Chapter 7

Autocrine regulation of re-epithelialization after wounding by chemokine receptors CCR1, CCR10, CXCR1, CXCR2 and CXCR3

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ABSTRACT

This study identifies chemokine receptors involved in an autocrine regulation of re-epithelialization after skin tissue damage. We determined which receptors, from a panel of thirteen, are expressed in healthy human epidermis and which mono-specific chemokine ligands, secreted by keratinocytes, were able to stimulate migration and proliferation. A reconstructed epidermis cryo-(freeze) wound model was used to assess chemokine secretion after wounding and the effect of pertussis toxin (chemokine receptor blocker) on re-epithelialization and differentiation. Chemokine receptors CCR1, CCR3, CCR4, CCR6, CCR10, CXCR1, CXCR2, CXCR3 and CXCR4 were expressed in epidermis. No expression of CCR2, CCR5, CCR7 and CCR8 was observed by either immunostaining or flow cytometry. Five chemokine receptors (CCR1, CCR10, CXCR1, CXCR2, CXCR3) were identified whose corresponding mono-specific ligands (CCL14, CCL27, CXCL8, CXCL1, CXCL10 respectively) were not only able to stimulate keratinocyte migration and/or proliferation but were also secreted by keratinocytes after introducing cryo-wounds into epidermal equivalents. Blocking of receptor-ligand interactions with pertussis toxin delayed re-epithelialization but did not influence differentiation (as assessed by formation of basal layer, spinous layer, granular layer and stratum corneum) after cryo-wounding. Taken together, these results confirm that an autocrine positive feedback loop of epithelialization exists in order to stimulate wound closure after skin injury.
CHAPTER 7

INTRODUCTION

Chemokines constitute a family of structurally related chemotactic cytokines. Many chemokines are constitutively expressed, and show increased secretion upon cutaneous damage. Chemokines were first described to direct migration of inflammatory cells (neutrophils, macrophages, monocytes, lymphocytes) into the wound bed (1,2). However, the presence of chemokine receptors on skin residential cells (e.g., keratinocytes, fibroblasts, endothelial cells) indicates that chemokines may also contribute to regulation of epithelialization, granulation tissue formation, angiogenesis and tissue remodeling.

Our previous study focused on the role of chemokines in dermal repair and in particular on mesenchymal stem cells (3). This study focuses on epidermal regeneration. Re-epithelialization occurs from a viable epidermal progenitor cell pool that resides in the basal layer of the epidermis and in dermal appendages such as hair follicles (4,5). Re-epithelialization involves keratinocyte migration and proliferation followed by differentiation in order to regenerate the epidermis during wound closure. Keratinocyte migration begins 3-6 hours after wounding and proceeds with proliferation and differentiation (6). Several hours after the onset of migration, keratinocyte proliferation is increased distal from the migrating edge (6, 7). Since wound healing processes are triggered very early after wounding and before infiltrating cells enter the wound area, it is possible that keratinocytes initiate re-epithelialization in an autocrine manner.

Chemokine receptor-ligand interactions have been described to be involved in re-epithelialization. Steude et al. reported in an in vitro skin model that CXCL1 and CXCL8 induce keratinocyte migration by binding to the receptor CXCR2 (8,9). Also several other ligand/receptor interactions (CXCL12/ CXCR4, CCL17/ CCR4 and CCL27/ CCR10) have been described to be involved in migration and proliferation of keratinocytes (10,11). In vivo results using CXCL11 -/- and CXCR3 -/- mice demonstrated a delayed re-epithelialization after wounding which suggested an autocrine regulation of re-epithelialization (12,13). Whereas all of these studies clearly indicate a role for chemokines in re-epithelialization, until now no distinction has been made between autocrine and paracrine regulation. Since most studies generally associate chemokine expression with inflammatory cell recruitment these studies often suggest paracrine regulation of re-epithelialization. We suggest that an early autocrine regulation would be favorable since it would result in an immediate response to tissue damage before infiltrating cells are able to induce a chemokine cascade and amplified wound healing response. Paracrine regulation of re-epithelialization would be expected to result in a delayed response since the cells would have to respond to a chemokine gradient originating from another cell type e.g. infiltrating cell or fibroblast. By using a reconstructed epidermis wound healing model we were able to determine whether autocrine chemokine regulation is involved in re-epithelialization and to identify putative chemokines involved.
In this study, we describe chemokine receptor expression and tissue location in native healthy human epidermis. We describe which mono-specific binding chemokines for these receptors are secreted by keratinocytes and whether these same chemokines can stimulate keratinocyte migration, proliferation and differentiation. An in vitro reconstructed epidermal equivalent wound healing model was used to determine which chemokines were secreted by keratinocytes and whether or not the chemokine receptor blocker pertussis toxin was able to inhibit re-epithelialization. We show that pertussis toxin is able to inhibit re-epithelialization by preventing chemokines secreted by epidermal keratinocytes from binding to their receptors. This study identifies chemokine receptors involved in an autocrine regulation of re-epithelialization after skin tissue damage.

