Oral wound healing and innate oral immune response studied in vitro

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CHAPTER 1

Introduction
Chapter 1
General introduction

The oral mucosa lines the inside of the mouth. Its primary function is to form a protective barrier for the body against harmful environmental factors e.g., pathogenic bacteria, mechanical abrasion from food, and harmful chemicals. As part of the oral mucosa, the gingiva (gums) surrounds the teeth. The gingiva directly attaches to the underling bone (Figure 1). The importance of maintaining the gingiva barrier is illustrated in the pathologies that occur when this barrier fails. For instance, when the gingiva is damaged bacteria can penetrate into the tissue. This will cause the secretion of wound healing factors, such as inflammatory cytokines, which activate residential cells of the gingiva and immune cells to remove the infection and restore tissue integrity. During this wound healing process, the gingiva becomes inflamed (gingivitis). When gingivitis is not resolved, it can cause loss of bone tissue (periodontitis) and eventually lead to tooth loss. This example makes it clear that maintaining the gingiva barrier is an important factor in human health.

Due to the constant environmental assault, oral mucosa has developed mechanisms to heal quickly and with relatively little scar formation. Indeed, oral tissue heals faster and with less scar formation than skin tissue [1–6]. 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 [3,4,7]. Finding the factors involved could lead to future wound healing therapies for patients with excessive skin scarring or large wounds. Besides the intrinsic properties of the cells there are many extrinsic factors which may contribute to the superior wound healing qualities of oral mucosa, such as the presence of saliva or the influence of commensal bacteria. Finding

Figure 1: Schematic representation of the gingiva and its relation to the teeth and bone.
out how all the different factors contribute to the oral wound healing process is not possible in human clinical studies. Therefore, animal or *in vitro* (culturing) experiments are required. Animal experiments have some obvious ethical issues and are not directly translatable to the human situation, e.g. the histatin salivary proteins, which stimulate oral wound healing in humans, are only present in primates. In contrast, *in vitro* experiments where human cells are cultured in a controlled environment are potentially suitable to answer fundamental questions about the human physiology. However, for translation of *in vitro* experiments to the human *in vivo* situation, the experimental models must be physiologically relevant. Therefore, complex organotypic models may be required.

In this chapter normal gingiva anatomy, factors involved in the wound healing process, gingiva environmental factors, and *in vitro* models will be further described. Thereafter, the goals and outline of this thesis will be explained.

**Figure 2: Histological overview of human gingiva.** A) Overview of the gingiva epithelium and lamina propria. B) Layered structure of the gingiva epithelium. Scale bar represents 50µm.

**Gingiva**

Human gingiva consists of multiple layers (Figure 2A). The upper layer is the epithelium which forms the protective barrier. This layer consists primarily of keratinocytes, which are responsible for maintaining the barrier. Other cell types within the epithelium are Langerhans cells and Merkel cells. Langerhans cells are immune cells that present antigens to the adaptive immune system. Merkel cells are involved in touch sensation. The epithelium is multi-layered (Figure 2B). In the basal layer of the epithelium, keratinocytes proliferate to provide a continuous supply of new cells. Keratinocytes from the basal layer move towards the surface of the gingiva. During this migration, the
keratinocytes stop proliferating and start to differentiate and in doing so start to flatten and express different proteins, such as keratins 10 and 13, which sustain mechanical stress, maintain structural integrity, and reduce proliferation [8,9]. By continuously shedding and renewing the top layers of the gingiva, the formation of bacterial biofilms on the gingiva suffice is restricted. The epithelium is attached to the basement membrane, which connects it to the underlying extracellular matrix (ECM) of the lamina propria. Within the lamina propria there are blood vessels, lymph vessels and nerves. The gingiva fibroblasts are the most abundant cell type in this layer of connective tissue. Fibroblasts maintain and restore the ECM by breaking down and secreting all components of the ECM, including the primary ECM-protein collagen [10].

**Wound healing**

When the gingiva is wounded it is imperative that the tissue is restored quickly and invading pathogens are removed. Tissue damage causes the release of different pro-inflammatory cytokines, such as interleukin 6 (IL-6) [11]. The inflammatory cytokines recruit and stimulate different cell types to start the wound healing process. The entire wound healing process is a complex interplay between all the different cell types native to the gingiva and the different cell types that are recruited to the site of injury. These complex cellular processes have been roughly subdivided into four successive overlapping stages: coagulation, inflammation, proliferation and remodelling. In general: immune cells such as neutrophils, macrophages and dendritic cells are recruited to remove debris and invading pathogens, keratinocytes close off the wound by increasing proliferation and migrating in a process called re-epithelialization, endothelial cells regenerate blood vessels, and fibroblasts proliferate and migrate into the wound where they remodel the wound tissue and lay down new ECM [11]. In each stage of the wound healing process all the different cell types communicate via the release of numerous cytokines to trigger the different cellular wound healing processes at the appropriate moment in time, severity and location. Each of these cytokines may be able to perform multiple functions, e.g. IL-6 can perform both pro-inflammatory and anti-inflammatory functions [12]. Moreover, some cytokines can trigger positive feedback loops which can stimulate the overall inflammation e.g. IL-6 stimulates fibroblasts to produce IL-6 [13]. It is important that after a stage is completed these signals are downregulated and that eventually tissue homeostasis is restored. When the balance is not restored this may lead to different pathologies such as chronic
inflammation and hypertrophic scarring [14]. The complex interplay between the
different cell types and wound healing factors is a subject of much research, since
knowledge of these processes may lead to new wound healing therapeutics. Taken
together, the gingiva wound healing process involves complex interactions between
different cell types and signalling proteins for the regeneration of the gingiva barrier.

