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Neovascularization and bone formation in the condyle during stepwise mandibular advancement

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SUMMARY The aims of this investigation were to identify the temporal expression of vascular endothelial growth factor (VEGF) in the mandibular condyle and to correlate it with the pattern of new bone formation during stepwise mandibular advancement. Two hundred and fifty female, 35-day-old Sprague–Dawley rats were randomly divided into 10 groups, with 10 rats allocated to the single-step bite-jumping subgroup, 10 rats to the stepwise advancement subgroup and five rats to the control subgroup. In the experimental groups, the mandibles were kept in a continuous forward position. The initial stepwise advancement commenced on day 35, whereas the second advancement started on day 65. The rats were sacrificed on experimental days 3, 7, 14, 21, 30, 33, 37, 44, 51 and 60. Sections (7 μm) were cut through the condyle in the parasagittal plane and stained with anti-VEGF antibody. Each section was counter-stained with haematoxylin for observation of the cellular response. The sections were digitized and quantitatively analysed with a computer-assisted image analysing system.

The results showed that the initial advancement in the stepwise group led to significantly less expression of VEGF when compared with single advancement. However, the second advancement on day 30 resulted in a significant increase in VEGF expression when compared with the one-step group and the natural growth control group. Thus, it was concluded that changes in the amplitude of mechanical loading, produced by stepwise advancement, have a significant effect on the production of VEGF by the chondrocytes. During the later stages of advancement, more VEGF and more condylar bone was produced.

Introduction

Vascular endothelial growth factor (VEGF) is a potent regulator of neovascularization expressed during endochondral ossification in long bone (Gerber *et al.*, 1999a,b; Horner *et al.*, 1999; Carlevaro *et al.*, 2000) as well as in mandibular condyles (Rabie and Hägg, 2002). Chondrocytes in the mandibular condyle express VEGF which stimulates neovascularization and marks the onset of cartilage replacement by bone (Rabie and Hägg, 2002). The expression of VEGF in the mandibular condyle increases upon forward mandibular positioning, with the pattern of expression closely correlated with that of new bone formation (Rabie *et al.*, 2002). The highest level of VEGF expression is present in the posterior region of the condyle during natural growth and during forward mandibular positioning, concomitant with the pattern of new bone formation (Rabie *et al.*, 2002). The close correlation between VEGF expression and new bone formation reported recently demonstrated the vital role of vascularization during endochondral ossification in the mandibular condyle and its influential role in the overall response of mandibular condylar growth to mechanical stimuli (Rabie *et al.*, 2002).

The potential for the mechanical stimulation of mandibular condylar growth was postulated by Petrovic *et al.* (1981), who conducted a study on rats with varying degrees of advancement and commented that mandibular length was greater with periodic advancement. Several clinical studies adapted the stepwise advancement treatment regime and reported reduced dentoalveolar effects with greater skeletal bone formation when compared with conventional single advancement (Pancherz *et al.*, 1989; Ömblus *et al.*, 1997; Kumar *et al.*, 1999). A recent clinical study (Du *et al.*, 2002) found that stepwise mandibular advancement led to greater improvement in the sagittal jaw relationship with the mandible being positioned more forward when functional appliances were used in a stepwise manner, compared with single-step bite-jumping. However, the mechanisms for these changes are not fully understood and factors regulating such changes are unknown. Changing the biophysical environment in the temporomandibular joint (TMJ) as a result of mandibular advancement has been shown to cause cellular changes that result in new bone in the TMJ (Rabie *et al.*, 2001).

Mechanical load is an important regulator of chondrocyte metabolism and is necessary to maintain cartilage matrix properties (Urban, 1994). Changes in the degree or frequency of loading significantly affect the production of matrix molecules such as type II collagen and proteoglycans (Buschmann *et al.*, 1995).

It is thus conceivable that stepwise advancement, with varying degrees of advancement, could solicit a different response from extracellular matrix in cells of the condyles.

Therefore, in order to understand the condylar tissue response to stepwise advancement of the mandible, this study was designed with the following objectives in mind:

1. To identify the expression of VEGF and to correlate it with bone formation in the condyle during stepwise mandibular advancement.
2. To compare the pattern of VEGF expression and bone formation during forward stepwise advancement with that occurring during natural growth and during single mandibular advancement.

Materials and methods

Experimental groups

Two hundred and fifty 35-day-old female Sprague–Dawley rats were randomly divided into 10 groups. Each group consisted of 10 rats with single-step bite-jumping appliances, 10 with stepwise bite-jumping appliances and five untreated. All groups were fed a soft diet. Different groups were sacrificed on days 3, 7, 14, 21, 30, 33, 37, 44, 51 and 60, respectively.

