SHS Web of Conferences **30**, 00008 (2016) DOI: 10.1051/shsconf/20163000008
Int. Conf. SOCIETY. HEALTH. WELFARE. 2014

Osteopontin, osteocalcin, and osteoprotegerin expression in human tissue affected by cleft lip and palate

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Abstract. Cleft lip and palate (CLP) is a common congenital anomaly with a complex etiology which has not been elucidated yet. This study investigated whether expression of osteopontin (OPN), osteoprotegerin (OPG), and osteocalcin (OC), which are essential for the normal craniofacial bone remodelling, is not regulated in children with CLP. Alveolar bone tissue samples were obtained from patients with complete bilateral (CB) CLP ($n = 14$) during corrective plastic surgery and unaffected control subjects ($n = 9$). OPN, OPG, and OC expression was assessed by immunohistochemistry, and data were analyzed with the Mann-Whitney test. OPN expression was observed only sporadically in the alveolar bone of 3 patients, in contrast to the control group ($z = -2.962$; $P < 0.003$). The number of OPG-positive bone cells varied from occasional to moderate, in contrast to the control group ($z = -2.247$; $P = 0.025$). OC-positive osteocytes were present in moderate to numerous numbers in both patients and controls, with no significant difference between them ($z = -1.356$; $P < 0.175$). The prominent expression of OC characteristic for CBCLP affected hard tissue indicates a high potential of bone mineralization. Few OPG-positive osteocytes in the bone tissue implicate the disregulation of osteoclast differentiation, maturation, and activity, but few OPN-containing cells may prove the common disregulation of bone remodelling during cleft morphopathogenesis.

Introduction

In humans, cleft lip and palate (CLP) is the fourth most common congenital malformation affecting approximately 1 per 600 newborns in European populations and causing significant medical, psychological, and social ramifications. CLP is characterized by the incomplete formation of structures separating the nasal and oral cavities including the lip, alveolus, and soft and hard palates, with the most severe clefts extending completely through both soft tissue and bone. Cleft lip and palate treatment is a complex procedure that may include multiple plastic surgical corrections, which may result in the formation of scar tissue that adversely affects the growth and development of facial and oral cavity tissues [1]. It has been reported that CLP in humans results in abnormal function and growth of the maxilla [2]. Following surgery, patients are left with acute wounds that will need to heal to allow restoration of function. The wound healing involves regeneration, cellular activity changes, and remodelling. Non-collagenous proteins play a major role in angiogenesis and osteoclastic bone remodelling, two vital processes for normal bone healing [3].

Bone is a highly dynamic tissue and an important site of continuous tissue remodelling during development, homeostasis, tissue remodelling and repair. Bone remodelling is a cyclic and continuous physiological process and depends on the tightly integrated activity of two cell types, the osteoblasts, which form bone, and the osteoclasts, which resorb bone. In addition, bone cells play an important role in bone homeostasis during wound healing [4].

Osteopontin (OPN) is a highly phosphorylated sialoprotein that is a prominent component of the mineralized extracellular matrix of bone. OPN is expressed by various human cell types in a variety of tissues, including bone, dentin, cement, cartilage, kidney, brain, vascular tissue, epithelial tissue [5]. In bone, OPN is produced by differentiated osteoblasts and osteocytes, and also by osteoclasts. It appears to be an important component of the communication between these cells and there is strong evidence for the involvement of OPN in the formation, migration, and attachment of osteoclasts and for their resorptive activity [6]. Bone cells secrete OPN physiologically during the process of bone remodelling. There is evidence suggesting that OPN acts as a pro-inflammatory cytokine and plays an important role in regulating inflammation process [7]. It has been shown that the OPN is expressed in selected tissues during embryonic development. Moreover, OPN may certainly play a role in the collagen deposition that is crucial for normal morphogenesis of the secondary palate [8].

Osteoprotegerin (OPG) is the main osteoclastogenesis modulator. It is a member of tumour necrosis factor receptor superfamily also known as osteoclast inhibitor factor produced by a variety of cells including osteoblasts, osteocytes, endothelial cells, vascular smooth muscle cells, fibroblasts as well as hematopoietic and immune cells. The cytokine OPG protects the skeleton by binding to RANKL and preventing it from interacting with RANK. It prevents the proliferation and differentiation of osteoclasts that increases bone density and volume [9]. The evidence showing the importance of OPG in bone biology was provided by studies based on genetic experiments [10–12].