**METHODS AND MATERIALS**

**Isolation and culture of human keratinocytes and epidermal equivalents.**

Human adult skin was obtained from healthy donors (with written informed consent) undergoing abdominal dermolipectomy and was used directly after surgery. The VU University medical center approved the experiments described in this paper. The study was conducted according to Declaration of Helsinki Principles.

Epidermal cells were isolated from human skin essentially as described earlier (27). In brief, epidermis was isolated from human skin by incubation overnight at 4°C in dispase (Roche, Mannheim, Germany). Hereafter, a cell suspension was made by incubation in trypsin (Gibco, Invitrogen, Paisley, UK) for 15 min at 37 °C. Freshly isolated keratinocytes were used for flow cytometry analysis or further cultured. Subconfluent, first-passage keratinocytes cultured in keratinocyte medium (DMEM/Ham's F12 (3:1), 1% UltroserG, 1% penicillin streptomycin, 1µmol L⁻¹ hydrocortisone, 1µmol L⁻¹ isoproterenol, 0.1µmol L⁻¹ insulin and 2 ng/ml KGF) were used for experiments to assess keratinocyte migration and proliferation and to construct the in vitro wound healing model.

To construct epidermal equivalents for the wound healing model and receptor analysis, keratinocytes were seeded onto acellular de-epidermized dermis or onto 24 mm diameter transwells (pore size of 0.4µm; Corning, NY, USA) respectively (14) and cultured submerged for one week in keratinocyte medium. After one week cultures were lifted to the air-liquid interface and cultured for a further week in DMEM/Ham's F12 (3:1), 0.2% UltroserG, 1% penicillin streptomycin, 1µmol L⁻¹ hydrocortisone, 1µmol L⁻¹ isoproterenol, 0.1µmol L⁻¹ insulin, 1.0 x 10⁻⁵ mol L⁻¹ L-carnitine and 1.0 x 10⁻⁵ mol L⁻¹ L-serine supplemented with 2 ng/ml KGF and 50µg ml⁻¹ ascorbic acid. Hereafter, UltroserG and KGF were omitted from the culture medium for 2 days prior to introducing the cryo-wound and for the duration of the experiment.
Medium was refreshed twice a week. Unless otherwise stated, all culture additives were obtained from Sigma (St Lois, MO, USA).

**Immunohistochemical analysis**

Human skin biopsies and epidermal equivalents were washed in phosphate-buffered saline, fixed in 4% paraformaldehyde and processed for conventional paraffin embedding. Sections (5µm) were cut, deparaffinized and rehydrated in preparation for immunohistochemical analysis of chemokine receptors. Immunohistochemical procedures were performed as previously described (3). Photographs were taken with a computer-assisted microscope (Nikon, Dusseldorf, Germany). Antibodies and corresponding isotypes used, were from BD Pharmingen generated in mice and reactive with human unless stated otherwise: CCR1 (53504, IgG2b); CCR2 (48607, IgG2b); CCR3 (5E8, IgG2b); CCR4 (1G1, IgG1); CCR5 (2D7, IgG2a); CCR6 (11A9, IgG1); rat anti-human CCR7 (3D12, IgG2a); rat anti-human CCR8 (191704, IgG2b, R&D systems); rat anti-human CCR10 (314305, IgG2a, R&D systems); CXCR1 (5A12, IgG1); CXCR2 (CDw128b, IgG2b); CXCR3 (1C6/cxcr3, IgG1); CXCR4 (12G5, IgG2a, R&D systems). Staining for each receptor was performed using skin or epidermal equivalents derived from the same three different donors and in duplicate.

