Apoptosis and MMP-2, TIMP-2 expression in cleft lip and palate

Liene Smane, Mara Pilmane, Ilze Akota

SUMMARY

Aim of our study was complex detection of appearance and distribution of specific signalling proteins and apoptosis in facial tissue of children with complete bilateral cleft lip and palate (CBCLP).

Materials and methods. Nineteen CBCLP patients and 11 unaffected subjects were involved in this study. All the tissue samples were proceeded for detection of matrix metalloproteinase-2 (MMP-2), tissue inhibitor of matrix metalloproteinase-2 (TIMP-2), and apoptosis. The intensity of immunostaining was graded semi-quantitatively. Results of the terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) method were obtained by counting apoptosis positive cells in five unintentionally chosen fields of vision. Groups were compared using the Mann-Whitney test.

Results. TUNEL-positive oral epithelial cells were significantly increased in the control group when compared with the CBCLP group. Connective tissue cells have a statistically significant lower expression of TIMP-2 in the control group compared to the CBCLP group.

Conclusions. TIMP-2 positive connective tissue cells increasingly found in oral mucosa lamina propria proves the decrease of local apoptosis in CLP patients. Prominent expression of MMP-2 in cleft affected soft tissue indicates a possible increase of tissue remodelling.

Key words: signalling proteins, apoptosis, cleft lip and palate, children.

INTRODUCTION

Cleft lip and palate (CLP) is among the most common genetically heterogeneous and multifactorial congenital malformation of the head and neck in humans. These defects account for approximately 65 % of all head and neck anomalies. The prevalence of CLP ranges from 3.4 to 22.9 per 10 000 births (1, 2).

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent proteinases that are responsible for tissue remodelling, cell migration, adhesion and proliferation. MMPs participate in biological processes, such as embryonic development, growth, morphogenesis and wound healing. MMPs can also process and regulate activity of

non – extracellular matrix proteins, such as growth factors, cytokines, cell receptors and other MMPs (3-5). Analysis showed that MMP-2, MMP-3 play an important role in palate development. Brown *et al.* (2002) found an immunohistochemical increases in MMP-2 during mice palatogenesis (6). MMP-2 plays a specific role in epithelial migration (7).

Tissue inhibitors of matrix metalloproteinases (TIMPs) are able to inhibit MMPs, cell growth activity and angiogenesis alike. TIMPs can also promote cell growth activity and modulate cell apoptosis. TIMP-2 is involved in the activation of pro-MMP-2 and stimulates the proliferation of dental pulp, fibroblast – like cells, gingival fibroblasts, as well as epithelial cells. In addition, TIMP-1 and TIMP-2 also have anti – apoptotic effects (8, 9). If MMP and TIMP balance is disrupted CLP can occur (10-13). Variation in MMP-2, MMP-3, TIMP-2 signalling that pathway genes are likely to be involved in the development of CLP (14).

Apoptosis is a morphological process that plays a central role in embryonic development, morphogenesis, tissue remodelling and normal tissue turnover

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(15). Oral epithelial cells apoptosis play a critical role in regulating oral mucosa development and homeostasis. Specific control of apoptosis is essential for correct formation of the lip and palate. Failure of apoptosis to occur in the epithelial edge can result in the development CLP (16). Genetic variations in genes of the mitochondrial – mediated apoptotic signalling pathway that block cell death may play an etiological role in CLP (17).

Many studies support that MMPs, TIMPs and apoptosis participate in the etiology of CLP. Moreover, still a little is known about local expression of MMP-2, TIMP-2 and apoptosis in facial tissue of children with CBCLP.

Thus, we aimed to determine the apoptotic cells by the TUNEL method and MMP-2, TIMP-2 expression in facial tissue of children with CBCLP.

MATERIALS AND METHODS

Patients

Nineteen CBCLP patients and 11 unaffected subjects were involved in this study. Samples of soft tissue were collected during the surgical correction of cleft at the age of 3 months to 8 years and 5 months or in the case of tooth extraction in controls at the age of 6 years and 9 months to 14 years and 5 months. All 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 parents after the nature of the study had been fully explained. Among affected patients there were 14 males and 5 females, whereas among controls there were 5 males and 6 females.

Methods

1) For conventional light microscopy and immunohistochemistry tissues were fixed for a day in mixture of 2% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.2). Following this, they were rinsed in 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.

2) Immunohistochemistry (IMH) (18).

