

Interleukin-1-stimulated Secretion of Interleukin-8 and Growth-related Oncogene- α Demonstrates Greatly Enhanced Keratinocyte Growth in Human Raft Cultured Epidermis

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The CXC chemokines, interleukin-8 and growth-related oncogene α , are known to play a prominent part in wound healing as well as inflammatory skin disorders, including psoriasis. Both chemokines are potent neutrophil activators and were discussed as potential stimuli in keratinocyte growth. We examined the action of growth-related oncogene α and interleukin-8 in organotypic raft culture, which resembles *in vivo* skin in several respects. Addition of growth-related oncogene α and interleukin-8 resulted in a time- and concentration-dependent epidermal hyperproliferation in organotypic cultures. In cryostat sections an increased number of epidermal layers as well as significantly elevated number of Ki-67-stained keratinocytes indicate marked hyperproliferation with no evidence for the reduction of apoptotic cells. Terminal differentiation was shown to proceed in a regular fashion with formation of a cornified layer and the expression of suprabasal keratins in addition to the presence of differentiation markers. Interleukin-8-mediated hyperproliferation was inhibited by a blocking human monoclonal antibody.

To demonstrate a specific receptor-mediated action of growth-related oncogene and interleukin-8, we used a CXC receptor 2 monoclonal antibody or a CXC receptor 2 selective nonpeptide antagonist, both of which lead to inhibition of interleukin-8-mediated hyperproliferation. Interleukin-1 α caused induction of interleukin-8 and growth-related oncogene α mRNA as well as marked epidermal hyperproliferation. The interleukin-1 α -mediated hyperproliferation was markedly reduced by both the interleukin-8-specific antibody and the CXC receptor 2 antagonist, indicating close correlation between the interleukin-8/CXC receptor 2 pathway and interleukin-1-induced keratinocyte growth stimulation. Our data indicate that interleukin-1 induces overexpression of interleukin-8 and growth-related oncogene α in human keratinocytes. These changes correlate with characteristic functional alterations of the epidermis as observed in psoriasis and wound healing. **Key words:** autocrine growth stimulation/epidermal raft culture/interleukin-1/interleukin-8. *J Invest Dermatol* 119:1254–1260, 2002

Interleukin (IL)-8 is a member of the CXC chemokine subfamily of chemotactic cytokines and plays a major part in the recruitment and activation of neutrophils at sites of inflammation (Schröder and Christophers, 1986). Members of this subfamily, including IL-8 and growth-related oncogene (GRO) α , are basic heparin-binding proteins and share a cysteine-X-cysteine motif near the amino terminus (Baggiolini *et al*, 1995).

In human skin, predominant cellular sources of IL-8 are activated monocytes, endothelial cells, and epidermal keratinocytes (Schröder *et al*, 1987, 1990; Schröder and Christophers, 1989). Although these cells are known to be potent producers of IL-8 *in vitro* following stimulation with primary cytokines, such as IL-1 or tumor necrosis factor α , little is known concerning the induction of IL-8 and GRO α *in vivo*. Increased release of biologically

active IL-1 has been shown in psoriatic keratinocytes and in chronic inflammatory skin diseases (Sauder *et al*, 1984; Bonifati *et al*, 1997). In keratinocytes, IL-1 α is stored intracellularly and can be quickly released in case of epidermal infection or injury. IL-1 can therefore be considered a possible inducer of IL-8 and GRO α *in vivo* (Sticherling *et al*, 1993).

In addition to its neutrophil chemotactic properties, IL-8 was shown to activate nonhematopoietic cells, including keratinocytes and endothelial cells. It was reported to stimulate angiogenesis (Nickoloff *et al*, 1994) and keratinocyte migration (Gyulai *et al*, 1994) as well as growth in submerged cultured keratinocytes (Tuschil *et al*, 1992).

Both CXC chemokines, IL-8 and GRO α , were isolated in large amounts from psoriatic scales (Schröder and Christophers, 1986) and could be shown to be both colocalized and overexpressed in psoriatic epidermis (Kulke *et al*, 1996). GRO α and IL-8-induced signaling is mediated by specific receptors on the surface of the target cells, which are designated as CXC receptors (CXCR) 1 and 2 (Ahuja *et al*, 1992). CXCR1 binds only IL-8 with high affinity (Ahuja and Murphy, 1996). CXCR2, which recently was shown to be overexpressed in psoriatic keratinocytes (Kulke *et al*, 1998), binds IL-8 as well as GRO α and other members of the CXC chemokine subfamily with similar affinities.

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Abbreviations: BM, basal medium; BrdU, bromodeoxyuridine; CXCR, CXC chemokine receptor; EGF, epidermal growth factor; GRO α , growth-related oncogene- α .