**Flow cytometric analysis**

Epidermal cells isolated from human epidermis or epidermal equivalents were examined for cell surface expression of chemokine receptors. Cells were incubated for 30 min with antibodies, washed in PBS supplemented with 0.1% BSA and 0.1% sodium azide and then resuspended in the same buffer for FACS analysis. Cells were measured on a FACScan and analysed with Cell Quest software (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA). PE labelled antibodies and corresponding isotypes were the same as those used for immunohistochemistry (see above). Staining for each receptor was performed using skin or epidermal equivalents derived from three different donors in duplicate.

**Chemotaxis assay**

Chemotactic migration of second-passage keratinocytes to chemokine ligands was assessed by the Boyden well chamber technique as described previously for dermal and adipose derived stromal cells (3). Prior to starting the experiment, the transwell inserts were coated with collagenIV and keratinocytes were incubated overnight in serum free medium (DMEM/Ham’s F12 (3:1), 1% penicilline streptomyccine, 1µmol L⁻¹ hydrocortisone, 1µmol L⁻¹ isoproterenol and 0.1µmol L⁻¹ insulin). The cell suspension (200µl; 5x10⁵/ml in serum free medium) was loaded into the upper well of the chamber and allowed to attach for four hours. After 4 hours differ-
ent concentrations of chemokine were placed in the lower well and migration was allowed for 24 hours. The number of migrated cells was determined by counting the number of nuclei in a 40-fold magnification area with a computer assisted fluorescence microscope (Nikon, Dusseldorf, Germany). Migration after exposure to chemokine is given relative to control. Experiments were performed using 3 different donors each in duplicate.

**Proliferation assay**

Proliferation of second-passage keratinocytes in response to chemokine ligands was assessed by a modified Lactate Dehydrogenase (LDH) assay. LDH released into the media after lysis of the cells is representative of the total number of cells. Second-passage keratinocytes were cultured in a collagen IV coated 48-well plate in DMEM/Ham's F12 (3:1), 0.1% UltroserG, 1% penicilline streptomycine, 1µmol L⁻¹ hydrocortisone, 1µmol L⁻¹ isoproterenol and 0.1µmol L⁻¹ insulin. Subconfluent cell monolayers (75%) were exposed to different concentrations of chemokine (0-500 ng ml⁻¹ medium) for 24 hours. After incubation cells were washed with phosphate buffered saline and lysed with 0.1% triton in PBS for 30 minutes at 4°C. LDH assay mixture was added to the lysate for 30 minutes at room temperature and then absorbance was measured at a wavelength of 492 nm. Proliferation after exposure to chemokines is given relative to control. Experiments were performed using 3 different donors each in duplicate.

**Wound healing model and chemokine receptor blocking**

Full thickness wounds were made in epidermal equivalents after 1 week of air-exposed culture as previously described (14). Wounds were defined as extreme cryo-wounds (freeze-wounds) (1mm wide and 2cm long) resulting in cell death of that entire region of the epidermis whilst leaving the rest of the culture intact and viable. One wound was introduced into each culture. After wounding, epidermal equivalents were further cultured air exposed. Directly after wounding, culture medium was supplemented with the chemokine receptor inhibitor (pertussis toxin) (0, 200, 400, 800 ng ml⁻¹), or left unsupplemented.

After 12 days, wound closure (re-epithelialization and differentiation of newly formed epidermis) was analyzed on haematoxylin/ eosin stained paraffin sections (5µm) with the aid of a Nikon microscope and Osteomeasure software (osteometrics, Atlanta, USA). Re-epithelialization was measured as the distance that the newly formed epidermis had migrated into the wound bed. Re-epithelialization after exposure to pertussis toxin-supplemented medium was compared to control unsupplemented medium and is given relative to control. As re-epithelialization occurred from both wound margins, duplicate readings were obtained for each wound and averaged. Differentiation was assessed by histology with respect to the presence or absence of a basal layer, stratum spinosum, stratum granulosum and stratum
corneum. Experiments were performed using epidermal equivalents constructed from three different pools of 5 skin donors in duplicate.

