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Expression of type X collagen and capillary endothelium in condylar cartilage during osteogenic transition—a comparison between adaptive remodelling and natural growth

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SUMMARY Adaptive remodelling of the condylar cartilage in response to mandibular protrusion constitutes the rationale for bite-jumping appliances to solicit growth modification. By investigating the expression of type X collagen and capillary endothelium, this study was designed to evaluate the osteogenic transition of chondrogenesis during adaptive remodelling of condylar cartilage and compare it with that under natural condylar growth.

One hundred female Sprague–Dawley rats, 35 days of age, were divided into five experimental groups (n = 15, fitted with bite-jumping appliances) where condylar adaptation was created by forward repositioning of the mandible, and five control groups (n = 5) where the condyles underwent natural growth. The animals were sacrificed at 3, 7, 14, 21 and 30 days and 7 μm serial sections of the condyles were processed for *in situ* hybridization and immunohistochemical analyses. The expression of type X collagen in the hypertrophic zone and capillary endothelium in the erosive zone of condylar cartilage were examined to evaluate osteogenic transition, a critical programme leading to endochondral ossification.

The results showed that (1) The temporal pattern of the expression of type X collagen and capillary endothelium during condylar adaptation coincided with that during natural condylar growth. (2) The amount of the expression of these two factors during condylar adaptation was significantly higher than that during natural growth ($P < 0.001$). It is suggested that condylar adaptation in growing rats triggered by mandibular forward positioning enhances osteogenic transition which eventually results in increased bone formation.

Introduction

Bite-jumping functional appliances have been widely used to obtain extra growth in the condyle and subsequently in the mandible, which partially contributes to the correction of jaw discrepancy in subjects with a severe overjet (Shen *et al.*, 2005). Condylar growth modification induced by mandibular advancement has been reported in a recent series of experimental studies, where increased bone formation was observed by either identifying the cellular response of mesenchymal progenitors (Rabie *et al.*, 2001) or detecting the increased expression of some growth factors regulating condylar chondrogenesis (Leung *et al.*, 2004; Shum *et al.*, 2004). It is well established that post-natal growth of the mandibular condyle is mainly supported by endochondral ossification of the condylar cartilage, a biological programme characterized by chondrogenesis and its transition into osteogenesis (Garant, 2003; Akiyama *et al.*, 2004). As the secondary cartilage, one of the most distinctive biological features of the condylar cartilage lies in its adaptive remodelling in response to external stimuli, such as changes in articulate functioning, mechanical loading, and mandibular positioning (Kantomaa and Rönning, 1997; Nakano *et al.*, 2003). The adaptation of condylar cartilage to mandibular forward positioning has been considered to constitute the biological basis for altered osteogenic transition of chondrogenesis that leads to increased endochondral ossification (Shen and Darandeliler, 2005).

It is agreed that in condylar cartilage, the highly matured chondrocytes in the hypertrophic zone indicate the termination of chondrogenesis, while degenerated cartilage in the erosive zone indicates the beginning of osteogenesis (Gerstenfeld and Shapiro, 1996; Hashimoto *et al.*, 1997; Garant, 2003). The phenotypic programme of chondrocytes in these two zones is therefore crucial to facilitate the conversion from chondrogenesis to osteogenesis (Chung *et al.*, 2001).

While the mechanisms that govern osteogenic transition of chondrogenesis in condylar cartilage during natural growth is well established (Cancedda *et al.*, 2000), the biological pathway through which chondrogenesis converts into osteogenesis due to condylar adaptation remains unknown.

Using an animal model where the condyle is deviated from the fossa, it has been found that mesenchymal cells in the articular layer are stretched and reorientated towards the pull, leading to an increased mesenchyme population and an enhanced differentiation into chondrocytes, which subsequently results in an adaptive remodelling (Rabie *et al.*, 2001; Shen *et al.*, 2003). An *in vivo* experiment where mouth-breathing posture was simulated to facilitate condylar adaptation suggested that accelerated maturation of chondrocytes are attributed to the remodelling of cartilage (Kantomaa and Hall, 1988). The theory of a multipotential phenotypic programme of chondrocytes is hypothesized in some studies, concluding that chondrocytes can undergo asymmetric division under adaptation, wherein one daughter cell undergoes apoptotic cell death in the hypertrophic zone, while the other gives rise to osteogenic cells, resulting in endochondral ossification (Roach *et al.*, 1995; Roach and Erenpreisa, 1996).