Because significant amounts of these chemokines could be recovered from psoriatic scales it appears likely that they play a part in causing the characteristic changes observed in this disease. These changes include epidermal hyperproliferation with increased mitotic activity of basal and suprabasal keratinocytes as well as altered keratinocyte differentiation accompanied by an inflammatory infiltrate in the epidermal and dermal compartments. In fact, these epidermal changes can be caused by local overexpression of growth factors and cytokines, which stimulate epidermal proliferation.

In vitro studies on epidermal keratinocytes employ culture systems in which the cells are submerged and thus surrounded by an artificial environment. Therefore, examination of keratinocyte proliferation in submerged cell culture appears of limited relevance.

We established an organotypic raft culture system that allows the study of epidermal growth regulation in close similarity to *in vivo* conditions. Using this culture system the effects of both chemokines, IL-8 and GRO α , in comparison with epidermal growth factor (EGF) and IL-1 were studied in organotypic raft cultures with regard to keratinocyte proliferation and differentiation.

MATERIALS AND METHODS

Reverse transcription-polymerase chain reaction analysis (reverse transcription-PCR) One microgram of the RNA prepared from frozen raft cultures was reverse transcribed using the Superscript RT-Kit (Gibco BRL, Eggenstein, Germany) according to standard protocols. Semiquantitative multiplex PCR with primers specific for the following was performed:

Glyceraldehyde-3-phosphate dehydrogenase and Ki-67 (5'-primer: 5'-ATTACAAGACTCGGTCCTG; 3'-primer: 5'-ACTGTCCCTATGACTTCTGG)

CXCR2 (5'-primer: 5'-AGCTGCTCTTCTGGAGGTGT; 3'-primer: 5'-TTAGAGAGTAG-TGGAAGTGTGC)

GRO α (5'-primer: 5'-TGAAGTGGCTGCCAGTGC; 3'-primer: 5'-GGCATGTTGCGCTGCCAGTGC)

Keratin 1 (5'-primer: 5'-CTTGACGAGTCCATCAG; 3'-primer: 5'-CAGAGGCATCTCTTTATC)

Keratin 5 (5'-primer: 5'-GTCAACTGTGCTCTTCC-GGAG; 3'-primer: 5'-CTGCGGTGGCAATCTCCTCA)

Keratin 16 (5'-primer: 5'-CTTCAGCAGCAGCAGCAG; 3'-primer: 5'-TCGTGGTTCTTCTCCTCAGG)

Laminin (5'-primer: 5'-CGGATCCTAGATGCAAAGAG; 3'-primer: 5'-ACAGAGTCCGTTGAAGGGTGG)

Small proline-rich protein 1 (5'-primer: 5'-GCATGAATTCTCAGCA GC; 3'-primer: 5'-CTTCTGCTTGGTCTTC-TG).

These were performed with the following specifications: denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min for 30 cycles. PCR products were separated on 1% agarose gels and stained with ethidium bromide. Quantification of the reverse transcription-PCR result was performed densitometrically using the BioDoc Analysis Software (Scanpack3, Biometra, Goettingen, Germany).

RNA isolation RNA was prepared from snap frozen raft cultures using 1 ml per culture Trizol (Gibco BRL) as described. The RNA yield was determined photometrically. RNA was stored at -70°C.

Immunohistochemistry Air-dried serial 5-8 μ m cryostat sections were fixed in acetone for 10 min, incubated with monoclonal mouse anti-Ki-67 antibody (Dianova, Hamburg, Germany) or with monoclonal mouse anti-CXCR2 or anti-CXCR1 antibody (both kindly provided by Genentech, San Francisco, CA) for 1 h, followed by incubation with a secondary biotin conjugated anti-mouse immunoglobulin antibody for 1 h. Detection of bound antibodies was performed with an avidin-biotin-peroxidase complex (ABC, Vector Laboratories, Burlingame, CA) and diaminobenzidine (Sigma, Deisenhofen, Germany) as chromogen. Sections were counterstained with hematoxylin. In the negative controls, the primary antibody was substituted by an isotype matched antibody with irrelevant specificity.

Organotypic raft culture Primary dermal fibroblasts and keratinocytes were prepared from foreskin and grown as previously reported (Mielke *et al*, 1990). Third passage fibroblasts ($5-7 \times 10^7$) were resuspended in 3 ml

ice-cold collagen solution containing 5.3 mg collagen type I (solved in 0.02 M CH₃COOH; Becton Dickinson, Bedford, MA), $1 \times$ Dulbecco minimal Eagle's medium (Gibco BRL), 2 mM L-glutamine (Gibco BRL), 0.5% NaHCO₃ (Merck, Darmstadt, Germany), 66.7 mM HEPES (Sigma), and 0.03 M NaOH (Merck), and submerged cultured in Dulbecco minimal Eagle's medium (Cell Concepts GmbH, Umkirch, Germany) + 10% fetal bovine serum (Kraeber GmbH & Co, Wedel, Germany) for 5 d. Third passage keratinocytes (5×10^5) were seeded on to these collagen lattices and submerged cultured for 4 d in keratinocyte growth medium (KGM Bullet Kit, BioWhittaker Europe, Verviers, Belgium) + 5% fetal bovine serum. Raft cultures were lifted to the air-medium-interphase and incubated in keratinocyte growth medium without bovine pituitary extract and EGF, but with additional 5% fetal bovine serum and 1.25 mM CaCl₂. Growth factors and antibodies were added into the medium. Before contacting the cultures, the IL-8 specific antibody was preincubated with medium-diluted IL-8. The receptor-specific antibodies and antagonists were added to the culture medium a minimum of 2 h earlier than the cytokines. The medium, including growth factors and antibodies, was renewed every 2-3 d. Raft cultures were harvested and frozen at -70°C. Serial cryostat sections (5-8 μ m) from frozen raft cultures were performed and stained with hematoxylin-cosin. Every raft series included three independent cultures and was repeated at a minimum twice.

