Original Article

Factors Regulating Endochondral Ossification in the Spheno-occipital Synchondrosis

Wai Yip Lei[a]; Ricky W. K. Wong[b]; A.B.M. Rabie[c]

ABSTRACT

Objectives: To identify the temporal pattern of core-binding factor α1 (Cbfa1) and vascular endothelial growth factor (VEGF) expressions in the spheno-occipital synchondrosis in vitro with and without tensile stress.

Materials and Methods: Sixty male BALB/c mice were randomly divided into an experimental group (with tensile stress) and a control group (without tensile stress) at each of five time points. Animals were sacrificed and the cranial base synchondroses were aseptically removed. In the experimental groups, mechanical stress was applied on the surgical explants with helical springs and incubated as organ culture for 6, 24, 48, 72, and 168 hours. In the control group, the springs were kept at zero stress. Tissue sections were subjected to immunohistochemical staining for quantitative analysis of Cbfa1 and VEGF expression.

Results: Quantitative analysis revealed that Cbfa1 and VEGF expressions reached a peak increase at 24 and 48 hours, respectively. Compared with the control groups, both Cbfa1 and VEGF were expressed consistently higher in the experimental groups at all time points.

Conclusion: Mechanical stress applied to the spheno-occipital synchondrosis elicits Cbfa1 expression and subsequently up-regulates the expression of VEGF. Increased levels of expression of both factors could play a role in the growth of the spheno-occipital synchondrosis.

KEY WORDS: Spheno-occipital synchondrosis; Cbfa1; VEGF; Endochondral ossification; Organ culture

INTRODUCTION

The spheno-occipital synchondrosis is an important growth center of the craniofacial skeleton. It is an important link between development of the cranial vault and that of the facial skeleton and is influential on the positions of the maxilla and mandible.[1] Normal development of the cranial base requires the coordinated growth and maturation of multiple skeletal elements. The embryonic cranial base, which is fully cartilaginous, is formed by the fusion of the parachordal plates around the notochord. During the postnatal period, endochondral ossification of the synchondrosis contributes largely to the expansion of the ossification centers and growth of the cranial base.[2] Any disruption in the growth of this synchondrosis can lead to craniofacial malformations such as achondroplasia,[3] Apert's syndrome,[4,5] and cleidocranial dysplasia.[6,7] Chondrocyte proliferation is enhanced by mechanical stresses, and the chondral growth is up-regulated by mechanical signals beyond naturally occurring chondrogenesis.[8,9]

Endochondral ossification plays a major role in bone formation that occurs as chondrocytes undergo proliferation, hypertrophy, cell death, and osteoblastic replacement; which could be traced by the expression of stage specific markers.[10]

Core-binding factor α1 (Cbfa1/Runx2), is a key transcription factor associated with chondrocyte maturation and osteoblast differentiation.[11] Endochondral ossification is accompanied by expansion of proliferating and hypertrophic chondrocytes within the cartilaginous growth plate. Cbfa1 appears to regulate endochondral ossification by controlling chondrocyte maturation and

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osteoblast differentiation.[11] Hypertrophic chondrocytes direct the mineralization of their surrounding matrix by inducing angiogenesis through the production of vascular endothelial growth factor (VEGF).[12] VEGF is a regulator of vascularization, and its maximum level of expression precedes the maximum level of new bone formation in the condyle.[13] Both Cbfa1 and VEGF are important factors for endochondral ossification. Rabie et al[11,14] reported that Cbfa1 and VEGF are critical for endochondral ossification of the condylar cartilage.

However, the factors governing the growth of the synchondrosis are yet to be elucidated. Whether the growth of synchondrosis can be affected by mechanical stress is still unknown. Therefore, we decided to examine the temporal pattern of expression of Cbfa1 and VEGF and to correlate their expression to cellular events and our understanding of the growth of the synchondrosis. This study was designed to identify the temporal pattern of Cbfa1 and VEGF expressions in the spheno-occipital synchondrosis with and without tensile stress.

