Oral wound healing and innate oral immune response studied in vitro

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Citation for published version (APA):

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CHAPTER 9

General discussion and summary
General discussion

The aim of the research described in this thesis was to provide insight into the oral wound healing process and innate oral immune response. These complex processes are influenced by numerous factors and are not easily unravelled. Both oral wound healing and the immune response have been intensively investigated before, but the exact mechanisms that give the oral mucosa its unique properties remain unclear. In this thesis, increasingly complex in vitro models were developed and used to contribute to the current understanding of the unique properties of the oral mucosa. The developed models can continue to be used in future research to provide more insight into the properties of oral mucosa. Here, the in vitro models were used to study: The expression and stimulation of chemokine receptors on oral fibroblasts; the stimulation of Toll-like receptors (TLR) on oral and skin keratinocytes; the wound healing potential of saliva; the chemokine secretion of skin and oral mucosa in response to an inflammatory, allergen or irritant stimulus; the potential of immortalized cells for constructing oral mucosa equivalents for the study of wound healing, oral implant attachment and response to oral biofilms; and the innate immune response against commensal and pathogenic biofilms.

Corresponding to the title of this thesis “Oral wound healing and innate oral immune response studied in vitro" the chapters can roughly be divided into the two main topics of this thesis: Chapters 2, 4, 6 and 7 focus on oral wound healing and Chapters 3, 5 and 8 focus on the innate oral immune response. Since the two topics are so closely related, there is overlap between the topics within the chapters e.g. the secretion of inflammatory cytokines during wound healing. As is also evident form the title of this thesis, only in vitro models were developed and used for all the research. In this final chapter we discuss the uses and limitations of the in vitro models developed and the results of the two main research topics.

In vitro models and assays

In the first two chapters conventional monolayer cultures of fibroblasts and keratinocytes were used to investigate the oral wound healing and innate immune response, thereafter tissue engineered three dimensional organotypic gingiva models were included for an improved translation to the in vivo situation. In the last three chapters these organotypic models were made with TERT-immortalized cell lines to
improve scalability and negate the need to isolate gingiva cells, thus speeding up research.

All the cells that were used in this research originated from humans. This is an advantage over the use of animal derived cells, because the results are more translatable to the human in vivo situation. Moreover, the mechanisms of wound healing and immune response have many important differences between humans and rodents e.g. a major inflammatory chemokine CXCL8, that has been an important factor in six of the seven chapters, is not expressed in mice or rats [1–3]. The advantage of in vivo animal models is that they are complex, containing the different cell types and tissue organisation of a mammal. However, because of ethical issues animal models should be used as little as possible. To reduce the number of animal needed for research, organotypic models provide an alternative by increasing the complexity of in vitro models.

The secretion of cytokines by a single cell type in a conventional cell culture plate was studied in six of the seven chapters and the cytokine secretion of gingiva equivalents in four chapters. This assay, where we collected culture supernatant and analysed cytokine content by ELISA, is a relatively easy and fast method to perform for single proteins, which provided a lot of new useful data. It is very suited to compare the release of specific cytokines between conditions, for example the release of IL-6 before and after the wounding of a gingiva equivalent. One of the strengths of the gingiva equivalents is that it facilitates intercellular communication and is thus more relevant to the in vivo situation, but this makes it impossible to distinguish which cell type was responsible for the production of the cytokines in the culture supernatant by ELISA. Therefore, the exact mechanism of the intercellular communication can’t be elucidated in this way and potential therapeutic (wound healing) targets may be missed. Other types of analysis, such as gene expression analysis of the separated epithelial and lamina propria tissues or in situ hybridisation would shed more light on this. This was not done in this thesis, but in the future these types of analysis could provide more data on the function of the cell types. For example, in Chapter 8 gene expression profiling after separation of the epithelium and lamina propria, could shed more light on the potential immune evasion of pathogenic oral biofilms by comparing the gene expression between the gingiva equivalents exposed to pathogenic biofilms or commensal biofilms. It would also be very interesting to know the full extent of cytokines secreted differentially between skin and gingiva that influence gingiva Langerhans cell migration in Chapter 5 since this is currently unknown, but analysing one cytokine at a time with the ELISA method is a time
and resource consuming process. Multiplex immunoassays can be used to analyse multiple cytokines simultaneously in a small volume and would be a useful improvement for future research to answer this type of questions, providing more insight into the innate oral immune response.

