 via a true-colour RGB computer-assisted image analysing system with a digital camera (Leica DC 300 V 2.0) and Leica Qwin version 2.4 software. In a pilot study, the major histological changes occurred in the posterior region of the condyle. Thus, the posterior area of the condyle was used in the present quantitative analysis. The amount of area was quantified within a fixed measurement frame of 1044 × 766 pixels. The statistical analysis was processed using SPSS for Windows (Release 11.0.0, standard version, SPSS Inc., Chicago, Illinois, USA) for one-way ANOVA with the Bonferroni multiple comparisons test.

Method error

The measurement error was calculated using Dahlberg's formula (Dahlberg, 1940):

$$Me = \sqrt{[(\Sigma d^2)/2n]}$$

where d is the difference between the two registrations of a pair, and n the number of double registrations. Ten rats were randomly selected for each evaluation of the method error. The size of the method error for the measurement of type II collagen, type X collagen and new bone formation was 0.10, 0.16 and 0.14, respectively. A paired t-test was performed to compare the two registrations. P values were found to be larger than 0.05, indicating that there were no statistically significant differences among the duplicate registrations.

Results

In the control groups, a thin cartilage layer covered the surface of the condyle (Figure 1). The arrangement of

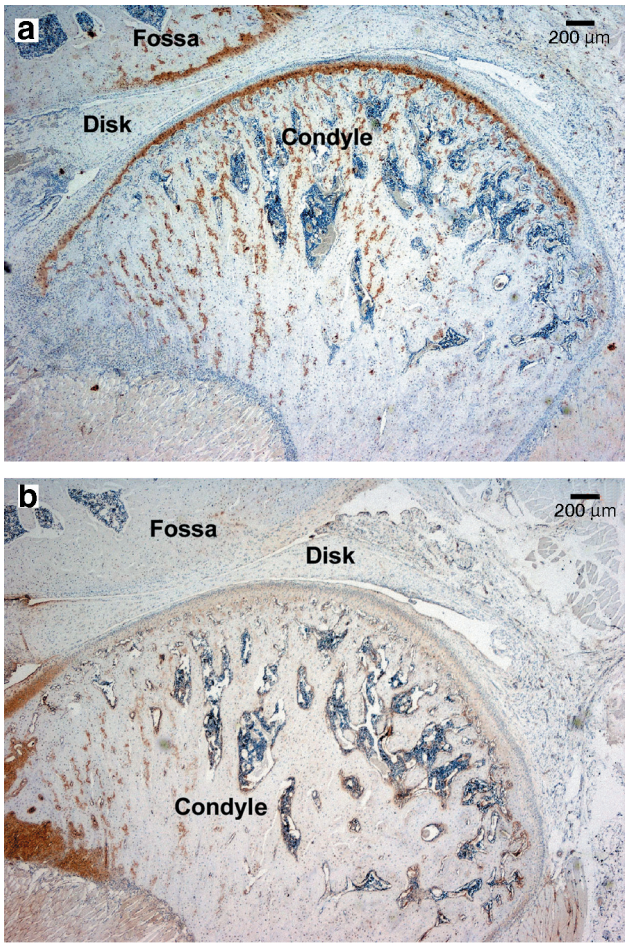

Figure 1 Histological characteristics and immunolocalization of types II and X collagen (120 days of age). (a) Staining of type II collagen, (b) staining of type X collagen.