Bite-jumping appliances

Bite-jumping appliances made from polymethylmethacrylate with identical inclined planes were cemented to the upper central incisors of the experimental group with Panavia-F™ cement (Kuraray Co. Ltd, Osaka, Japan). The appliances were worn for 24 hours producing a continuous forward and downward positioning of the mandible according to the method reported recently (Rabie *et al.*, 2001). The single-step advancement produced a 3.5 mm advancement; an initial 2 mm advancement followed by a 1.5 mm advancement on experimental day 30. The second advancement was in the form of a veneer, adhered to the top of the initial advancement appliance by monomer in order to minimize the thickness of the adhering medium.

Tissue preparation

After the rats were sacrificed by intraperitoneal injection of Dominal 20 per cent solution (200 mg of pentobonbital sodium, Alfasan), tissues were collected and prepared following the method of Rabie *et al.* (2001). Sections (7 μm) were cut through the condyle at the parasagittal plane. The sections from the 250 specimens were obtained from a similar parasagittal plane of the condyle in order to standardize the location of where the condyles were measured.

VEGF immunostaining

The sections were prepared and immunostained with VEGF according to the method reported by Rabie *et al.* (2002). The sections were submerged in 3 per cent H_2O_2 for 10 minutes to block endogenous peroxidase activity. After washing, antigenic sites were exposed by digestion with trypsin for 20 minutes. The sections were then washed and non-specific binding was blocked by incubation in normal rabbit serum [Dako A/S, Denmark, 1:10 diluted with ×1 Tris-buffered saline (TBS)] for 30 minutes, followed by incubation with primary polyclonal goat anti-VEGF antibody (Sigma Chemical Co., St. Louis, Missouri, USA) at a concentration of 2 μg/ml (1:50) overnight below 4°C. After washing, the sections were incubated with secondary biotin-conjugated rabbit anti-goat IgG (Dako, 1:400 diluted with phosphate-buffered saline) for 30 minutes at 37°C, followed again by washing. Strept ABComplex (HRP, Dako, 1:100 diluted with ×1 TBS) was applied for 1 hour at 37°C and washed with ×1 TBS plus 0.1 per cent Tween-20, before developing in 0.05 per cent 3,3-diaminobenzidine tetrahydrochloride (Sigma) for 5 minutes to identify the binding sites. Positive VEGF was indicated by brown staining. The sections were then counter-stained with Mayer haematoxylin for background staining.

Quantitative analysis

The amount of VEGF expression and new bone formation were quantified via a computer-assisted image analysing system (Leica Q5501W, Leica Microsystems Imaging Solutions Ltd, Cambridge, UK) with Leica Qwin Pro. (version 2.2) software, following the method of Rabie *et al.* (2001). This system acquires high-definition digital images of the specimen with features from the acquired images being selected by the operator. The sections were evaluated through a light microscope (Leitz Orthoplan, Wetzler, Germany) connected to the computer for digital analysis through a three-channel system (3CCD) red–green–blue colour video camera (JVC TK-C1380, Victor Company of Japan, Yokohama, Japan), which included special optics that were colour neutral. The 3CCD component signal was caught on television real-time by the hardware of the image analyser. The amount of positive staining was recognized and quantified by the computer software according to the colour, shade and contrast of the feature selected. The colour boundaries selected were kept constant throughout the section analysis. The VEGF expressions in the hypertrophic zones (Garcia-Ramirez *et al.*, 2000) in the posterior, middle and anterior regions were quantified separately under the fixed measurement frame, using ×360 magnification (Leitz Orthoplan). The measurement frame was orientated parallel to the articular surface,

Table 1 Results of the method error for digitizing vascular endothelial growth factor expression in the condyle with the computer-assisted image analysing system.

Mean of the differences	Standard deviation of the differences	*P*	Size of the method error
–0.00003	0.00042	0.255	0.0003

and the superior margin of the measurement frame was set at the superior surface of the articular surface for standardization. The data were processed with SPSS® for Windows (version 10.01, SPSS Inc., Chicago, Illinois, USA) for both *t*-test and ANOVA. Ten randomly drawn histological slides from the 250 specimens were quantified on two separate occasions to calculate the method error (Table 1). A paired *t*-test was performed to compare the two registrations.

Results

Mandibular stepwise advancement versus condylar growth

After the initial advancement, the pattern of VEGF expression in the posterior region of the condyle followed that of the control with a significant difference only on day 21 (Figure 1). After the second advancement there was a significant increase in all three regions of the condyle, with the highest increase on day 51 in the middle and anterior regions and on day 60 in the posterior region. Although the level of VEGF expression was highest in the posterior condyle, the largest difference was found in the anterior region.

