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LOCAL EXPRESSION OF INFLAMMATORY CYTOKINES IN THE FACIAL TISSUE OF CHILDREN WITH A CLEFT LIP AND PALATE

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ABSTRACT

The cleft lip and/or palate are among the most common congenital anomalies that occur in early development. Cytokines play an important role in the proliferation, growth, differentiation, survival and the functional activity of many cells and the upregulation of cytokines might be involved in the pathological processes of the maxillofacial region. The purpose of our study was to evaluate the expression of pro-inflammatory and anti-inflammatory cytokines in the facial tissue of children with the cleft lip and palate.

The study involved 14 patients with the unilateral cleft lip and palate at the age of three months to 10 years and five months. Soft and hard palate tissue samples were collected during the primary cleft operation if the parents had given their informed written consent. All the tissue samples were stained with hematoxylin and eosin and by immunohistochemistry for IL-1α, IL-6, IL-8, IL-10 and TNF-α. The intensity of immunostaining was graded semiquantitatively.

IL-1α containing structures were not detected in any of soft, cartilage and bone tissue samples, meanwhile IL-6, IL-8, IL-10 and TNF-α showed explicit expression. The expression of IL 6 was observed in the tissues of all the patients. IL-6 positive cells were found in the range from no positive to moderate of positive structures in the visual field. IL-8 positive osteocytes were found in the range from few positive to moderate, but IL-8 positive chondrocytes were found in abundance. IL-10 was richly secreted by osteocytes in bone and by chondrocytes in cartilage obtained from all the cases of plastic surgery. In all the cases we also observed numerous IL-10 positive gingival epithelial cells.

Despite these data obtained, moderate to numerous macrophages and neutrophils expressed TNF-α.

The sporadical and scarce expression of IL-6 indicates its insignificant role in the cleft lip and palate affected tissue. Rich cytokine IL-10 expression proves the compensated local anti-inflammatory effects of the cleft affected soft and hard tissue.

Key words: *cytokines, cleft lip and palate, human.*

INTRODUCTION

The morphogenetic cleft lip and/or palate (CL/P) affected tissue study has become very significant nowadays. CL/P is among the most common congenital anomaly that arise in the early development and affecting approximately 2 per 1,000 newborns worldwide [1, 2]. Abnormal facial tissue development during gestation is caused by multiple genetic and environmental factors and this may result in local changes in growth factors, the extracellular matrix, cell adhesion molecules and cytokines [1, 3].

Cytokines are pleiotropic peptides, which are involved in numerous biological processes such as in the proliferation, growth, differentiation, survival and functional activity of many cells [4]. There are data about the cytokines role in the embryo growth and differentiation, as well as inflammation and the tissue remodeling [5, 6]. Traditionally, these small, nonstructural proteins are subdivided into following families: interleukins, growth factors, chemokines, colony-stimulating factors, interferons, the transforming growth factor and the tumor necrosis factor families [7]. It should be noted that interleukins and growth factors are present in skull tissues at the time of active differentiation and morphogenesis [8]. Further cytokines are polymorphic, which means that the expression can vary widely between individuals, and this may be genetically controlled [9]. A number of studies showed that the damages in different sections of immunity are significant causes of the pathological processes of maxillofacial region [10, 11]. The analyses of cytokines distribution in the orofacial region constitute the key to understanding the etiopathology of various diseases. Therefore, using an immunohistochemical method many studies reported the expression of interleukin – 1 (IL-1), interleukin – 6 (IL-6), interleukin – 8 (IL-8) and tumor necrosis factor alpha (TNF-α) in the squamous cell carcinoma of the palate tissue and diseased periodontal tissues [4, 12, 13]. Inoyatov et al. (2012) reported that the cytokine level for IL-1,

TNF, interferon-gamma in the blood serum increasing under the congenital cleft [10]. Moreover, still a little is known about the local expression of inflammatory cytokines in the facial tissue of children with the cleft lip and palate.

