CHAPTER 5

The number of immune cells is lower in healthy oral mucosa compared to skin and does not increase after scarring

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ABSTRACT

Depending on the location of injury, wounds can heal with different outcomes. Fetal wounds heal fast without scar formation, while scars are a common feature of regular skin repair. Absence of immune cells might be responsible for scarless fetal wound healing, since inflammation is very limited in these wounds. Similar to fetuses, oral wounds have comparable healing properties by means of accelerated reepithelialization and negligible scar formation. Since fetal and oral wounds share healing properties, immune cells might also be diminished in oral wounds compared to skin wounds. Here we investigated the presence of various immune cells in human skin and oral mucosa, with or without scars. Presence or absence of these cells may play a role in the different modes of healing observed between the two types of tissue. Mast cells, neutrophils, M1/M2 macrophages, T-cells and blood vessels were localized in healthy and scarred skin and oral mucosa (scars >1 year old). Oral mucosa had significantly fewer neutrophils, macrophages, mannose receptor-positive M2 macrophages, but more blood vessels. Scars contained similar numbers of immune cells compared to healthy tissues. Less immune cells in the healthy oral mucosa may induce a diminished immune reaction when wounding occurs, and could improve repair.
INTRODUCTION

A plethora of cells and molecules participate in the process of wound healing. The sequence of events during wound healing passes different phases (inflammation, proliferation, and remodelling), and immune cells fulfil a notable task during each phase. Both mast cells and neutrophils enter the wound quickly and accumulate at the site of injury. Upon activation, mast cells secrete mediators that increase vascular permeability (e.g. interleukin (IL)-6, IL-8, vascular endothelial growth factor (VEGF) and recruit leukocytes, such as neutrophils, attracted by chymase. Furthermore, mast cells contributed to angiogenesis during the proliferation phase, by secretion of VEGF and fibroblast growth factor-2.

Neutrophils form a first line of defence against invading pathogens and release antimicrobial peptides, phagocytose pathogens, and produce reactive oxygen species. Additionally, neutrophils secrete cytokines and chemokines to recruit additional immune cells. Neutrophils also contribute to angiogenesis by secretion of angiogenic factors such as VEGF and IL-8. In addition, neutrophils mediated proliferation of fibroblasts and keratinocytes. Neutrophil depletion had no effect on collagen deposition and wound strength, but led to accelerated reepithelialization.

Monocyte-derived macrophages enter the wound approximately 48 hours post-injury. During the inflammatory phase, macrophages accomplish their main function: phagocytosis of damaged tissue components and apoptotic neutrophils. Early macrophages (classically activated or M1 macrophages) secrete pro-inflammatory cytokines such as IL-1, tumour necrosis factor (TNF)-α, and IL-6. Impaired reepithelialization and minimally vascularised granulation tissue were consequences of macrophage depletion during the inflammatory phase, although scar tissue was reduced. As the proliferation phase progresses macrophages switch to an alternative M2 phenotype. The cells start to produce anti-inflammatory cytokines and growth factors like VEGF, IL-10, and transforming growth factor (TGF)-β. Phenotype switching may be induced by phagocytosis of damaged tissue and dead cells. In chronic wounds, persistence of M1 macrophages together with an incomplete switch to M2 macrophages led to tissue breakdown and impaired healing.

The role of T-cells during wound healing is not well defined yet, although a few effects of these cells during repair have been described. T-cell suppression and depletion resulted in impaired wound healing, while enhancement of T-cell function increased wound strength and collagen deposition.
Depending on the location, wounds can heal with different outcomes. Fetal wounds heal fast without scar formation, while the adult cutaneous healing process progresses slower and may cause scars. The absence of immune cells is one of the factors hypothesized to be responsible for scarless fetal wound healing since fetal wounds had very limited, if any, inflammation. Fetal mice deficient for IL-10 generated scars after wounding, whereas addition of IL-10 mediated tissue regeneration in adult mice. On the contrary, immune cells were associated with fibrotic scarring. Increased numbers of mast cells, macrophages, and CD4+ T-cells were found in hypertrophic scars as compared to normotrophic scars. Furthermore, mast cells contributed to scar formation in a fetal wound healing model.

Similar to the fetus, oral wounds have comparable healing properties by means of accelerated reepithelialization and negligible scar formation. Because of the similarities between fetal and oral repair, it is conceivable that oral wounds contain reduced numbers of immune cells compared to skin wounds. Therefore, presence and amount of various immune cells in human tissue (healthy and scarred skin and oral mucosa) was investigated. Presence or absence of these cells possibly plays a role in the different modes of healing observed between the two types of tissue.