Type X collagen is synthesized exclusively by hypertrophic chondrocytes and its expression indicates the termination of chondrogenesis (Nishida *et al.*, 2002). Invasion of the new vasculature in the erosive cartilage, on the other hand, produces osteogenic progenitor cells and therefore is associated with the onset of osteogenesis (Luder *et al.*, 1988; Garant, 2003). These two factors therefore are reliable markers to depict transition between chondrogenesis and osteogenesis.

Using an experimental model with growing rats where adaptive remodelling of condylar cartilage was created by mandibular forward positioning, the aim of the present study was to compare the expression of type X collagen and capillary endothelium between condylar remodelling and natural growth, and therefore evaluate the effects of bite-jumping therapy on condylar growth. Cellular responses in the hypertrophic and erosive zone of condylar cartilage were also examined to complement the biochemical analyses.

Materials and methods

Animal model

This research was approved by The University of Hong Kong Committee on the Use of Live Animals in Teaching and Research, No. 26398. One hundred female Sprague–Dawley rats, 35 days of age, were divided randomly into five experimental (n = 15) and five control (n = 5) groups. Bite-jumping appliances were fitted to the upper incisors of the animals in the experimental groups to facilitate mandibular protrusion. The animals in the control groups were under natural growth. The experimental animals, together with their age-matched controls, were sacrificed 3, 7, 14, 21, and 30 days after mandibular repositioning. The mandibles were removed and the entire fresh temporomandibular joints was cut at the sagittal plane and prepared for paraffin wax embedding. Serial sections, 7 μm, were cut using a rotary microtome (Leica RM 2155, Wetzlar, Germany) and floated onto glass slides.

In situ hybridization examination of type X collagen mRNA

Sense and antisense [α-^{35}S]uridine triphosphate ([α-^{35}S]-UTP) riboprobes were generated by *in vitro* transcription from linearized DNA template using RNA polymerase T3, T7, and SP6 in transcription buffer with 10 mM dithiothreitol, 250 μM adenosine triphosphate, cytidine triphosphate, and guanosine triphosphate, 50 units of RNasin, and 100 μCi [α-^{35}S]-UTP. The RNA probe was precipitated out from the tube with the highest radioactivity by the addition of 0.5 volume of 6 M NH_4Ac (pH 5.3) and two volumes of absolute ethanol. The labelled riboprobe was resuspended in 0.1 M DTT to give a radioactivity of 2×10^6 cprn/μl followed by the addition of nine volumes of hybridization buffer and stored at −20°C. *In situ* hybridization using paraffin sections was performed as described by Kwan *et al.* (1997) to determine the spatial expression of type X collagen mRNA transcripts. After the pre-hybridization treatment of tissue sections, the sense and antisense ^{35}S-labelled riboprobes dissolved in hybridization buffer were heated at 80°C for 3 minutes and then, respectively, applied onto the pre-treated sections on the glass slide. A clean cover slip was then placed onto the sections to enable the riboprobe to be spread uniformly over the sections. The hybridization was carried out in a slide box placed in a sealed jar with the presence of 50 per cent (v/v) deionized formamide and ×5 standard saline citrate at 50°C for 18 hours. The slides treated with sense riboprobe served as the negative control. The sections were then processed for post-hybridization washing. The hybridization signal was detected by autoradiography by dipping the slide in liquid emulsion K5 (Ilford, Paramus, NJ, USA).

Immunohistochemical examination of type X collagen protein

The antisera raised against type X collagen were found not to cross-react with other collagen types when tested either by enzyme-linked immunosorbent assay or by immunoblotting as reported by Rucklidge *et al.* (1996). The secondary antibodies were anti-rabbit immunoglobulin (Ig) G–peroxidase conjugate (Sigma, St Louis, Missouri, USA, Code No. A-4914). After incubation with 0.5 U/ml hyaluronidase (Sigma Code No. H-6254) and 0.5 U/ml chondroitinase avidin-biotin HRP complex (Sigma Code No. C-2905) for 1 hour at 37°C, the sections were washed in phosphate-buffered saline and then incubated with normal foetal bovine serum (GibcoBRL, Carlsbad, California, USA, Code No. 16000-044) for 30 minutes. Sections were then incubated overnight at 4°C with the primary antibody.