MATERIALS AND METHODS

This experiment was approved by the Committee on the Use of Live Animal in Teaching and Research of the University of Hong Kong (CULTAR 1106-05). Sixty 1-day-old male BALB/c mice were randomly divided into experimental and control groups. Each group was subdivided into five subgroups of different time frames—6, 24, 48, 72, and 168 hours. Each subgroup consisted of six mice.

Surgical explants of cranial base

Methods of animal sacrifice were performed in accordance with the guidelines for the euthanasia of rodent feti and neonates from the Office of Animal Care and Use of the U.S. Department of Health and Human Services and the Laboratory of Animal Unit, The University of Hong Kong. The cranial base synchondroses were aseptically removed and incubated in a 24-well plate with or without mechanical stress in organ culture as described below.

Mechanical stress device

In the experimental groups, tensile stress was applied across the spheno-occipital synchondrosis using a helical spring made of Elgiloy orthodontic wire (Elgiloy 0.01 inch, semiresilient, Rocky Mountain, Denver, CO) according to the methods of Hickory and Nanda,[15] and Ikegame et al.[16] The magnitude of each helical spring was set at 0.2 g tensile strength. The arms of the spring were set 5 mm apart. In the control groups, springs were maintained at a 5 mm distance by means of adhesive tape to give 0 g tensile strength.[16]

Organ culture

The serum-free protocol was modified from Shum et al[2] using BGJb medium (Gibco, Invitrogen, New York, NY) supplemented with 2 mg/mL bovine serum albumin (BSA; A-9647, Sigma, St Louis, MO), 100 μg/mL sodium ascorbate (A-4034, Sigma), 1 mM beta-glycerophosphate (Fluka 50020, Buchs, Switzerland), antibiotics and antimycotics (Gibco, Invitrogen, New York, NY). The culture medium was maintained at 37°C and 5% CO_2 in air and changed daily.

Tissue preparation and immunohistochemistry

Tissue preparation and sectioning were performed using the method described by Rabie et al.[17] Immunolocalization of the factors Cbfa1 and VEGF were performed to localize their expression in the spheno-occipital synchondrosis after incubation in medium culture with different time intervals. Goat polyclonal PEBP2αA antibody IgG (sc-8566, Santa Cruz Biotechnology, Santa Cruz, CA) that was raised against a peptide mapping at the C-terminus of PEBP2αA was used for immunolocalization of Cbfa1, and goat polyclonal antibody IgG (sc-152, Santa Cruz Biotechnology) that was raised against a peptide mapping at the N-terminus of VEGF-A was used for the detection of VEGF. The immunohistochemical staining procedures of Cbfa1 and VEGF were performed after the standard three-step avidin-biotin complex method,[18] which was discussed in details by Rabie et al.[19]

Quantitative Analysis

The sections were evaluated at a magnification of ×20 (Leitz Orthoplan, Wuerttemberg, Germany) with software (Qwin, Leica, Cambridge, UK). To localize the expression, Cbfa1 positive stained cells were determined. Cells with at least 80 pixels of positive staining were regarded as positive. The level of VEGF expression was evaluated as the percentage of positive staining area in the measurement frame. The expressions were measured using a true-color RGB (red-green-blue) computer-assisted image-analyzing system (Q5501W; Leica Microsystems Imaging Solutions, Cambridge, UK) with Leica Qwin software Pro (version 2.6).

For each subject, five sections were prepared for quantification. Twenty sections for each group were randomly chosen, and the measurements were used for statistical analysis. In total, 400 sections were measured and quantified. The amount of staining for all sections was quantified and evaluated by one exam-

iner. The difference between experimental and control groups was tested by paired *t* test and one-way analysis of variance using SPSS statistical analysis softward (version 13.0 for Windows, SPSS, Chicago, IL).

Method error

The measurement error (Me) was calculated using Dahlberg's formula[20]: $Me = \sqrt{(\Sigma\ d^2)/2n}$, where *d* is the difference between the two registrations of a pair and *n* is the number of double registration. Ten randomly selected histologic sections were digitized on two separate occasions. The size of the method error was 0.00518. A paired *t*-test was also performed to compare the two registrations. There was no significant difference ($P = .2662$) among the duplicate registrations of the 10 randomly drawn sections.