Osteocalcin (OC) or bone Gla-protein is the most abundant osteoblast-specific non-collagenous protein. OC is predominantly synthesized by mature osteoblasts, osteocytes, cementoblasts, odontoblasts and chondrocytes. It has an important role in both bone resorption and mineralization. While OC is detected of high concentration in the bone extracellular matrix, it also possesses several characteristic of a hormone [13].

To date, the physiological significance of non-collagenous proteins in CBCLP affected alveolar bone is still unknown. This study investigated the expression of OPN, OPG, and OC in complete bilateral (CB) CLP to identify possible changes in signalling pathways that could lead to the aberrant maxillofacial bone remodelling observed in CLP.

Materials and methods

Study population

Samples were collected from the Cleft Lip and Palate Centre at the Institute of Stomatology of Riga Stradins University from 14 patients with CBCLP and 9 unaffected control subjects. The patient group comprised 9 males and 5 females. Samples of hard tissue were collected during osteoplasty from children ranging in age from 6 years 4 months to 15 years and 4 months, or upon tooth extraction from control subjects ranging in age from 6 years to 9 years. The control group comprised 4 males and 5 females. This study was independently reviewed and approved by the local Ethical Committee of the Riga Stradins University, and written informed consent was obtained from all parents after the nature of the study had been fully explained.

Tissue sample preparation

For conventional light microscopy and immunohistochemistry, the tissue specimens were fixed for 1 day in a mixture of 2% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.2). They were then rinsed in thyroid buffer containing 10% saccharose for 12 h and embedded in paraffin. Each block was cut into 4-μm sections that were mounted on glass slides, deparaffinized, rehydrated through a graded alcohol series, and stained with hematoxylin and eosin.

Immunohistochemistry

Tissue sections were labelled with the following primary antibodies: mouse anti-OPN at 1:100 (sc-73631; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-OC at 1:50 (sc-30044; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and goat anti-OPG at 1:50 (sc-8468; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The signal was visualized by biotin-streptavidin immunohistochemistry [14]. Images were captured using a DC 300F camera (Leica, Wetzlar, Germany) and the image processing and analysis software Image Pro Plus v.6.0 (Media Cybernetics, Silver Spring, MD, USA). The intensity of immunostaining was graded semi-quantitatively on a scale of 0 to ++++, as follows: 0, no positive structures; 0/+, occasional positive structures; +, few immunoreactive structures; ++, a moderate number of immunoreactive structures; +++, numerous immunoreactive structures; and ++++, an abundance of immunoreactive structures in the visual field [15]. The localization of staining was assessed at both tissue and cellular levels.

Statistical analysis

Statistical analyses were performed using SPSS v.20.0 (IBM Corp., Armonk, NY, USA). Results are expressed as mean ± SD. A Mann-Whitney test was used to compare groups and a P value < 0.05 was considered statistically significant.

Results

OPN-positive structures were detected in 3 CBCLP cases, and the presence of OPN-positive osteocytes was occasional (Fig. 1). OPN immunoreactivity was observed in osteoblasts and osteocytes. In all control specimens, few OPN-positive cells were seen (Fig. 2). The total number of OPN-positive the bone forming cells was significantly lower in the CBCLP than in the control group ($z = -2.962$; $P < 0.003$) (Fig. 3).

OPG-positive structures were present in all hard tissue samples, and immunoreactivity was detected in osteoblasts and osteocytes (Fig. 4). The number of OPG-positive bone cells varied from occasional to moderate in all CBCLP cases. In controls, the number of OPG-positive bone cells varied from few to numerous. There was significant difference between the groups in the mean numbers of OPG-positive osteocytes ($z = -2.247$; $P = 0.025$) (Fig. 5).

Of the 19 CBCLP specimens, all were positive for OC, although the signal was detected in a moderate to numerous cells in both patients and controls, with no significant difference between them ($z = -1.356$; $P < 0.175$) (Fig. 6). Moderate to numerous OC-positive osteocytes were present in CBCLP specimens (Fig. 7), and there was no immunoreactivity present in other structures of the alveolar bone.