Growth factors and cytokines EGF, IL-1 α , IL-8, and GRO α (Cell Concepts) were solved in water in a concentration of 100 μ g per ml and stored as aliquots at -20°C.

Antibodies EGF receptor and IL-8-specific antibodies were kindly provided by Abgenix Inc. (Fremont, CA). The CXCR2-specific antibody was provided by Genentech. In the negative controls, the antibody was substituted by an isotype matched antibody in a similar concentration, but with irrelevant specificity.

CXCR2 selective receptor antagonists The two antagonists (SB-225002 and SB-236210, kindly provided by SmithKline Beecham Pharmaceuticals, King of Prussia, PA) were dissolved in dimethyl sulfoxide at a concentration of 10 mM and used at a final concentration of 300 nM-1 μ M in culture medium. Equal amounts of dimethyl sulfoxide were used as negative control.

Detection of apoptotic cells in raft epidermis Apoptotic cells in serial cryostat sections were detected by TUNEL (terminal deoxynucleotidyl transferase-mediated nick end labeling) using the ApoAlert™ DNA fragmentation assay kit (Clontech, Palo Alto, CA) as recommended by the manufacturer's. 3'-OH DNA ends were labeled with fluorescent deoxyuridine triphosphate. Nonapoptotic cells were counterstained with propidium-iodide (Sigma).

Detection of proliferating cells in raft epidermis Raft cultures were grown in normal medium or supplemented with 100 ng IL-8 per ml. After 4d of culture, the medium was supplemented with 10 μ M bromodeoxyuridine (BrdU) for 24 h. The cultures were washed twice with phosphate-buffered saline, harvested, and fixed in 7.5% formaldehyde. Serial cryostat sections (4 μ m) were performed from paraffin-embedded raft cultures, dried at 37°C, and incubated with a BrdU-specific antibody (Amersham Life Science, Braunschweig, Germany). Detection of bound antibodies was performed using the Universal DAKO®-APAAP Kit (DAKO® Diagnostika, Hamburg, Germany). The sections were counterstained with hematoxylin.

RESULTS

Keratinocyte differentiation and epidermal stratification Rafts cultured in basal medium (BM) supplemented with 100 ng IL-8 per ml show regular epidermal differentiation and stratification, including formation of a stratum basale, as well as multilayered stratum spinosum. The granular zone consists of one to three cell layers covered by an orthokeratotic packed stratum corneum (Fig 1D). Differentiation and stratification was observed over a period of 16 d. On day 4 the epidermis consists of one basal cell layer, one to two suprabasal layers, and a thin stratum corneum with one to two layers (Fig 1B). On day 8 there was an increased epidermal thickness visible with two to three suprabasal layers (Fig 1C), which increased to about five layers on day 12 (picture not shown). Mitotic figures were occasionally seen within the basal layer at all investigated time-points. The dermis

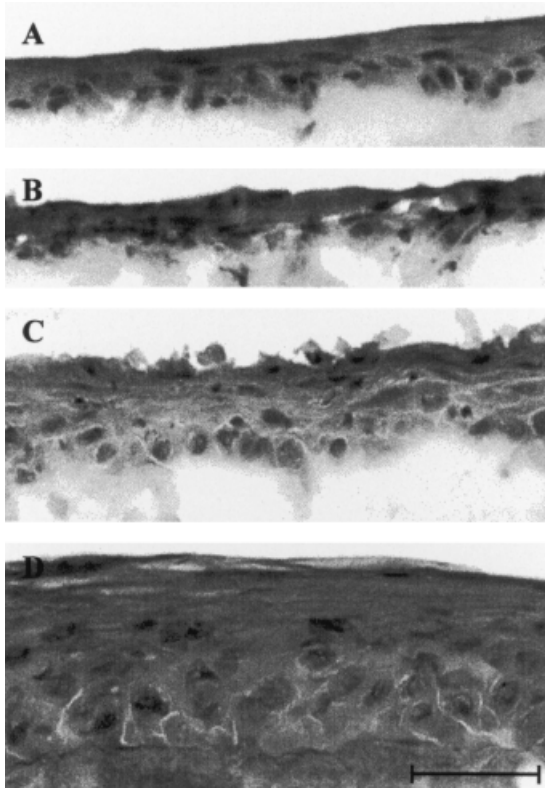


Figure 1. Time-dependent epidermis stratification in organotypic raft culture. Raft cultures were grown over a period of 16 d in BM (A) or in medium supplemented with 100 ng IL-8 per ml (B,C,D). Cultures were harvested on day 4 (B), day 8 (C), and day 16 (A+D). Cryostat sections from frozen raft cultures were performed and stained with hematoxylin–eosin. Scale bar = 50 μ m.

equivalent can be described as loose collagenous formation containing isolated fibroblasts (Fig 1A–D).

