Epidermal Reactions to Injury with Implications for Innate Immunity

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EPIDERMAL REACTIONS TO INJURY WITH IMPLICATIONS FOR INNATE IMMUNITY

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Abstract

The epidermis is one of our primary interfaces towards the external milieu. Following injury, the physical barrier function of the skin is destroyed and the epidermis is left vulnerable to microbial invasion. Inducible innate immune response mechanisms exist to keep the wound site free from infection, thus allowing the wound to heal and the epidermis to re-establish its barrier function. The main focus of the present thesis has been to investigate the regulatory mechanisms of some of these responses. In paper I we present an injury-induced mechanism for increasing the production of antimicrobial peptides (AMPs) in the skin mediated by the epidermal growth factor receptor (EGFR) transactivation process. In paper II we further highlight the importance of this mechanism by demonstrating that it is responsible for the bulk of the expression of the AMPs known to be induced in epidermis during the proliferative phase of wound healing. In addition we show that the EGFR-mediated increase in interleukin-8 (IL-8) production represents the primary source of chemotactic activity towards neutrophils generated in injured human epidermis. Thus a novel molecular link between cutaneous injury and neutrophil accumulation is provided. In paper III we disclose a possible role of the prion protein as an AMP in host defense, based on its antimicrobial properties and EGFR-dependent induction in response to injury. In paper IV we furthermore report an increased expression of several protease inhibitors during wound healing and a change in gene expression in the epidermal tissue representing a shift in the apoptotic balance. The shift indicates a reduced sensitivity to the extrinsic pathway of apoptosis and concomitantly, an apparently increased sensitivity to the intrinsic pathway of apoptosis. Taken together we hypothesize that this represents an epidermal response to cope with the external detrimental effects of inflammation while safeguarding itself against the increased risk of malignant transformation accompanying the increased proliferation. Finally we identify a highly significant overrepresentation of transcription factor binding sites for forkhead box O1 (FOXO1), FOXO4 and STAT5A, in the most differentially expressed genes in injured skin. This indicates, for the first time, that these transcription factors might play a major role in the wound healing process.

Key words: Wound healing, injury, epidermis, keratinocyte, EGFR, antimicrobial peptide, inflammation, interleukin-8, neutrophil, apoptosis, epidermal differentiation, serpin, transcription factor

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Electron micrograph of the antimicrobial peptide human beta defensin-3, released from keratinocyte granules at the cell surface. Black dots represent gold labeled antibodies against human beta defensin-3. The section is from a human *in vivo* skin wound four days after wounding.

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To my Family

Abstract

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Contents

List of Papers

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Paper I
Ole E. Sørensen, Dharma R. Thapa, **K. Markus Roupé**, Erika V. Valore, Ulf Sjöbring, Alice A. Roberts, Artur Schmidtchen and Tomas Ganz
Injury-induced innate immune response in human skin mediated by transactivation of the epidermal growth factor receptor
J Clin Invest, 2006 Jul, 116(7):1878-85

Paper II
K. Markus Roupé, Mads Nybo, Ulf Sjöbring, Per Alberius, Artur Schmidtchen, Ole E. Sørensen
Injury is a major inducer of epidermal innate immune responses during wound healing
J Invest Dermatol, (Epub ahead of print)

Paper III
Mukesh Pasupuleti, **K. Markus Roupé**, Witold Surewicz, Martin Malmsten, Ole E. Sörensen, Ania Chalupka, Artur Schmidtchen
Antimicrobial activity of human prion protein is mediated by its N-terminal region
PLoS One (in press)

Paper IV
K. Markus Roupé, Per Alberius, Artur Schmidtchen, Ole E. Sørensen
Gene expression demonstrates increased resilience toward harmful inflammatory stimuli in the proliferating epidermis of human skin wounds
Submitted to *Exp Dermatol*

Abbreviations

ADAM	A disintegrin and metalloprotease
AMP	Antimicrobial peptide
AR	Amphiregulin
BTC	Betacellulin
CE	Cornified envelope
CGD	Chronic granulomatous disease
DAMP	Danger associated molecular pattern
ECM	Extracellular matrix
EDC	Epidermal differentiation complex
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EPR	Epiregulin
FGF	Fibroblast growth factor
FOSL-1	FOS-like antigen 1
FOXO	Forkhead box O
hBD	Human beta defensin
HB-EGF	Heparin-binding epidermal growth factor
hCAP-18	Human cathelicidin protein 18
HD	Human defensin
HGF	Hepatocyte growth factor
HMGB1	High mobility group box 1
HNP	Human neutrophil peptide
IGF-I	Insulin like growth factor I
INF-γ	Interferon gamma
IL	Interleukin
IP-10	Interferon gamma inducible protein 10
LTB_4	Leukotriene B_4
LXB_4	Lipoxin B_4
MMP	Matrix metalloprotease
NGAL	Neutrophil gelatinase associated lipocalin
NLR	Nodd-like receptor
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen associated molecular pattern
PDGF	Platelet-derived growth factor
PG	Prostaglandin
PLGF	Placenta like growth factor
PMN	Polymorphonuclear neutrophil
PrP^c	Cellular prion related protein

PRR	Pattern recognition receptor
SC	Stratum corneum
SLPI	Secretory leukocyte protease inhibitor
SPRR	Small proline rich repeat
TFBS	Transcription factor binding site
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TLR	Toll-like receptor
VEGF	Vascular endothelial growth factor

Introduction

Background

The skin is the largest organ of the human body and the integrity of the skin is a prerequisite for normal homeostasis (Proksch et al., 2008). The epidermis, being the primary interface between the human body and the environment, is colonized with a multitude of different fungi and bacteria. Recent studies of ribosomal 16 RNA have revealed a great diversity with the species of bacteria alone, ranging over 19 different phyla (Grice et al., 2009). During the course of evolution, the epidermis has developed several innate defense systems to control this diverse microflora in order to prevent the spread and invasion of different pathogenic bacteria and fungi. A fundamental part of this outer defense system is the presence of antimicrobial peptides (AMPs) which can be found in all forms of life (Boman, 2000; Zasloff, 2002) (Ganz et al., 1985) (Hancock and Diamond, 2000). During non-pathological conditions we live in a finely tuned balance with our microbes. When the anatomical barrier preventing the entry of micro-organisms is disrupted by cutaneous injury this balance is broken. If the wound is not healed quickly and the barrier function re-established this imbalance can result in severe infections (Robson, 1997). This has serious consequences for patients with burn wounds or patients with underlying conditions such as diabetes or venous insufficiency, who demonstrate a weakened barrier function and a reduced capacity to heal wounds (Proksch et al., 2008; Falanga, 2005). These patients often suffer from chronic wounds with recurrent infections, and require prolonged treatments with antibiotics (Howell-Jones et al., 2005). Patients suffering from venous ulcers constitute a big health problem with an incidence of over 50 000 patients in Sweden per year (Nelzen, 1994). The prolonged use of antibiotics in the treatment of chronic wounds is generating another big medical problem by creating a biological niche for multi-resistant bacteria. This drives the development of bacterial resistance which extends to some of the last effective antibiotics we have today (Howell-Jones et al., 2005). An increased knowledge of innate immunity of the skin and other processes occurring during normal acute wound healing could contribute to resolving the malfunctions associated with chronic wounds and facilitate our fundamental understanding of wound healing. During infection, inflammation and wound healing the epidermis increases its production of AMPs (Frohm et al., 1997; Liu et al., 1998; Liu et al., 2003; Sorensen et al., 2005; Dorschner et al., 2001) and cytokines. (Giustizieri et al., 2001; Goebeler et al., 2001; Wood et al., 1996; Nickoloff and Naidu, 1994) Our work has focused on delineating the mechanisms that initiate this increase and on elaborating the behavior of the epidermis during the proliferative phase of wound healing.

The skin

The skin consists of two distinct layers, the dermis and the epidermis. They are both firmly attached to and separated by the basement membrane, a thin resilient sheet of crosslinked extracellular matrix (ECM) proteins (Kleinman et al., 1986; Timpl, 1996). The dermis is situated below the epidermis and consists mainly of connective tissue. It is primarily populated by ECM-producing fibroblasts but it also contains blood vessels and harbors the appendages of skin, e.g. hair follicles, sweat glands, sebaceous glands and apocrine glands (Sorrell and Caplan, 2004). Nerve endings and mechanoreceptors for sensing heat and touch are also situated in the dermis. The blood supply of the dermis is far greater than required which enables additional heat regulation by the increase or decrease of cutaneous blood flow as a complement to thermoregulation via perspiration. The blood vessels in the dermis also provide nourishment and waste removal not only for the dermis but also for the cells of the avascular epidermis.

Epidermis

The epidermis constitutes a semi-permeable barrier allowing a modest perspiration while effectively preventing micro-organisms from entering the body. It constitutes our primary interphase to the environment and it is tightly anchored to the basement membrane via integrins. To withstand the wear and tear from our external, often hostile, surroundings the epidermis is built up of several layers of squamous epithelium (keratinocytes) which are maintained by cell division within the basal layer (Figure 1). Proliferative keratinocytes in the basal layer continuously detach from the basement membrane and begin the process of terminal differentiation. This involves the sequential expression of several major protein products necessary for upholding the barrier function (Candi et al., 2005). Detached suprabasal cells begin expressing keratin 1 and 10, which replaces the pool of keratin 5 and 14 as the principal intermediate filaments (Fuchs and Green, 1980) (Fuchs and Cleveland, 1998). As the keratinocytes differentiate and slowly move outwards through the five layers of the epidermis they acquire keratohyalin granules which contain profillagrin. Once released the profilaggrin aggregates keratin 1 and 10 into tight bundles promoting a collapse of the cells into a flattened shape. Meanwhile proteins (e.g involucrin, loricrin, trichohyalin and small proline rich repeat proteins (SPRRs)) are deposited and covalently crosslinked by transglutaminases to the inside of the plasma membrane forming the cornified envelope (CE) (Kalinin et al., 2002). The majority of the genes corresponding to the above mentioned proteins are clustered closely together on a small stretch of 2.5 kilo base pairs on chromosome 1q21. This chromosomal region is called the epidermal differentiation complex (EDC) and contains several of the genes coding for the structural components of the CE and the S100 family of calcium binding proteins (Mischke et al., 1996). Apart from proteins, various lipids such as ceramides, are also produced that become covalently attached to the CE. The

keratinocytes end their journey as corneocytes, dead anucleate, flattened cells in the stratum corneum (SC). This rigid structure of lipids and tightly crosslinked corneocytes provides physical resistance and acts as a water barrier. In healthy skin the corneocytes in the outermost layer of the SC are constantly being shed off and replaced by new cells from underneath in a rate matching the expenditure. The stratified epidermis is thus constantly being rejuvenated (Candi et al., 2005).