RESULTS

Immunostaining for Cbfa1 expression

In the control group, the spheno-occipital synchondrosis was put under zero tensile stress, the level of Cbfa1 expression increased progressively from 6 to 72 hours, reaching a peak at 72 hours, after which the expression declined gradually until 168 hours. The level of expression of Cbfa1 increased 30% at 48 hours and a further 8% upon reaching a peak at 72 hours.

In the experimental groups, tensile stress applied across the spheno-occipital synchondrosis led to a statistically significant increase of Cbfa1 expression. The increase was most remarkable after 24 hours ($P < .001$), showing a 30% increase, and it continued to increase further at 48 hours onwards up until 168 hours. When under tensile stress, the level of Cbfa1 expression is consistently higher compared with that of the control group throughout the same time frame. The patterns of expression of Cbfa1 in both groups were similar, and tensile stress intensified the expression in the experimental groups (Table 1).

Immunostaining for VEGF expression

VEGF expression in the control group, as shown by immunohistochemical staining, in the spheno-occipital synchondrosis under no tensile stress, showed a minimal increase within the first 24 hours. A more gradual increase (12.7%) was detected at 48 hours up to 72 hours (7.7% increase). Thereafter, the increase remained at this elevated level until 168 hours.

For the experimental groups, under tensile stress, quantitative analysis showed a progressive increase throughout the whole experimental period, and a statistically significant increase (21.5%) was detected at the 48 hours ($P < .001$). This showed that the tensile stress induced the expression of VEGF. In addition, the level of VEGF expression was noted to be higher in the experimental groups than in the control groups (Table 2).

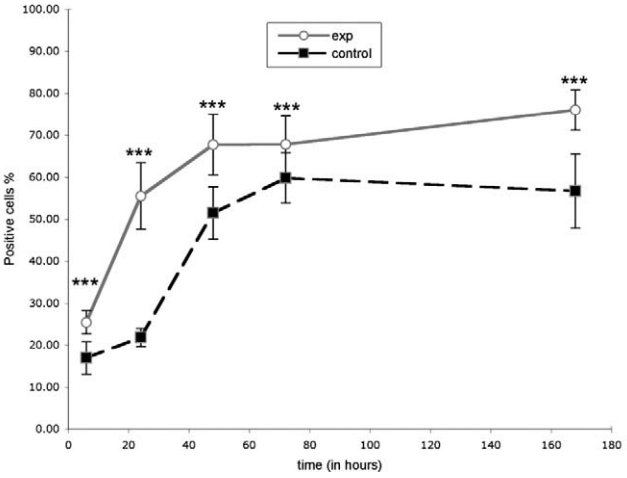

Figure 1. The temporal pattern of Cbfa1 expression with and without mechanical stress (***$P < .001$).

DISCUSSION

To our knowledge, this is the first study to investigate the growth of spheno-occipital synchondrosis in an organ culture model. The inaccessibility of the spheno-occipital synchondrosis made in vivo investigation difficult. Results of the present study showed that tensile stress triggers cartilage synthesis and increased vascularization in the spheno-occipital synchondroses in vitro (Figures 1 and 2). Eventually, this contributed to osteogenesis and induced the growth of the synchondrosis. Our research provides evidence that the spheno-occipital synchondrosis from a neonatal animal expressed the Cbfa1 gene. The results further suggest that chondrocyte differentiation seemed to be accompanied by an increase in Cbfa1 expression in the cultured spheno-occipital synchondrosis (Figures 1 and 3), which points to a role of Cbfa1 in cartilage formation in the synchondrosis.