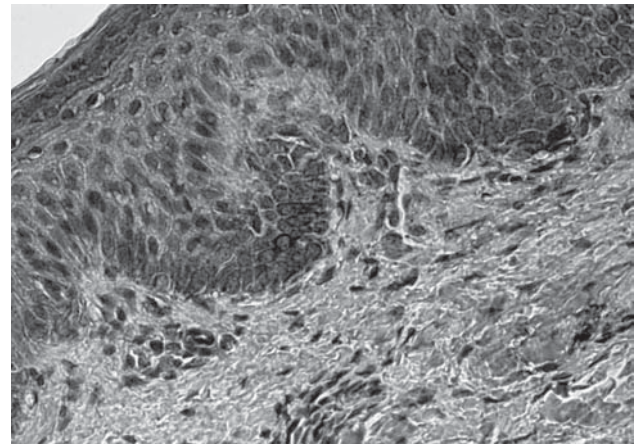Five-micrometer thick sections were cut from the same blocks and placed on polyli-

Fig. 1. Numerous MMP-2 immunoreactive oral epithelial cells, especially in the basal cell layer from cleft lip region of 3 months old child. MMP-2 IMH, ×250

sine coated slides for immunohistochemical analysis. MMP-2 (cat No AF902, LOT DUBO 34081, obtained from goat, 1:100 dilution, R&D Systems, Germany) and TIMP-2 (cat No 3A4, sc – 21735, obtained from mouse, 1:200 dilution, Santa Cruz Biotechnology, INC) were used by biotin-streptavidin IMH.

3) Paraffin sections were also placed on polylisine coated slides to perform TUNEL method. For TUNEL method we used In situ Cell Death Detection Kit (Pod cat No 11684817910, Roche Diagnostics, Germany) in accordance to Negoescu *et al.* (19). The

Table. Information about the patients

Patient	Gender	Age	Plastic surgery procedure	Material
No. 1	M	3 months	Lip plastic	Cleft lip region
No. 2	F	3 months	Lip plastic	Cleft lip region
No. 3	M	4 months	Lip plastic	Cleft lip region
No. 4	M	4 months	Lip plastic	Cleft lip region
No. 5	M	4 months	Lip plastic	Cleft lip region
No. 6	M	4 months	Lip plastic	Cleft lip region
No. 7	M	4 months	Lip plastic	Cleft lip region
No. 8	F	5 months	Lip plastic	Cleft lip region
No. 9	M	6 months	Lip plastic	Cleft lip region
No. 10	M	6 months	Lip plastic	Cleft lip region
No. 11	M	7 months	Lip plastic	Cleft lip region
No. 12	M	7 months	Lip plastic	Cleft lip region
No. 13	F	7 months	Lip plastic	Cleft lip region
No. 14	M	10 months	Lip plastic	Cleft lip region
No. 15	M	10 months	Lip plastic	Cleft lip region
No. 16	F	11 months	Lip plastic	Cleft lip region
No. 17	F	11 months	Lip plastic	Cleft lip region
No. 18	M	8 years 5 months	Lip plastic	Cleft lip region
No. 19	M	8 years 5 months	Lip plastic	Cleft lip region

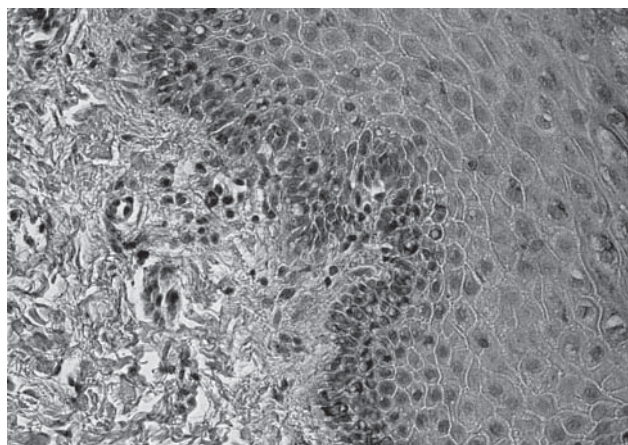**Fig. 2.** Marked distribution of MMP-2 in subepithelial tissue from cleft lip region of 4 months old child. MMP-2 IMH, ×250
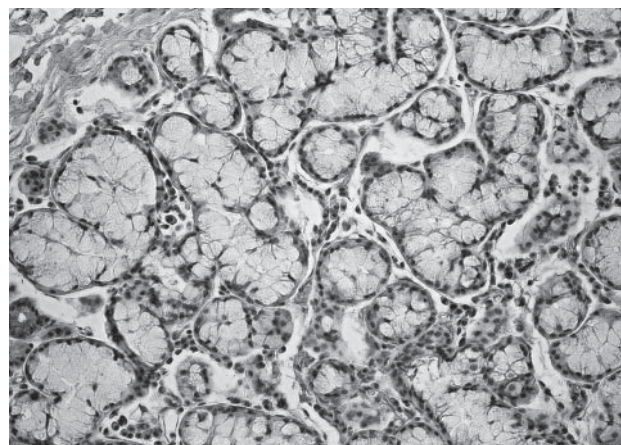