The incubation with secondary antibodies was performed for 1 hour at 37°C followed by Tris-buffered saline washing and dips in 3,3-diaminobenzidine (Sigma Code No. D-5637) for 2 minutes. The sections were then stained with Mayer haematoxylin for 3 minutes as background staining.

Immunohistochemical examination of capillary endothelium

Monoclonal antibody EN 7/44 (anti-human angiogenesis related to endothelial cells; BMA, Augst, Switzerland, Code No. T-1107) used in this study positively reacts with budding endothelial cells where the antigen is only found on the apical part of the vessels. Finally differentiated endothelial cells are not recognized by these antibodies. The secondary antibodies used were mouse Ig (biotin; Dako, Glostrup, Denmark, Code No. E0354). The immunostaining process was similar to that for type X collagen.

Quantitative evaluation

The quantitative evaluation was conducted via a computer-assisted image-analysing system (Leica Q550IW, Leica Microsystems Imaging Solutions Ltd., Cambridge, UK) with Leica Qwin Version 2.2 software. This system can acquire high-definition digital images from the specimens. The designated features from the acquired images can be automatically selected and recognized by identifying colour, shade, shape, or texture. A three-channel system three charge-coupled device (3CCD) red–green–blue true-colour video camera (JVC TK-1281 EG) was connected to the microscope (Leitz Orthoplan, Wetzlar, Germany), from which the 3CCD component signal was captured in real time by the hardware of the image analyser. Thus, the type X collagen mRNA signals and immunostaining were captured and their area was then measured. Immunostaining against capillary endothelium in the erosive zone was also quantified using this system. As the positive reaction of both *in situ* hybridization and immunostaining was distinctive, only one fixed threshold was set to capture all the positive targets. Measurements were conducted in the posterior region of the condyle, due to the fact that this region connects with the disc ligaments where major chondrogenesis and osteogenesis occur in response to mandibular repositioning (Kantomaa and Hall, 1988; Rabie *et al.*, 2004). The sections were evaluated at a total magnification of ×40, with fixed measurement frame of 450 × 450 μm.

The data were processed with GraphPad InStat (San Diego, California, USA, Version 3.00) for both paired *t*-test and analysis of variance.

Method error analysis

The area measurements for all the specimens were conducted twice by two independent examiners on two separate occasions with a time interval of approximately 2 months. Method error (ME) analysis in measuring the signal area was calculated by the formula:

$$\mathrm{ME} = \sqrt{\frac{\sum d^2}{2n}},$$

where d is the difference between the two registrations of a pair and n the number of double registrations. Ten readings were drawn randomly from the readings of each observer for ME analysis. Paired *t*-tests were performed to compare the two registrations. There was no significant difference among the inter- and intraobserver registrations.

Results

In situ hybridization and immunohistochemical examination revealed that type X collagen was invariably expressed in hypertrophic chondrocytes (Figure 1) and capillary endothelium in the erosive cartilage (Figure 2). Computer-assisted image analysis quantified the amount of the expression of these two factors (Tables 1 and 2), and subsequently revealed their temporal patterns (Figure 3).

Day 3 (38 days of age)

There were few signals of type X collagen mRNA within the hypertrophic chondrocytes in either the experimental or control groups with *in situ* hybridization. Positive immunostaining for type X collagen was not evident (Table 1). Very little positive immunostaining for capillary endothelium was observed in the erosive zone in either group, indicating a weak neovascularization at this stage (Table 2).

Day 7 (42 days of age)

In situ hybridization showed that type X collagen mRNA signals were localized in the hypertrophic zone and were more abundant than those at day 3. Positive immunostaining for type X collagen was detected in the upper region of the hypertrophic zone. There was positive immunostaining for endothelial cells in the erosive zone, indicating the formation of new blood vessels. Quantitative analysis showed significant differences between the mandibular protrusion and natural growth groups in the amount of type X collagen mRNA signals and immunostaining for both type X collagen and capillary endothelium (Tables 1 and 2).