In contrast to nonsupplemented cultures (Fig 1A), organotypic raft cultures grown with 100 ng IL-8 per ml developed a markedly thickened epidermis with an increased number of suprabasal and cornified layers. Epidermal thickness was increased by a minimum of 50%. Above the stratum basale the stratum spinosum is 10 layers thick, followed by two to three granular layers and a loose multilayered stratum corneum. Epidermal thickening is visible at all investigated time-points.

Regular keratinocyte differentiation in artificial epidermis can be demonstrated by expression of differentiation markers. As shown in Fig 2, raft cultures were analyzed by semiquantitative reverse transcription–PCR in order to investigate the expression of typical keratinocyte differentiation markers.

As a basal lamina marker, laminin mRNA is regularly expressed at all time-points (4–16 d). A slight downregulation of mRNA expression occurs after day 4 (Fig 2A).

Keratin 5, a marker for basal keratinocytes, is continuously downregulated in raft cultures during growth from day 4 to day 16 (Fig 2C).

Expression of keratin 1, a suprabasal keratinocyte marker, is upregulated dependent on the time of culture. As shown in Fig 2(B), mRNA expression is strongly induced on the eighth day of growth.

Small proline-rich protein-1, which is known to be a marker for terminal differentiation, is equally expressed over the entire time period of epidermis development (Fig 2D).

As seen in Fig 2(E), keratin 16 shows a permanent expression pattern at every stage of growth. Keratin 16 is described as a protein only expressed in hyperproliferative epidermis.

Expression of Ki-67, a known nuclear antigen, is restricted to proliferating cells, whereas in resting cells Ki-67 is not detectable.

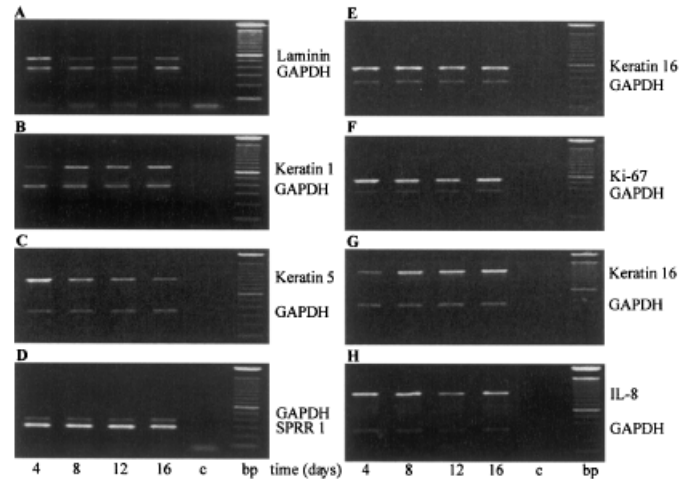


Figure 2. Expression of differentiation markers in artificial epidermis. Total RNA of frozen raft cultures grown for 4–16 d in unsupplemented raft medium was prepared and reverse transcribed. Semiquantitative multiplex reverse transcription–PCR was performed with specific primers for glyceraldehyde-3-phosphate dehydrogenase (A–H) and specific primers for laminin (A), keratin 1 (B), keratin 5 (C), small proline-rich protein 1 (D), keratin 16 (E), Ki-67 (F), CXCR2 (G), or IL-8 (H). PCR fragments were separated on 1% agarose gels and stained with ethidium bromide. c = control reaction, bp = 100 base pair ladder.

Proliferation of keratinocytes in artificial skin can be demonstrated by mRNA expression of Ki-67 in every analyzed raft culture (Fig 2F). Expression of Ki-67 mRNA in raft epidermis shows a time-dependent downregulation from day 4 to day 16.

In addition, the mRNA expression of CXCR2 (Fig 2G) and IL-8 (Fig 2H) can be demonstrated in all analyzed cultures.

Effect of chemokines

Effect of IL-8 and GRO α When the chemokines IL-8 or GRO α were added to the cultures this resulted in marked epidermal hyperproliferation as demonstrated by an increased number of suprabasal and cornified layers and an elevated number of Ki-67 expressing keratinocytes. This hyperproliferative chemokine effect is concentration dependent ranging from 50 to 250 ng per ml.