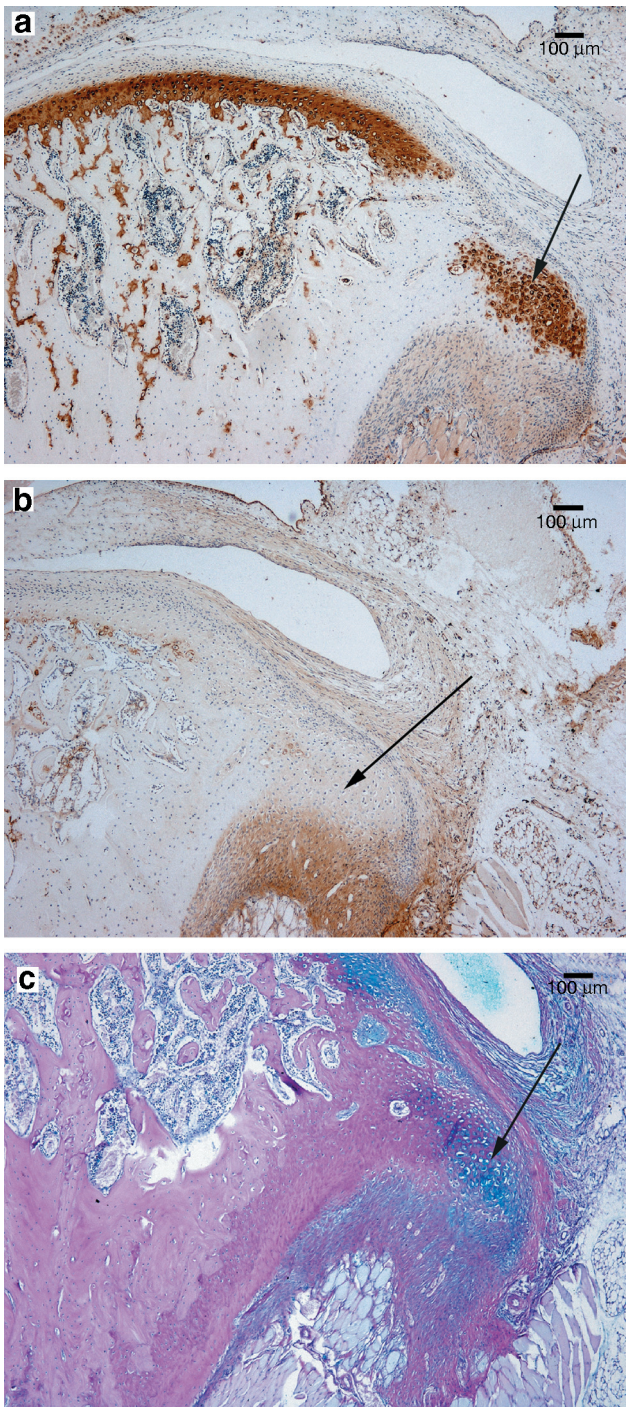

Figure 2 New cartilage was induced near the retrodiscal pad on experimental day 7. (a) Staining of type II collagen revealed new cartilage near the retrodiscal pad (arrow), (b) negative staining of type X collagen of the newly formed cartilage revealed that the new cartilage did not enter the hypertrophic stage, (c) positive staining with Alcian blue of the newly formed cartilage.

cells was not distinct compared with that of the condylar cartilage of young rats. Chondrocytes in the cartilage had only two to three layers and were distinguished by fewer hypertrophic chondrocytes. There was no obvious hypertrophic layer in the condylar cartilage of adult rats. Positive staining of type II collagen was located beneath the proliferative layer (Figure 1a). The staining for type X collagen was weak and some sections showed negative staining (Figure 1b). The expression of type II collagen, type X collagen and new bone formation of control animals showed no significant change during the experimental period.

In the experimental groups, typical endochondral ossification occurred in the posterior of the condyles. There were no obvious changes at experimental day 3. From day 7, new cartilage appeared near the region attached to the retrodiscal pad (Figure 2). The expression of type II collagen increased and type X collagen could be immunolocalized later in the hypertrophic layer. The highest levels of expression of types II and X collagen were present on day 21 (Figure 3); the increases were 248 and 540 per cent, respectively (Figure 4A, B). The highest level of new bone formation was found on day 30 (Figure 5) of advancement, when the amount of increase of new bone formation was 319 per cent

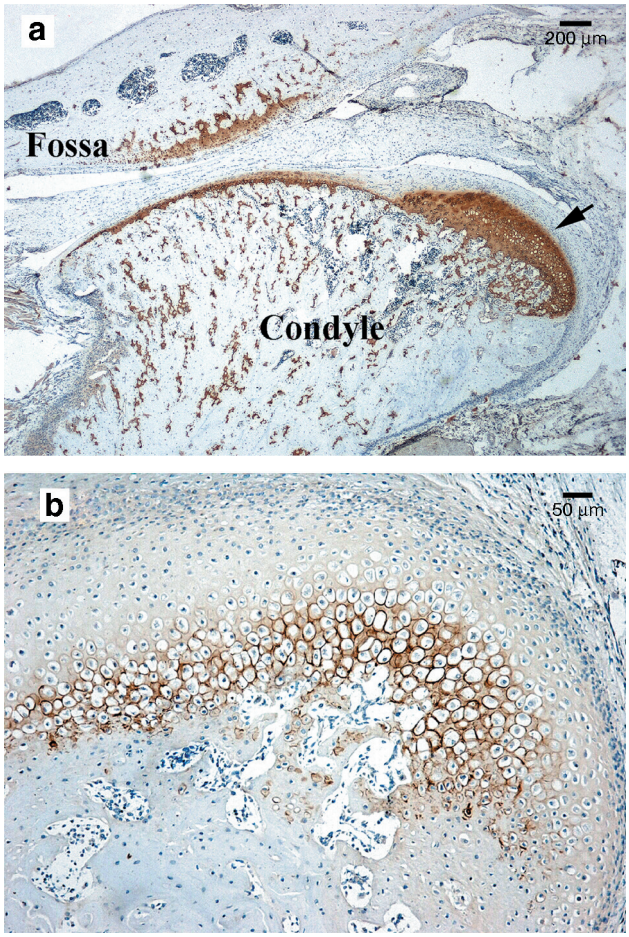

Figure 3 The highest expression of (a) type II and (b) type X collagen in the posterior condyle.

