8. Summarizing discussion
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The aim of the research described in this thesis was to investigate differences in human cutaneous and oral mucosa wound healing and to determine the role of cytokines and saliva peptides (histatins, LL-37) in this process, using a variety of *in vitro* models.

In chapter 2 *in vitro* methods to study differences in cell mobility of keratinocytes, melanocytes, fibroblasts and endothelial cells during cutaneous wound healing are described. These methods included culture of cell monolayers in the scratch assay and chemotactic assay, as well as organotypic cultures (see Fig A, C, F and I).

The scratch wound healing assay is a relatively simple migration assay, of which we showed that it can be used to determine cell migration rates, and to investigate whether cells migrate over a surface as single cells (fibroblasts) or as an intact sheet (endothelial cells). This assay has been used for multiple experiments throughout this thesis. To study migration of fibroblasts into the dermis and to investigate the expansion of keratinocytes and melanocytes over this fibroblast populated dermis, human, physiologically relevant tissue-engineered skin models were used. Together these tissue models provide a platform ranging from low to high throughput assays depending on the complexity of the model for investigating skin biology, wound healing and for testing the mode of action of novel compounds which may positively influence wound healing. It is important when choosing an assay that the complexity of the model is appropriate for the particular research question.

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In chapter 3 we investigated chemokine receptors involved in an autocrine regulation of re-epithelialization after skin tissue damage, using in vitro skin models (see fig B, C and G). We identified five chemokine receptors (CCR1, CCR10, CXCR1, CXCR2, and CXCR3) that are expressed on the surface of skin keratinocytes, of which their monospecific corresponding ligands (CCL14, CCL27, CXCL8, CXCL1 and CXCL10) are also secreted by keratinocytes and stimulate keratinocyte migration and/or proliferation. Chemokine receptors were already expressed in normal unwounded epidermis and were not further upregulated after wounding. In conclusion, these results show that autocrine regulation of re-epithelialization occurs, and stimulates wound closure of the skin.

CCL22, inducing keratinocyte migration, and CCL24 and CXCL12, inducing keratinocyte proliferation in vitro, were not secreted by keratinocytes in the wound-healing model, despite that their corresponding receptors (CCR4, CCR3, and CXCR4) are present on epidermal keratinocytes in vivo, suggesting a role in paracrine regulation.

We found that CCL20 did not influence keratinocyte migration or proliferation in any way, even though its secretion was increased upon wounding, and its chemokine receptor (CCR6) was present on the surface of differentiated keratinocytes in the upper layers of the epidermis. It is therefore possible that CCL20/CCR6 has no role in wound closure, but rather to control pathogen infection after wounding.

Our study shows that autocrine mechanisms in wound healing can be distinguished from paracrine mechanisms, enabling us and others to identify target molecules and cells for developing new wound-healing strategies in the future.

Intrinsic differences between wound healing in skin and oral mucosa

It has long been recognized and clinically appreciated that oral wounds tend to heal faster and with less scar tissue formation than skin wounds [1;2]. Wounds created in the skin took several weeks to heal [3], whereas similar oral wounds healed within 7 days [4]. There are several differences between skin and oral mucosa that can account for better oral wound healing, consisting of intrinsic differences (those that originate from the tissues itself) and extrinsic differences (those which are mediated via influence from the environment). In chapter 4 and 5 the intrinsic differences in wound healing processes between skin and oral mucosa were investigated; extrinsic differences were investigated in chapter 4, 6 and 7.

In chapter 4 the scratch assay (fig A) was used to determine whether mesenchymal stromal cells (MSC) derived from three different tissues (dermis, adipose tissue and gingival) showed different intrinsic migration rates. We showed that all MSC types migrated as individual cells, rather than as a front in the 4 day study period. The intrinsic migration rate of dermis- and adipose-derived MSC was similar, whereas gingival MSC migrated twice as fast as either dermal or adipose MSC.

Next we investigated proliferation (fig B). Gingival epithelium is highly proliferative when compared to skin epidermis [5], but studies that compare MSC proliferation of skin and gingiva are scarce. We found that the proliferation rate of dermal and gingival MSC was not statistically different, but the proliferation rate for adipose MSC was lower compared to either gingival or dermal MSC. That adipose derived MSC have a lower proliferation rate has also been described in literature [6].