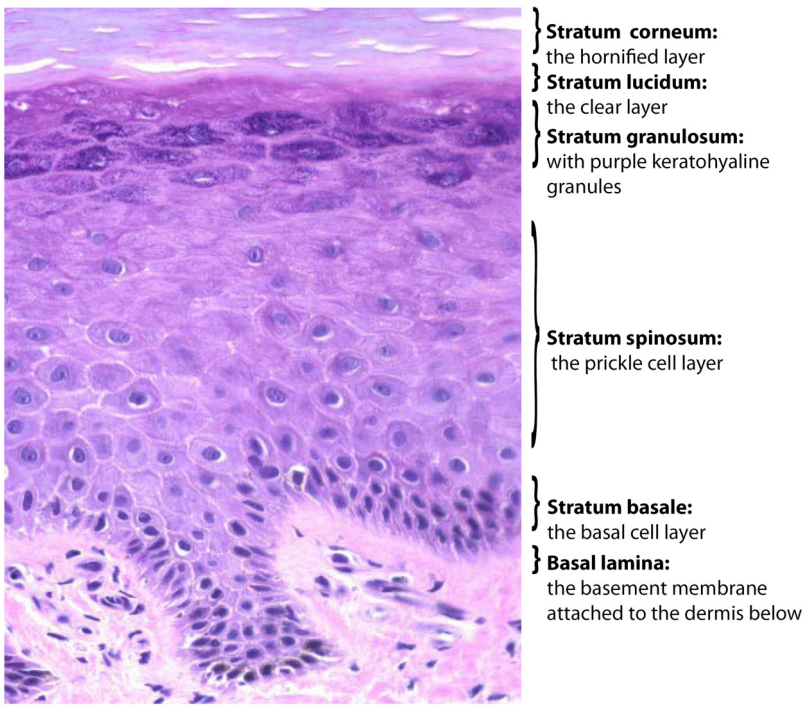

Figure 1. The different layers of epidermis.

Epidermis and innate immunity

In contrast to adaptive immunity, the cells and mechanisms comprising innate immunity do not require prior exposure to an infectious agent in order to prevent infections. Being the primary interface between the body and the environment the human epidermis per se displays several features that can be viewed as innate immune defenses. The limited presence of water, the high content of lipid and the acidic pH in the SC, are all limiting factors for bacterial growth (Wickert and Visscher, 2006; Fuchs, 2007). The tight crosslinking of corneocytes and the presence of incorporated antimicrobial peptides and lipids both in the ECM and

within the corneocytes in the SC are further examples of default obstacles preventing colonization and infection (Elias, 2007). The epidermis thus constitutes a formidable physical and biochemical barrier that few microbes can penetrate. Therefore skin infections rarely arise in the intact epidermis. However, the skin barrier is constantly being subjected to a spectrum of different trauma ranging from chemical agents and electromagnetic radiation to thermal and mechanical injury. This generates footholds for pathogenic or opportunistic bacteria and a possible point of entry for infection of the underlying viable tissue. In order to rapidly respond to infection specialized dendritic cells (Langerhan´s cells) (Wickert and Visscher, 2006), mast cells and tissue macrophages reside in the epidermis. These cells are important initiators of inflammation and are also involved in the recruitment of T and B cells of the adaptive immune system (Medzhitov, 2008). Our increasing knowledge of the innate immune responses in the skin indicate that keratinocytes are far from just bricks in a wall or simple passive bystanders in this process. Therefore, it is now under debate as to which cell types are most important for skin immunity (Schroder et al., 2006). In fact, since keratinocytes carry pattern recognition receptors, both Toll like receptors (TLRs) and Fc receptors, (Cauza et al., 2002; Cauza et al., 2004) and are capable of producing antimicrobial peptides and cytokines, they appear to play a central role in skin immunity (Schroder et al., 2006). Activation of various pattern recognition receptors have also been shown to induce the expression of both AMPs (Abtin et al., 2008) (Schauber et al., 2007; Buchau et al., 2007; Miller et al., 2005; Voss et al., 2006) (Kawai et al., 2002; Mempel et al., 2003) and cytokines (Pivarcsi et al., 2003) (Lebre et al., 2007) in keratinocytes.

Pattern recognition receptors

"Know your enemy" is a saying derived from Sun Tzu's The Art of War (6th century BC) (Sun Tzu, 1910). Accordingly cells involved in innate immunity are able to recognize and respond to microbial components containing pathogen associated molecular patterns (PAMPs) (Akira et al., 2006). These patterns are identified via germ-line encoded cell surface pattern recognition receptors (PRRs) that have evolved to target common molecules essential for microbial survival. These molecules remain evolutionarily conserved as alterations often cause a substantial loss of microbial viability. Of these receptors, the group of TLRs originally found in *Drosophila* (Nussleinvolhard and Wieschaus, 1980), are perhaps the most extensively studied (Medzhitov et al., 1997; Iwasaki and Medzhitov, 2004; Kumar et al., 2009). These transmembrane proteins recognize general bacterial components such as lipopolysaccharide, flagellin, lipoproteins, lipoteichoic acid, and unmethylated CpGDNA (Akira et al., 2006). Apart from recognizing PAMPs, TLRs may also recognize endogenous ligands induced during an inflammatory response such as hyaluronan (Jiang et al., 2005; Scheibner et al., 2006) and hBD2, (Biragyn et al., 2002) categorized as danger associated molecular

patterns (DAMPs) (Matzinger, 1994). While TLRs sense bacterial products at the outer cell surface or within endosomes, another type of PPRs is capable of mediating the cytoplasmic recognition of bacterial products: nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) recognize bacterial cell wall fragments produced during the synthesis or degradation of peptidoglycan (Meylan et al., 2006). Following activation of TLRs and NLRs by ligands of bacterial origin, a cascade of reactions takes place in the host cells encompassing an increased production and release of cytokines and AMPs. The response may also vary depending on which particular receptor that is activated and on the particular cell type involved (Janeway and Medzhitov, 2002; Flacher et al., 2006; Barton, 2008). Expression of TLR-1, 2, 3, 4, 5, 6, 9, and 10 mRNA, but not TLR-7 and 8 have been found in human keratinocytes (Lebre et al., 2007). Recently functional expression of NOD-1 and NOD-2 has also been found (Harder and Nunez, 2009; Voss et al., 2006). The functional responses to different PAMPs have not yet been fully established in keratinocytes, however an increased expression of AMPs has been demonstrated in response to microbial stimuli albeit in vitro (Voss et al., 2006; Kawai et al., 2002).

Antimicrobial peptides

-History

In the end of the 1960s it was discovered that patients with chronic granulomatous disease (CGD) had nonfunctional NADPH oxidase rendering their neutrophils unable to generate a respiratory burst (Steiner et al., 1981). The respiratory burst was believed to be the major mechanism by which neutrophils killed microbes. However, the observation that they were still quite capable of neutralizing microbes indicated that other oxygen independent mechanisms of killing were active. Intense research on these patients soon led to the finding that this killing could be attributed to several basic proteins and peptides (Odeberg and Olsson, 1975). Parallel to this research, Hans G Bohman was researching the immunity of insects, which lack both T and B cells and therefore possess no adaptive immunity. Particularly fascinated by the fact that despite this, insects could live in the feces of animals without succumbing to bacterial infections, he studied innate immunity in butterflies. He was the first to sequence an antimicrobial peptide, which he isolated from the hemolymph of the butterfly Hyalophora cecropia, hence the name cecropin (Steiner et al., 1981). Concomitantly a research group at the University of Los Angeles followed up on the findings from the CGD patients and identified some of the basic peptides in neutrophils and called them neutrophil peptides or defensins (Ganz et al., 1985). When Michael Zasloff later discovered several antimicrobial peptides produced by skin cells of the African clawed frog Xenopus Laevis (which he called magainins from the Hebrew word for shield) it became apparent that these peptides were commonly used in nature as a defense against microbes (Zasloff, 1987).