For the analysis of the wound healing potential of fibroblasts, a wound healing scratch assay was used in Chapter 2 and Chapter 4. Even though low serum concentrations were used which should stop proliferation in theory, this was not verified, and therefore the wound closure could have been the result of a combination of proliferation and migration [4]. For keratinocytes the scratch method could not be used since keratinocytes attach to each other, making it impossible to make a scratch wound with straight edges. Therefore, we developed an epithelialization model in Chapter 4 which measures the outgrowth of a confluent layer of keratinocytes over a culture plate. In this model epithelialization is likely also a combination of migration and proliferation, even though culture medium containing low serum concentrations was used. The wound healing scratch assay and epithelialization model are relatively simple to perform making it possible to scale up the research, as was done in Chapter 2, where fourteen different chemokines were tested in five concentrations in triplicate with an intra-experimental duplicate. This gave us a lot of new knowledge about the gingiva fibroblast in a relatively short amount of time. The wound healing scratch assay and epithelialization model were used to measure the influence of chemokines or saliva on the speed of recellularization, which is an important factor in wound healing. These models do not show if a cell is migrating towards a gradient, and therefore if they are actively migrating towards the wound, because the models do not distinguish chemotaxis from chemokinesis since there is no chemical gradient [4]. A Boyden chamber assay, which does have a chemical gradient, could be used for future studies to determine which chemokines stimulate fibroblast chemotaxis towards a wound and which stimulate chemokinesis. It is also possible to visualize migration in a three dimensional collagen gel, which would be more translatable to the in vivo situation, and visualize migration over time using time-laps imaging [5]. These methods would provide new data on the influence of chemokines on migration, but are not suited for large scale experiments and should therefore only be used for a few chemokines of special interest, for example when you want to know if gingiva fibroblast are actively migrating towards the mucosa specific CCL28.

A major goal of this thesis was to develop organotypic gingiva models for the investigation of oral wound healing and interaction with oral biofilms and dental
materials. One important obstacle was that the availability of primary human oral tissue for research is very limited, biopsies are small and due to the origin of the material it is often infected. Furthermore, once isolated the primary cells enter senescence after only a small number of passages. The use of physiologically relevant immortalized cell lines would solve these problems. Therefore, to achieve the major goal of this thesis and simultaneously find an answer to the limited supply of oral tissue, fully differentiated human gingiva equivalents constructed entirely from cell lines were developed in Chapter 6. Gingiva equivalents were constructed with TERT-immortalized gingiva fibroblasts and TERT-immortalized or HPV-immortalized gingiva keratinocytes. These equivalents were then compared to native gingiva tissue and gingiva equivalents constructed with primary cells. It was important that the gingiva equivalents were made with tissue-specific cells, as is emphasized by the results from Chapter 5 which showed the difference in histology and cytokine release between skin and gingiva equivalents. This is further illustrated by the unique phenotype of oral mucosal fibroblasts and the distinct differentiating keratin expression patterns of oral keratinocytes.\[6,7\] To investigate the physiological relevance and characterize the different gingiva equivalents, immunohistochemical staining for proliferation (Ki67), mucosal epithelial differentiation (K10, K13) and basement membrane (collagen type IV and laminin 5) was performed [7–9]. The gingiva equivalents made with TERT-immortalized cells formed a multilayered epithelium and histologically very closely represented native gingiva and equivalents made with primary gingiva cells. In contrast, the epithelium of gingiva equivalents made with HPV-immortalized keratinocytes showed characteristics of squamous cell carcinoma indicating that the model with HPV-immortalized keratinocytes may be suitable as an oral mucosa tumor model. Taken together, immortalized human gingiva keratinocytes and fibroblasts can be used to make physiologically relevant gingiva equivalents which resemble either the healthy gingiva or a neoplastic disease model. These organotypic models will provide valuable tools to investigate oral mucosa biology and can also be used for testing new therapeutics, oral implant material (Chapter 7) and microbial biofilm studies (Chapter 8).

Previously, a skin equivalent constructed from TERT-immortalized human skin cell lines was developed [10]. This skin equivalent model also showed excellent physiological similarity to native skin. In the future, the gingiva and skin equivalents constructed from TERT-immortalized cells will provide valuable physiologically relevant tools to investigate intrinsic differences in wound healing and innate immunology
between these two tissues, bypassing the complicated logistics and ethics related to a steady supply of fresh biopsies to the research laboratory.

Taken together, this thesis utilizes different in vitro techniques, ranging from relatively simple assays, to complex tissue engineered three-dimensional tissue equivalents. By expanding these in vitro methods with novel techniques in the future, the acquired knowledge about the oral wound healing and innate oral immune response can be expanded even further.