Fig. 3. Moderate positive for MMP-2 salivary gland cells in cleft lip region of 4 months old child. MMP-2 IMH, ×200
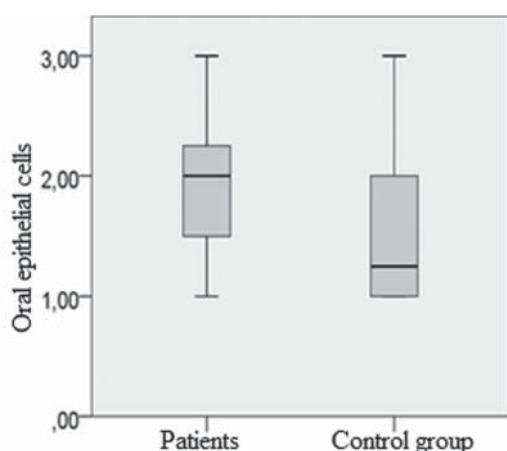

Fig. 4. The graphs represent MMP-2 expression in CBCLP affected epithelium and control group epithelium. No statistically significant differences were identified
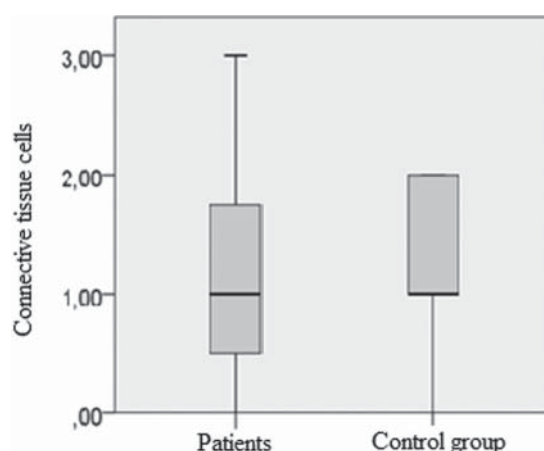

Fig. 5. Comparison of MMP-2 expression in CBCLP affected connective tissue and control group connective tissue cells. No statistically significant differences were identified

sections were pre – treated with Proteinase K (10 µg/ml), incubated with the TUNEL reaction mixture with TdT Enzyme and then Anti-FITC HRP conjugated at 37˚C. DAB was also used as the chromogen. TUNEL-positive if the nucleus showed a dark brown peroxidase stain.

4) For visual illustration of our finding we used 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. Scale was as following: "0" – no positive structures found in visual field, "0/+" – occasional positive structures seen in visual field, "+" – few immunoreactive structures seen in visual field, "++" – moderate number of immunoreactive structures seen in visual field, "+++" – numerous immunoreactive structures seen in visual field, and "++++" – abundance of immunoreactive structures seen in visual field (20).

Results of TUNEL method were obtained by counting apoptosis positive cells in five unintentionally chosen fields of vision.

Analysis of the data

Statistical analyses were performed with SPSS software, version 20.0 (IBM Company, Chicago, USA). Results were expressed as mean±SD. The Mann-Whitney test for comparison of study groups was conducted. P values <0.05 were considered statistically significant.

RESULTS

Number of MMP-2 positive oral epithelial cells, especially in the basal cell layer, connective tissue cells and salivary gland cells varied from few to numerous in all specimens of the control group and CBCLP group (Figs. 1, 2 and 3). There was no significant difference in mean between the CBCLP group and the control group (z=1.65; p=0.09, z=0.32; p=0.77, Figs. 4 and 5).