In comparison with rafts grown in BM without chemokine supplementation, cultures displaced with 50 ng IL-8 per ml show an increase of approximately three times in the number of epidermal layers. The stratum corneum contains a minimum of five layers in contrast to two to three layers present in unsupplemented cultures. Addition of 250 ng IL-8 per ml leads to a five to seven times increase in epidermal thickness with three times as many cornified layers (pictures not shown). The same results were obtained, when GRO α was used. Both IL-8 and GRO α promote epidermal cell proliferation to nearly the same extent.

Ki-67 protein expression is clearly upregulated under the influence of IL-8. In contrast to unsupplemented cultures, 100 ng IL-8 per ml leads to a two times, and 250 ng IL-8 per ml to a four times increase in the number of proliferating Ki-67 expressing keratinocytes in raft epidermis (Fig 3).

In addition to the increased cellularity and an upregulated Ki-67 expression, hyperproliferation was also shown by elevated BrdU incorporation in IL-8 supplemented raft cultures. After 4 d of culture, with 100 ng IL-8 per ml supplemented raft cultures show six times more replicating keratinocytes than cultures grown in BM without any growth-stimulating chemokines (Fig 4).

When a human anti-IL-8 monoclonal antibody was added to the raft cultures, the IL-8-associated keratinocyte hyperproliferation became inhibited, as demonstrated by a decreased number of

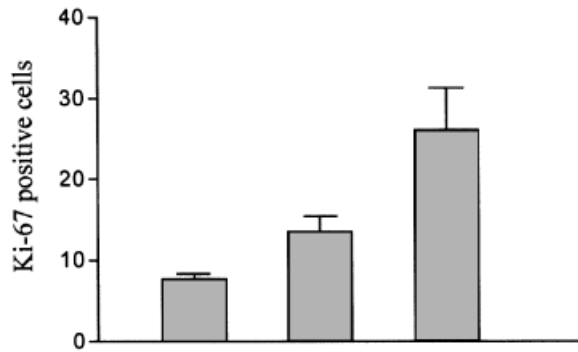


Figure 3. Increased number of Ki-67-positive stained keratinocytes in raft epidermis under the influence of IL-8. Raft cultures were grown for 14 d in BM or with different concentrations of IL-8 (100 or 250 ng per ml). Serial cryostat sections were stained immunohistochemically with a Ki-67-specific antibody. Bars show number of stained keratinocytes (mean ± SD) counted in a defined epidermal area.

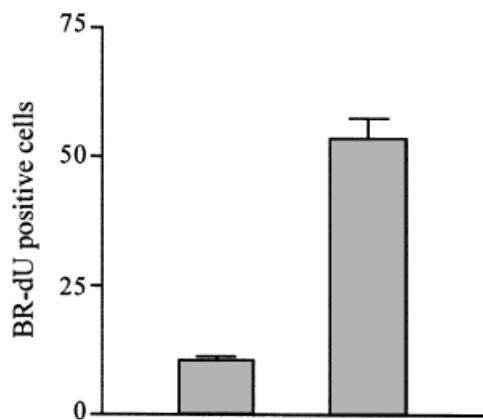


Figure 4. Increased number of BrdU incorporating keratinocytes in raft epidermis under the influence of IL-8. After for 4 d of culture in BM or in medium containing 100 ng IL-8 per ml, raft cultures were supplemented with 10 μ M BrdU for 24 h. Incorporated BrdU was detected immunohistochemically with a BrdU-specific antibody. Bars show number of stained keratinocytes (mean ± SD) counted in a defined epidermal area.

epidermal cell layers (**Fig 5B**) and a reduced number of proliferating Ki-67 expressing cells (**Table I**). Epidermal thickness and number of proliferating cells were reduced to the same level observed in cultures grown in normal, unsupplemented medium. Inhibition of IL-8 could not be detected with a control antibody of irrelevant specificity added to the IL-8 containing culture medium.

Effect of EGF Addition of 10 ng EGF per ml resulted in a markedly increased keratinocyte growth similar to the effects of 100 ng IL-8 per ml on epidermal growth. The number of suprabasal cell layers increased 5-fold when 10 ng EGF per ml was added. Interestingly, there was no increase of Ki-67 mRNA expression in EGF-stimulated keratinocytes detectable.

In the presence of an EGF receptor specific monoclonal antibody, complete inhibition of keratinocyte growth was noted and the epidermal thickness was reduced to a minimum in cultures with blocked EGF receptor. This strong inhibitory effect of the EGF antibody on keratinocyte growth could not be detected, when a control antibody was added to the EGF containing culture medium (data not shown).