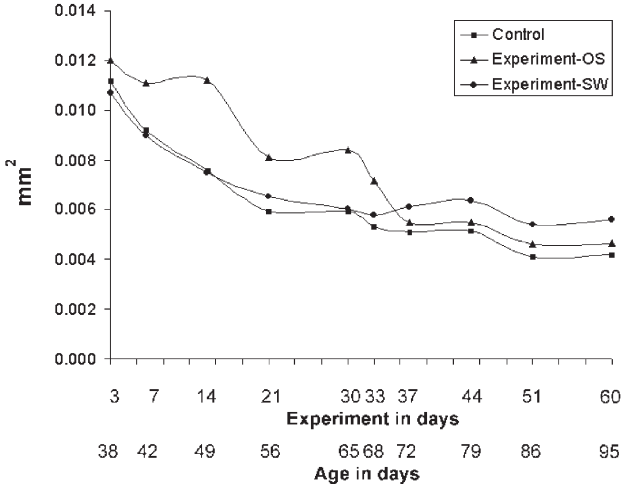

Figure 1 The temporal pattern of vascular endothelial growth factor expression in the hypertrophic zone of the posterior region of the condyle during natural growth, one-step (OS) and stepwise (SW) advancement.

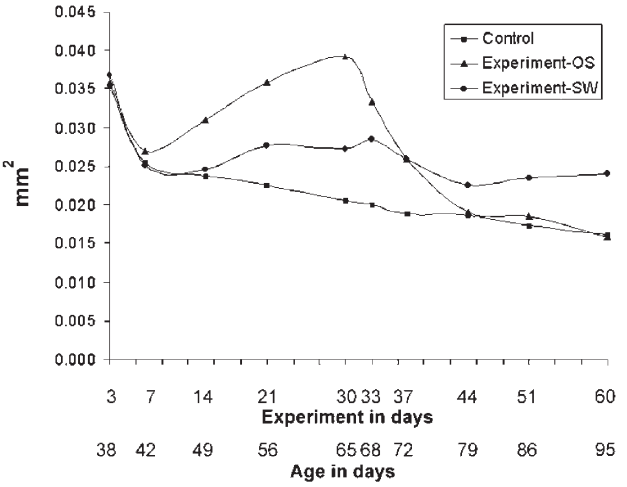

Figure 2 The temporal pattern of new bone formation in the posterior region of the condyle during natural growth, one-step (OS) and stepwise (SW) advancement [reprinted with permission from Chayanupatkul *et al.* (in press)].

The temporal sequence of new bone formation was later than VEGF expression (Figures 1 and 2). The peaks of bone formation in the posterior region occurred on days 33 and 60 (Figure 2) whereas VEGF expression showed the highest percentage increase on days 21 and 60 (Figure 1).

Mandibular stepwise advancement versus single-step advancement

Single-step mandibular advancement led to a constant increase in VEGF expression during the early days when compared with normal growth. The levels then gradually returned to normal after day 37 (Figure 1). Although the amount of VEGF expression upon initial stepwise advancement was higher on day 21, the amount of increase was substantially lower than that after single-step advancement (Figure 1). A similar trend of increase in bone formation was evident in the mandibular condyle, with the amount of new bone formation substantially higher during single-step advancement when compared with the amount after initial stepwise advancement (Figure 2). Both regimes of mandibular advancement produced the greatest levels of VEGF expression and new bone formation in the posterior region of the mandibular condyle.

Discussion

It has been reported that functional appliance therapy delivers more stable results when used at or after the peak pubertal period (Hansen *et al.*, 1991; Ruf and Pancherz, 1999; Von Bremen and Pancherz, 2002). Therefore, in the present study, mandibular advancement

was carried out on 35-day-old rats because at this age they are at the post-pubertal period, which begins on day 31.5 (Luder, 1996).