Day 14 (49 days of age)

In situ hybridization revealed that positive signals of type X collagen mRNA were distributed within the hypertrophic zone (Figure 1a,b). Quantitative analysis showed that the amount of type X collagen mRNA, in both the experimental and control groups, was at maximum compared with that at other time points, with that in the protrusive group being significantly higher than in the natural growth group (Table 1). In the experimental groups, endothelial cells in

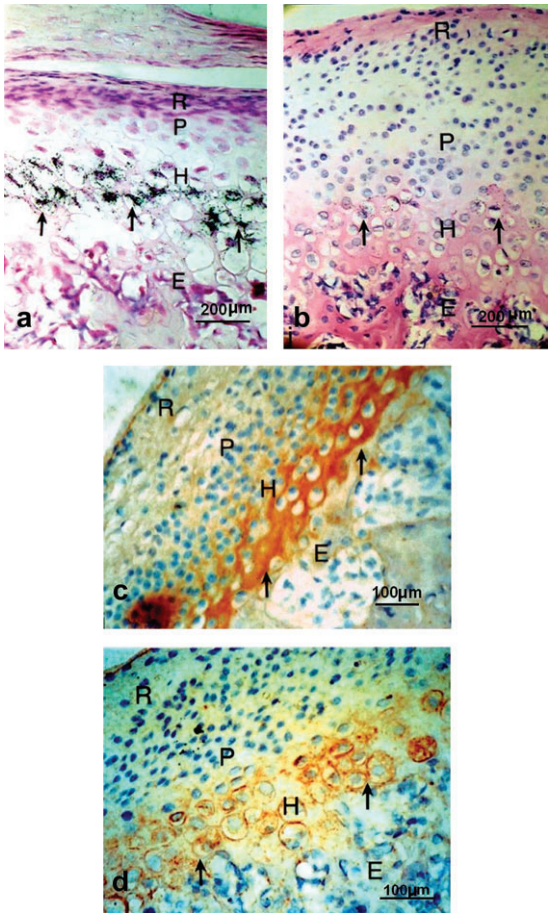

Figure 1 Photomicrographs showing biochemical examination of the expression of type X collagen in the hypertrophic zone of the condylar cartilage. The positive *in situ* hybridization signals for type X collagen mRNA (arrows) at day 14 are more intense in the experimental (a) than in the control (b) groups. The positive immunostaining against type X collagen protein (arrows) at day 21 is much stronger in the experimental (c) than that in the control (d) groups, where there is only moderate immunoreactivity. R, resting zone; P, proliferative zone; H, hypertrophic zone; E, erosive zone.

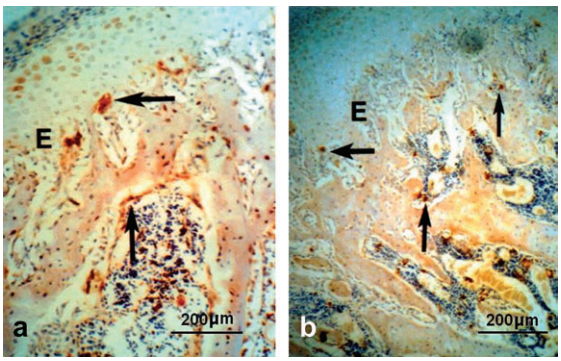

Figure 2 Photomicrographs showing biochemical examination of the expression of capillary endothelium in the erosive zone of the condylar cartilage at day 21. Distinctive immunostaining against capillary endothelium (arrows) is evident in the experimental groups (a), while fewer endothelial cells are immunolocalized (arrows) in the control groups (b). E, erosive zone.

the erosive zone were positively stained with EN 7/44 antibody. Quantitative analysis showed that the amounts of type X collagen and endothelium were significantly higher than those at previous stages (Tables 1 and 2).

Day 21 (56 days of age)

Quantitative analysis showed that the amount of *in situ* hybridization signals for type X collagen mRNA had decreased compared with that at day 14 (Table 1). In both the experimental and control groups, the amount of immunostaining for type X collagen, however, was at its highest level compared with other time points, with that in the experimental groups being much higher than in the control (Table 1, Figure 1c,d). The expression of immunostaining against capillary endothelium in both groups also reached its maximum level, evidenced by strong immunoreactions in the erosive zone (Table 2, Figure 2a,b).