The aim of this study was to evaluate the expression of pro-inflammatory cytokines such as interleukin 1 alpha (IL-1α), IL-6, IL-8, TNF-α and anti-inflammatory cytokine interleukin-10 (IL-10) in the facial tissue of the children with the unilateral cleft lip and palate.

MATERIAL AND METHODS

Patients

The study involved 14 children with the unilateral cleft lip and palate at the age of three months to 10 years and five months. The samples of soft and hard palate tissue were collected during the surgical procedure from the borders of the cleft region. All the information about the patients is summarized in Table 1. This study has been independently reviewed and approved by the local Ethical Committee of Riga Stradins University (2007), and written informed consent was obtained from all the parents after the nature of the study had been fully explained.

Table 1. Information about the patients

Patient	Gender	Age	Plastic surgery procedure	Material
No. 1	F	9 years 4 months	Rhinoplasty	Bone tissue from *spina nasalis anterior maxillae*
No. 2	F	9 years 4 months	Rhinoplasty	Cartilage from *septum nasale*
No. 3	M	7 years 7 months	Rhinoplasty	Bone tissue from *spina nasalis anterior maxillae*
No. 4	M	7 years 7 months	Rhinoplasty	Cartilage from *septum nasale*
No. 5	M	10 years 5 months	Osteoplasty	Bone tissue from processus alveolaris
No. 6	F	7 years 3 months	Osteoplasty	Bone tissue from processus alveolaris
No. 7	F	8 years	Rhinoplasty	Cartilage from septum nasale

Table 1. Continuation

Patient	Gender	Age	Plastic surgery procedure	Material
No. 8	F	8 years	Rhinoplasty	Bone tissue from *spina nasalis anterior maxillae*
No. 9	M	8 years 5 months	Osteoplasty	Bone tissue from processus alveolaris
No. 10	F	7 years 10 months	Osteoplasty	Bone tissue from processus alveolaris
No. 11	M	4 months	Lip plastic	Cleft lip region
No. 12	M	4 months	Lip plastic	Cleft lip region
No. 13	M	3 months	Lip plastic	Cleft lip region
No. 14	M	4 months	Lip plastic	Cleft lip region

Methods

For conventional light microscopy and immunohistochemistry tissues were fixed for a day in the mixture of 2% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.2). Following this, they were rinsed in the thyroid buffer, containing 10% sacharose for 12 hours, and then samples were embedded into paraffin. Five micrometer thick sections were cut from each block, mounted on glass slides, then de-paraffinized, rehydrated through graded alcohol solutions and colored with hematoxylin-eosin.

Five-micrometer thick sections were cut from the same blocks and placed on polylisine coated slides for the immunohistochemical analysis. Sections were proceeded for the detection of the following interleukins: interleukin-1 alpha (IL-1α (B-7): sc-9983, obtained from the mouse, working dilution 1:50, Santa Cruz Biotechnology, Inc., USA), interleukin-6 (IL-6 (NYRhIL6): sc-73319, obtained from the mouse, working dilution 1:50, Santa Cruz Biotechnology, Inc., USA), interleukin-8 (IL-8 (C-19): sc-1269, obtained from the goat, working dilution 1:50, Santa Cruz Biotechnology, Inc., USA), interleukin-10 (IL-10, code ab 34843, obtained from the rabbit, working dilution 1:400, Abcam, Cambridge, UK), tumor necrosis factor alpha (TNF-α, code ab 6671, obtained from the rabbit, working dilution 1:100, Abcam, Cambridge, UK) by use of Hsu et al. (1981) biotin – streptavidin immunohistochemical method.

Our findings were illustrated using Leica DC 300F camera and the image processing and analysis software Image-Pro Plus Version 6.0.