Chemokines

During wound healing, it is important that the different cells involved e.g., keratinocytes,
fibroblasts and immune cells, migrate towards the site of injury [15–17]. This directional
cellular movement is regulated by small proteins called chemokines. Chemokine
receptors located on the cell membrane allow the cells to follow the chemokine gradient
towards its source. Different cell types express different chemokine receptors. In
humans, there are 18 known chemokine receptors. Besides the directional migration of
cells, chemokines have been shown to, increase proliferation and modulate
inflammatory cytokine release [15,18–20]. Therefore, chemokines are key players which
regulate the process of wound healing.

Chemokines are classified according to their protein structure (CC, CXC, XC or CX3C),
followed by L for a ligand or R for a receptor, and then by a number [21]. Some
chemokines interact with only one specific receptor, while others can bind to multiple
receptors (e.g. CXCL8 can bind to CXCR1 and CXCR2). A single receptor may also be able
to interact with multiple ligands. It is of interest to determine how chemokine receptor-
ligand pairs stimulate the different cell types involved in oral wound healing as this will
provide valuable information on the superior wound healing characteristics of oral
mucosa.

Within the gingiva epithelium reside innate immune cells called Langerhans cells.
These cells are a subset of dendritic cells, which capture antigens and present them to
the T-cells of the adaptive immune system. The Langerhans cells have to follow a
chemotactic gradient to come into contact with the T-cells [22]. Little is known about
this migratory process for gingiva Langerhans cells, despite their importance within the
immune response. In contrast, for their skin counterparts it is known that the
chemokines CCL2, CCL5, CCL20 and CXCL12 are responsible for the directional migration
of these cells [23–25]. Therefore, it is interesting to investigate whether Langerhans cell
migration in the gingiva is regulated in the same way.
Another chemokine of particular interest for oral wound healing is CCL28, also named mucosae-associated epithelial chemokine, because of its association with mucosal inflammation [26–28].

Oral bacteria
The oral cavity is home to many different species of bacteria [29]. Per individual hundreds of different bacterial species can live together in the oral cavity. These bacteria interact with each other and can form complex biofilms by producing extracellular matrix. Dental plaque, a biofilm of the oral cavity, may cause different pathologies via the destruction of oral tissue [30]. In gingivitis, bacteria invade and damage the soft gingiva tissue, causing bleeding gums. In cariogenesis, bacteria damage the hard tissues, the teeth, causing caries. In a healthy mouth oral bacteria are also present, however in this case the bacteria do not cause clinical problems. Currently little is known about how these commensal bacteria interact with the host tissue [31]. The complex interaction between the biofilm and the host tissue has a great impact on oral health. Therefore, physiological relevant models are required to study this interaction between oral biofilms and oral tissue. These models may be of use for the discovery and validation of new drugs and antimicrobials.

Saliva
Saliva has many functions which are important for maintaining oral and general health [32]. It contains over a thousand different proteins, such as antimicrobials which reduce the number of bacteria in the mouth, mucins which assist lubrication, and proteins which form a pellicle over the teeth to protect them [33]. It has also been found in animals, such as mice and rats, that saliva can stimulate wound healing. Different proteins in the saliva, such as epidermal growth factor (EGF) or nerve growth factor (NGF), can stimulate keratinocyte and fibroblast wound healing characteristics. However, the concentration of these proteins is more than ten thousand times lower in human saliva than in rodent saliva. In contrast, histatin proteins, which have been proven to stimulate cellular wound healing characteristics, are only present in primate saliva. Considering these physiological differences between humans and rodents, salivary stimulation of wound healing is likely not regulated in the same way. Primarily it is of interest to find out if human saliva has a positive influence on human wound
healing. If this is the case, saliva could lead to new therapies that enhance wound healing.