Day 30 (65 days of age)

Few signals for type X collagen mRNA were positively labelled by ^{35}S-labelled riboprobe hybridization. This was further confirmed by quantitative analysis which showed that the amount of type X collagen mRNA signals considerably decreased (Table 1). The amount of immunostaining for type X collagen and for endothelial cells also decreased (Tables 1 and 2). Histological observation demonstrated that the erosive zone in the experimental groups became thinner due to the newly formed bone advancing and replacing part of the degenerating cartilage in this zone (Figure 4).

Discussion

A recent series of animal experiments have reported that active mandibular protrusion produces adaptive remodelling of condylar cartilage evidenced by increased endochondral bone formation (Rabie *et al.*, 2003, 2004; Leung *et al.*, 2004; Shum *et al.*, 2004). In the present study, an experimental model where adaptation of condylar cartilage was created during the pubertal growth of rats was used, and was compared with natural condylar chondrogenesis in age-matched controls. The expression of type X collagen and capillary endothelium, two factors that indicate terminal hypertrophic phenotype of chondrogenesis and the beginning of endochondral ossification, was investigated. The expression of type X collagen was examined at both the genetic and molecular levels. It was clearly shown that the temporal patterns of type X collagen mRNA expression under both adaptive remodelling and natural growth were similarly characterized by an increase between days 3 and 14, a peak level at day 14 followed by a decrease (Figure 3). However, a marked discrepancy in quantity between the two groups was evident, with *in situ* hybridization signals expressed during adaptive remodelling (8541 μm^2 at peak)

Table 1 Quantitative analysis of type X collagen expression in the hypertrophic zone of the experimental (Exp.) and control (Con.) groups (*in situ* hybridization signals and immunostaining, μm²).

	Day 3	Day 7	Day 14	Day 21	Day 30	ANOVA
mRNA signals						
Exp. (n = 15)	862 ± 36	2249 ± 78	8541 ± 74	5038 ± 93	3177 ± 93	***
Con. (n = 5)	825 ± 25	1297 ± 55	3543 ± 68	1389 ± 21	942 ± 52	***
t-test	NS	***	***	***	***	
Immunostaining						
Exp. (n = 15)	6491 ± 80	17456 ± 188	29748 ± 165	54864 ± 134	27941 ± 177	***
Con. (n = 5)	6084 ± 33	8807 ± 61	10493 ± 81	15470 ± 121	7381 ± 75	***
t-test	NS	***	***	***	***	

ANOVA, analysis of variance. Data are presented as the mean ± standard deviation. NS, $P > 0.05$; ***$P < 0.001$.

Table 2 Quantitative analysis of expression of capillary endothelium in the erosive cartilage of the experimental (Exp.) and control (Con.) groups (Immunostaining, μm²).

	Day 3	Day 7	Day 14	Day 21	Day 30	ANOVA
Exp. (n = 15)	943 ± 33	2727 ± 80	5756 ± 82	10000 ± 94	5471 ± 41	***
Con. (n = 5)	888 ± 34	1886 ± 42	2622 ± 29	3415 ± 67	2092 ± 71	***
t-test	NS	***	***	***	***	

ANOVA, analysis of variance. Data are presented as the mean ± standard deviation. NS, $P > 0.05$; ***$P < 0.001$.

being significantly more intense than those expressed during normal growth (3543 μm² at peak). The consistency in the temporal pattern and the discrepancy in quantity between the experimental and control groups were also evident with type X collagen immunolocalization (Figure 3).

It is worth noting that the temporal pattern of type X collagen expression revealed in the present study is in agreement with other findings that the condylar growth puberty for Sprague–Dawley rats occurs between 35 and 56 days of age, followed by decrease and then a cessation (Lane-Petter, 1976; Rao and Luo, 1990).