Effect of IL-1 α IL-1 α at a concentration of 100 ng per ml strongly promoted keratinocyte growth resulting in an increase of cell proliferation and epidermal thickening. Additionally, an approxi-

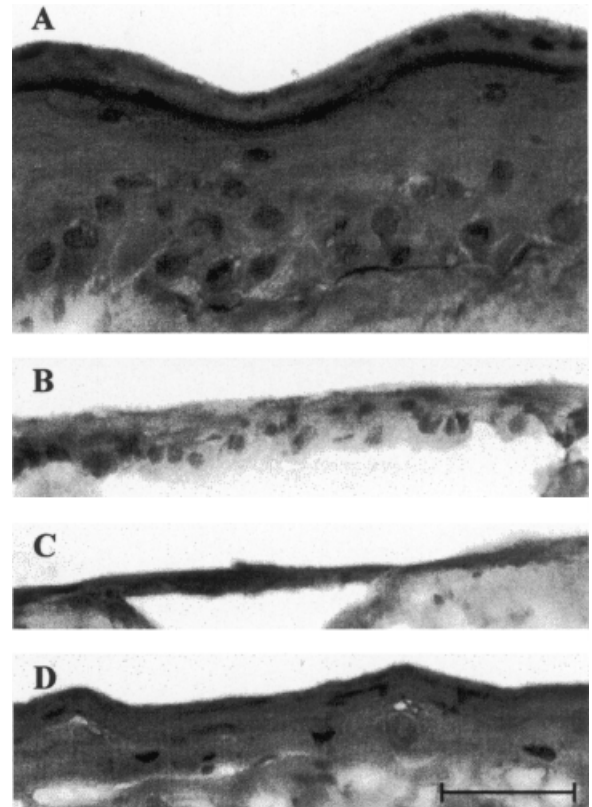


Figure 5. IL-8 induced hyperproliferation can be inhibited by blocking of CXCR2. Raft cultures were grown over a period of 15 d in medium supplemented with 100 ng IL-8 per ml (**A**), 100 ng per ml IL-8+82.5 μ g per ml anti-IL-8 antibody (**B**), 100 ng per ml IL-8+250 μ g per ml anti-CXCR2 antibody (**C**), or 100 ng per ml IL-8+1 μ M SB22 (**D**). Cryostat sections from frozen raft cultures were performed and stained with hematoxylin-eosin. Scale bar = 50 μ m.

mately five times raise of Ki-67 expressing cells was detected. (**Table I**).

Additionally, reverse transcription-PCR analysis of IL-1 α -stimulated raft cultures revealed an induction of IL-8 and GRO α mRNA. As shown in **Fig 6**, expression of GRO α mRNA is twice, whereas expression of IL-8 mRNA five times higher than in unstimulated raft cultures.

In order to investigate further the mechanism by which IL-1 α acts on keratinocytes, a human IL-8-specific monoclonal antibody was added to hyperproliferating IL-1 α -stimulated cultures. This resulted in a reduction of the number of epidermal layers by approximately 50%. The morphologic changes of epidermal thickness were ascertained by measuring the rates of proliferating Ki-67-positive keratinocytes. As can be seen in **Table I**, addition of the anti-IL-8 antibody resulted in a decrease of approximately 50% of the number of proliferating keratinocytes in IL-1 α -stimulated raft cultures.

Receptor-mediated effects of IL-8 on keratinocytes The IL-8-mediated keratinocyte hyperproliferation in raft epidermis was inhibited by blocking the receptor protein CXCR2. Whereas both receptors, CXCR1 and CXCR2, were expressed at the mRNA level only CXCR2 could be detected at the protein level by immunohistochemistry (data not shown). Receptor blocking was performed by using either a CXCR2 specific monoclonal antibody, or the nonproteinous CXCR2 selective receptor antagonist SB-225002. Both additives led to similar results: inhibition of IL-8-mediated growth promotion of keratinocytes in organotypic culture. This inhibition can be demonstrated by reduction of IL-8-induced epidermal thickness (**Fig 5C + D**) and additionally by a decreased amount of

Table I. Up/downregulation of Ki-67 mRNA and protein in organotypic culture. Raft cultures were grown in BM or supplemented with growth factors and inhibitors as indicated. Induction of Ki-67 mRNA is demonstrated by semiquantitative multiplex reverse transcription-PCR with specific primers for glyceraldehyde-3-phosphate dehydrogenase and Ki-67. PCR products were separated on a 1% agarose gel, stained with ethidium bromide and analyzed densitometrically. Proliferating keratinocytes in artificial epidermis were detected by immunohistochemistry with a Ki-67 specific antibody and counted in a defined epidermal area

Raft culture	Ki-67 mRNA expression (PCR-data)	Ki-67 mRNA expression (relative to BM)	Total no. of Ki-67 positive cells per area	No. of Ki-67 positive cells (relative to BM)
BM	0.08	1.00	4.40	1.00
IL-8	0.94	11.40	26.00	5.90
IL-8 + α IL-8 antibody	0.07	0.88	7.33	1.67
IL-8 + SB22	0.23	2.80	0.90	0.20
IL-8 + DMSO	0.50	6.00	26.00	5.90
IL-8 + α CXCR2 antibody	0.21	2.48	—	—
IL-8 + control antibody	0.86	10.31	22.67	5.15
BM	0.08	1.00	8.60	1.00
IL-1 α	1.76	22.00	39.80	4.63
IL-1 α + SB22	0.04	0.48	—	—
IL-1 α + α IL-8 antibody	1.03	12.88	—	—