**Enzyme-linked immunosorbent assay**

Freshly excised adult skin (4cm²), and epidermal equivalents with and without full thickness cryo-wounds (see below) were placed in transwells (2.4cm diameter, 3µm pore size) with 1.5ml culture medium in the lower compartment for 24 hrs (14). Culture supernatants were collected and stored at -20ºC for further analysis. For chemokine quantification in culture supernatants enzyme-linked immunosorbent assay (ELISA) reagents were used in accordance to the manufacturer’s specifications. CCL14, CCL20, CCL22, CCL27 and CXCL1, CXCL10 and CXCL12 were measured by commercially available paired ELISA antibodies and recombinant proteins obtained from R&D System Inc. (Minneapolis, Minnesota). For CXCL8/ IL-8 a Pelipair reagent set (CLB, Amsterdam, The Netherlands) was used. For CCL24 a Quantikine Immunoassay (R&D Systen Inc. Minneapolis, Minnesota) was used.

**Statistics**

All experiments were performed in duplicate using skin cells derived from 3 different donor pools, each pool derived from 3 different donors. When intact freshly excised skin was used, 3 different donors were used with an intra-experiment/donor duplicate. All data are presented as mean ± standard error mean. Differences were evaluated by one-way ANOVA post hoc dunnet’s, using computer program GraphPad Prism (San Diego, CA, USA).

**RESULTS**

**Chemokine receptor expression on epidermal cells**

If autocrine chemokine regulation initiating re-epithelialization occurs it would be expected that chemokine receptors would already be present on the surface of cells within normal healthy epidermis in order for these cells to immediately respond to tissue damage without delay. Therefore we first determined the tissue location (immunohistochemical staining) and cell surface expression (flow cytometry) of chemokine receptors in human epidermis (Figure 1). Chemokine receptors CCR1, CCR3, CCR4, CCR6, CCR10, CXCR1, CXCR2, CXCR3 and CXCR4 were expressed in the epidermis (Figure 1, Table 1). Immunohistochemical staining occurred throughout the epidermis for CCR1, CCR4, CXCR1, CXCR2 and CXCR4 in line with flow cytometry which showed a similar homogenous expression for these receptors. CCR10 was also expressed throughout the epidermis, but flow cytometry identified a heterogenous
Figure 1: Chemokine receptor expression on human epidermal keratinocytes.

Immunohistochemical staining was used to locate chemokine receptors in the epidermis of human skin and epidermal equivalents. Flow cytometry was used to determine chemokine receptor expression on keratinocyte cell surface. Tissue sections or keratinocytes isolated from fresh skin tissue or epidermal equivalents were stained with monoclonal antibodies against the indicated PE labeled chemokine receptors (thick line in histogram). Each histogram plot contains a PE labeled isotype matched control (thin line). Data shown are from one individual donor (skin biopsy/epidermal equivalent and keratinocyte isolation matched) and are representative experiments from 3 donors performed in duplicate. bar = 250μm.
Table 1: Summary of chemokine receptor expression on epidermal keratinocytes and the effect of receptor specific chemokines on chemotaxis and proliferation

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<th>Chemotaxis</th>
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* monospecific binding chemokines investigated in this study.

* data derived from: The chemokine and chemokine receptor superfamilies and their molecular evolution. Albert Zlotnik, Osamu Yoshie and Hisayuki Nomiyama. Genome Biology 2006;7;243

*also a ligand for CXCR2;

*chemokinesis instead of chemotaxis;

KC, keratinocyte; BL, basal layer; SB, suprabasal layer; SG, stratum granulosum/granular layer; SS, upper stratum spinosum/ spinous layer, SS, lower stratum spinosum/ spinous layer.
intensity of expression of CCR10. Chemokine receptors CCR3, CCR6, and CXCR3 showed a differential expression within the epidermis; CCR3, and CCR6 were expressed predominantly in suprabasal epidermal layers, whereas CXCR3 was expressed in basal and lower spinous layers. The heterogenous expression of these receptors was confirmed with flow cytometry. No expression of CCR2, CCR5, CCR7 and CCR8 was observed by either immunostaining or flow cytometry.

Chemokine receptor expression was also studied in primary cultured keratinocytes which had been incorporated into a three dimensional reconstructed epidermis in vitro (Figure 1). These epidermal equivalents demonstrated similar results with regards to chemokine receptor tissue location and cell surface expression to that observed in normal human epidermis. Therefore the epidermal equivalent closely resembles in vivo human skin (epidermal tissue architecture as well as chemokine receptor expression) and ascertains the use of this model for further experiments.