Appearance of TIMP-2 positive oral epithelial cells, macrophages and fibroblasts in current study varied from few positive structures to numerous of positive structures in visual field in patient with CLP
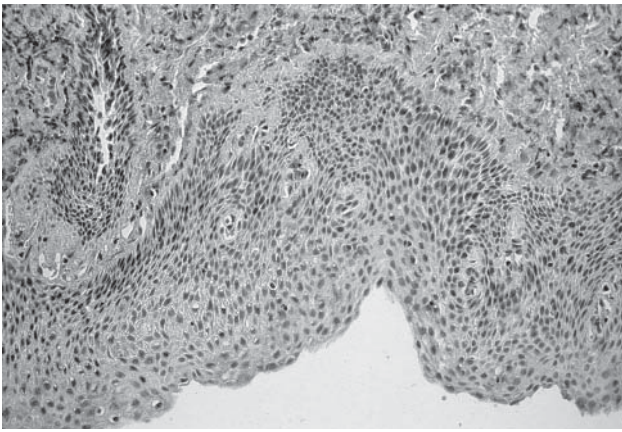
Fig. 6. Moderate number of TIMP-2-containing oral epithelial cells in the soft tissue from lip region of 11 months old child. TIMP-2 IMH, ×200
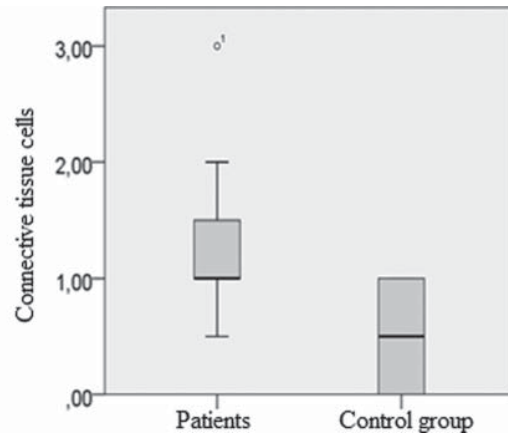

Fig. 7. Comparison of TIMP-2 expression in CBCLP affected connective tissue and control group connective tissue cells. Significant differences between groups were observed
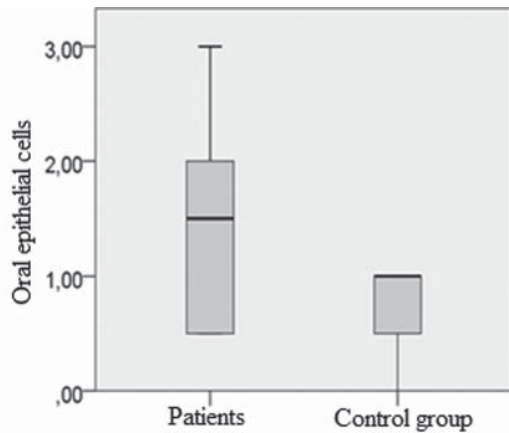

Fig. 8. The graphs demonstrate TIMP-2 expression in CB-CLP and control groups epithelial tissue. No statistically significant differences were identified.
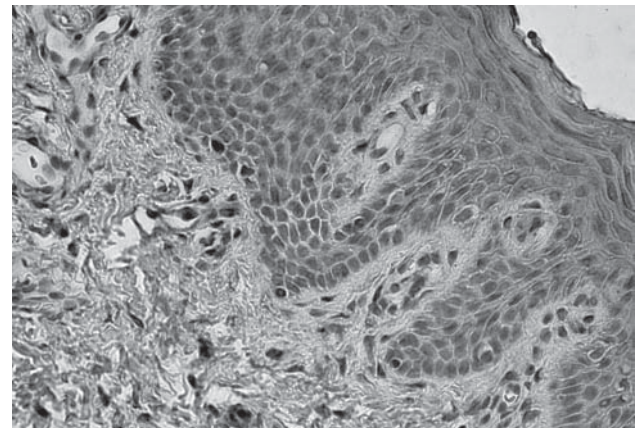

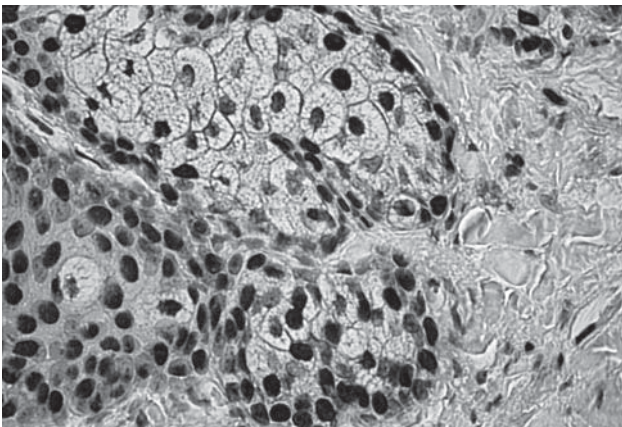**Fig. 9.** Marked apoptosis of oral epithelial cells and connective tissue cells in patient. TUNEL, ×200

Fig. 10. Marked apoptosis in sebaceous gland cells of patient. TUNEL, ×400
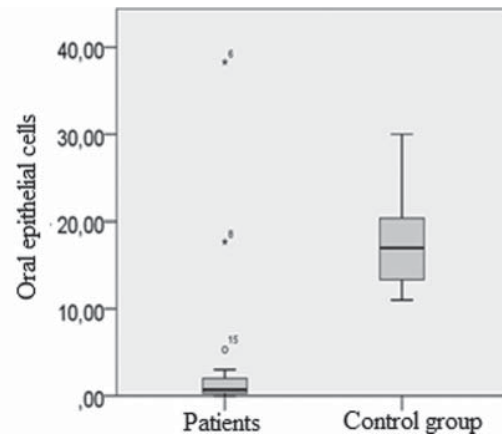

Fig. 11. Comparison of apoptotic epithelial cells using TUNEL method in patient and control groups. Significant differences between groups were observed.