It is believed that type X collagen is specifically synthesized by hypertrophic chondrocytes and its expression is correlated with the hypertrophic phenotype and subsequently to endochondral ossification (Kong *et al.*, 1993). A similarity in temporal pattern but a significant difference in quantity of type X collagen synthesis between the two groups indicates that the rate of chondrocyte maturation under adaptive remodelling is consistent with that under natural growth, but to a more enhanced degree. This is contrary to some studies which have suggested that forward positioning of the mandible results in an enhanced condylar growth by accelerating the differentiation of mesenchymal cells into chondroctytes and subsequently provoking an earlier formation of cartilage matrix (Rabie *et al.*, 2003), it is also not supportive of the hypothesis that an accelerated process of cartilaginous maturation is associated with condylar adaptation (Kantomaa and Hall, 1988, 1991). An enhanced, but not accelerated, transition into osteogenesis in condylar cartilage during adaptive remodelling was further verified in this study by detecting capillary invasion within the erosive cartilage. Interestingly, the temporal pattern of the emergence of capillary endothelium during condylar adaptation occurred at a concomitant rate with that during natural growth, with the amount of capillary endothelial cells expressed in the former (10 000 μm² at peak) being much more (3415 μm² at peak) than the latter (Figure 3c). These phenomena indicated that mandibular forward positioning triggers the adaptation of condylar cartilage which is characterized by a significantly enhanced osteogenic transition of chondrogenesis. It is further suggested that condylar adaptation follows the same biological pathway as that governing chondrocytes during natural condylar growth. The biochemical analyses in the present study were also complemented by histological observations on the sequence of cellular responses in the transition zone. Histological structures observed at the same time point between the two groups confirmed the similar advent of cartilage chondrogenesis but with a different intensity, indicated by the thickness of the hypertrophic and erosive cartilage and the amount of endochondral bone deposition (Figure 4a,b).

The phenotypic programme that governs the pathway of chondrogenesis and its transition into osteogenesis during condylar growth has been well documented (Erenpreisa and Roach, 1996; Cancedda *et al.*, 2000; Ogawa *et al.*, 2003). It is widely accepted that natural condylar growth is governed by an intrinsic programme of condylar chondrocytes which is genetically predetermined (Inoue *et al.*, 2002) but could

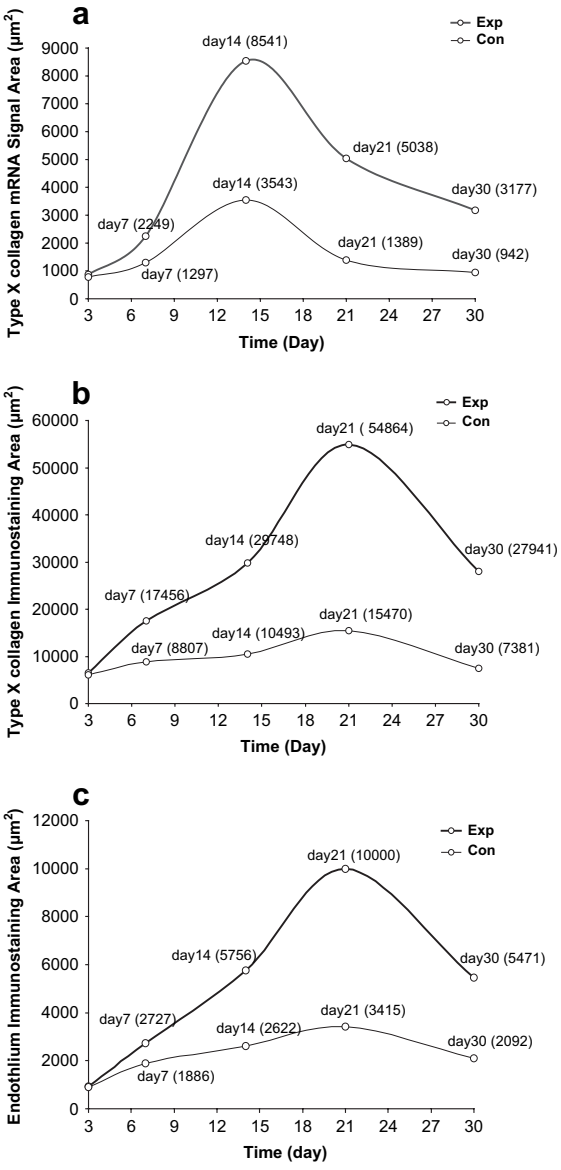

Figure 3 Diagrams depicting temporal patterns of the expression of type X collagen and capillary endothelium. The expression of these two factors during condylar adaptation occurs in the same pattern as those expressed during natural condylar growth. The marked discrepancy in quantity, however, is well manifested by the peak levels between two groups. (a) The expression of type X collagen mRNA revealed by *in situ* hybridization in the hypertrophic cartilage. (b) The expression of type X collagen revealed by immunostaining. (c) The expression of capillary endothelium in the erosive cartilage revealed by immunostaining.