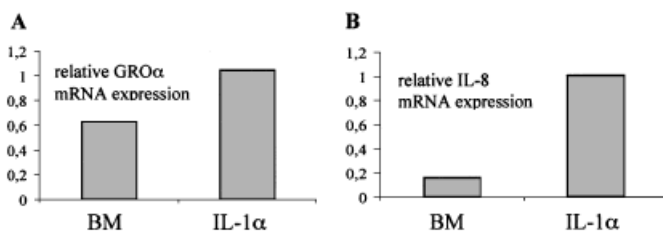


Figure 6. IL-1 α induces upregulation of IL-8 and GRO α mRNA in organotypic culture. Raft cultures were grown in BM or supplemented with 100 ng IL-1 α per ml. Induction of chemokines is demonstrated by semiquantitative multiplex reverse transcription-PCR with specific primers for glyceraldehyde-3-phosphate dehydrogenase and GRO α (A) or IL-8 (B). PCR products were separated on 1% agarose gels, stained with ethidium bromide and analyzed densitometrically.

proliferating Ki-67-positive keratinocytes in raft epidermis (Table I).

The number of epidermal layers as well as the quantity of proliferating cells in CXCR2-blocked raft cultures was reduced to the same level observed in organotypic cultures grown in basal unsupplemented medium. This effect was not seen with an appropriate concentration of isotype matched control antibody with irrelevant specificity or with dimethyl sulfoxide. Control cultures developed a similarly thickened epidermis seen in IL-8-stimulated raft cultures. The SB-225002 related substance SB-236210 did not show any visible effects on keratinocyte growth in raft cultured epidermis (data not shown).

Addition of the CXCR2 selective receptor antagonist SB-225002 to IL-1 α -stimulated organotypic raft cultures resulted in a similar keratinocyte growth inhibition to cultures grown in IL-8 containing medium. Cell proliferation (Table I) as well as epidermal thickening returned to the basal level of unstimulated cultures. [Ongoing studies using the inhibitor of CXCR2 SB-225002 and also anti-EGF receptor antibodies showed that these were able to block epidermal thickening stimulated with IL-8 as well as with GRO α (unpublished).]

DISCUSSION

In this study we demonstrate that IL-8 and GRO α both markedly stimulate keratinocyte growth in organotypic cultures of

human skin. Also it is shown that IL-1 acts as a powerful growth promoter by stimulating the secretion of IL-8 and GRO α .

Raft cultures of human skin have provided a useful tool for the study of human skin *in vitro* (Parenteau *et al*, 1992). They consist of a dermal equivalent of collagen-embedded fibroblasts covered by keratinocytes. Being located exactly at the air-liquid interphase, keratinocytes are able to form a multilayered epithelium within days after seeding (Bell *et al*, 1991). In addition, stratification and the formation of keratinized cell layers ensues and, as revealed by morphology, horny cell layers result from further differentiation of granular cells in the stratum granulosum (Fig 1).

Evidence for an *in vivo*-like terminal differentiation of these keratinocytes is provided by expression of distinct markers of keratinocyte maturation. Within 4 d after seeding the so-called small proline-rich protein 1 as a marker for cellular envelope formation was noted in addition to mRNA for keratin 10, involucrin (Fig 2), small proline-rich protein 2, transglutaminase, and protein kinase C (data not shown). The synthesis of these markers is indicative for cell-specific terminal differentiation of the human keratinocyte system (Watt, 1983; Bayerl *et al*, 1995; Fischer *et al*, 1998).

A concentration-dependent increase in keratinocyte numbers and epidermal thickness is seen following the addition of ELR(+) (glutamic acid-leucine-arginine motif positive) CXC chemokines IL-8 and GRO α . Augmented cell members include suprabasal cells as well as cornified cells and are likely to be the result of increased proliferative activity of the basal layer shown by the raised number of Ki-67-stained cells and Ki-67 mRNA. This is further ascertained by BrdU staining demonstrating that keratinocyte new cell production was raised two to four times above controls (Fig 4). Therefore the increased epidermal thickness seen in these raft cultures appears to result from IL-8 (or GRO α) induced stimulation of cell division in the basal cell layer.

Specificity of this process was supported by blocking experiments using an anti-IL-8 antibody that completely abrogated an IL-8-induced increase in epidermal cellularity (Fig 5). Also, the expression of Ki-67 mRNA remained within the control range indicating that the human antibody shows marked inhibitory effects in CXCR2-mediated hyperproliferation (Table I).