(Fig. 6). TIMP-2 positive connective tissue cells were significantly increased in the CBCLP group when compared with the control group (z=2.16; p=0.03, Fig. 7). Number of TIMP-2 positive oral epithelial cells, especially in the middle layer of oral mucosa, varied from few to moderate in all specimens of the CBCLP and the control group, showing no significant

difference in mean value between the study groups (z=1.33; p=0.21, Fig. 8).

Marked apoptosis affected oral epithelial cells, connective tissue cells, sebaceous gland cells and inflammatory cells focally (Figs. 9 and 10). TUNEL-positive cells were observed in both groups; however, when the cells were counted, TUNEL-positive oral

epithelial cells were significantly increased in the control group (17.80±6.20) when compared with the CBCLP group (1.07±1.36) (z=3.98; p<0.001, Fig. 11). In the cleft disordered tissue sections, TUNEL-positive cells were detected in basal and middle layers of oral mucosa. TUNEL-positive cells were observed in all layers of oral mucosa in the control group.

DISCUSSION

Oral clefts appear to be caused by multiple genetic and environmental factors during palatogenesis. Multiple environmental factors that cause alterations in specific signalling proteins may induce CLP (21, 22). MMPs and their inhibitors are widely expressed during embryonic development. These proteinases responsible for extracellular matrix turnover may be implicated in cleft palate. The enzyme MMP-2, also known as gelatinase A, significantly contribute to mouse palate development and the aberrant expression or function of the enzyme could be involved in causing facial abnormalities, such as CLP (23, 24). MMP-2 is expressed in almost all human tissues, and chiefly present in connective tissue cells, endothelial and epithelial cells. The MMP-2 activity is regulated by TIMP-2, TIMP-3 and TIMP-4. Appearance of MMP-2 positive cells in current study varied from moderate positive structures to numerous of positive structures in visual field in previously mentioned patient with CLP. Expression of MMP-2 was observed in the tissues of all patients. Considering the foregoing, we suggest that MMP-2 expression in material from clefts areas might be explained by its demand for extracellular matrix turnover and beneficial for the wound healing outcome.

In the present study, the number of TIMP-2 positive oral epithelial cells and connective tissue cells was found at an average amount of numerous positive structures in the visual field. Also Letra *et al.* (2012) discovered connection existing between the TIMP-2 gene and oral clefts in population (25). TIMPs are multifunctional proteins, physiological tissue inhibitors of MMPs. These multifunctional proteins are widely distributed in tissues. Tissue remodelling is an important process during embryogenesis and the balance between activated MMPs and TIMPs controls the extent of extracellular matrix remodelling. MMPs and TIMPs may play a crucial role in the formation of the palate and an imbalance in favour of TIMP-2 might increase the risk of a cleft. Some polymorphisms of TIMP-2 gene have been found to be associated with all cleft types (26). Furthermore, TIMP-2 seems to have anti-apoptotic effects in the regulation of apoptosis (27).

Apoptosis was seen in rare cells of cleft affected soft tissue, while the control group tissue demonstrated abundance of cells in programmed cell death condition. Feasibly it is dependent apoptosis, which is related to abundant secretion of MMP-2 and TIMP-2. Apoptosis is a genetically regulated process of cell elimination that plays role in biological processes, such as embryonic development to aging, normal and pathological tissue homeostasis, as well as normal tissue turnover. Thus, development of the lip and palate is a complex process in which apoptosis plays a vital role in craniofacial development. Failure of programmed cell death to occur in the epithelial edge could contribute to the development of CLP. It is possible that variation in anti-apoptotic genes involved in apoptosis may be responsible for some of the predisposition to CLP (28-29).

CONCLUSIONS

TIMP-2 positive connective tissue cells increasingly found in oral mucosa *lamina propria* proves the decrease of local apoptosis in CLP patients. Prominent expression of MMP-2 in cleft affected soft tissue indicates a possible increase of tissue remodelling.

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