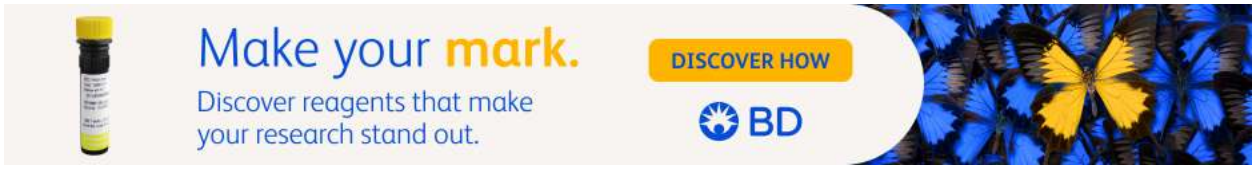


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# Wound Healing and Expression of Antimicrobial Peptides/Polypeptides in Human Keratinocytes, a Consequence of Common Growth Factors[1]

**Ole E. Sørensen,[2]*† Jack B. Cowland,* Kim Theilgaard-Mönch,* Lide Liu,† Tomas Ganz,† and Niels Borregaard***

**In addition to acting as a physical barrier against microorganisms, the skin produces antimicrobial peptides and proteins. After wounding, growth factors are produced to stimulate the regeneration of tissue. The growth factor response ceases after regeneration of the tissue, when the physical barrier protecting against microbial infections is re-established. We found that the growth factors important in wound healing, insulin-like growth factor I and TGF-$\alpha$, induce the expression of the antimicrobial peptides/polypeptides human cationic antimicrobial protein hCAP-18/LL-37, human $\beta$-defensin 3, neutrophil gelatinase-associated lipocalin, and secretory leukocyte protease inhibitor in human keratinocytes. Both an individual and a synergistic effect of these growth factors were observed. These findings offer an explanation for the expression of these peptides/polypeptides in the skin disease psoriasis and in wound healing and define a host defense role for growth factors in wound healing.** ***The Journal of Immunology,*** **2003, 170: 5583–5589.**

Epithelia constitute an important barrier against invading microorganisms. Epithelial cells produce antimicrobial peptides and polypeptides, important effector molecules in the innate immune response in all species from insects to man (1, 2). Most antimicrobial peptides are active against a broad spectrum of bacteria, some enveloped viruses, and fungi. They may also play a role in the regulation of normal microflora (3). Four antimicrobial peptides have been identified in the human skin and keratinocytes: the human $\beta$-defensins (hBD)[3] hBD-1 (4), hBD-2 (5), hBD-3 (6), and human cationic antimicrobial protein 18 kDa (7) (hCAP-18), the only human member of the cathelicidin family of antimicrobial peptides (hCAP-18 is also named LL-37 for the 37-aa active antimicrobial peptide liberated from the C terminus of the protein). Both hCAP-18 and its mouse analog cathelin-related antimicrobial peptide (CRAMP) have been shown to be up-regulated in the skin following cutaneous injury (8). The CRAMP knockout mouse has increased susceptibility to necrotic skin infection caused by group A streptococcus (9), clearly demonstrating a role of cathelicidins in skin immunity. Keratinocytes also express secretory leukocyte protease inhibitor (SLPI) (10) and neutrophil gelatinase-associated lipocalin (NGAL) (11), polypeptides with antimicrobial activity (12–14).

Keratinocytes in the hyperproliferative skin disease psoriasis are also known to express antimicrobial peptides/polypeptides (5–7, 10, 11). This prompted us to examine the role of growth factors involved in wound healing in the regulation of expression of antimicrobial peptides/proteins in human keratinocytes.

We here demonstrate that two of the important growth factors in wound healing, insulin-like growth factor I (IGF-I) and TGF-$\alpha$, induce the expression of the antimicrobial peptides/polypeptides hCAP-18, hBD-3, NGAL, and SLPI in human keratinocytes. Both an individual and a synergistic effect of these growth factors were observed.

Furthermore, we demonstrate that each of the tested antimicrobial peptides/polypeptides had an individual pattern of induction of expression in response to growth factors and proinflammatory cytokines, and that each cytokine/growth factor induces a distinct expression profile of the antimicrobial peptide/polypeptides tested.

## Materials and Methods

### *Reagents*

Primary human keratinocytes were purchased from BioWhittaker (Walkersville, MD). Anti-SLPI Abs were provided by P. Hiemstra (Leiden, The Netherlands). IGF-I, TGF-$\alpha$, TGF-$\beta$1, basic fibroblast growth factor (bFGF), IL-1$\beta$, and TNF-$\alpha$ were purchased from Sigma-Aldrich (St. Louis, MO). IL-6 was purchased from Sandoz (East Hanover, NJ). Anti-hBD-3 Abs were purchased from Orbigen (San Diego, CA), and synthetic hBD-3 was obtained from PeproTech (Rocky Hill, NJ). The Abs for NGAL and hCAP-18 have been described previously (15, 16).

