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CASE REPORT

Immunohistochemical evaluation of the cleft-affected scar tissue three decades post-corrective surgery: A rare case report

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ABSTRACT

Cleft lip and palate are the most common congenital malformations which require early primary cleft surgery in all infants. The surgical treatment of cleft lip and palate deformities results in the formation of scar tissue. However, the scarred tissue has sparsely been immunohistochemically evaluated to date. Herein, we report the differences in the cellular expression of various proteins in the scar and nearby healthy tissue of a 35-year-old patient who underwent cleft correction surgery during infancy. The scar tissue showed basal cell proliferation, prominent cysts, and fibrotic connective tissue. An increased expression of both interleukin 1α (IL-1α) and IL-10 in the scar tissue was noted, although the balance favored an anti-inflammatory environment. No differences in the expression of matrix metallopeptidase-2 (MMP-2) and TIMP-2 were found whilst an increased expression of PAX-9 and MSX-1 was observed in the scar tissue, which co-localized with RYK expression. Temporal studies like the present one can aid in advancing our understanding of the longitudinal processes governing wound healing morpho-pathogenesis in cleft-affected patients.

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Cleft palate; Scar tissue; Wound repair; Immunohistochemistry; Interleukins; Growth factors

1. Introduction

Cleft lip and palate are one of the most common congenital malformations in the world [1] which manifest with variable severity and range. The gold standard of care remains primary cleft repair in all infants, which is followed by secondary orthognathic surgical interventions. These surgeries are followed by periods of long-term sequalae monitoring and multiple exhaustive medical sessions addressing the associated conditions like difficulty in swallowing and speech impediments [2].

In recent times, however, the outcomes of the primary cleft palate repair surgeries have been drastically enhanced by the application of modern principles including the reduction of tension during midline closure to avoid fistula formation, retro-positioning of the velar musculature to allow proper speech development, and avoidance or reduction of lateral bone raw surfaces and healing by secondary intention to attenuate maxillary growth interference [3–6]. Furthermore, the latest studies have shown enhanced healing of the lateral raw surfaces by using pedicled buccal fat pad flaps [3–6]. Yet, slow wound healing and formation of hypertrophic scars [7] remain an unsolved problem in the cleft literature.

Tissue healing and repair is a complex and dynamic process that involves a collective response from different types of cells and their extracellular products in a tightly regulated spatiotemporal cascade of interactions [8–9]. These processes are regulated by a fine balance between various factors including cytokines like interleukins IL-1α (pro-inflammatory) and IL-10 (anti-inflammatory) [10–11] and tissue remodeling factors like matrix metallopeptidase-2 (MMP-2) and its inhibitor TIMP-2 [12–13]. Additionally, transcriptional factors proteins like paired box PAX-9 and Msh homeobox MSX-1 have been shown to participate in both pathogenesis of clefts and wound healing and repair functions, with their function most prominent in the craniofacial region [14–15]. An associated

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protein, receptor-like tyrosine kinase (RYK) has been also implicated in both these processes [16–17].

However, the function of these factors is still under investigation in the wound healing process, and little is known regarding their expression patterns in wound healing processes in cases of congenital pathologies. There is a lack of investigations that observe the long-lasting changes in the scar tissue after corrective surgery. Henceforth, in the present study, we report for the first time, a morpho-immunohistochemical investigation of the expression of various key regulators of wound healing in post-surgical scar tissue three decades post-surgery.

2. Case report

A 35-year-old healthy male patient reported to our department for endoscopic sinus septoplasty indicated due to the nasal septum deviation. The patient had previously undergone cleft lip and palate correction surgeries at our center at the age of one year. He suffered from a unilateral incomplete cleft of the lip and soft palate close to the midline (Veau II). For the primary correction of the cleft lip, Millard's rotation-advancement technique was performed. Six months later, the patient underwent another surgery for the correction of the soft palate (Krien's intravelar veloplasty). The veloplasty was performed near the junction of the hard and soft palate near the oro-nasal region. No raw surfaces (exposed bony tissue) were noticed at the time of the surgery.