-Structure and mode of action

Antimicrobial peptides are present in organisms ranging from primitive fungi to man and to date more than 880 AMPs have been identified (Tossi et al., 2009). They often have a broad spectrum of antimicrobial activity encompassing bacteria, eukaryotic parasites, viruses and fungi. Despite being essential components of ancient defense systems and varying considerably in sequence and structure these peptides nonetheless share some common features. Most AMPs are cationic due to an excess of lysine and arginine residues; they range from 12 to 150 amino acids in length of which approximately 50 % are hydrophobic and generally fold into three dimensional structures that are amphipathic (i.e. positively charged, hydrophilic surfaces well separated from hydrophobic ones) (Hancock and Diamond, 2000; Yount et al., 2006). These properties enable them to interact with

membranes, primarily bacterial cell membranes which are hydrophilic and negatively charged on their outer surface and have a hydrophobic core (Hancock, 2001). Based on structural characteristics most AMPs can be broadly categorized as belonging to one of the following four groups; Amphipatic α-helical peptides, β-sheet peptides stabilized by disulphide bonds, peptides enriched in one or two amino acids, and peptide fragments of larger proteins. The exact molecular basis for a given antimicrobial activity is often difficult to determine. Most peptides are believed to exert their activity by a general destabilization of the integrity of fungal, bacterial or viral membranes (Brogden, 2005). This is due to the detergent-like effects of these peptides acting on the phospholipid bilayer. Other peptides are known to form direct pores when they are integrated into membranes, while yet others are not lytic but are translocated over membranes and into the cytoplasm of fungi or bacteria where they inhibit protein synthesis, metabolic pathways or other pathways essential for microbial survival (Gennaro et al., 2002; Hancock and Rozek, 2002; Brogden, 2005). In addition to their antimicrobial activity they often exhibit several other properties including chemotactic (Territo et al., 1989; Yang et al., 2000; Agerberth et al., 2000), growth promoting and angiogenic effects (Koczulla et al., 2003). There are two large families of AMPs in mammals; cathelicidins and defensins. Cathelicidins belong to the α-helical AMPs and in humans the only characterized member is LL-37, which is generated from the proteolytic cleavage of human cathelicidin protein 18 (hCAP-18) by proteinase 3 (Sorensen et al., 2001). hCAP-18 is mainly expressed in leukocytes and in the epithelial cells of the epididymus but has also been found in the gastrointestinal and respiratory tracts and in keratinocytes during inflammatory conditions (Zaiou and Gallo, 2002; Frohm et al., 1997; Bals et al., 1998; Malm et al., 2000; Nilsson et al., 1999). AMPs known to be expressed in the epidermis are listed in Table I. Defensins belong to the β-sheet peptides and are stabilized by three disulphide bridges between six conserved cysteine residues. Depending on the arrangement of these sulphide bridges defensins can be further subdivided into α-defensins and β-defensins (See table I). In humans six α-defensins are expressed; the human neutrophil peptides (HNPs) HNP-1 to 4 found in the azurophil granules of polymorphonuclear neutrophils (PMNs) (Ganz et al., 1985; Wilde et al., 1989) and the human defensins (HD) HD-5 and HD-6 present in the Paneth cells of the small intestine (Mallow et al., 1996). Of the over 30 β-defensins in the human genome only four defensins, human beta defensins (hBDs), hBD-1 to 4, have so far been found at the protein level (Schutte et al., 2002). Pertinent to this thesis, all have been found at epithelial sites of the integument and mucosal surfaces (Bensch et al., 1995; Harder et al., 1997; Harder et al., 2001; Yanagi et al., 2005). In human epidermis hBD-1 to 3 are the most well studied. However keratinocytes also express several other AMPs that are not members of either the defensin or the cathelicidin family. This includes psoriasin, important for the remarkable activity of the skin against *Escherichia coli* (Glaser et al., 2005), RNAse 7 (Harder and

Schroder, 2002), neutrophil gelatinase associated lipocalin (NGAL) (Mallbris et al., 2002), secretory leukocyte proteinase inhibitor (SLPI) (Wingens et al., 1998) the calgranulins (MRP8/MRP14) (Sohnle et al., 1991) elafin (van Bergen et al., 1996) and S100A15 (Buchau et al., 2007). Although hBD-1 and psoriasin are constitutively expressed most of the AMPS of the skin are induced during inflammation and wound healing. Several of these are induced by pro-inflammatory cytokines and growth factors (Liu et al., 2003; Sorensen et al., 2005; Tsutsumi-Ishii and Nagaoka, 2003; Sorensen et al., 2003).

Table I **Antimicrobial peptides and their localization**

Antimicrobial peptide	**aa.***	**Localization**
Alfadefensins		
HNP-1	30	Leukocytes
HNP-2	29	Leukocytes
HNP-3	30	Leukocytes
HNP-4	34	Leukocytes
HD-5	31	Paneth cells, small intestine
HD-6	30	Paneth cells, small intestine
Betadefensins		
HBD-1	36	Epithelium, mucous membranes
HBD-2	41	Epithelium, mucous membranes
HBD-3	45	Epithelium, mucous membranes
HBD-4	49	Epithelium, mucous membranes
Cathelicidins		
LL-37	37	Epithelium, testis, leukocytes
Others		
Psoriasin	100	Epithelium
S100A15	100	Epithelium
Calgranulin A	93	Epithelium, leukocytes
Calgranulin B	114	Epithelium, leukocytes
SLPI	102	Epithelium, mucous membranes
SKALP/Elafin	95	Epithelium, mucous membranes
RNAse7	128	Epithelium
Lysozyme	130	Lekocytes
Lactoferrin	691	Epithelium, leukocytes
Haptocorrin	410	PMNs, mammary epithelium

*Number of amino acids in the mature peptide

Table modified from (Bergman et al., 2008) and supplemented with recent data from data bases

Growth factors and Cytokines

Not surprisingly, the constantly rejuvenating epidermis is a rich source of growth factors and cytokines important for innate immunity, particularly during wound healing and inflammation (Werner and Grose, 2003). Growth factors play a crucial role in promoting the growth and differentiation of cells necessary for the re-establishment of the barrier function of the epidermis while cytokines are important mediators of inflammation. Although fibroblasts, platelets, infiltrating macrophages, neutrophils and other inflammatory cells are major contributors to the multitude of growth factors present at a wound site, keratinocytes are known to greatly increase the production of several of these factors during inflammation and wound healing (Werner and Grose, 2003). This includes: platelet derived growth factor (PDGF) (Ansel et al., 1993), fibroblast growth factors (FGF1,FGF2) (Antoniades et al., 1993), transforming growth factor-α (TGF-α) (Nickoloff and Naidu, 1994), heparin binding growth factor (HB-EGF) (Cribbs et al., 2002; McCarthy et al., 1996; Marikovsky et al., 1993), vascular endothelial growth factor-A (VEGF-A) (Frank et al., 1995; Brown et al., 1992), placenta like growth factor (PLGF) (Failla et al., 2000), insulin like growth factor (IGF-I) (Jennische et al., 1987; Gartner et al., 1992), hepatocyte growth factor (HGF) (Cowin et al., 2001), transforming growth factor β (TGF-β) (Okane and Ferguson, 1997) and activin β_B (Hubner et al., 1996b). Keratinocytes also express proinflammatory cytokines during wound healing such as interleukin-1 alpha (IL-1α), tumor necrosis factor α (TNF-α) (Hubner et al., 1996a), IL-6 (Grossman et al., 1989) and interferon gamma (INF-γ) as well as the anti-inflammatory cytokine IL-10 (Nickoloff and Naidu, 1994). In addition to these cytokines, the epidermis increases production of chemotactic cytokines (chemokines) during wound healing, including interferon gamma inducible protein 10/CXCL-10 (IP-10) (Satish et al., 2003) and, pertinent to this thesis, CXCL-1 (Nanney et al., 1995) and interleukin-8 (IL-8) (Nickoloff and Naidu, 1994). Both CXCL-1 and IL-8 are known to be potent chemotactic agents for PMNs. A summary of the effect of growth factors and cytokines originating from the epidermis during wound healing is found in Table II. Much effort is currently being focused on understanding both the spatial and temporal regulation of the expression pattern of growth factors and cytokines during wound healing since certain expression patterns are associated with healing impairment. However, an altered level of expression of one factor often influences the expression and production of several others. This makes it difficult to delineate the direct effect of a particular growth factor or cytokine on the process of wound healing (Werner and Grose, 2003).

Table II Growth factors and cytokines originating from the epidermis and their effect during wound healing

Process	Growth factors/cytokines involved
Neutrophil infiltration	TGF-β, CXCL1, IL-8, IL6, IL-10 (-)*
Macrophage infiltration	TGF-β, IL-10 (-)
Angiogenesis	VEGF-A, PLGF, FGF-2, HGF , IL-8, CXCL-1, IP-10 (-)
Fibroplasia	PDGF, TGF-β, IGF-I
Matrix deposition	FGF2, IGF-I, TGF-β, Activin
Scarring	IGF-I, TGF-β, Activin, IL-6, IL-10 (-)
Re-epithelialization	FGF-2, TGF-α, HB-EGF, IGF-I, Activin, IL-6, TGF-β (-), IP-10 (-)

* The growth factor/cytokine exerts a negative influence on the process

Table modified from (Werner and Grose, 2003)

Wound healing

Cutaneous injury disrupts the structural integrity of the skin and causes the loss of its two major functions; i.e. preventing dehydration and barring entry of micro-organisms. A rapid re-establishment of these two functions is therefore of outmost importance to prevent infection and to maintain homeostasis. Traditionally the complex process of wound healing is divided into four sequential yet overlapping phases; coagulation, inflammation, proliferation and the more long term remodeling of the scar tissue (Falanga, 2005).

-Coagulation

Immediately upon injury the repair process is set in motion by the plasma exudate. A rapid activation of both the coagulation system and the contact system leads to the release of various growth factors, cytokines and low molecular weight compounds. Platelets degranulate and aggregate at the site of injury forming a mass and the crosslinking of fibrin-fibronectin forms a clot stopping further blood loss. The clot also provides structural support for the wound until collagen is deposited and forms a matrix for migratory cells to crawl across. In addition, the clot functions as a reservoir of growth factors. Furthermore, the degranulating platelets and the proteolytic cascades of the coagulation and contact systems release AMPs (Nordahl et al., 2004; Tang et al., 2002) as well as proinflammatory and chemotactic factors such as thromboxane, C3a, C5b, serotonin, bradykinin,

prostaglandins and histamine. This contributes to the recruitment of inflammatory cells and the initiation of the inflammatory phase (Falanga, 2005).