**MATERIALS & METHODS**

**Tissue samples**
Human skin was obtained from five healthy individuals undergoing abdominal dermolipectomy, and scars (>1 year old) were derived after scar revision. Oral mucosa was obtained from five patients with a history of open cleft palate undergoing pharyngoplasty. These oral tissues contain both healthy structures as well as scarred (>1 year old) regions. Tissues were embedded in Tissue Tek OCT Compound (Sakura Finetek, Alphen aan den Rijn, The Netherlands) and stored at -80°C until sectioning. All donors provided informed consent according to institutional and national guidelines.
Immunofluorescence

Of all tissues cryosections (5 µm) were made and mounted on collagen coated glass slides. Sections were fixed in acetone for 10 minutes and blocked with normal goat serum. Next, sections were incubated with primary antibodies (Table 1) or non relevant isotype control antibodies (negative controls) for one hour at room temperature. Mast cells were identified by tryptase, neutrophils by CD66b, and T-cells by CD3. CD68 was used as pan-macrophage marker, CD40 and CD64 for M1 macrophages, and mannose receptor (MR or CD206), CD163, stabilin-1, and platelet derived growth factor (PDGF)-CC for M2 macrophages. Blood vessels were stained for CD31 and α-smooth muscle actin (SMA). After washing with PBS-tween, corresponding Alexa Fluor® labelled secondary antibodies (Molecular Probes®, Life Technologies, Bleiswijk, The Netherlands) were applied to the tissue sections. Hoechst (Invitrogen, Breda, The Netherlands) was used for nuclei staining and sections were embedded in glycergel (Dako, Glostrup, Denmark).

Analysis

Five randomly taken pictures were made of every staining, by use of a Leica CTR6000 microscope (Leica-microsystems, Wetzlar, Germany). The number of positive cells was counted by Cell Profiler software. For evaluating the amount of CD40, CD64, MR, CD163, or stabilin-1 positive macrophages, only co-localization with CD68 was measured. CD31 and α-SMA were analysed by Image J, and positive pixels were counted and designated to ‘intensity score’. Statistical significance was determined by Mann Withney U test analysis. * P<0.05, ** P<0.01, *** P<0.001
RESULTS

Mast cells and neutrophils
With respect to mast cells, no significant differences were observed between skin and oral mucosa (Fig. 1A, B). Furthermore, both skin and oral mucosa scars contained equal numbers of mast cells when compared to healthy tissue. Only few neutrophils were observed, which were significantly lower in oral mucosa (Fig. 1A, B). No difference in numbers of neutrophils between scars and healthy tissues was found.

Figure 1: Mast cells and neutrophils in skin and oral tissue with or without scars.
A) Mast cells were identified by tryptase (red) and neutrophils by CD66b (green) fluorescent staining. Insert CD66b: magnification of neutrophil staining. B) Quantification of 5 donors. OM = oral mucosa. Magnification: 100x.
Macrophages

CD68 positive macrophages were significantly more present in skin than oral mucosa (Fig. 2). Scars contained equal numbers of macrophages compared to healthy skin. In the oral mucosa the same observations were found: healthy and scarred oral mucosa had similar numbers of macrophages.

Figure 2: CD40 and MR positive macrophages in skin and oral tissue with or without scars. A) Macrophages were identified by CD68 (red) fluorescent staining, M1 by CD40 (yellow) and M2 by MR (green). Blue: nucleus. B) Quantification of 5 donors. OM = oral mucosa. Magnification: 100x.
For discrimination of classically (M1) and alternatively (M2) activated macrophages a number of markers were used. CD40 and mannose receptor (MR or CD206) are until now the best defined markers for M1 and M2 macrophages, respectively. CD40 was scarcely present in these tissues and no differences were observed (Fig. 2A, B). In addition to CD40, CD64 is used as marker for M1 macrophages, and present in all tissues (Fig. 3). In oral mucosa presence was slightly reduced compared to skin, although not significant (p=0.09). Scars contained equal numbers of CD64 positive macrophages, compared to healthy tissues (Fig. 3B).

For M2 identification, co-localization of MR, CD163, stabilin-1, or PDGF-CC together with CD68 was examined. MR positive macrophages were significantly less present in both oral mucosa and oral scar in contrast to skin (Fig. 2). As compared to skin scar, oral scar contained less MR positive macrophages. Distribution of MR did not differ between healthy tissue and scar which was the

Figure 3: CD64 positive macrophages in skin and oral tissue with or without scars.
A) Macrophages were identified by CD68 (red) fluorescent staining, and M1 (green) by CD64. B) Quantification of 5 donors. OM= oral mucosa. Magnification: 100x.
case for both skin and oral mucosa (Fig. 2B). Remarkably, the scavenger receptor CD163 was highly expressed in oral mucosa, scar, and oral scar (Fig. 4A). Here it seemed to be more present around blood vessels (based on the structure) and other cells. Co-localization of this molecule with macrophages (CD68) showed something different: As seen previously with MR, its expression was slightly higher in skin compared to oral tissue, although not significant (p=0.09) (Fig. 4B). Finally, stabilin-1 expressing macrophages were evenly distributed between all tissues and scars (Fig. 5). PDGF-CC was not detectable.