During the septoplasty, the palatal region was found to be normal. The presence of scar tissue was noted as a slightly depressed, pinkish, and paler region near the hard-soft palate junction at the midline. Palatal mucosal samples were collected during the surgery from this scar tissue and from the nearby healthy palatal tissue. The surgery went without any complications and patient was discharged with routine instructions and medications. The follow-up for the patient was uneventful.

The collected mucosal samples underwent routine biotin-streptavidin immunohistochemical reactions to detect the expression of various proteins of interest. The tissue sections were incubated with primary antibodies against IL-1α, IL-10, MMP-2, TIMP-2, MSX-1, PAX-9, and RYK (see supplementary material for detailed methodology). Routine hematoxylin and eosin staining of the palatal scar tissue revealed presence of basal cell proliferation in the palatal epithelium and inflammatory infiltration of the subepithelium with mostly lymphocytes and macrophages (Figure 1(A)) along with hyperplastic and hypertrophic mixed salivary glands. Fibrotic tissue with a patchy distribution was visible in the scar tissue (Figure 1(B)) along with prominent cysts-like structures in the epithelium (Figure 1(C)).

The scar tissue had an increased expression of IL-1α in all cell types – epithelium, connective tissue cells, and endothelium (Figure 2(B); see supplementary material). Expression of IL-10, however, was comparable in both tissues. Both connective tissue cells and endothelium had similar expression of IL-10 whilst epithelial cells in the scar tissue had an increased protein expression of IL-10 in comparison with healthy tissue (Figures 2(C,D)).

There were no differences in the intensity of staining (protein expression) of both MMP-2 and TIMP-2 (not shown). Both PAX-9 and MSX-1 were found to be more prominently expressed in the scar tissue than the healthy tissue. In the scar tissue, there were an abundant number of epitheliocytes that were found to be positive for both PAX-9 and MSX-1. In the healthy tissue, however, only a moderate number of epithelial cells were found to be positive for both PAX-9 and MSX-1 (Figure 3(A,B)). Finally, for the RYK receptor, we found that there was an increased expression in the scar tissue. Whilst a moderate number of epitheliocytes were immunoreactive for RYK in healthy tissue, numerous epithelial cells were found to be RYK immunopositive in the scar tissue of the patient (Figure 3(E,F)).

3. Discussion

Post-corrective surgery complications including surgical-site infections, airway obstructions, feeding difficulties, hypertrophic scar formation, etc. are not an uncommon occurrence in cleft patients [18], most of which occur due to dysregulations in the wound healing process. As an immunomodulator IL-10 can downregulate various pro-inflammatory cytokines including IL-1, thereby protecting the host from immune-mediated tissue damage [19]. It has also been associated with anti-fibrotic and scarless healing, since it maintains and induces postnatal production of hyaluronan, a glycosaminoglycan that promotes ECM expansion and rapid cellular migration [20]. Overexpression of IL-10 in the early phases of wound healing has been associated with long-lasting positive effects [21].

Previously, we have reported undetectable to low levels of IL-10 in cleft-affected tissue in infants [22]. Upon comparison of the results, it appears that IL-10