**Chemokine mediated keratinocyte migration**

Re-epithelialization after wounding involves both keratinocyte migration and proliferation. In order to determine whether the chemokine receptors identified on the cell surface of epidermal keratinocytes could be involved in epithelialization we next determined the effect of monospecific chemokines (ligands which bind to only one receptor) (CCL14/ CCR1; CCL20/ CCR6; CCL22/ CCR4; CCL24/ CCR3; CCL27/ CCR10; CXCL1/ CXCR2; CXCL10/ CXCR3; CXCL12/ CXCR4) on keratinocyte migration in a chemotaxis transwell assay (Figure 2). For CXCR1, no monospecific chemokine has been identified and therefore CXCL8 was used which also binds to CXCR2.

Three different types of response were observed: i) Chemotaxis - a strong dose-dependent increase in migration of keratinocytes toward CCL14, CCL22, CCL27, CXCL1 and CXCL10 was observed (Figure 2a). When an equal concentration of each of these chemokines (125 ng ml⁻¹) was placed in the upper – and lower well no increase in keratinocyte migration occurred compared to the medium control, indicating that chemotaxis rather than chemokinesis was involved; ii) Chemokinesis - a strong dose-dependent increase in migration of keratinocytes was also observed towards CXCL8 (Figure 2b). However the mode of action was chemokinesis rather than chemotaxis since equal concentrations of CXCL8 (125 ng ml⁻¹) in the upper – and lower compartments still induced a greater migratory response compared to control; iii) No effect on migration - CCL20, CCL24 and CXCL12 were not able to increase cell migration compared to the medium control (Figure 2c).
Figure 2: Migration of keratinocytes upon chemokine exposure.

Cultured keratinocytes were seeded into the upper chamber of a chemotaxis transwell with chemokines in the lower well. As a control, an equal concentration of chemokine (125ng ml⁻¹) in the upper and lower well distinguished chemotaxis from chemokinesis (125-125). Keratinocyte migration from the upper to the lower transwell surface is expressed relative to unsupplemented cultures. Three groups of chemokines could be distinguished based upon their effect on keratinocyte migration; (a) inducing chemotaxis; (b) inducing chemokinesis; (c) no effect on keratinocyte migration. Statistical significant differences between supplemented and unsupplemented keratinocytes were calculated using one-way ANOVA test followed by a Dunnett’s test. Differences were considered significant when *P<0.05, **P<0.01.
Chemokine mediated keratinocyte proliferation

Having identified the chemokine ligand receptor pairs involved in initiating keratinocyte migration, we next determined which chemokine ligand receptor pairs could stimulate keratinocyte proliferation.
A two fold increase in proliferation compared to unsupplemented cultures was observed after exposure of keratinocytes to CCL24, CXCL1, CXCL8 and CXCL12. Characteristic peaks in the dose response data indicated that an optimal working concentration existed for each chemokine. (Figure 3a). In contrast, CCL14, CCL20, CCL22, CCL27 and CXCL10 were not able to increase cell proliferation (Figure 3b).

Taken together our results on mono-specific binding chemokines can be subdivided into 4 groups depending on how they mediate keratinocyte migration and proliferation: i) CXCL1/ CXCR2; CXCL8/ CXCR1 and CXCL2; and CXCL12/ CXCR4 interactions increase both keratinocyte migration and proliferation; ii) CCL14/ CCR1, CCL22/ CCR4, CCL27/ CCR10 and CXCL10/ CXCR3 interactions increase only cell migration; iii) CCL24/ CCR3 interaction increases only proliferation and iv) CCL20/ CCR6 has no effect on migration or proliferation (Table 1). Of note, additional non-specific chemokines which are able to bind to multiple chemokine receptors are also summarized in Table 1. However, due to their non-specific binding nature, these chemokines were not used to investigate specific receptor function.