**Dental implants**

When the teeth are lost, due to periodontitis, caries or trauma, they can be replaced with dental implants. The dental implants restore the aesthetics and function of the teeth and are therefore becoming an increasingly popular dental procedure [34]. Unfortunately dental implants can cause some serious medical problems. They can cause a chronic inflammation or in some cases a suspected allergic reaction due to continuous release of low concentrations (leachables) of metals from the implant. In the case of an allergic reaction dendritic cells take up the allergen and migrate towards the lymph nodes to present it to the T-cells. When T-cells become activated after recognition of the allergen they will start to proliferate and initiate an allergic reaction. Which chemokines exactly stimulate the migration of oral dendritic cells, is not known yet. Another major problem that can be caused by dental implants is peri-implantitis [34]. Because the natural attachment of the oral mucosa and the tooth has been removed the normal barrier function is disrupted, bacteria can invade more easily, which causes inflammation and can subsequently lead to bone loss and eventually implant failure. Therefore, it is important that a barrier between the implant and the surrounding gingiva is restored. *In vitro* models to study and improve implant and soft tissue attachment are currently lacking [35]. There is a need to properly define valid and reproducible pre-clinical models for the assessment of soft tissue regeneration procedures around teeth and implants [36].

**Tissue models**

For the study of gingiva wound healing, it is necessary to investigate cellular responses to specific stimuli. Assays on two-dimensional cellular monolayers are very well suited to find out the cellular responses of one specific cell type to external stimuli, e.g., the cytokine secretion profile of keratinocytes after stimulation with TNF-α, or the migratory speed of fibroblasts after stimulation with CXCL8. Furthermore, monolayer cultures are suitable for high throughput assays, allowing for the fast screening of many different compounds. These properties make monolayer cultures of great value for the investigation of cellular processes during wound healing. Since, as described before, different cell types interact during wound healing, it is also important to investigate the
effects of stimuli on co-cultured cell types. Co-culturing different cell types and tissue engineering three-dimensional organotypic models which closely represent the native tissue (tissue equivalents) is better suited to provide information on whole tissue characteristics than monolayer cultures. However, these complex models make it difficult to elucidate the exact mechanisms behind the observed responses and since they are more difficult to make, these models are less suited for high throughput screening. Taken together, both monolayer cultures and tissue engineered organotypic models are needed to elucidate the processes of oral wound healing.

The different cellular models for the investigation of gingiva wound healing, requires a large number of gingiva cells. Gingiva tissue for the isolation of cells is unfortunately scarce and the yield of the small biopsies is low and regularly infected. Therefore, large scale experiments require an alternative cell source. Cell lines are such an alternative source since these cells keep proliferating and can readily be amplified in bulk culture. However, because cell lines often originate from tumours or acquire tumour characteristics, care must be taken that the obtained results are physiological relevant.

**Thesis outline**

The overall aim of this thesis was to provide insight into the oral wound healing process and innate oral immune response. To achieve this, novel tissue engineered models that can provide such insights have to be developed. Therefore, a major goal was to develop organotypic gingiva models for the investigation of oral wound healing and interaction with oral biofilms and dental materials.

The first two chapters utilise conventional monolayer cultures to provide insight into the individual cellular responses of the gingiva. Fibroblasts, the most abundant cell type of the lamina propria, play an important role in the wound healing process. In Chapter 2 the expression of chemokine receptors on gingiva fibroblasts was investigated. Subsequently these receptors were activated with corresponding ligands to find out if these chemokine receptors could stimulate fibroblast wound healing characteristics: proliferation, migration and the secretion of wound healing mediators. In Chapter 3 the role of keratinocytes, the most abundant cell type of the epithelium, in pathogen recognition was investigated. Recognition of pathogens by cells occurs via different Toll-like receptors (TLR). Which TLRs are involved in skin and gingiva keratinocyte signalling is determined by exposing keratinocyte monolayers to TLR ligands. Furthermore,
different metals were tested to find out if they activate an immune response in skin keratinocytes.

The next two chapters make use of both monolayer cultures and organotypic models to gain insight into the oral and skin wound healing and innate immune response. Since saliva has been shown to be beneficial for oral wound healing in animals, Chapter 4 evaluates whether human saliva contributes to human gingiva and skin wound healing, using monolayer wound healing assays and organotypic wound healing models. Chapter 5 investigates whether the chemokines involved in skin Langerhans cell migration are also secreted in gingiva. Keratinocyte and fibroblast monolayers are stimulated to evaluate their individual cytokine release. Additionally, to incorporate the complex communication between different cell types, we evaluated cytokine release of organotypic skin and gingiva cultures after stimulation with a pro-inflammatory cytokine, irritant and allergen.

To enable large scale experiments with organotypic gingiva cultures for further investigation into the innate immune response and the wound healing of gingiva, Chapter 6 describes the development of gingiva equivalents constructed with gingiva cell lines. Histology and inflammatory cytokine secretion of the cell lines and gingiva equivalents are compared with native tissue counterparts, and the equivalent is used to establish an oral wound healing model. The gingiva equivalents developed in this chapter were used in the following two chapters. Soft tissue attachment to dental implants was evaluated in Chapter 7 after implantation of different materials into the gingiva equivalents. In Chapter 8 the gingiva equivalents were exposed to physiological relevant commensal, gingivitis and cariogenic biofilms for the study of the interaction between gingiva host tissue and pathogens by histology and inflammatory cytokine release. Finally, Chapter 9 summarizes and discusses the results and future perspectives.
References


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