-Inflammation

Although considerable progress has been made in our understanding of what triggers and perpetuates inflammation in response to infection, less is known about the mechanisms initiating and driving inflammation in response to tissue injury (Medzhitov, 2008). Activation of PPRs on tissue-resident mast cells and macrophages is the main trigger of release of inflammatory mediators from the intracellular granules of these cells during infection. The triggering mechanisms after injury are more elusive and are incompletely understood. Endogenous inducers originating from stressed and damaged cells are believed to play an important role. These include cellular constituents released from necrotic cells like ATP, K^+ ions, uric acid and high mobility group box 1 protein (HMGB1). S100 calcium binding proteins like the calgranulins (S100A8, S100A9) and S100A12 also belong to this category (Rock and Kono, 2008; Bianchi, 2007). Other contributing stimuli, like the general ischemia or components released from the damaged ECM, likely also play a role (Medzhitov, 2008; Jiang et al., 2005; Jiang et al., 2007). Fundamentally, acute inflammation is perhaps best described as a response, which is triggered by infection or tissue injury and involves the coordinated delivery of blood components (i.e. plasma and leukocytes) to a site of infection or injury. This is accomplished by the release of inflammatory mediators such as chemokines and pro-inflammatory factors e.g histamine, serotonin, TNF-α leukotrienes, and prostaglandins. These mediators subsequently give rise to the characteristics of an acute inflammation; i.e. vasodilation, increased permeabilization of blood vessels, sensitization of nocireceptors and activation of endothelial cells. Plasma proteins normally restricted to the blood vessels exude through the permeabilized vessels. Activated endothelial cells allow selective extravasation of neutrophils while preventing the exit of erythrocytes. By expressing selectins and later integrins on their luminal cell surfaces, endothelial cells enable neutrophils to stick to the blood vessel wall. Attracted by fibronectin, prostaglandins (e.g prostaglandin E_2 and D2 (PGE_2, PGD_2)) and leukotrienes e.g (leukotriene B_4 (LTB_4)), kinins and chemokines (e.g IL-1, IL-8 and TNF-α), neutrophils extravasate into the tissue and start migrating towards concentration gradients of chemokines and the actual site of infection or injury (Medzhitov, 2008; Levy et al., 2001).

-Polymorphonuclear neutrophils

Polymorphonuclear neutrophils (PMNs) are thus the first leukocytes to infiltrate the inflamed tissue arriving within a few minutes and becoming the predominant cell type at the wound site during the first two days after injury. Neutrophils are

professional phagocytes specialized at clearing the tissue from cell debris, foreign particles and microbes. In order to rapidly kill pathogens and clear the wound from damaged tissue they release the toxic contents of their granules that include reactive oxygen and nitrogen species, and potent proteases; proteinase 3, cathepsin G and elastase (Nathan, 2006). These potent effector molecules do not distinguish between host cells and pathogens, healthy or damaged cells. Collateral damage to host tissue is therefore an intrinsic part of inflammation (Nathan, 2002). Neutrophils produce and secrete growth promoting cytokines and growth factors which likely represents a compensatory mechanism for these effects (Theilgaard-Monch et al., 2004). However, if left unchecked they can cause further tissue damage and increase the risk of the injury resulting in a chronic non-healing wound (Wlaschek and Scharffetter-Kochanek, 2005). Resolving inflammation is thus important for successful wound healing (Medzhitov, 2008). Several findings also indicate a built-in self-limiting signaling program in neutrophils. Apart from having a chemotactic effect on PMNs, PGD_2 also has been shown to override granulocyte survival signals, which eventually leads to caspase mediated apoptosis (Ward et al., 2002). Furthermore PGE_2 and PGD_2 initiate a gradual switch in the neutrophilic production of lipid mediators. The switch from the pro-inflammatory leukotriene, LTB_4 to the anti-inflammatory lipid, Lipoxin B_4 (LXB_4) causes reduced vascular permeability and limits further recruitment of neutrophils (Levy et al., 2001). LXB_4 also promotes the infiltration of monocytes and stimulates their ability to phagocytose and clear the wound of apoptotic neutrophils. Thus the beginning of inflammation has also been proposed to mediate the end (Serhan and Savill, 2005).

-Macrophages

Approximately two days after injury the infiltrating monocytes that mature into macrophages replace neutrophils as the predominant cell type at the wound site. The macrophages continue the task of clearing the wound by the phagocytosis of any lingering bacteria, damaged tissue and the apoptotic neutrophils. The uptake of apoptotic cells promotes a reprogramming of the macrophages and a transformation from an inflammatory phenotype to either a reparative or an emigratory type. Phagocytosis of the apoptotic cells thus leads to increased macrophage production and secretion of angiogenic VEGF and anti-inflammatory TGF-β (Fadok et al., 1998; Huynh et al., 2002; Voll et al., 1997). Stimulated by low oxygen concentrations, macrophages release several growth factors and cytokines that increase the pace of angiogenesis. Other growth factors and cytokines released by macrophages promote migration and proliferation of fibroblasts and keratinocytes. Neutrophils and macrophages have thus long been considered to be crucial coordinators of the repair process (Rappolee et al., 1988). Interestingly it should be noted that PU.1-knockout mice which essentially are deficient in both neutrophils and macrophages display a similar time course or even

an enhanced rate of wound repair, in the absence of microbes. This results in what appears to be a scar-free healing of the wound (Martin et al., 2003) and implies that other sources of growth factors must be more involved in the wound healing process.

-The proliferative phase

During the proliferative phase the secretion of inflammatory mediators subsides and the numbers of neutrophils and macrophages decline. Stimulated by growth factors and hypoxia, dermal fibroblasts proliferate and enter the wound site migrating across the fibrin scab while depositing large amounts of a provisional ECM consisting mainly of hyaluronan and fibronectin (Singer and Clark, 1999). This highly hydrated matrix facilitates migration and is gradually reinforced with type III collagen (Falanga, 2005). Meanwhile endothelial stem cells from uninjured blood vessels push through the ECM attracted by growth factors released both by platelets and macrophages. The hypoxia and the presence of lactic acid stimulate their proliferation, sprouting new blood vessels necessary for sustaining the proliferating fibroblasts and the increasing amounts of keratinocytes migrating in from the edges of the wound. The keratinocytes move in between the fibrin scab and the rudimentary granulation tissue. The keratinocytes behind the leading edge proliferate in response to the growth factors released by the platelets, fibroblasts and macrophages. Basal keratinocytes disassemble their hemidesmosomes anchored in the basement membrane and their desmosomes that are attached to neighboring keratinocytes. To facilitate their migration keratinocytes also express matrix metalloproteases (MMPs) capable of degrading and modifying ECM proteins. Once the leading edges of keratinocytes meet under the scab the migration is inhibited by contact inhibition (Perrais et al., 2007). The keratinocytes produce basement membrane proteins, revert to their normal phenotype and start the process of re-epithelialization. They re-establish their hemidesmosomes and desmosomes and the basal keratinocytes once again begin to differentiate, increasing the gene expression of several of the genes of the EDC including the specific gene products of the cornified envelope that restore the barrier function. During the intervening time, stimulated by TGF-β, several of the fibroblasts differentiate into myofibroblasts that are similar to smooth muscle cells. They establish multiple attachments to the ECM and to other myofibroblasts and start to contract, pulling the wound edges together reducing the wound size and the distance that keratinocytes need to travel to close the wound. The contraction continues after re-epithelialization and is reinforced by collagen-deposition from fibroblasts (Falanga, 2005).

- The remodeling phase

The wound now enters the remodeling phase in which the granulation tissue matures into tissue more resembling the original tissue. The temporarily laid down type III collagen is degraded by collagenases and replaced by the stronger type I collagen. Disorganized collagen fibers are crosslinked, rearranged into larger collagen bundles and aligned along tension lines increasing the tensile strength in the new tissue. The provisional matrix is supplemented by glucosaminoglycans and elastin. Fibroblasts and redundant blood vessels undergo apoptosis leaving behind a tissue mainly consisting of extracellular matrix. This phase continues for a considerable time lasting for a year or longer and the tissue can regain as much as 60 % of its initial tensile strength (Falanga, 2005).

The Epidermal growth factor receptor

An important receptor during growth and development and during the wound healing process is the epidermal growth factor receptor (EGFR) (Pastore et al., 2008). Originally named for its ability to bind epidermal growth factor (EGF) (Carpenter et al., 1975) it was soon also discovered that ligand binding caused autophosphorylation of the receptor (Carpenter et al., 1978). Since then a variety of ligands apart from EGF have been shown to bind and stimulate the EGFR. This includes its more potent agonist, TGF-α (Derynck, 1992), HB-EGF (Higashiyama et al., 1991), betacellulin (BTC) (Shing et al., 1993) amphiregulin (AR) (Yarden and Sliwkowski, 2001) and epiregulin (EPR) (Shelly et al., 1998) among others. The EGFR belongs to the tyrosine kinase superfamily and it is also the founding member of a smaller subfamily of four receptors, all with homology to the EGFR and therefore named HER receptors. HER receptors form homo - or hetrodimers with each other when binding ligands and this activates intrinsic tyrosine kinase domains, resulting in autophosphorylation of tyrosine residues on the cytoplasmic tail of the dimerized HER receptors. Adaptor proteins containing Src homology 2 - and phosphotyrosine binding domains subsequently bind to the receptors and initiate multiple signaling pathways within the cell (Normanno et al., 2006). An alternative pathway of activation occurring through intracellular signaling via G-protein coupled receptors was reported in 1997 (Prenzel et al., 1999). This mechanism was independent of exogenously added EGFR ligands and was thus called transactivation of the EGFR. However the same research group later discovered that the transactivation required cleavage of endogenous membrane-bound ligands that once released bound and activated the EGFR (Daub et al., 1996). A disintegrin and metalloprotease (ADAM) proteins were also found to be capable of shedding the ectodomains of ligands of the EGFR. These membrane-anchored proteases have been implicated in the shedding of six of the seven EGFR ligands (TGF-α, EGF, HB-EGF, BTC, EPR and AR) in response to several diverse physiological stimuli (Blobel, 2005). The released ligands can then either be involved in autocrine, juxtacrine or paracrine signaling. Salient evidence for the in vivo relevance of this signaling came from experiments with ADAM 17 $^{-/-}$ knockout mice (Peschon et al., 1998). These mice were found to have a similar phenotype as mice lacking either TGF-α or EGFR. This implies that the transactivation of the EGFR via ADAMs may constitute the principal mechanism of EGFR-activation. The EGFR is expressed in most cells apart from mature hematopoietic cells. In skin, the EGFR can be found throughout the epidermis but it is more pronounced in the basal cell layers (Nanney et al., 1984). Skin injury is one of the physiological stimuli that activates ADAMs and leads to transactivation of the EGFR. Although the exact mechanism is not yet known, it has been demonstrated that a simple breach in a monolayer of keratinocytes is sufficient to cause transactivation of the EGFR (Tokumaru et al., 2000).