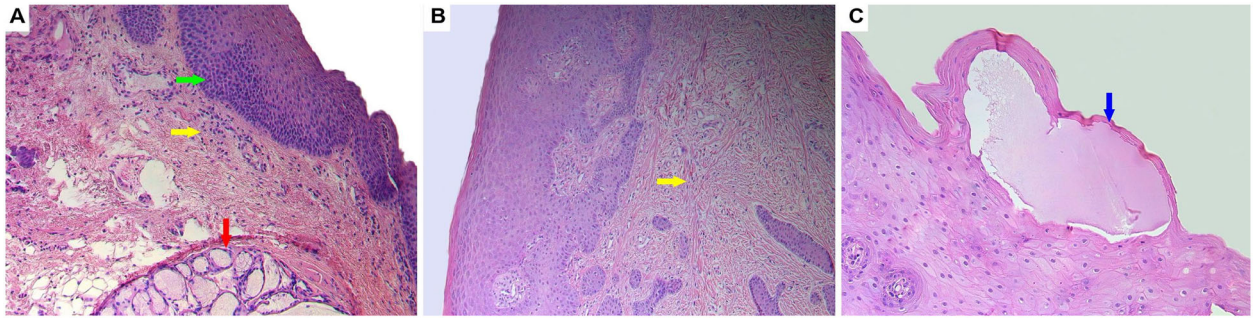

Figure 1. Microphotographs from routine hematoxylin and eosin (H&E) staining of the palatal scar tissue. (**A**) Subacute inflammatory infiltration in the subepithelium (yellow arrow), basal cell proliferation of palatal epithelium (green arrow), and mixed salivary glands (red arrow) can be visualized in the scar tissue. Original magnification, 100×; (**B**) Patchy distribution of fibrotic tissue is seen in the scar tissue (yellow arrow). Original magnification, 100×, and (**C**) Prominent cyst found in the scar palatal epithelium (blue arrow). Original magnification, 200×.

