Concentrations of MMP-9 and TIMP-1 in lip tissue and their impact on cleft lip surgery healing

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Aim. To compare aspects of wound healing after cleft lip surgery performed within one week of age and wound healing after surgery performed within 2 - 4 months of age, especially concentrations of matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinases-1 (TIMP-1) in tissue removed during surgery.

Methods. 34 tissue samples (26 boys and 8 girls) were removed during surgery within one week of age (n=19) or within 2 - 4 months of age (n=15). Tissue samples were separated into epidermis, dermis and mucous membrane. Proteins were extracted in cacodylic buffer for 24 h at a temperature 2 - 8 ºC. Total protein concentrations were examined using a modification of the Lowry method. Samples were examined using ELISA kit Amersham Biotrak Activity Assay (GE Healthcare UK) for detection of MMP-9 and TIMP-1 concentrations.

Results. MMP-9: early surgery - epidermis 2.168±3.303 μg/g of protein (mean±SD), dermis 1.251±1.848 µg/g, 2 - 4 months surgery - epidermis 0.347±0.212 μg/g, dermis 0.555±0.276 µg/g. TIMP-1: early surgery - epidermis 1.762±2.162 μg/g, dermis 1.628±0.822 µg/g, mucous membrane 2.066±1.717 µg/g, 2 - 4 months surgery - epidermis 1.881±2.810 µg/g, dermis 3.117±1.540 µg/g, mucous membrane 4.833±6.550 µg/g.

Conclusions. There were no significant differences in concentrations of protein MMP-9 in epidermis and dermis and TIMP-1 in epidermis and mucous membrane according to time of surgery. Significantly decreased levels of TIMP-1 in dermis were found in samples obtained from early surgery compared to levels in samples obtained from 2 - 4 months surgery.

Key words: matrix metalloproteinases, cleft lip, wound healing

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INTRODUCTION

Cleft lip is one of most common congenital malformations in humans. Labioplasty performed on infants to repair such defects often results in severe scar formation1.

Wounds in fetuses heal rapidly and generally without scar formation until late gestation. Adult skin wounds heal by scar formation, while fetal skin wounds heal without scar formation by a process resembling regeneration rather than repair2. Relative to that in adults, fetal wound repair is characterized by more epithelization, fibroblast migration, extracellular matrix (ECM) deposition, and ultimate restoration of normal tissue architecture2. In early gestation, the amount and organization of ECM may be associated with scarless repair of fetal skin wounds2. The amount and organization of normal wound ECM are determined by a dynamic balance among overall matrix synthesis, deposition, and degradation3.

The composition and organization of ECM are governed by matrix metalloproteinases2. Matrix metalloproteinases (MMPs) play major roles in tissue regeneration and remodeling2. MMPs comprise a group of structurally related enzymes that are collectively able to cleave practically all types of ECM molecules and various other substrates including other proteases, growth factors and cytokines4. MMP-9 (92 kDa gelatinase) protein expression has been reported in the epidermal cells of acute wounds5. MMP-9 catalyzes cleavage of denatured collagens of all types and native basement membrane components2. MMP activity is further controlled by a group of protein inhibitors called tissue inhibitors of metalloproteinases (TIMPs) (ref.3). Four TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been identified in vertebrates, and their expression is regulated during development and tissue remodeling5. All TIMPs can inhibit all the MMPs, but with different levels of potency, whereas TIMP-1 preferentially inhibits MMP-9 (ref.7).