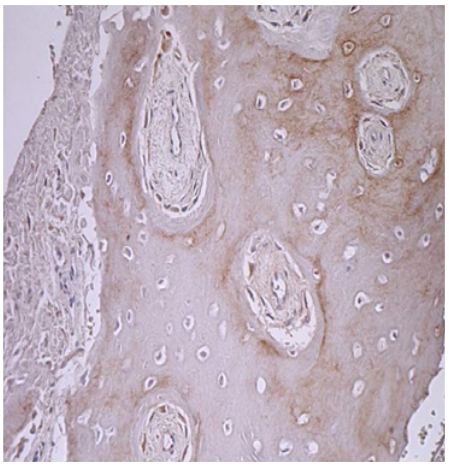

Figure 1. Occasional OPN-possitive osteocytes from the alveolar bone tissue of a 6-year-old child with CBCLP. OPN IMH, X 200.

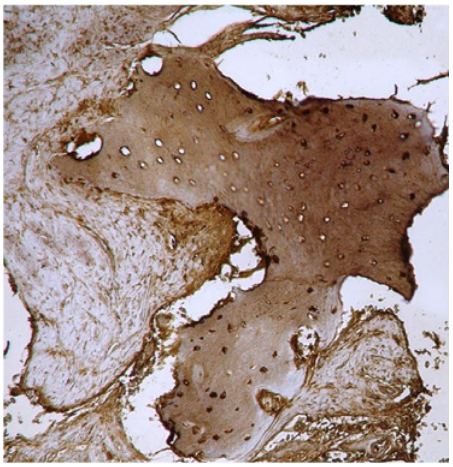

Figure 2. Abundant OPN-positive osteocytes in control group specimen. OPN IMH, X 100.

Discussion

Non-collagenous proteins play a key role in different physiological and pathological conditions, including embryonic development, wound healing, inflammation, tumour growth, calcification, and bone remodelling. The alveolar bone undergoes constant remodelling process [16]. Various molecules contribute to the tissue regeneration associated with acute bone wound healing, including OPN, OPG and OC molecules. Such molecules can be identified to allow analysis of bone remodelling, and can be useful to determine the rate or outcome of states of bone remodelling and therefore signal the success of surgical or pharmaceutical interventions [17].

It was demonstrated that OPN has a role in cell adhesion, migration, cell survival and bone remodelling [18–20]. Research in animal models has indicated that OPN deficiency alters the functionality of multiple cell types, resulting in delayed early vascularization, altered matrix organization and late bone remodelling [21]. Furthermore, OPN has been emphasized

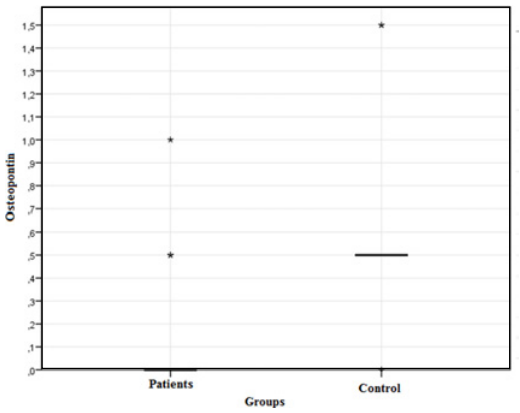

Figure 3. Comparison of OPN expression between study groups. Statistically significant differences were observed.

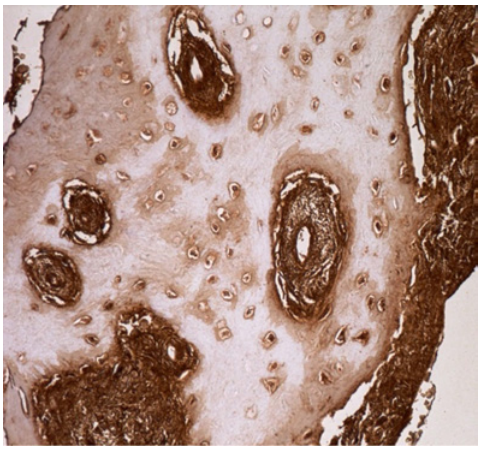

Figure 4. Numerous OPG-possitive osteocytes in the hard tissue of a 8-year-old child with CBCLP. OPG IMH, X 200.

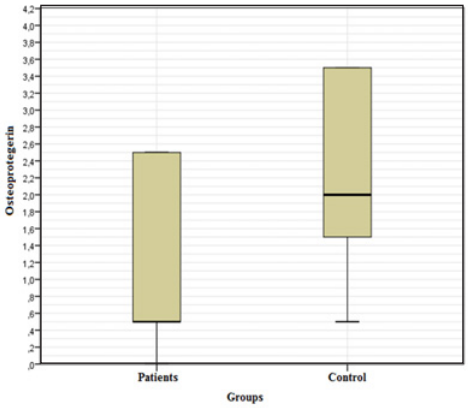

Figure 5. Comparison of OPG expression between study groups. Statistically significant differences were observed.