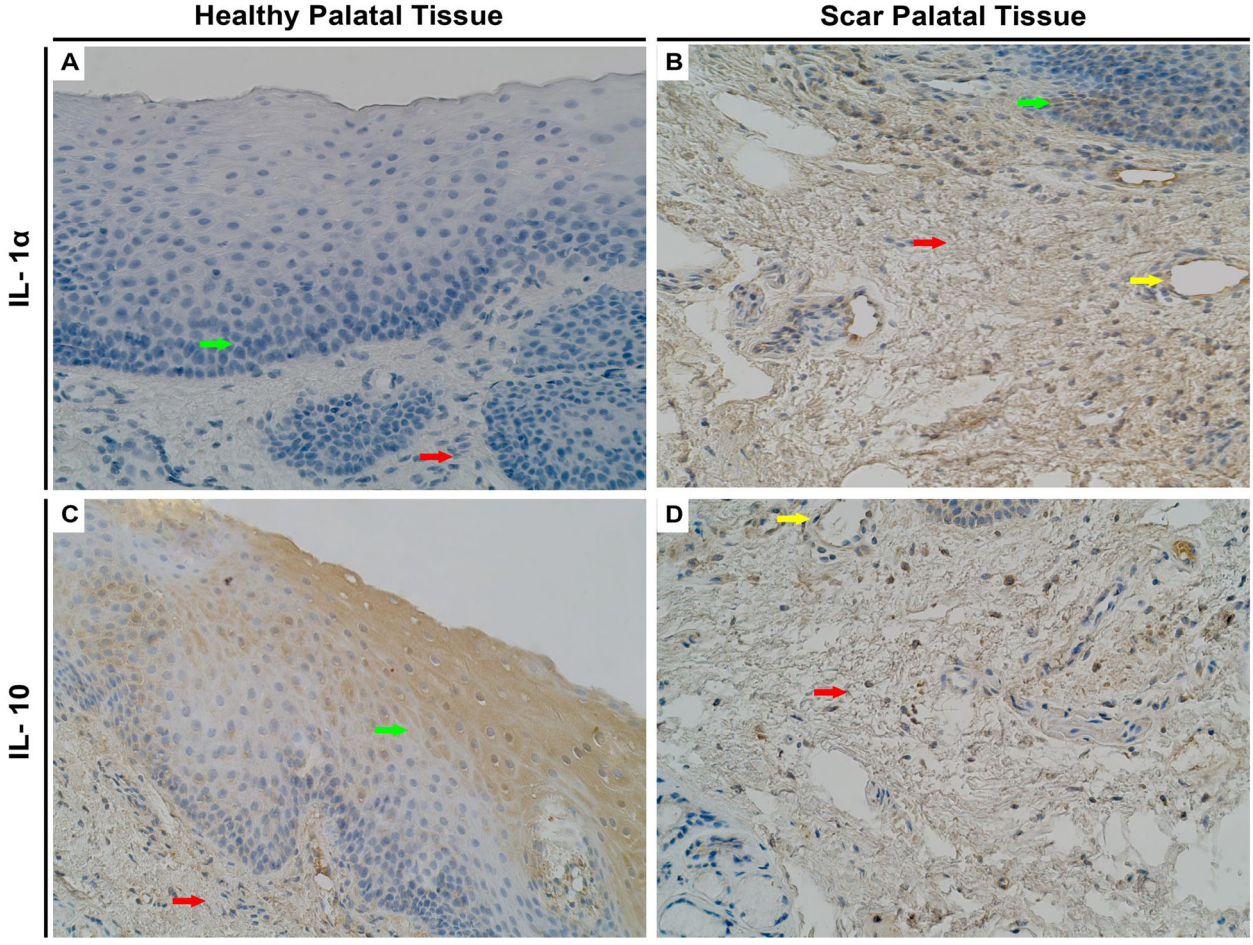

Figure 2. Representative microphotographs from healthy (**A,C**) and scar (**B,D**) palatal mucosal tissue immunostained for interleukins. Epithelial cells are shown with green arrows; Connective tissue cells are shown with red arrows and Endothelial cells are shown with yellow arrows. (**A**) Absence of IL-1α positive structures in the healthy tissue. Original magnification, 200×; (**B**) Few to moderate IL-1α containing epithelial and endothelium cells in the scar tissue. Original magnification, 250×; (**C**) Moderate number of IL-10 positive epitheliocytes in the healthy tissue. Original magnification, 200× and (**D**) Numerous IL-10 positive connective tissue cells in the scar tissue. Original magnification, 250×.

levels in scar tissue normalized to the levels of healthy tissue, however, this normalization seems to occur not during the early phases of wound healing, which could be responsible for the formation of the scar tissue. This is also supported by our findings of inflammatory infiltration in the subepithelium along with the presence of patchy fibrotic connective tissue (Figure 1). An increased expression of IL-1α was