Cbfa1 regulates chondrocyte hypertrophy,[11] which is directly related to chondrocyte functional maturation and terminal differentiation in the mandibular condyle. Rabie et al[11] demonstrated that Cbfa1 was strongly expressed in hypertrophic chondrocytes and osteoblasts during natural growth of the mandibular con-

Table 1. Summary of the temporal pattern of levels of Cbfa1 expression (positive cells %)

	Time				
	6 h	24 h	48 h	72 h	168 h
Control group	0.170	0.219	0.515	0.598	0.557
Experimental group	0.255	0.555	0.678	0.678	0.76

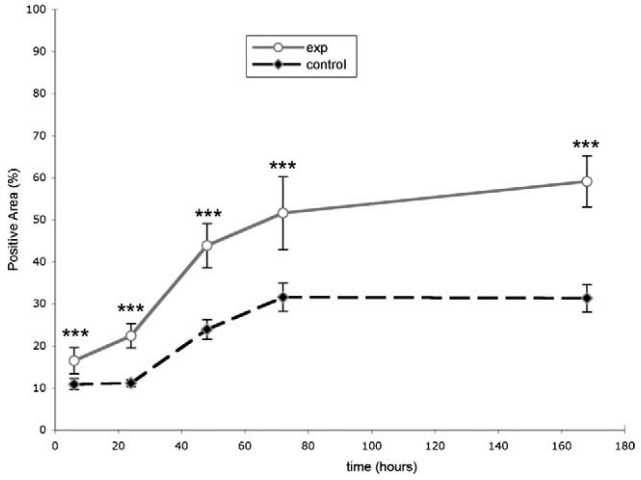

Figure 2. The temporal pattern of VEGF expression with and without mechanical stress (***$P < .001$).

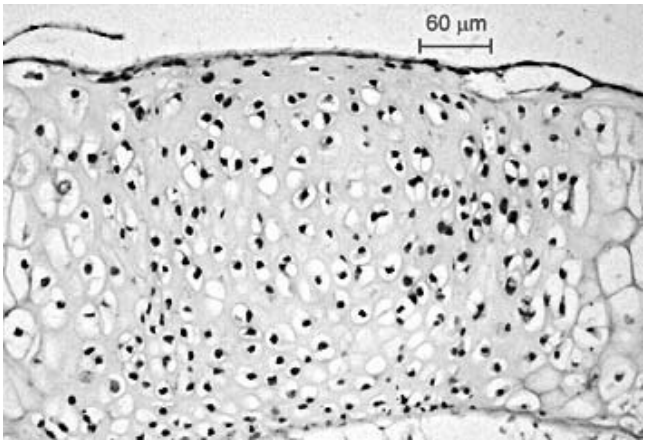

Figure 3. Immunostaining with expression of Cbfa1 gene in mouse spheno-occipital synchondrosis, experimental, 24 hours, ×20.

dyle. Cbfa1-deficient mice showed chondrocyte maturational disturbance and the absence of hypertrophic chondrocytes.[21–25] Overexpression of Cbfa1 caused acceleration of endochondral ossification because of precocious chondrocyte maturation.[26,27] Considering the present data in light of the functional analysis of Cbfa1, it is clear that the amount of Cbfa1 would affect cartilage formation in the synchondrosis. Furthermore, Cbfa1 could have a direct role in endochondral ossification of the synchondrosis.

Cbfa1 is involved in extracelluar matrix mineralization during endochondral ossification in the mandibular condyle, regulating the transcription of bone sialoprotein and osteopontin.[11,21,23] Studies have shown that Cbfa1 stimulates matrix calcification in vitro[25] and that Cbfa1-deficient animals show limited calcification in cartilage in vivo.[23]

Cbfa1 is an absolute requirement for osteoblast differentiation and function by transcriptionally up-regulating all the major osteoblast-specific genes[28–33] during endochondral bone formation of the mandibular condyle.[11] The formation of osteoblasts and osteoclastogenesis during endochondral ossification was blocked in Cbfa1 mutant mice and recovered when using transgene to restore its expression.[21,22,26,34] Cbfa1-deficient mice completely lacked both intramembranous and endochondral ossification. In addition, the expression of PTH/PTHrP receptor, Indian hedgehog, type X collagen, and BMP6 was also not detected in the Cbfa1-deficient mice.[23] Moreover, Cbfa1 also regulates osteoclast differentiation and function.[35,36] Therefore, Cbfa1 could be a coupling agent of chondrocyte maturation and endochondral ossification of the spheno-occipital synchondrosis.

To further elucidate the factors that regulate endochondral ossification of the spheno-occipital synchondrosis, we examined the temporal pattern of expression of VEGF during induced growth of the synchondrosis.