**Chemokines secreted by keratinocytes in response to wounding**

Once we had identified the key chemokine receptors responsible for initiating proliferation and migration it was next important to determine whether the mono-specific ligands for these receptors were indeed secreted by keratinocytes after epidermal wounding. In order to investigate this we used our previously described *in vitro* epidermal wound healing model which consists of reconstructed epidermis (keratinocytes) grown on human acellular donor dermis (14). Due to the air exposed culture method complete epidermal differentiation occurs, resulting in a basal layer, spinous layer, granular layer and stratum corneum similar to native healthy skin. In this model a full thickness standardized wound was introduced by cryo-freezing a defined region of the epidermis. Chemokine secretion before and after wounding was analyzed. Figure 4 shows that the epidermal equivalent secretes basal levels of CCL14, CCL20, CCL27, CXCL1, CXCL8, and CXCL10. Increased secretion of CCL14, CCL27 and CXCL10 (and increased trend for CCL20, CXCL1 and CXCL8) was observed 24 hrs after wounding. Of note, these chemokines were also secreted by excised healthy human skin, confirming the relevance of the *in vitro* model (Table 2). Chemokines CCL22, CCL24 and CXCL12 were not secreted by epidermal equivalents either before or after wounding and CCL24 and CXCL12 were also not secreted at detectable levels by excised skin (15 pg/ml = detection limit of ELISA). CCL22 however was detectable in excised skin.

Of note, chemokine receptor expression remained unaltered after wounding as observed by immunohistochemical analysis of the migrating epithelial tongue and flow cytometric analysis of keratinocytes incorporated into a scratch wound healing assay (see 3 for method) (data not shown).
Autocrine regulation of re-epithelialization after wounding by chemokine receptors CCR1, CCR10, CXCR1, CXCR2 and CXCR3

Since keratinocytes secrete a number of chemokine ligands (CCL14, CCL20, CCL27, CXCL1, CXCL8, and CXCL10) which are able to bind to a single receptor present on their cell surface, it is most probable that an autocrine regulation of re-epithelialization occurs. In order to confirm this, the degree of re-epithelialization in the wound healing model was determined after wounding in the presence or absence of a chemokine receptor blocker (pertussis toxin, pertussis toxin).
Ptx). In this model the regenerating epidermis forms underneath the dead epidermal tissue (14). Re-epithelialization occurs from the wound margins. A representative photograph of a wound margin is shown in Figure 5a. Incubation with pertussis toxin showed a dose dependent inhibition of re-epithelialization (Figure 5). A 50% decrease in re-epithelialization of the dermal matrix was observed after supplementing culture medium with 800 ng/ml pertussin toxin. This finding confirms that chemokines secreted by keratinocytes do indeed provide an immediate autocrine feedback loop to initiate wound closure upon tissue injury.

Whereas re-epithelialization was clearly inhibited by pertussis toxin, epidermal differentiation was not affected (Figure 5a). Even though the outgrowth of the epidermal tongue was less, the epidermis which did regenerate showed an equal number of cell layers consisting

![Figure 5](image)

**Figure 5: Pertussis toxin inhibits re-epithelialization in an epidermal wound model.**

Cryo-(freeze) wounds were introduced into epidermal equivalents and medium was supplemented with 0, 200, 400 and 800 ng/ml pertussis toxin. Cultures were harvested twelve days later. (a) a representative photograph showing histology of re-epithelialization in the epidermal equivalent cultured in unsupplemented and supplemented (400 ng/ml pertussis toxin) medium. a = unwounded epidermis; b = newly formed epidermis; arrow = wound margin; bar = 250μm. (b) Re-epithelialization was measured as the distance the newly formed epidermis migrated into the wound bed. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Statistical significant differences between supplemented and unsupplemented epidermal equivalents were calculated using one-way ANOVA test followed by a Dunnett’s test. Differences were considered significant when **P<0.01. PTx = pertussis toxin.
of a newly formed basal layer, spinous layer, granular layer and stratum corneum similar to control cultures (without pertussis toxin). Therefore, chemokines did not regulate differentiation in an autocrine manner.

**DISCUSSION**

Here we show that an autocrine regulation of re-epithelialization exists which has a direct effect on stimulating wound closure. Keratinocytes secrete chemokines which in turn are able to bind to receptors already present on their cell surface. This autocrine loop initiates migration and/or proliferation, but does not influence epidermal differentiation.