**Oral wound healing**

Oral tissue heals faster and with less scar formation than skin tissue [11–16]. Even though intrinsic differences have been found between the cells residing within the skin and the gingiva, the exact reason for the superior oral wound healing qualities remain unknown. [12,13,17] Finding the factors involved could lead to future wound healing therapies for patients with excessive skin scarring or large wounds. In Chapter 4 we have shown notable intrinsic factors of the oral mucosa that are part of the reason why oral mucosa has superior wound healing qualities. The gingiva keratinocytes showed faster epithelialization than skin keratinocytes and gingiva fibroblasts showed faster closure of a scratch in a wound healing scratch assay than skin fibroblasts. The faster wound closure and therefore a faster return to tissue homeostasis may well be an important factor in reducing scar formation [18].

Oral wound healing is caused by both the intrinsic properties of the cells within the oral mucosa and the interactions with the surrounding environment [13,19]. To identify targets for wound healing strategies, Chapter 2 focused on the responsiveness of oral fibroblasts to chemokines. Primary isolated gingiva fibroblasts and TERT-immortalized gingiva fibroblasts both expressed the same 12 chemokine receptors (CCR3, CCR4, CCR6, CCR9, CCR10, CXCR1, CXCR2, CXCR4, CXCR5, CXCR7, CX3CR1 and XCR1). Fourteen corresponding chemokines were used to study the activation of these receptors on the TERT-immortalized gingiva fibroblasts. Twelve of these fourteen chemokines stimulated migration (all except for CXCL8 and CXCL12). Five of the chemokines stimulated proliferation (CCL5, CCL15, CCL22, CCL28 and XCL1). Furthermore, CCL28 and CCL22 stimulation increased IL-6 secretion and CCL28 together with CCL27 upregulated HGF secretion. Moreover, TIMP-1 secretion was reduced by CCL15. Taken together, the chemokine receptors CCR3 and CCR4, and the chemokine CCL28, had a predominant role in stimulating the oral fibroblast wound healing response. The identification of
these potent wound healing targets can be used for future research of therapeutics and regenerative therapies. Potentially the gingiva equivalents can be used to investigate whether topical or systemic application of CCR3 ligands, CCR4 ligands or CCL28 stimulates oral wound healing.

A potential stimulating factor of oral wound healing is saliva. Saliva can potentially be a low cost and easy obtainable wound healing therapeutic. It is thought to be one of the main contributing factors to the superior oral wound healing qualities compared to skin. Therefore, Chapter 4 investigated the therapeutic wound healing properties of human saliva. For both skin and oral mucosa, fibroblast wound healing in a scratch assay and keratinocyte epithelialization over a cell culture surface was stimulated by saliva. These models represented an open wound. To increase tissue complexity, full thickness blister wounds were introduced into human skin and gingiva equivalents. Inflammatory mediator secretion (CCL20, IL-6 and CXCL8) was increased by saliva. Moreover, saliva also stimulated faster re-epithelialisation in gingiva equivalents after wounding, but this effect was not found for the skin equivalents, probably due to the dense layer of dead stratum corneum and keratinocytes forming the blister restricting penetration of saliva to the migrating epithelial front. Clearly saliva is a potent stimulator of oral wound healing and may be beneficial for treating open skin wounds. The observed effect of saliva for skin wound healing is therefore potentially extremely beneficial for burns patients. Whether saliva has the ability to reduce scar formation, or stimulate chronic wound closure still has to be determined. The reduction of scar tissue formation can be studied in specialized scar models or by quantifying the production of scar tissue protein type I collagen [20]. Similarly, there are models for chronic wounds, and it may be possible to start a patient trial once optimal conditions have been found [21].

To speed up research into oral wound healing, the gingiva equivalent constructed with human TERT-immortalized keratinocytes and fibroblasts was developed in Chapter 6. This model was functionality tested by introducing full-thickness wounds. Re-epithelialization, fibroblast re-population of the hydrogel, metabolic activity and cytokine release (ELISA) was assessed during wound closure over seven days. An immediate inflammatory response (IL-6, CCL2 and CXCL8) was observed followed by complete re-epithelialization. Seven days after wounding, metabolic activity and cytokine levels had returned to the pre-wounded state. These results show the potential that this gingiva model holds for the investigation of oral wound healing. To further determine if the model is also suited to study dental implant soft tissue attachment, Chapter 7 established a proof of concept where dental implant material was implanted
into the gingiva equivalents. Histological evaluation after two weeks showed epithelial attachment and hemidesmososome formation. The ultimate success of dental implant therapy depends on the soft tissue attachment to the implant material. Therefore, this model holds great potential for future research into implant biocompatibility and implant survival, possibly leading to a reduction in animal experiments.