Figure 4: CD163 positive macrophages in skin and oral tissue with or without scars. A) Macrophages were identified by CD68 (red) fluorescent staining, and M2 (green) by CD163. B) Quantification of 5 donors. OM= oral mucosa. Magnification: 100x.
T-cells

T-cells were identified by CD3 expression and no significant differences were observed, only a trend. Presence of these cells was slightly lower (p=0.09) in oral mucosa compared to skin (Fig. 6). T-cells were diminished in scars in contrast to healthy skin although in oral mucosa, T-cells did not differ between healthy and scar tissue. Distribution of T-cells differed between the two types of tissue. Besides the lamina propria/dermis, T-cells of the oral mucosa and oral scar were also located in the epithelial layer (Fig. 6A).

Blood vessels

Finally the presence of blood vessels in these tissues was evaluated by identifying CD31 and α-SMA. Furthermore, α-SMA is expressed by myofibroblasts, the cells contributing to contraction in scars. CD31 and α-SMA were higher expressed in oral mucosa (Fig. 7). In both scars CD31 and α-SMA were equally expressed as healthy tissue. Additionally, location of the blood vessels differed between skin and oral mucosa. CD31 and α-SMA positive blood vessels of the oral mucosa assemble very close to the basal lamina (Fig. 7A). In oral scar these vessels are positioned over a large proportion of the scar parallel to the basal lamina (Fig. 7A). With respect to myofibroblasts, which are associated with scar formation, none of these cells were observed; α-SMA was only present in blood vessels.
**DISCUSSION**

In this study, the presence and number of different immune cells was quantified in scars and healthy tissue of human skin and oral mucosa. An altered cell distribution between the two types of scars can eventually give rise to scarless or scar forming healing. Significant differences were found between skin and oral mucosa, although no differences were observed between scars and healthy tissue.

Mast cell numbers were similar between skin and oral mucosa, in both healthy and scarred tissue. In pigs however, mast cells were found to be less dispersed in oral compared to dermal wounds. An explanation for this controversy might be that we investigated scars while in the pig study wounds were examined.

Fewer neutrophils were detected in oral mucosa compared to skin. Upon neutrophil depletion wound reepithelialization was accelerated in mice. Genetically
diabetic mice demonstrate delayed wound healing and neutrophils are excessively present in these wounds. Neutrophil depletion however, increased re-epithelialization.\textsuperscript{5} This may indicate that neutrophils contribute to delay wound healing.

Total macrophage numbers were significantly lower in oral mucosa compared to skin. Discrimination between M1 and M2 macrophages was made using a panel of markers, although \textit{in vitro} studies showed that these markers are not strictly designated to one another.\textsuperscript{21} Based on CD64, M1 macrophages were not differently present between the two tissues or scars and CD40 was barely present on macrophages. CD40 is a co-stimulatory molecule for antigen
presentation, induced by Th1 factors such as IFN-γ, TNF-α, or bacterial components (lipopolysaccharide). Since these tissues harbour a sterile environment, it seems plausible that CD40 is hardly present here.

MR positive M2 macrophages however, were lower in oral than in dermal tissues. M2 macrophages were shown to be involved in fibrotic processes and the diminished occurrence in oral mucosa may give rise to scarless healing during repair. Furthermore, no differences were observed in scars regarding MR expression on macrophages. The scars investigated during this study were obtained >1 year after wounding and possibly the cells have performed their actions during wound healing, such as production of factors provoking extracellular matrix synthesis by fibroblasts, and withdraw during the later stages of healing. Besides macrophages, CD163 was present on other cells, possibly dendritic cells, since this molecule is also present on a subset of these cells. Stabilin-1 positive macrophages were similarly present in all tissues. In vitro macrophages require IL-4 and dexamethasone for expression of stabilin-1, although we did not observe stabilin-1 induction by IL-4 or dexamethasone separately (unpublished data). Therefore, we suggest that stabilin-1 is not a very accurate marker for identification of M2 macrophages.

T-cells were not significantly distributed between skin, oral mucosa, and scars. However, T-cells were prominently located in the epithelium of the oral mucosa whereas not in the epidermis of the skin. We investigated the palatum, and also in the buccal mucosa many more T-cells were found in the epithelium. Human resident epidermal T-cells were shown to contribute to accelerated wound closure by secretion of insulin-like growth factor 1 (IGF-1). IGF-1 regulates keratinocyte shape and migration, in order to mediate reepithelialization. Increased numbers of oral epidermal T-cells may be a factor involved in the faster reepithelialization observed in these wounds.

Finally, blood vessels were examined and found to be increased in the oral mucosa, which was also found in animal studies. Remarkably, nearby the oral mucosal basal lamina blood vessels were established, while in the skin blood vessels were clearly further situated from this lamina. Location of blood vessels may influence the outcome of repair, by faster delivery of growth factors thereby affecting the rate of reepithelialization.

In summary, most of the investigated immune cells were less present in healthy oral mucosa as compared to skin. Scars enclosed similar levels of immune cells compared to healthy tissues. Fewer immune cells in the healthy oral mucosa may induce a diminished immune reaction when wounding occurs, and could give rise to improved repair.
REFERENCE LIST