The intensity of immunostaining was graded semi-quantitatively. The scale was the following: "0" – no positive structures found in the the visual field, "0/+" – occasional positive structures seen in the visual field, "+" – few immunoreactive structures seen in the visual field, "++" – moderate number of immunoreactive structures seen in the visual field, "+++" – numerous immunoreactive structures seen in the visual field, and "++++" – the abundance of immunoreactive structures seen in the visual field (Pilmane et al. 1998).

RESULTS

Routine haematoxylin and eosin slides showed patchy infiltrates with inflammatory cells vacuolization in the polymorphic epithelial cell layer.

IL-1α-containing structures were not detected in any of the soft, cartilage and bone tissue samples, meanwhile IL-6, IL-8, IL-10 and TNF-α showed explicit expression.

The expression of IL-6 was also observed in the tissues of all the patients. IL-6 positive cells were found in the range from the absence to moderate (++) of positive structures in the visual field. We observed few (+) to moderate (++) positive osteocytes (Figure 1), few (+) to moderate (++) immunoreactive hyaline cartilage cells and many positive cells in the cartilage growth zone (Figure 2) and few positive epithelial cells (Figure 3) into the gingival epithelium.

IL-8 also was seen in the tissue samples of all the patients. IL-8 positive osteocytes were found in the range from few (+) positive to moderate (++) of positive structures in the visual field (Figure 4). It should be noted that IL-8 presented the abundance of richly stained hyaline cartilage chondrocytes (Figure 5).

Interestingly, IL-10 was richly secreted by osteocytes in bone obtained from all the cases of plastic surgery, as well as by chodrocytes in the hyaline cartilage obtained from all the cases of plastic surgery. IL-10 positive structures mostly varied from numerous (+++) positive structures to abundance (++++) of positive structures in the visual field. Therein we detected many immunoreactive hyaline cartilage cells and many positive cells in the cartilage growth zone as well as many osteocytes (Figure 6). In all the cases we also observed numerous (+++) IL-10 positive gingival epithelial cells.

Finally, despite these data moderate (++) to numerous (+++) macrophages and neutrophils expressed TNF-α (Figure 7).

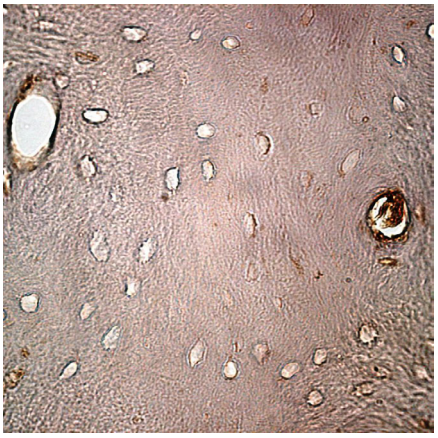

Figure 1. Few IL-6 positive osteocytes in the bone from *spina nasalis anterior maxillae* of 9 years and 4 months old child. IL-6 IMH, X 400.

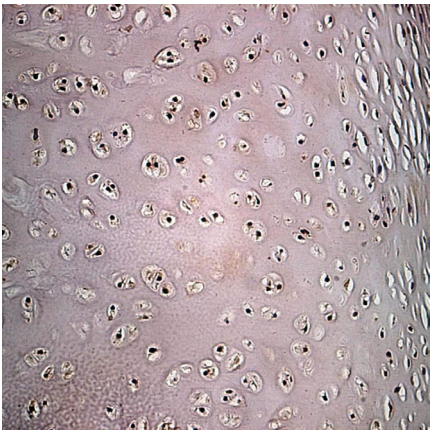

Figure 2. Moderate number of IL-6 positive chondrocytes in the mature and proliferation zone. IL-6 IMH, X 250.