Present investigation

-The forming of a hypothesis

The basis for this thesis emanated from the finding in 2003 that common growth factors known to be present during wound healing, such as IGF-I and TGF-α induced expression of antimicrobial peptides in human keratinocytes. (Sorensen et al., 2003). Furthermore Tokumora and coworkers determined that injury of the epidermis, or even a simple breach in a monolayer of keratinocytes, results in shedding of EGFR ligands by membrane bound metalloproteases (ADAMs) and transactivation of the EGFR (Tokumaru et al., 2000). Since TGF-α is an EGFR ligand, we therefore hypothesized that keratinocytes could be capable of initiating AMP production as a direct reaction to sterile injury and that this response could rely on the transactivation process of the EGFR.

-Increased production of three antimicrobial peptides are induced via transactivation of the EGFR in the skin in response to injury (Paper I)

Our hypothesis was investigated in a novel model of wound healing using skin obtained from surgical residua. We "sterilized" the skin in ethanol before cutting the skin into small pieces and incubating it in serum free keratinocyte medium. We found prominently increased epidermal expression and production of the human AMPs: NGAL, hBD-3 and SLPI in keratinocytes and the expression peaked after four days. This was investigated both with Northern blot, Western blot and immunohistochemistry. Consistent with our hypothesis the increase could be abolished by adding EGFR-neutralizing antibodies or the specific EGFR inhibitor, AG-1478, which blocks the cytosolic tyrosine kinase activity of the EGFR. However, it could be justifiably argued that there is no such thing as a completely sterile wound nor is it possible to fully sterilize skin with ethanol. Propionobacteria residing in the sweat glands and the sebaceous glands (Bruggemann et al., 2004) and the commensal *Finegoldia magna* dwelling beneath the epidermis (Karlsson et al., 2007) are barely effected by this treatment. In retrospect it was perhaps unfortunate to denote the model as being sterile wounded skin in culture. A better phrasing might have been wounded skin under antiseptic culture conditions. Irrespective of the semantics, a considerable drawback with our model system was that it could be disputed whether the release of EGFR-ligands and induction of AMPs was elicited by keratinocytes in response to injury. Langerhans cells and dendritic cells residing in the tissue could, via their pattern recognition receptors, respond to the microbial presence and initiate a response (Liu et al., 2003) (Sorensen et al., 2005). Injury could also promote the contact between resident bacteria and TLRs on keratinocytes leading to their activation. Thus, it was neither

clear what cell type nor whether it was the actual breach of the epithelial lining or the presence of bacteria that triggered the response. In order to identify the source of the response we used a keratinocyte organotypic airlifting model where we could make an incision in a differentiated keratinocyte culture without the presence of confounding immune cells and microbes. Indeed, in this model prominent staining of hBD-3 was found around the edges of the sterile incision after four days. Hence, we were convinced that the induced expression of AMPs could be ascribed to the sterile breach of the epithelium. To validate that our novel antiseptic *ex vivo* wound model reflected *in vivo* wounding we performed "sterile" wounding in mice. Here we saw a similar increase in the mouse orthologs of NGAL, (termed 24p3 in mice) and SLPI. This could also be observed in the equivalent mouse *ex vivo* model. However no functional murine beta defensin had so far been identified to provide us with a mouse ortholog of hBD-3. Based on homology in the primary sequence, mBD-14 was suggested to be the murine ortholog (Boniotto et al., 2003; Hinrichsen et al., 2008). This was also recently proposed by Rohrl et al (Rohrl and Hehlgans, 2008). However we could not detect any mBD-14 in murine skin nor any induction in response to wounding *in vivo*. Instead, we confirmed our hBD-3 *ex vivo* findings in a human *in vivo* skin wound model. This demonstrated that the *ex vivo* model was mirroring the *in vivo* situation. In the *ex vivo* model we found that endogenously shed HB-EGF was the main cause of the activation of the EGFR and chiefly responsible for the subsequent increased expression of hBD-3. In *ex vivo* wounded skin, we found relevant antimicrobial concentrations of hBD-3 and consequently we could demonstrate an EGFR dependent antibacterial effect against *Staphylococcus aureus*, in extracts from organotypic epidermal cultures.

-The majority of antimicrobial peptides induced during wound healing are dependent on EGFR-activation (Paper II)

The EGFR dependent induction of SLPI, NGAL and hBD-3 became the starting point for our next study, spawning the idea that there might be additional innate immune responses induced by this pathway. To investigate this we utilized mRNA microarray on *in vivo* wounded skin. To assess if induced genes were dependent on injury-induced EGFR-activation we also performed microarray analysis on human *ex vivo* wounded skin. The *ex vivo* model was associated with two major benefits; A) it was possible to block the EGFR and B) the infiltrating inflammatory cells would be absent from this model. This model allowed us to determine that the majority of the AMPs previously known to be induced during wound healing had EGFR dependent expression. This was confirmed in keratinocyte cultures where we found increased expression of all these AMPs in response to the potent EGFR ligand TGF-α. Furthermore, we found induced expression of three additional AMPs not previously known to be induced in skin during wound healing: lactoferrin, S100A15, and haptocorrin.

-The major chemotactic activity for neutrophils in wounded epidermis can be ascribed to injury induced EGFR dependent IL-8 production (Paper II)

While examining the expression of cytokines in skin wounds we found strong EGFR dependent induction of IL-6 (Grossman et al., 1989), CXCL-1 and IL-8. The latter two are both well known potent chemoattractants for neutrophils (Anisowicz et al., 1987; Richmond et al., 1988; Schroder and Christophers, 1986) (Baggiolini et al., 1989; Gillitzer et al., 1996). Consequently we found potent chemotactic activity towards neutrophils in the medium generated from the *ex vivo* injured skin. The addition of IL-8 neutralizing antibodies to the medium almost abolished the chemotactic activity whereas antibodies against CXCL-1 had no significant inhibitory effect. The generation of chemotactic activity in the injured skin could also be strongly inhibited by adding an EGFR inhibitor to the culture. By stimulating keratinocyte cultures with TGF-α we also found a rapid increase in CXCL-1 and IL-8 mRNA expression levels. We could thus demonstrate that injury induced EGFR-activation was responsible for the majority of the chemotactic activity towards neutrophils in the *ex vivo* injured skin.

-The injury-induced expression of antimicrobial peptides and chemotactic activity during wound healing is differently regulated in murine skin (Paper II)

To determine the functional importance of the EGFR dependent induction of chemotactic activity and AMPs found in human skin we investigated these injury induced responses in murine skin. However, although we found both induced expression of AMPs and increased chemotactic activity in response to injury in murine skin, we found that neither of these responses were dependent on the EGFR-activation in murine skin. Furthermore, we found that unlike the human epidermis the murine epidermis did not trigger an increased expression of AMPs in direct response to microbial stimuli, neither *ex vivo* nor *in vivo*. Taken together this emphasized the importance of injury for initiating epidermal innate immune responses but also demonstrated that the pathway regulating these responses may vary between species. Caution should therefore be taken when extrapolating conclusions drawn from epidermal mouse data to the human condition.

-A novel possible function identified for the cellular prion-related protein (Paper III)

The prion related protein (PrP^c) is ubiquitously expressed in the human body but so far the exact physiological role of PrP^c has not been established. In paper III we disclose a potential novel role for PrP^c in host defense. The exposed cationic and heparin-binding N-terminus of the protein is shown to have antimicrobial and antifungal effects. The recombinant prion protein is capable of killing

Pseudomonas aerginosa, Escherichia coli, Staphylococcus aureus and *Bacillus subtilis* as well as the fungi *Candida albicans* and *Candida parapsilosis*. In this study, we demonstrated increased EGFR-dependent expression of PrP^c during wound healing. This expression pattern of PrP^c is consistent with our previous findings in paper II where we found that the majority of AMPs induced in response to injury were induced via the EGFR pathway. Together with the antimicrobial effect against both Gram positive and Gram negative bacteria as well as against fungi we therefore propose a novel possible role for PrP^c as an AMP in host defence.

-The epidermis elicits protective countermeasures against inflammation in response to injury (Paper IV)

In order to re-epithelialize the wound bed and re-establish the barrier function, keratinocytes need to proliferate and differentiate in the presence of inflammatory cells. In paper II we found that in response to injury the epidermis recruits neutrophils to the wound via EGFR dependent IL-8 production. We therefore hypothesized that injury might also elicit an epidermal response as a countermeasure to the detrimental effects of the ensuing inflammation. Accordingly, we found increased expression of several serine protease inhibitors including members of the intracellular serpin B family. Interestingly we found induced expression levels of serpin B1, which is able to inhibit all known proteases from the azurophilic granules of neutrophils and of serpin B9 known to limit the cytotoxic effects of both granzyme B and elastase released from inflammatory cells (Silverman et al., 2004; Sun et al., 1996). We also found extracellular protease inhibitors of neutrophil proteases such as PAI-1, α_1-antitrypsin and also the EGFR-dependent expression of SLPI and elafin. The majority of the protease inhibitors induced *in vivo* were also induced in the *ex vivo* skin injury model, indicating that this represented an inherent epidermal response to injury activated even in the absence of infiltrating inflammatory cells. As expected, the data also revealed that there was a significant overrepresentation of genes associated with the cell cycle and mitosis. However the expression of markers from the basal proliferative layer was not increased. Interestingly, markers for the spinous layers and for the cornified layer were instead found to be increased. Thus there did not seem to be the simple inverse relationship between proliferation and differentiation that we might have anticipated. In line with this we also found induction of several of the genes belonging to the EDC in injured skin *ex vivo*. Several of the EDC-genes were also shown to be EGFR-dependent demonstrating that EGFR-activation in human skin is not limited to activation of innate immune responses and migration but also plays a role in the differentiation and formation of the cornified envelope (Mischke et al., 1996). Thus proliferation and differentiation occurs concomitantly during the proliferative phase of wound healing. We were surprised

to find that genes responsible for the execution of the extrinsic pathway of apoptosis were down-regulated. Conversely the opposite was found for genes involved in the intrinsic pathway of apoptosis. This shift likely causes the epidermis to be less sensitive to harmful external stimuli while rendering it more sensitive to internal apoptotic cues e.g. DNA-damage or intracellular stress. We also found matrix metallo-protease 1 (MMP-1) and MMP-3 to be EGFR dependent in their expression. A transcription factor binding site (TFBS) analysis of the 100 most differentially expressed genes further revealed possible undisclosed roles for STAT-5, SRY and members of the FOXO family of transcription factors in the process of wound healing. None of them have previously been associated with wound healing but they were all found to have binding sites in more than 99% of the promoters of the genes analyzed.