Interestingly, induction of epidermal growth was nearly identical with addition of either IL-8 or GRO α at the same concentrations. Both chemokines act as ligands for CXCR2, in contrast to CXCR1, which shows high affinity for IL-8 only (Murphy, 1997). CXCR1 and CXCR2 appear to be differentially expressed

in raft cultures, as CXCR1 mRNA is detected, no CXCR1 protein is expressed (Michel *et al*, 1992; Mueller *et al*, 1994). Also, in a previous study Kulke *et al* (1998) were unable to detect CXCR1 in normal human or in psoriasis epidermis. Thus, *in vivo* as well as in organotypic cultures the receptor for IL-8 and GRO α appears to be CXCR2. This observation was further supported by use of CXCR2-specific antibody (Fig 5), which completely blocked chemokine (IL-8, GRO α) induced keratinocyte growth.

Among the various chemokines/cytokines produced by human keratinocytes IL-8 plays a major part. Whereas IL-8 initially was detected as a powerful chemoattractant for neutrophils (Schröder and Christophers, 1986) subsequent work by various groups has revealed a comparatively broad spectrum of biologic activities (for review see Schröder, 1995). These include proinflammatory effects by activating inflammatory cells (Barker *et al*, 1991), growth promoting activities in tumor cell lines (Schadendorf *et al*, 1993) and keratinocytes, and angiogenesis (Koch *et al*, 1992).

Previous studies (Sticherling *et al*, 1991) have demonstrated that in human keratinocytes preformed IL-8 is present and is secreted following stimulation. Normal keratinocytes were shown to contain significant amounts of IL-8 protein as revealed by immune histology (Sticherling *et al*, 1991) and this immunoreactivity was readily lost following wounding or inflammation (Sticherling *et al*, 1992). Production of IL-8 by epithelial cells can be upregulated by the addition of various cytokines (e.g., IL-1 α , IL-1 β , tumor necrosis factor α ; Larsen *et al*, 1989) or irritants (e.g., phorbol ester; Schenk *et al*, 1996). In human keratinocytes cultured *in vitro* we could show that IL-8 production was greatly augmented by the addition of IL-1 and tumor necrosis factor α to the culture medium (Schröder *et al*, 1990).

Furthermore, early and significant secretion of IL-8 was observed when human keratinocytes were confronted with inactivated bacterial membranes *in vitro*. Next to the generation of anti-microbial defense peptides (e.g., hBD-2; Harder *et al*, 1997), IL-8 production could be stimulated when living bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*; Xue *et al*, 2000) were in close physical contact with keratinocytes.

More recently, exposure of epithelial cells with *P. aeruginosa* resulted in early expression of IL-8 (and IL-6) and was followed by IL-1 β and tumor necrosis factor α (Xue *et al*, 2000). Also, *in vivo* exposure of epithelium to *P. aeruginosa* caused marked production of CXCR chemokines (Tsai *et al*, 2000). When in these mice the CXCR 2 was neutralized a marked increase in mortality ensued.

In this study it has been shown that with 100 ng IL-1 per ml an increase in epidermal thickness can be obtained, which compares with the effects of high concentrations of IL-8 and GRO α . This effect again was caused by stimulated cellular growth of basal cells shown by greatly elevated levels of Ki-67 mRNA and protein (Table I). In order to analyze more closely this IL-1 effect in keratinocytes mRNA levels for IL-8 and GRO α were determined and were found to be elevated, whereas IL-6 mRNA was not affected by exposure of the cells to IL-1 (data not shown).

Furthermore, when organotypic raft cultures were stimulated in the presence of the CXCR2 receptor antagonist, both proliferative activity as well as epidermal thickness remained at control levels (Fig 5). These data indicate that IL-1 indirectly stimulates keratinocyte growth via the production of both IL-8 and GRO α and that these effects are mediated by CXCR2. Indeed, the addition of an IL-8-specific antibody to the IL-1-stimulated culture system caused a near 50% reduction of growth (Table I). The remaining growth potential is likely to be due to GRO α , which after being induced by IL-1 may still cause signaling by binding to CXCR2.

Taken together our data provide strong evidence for potent growth promoting effects of both chemokines, IL-8 and GRO α , on human keratinocytes. This observation is of help in further understanding the pleiotropic activities of IL-8.

In skin, the chemotactic and growth promoting effects of IL-8 appear to play a major part especially in wound healing. Previous work has shown that in skin wounding the concentrations of IL-1 as well as IL-8 and GRO α are markedly increased (Sauder *et al*,

1990; Nanney *et al*, 1995). Similarly elevated amounts of these cytokines are seen in psoriatic epidermis and inflammatory conditions (e.g., contact dermatitis; Corsini and Galli, 1998). In psoriasis the amounts of IL-8 recovered from scale material were higher than any other cytokine studied (Schröder *et al*, 1992) suggesting that this chemokine is of primary importance in epithelial growth and defense. As shown (Schröder and Christophers, 1989), potent stimuli for IL-8 are provided by microbial agents in contact with keratinocytes as well as the dominating proinflammatory cytokine IL-1. Thus, under wound healing conditions at least two stimuli for the secretion of IL-8 are operative.

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