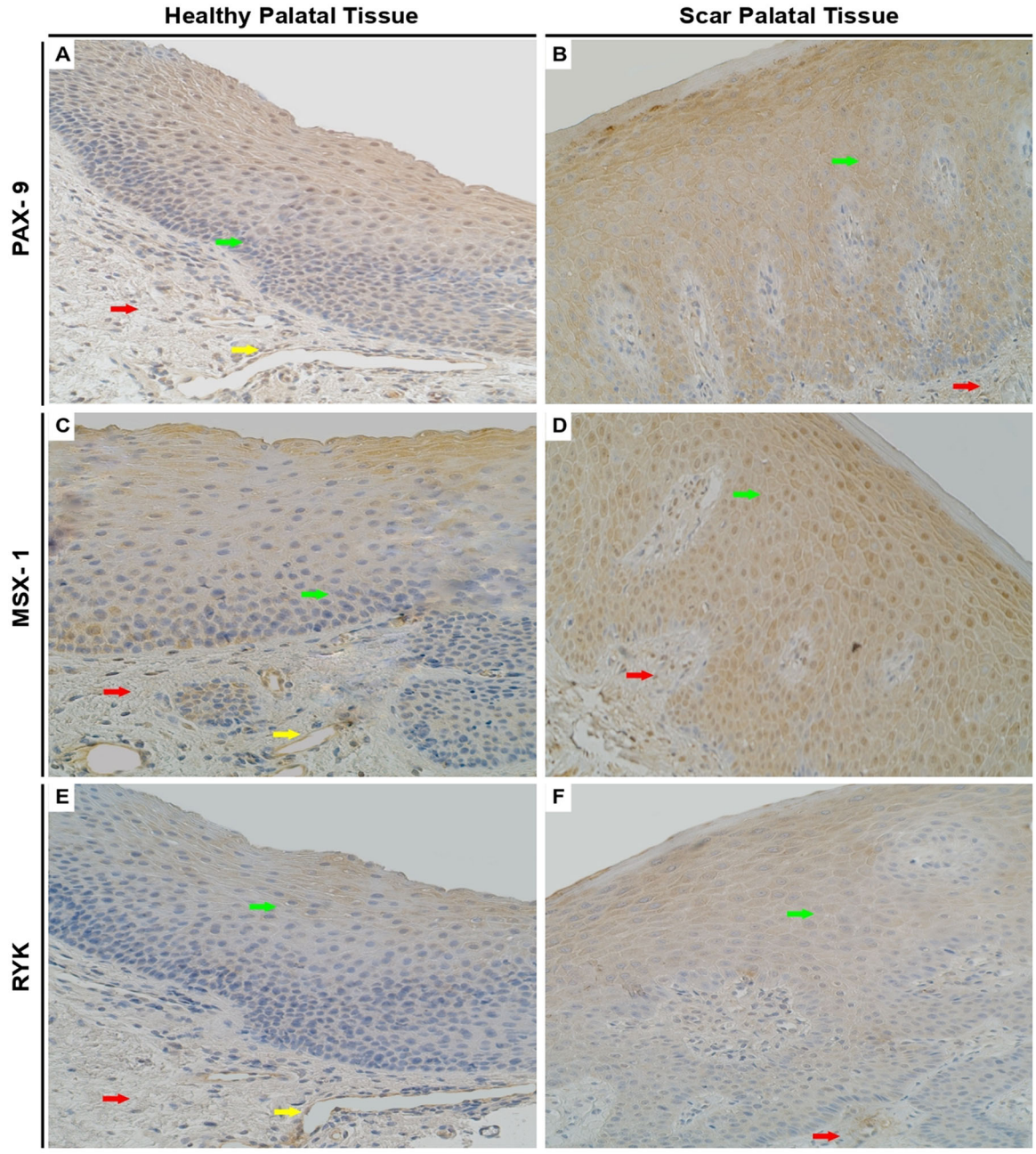

Figure 3. Representative microphotographs from healthy (**A,C,E**) and scar (**B,D,F**) palatal mucosal tissue immunostained for transcription factors (PAX-9; paired box-9 and MSX-1; Msh homeobox-1) and Wnt receptor (RYK; receptor-like tyrosine kinase). Epithelial cells are shown with green arrows; Connective tissue cells are shown with red arrows and Endothelial cells are shown with yellow arrows. (**A**) Moderate weakly stained PAX-9 positive epithelial cells in the healthy palatal tissue. Original magnification, 200×; (**B**) Abundant PAX-9 positive epitheliocytes can be seen in the scar tissue of the patient. Original magnification, 200×; (**C**) Moderate number of immunoreactive MSX-1 cells seen in the epithelium of the healthy palate. Original magnification, 250×; (**D**) Numerous immunoreactive MSX-1 epithelial cells are seen in the scar-affected tissue. Original magnification, 200×; (**E**) Moderate number of weakly stained RYK positive epithelial and endothelial cells in the healthy tissue. Original magnification, 250× and (**F**) Numerous weakly stained RYK positive epithelial cells in the scar palatal tissue. Original magnification, 200×.

noted though the expression remained lower in comparison with IL-10, indicating indicates an anti-inflammatory microenvironment. Keratinocyte-produced IL-1 in the wounded tissue leads to the inhibition of keratinocyte mitotic activity *via* stimulation of IL-1Ra (IL-1 receptor antagonist) [23]. This leads to an autocrine decrease in IL-1 signaling and decreases the mitogenic factors secreted by the fibroblasts, thereby regulating keratinocyte proliferation [23]. It seems that the sustained IL-1α levels are due to the increase in number of fibroblasts in the tissue (due to IL-10), which stimulates keratinocytes, though

the expression per cell is limited due to direct inhibition from IL-10. In terms of the probable role of IL-1α, it has been shown to cause accelerate wound healing, along with causing expansion of cystic cavity [24].