General discussion

-Toll-like receptors and antimicrobial peptides

Antimicrobial peptides were originally found in insects by Hans G. Boman and coworkers (Steiner et al., 1981) and were later also discovered in mammals (Lehrer et al., 1983). Shortly after the delineation of the toll receptor pathway in *Drosophila melangaster*, by Christiane Nüsslein-Volhard and others (Nussleinvolhard and Wieschaus, 1980) it was discovered that the toll receptor was not just important for embryogenesis, growth and development but was also highly involved in innate immunity (Lemaitre et al., 1996). The currently prevailing view is that two different proteolytic pathways are activated in the hemolymph of *Drosophila* either upon binding of microbial molecules to three different PPRs or by the abnormal proteolytic activity of microbial proteases. Both cascades converge, eventually cleaving the circulating cytokine-like protein pro-Spätzle, generating considerable amounts of the toll ligand Spätzle. Activation of the Toll receptors on hemocytes and the cells in the fat body leads to the nuclear translocation of the NF-κB-related transcription factors, Relish and Dorsal-related immune factor, respectively. This results in the transcription of hundreds of genes, including those encoding effector antimicrobial peptides directed against the intruding micro-organisms (El Chamy et al., 2008). The early findings that the Toll receptor could induce AMPs in *Drosophila* and the cloning of the first human TLR with its interleukin-1-homologous cytoplasmic domain greatly influenced the emerging field of AMPs (Hultmark, 2003; Lemaitre et al., 1996; Medzhitov et al., 1997). This came to associate the TLRs with a direct induction of AMPs. It should however be noted that these two receptor types are more different then their name implies. Whereas the human and mammalian TLRs are in fact PPRs activated upon the direct binding of microbial derived molecules, the Toll receptor in *Drosophila* relies on the binding of an endogenous cytokine/growth factor-like molecule for its activation. Here the pattern recognition step has already occurred in the hemolymph. In *Drosophila* it was soon elegantly illustrated that specific innate immune responses were initiated depending on the microbial molecules recognized by the PPRs in the hemolymph, with a differential, particular release and production of AMPs (Lemaitre et al., 1997). In spite of the differences between the toll receptor and the TLRs this generated high expectations that different TLRs could confer a similar directed AMP production against various pathogens during infection in mammals.

-AMPs as a first line of defense

Soon after Boman's discovery, it was hypothesized that epidermal-derived AMPs, being germ-line encoded and effective against a broad spectrum of microbes,

would be the ideal way of achieving a rapid protection of a wound site. Amphibian evidence indicating this possibility was already provided by Zasslof in 1990 (Bevins and Zasloff, 1990). Having previously found the presence of antimicrobial magainins in the skin of the frog *Xenopus Laevis* (Zasloff, 1987) he also reported that large amounts of these AMPs were released from intradermal glands in frog skin upon nervous stimuli in response to stress or injury (Bevins and Zasloff, 1990). Induced expression was also found for the lingual AMP (LAP) surrounding lesions in bovine tongue (Schonwetter et al., 1995). These findings had a big impact and gave rise to the idea that induced epidermaly derived AMPs could function as a first line of defense following stress, infection/inflammation and injury also in mammals.

-Inducible expression of AMPs found in human skin

Indeed several human AMPs were subsequently found in human skin and at other epithelial sites. Harder and Schröder hypothesized that the inflamed skin of psoriasis patients could be a rich source of AMPs. They argued that this would explain the low incidence of skin infections in these patients in spite of their compromised skin barrier. Thus the perhaps most well studied human AMPs, hBD-2 and hBD-3, were originally found and isolated from psoriatic scales (Harder et al., 1997; Harder et al., 2001). While some AMPs, like hBD-1 (Zhao et al., 1996) and psoriasin (Glaser et al., 2005) appeared to be constitutively expressed, several others were indeed found to be generated by induced *de novo* synthesis at sites of inflammation/infection and wound healing, either by epithelial cells e.g. like the hBD-2 and hBD-3 peptides or by monocytes/macrophages e.g. hCAP-18/LL37 (Frohm et al., 1997; Liu et al., 1998; Liu et al., 2003; Sorensen et al., 2005; Dorschner et al., 2001).

-TLRs induce the expression of human AMPs in vitro

AMP expression in epithelial cells was also found to be induced by direct stimulation with microbes and microbe-derived molecules, at least experimentally in cell cultures (O'Neil et al., 1999; Harder et al., 2000; Krisanaprakornkit et al., 2000; Hertz et al., 2003; Zilbauer et al., 2005). Induced expression was also observed through signaling of TLRs (Hertz et al., 2003; Miller et al., 2005), NOD receptors (Boughan et al., 2006) and through protease activated receptors (Chung et al., 2004) *in vitro*.

-Cytokines and growth factors induce human AMPs

Despite the constant presence of microbes at epithelial sites only limited or low expression levels of AMPs are for instance found in intact, non-inflamed, healthy skin. Other investigations have also emphasized that direct microbial stimuli do not yield a significant production of AMPs in epithelial cells (Liu et al., 2003). A more prominent induction was instead found via indirect microbial stimuli through

activated Langerhans cell, dendritic cells or monocyte-derived cells residing in epithelial tissues. Upon recognition of microbial patterns via their PRRs these cells release various mediators and inflammatory cytokines that in turn induce significant levels of AMPs in the epithelial cell (Liu et al., 2003; Sorensen et al., 2005; Tsutsumi-Ishii and Nagaoka, 2003). This induction of AMPs is indeed more similar to the original findings in *Drosophila* where the pattern recognition step is separated from the AMP-inducing step and where the activation is triggered by the binding of a cytokine/growth factor-like molecule to the receptor. Interestingly, different inflammatory mediators elicit production of different AMPs which again is similar to the original findings in *Drosophila* (Sorensen et al., 2005). However the epidermal induction of human AMPs following stimulation with inflammatory mediators requires *de novo* synthesis of AMPs. Human skin furthermore lack the neuroendocrine secretory glands found in *Xenopus Laevis* with their large amounts of premade AMPs (Bevins and Zasloff, 1990).

-Injury is via activation of EGFR a major inducer of human AMP expression during wound healing

Since microbes colonize the skin, it is not a question of if but rather when micro-organisms colonize a wound site. Accordingly, we reasoned from an evolutionary perspective that the production of AMPs should be initiated immediately as a direct reaction to injury. Indeed in paper I we found a highly increased production of AMPs in response to injury which was independent of TLRs and instead dependent on transactivation of the EGFR. Having found that an injury-induced mechanism increased the AMP-production we wanted to investigate whether it was a minor or a major contributor to the overall expression of AMPs during wound healing. Inflammation is a potent inducer of AMPs and likely enhances the transactivation of the EGFR. LL-37 generated through the proteolytic cleavage of hCAP-18 (Sorensen et al., 2001) released from infiltrating neutrophils has for instance been shown to cause transactivation of the EGFR (Tjabringa et al., 2003). Neutrophil elastase from the azurophil granules can also directly cleave off EGFR ligands from keratinocytes resulting in EGFR-activation (Meyer-Hoffert et al., 2004). In paper I we found hBD-3 to be solely dependent on EGFR-activation during wound healing and its expression was also previously known to be EGFR-dependent after stimuli from inflammatory cells (Sorensen et al., 2005). However, other AMPs like SLPI and Elafin are known to be induced by pro-inflammatory cytokines (Sallenave et al., 1994). When looking at the time course of expression of AMPs during wound healing, we found that the majority reached their peak mRNA levels during the proliferative phase, following the inflammatory phase of wound healing. Thus it was unclear whether injury or stimulation from inflammatory cells was the major inducer of the increased expression of AMPs in keratinocytes during wound healing. In paper II we found that the induction of all AMPs known to be significantly expressed in wounded skin was EGFR-dependent with the exception

of hBD-2, S100A15, haptocorrin and lactoferrin. We also found that the EGFR-dependent AMPs were prominently induced in injured skin *ex vivo* where the amount of infiltrating inflammatory cells was very limited. We thus concluded that the injury in itself was the major inducer of AMP expression through EGFR-activation.

-The identification of a novel epidermal AMP

The microarray allowed us to look at all AMPs known to be induced during wound healing in skin and identify most of them as EGFR dependent in their expression. Many AMPs also have structural motifs in common and several of them are heparin-binding (Andersson et al., 2004). However to be physiologically relevant they of course also need to be expressed at the right time and place and also at a high enough concentration. Due to the presence of a heparin-binding motif in PrP^c (Pan et al., 2002) and reports demonstrating increased expression of PrP^c during both bacterial infection (Konturek et al., 2005) and inflammation (Pammer et al., 1998) we therefore investigated PrP^c for antimicrobial activity in paper III. The N-terminal part was found to be antimicrobial to both Gram negative and Gram positive bacteria as well as against fungi. We further investigated whether it was induced during wound healing and found that it had an EGFR-dependent expression.

-Induced expression of epidermal AMPs does probably not represent first line of defense against microbes.