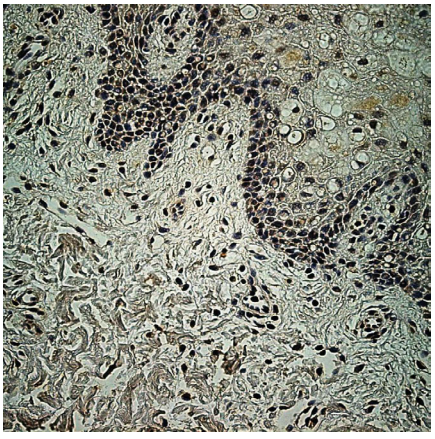

Figure 3. Few IL-1α positive epithelial cells into the gingival epithelium of 4 months old child. IL-1α IMH, X 250.

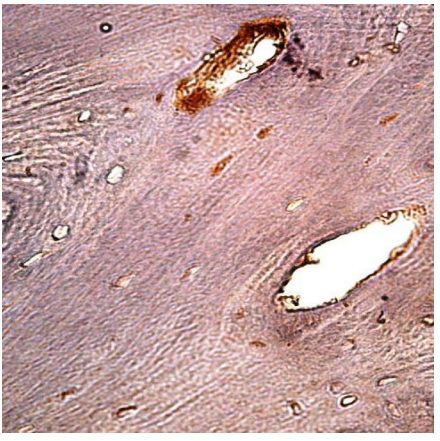

Figure 4. Moderate number of IL-8 positive osteocytes in the bone from *spina nasalis anterior maxillae* of 9 years and 4 months old child. IL-8 IMH, X 250.

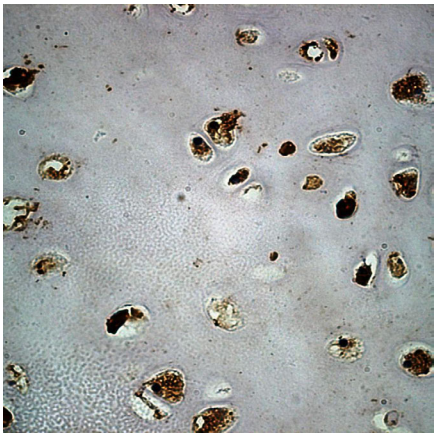

Figure 5. Abundance of IL-8 positive chondrocytes in the hyaline cartilage from *septum nasale* of 7 years and 7 months old child. IL-8 IMH, X 250.

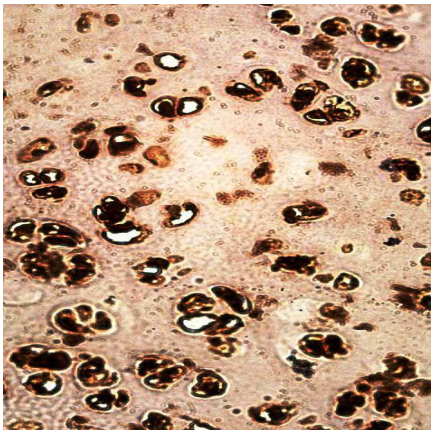

Figure 6. Abundance of IL-10 positive chondrocytes in the hyaline cartilage from *septum nasale* of 7 years and 7 months old child. IL-10 IMH, X 250.

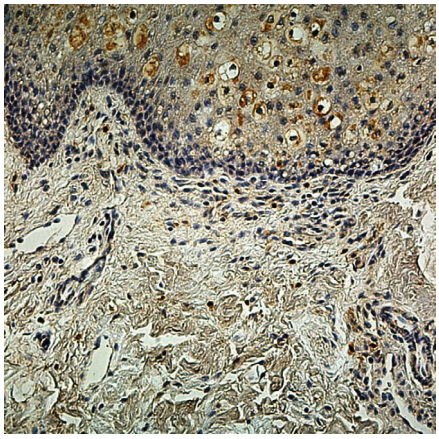

Figure 7. Moderate number of TNF-α containing macrophages and neutrophils in the soft tissue from lip region of 4 months old child. TNF-α IMH, X 200.