The last process investigated was wound contraction, which is part of the healing process and decreases the wound area. The different MSC types (dermis-, adipose-, and gingiva derived) were seeded into a collagen gel to explore possible differences in their ability to contract a connective tissue matrix (see fig D). The three MSC types induced comparable levels of contraction of the collagen gels. This was also the case after addition of TGFβ1, known for its capacity to enhance dermal matrix contraction, which resulted in equal enhancement of collagen gel contraction. We also used tissue equivalents, in which only MSC (connective tissue equivalent) or MCS together with keratinocytes of the corresponding tissue (full thickness tissue equivalent, see fig H) were incorporated to investigate contraction. We found that the extent of matrix contraction and number of αSMA-expressing myofibroblasts...
In further research is required to identify these. The clinical observation that a higher risk of hypertrophic scar formation exists after deep cutaneous wounding (where adipose tissue is exposed) and that there is rarely a scar visible was almost similar for all three MSC types in connective tissue equivalents. However, the full tissue equivalents of skin keratinocytes constructed with adipose-derived MSC showed surprisingly high amounts of αSMA expressing myofibroblasts underneath the epidermis, and significantly more contraction than gingiva equivalents or skin equivalents constructed with dermal-derived MSC. This indicates that a very specific crosstalk occurs in our model between epidermal keratinocytes and adipose MSC, which may play a pivotal role in hypertrophic scar formation. This crosstalk occurs to a lesser extent between dermal MSC and seems absent between gingiva keratinocytes and MSC. Our observations are in line with the clinical observation that a higher risk of hypertrophic scar formation exists after deep cutaneous wounding (where adipose tissue is exposed) and that there is rarely a scar visible after wounding of oral mucosa [1,7]. The soluble mediators involved are still unknown, so further research is required to identify these.

In chapter 5 we compared the wound healing potential of skin and gingival tissue-engineered substitutes, which are both fully compliant with the new law referring to application of cell based products covered by the European Advanced Therapy Medicinal Product (ATMP) law. No human tissue-engineered oral substitutes for clinical applications are yet commercially available, while there are skin substitutes available to close hard-to-heal wounds; for example several autologous and allogeneic dermal and bi-layered (epidermis and dermis) constructs have been described over the past years to heal venous and arterial leg ulcers, diabetic foot ulcers, pressure ulcers and large burn wounds [8-17]. Autografts still remain the gold standard for oral hard-to-heal wounds (arising from tumor excision, cleft palate repair and large trauma). Because the amount of donor material available for grafting is often limited, solutions are also being sought in the area of tissue engineering. We characterized and compared the mode of action of our skin- and gingiva substitutes (see fig 1).

First we looked at the histology of both skin and gingival substitutes. Both substitutes consisted of a differentiated epithelium growing out from the original epithelial sheet and a fibroblast populated human connective tissue matrix, histologically resembling the native tissue biopsies. Skin substitutes had approximately the same number of cell layers as the native tissue biopsy, while in gingiva substitutes this was clearly less than in the native tissue biopsy. The keratins 6, 10, 13, and loricrin, involucrin and SKALP were expressed in the SS and GS in a way that was comparable with the native tissue biopsies. Keratin 16 in skin substitutes and keratin 17 in both skin and gingiva substitutes showed higher expression compared to the native tissue biopsies.

Keratinocyte migration and proliferation was investigated since this is key in epithelization. Gingiva substitutes had a larger epithelial outgrowth (keratinocyte migration), and a higher percentage of Ki-67 positive cells (keratinocyte proliferation). The metabolic activity of skin and gingiva substitutes was not statistically different. Our results clearly show that the increased proliferation and migration capacity of gingiva compared to skin is an intrinsic property of the epithelium, while the transition of proliferating keratinocytes into terminally differentiating keratinocytes and the following stratification into a multilayered epithelium is regulated in part by extrinsic factors, since we did not observe an increased number of epithelial cell layers in gingiva substitutes.

Next the secretion of cytokines, chemokines and growth factors described to play a role in wound healing was determined. The secretion of IL-6, CXCL8 and hepatocyte growth factor (HGF) was much higher by gingiva substitutes compared to skin substitutes. CCL2, CCL5, CCL27 and vascular endothelial growth factor were not statistically different between skin and gingiva substitutes. Gingiva not only heals faster than skin, but it also heals with negligible final scarring [1,18,19]. HGF has an anti-fibrotic effect, it acts as mitogen and motogen, and promotes myofibroblast apoptosis [20], which would clearly be expected to increase the quality of a scar. The increased amount of HGF that is secreted by gingiva substitutes compared to skin substitutes may contribute to the intrinsic property of gingiva to heal with superior scar quality. Indeed in our pilot study with the 1st generation gingiva substitutes (prior to ATMP regulations), the three tooth extraction sites that were treated healed with negligible scarring [21].

**Extrinsic differences between wound healing in skin and oral mucosa: the role of salivary histatin peptides**

One of the major extrinsic differences between skin and oral mucosa is the presence of saliva in the mouth. Saliva contains multiple substances that can accelerate wound healing, as described in the introduction (also see thesis of M.J. Oudhoff, 2010). In chapter 4, 6 and 7 the influence of histatins on different wound healing processes was investigated.