In the gingiva equivalent wound healing model there is no blood flow, and therefore no blood clot, as there is in an *in vivo* wound. The fibroblasts and keratinocytes thus have no substrate to migrate into and over when the ECM is destroyed. This problem was solved by applying freeze wounds, which leaves the ECM intact, but kills the cells. To mimic a cutting wound with a blood clot it may be possible to apply a cutting wound on the gingiva equivalent and fill this with fibrin glue. The fibrin glue can potentially be preloaded with innate immune cells, such as neutrophils and monocytes, to resemble the influx of these cells into a wound. The introduction of more cell types in the gingiva equivalents will make it more translatable to the *in vivo* situation, but it will also make the experiments more difficult to perform and analyze. This reduces the potential for large scale experiments, which goes against what we were trying to accomplish by introducing the immortalized cells. Therefore, it is important to select the appropriate model for each research question.

In conclusion, we have found clear intrinsic differences between skin and oral mucosa, which most likely contribute to the superior wound healing qualities of oral mucosa. We have identified CCR3, CCR4 and CCL28 as potential wound healing targets, and established that saliva contributes to oral wound healing and is a potential wound healing therapeutic. For future oral wound healing studies, we have established a physiologically relevant gingiva equivalent model that is suited for large scale experiments and can be used to study soft tissue implant attachment.

**Innate oral immune response**

Both oral mucosa and skin have the capacity to maintain immune homeostasis and regulate immune responses upon environmental assault. They both provide an important barrier and a first line of defence against, trauma, toxic substances (allergens and irritants) and pathogen invasion. Whereas much is known about key innate and adaptive immune events in skin [22–24], relatively little is known in this respect about oral mucosa [25,26]. In this thesis we aimed to gain insight into the innate oral immune response under influence of trauma, toxic substances and pathogen invasion.
Metal ions leached from dental implant materials may create local irritation, itching and swelling by triggering an innate immune response, which potentially also facilitates the development of metal specific adaptive immunity. Chapter 3 explored the potential of metals to activate the innate immune pathway in keratinocytes. By stimulating the Toll Like Receptors (TLR) with a panel of different ligands it was found that TLR3 is a key innate immune receptor of both skin and oral keratinocytes. Potentially, TLR3 plays a key role in both skin and oral localized irritation to metals. Stimulation of skin keratinocytes with gold, mercury, copper and nickel salts activated the innate immune response, although the pathways involved remain unclear. It is known that some TLRs are expressed in distinct layers of the epithelium [27,28]. The research in Chapter 3 was performed with keratinocytes grown in a well plate, thus they may not be expressing all relevant TLRs. It may therefore be interesting to expose gingiva equivalents with a fully differentiated epithelium to the metals, as we have done with allergens in Chapter 5. It would be even more physiologically relevant to study this with the implant models created in Chapter 7, because the peri-implant epithelium may also have a distinct TLR expression pattern compared to gingiva epithelium. It may even be possible to study the effect of leachables from dental implants on the immune activation in this model, but that would most likely require a long culture period of more than several months. Possibly dental implant material that was causing an allergic reaction in patients and was subsequently removed can be used. This would make the results directly relatable to clinical problems.

The TLRs recognize pathogens and are quite certainly part of the innate immune activation observed in Chapter 8, where gingiva equivalents were exposed to commensal, gingivitis and cariogenic biofilms grown under defined conditions from human saliva. The in vitro grown biofilms contained physiological numbers of bacterial species, averaging over 70 operational taxonomic units (OTUs), including twenty differentiating OTUs. When the biofilms were applied topically to the gingiva equivalents for 24 hours, the gingiva epithelium increased its expression of elafin, a protease inhibitor and antimicrobial protein. This increased elafin expression was observed as a response to all three biofilm types. Biofilm exposure also increased secretion of the antimicrobial cytokine CCL20 and inflammatory cytokines IL-6, CXCL8 and CCL2 from gingiva equivalents. This inflammatory response was far greater after commensal biofilm exposure than after pathogenic biofilm exposure. These results show that pathogenic oral biofilms have early immune evasion properties compared to commensal oral biofilms. The underlying mechanisms are currently not fully
understood. It would in the future be interesting to investigate longer biofilm exposure times. In contrast to the reduction in IL-6, CXCL8 and CCL2, the dose dependent increased secretion of the chemokine CCL5 was similar after exposure to the different biofilms. CCL5 is known to be particularly associated with viral infections and induce the in vitro migration and recruitment of T cells, dendritic cells, neutrophils, eosinophils, NK cells, mast cells and basophils \([29,30]\). CCL5 was in contrast to CXCL12, CCL2 and CCL20, also consistently expressed between skin and gingiva equivalents in Chapter 5. The secretion of CCL5 by gingiva or skin equivalents was stimulated by a pro-inflammatory cytokine, but not by an allergen or irritant. CCL5 was not upregulated after wounding of the gingiva equivalent in Chapter 6. The receptor of CCL5 is CCR3, which was concluded in Chapter 2 to be one of the two receptors to play a dominant role in stimulating oral fibroblasts. Since CCL5 is consistently expressed between skin and gingiva, is not affected by the potential immune evasion mechanism of the biofilms, and is a potent stimulator of oral fibroblasts, it may be a key factor in the immune response against infections.