Previous reports investigating the expression of MMP-2 in control and cleft-affected surgical tissue found no differences in the expression [25]. However, in another study, the authors reported significantly lower expression of MMP-2 in fibroblasts, macrophages, and osteocytes in the control group when compared with the cleft-affected surgical tissue [26]. It is understandable that the expression of MMP-2 was reported to be higher in the osteocytes due to the constant bone turnover that is driven by the mechanical loading stimulus. This constant remodeling affects not only the bone architecture but also its density [27]. Since the oral cavity soft tissue including palatal and lip mucosa are under constant stress and minor trauma from chewing and speaking activities, the expression of MMP-2 is needed to allow for tissue remodeling and repair. It is interesting to note that there are no differences in expression of both MMP-2 and TIMP-2 in healthy palatal tissue and scar-affected tissue after several decades of uninterrupted healing. This potentially indicates that the cleft-affected tissue doesn't undergo excessive tissue remodeling post-surgical correction and matures in the same way as the healthy tissue.

A downregulation in the expression of PAX-9 in the cleft-affected tissue when compared with the control tissue has been reported [28]. This is contrary to the results we obtained. PAX-9 is a critical regulator of mesenchymal-epithelial crosstalk during the palatogenesis process. We suspect that surgical correction of the cleft lip and palate somehow affects the protein expression which promotes accelerated cellular differentiation in the scar tissue [14]. Along with PAX-9, MSX1 has been shown to synergistically affect cellular proliferation in the dental epithelium and mesenchyme [29]. The expression of MSX-1 in the scar-affected tissue seemed to be like its expression reported in the surgical tissue obtained during the cleft-corrective surgery in children [30], indicating a rather stable yet elevated expression in the cleft-affected tissue.

Lastly, RYK was also found to be elevated in the scar tissue and co-localized with the expression of PAX-9. This elevated expression looks like remnant from infancy, since RYK expression was found to be elevated in surgical tissue during the corrective surgery as well [28]. The expression of RYK mRNA has been thought to occur in a differentiation-specific manner in the epithelial tissues with differences in spatiotemporal expression amongst different tissues [31]. The exact role of RYK remains to be completely understandable. A limitation of our case report is the number of proteins and pathways that we investigated. This was due to the limited tissue sample quantity and hence our choice our choice of immunomarkers were dictated from previous evidence of the role of these proteins in cutaneous wound healing. Additionally, we cannot at this time predict the differences or fluctuations in the expression of these proteins that occurred over the years in the scar tissue. We also are not aware of the expression patterns in the pre-surgery cleft-affected tissue. Since maternal, paternal, as well as environmental factors can modulate the protein expression, it is challenging to completely encapsulate the underlying causative mechanisms behind scarring.

4. Conclusions

An increased IL-1α expression in the scar tissue can stimulate fibroblastic proliferation and formation of cysts, whilst the delayed expression of IL-10 could promote scarring. Stronger MMP-2 expression could be a result of the constant tissue remodeling in the oral cavity due to stress from masticatory and speech-related activities. Finally, increased expression of transcriptional factors PAX-9 and MSX-1 in the scar tissue can accelerate cellular proliferation and differentiation whilst dysregulating the mesenchymal-epithelial crosstalk.

Acknowledgment

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Ethical approval

The study protocol was approved by the local ethics committee of the Riga Stradins University (dated 25 June 2018 vide no. 5/25.06.201).

Informed consent

Written informed consent to publish the study was collected from the patient. The full nature and extent of the study was explained to the patient prior to signing of the consent.

Author contributions

MP and GS conceptualized the study whilst MP, EN, and GS were responsible for methodology, data curation, and validation of the results. Software, visualization, formal analysis, and investigations were done by MP and NJ. MP was responsible for resources, supervision, funding acquisition and project administration. Original draft was written by MP and NJ, while revisions and editing was done by MP, NJ, EN, PF, and GS. All authors have read and approved the final version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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