We hypothesized that MMP-9 or TIMP-1 may have a role in scarless healing after cleft lip surgery. We compared levels of MMP-9 and TIMP-1 in tissue removed during surgery performed within one week where children were healing with reduced scarring with levels in tissue removed during surgery performed within 2 - 4 months where healing was by scar formation.
MATERIAL AND METHODS

34 tissue samples (26 boys and 8 girls) were obtained from children suffering from cleft lip. The patients were divided into two groups according to the time of the surgery. Surgery was performed during early gestation period - within one week of age (n=19) or during later gestation period - within 2 - 4 months of age (n=15). Assays were performed in samples of excessive tissue removed during surgery. After collection, samples were stored at -70 °C until assays were performed. Tissue samples were separated to epidermis, dermis and mucous membrane. MMP-9 was not investigated in mucous membrane. Separation was performed mechanically using scissors under microscope. Samples were placed in cacodylic buffer (cacodylic acid C₆H₈AsO₃ 10 mmol/L, NaCl 0.15 mol/L, CaCl₂ 20 mmol/L, ZnCl₂ 0.001 mmol/L, NaN₃ 1.5 mmol/L and 0.01% Triton X-100), and then homogenized. Proteins were extracted in cacodylic buffer for 24 h in temperature 2 - 8 °C. We used a volume of four times sample weight. After extraction, the supernatant was separated by centrifugation (30 min at 13000 G).

MMP-9 and TIMP-1 concentrations were adjusted to total protein concentrations. Total protein concentrations were examined using a modification of the Lowry method (Na₂CO₃ 0.19 mol/L, NaOH 0.1 mol/L, CuSO₄ 0.125 mol/L, C₆H₈KNaO₆ 0.095 mol/L, Folin reagent (Na₂WO₄, Na₂MoO₄, H₃PO₃, HCl, Li₂SO₄, Br), working range 0.01 - 1 mg/L, wavelength 750 nm). The method was validated at concentration levels of 0.040 mg/mL (Coefficient of variation - CV₁ = 13.43%) and 0.085 mg/mL (CV₂ = 18.25%). Samples were examined using ELISA kit Amersham Biotrak Activity Assay (GE Healthcare UK) for detection of MMP-9 and TIMP-1 concentrations.

Statistical analysis

Unpaired tests (Mann-Whitney) were used to compare MMP-9 (epidermis, dermis) and TIMP-1 (epidermis and mucous membrane) concentrations. Unpaired t-tests were used to compare TIMP-1 concentrations in dermis. Value of P<0.05 was assumed significant. For analysis, the software GraphPad 5.03 (San Diego, California) was used.

RESULTS

Table 1. shows concentration of MMP-9 isolated from the lip tissue related to the age of children in the time of surgery. (Results expressed as mean ± SD).

![Fig. 1. Data presented as boxes (mean, 2.5 and 97.5 percentiles) and whisker (higher and lower values) plots. Differences between groups are expressed as P=0.200 in epidermis and P=0.866 in dermis.](image)

<table>
<thead>
<tr>
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<th>Epidermis (μg/g of total protein)</th>
<th>Dermis (μg/g of total protein)</th>
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<tbody>
<tr>
<td>1 week</td>
<td>2.168 ± 3.303</td>
<td>1.251 ± 1.848</td>
</tr>
<tr>
<td>2 - 4 months</td>
<td>0.347 ± 0.212</td>
<td>0.555 ± 0.276</td>
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Results are expressed as mean ± SD.
Fig. 2 shows data expressed as boxes (mean, 2.5 and 97.5 percentiles) and whisker (higher and lower values) plots. Differences between groups are expressed as $P=0.720$ in epidermis, $P=0.018$ in dermis and $P=0.693$ in mucous membrane.

**DISCUSSION**

The cleft defect severely deforms the face aesthetically, significantly complicates food intake, breathing and unoperated defects may negatively affect the development of speech. Intellect is mostly unaffected. The technique of cleft repair and surgical skills play a very important role in obtaining good results, but we assume there are other aspects which could influence aesthetics and healing after the surgery. Of these, timing of the operation plays an important role.

According to traditional treatment protocols, surgical cleft lip repair is performed at the age 3 - 4 months. Surgical repair of the cleft lip at a standard age of 3 months often results in severe residual scar formation. Cleft lip repair performed as soon after the birth as possible according to the general health of the patient is believed to be beneficial especially in scarless healing. Scars on the upper lip in patients treated shortly after the birth are visibly more aesthetic.