In chapter 4 the scratch assay has been used to determine the influence of histatins on migration of MSC derived from dermis, adipose tissue and gingiva. Stimulation of MSC with histatins enhanced the migration of all three MSC types. Surprisingly, histatins had a greater effect on adipose MSC migration, while rhEGF had a greater effect on dermal MSC migration. Gingival MSC showed a higher intrinsic migration, which is probably the reason why little additional migration upon stimulation with histatin and EGF was observed. Since histatins do not influence collagen gel contraction or αSMA expression, while they do stimulate skin MSC migration, it may possibly be used as a healing therapeutic for skin wounds.

Protein-based therapeutics to enhance wound healing (e.g. histatins or cytokine) will be broken down by the multitude of proteases present in the chronic wound bed. Therefore, we tested the proteolytic breakdown of histatins, rhIL-6 and rhCXCL8 in diluted chronic wound extracts by capillary zone electrophoresis in vitro in chapter 6, to find out whether these peptides would survive long enough to exert their migration-stimulating activity. All histatins proved stable in PBS at 37°C for at least 24 h, which makes them ideal for easy storage as an off-the-shelf product. However, in the presence of wound extracts half of the original amount of Hst1 and Hst2 was degraded within 8 h. A cyclical form of Hst1 and the minimal active domain of Hst1 were much more stable under identical conditions, and the cytokines IL-6 and CXCL8 were even more stable. Degradation of histatins and cytokines was diminished by addition of a protease inhibitor cocktail.
To find out whether a significant concentration of histatins and cytokines is essential during the entire migration process, or whether they act as signalling or initiating molecules that are only necessary to start this process, we performed another series of scratch assays. We found out that an initial 8 h pulse of either histatins or cytokines during a 96 h study period resulted in a moderate increase in cell migration compared to unexposed fibroblasts; this increased cell migration was not significantly different from continuous exposure to histatins and cytokines. From this we could conclude that prolonged exposure to histatins and cytokines would not be necessary and degradation is therefore less critical for its migration stimulating effect.

In chapter 7 the inflammatory and antimicrobial response of skin and gingival fibroblasts and keratinocytes to histatins and LL-37, another peptide present in human saliva, was investigated (see fig B and E). We found that secretion of IL-1α, CCL20 and CCL27 by keratinocyte monocultures increased upon histatin and LL-37 exposure. Secretion of IL-6, CXCL1, CXCL8, CCL2 and CCL20 by fibroblast monocultures was not increased upon exposure to histatins and LL-37. When keratinocytes were co-cultured together with fibroblasts, these cytokines were upregulated. This means that histatins and LL-37 have an indirect antimicrobial effect. The increased secretion of IL-6, CXCL1, CXCL8 and CCL2 by keratinocyte-fibroblast co-cultures was blocked by neutralizing IL-1α antibodies, while increased secretion of CCL20 was not.

Translational considerations

In general, care has to be taken with extrapolation of in vitro experiments to the clinical situation. Our results give an indication of the mode of action and expected efficacy of histatins, which can form the basis of designing a clinical phase 1 study. In contrast to our studies, in clinical practice the debrided wound bed would be a non-diluted protease-rich environment, cells might not be as healthy and active [22,23], and hypoxia may be present [24,25]. Therefore, in a follow-up in vitro study, it would be important to compare fibroblasts and keratinocytes derived from healthy skin with those derived from chronic wounds (venous and arterial ulcers, pressure ulcers and diabetic foot ulcers). Furthermore, a phase 1 study is important for further investigation on effectiveness and safety of histatins, as well as optimization on the in vivo peptide dose finding.

Since wound healing is a complex and dynamic process, it is not expected that histatins would form a stand-alone therapy, but would rather be used in combination with other wound treatments. Improving the healing of a chronic wound by addition of peptides will be a challenge, as it is difficult to mimic the bodies capacity to secrete or inhibit secretion of factors according to the physiological needs, and will probably depend on timing.

The focus of this thesis was to study skin and gingival residential cells during wound healing processes. In our models, no immune cells, lymph- and blood vessels are present, but these are important during wound healing. A skin equivalent model with integrated Langerhans cells in the epidermis is developed and is shown to mimic the first part of sensitization and the innate immunology, by Langerhans cell maturation and migration to the dermis upon allergen exposure [26,27]. The future may lie in the development of immune competent organ-on-a-chip models [28], where lymph- and blood vessels are mimicked by integrated microfluidic compartments, which allows study of immune cells into and out of the tissue being studied, as well as the effect of these cells on wound healing.
Reference List