As mentioned above, the secretion of cytokines and chemokines involved in LC migration and general inflammation by oral mucosa and skin equivalents were compared in Chapter 5. Basal secretion, representative of homeostasis, and also secretion after stimulation with a pro-inflammatory cytokine, an allergen or an irritant was assessed. We found that pro-inflammatory IL-18 and chemokines CCL2, CCL20, CXCL12, all involved in Langerhans cell migration, were predominantly secreted by skin as compared to gingiva. Furthermore, CCL27 was predominantly secreted by skin keratinocytes whereas CCL28 was predominantly secreted by gingiva keratinocytes. In Chapter 2 we found that CCL28 is a key chemokine in stimulating oral fibroblasts thus it is likely that CCL28 is an important factor in the paracrine signalling between oral keratinocytes and fibroblasts. Moreover, CCL28 has been found to be a major factor in both the innate and adaptive immune response, combining antimicrobial properties and chemoattractant properties to B and T cells, making it a potential candidate for use as a therapeutic \([31]\).

The general inflammatory cytokine IL-6 and the chemokine CXCL8 were secreted in higher amounts by the gingiva keratinocytes than by the skin keratinocytes in Chapter 3 and Chapter 5. This is consistent with the work of others \([32,33]\). In gingiva equivalents, IL-6 and CXCL8 were up-regulated after wounding (Chapter 6 and Chapter 7), stimulation with saliva (Chapter 7) or general inflammatory cytokine TNF-α (Chapter 5), exposure to a biofilm (Chapter 8), allergen (IL-6) or irritant (CXCL8) (Chapter 5). IL-6 has many
functions and can be pro-inflammatory or anti-inflammatory [34]. CXCL8 is an important factor in attracting neutrophils to the site of inflammation [35]. Clearly our results show that IL-6 and CXCL8 play a constant role in regulating the acute innate oral immune response. This is in contrast to CCL5 that was, as discussed before, only upregulated in response to infection and not after wounding.

In conclusion, these results indicate that the cytokines and chemokines involved in triggering and mediating the innate immune response are different in skin and gingiva. Important chemokines in skin were not expressed in gingiva, while IL-6 and CXCL8 were higher expressed in gingiva than in skin.
Summary

In this thesis we provide insight into the oral wound healing process and innate oral immune response. In summary:

- Chemokine receptors on gingiva fibroblasts were identified.
- CCR3, CCR4 and CCL28 are potential wound healing targets.
- Saliva is a potential wound healing therapeutic.
- Gingiva intrinsically has a higher wound healing potential than skin.
- TLR3 is a key innate immune receptor of both skin and oral keratinocytes, potentially playing a key role in localized irritation to metals.
- The expression of chemokines, stimulating immune cell migration, is differently regulated in gingiva than in skin.
- Inflammatory cytokine secretion by gingiva models is higher after commensal biofilm exposure than after pathogenic biofilm exposure, which may be explained by an immune evasion mechanism of the pathogenic biofilms.

We can conclude that: The superior wound healing qualities of oral mucosa compared to skin are caused by intrinsic characteristics of oral cells in combination with external stimulating factors, such as saliva; CCR3, CCR4, CCL28 and saliva should be investigated for their potential to stimulate wound healing; Not only the wound healing potential of oral mucosa is intrinsically different from skin, but also the innate immune response is differently regulated.

The major goal to develop organotypic gingiva models for the investigation of oral wound healing and interaction with oral biofilms and dental materials was achieved with the use of TERT-immortalized gingiva cells.

Future directions

The in vitro models used in this thesis were focused on the innate properties of the oral mucosa. To be able to incorporate the adaptive immune response, more complex systems are required. By the development of organ-on-chip models that incorporate a flow system, immune cells can be introduced in a way that mimics normal blood and lymph flow [36]. The incorporation of the adaptive immune response will lead to a better translation to the in vivo situation and may in the future lead to improved understanding of oral wound healing and oral immune response.
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