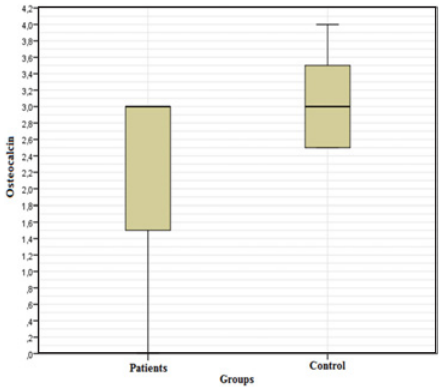

Figure 6. Comparison of OC expression between study groups. No significant differences between groups were observed.

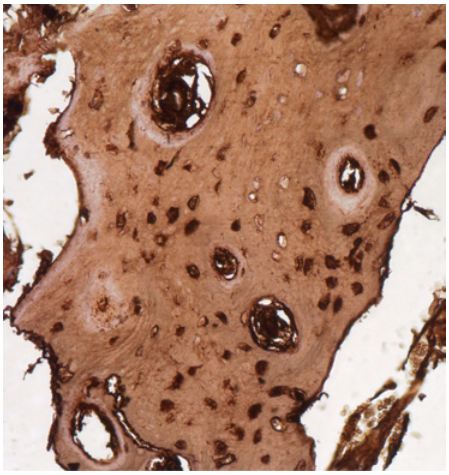

Figure 7. Abundant OC-positive osteocytes from cleft region of a 6-year-old child. OC IMH, X 200.

as important for palate formation in recent study on foetal murine palates, which showed significant changes in expression of OPN during craniofacial development [22]. Jakobsen et al. by Gene chip analysis and staining with selected antibodies in human embryonic palates found supportive evidence that OPN may play in the development of the normal palate [23]. OPN-positive cell distribution varied from occasional to few positive structures in the patients examined, and OPN expression in the alveolar bone was lower in the CBCLP compared to the control group. These our results suggest an involvement of *OPN* in decreased cell mobilization, protection, survival, resistance to apoptosis, and/or cell migration during alveolar bone remodelling.

OPG is a powerful inhibitor of bone remodeling. It was previously demonstrated that OPG knockout mice show severe osteoporosis, whereas overexpression of OPG causes osteopetrosis [24]. Research in animal models has indicated that local delivery of OPG inhibits bone modelling in tooth movement and during maxillary expansion [25, 26]. While OPG is presumed to have a role in maxilla tissue remodelling, there has been no evidence in support of this possibility in non-syndromic CBCLP affected hard tissue. The present

study targeted the localization of OPG in the CLP affected alveolar bone. During the light microscopic examination of immunohistological sections of the alveolar bone sections, there were fewer OPG positive bone cells than unaffected individuals.

OC has been implicated in the mineralization of embryonic and adult bone [27], but the changes in OC expression or function in CBCLP affected alveolar bone have not yet been defined. Its absence leads to increase bone formation with bones of improved functional quality without impairing bone resorption [28]. Furthermore, Cantatore et al. (2005) have reported that osteocalcin stimulates angiogenesis and may play a role in bone remodeling [29]. An immunohistochemistry study has suggested a role of OC during alveolar bone healing process [30]. However, it is not known how the bone protein OC appear in the CLP affected alveolar bone tissue. In the present study, OC expression was analogical in CBCLP tissue compared to control tissue, providing evidence that extracellular matrix mineralization is well regulated locally by OC in CBCLP patients.

Conclusions

The prominent expression of OC characteristic for CBCLP affected hard tissue indicates a high potential of bone mineralization. Few OPG-positive osteocytes in the bone tissue implicate the disregulation of osteoclast differentiation, maturation, and activity, but few OPN-containing cells may prove the common disregulation of bone remodelling during cleft morphopathogenesis.

We acknowledge D.D.S., MSc (Oslo), Ph.D., prof. Ilze Akota from Institute of Stomatology, Riga Stradins University, Latvia for supplying of patient material. This study was funded by the Support for Doctoral and Post-doctoral Investigations Riga Stradins University fellowship (Nr.2009/0147/1DP/1.1.2.1.2/09/IPIA/VIAA/009) and RSU project "Longitudinal research on cleft morphopathogenesis".

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