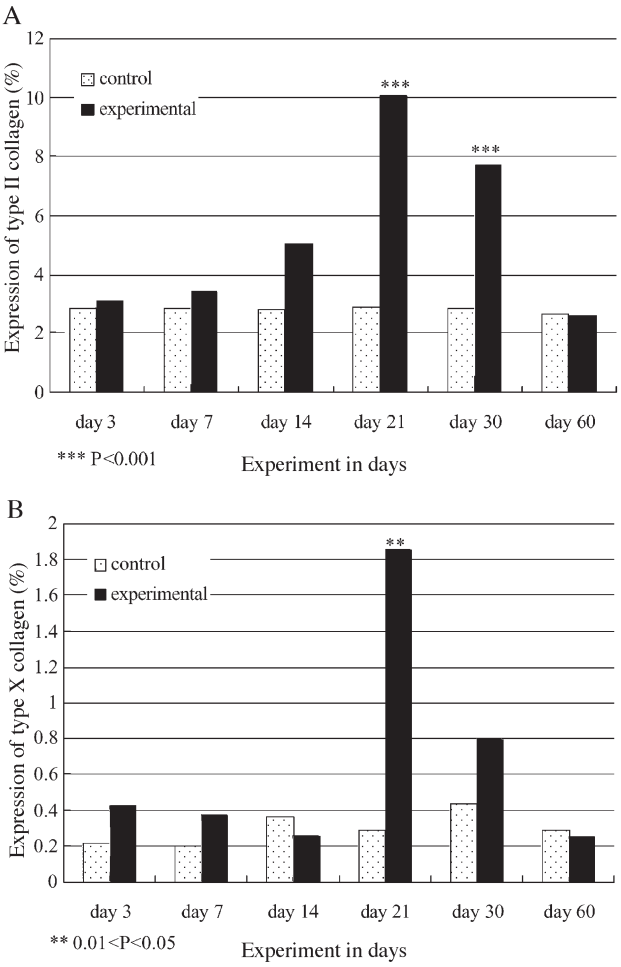

Figure 4 Graphs showing the increase in (A) type II and (B) type X collagen in the posterior condyle during the experimental period.

(Figure 6). New bone formation was reduced from experimental day 30 onwards, but was still more than that of the controls on experimental day 60.

Discussion

In a study of the morphological changes as a result of bite-jumping in adult rats, it has been shown that active forward mandibular positioning results in an elongated condylar head (Xiong *et al.*, 2004). A critical issue in the present study was: how to measure the changes in the adult condyle that would be indicative of condylar growth? One possibility would be to quantify known extracellular matrix components for the stages of bone growth in the condyle, then to correlate the amount of those components with the ultimate amount of bone formed in the condyle in response to mandibular advancement.

The present study has successfully addressed all of these issues to identify a quantitative measure for what could be called 'induced condylar adaptation in non-growing animals'. First, the amount of type II collagen, the major component of the cartilage matrix expressed in the adult condyle, was measured and compared with its level of expression during forward mandibular positioning (Figure 4a). Healy *et al.* (1999) and De Luca *et al.* (2001) reported that the rate of longitudinal bone growth depends primarily on the rate of growth plate chondrogenesis. Similarly, Rabie *et al.* (2003a) found that bone growth in the condyle was closely related to cartilage formation in growing rats. It was reported that an increase of 98 per cent in the cartilage matrix in response to mandibular advancement in growing rats was accompanied by a 90 per cent increase in the amount of bone formed in the condyle (Rabie *et al.*, 2003a). Therefore, to determine the enhanced chondrogenesis in the adult condyles in response to mandibular advancement, a quantitative assessment was carried out for type II collagen, the main constituent of the cartilage matrix. The results clearly showed that a significant increase in the amount of type II collagen (248 per cent) was expressed in adult