In this study we have identified five chemokine receptors (CCR1, CCR10, CXCR1, CXCR2, and CXCR3) which are expressed on the surface of keratinocytes. Their corresponding ligands (which notably can only bind mono-specifically to these receptors) are not only able to stimulate keratinocyte migration and/or proliferation but are also secreted by keratinocytes. Additionally, the chemokine receptor blocker, pertussis toxin, was able to partially block re-epithelialization in a fully defined (serum free) *in vitro* epidermal wound healing model consisting only of keratinocytes. Chemokine receptors were already expressed in normal unwounded epidermis allowing for immediate re-epithelialization after wounding and were not further up-regulated after wounding. Taken together, these results confirm that an autocrine regulation of epithelialization exists in order to stimulate wound closure after skin injury.

In addition to autocrine regulation, our results also identify chemokine receptors which may be associated with paracrine regulation. Three of the nine chemokines in our study, namely CCL22, CCL24 and CXCL12, were not secreted by keratinocytes in the wound healing model. However, their corresponding receptors (CCR4, CCR3 and CXCR4) were present on epidermal keratinocytes *in vivo* and their ligands (CCL22, CCL24 and CXCL12) are able to induce migration and/or proliferation *in vitro*. In line with these results, chemokine CCL24 has been reported to be secreted by dermal fibroblasts and is known to induce keratinocyte proliferation (15,16). For CXCL12 we and others have previously shown that dermal fibroblasts secrete this ligand, which in turn stimulates keratinocyte proliferation via its only receptor CXCR4 (10,17). With regards to CCR4, Katou et al. reported that its ligand CCL22 is secreted by macrophages and epidermal Langerhans cells (18). In addition to CCL22, CCR4 has two other ligands (CCL5 and CCL17; not monospecific). Both of these ligands are produced by keratinocytes and act as mitogens for keratinocytes suggesting that CCR4 may be involved in the autocrine regulation of keratinocyte migration but via CCL5 and CCL17 rather than via CCL22 (11,19,20). Since we were only able to detect secretion of CCL22 from excised skin it is possible that the ligands CCL24 and CXCL12 were secreted at very low amounts, were directly
taken up by adjacent cells or were bound to the dermis rather than being released into the culture supernatant.

We identified one chemokine receptor (CCR6) which was present on the surface of differentiated keratinocytes in the upper layers of the epidermis and whose ligand (CCL20) did not influence keratinocyte migration or proliferation in any way, even though its secretion was increased upon epidermal damage. This finding is in line with others who have identified CCL20 as a chemokine with antimicrobial properties which is secreted from differentiated keratinocytes (21,22). It is therefore possible that CCL20/CCR6 is regulated in an autocrine manner with its role being to control pathogen infection after wounding rather than wound closure.

Whereas reports by others have emphasized the important role of chemokines in wound healing, these reports did not distinguish between autocrine and paracrine regulation of re-epithelialization and only focused on 1 or 2 ligand-receptor interactions. For example reports describe up-regulation of CXCR2 (receptor for CXCL1 and CXCL8) early after wounding in undifferentiated keratinocytes (23), and mice lacking CXCR2 show impaired re-epithelialization after wounding (24). Also, expression of CXCR3 (receptor for CXCL10) on keratinocytes (25) and a delayed re-epithelialization in mice lacking CXCR3 and CXCL11 has been reported (12,13). Low et al. (26) described delayed wound healing in MCP1 knock-out mice. Fujimoto et al. (11) described CCR10 expression on epidermal keratinocytes and increased migration to its recombinant ligand CCL27 and Petering et al. (15) described proliferation of human keratinocytes after stimulation with CCL24, which may be inhibited by anti-CCR3. Our study clearly distinguishes the autocrine from the paracrine mechanisms involved in wound healing and this information will enable us and others to identify target molecules and cells for developing new wound healing strategies in the future.

Importantly, in our study we show that multiple chemokine receptors are involved in the autocrine regulation of re-epithelialization. Therefore, mutation or blocking of a single receptor would enable a by-pass mechanism via the other receptors to take place thus ensuring that wound closure still occurs even if a single gene should malfunction. Indeed, studies using knockout mouse show delayed wound healing but not absence of wound healing (12,13,26).

In conclusion, we show that autocrine regulation of re-epithelialization occurs via chemokine receptors and their ligands. Such a response may facilitate early triggering of wound closure. This early autocrine response is most probably combined with paracrine responses from infiltrating cells and neighbouring skin cells in order to amplify the wound healing cascade.
REFERENCES