A most interesting finding in this study was that varying degrees of mechanical stimuli produced different tissue reactions. A smaller advancement, as in the initial step of a 2 mm forward movement in the stepwise group, resulted in considerably lower levels of expression of VEGF (Figure 1). Human and bovine chondrocytes have been shown to produce different levels of extracellular matrix proteins as a result of varying forms and degrees of mechanical stimulation (Sah *et al.*, 1992). Those authors, in an investigation on chondrocytes isolated from human and bovine articular cartilage cultured on flexible dishes and exposed to mechanical stimulation by the flexercell technique, found that stretching the chondrocytes by 5, 7 and 10 per cent resulted in an increase in the surface area, with the largest response produced with the 10 per cent stretch, clearly demonstrating that the degree of stretching influences chondrocyte metabolism. Similarly, in the present study, the varying degrees of mandibular advancement resulted in different levels of expression of VEGF. The 3.5 mm advancement produced significantly more VEGF and subsequently more bone during the first 30 days of advancement than the initial step (2 mm) of the stepwise group (Figure 1). Thus, the smaller amount of vascular tissue invasion observed in the initial advancement in the stepwise group compared with the single-step group was possibly due to the fact that with stepwise advancement the threshold value to elicit an angiogenic response had not yet been reached. Although a 2 mm advancement is substantial when considering the length of the mandible, the anatomy of the rat displays a less prominent mandibular fossa with only an articular eminence. Moreover, the posterior attachment of its fibrous capsule is also lax to allow for extensive mandibular movement (Luder, 1996). Thus, greater advancement may be needed to solicit a greater angiogenic response.

The second-step advancement in the stepwise group produced significantly more VEGF and more bone formation during the later days when compared with the single advancement (Figures 3a–c). This can be explained based on the results of the present study and a previous investigation (Rabie *et al.*, 2002). Mandibular advancement led to an increase in VEGF by the chondrocytes, which is the regulator of the process of recruiting new blood vessels into the hypertrophic cartilage matrix of the condyle (Figures 1 and 3). The newly recruited blood vessels deliver mesenchymal cells required to replenish the population size of the osteoprogenitor cells needed for differentiation into bone-making cells in order to replace the hypertrophic cartilage matrix in the condyle with bone. The invasion of new blood vessels into the hypertrophic cartilage matrix marks the onset of endochondral ossification. This cycle of tissue responses was obviously re-triggered by the second advancement on day 30, which led to an increase in VEGF expression from days 37 to 60 (Figure 1). This is why the level of VEGF expression continued to significantly increase with the second advancement while VEGF in the single advancement group dropped to a level closer to that expressed during natural growth (Figure 1). When the pattern of VEGF was correlated with that of bone formation, a second cycle of increase in bone formation was also seen after day 44 in the stepwise group, which led to an increase in bone formation a few days later. This differed from the single advancement, where the amount of bone formation showed no significant difference when compared with the controls (Figure 2).

Moreover, the increase in VEGF expression in the single advancement group which also preceded that of bone formation further supports the hypothesis that the cascade of events leading to a greater amount of bone formation is triggered by the increase in VEGF after mandibular advancement.

Conclusions

Stepwise advancement of the mandible triggers a repeated cycle of events that leads to an increase in vascularization and subsequently to an increase in bone formation in the mandibular condyle.

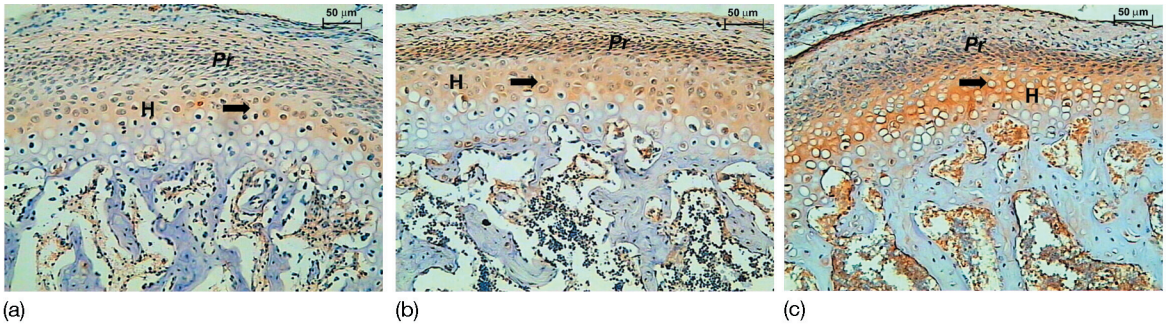

Figure 3 Photomicrographs showing positive immunostaining of vascular endothelial growth factor expression (→) in the posterior region of the mandibular condyle on day 60. Pr, proliferative zone; H, hypertrophic zone. Magnification ×360. (a) Natural growth. (b) Single-step advancement. (c) Stepwise advancement. Scale bar = 50 μm.

Changes in the amplitude of mechanical loading, produced by stepwise advancement, have a significant effect on the production of VEGF by chondrocytes. The later stages of advancement produce more VEGF and subsequently more bone formation in the condyle.

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