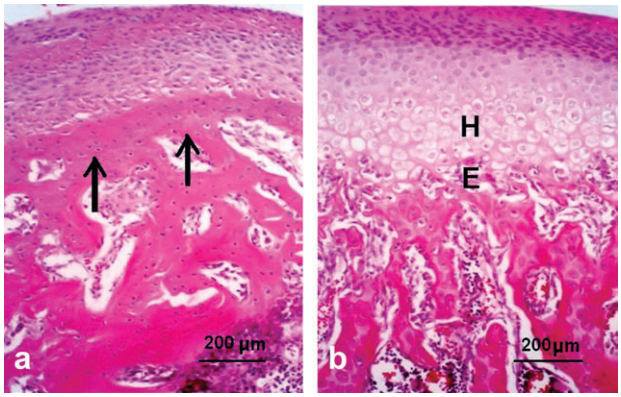

Figure 4 Photomicrographs showing histological examination of cellular response during osteogenic transition of condylar cartilage at day 30. (a) In the experimental groups, the thickness of cartilage is decreased and the erosive zone is occupied by the newly formed bone, indicating an aggressive advancement of endochondral bone formation (arrows) replacing the degenerating cartilage during adaptive remodelling. (b) In the control groups, the thicker proportion of cartilage is evident with distinct hypertrophic and erosive zone, indicating a moderate osteogenic transition. H, hypertrophic zone; E, erosive zone.

also be microenvironmentally influenced (Fuentes *et al.*, 2003; Akiyama *et al.*, 2004). The programme starts with the differentiation of mesenchymal cells into chondroctyes, which then mature into terminally hypertrophic phenotype (chondrogenesis) followed by degeneration of chondroctyes and subsequent advancement of endochondral bone formation (osteogenesis). It remains unclear, however, as to whether or not the adaptive remodelling of condylar cartilage also follows this biological programme. The effect of articulate functioning on condylar cartilage, especially its maturation, has been studied *in vitro* and *in vivo*. When functioning of the posterior part of the condyle is inhibited, a situation similar to mandibular forward positioning, maturation of the cartilage is found to be accelerated (Kantomaa and Hall, 1988; Nakano *et al.*, 2003). It is therefore suggested that condylar unloading stimulates condylar growth, while articulate functioning, on the other hand, slows down condylar growth (Kantomaa and Rönning, 1997). It has been hypothesized that, because prechondroblasts in condylar cartilage are multipotential, they switch their biomolecular pathway towards the direction of osteoblasts in the absence of articulate functioning, and growth increases. If articulate functioning is not present, maturation advances rapidly and the highly matured cartilage proceeds to induce bone formation (Kantomaa and Hall, 1991; Takahashi *et al.*, 1995). The present findings, however, suggest otherwise; quantitative analysis clearly demonstrated that mandibular forward positioning causes adaptation of condylar cartilage by enhancing, but not accelerating, hypertrophy of chondrocytes and then the subsequent osteogenic transition of chondrogenesis.

Conclusions

Type X collagen and capillary endothelium are factors that are expressed in the hypertrophic and erosive zones of condylar cartilage where osteogenic transition takes place to facilitate endochondral ossification. Compared with that during natural condylar growth, the expression of these two factors during condylar adaptive remodelling significantly increases. Interestingly, both of these two factors are expressed in the same temporal pattern during natural

growth and during responsive adaptation of the condyle. These phenomena suggest that bite jumping causes condylar adaptive remodelling where the transition from chondrogenesis into osteogenesis is enhanced, but not accelerated, to encourage bone formation in the condyle.

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