De novo synthesis of epidermal human AMPs is a time-consuming process and only results in adequate antimicrobial levels for most AMPs days after wounding. hBD-2 expression induced by IL-1 reaches relevant antimicrobial levels first 48 hours after stimulation under optimal conditions in keratinocyte cell cultures (Liu et al., 2003). The epidermal levels of LL-37 have been described to increase at a quicker pace. However, maximum hCAP-18 levels with release of active LL-37 are found in the wound first 12-48 hours after wounding in skin (Heilborn et al., 2003). Furthermore, it should be noted that it is unclear how the epidermal contribution to these levels compares with the quantities released from infiltrating neutrophils. Neutrophils are known to possess an abundant amount of LL-37 (Sorensen et al., 1997b; Sorensen et al., 1997a). Regardless of this, considering that some strains of *Escherichia coli* are capable of dividing every 20 min (Helmstetter, 1968; Skarstad et al., 1986) it is therefore probably not accurate to view the epidermal induction of AMPs as a first line of defense. The rapid generation of AMPs from the catalytic cascades of the contact - and the coagulation system (Frick et al., 2006; Frick et al., 2007; Nordahl et al., 2004) in the plasma exudates would fit this description better. AMPs are also released from degranulating platelets (Tang et al., 2002). Together these locally generated peptides are likely to play an important role in the initial defense of the wound until the neutrophils arrive. Why the gradual induction of

AMPs in keratinocytes then? Looking at the time point of maximum expression for the bulk part of AMPs it coincides with the departure of neutrophils and macrophages from the wound site. It is therefore possible that the gradual increase in AMP levels in human skin, instead of being seen as a first line of defense mechanism, more should be viewed as a reinforcement of the defense of the epidermis, an epidermis which is still vulnerable without the re-established barrier function, when the numbers of neutrophils and macrophages begin to decline. A protection based on antimicrobial peptides instead of inflammatory cells is likely less effective but offers a better environment for the proliferating keratinocytes and thus facilitates the healing process.

-A molecular link between injury and inflammation

Although the release of neutrophil proteases and reactive oxygen species may cause damage to the tissue the recruitment of PMNs is essential for keeping the wound free of infection (Martin and Leibovich, 2005). IL-8 has previously been found to be the major neutrophil attractant in human wound fluid (Rennekampff et al., 2000). Contrary to a previous report by Nickoloff et al (Nickoloff and Naidu, 1994), Rennekampff et al found no signal of IL-8 mRNA in the epidermis during wound healing and considered release from intracellular epidermal stores to be only an immediate source of IL-8 after injury. Accordingly, they proposed PMN-derived IL-8 to be the likely source of the prolonged and increasing amounts of IL-8 during wound healing. However, the number of neutrophils peak at day 1-2 after wounding whereas the levels of IL-8 in wound fluid peak later at day 5 (Rennekampff et al., 2000). IL-8 has also been shown to be up-regulated in keratinocytes by proinflammatory cytokines such as IL-1 and TNF-α (Larsen et al., 1989) and by various other pathways and agonists *in vitro,* including TLRs (Pivarcsi et al., 2003), retinoic acid (Dai et al., 2004), TRAIL (Leverkus et al., 2003), LL-37 (Murakami et al., 2004) and ligands of EGFR (Miller et al., 2005) (Pastore et al., 2005). In paper II we discovered, contrary to Renekampff et al, a rapid increase in IL-8 mRNA levels and protein levels both in *in vivo* and in *ex vivo* wounded epidermis. We also found that the majority of the chemotactic activity generated in *ex vivo* injured epidermis was both IL-8 and EGFR-dependent. Thus we find that injury and the subsequent activation of EGFR rapidly induces production of IL-8 in keratinocytes recruiting neutrophils to the wound site. This both emphasizes the role of the keratinocyte in the initiation of the inflammatory phase of wound healing and provides a novel molecular link between injury and neutrophil accumulation in cutaneous wounds.

-The response to injury increases the protection of the tissue against inflammation

Although keratinocytes appear to play a major role in initiating innate immune responses, their primary objective after cutaneous injury must be to cover the

wound bed and re-establish the barrier function. However, the epidermal recruitment of neutrophils, which release reactive oxygen species, cytotoxic granzyme B (Wagner et al., 2004; Wagner et al., 2008) and potent proteases (Nathan, 2002) from their azurophilic granules, likely impairs this process. Embryos, which still lack neutrophils with functional chemotaxis, have a virtually scar free wound healing (Ferguson and O'Kane, 2004). Under sterile conditions adult knockout mice lacking neutrophils and macrophages also display a similar scar free wound healing which is faster than the one found in the wild type littermates. However, under non-sterile conditions the knockout mice do not heal their wound but succumb to bacterial infections. Increased fibrosis and the cosmetic disadvantage of a disfiguring scar therefore likely represent a compromise for minimizing the increased risk of serious infections following injury. Thus we found that the epidermal response to injury both encompassed recruitment of neutrophils and countermeasures in order to be ready for the detrimental activities of these inflammatory cells. This included an increased production of serine protease inhibitors against neutrophil proteases and a reduced sensitivity against apoptotic cues from the extrinsic pathway of apoptosis. EGFR-activation further stimulates keratinocyte survival and proliferation (Jost et al., 2000). In Figure 2, a summary of the role of the EGFR-activation together with other injury-induced responses is presented. EGFR-activation may also indirectly stimulate proliferation by increasing the expression of IL-6 (Sato et al., 1999; Grossman et al., 1989). Concomitantly there was an increased receptiveness to internal apoptotic signals likely representing a defensive reaction to avoid the risk of malignant transformation in the highly proliferating epidermis.

-Transcription factors previously not described to be involved in wound healing

In paper IV we could identify significant changes in the expression levels of several transcription factors including FOS-like antigen 1 (FOSL1) known to be important in wound healing (Schafer and Werner, 2007). However, the activity of many transcription factors is not regulated at the transcriptional level but by posttranslational modifications e.g. by phosphorylation and proteasomal degradation. Not to overlook transcription factors important for wound healing we therefore investigated the frequency of TFBS for various transcription factors in the most differentially expressed genes in the injured skin. This revealed a highly significant overrepresentation of TFBS for the FOXO family of transcription factors. These transcription factors have previously not been associated with the wound healing process (Schafer and Werner, 2007) and hopefully future studies will determine their role in this process. However, the regulation of these transcription factors is very complex with at least six different mechanisms of regulation and it will therefore be a considerable challenge to elucidate their importance for wound healing (van der Vos and Coffer, 2008).

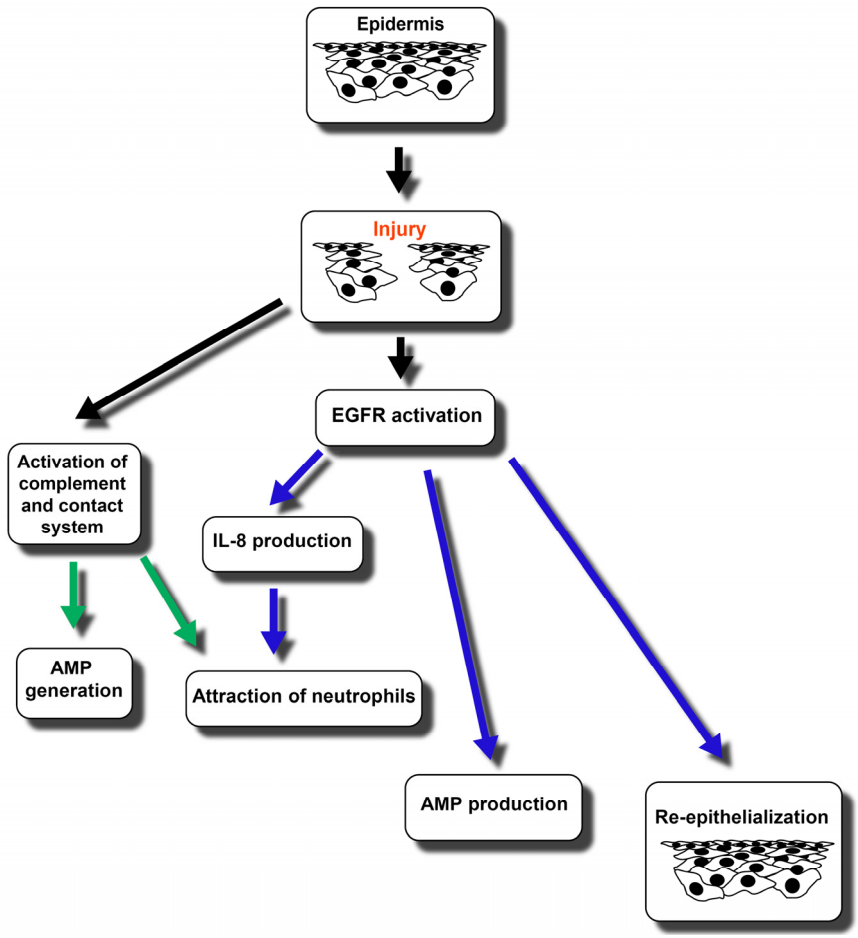

Figure 2. Injury induced innate immune responses during wound healing
After injury the exudation of plasma proteins lead to a rapid activation of the complement system and the intrinsic pathway of coagulation. This generates AMPs and chemotactic factors, such as C5a attracting neutrophils to the wound. Concurrently the breach in the epidermal barrier causes transactivation of the EGFR, which elicits increased IL-8 production and thus a sustained recruitment of neutrophils. EGFR-activation is at later stages responsible for the major expression of epidermal AMPs and is also involved in the re-epithelialization process and the re-establishment of the physical barrier. The bulk part of epidermal AMPs coincides with the departure of neutrophils and macrophages from the wound site and probably represents an antibacterial reinforcement of epidermis before the physical barrier is re-established. Therefore, AMPs, generated from coagulation and complement, together with neutrophils are probably responsible for the initial clearance of microbes from the wound. Figure modified from (Sorensen et al., 2008)

Summary

The findings in this thesis show that epidermal innate immune responses are initiated by injury and that these responses are an integrated part of the cutaneous wound healing process. From an evolutionary point of view this makes sense. The epidermis constitutes the primary interface towards our external milieu full of micro-organisms. After cutaneous injury, the physical barrier preventing micro-organisms from entering the body is destroyed. This greatly increases the risk of infection either by opportunistic commensals, already present in our microflora, or by pathogenic micro-organisms existing in our surroundings. A rapid and massive initiation of immune responses already in reaction to a breach in the epidermis is therefore necessary to prevent subsequent infection. Accordingly, we found that injury-induced EGFR-activation was the major cause of both the chemotactic activity rapidly generated in the epidermis and the gradually increasing epidermal AMP-production. Firstly, the epidermis thus recruits inflammatory cells and then subsequently reinforces the epidermal defense with AMPs in preparation for the time when neutrophils depart. Secondly, it braces itself against the impact of the detrimental effects of the inflammatory process both by a decreased sensitivity to extrinsic apoptotic stimuli and by an increased production of proteinase inhibitors to counter the potent proteases from inflammatory cells. Concomitantly, the proliferating epidermis is protected from the risk of malignant transformation by a modified expression of the genes involved in the intrinsic pathway of apoptosis. This renders keratinocytes more susceptible to apoptosis mediated through DNA damage and intracellular stress signals. Concurrently, sustained EGFR-activation promotes proliferation and also differentiation by increasing the expression of genes belonging to the epidermal differentiation complex. This increases both the proliferation rate and the differentiation rate, which facilitates the re-epithelialization and the re-establishment of the barrier function.