During endochondral ossification, an invasion of blood vessels into cartilage was associated with up-regulation of VEGF in hypertrophic chondroctyes and increased expression of VEGF receptors in the perichondrium. Cbfa1 has been stated to up-regulate VEGF expression.[37] In Cbfa1-deficient mice, up-regulation of VEGF is lacking, and cartilage angiogenesis does not occur.[37] Overexpression of Cbfa1 induces an increase in VEGF mRNA level and protein production by stimulating VEGF transcription.[37] Rabie et al[12] have shown that VEGF expression causes subsequent neovascularization that is required to trigger the onset of ossification in the hypertrophic condyle cartilage. The invading new blood vessels recruit osteoprogenitor mesenchymal cells in the perivascular sites for later osteoblast differentiation to form bone in the growing condyle.[12] Inactivation of VEGF resulted in suppressed blood vessel invasion, impaired trabecular bone formation, and expanded hypertrophic chondrocyte zone in the growth plate of growing mice.[38] Inactivation of VEGF also suppresses the resorption of cartilage matrix through restraining the recruitment and differentiation of osteoclasts. Cessation of inactivation resulted in the recovery of capillary invasion, resorption of hypertrophic cartilage, and the normal architectural properties of the growth plate.[38]

The present study demonstrated that tensile stress

Table 2. Summary of the temporal pattern of levels of VEGF expression (area %)

	Time				
	6 h	24 h	48 h	72 h	168 h
Control group	0.1091	0.1116	0.2392	0.3157	0.3131
Experimental group	0.1648	0.2239	0.4385	0.5159	0.591

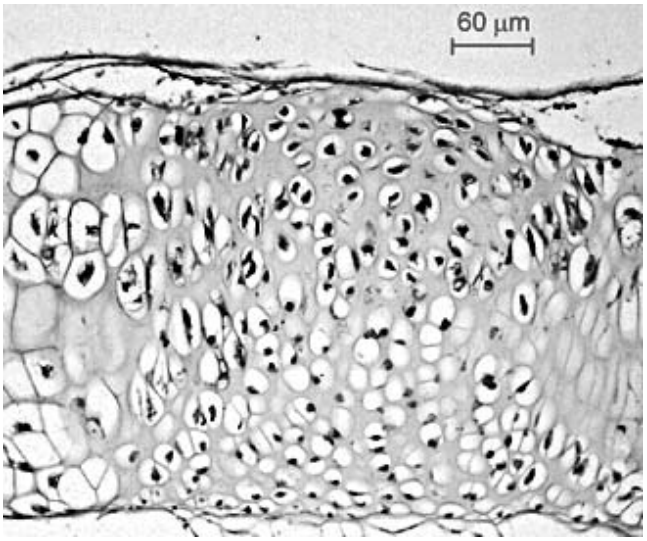

Figure 4. Immunostaining with expression of VEGF gene in mouse spheno-occipital synchondrosis, experimental, 48 hours, ×20.

solicited cellular responses that led to a significant increase in VEGF expression in the spheno-occipital synchondrosis compared with the control group (Figure 4). Rabie et al[13] reported similar findings that mechanical stress applied in the condyle solicits an increase in the expression of VEGF, the key regulator of neovascularization. The peak increase of VEGF expression was noted at 48 hours (Figure 2) in the spheno-occipital synchondrosis. This meant that the invasion of new blood vessels, with their perivascular sites rich in mesenchymal cells, contributes to a significant increase in the number of skeletal progenitors in the erosive zone. The increase in Cbfa1 expression triggered by mechanical stimulation could induce mesenchymal cell differentiation into osteoblasts, while the hematopoietic cells differentiated into chondroclasts. Thus, the increase in the recruitment of osteoblasts and chondroclasts directly contributed to the removal of cartilage and the deposition of bony tissues, leading to enhanced endochondral ossification in the spheno-occipital synchondrosis.

CONCLUSION

- Mechanical stress applied to the spheno-occipital synchondrosis elicits Cbfa1 expression and subsequently up-regulates the expression of VEGF. Increased levels of expression of both factors could play a role in the growth of the spheno-occipital synchondrosis.

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