DISCUSSION

Cytokines are of central importance for the regulation of inflammation, tissue remodeling, and embryogenic development [5, 6]. Yet little is known about the local expression of pro- inflammatory cytokines in the cleft lip and palate tissues.

The expression of interleukin-6 (IL-6) was observed in the tissues of all the patients. Therein IL-6 showed slight elevated expression in gingival epithelial,

hyaline cartilage and hyaline cartilage growth zone cells as well as osteocytes. IL-6 is a well known major inflammatory and fibrogenic cytokine and it has a wide range of biological activities including the regulation biological functions of connective tissue cells (e.g. fibroblasts), the expression of proteases, inhibiting the formation of the extracellular matrix, the immune regulation and the stimulation of acute-phase reactants [14, 15, 16]. IL-6 is involved in the regulation of bone and cartilage cells functions, but its role in supportive tissue metabolism is uncertain [17]. Bodo et al. (1998) reported that changes between TGF-β3 and IL-6 signal transduction pathways are involved in the induction of the cleft palate [18]. The role of tumor growth factor beta3 (TGFβ3) expression in orofacial clefts has been investigated for several years. TGF-β3 significantly down-regulates IL-6 secretion in the cleft lip and palate fibroblasts [19]. Further IL-6 reduces connective macromolecule production unlike TGF-β [20, 21]. IL-6 expression in the material from clefts areas possibly might be explained with the necessity for an inhibitory effect on extracellular matrix components levels. In our material the sporadical and scarce expression of IL-6 indicates its insignificant role in the cleft lip and palate affected tissue. A number of studies reported that the TGF-β is involved in regulating not only the IL-6 network, but also interleukin-1 alpha (IL-1α) [22].

It should be noted that IL-1α containing structures were not detected in any of soft, cartilage and bone tissue samples from the patients with the clefts lip and palate. IL-1α is a major pro-inflammatory cytokine. In addition, this cytokine has been supported to be a potent regulator of fibroblast proliferation, and is also known to induce the synthesis of the basic fibroblast growth factor (bFGF) in human osteoblasts and gingival fibroblasts [23, 24]. In its turn, bFGF is involved in various cellular processes such as in cell proliferation associated with wound healing, differentiation and cell migration [25]. In this study TNF-α was observed in macrophages and neutrophils. Like IL-1, TNF-α induces inflammatory response and is a central regulator of the innate immune response as well as induces the expression of proteases and inhibits the formation of the extracellular matrix and promotes cells to secrete pro-inflammatory cytokines [15, 26, 27, 28], and these functions may play a role in our patients also.

Interleukin-8 (IL-8) was observed in the cells of hyaline cartilage, bone and soft tissue. Some variations in the distribution of cytokines may be explained by polymorphism of interleukins genes [9]. This pro-inflammatory cytokine is a neutrophil chemoatractant and activator, play a critical role in inflammation

and host defense and is synthesized by fibroblasts from different tissues, chondrocytes and several types of epithelial cells [29, 30]. A moderate number of IL-8 positive cells in our patients my play a role in these functions. IL-8 immunoreactivity is associated with many orofacial region diseases such as the periodontal disease [31]. In support of this, Huang et al. (2001) showed that the expression of IL-8 by gingival epithelial cells increases the following interaction with several periodontal microbes [31].

Interestingly, interleukin-10 (IL-10) presented the abundance of richly stained chondrocytes, osteocytes, fibroblasts, neutrophils, and macrophages, the cells of gingival epithelium, sebaceous glands and hair follicles in all the patients. This cytokine is a true anti-inflammatory cytokine [32]. Its presence suggests the presence of inflammation in the bone, cartilage and soft tissue, even though IL-1α absence may suggests the absence of inflammation in the same tissues.

We concluded that the sporadical and scarce expression of IL-6 indicates its insignificant role in the cleft lip and palate affected tissue. Rich cytokine IL-10 expression proves the compensated local anti-inflammatory effects of the cleft affected soft and hard tissue.

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