Studies of the role of MMPs and TIMPs in scarless healing have been described in mice, human fetuses and adult humans. No previous studies of the role of MMP-9 and TIMP-1 in scarless healing after cleft lip surgery on human neonates have been published.

The purpose of this study was to compare the aspects of wound healing after cleft lip surgery performed within one week of age and wound healing after surgery performed within 2 - 4 months of age. The main aim was to examine ECM involved in wound healing, especially concentrations of MMP-9 and TIMP-1 in tissue removed during surgery. While MMP-9 and TIMP-1 play a role in healing, we hypothesized that levels of MMP-9 and TIMP-1 could be different in the two groups.

Our results show that levels of MMP-9 were increased in samples received from early surgery compared to samples received from 2 - 4 months surgery (epidermis, dermis), but the differences were not significant ($P=0.200$, $P=0.866$). This fact is caused by small group of samples and nonparametric data distribution resulting in SDs overlapping means as is shown in table 1.

Scars after surgical repair of the cleft lip in neonates are composed of heavily deposited type I collagen fibers. MMPs are required for regular skin wound healing and the distinctive pattern of their expression has been implicated in promoting scarless healing. The family of collagens is a major component of the wound extracellular matrix and the major substrate for MMPs action. Type I collagen can be remodeled by several MMPs including MMP-9 (ref. 1). High expression of MMP-9 during the skin repair process was described on mice models. MMPs are required for regular skin wound healing and the distinctive pattern of their expression has been implicated in promoting scarless healing. The family of collagens is a major component of the wound extracellular matrix and the major substrate for MMPs action. Type I collagen can be remodeled by several MMPs including MMP-9 (ref. 1).

High expression of MMP-9 during the skin repair process was described on mice models. These studies show that high expression of MMP-9 during skin repair process lead to scarless healing. Similar results were found in our group of children where healing without scar formation correlated with high levels of MMP-9. High levels of MMP-9 expressions can at least partially explain collagen
turnover (production vs degradation) and different kinetics of wound reorganization. According to Hosokawa MMP-9 expression and activity implicates the promotion of type I collagen degradation at the site of surgery. MMP-9 activity is low or undetectable in hypertrophic scars where collagen is excessive.

Fetal wounds are characterized by highly organized collagen deposition. Chen investigated the expression profiles of MMP-9 and TIMP-1 in human fetal and adult skins in order to examine the hypothesis that these enzymes are involved in skin development and wound healing, as well as the possible mechanism regulating fetal scarless healing. According to this study endogenous MMP-9, and endogenous inhibitors might be involved in skin development and in maintenance of cutaneous structure and function. Lower protein content of TIMP-1 in early gestational skin might provide a predominantly antiscarring signal while higher protein expression of this inhibitor might be associated with scar-forming healing in late gestational and adult skins.

This finding correlates with results of our study. Our results show that levels of TIMP-1 in dermis were significantly lower in samples from early surgery compared to samples from 2-4 months surgery ($P=0.018$). Levels of TIMP-1 were decreased in samples received from early surgery compared to samples received from 2-4 months surgery (epidermis, mucous membrane) but the differences were not significant ($P=0.720$, $P=0.693$).

**CONCLUSION**

While concentrations of MMP-9 were increased in tissue samples removed during surgery performed within 1 week, no significant differences in concentrations of protein MMP-9 between the two groups according to the time of the surgery were found. Lower levels of TIMP-1 in dermis in samples received from early surgery compared to levels in samples received from 2-4 months surgery were found. No significant differences in concentration of TIMP-1 in epidermis and mucous membrane according to time of surgery were found. Investigation of TIMP concentrations in tissue samples at different times of cleft lip surgery seems to be promising in wound healing.

**ABBREVIATIONS**

CV: Coefficient of variation, ECM: Extracellular matrix, MMP: Matrix metalloproteinase, SD: Standard deviation, TIMP: Tissue inhibitor of metalloproteinases

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**CONFLICT OF INTEREST STATEMENT**

Author’s conflict of interest disclosure: None declared.

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