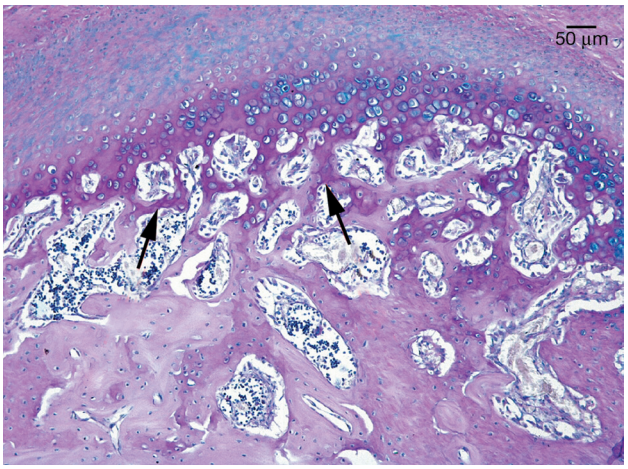

Figure 5 New bone formation in the experimental group at day 30: obvious new bone formation (arrow) located in the erosive zone.

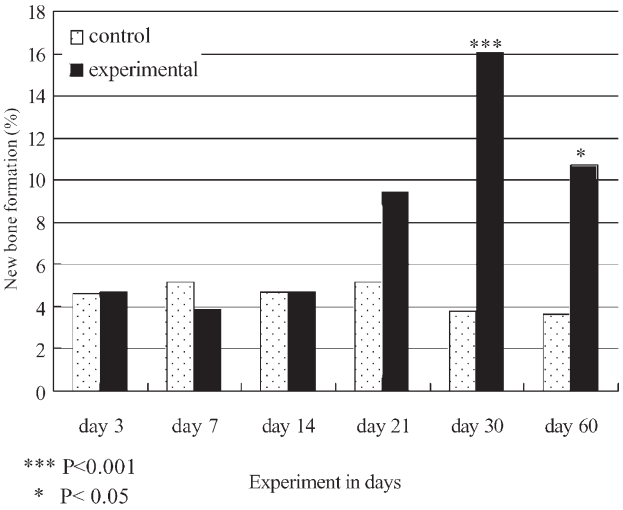

Figure 6 Graph showing the amount of new bone formation at the posterior condyle during the experimental period.

condyles in response to advancement. A most interesting finding was that in the non-growing animals, the condyle expressed a very thin film of type II collagen (Figure 1a). On mandibular advancement, a significant increase in the expression of type II collagen in the posterior region of the condyle was found, indicating increased chondrogenesis (Figure 3a). The cells constituting this newly formed cartilaginous matrix in the growing condyle would undergo hypertrophy and synthesize the hypertrophic matrix. The framework of the hypertrophic matrix is type X collagen (Rabie *et al.*, 2000; Rabie and Hägg, 2002). Type X collagen has been used as a marker for endochondral ossification and its expression precedes the onset of endochondral ossification in long bones and mandibular condyles (Salo *et al.*, 1996; Rabie *et al.*, 2000). Rabie *et al.* (2000) reported that in growing rats, type X collagen expression was greatly enhanced upon mandibular advancement. Therefore, to determine the progressive change of the newly formed cartilage matrix into bone as a result of mandibular advancement in the condyles of adult rats, the level of expression of type X collagen was identified (Figure 4B). Type X collagen expression was significantly increased in the experimental group (541 per cent), whereas its level was extremely low or even absent in the condyles of older rats (Figure 1b). In the present study, the temporal expression of type X collagen during endochondral ossification in the condyles of non-growing rats was correlated. A close correlation existed between type X collagen, the framework of the hypertrophic matrix and the amount of bone replacing such a hypertrophic matrix. Such a result is not totally unexpected as growth is regulated by factors expressed by cells of the condyles. Furthermore, the level of expression of these regulatory factors is enhanced by changing the biophysical environment of the TMJ regardless of age. It has been shown that mechanical signals are converted by the mechanotransduction mediator, Ihh, into increased mesenchymal cell proliferation (Tang *et al.*, 2003). It is thus conceivable that, regardless of age, mechanical signals trigger a response leading to increased bone formation in the condyle.

In order to correlate the molecular changes in response to adaptive changes in the condyles of non-growing rats, the amount of bone formed in the condyle of non-growing rats between the ages of 120 and 180 days was measured (Figure 6). The results were then compared with the amount of bone formed in response to mandibular advancement. Mandibular advancement led to a significant increase in the amount of bone formed in the posterior part of the condyle (Figure 5). The current results show that in non-growing rats, mandibular advancement led to a 540 per cent increase in type X collagen and a 319 per cent increase in new bone in the posterior of the condyle.

Conclusion

Forward mandibular positioning causes changes in the biophysical environment of the TMJ of adult rats that leads to condylar adaptation.

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