Conclusions

- hBD-3, SLPI and NGAL are induced in response to injury in human skin through transactivation of the EGFR.

- Endogenously shed HB-EGF is the growth factor contributing most to the EGFR mediated expression of hBD-3 in response to injury in human skin.

- Injury independent of inflammation induces the majority of the known inducible AMPs in the skin through activation of the EGFR.

- EGFR-dependent IL-8 production in response to injury was the major contributor to the potent chemotactic activity toward neutrophils generated in injured epidermis.

- Intact recombinant PrP^c exerts antibacterial and antifungal effects both at normal and low pH. The antimicrobial activity is mediated by the heparin-binding N-terminal part of the protein.

- The expression of PrP^c is increased in response to injury of human skin *ex vivo* and *in vivo*. Like the majority of known inducible AMPs found in human skin PrP^c is induced through the activation of the EGFR.

- Changes in gene expression in the epidermal tissue during wound healing indicate a reduced sensitivity to the extrinsic pathway of apoptosis and concomitantly an increased sensitivity to the intrinsic pathway of apoptosis.

- The FOXO family of transcription factors have a highly significant overrepresentation of transcription factor binding sites in the most differentially expressed genes in injured human skin.

Populärvetenskaplig Sammanfattning

En värld av mikrober
Vi lever i en värld full av olika bakterier och svampar, så kallade mikrober. Med tanke på artrikedomen och mängden av infektiösa mikrober vi dagligen kommer i kontakt med kan det nästan tyckas som ett smärre mirakel att vi trots allt är så friska som vi är. Detta är dock nästan helt och hållet vårt medfödda immunförsvars förtjänst. Immunförsvaret kan delas in i det förvärvade (adaptiva) och det medfödda immunförsvaret. Dock är distinktionen mellan dem ibland svår att göra eftersom de till stor del är integrerade med varandra och fungerar som en helhet. Det adaptiva immunförsvaret är uppbyggt kring antikroppar och immunologiskt minne och är en viktig förstärkning av det medfödda försvaret. Den här avhandlingen har främst fokuserat på det medfödda immunförsvaret i epidermis, de fem yttersta lagren av huden, och epidermis produktion av antimikrobiella peptider i synnerhet.

Epidermis
Vår yttersta fysiska barriär mot omvärlden utgörs av epidermis. En av epidermis mycket viktiga uppgifter är att hålla omgivningens mikroorganismer åtskilda från den sårbara underliggande vävnaden och på så vis förhindra infektion. Epidermis är emellertid inte bara en fysisk barriär utan utgör även en kemisk barriär vilket gör den ytterst svårgenomtränglig för bakterier och svampar. Det låga pH som råder i epidermis gör det besvärligt för bakterier att växa och etablera sig, vidare producerar hudceller antimikrobiella peptider och fetter som ytterliggare försvårar överlevnaden för mikrober. Ännu en viktig mekanism är epidermis förmåga att ständigt förnya sig självt. Genom celldelning i de understa lagren fylls de övre lagren ständigt på. Cellerna utmognar successivt medan de rör sig upp genom de olika lagren tills de slutar som döda, platta, hårt packade och sammanlänkade hornceller i det yttersta hornlagret. I takt med att horncellerna flagnar av fylls de på underifrån. Detta gör det ännu svårare för mikroorganismer att få fäste i huden. Vid sårskada i huden förstörs emellertid den fysiska barriären med en ökad risk för infektion som följd. Kroppen har därför utvecklat flera försvarssystem för att förhindra infektioner och därmed ge vävnaden och huden tid och chans att reparera sig och återupprätta barriärfunktionen. Att dessa försvarssystem samt förmågan att läka en sårskada fungerar är livsviktigt för oss människor. Hos diabetespatienter och andra patientgrupper med försämrad blodcirkulation samt hos svårt brännskadade är hudens läkningsförmåga nedsatt och patienten har även en ökad infektionskänslighet. För att kunna förbättra behandlingsmöjligheterna vid svårläkta sår är det viktigt att studera hur huden normalt reagerar vid sårskada.

Sårläkning i korthet
Vid akut sårskada koagulerar blodet och bildar en blodpropp som förhindrar fortsatt blodförlust. Vita blodkroppar, i huvudsak neutrofiler som är specialiserade på att äta och oskadliggöra mikroorgansismer, anländer snabbt till det skadade området. Dessa bryter ner skadad vävnad och håller såret fritt från mikroorganismer. För att städa upp efter neutrofilerna som efter ett tag börjar självdö rekryteras en annan typ av vita blodkroppar kallade makrofager. Dessa äter upp döende neutrofiler och de sista resterna av skadad vävnad. Samtidigt och innan makrofagerna lämnar såret utsöndrar de och andra celler protein, så kallade tillväxtfaktorer. Tillväxtfaktorerna sätter igång en ökad celldelning i huden vilket slutligen leder till att sårskadan återigen täcks av hudceller och att hudbarriären återskapas.

Vad sätter igång produktionen antimikrobiella peptider?
Förutom skyddet från de vita blodkropparna har forskning kring sårläkningsprocessen även påvisat att epidermis och hudcellerna ökar produktionen av antimikrobiella peptider som utgör en viktig del i försvaret mot mikroorganismer. I arbetet med denna avhandling har vi försökt utreda vad det är som sätter igång denna produktion i hudceller samt hur epidermis i övrigt reagerar i samband med sårskada. Då den övervägande delen av antimikrobiella peptider ifrån epidermis produceras först efter att neutrofilerna anlänt har den allmänna uppfattningen varit att det är neutrofilerna som sätter igång den ökade produktionen i hudcellerna. Våra data visar dock att hudcellerna, till skillnad från vad man tidigare trott, reagerar direkt på själva sårskadan. Sårskada leder till en aktivering av en receptor kallad epidermala tillväxt receptorn (EGFR) på ytan av hudcellerna vid sårkanten. I våra studier ser vi att sårskadan i sig via EGFR-aktivering faktiskt står för merparten av den ökade produktionen av antimikrobiella peptider i huden.

Antimikrobiella peptider bildar eftertrupp
Hudens ökade produktion av antimikrobiella peptider vid sårskada har ansetts vara en snabb försvarsmekanism. När vi studerat sårläkningsförloppet fann vi emellertid att de flesta av hudens producerade antimikrobiella peptider når bakteriedödande koncentrationer först efter två till fyra dygn. Således menar vi att denna mekanism snarare bör ses som en förstärkning av hudens försvar. När de vita blodkropparna lämnar såret är den skadade hudbarriären ännu inte helt återställd och risken för infektion kvarstår. Det är nu antimikrobiella peptider producerade i huden når högst koncentrationer och kan ge huden skydd medan barriären återetableras.

Återuppbyggnad
EGFR-aktivering är sedan tidigare känd för att vara involverad i återuppbyggnaden av hudskada genom att öka celldelningen av hudceller vilket är en förutsättning för att snabbt kunna täcka sårbädden. Vi visar även att EGFR-aktiveringen indirekt bidrar till celldelning via en ökad produktion av andra tillväxtfaktorer. Vidare fann vi att EGFR-aktivering även leder till en ökad produktion av proteiner som behövs för att bygga upp den yttre hudbarriären.

Hudceller rekryterar vita blodkroppar
Hudcellerna är alltså vid sårskada fullt kapabla att själva sätta ingång både hudens återuppbyggnadsprocess samt en ökad produktion av antimikrobiella peptider utan att bli aktiverade av neutrofiler. Vi fann att hudcellerna, till skillnad från vad som visats i tidigare studier även ansvarar för en stor del av rekryteringen av neutrofiler. Detta sker genom en ökad produktion av ett så kallat kemotaktiskt protein som lockar till sig neutrofiler. Även produktionen av detta protein visade sig vara beroende av EGFR aktivering.

Sammanfattning
Sårskada är i sig självt ett kraftfullt stimuli som via EGFR-aktivering i epidermis leder både till rekrytering av neutrofiler samt till en ökad produktion av antimikrobiella peptider. De antimikrobiella peptiderna skyddar den läkande sårbädden efter att de vita blodkropparna återgått till blodbanan. EGFR-aktiveringen påskyndar även återskapandet av hudbarriären genom att sätta fart på celldelningen och genom att öka uttrycket av protein som bygger upp det yttersta lagret i huden. En ökad förståelse för hur den normala sårläkningsprocessen går till skulle i förlängningen kunna leda fram till förbättrade behandlingsstrategier för patienter med nedsatt sårläkningsförmåga. Min förhoppning är att denna avhandling kan bidra med en del av den kunskap som behövs för att nå det målet.

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då blir jag pigg och orkar lite till... Bob Hund

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