### *SDS-PAGE and immunoblotting*

SDS-PAGE (17) and immunoblotting (18) were performed with Bio-Rad systems according to instructions given by the manufacturer (Bio-Rad, Hercules, CA). For immunoblotting, after the transfer of proteins from the 14% polyacrylamide gels, the polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) were blocked for 1 h with 5% skimmed milk in PBS. For detection of hCAP-18, NGAL, and SLPI, the PVDF membranes were incubated overnight with primary Abs. The following

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[3] Abbreviations used in this paper: hBD, human $\beta$-defensin; AU, acid urea; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; hCAP-18, human cationic antimicrobial protein; IGF-I, insulin-like growth factor I; NGAL, neutrophil gelatinase-associated lipocalin; PVDF, polyvinylidene difluoride; SLPI, secretory leukocyte protease inhibitor; CRAMP, cathelin-related antimicrobial peptide.

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day, the membranes were washed and incubated for 2 h with alkaline phosphatase-conjugated secondary Abs (DAKO, Glostrup, Denmark) then washed and visualized by 5′-bromo-chloro-indolyl phosphate (Sigma-Aldrich) and nitro blue tetrazolium (Sigma-Aldrich).

### *Extraction and detection of hBD-3*

Medium from keratinocytes was extracted with MacroPrep CM Support beads (Bio-Rad) overnight at 4°C. The beads were subsequently washed, and bound material was eluted with 30% acetic acid. The eluted material was dialyzed in 5% acetic acid and lyophilized before resuspension in sample buffer for acid urea (AU)-PAGE.

AU-PAGE and immunoblotting were performed according to instructions given by the manufacturer (Hoeffer, San Francisco, CA). After transfer of proteins from the 12.5% acrylamide gels, the PVDF membranes were fixed for 30 min in TBS with 0.05% glutaraldehyde (Sigma-Aldrich), followed by blocking with Superblock Blocking Buffer (Pierce, Rockford, IL) For visualization of hBD-3, the PVDF membranes were incubated overnight with primary Abs. The following day, the membranes were incubated for 2 h with HRP-conjugated secondary Abs, (Pierce) and visualized by Immun-Star HRP luminal/enhancer and Immun-Star peroxide buffer (Bio-Rad).

### *Growth and stimulation of primary keratinocytes*

Cells were grown in serum-free keratinocyte medium from Clonetics (KGM-2 Bullet Kit; San Diego, CA) with bovine pituitary extract, transferrin, human epidermal growth factor (EGF), hydrocortisone, gentamicin, amphotericin B, and epinephrine, but without insulin. Cells were stimulated beginning 24 h after complete confluence was reached.

Cells and medium was harvested 0, 3, 6, 12, 24, and 48 h after stimulation with IGF-I (100 ng/ml), TGF-$\alpha$ (50 ng/ml), TGF-$\beta$1 (10 ng/ml), bFGF (100 ng/ml), IGF-I/TGF-$\alpha$, IL-1$\beta$ (20 ng/ml), IL-6 (100 ng/ml), EGF (100 ng/ml), and TNF-$\alpha$ (20 ng/ml). For demonstration of hCAP-18, medium and cells were harvested 0, 24, 48, 72, and 96 h after stimulation with IGF-I.

### *Organotypic culture and stimulation*

Primary epidermal cultures EPI-200–3S (MatTek, Ashland, MA) containing human epidermal keratinocytes were grown on a collagen-coated Millicel CM membranes. The cultures were placed in 12-well plates with medium supplied by the manufacturer (which contains no bovine pituitary extract). On day 4 the epidermal cultures were lifted to the air-liquid interface and then cultured in air-liquid interface for another 4 days according to the instructions of the manufacturer. On day 2 after airlifting the cultures the medium was changed to medium without insulin or EGF. On day 4 after airlifting the cultures were stimulated with IGF-I (100 ng/ml), TGF-$\alpha$ (50 ng/ml), or a combination of IGF-I and TGF-$\alpha$. Cells were harvested after 48 h of stimulation.

### *RNA isolation*

Total RNA was isolated with TRIzol (Life Technologies, Gaithersburg, MD) according to recommendations of the manufacturer. RNA was precipitated with ethanol and resuspended in 0.1 mM EDTA. The concentration was determined by spectrophotometric measurement, and the integrity of the RNA assessed by running a sample on an agarose gel.

### *Northern blotting*

For Northern blotting, 5 $\mu$g of RNA was run on a 1% agarose gel with 6% formaldehyde dissolved in 1× MOPS for size separation. The RNA was transferred to a Hybond-N membrane (Amersham Pharmacia Biotech, Little Chalfont, U.K.) by capillary blotting and was fixed by UV irradiation. The filters were prehybridized for a minimum of 30 min at 42°C in 10 ml of ULTRAhyb (Ambion, Austin, TX) and hybridized overnight at 42°C after the addition of an additional 5 ml of ULTRAhyb containing the $^{32}$P-labeled probe. The membranes were washed twice for 5 min each time at 42°C in 2× SSC (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0)/0.1% SDS, followed by twice for 15 min each time in 2× SSC/0.1% SDS, once for 15 min in 0.2× SSC/0.1% SDS, and once for 15 min in 0.1× SSC/0.1% SDS at 42°C. The blot was developed and quantified by a phosphorimager (Fuji Imager Analyzer BAS-2500, Image Reader version 1.4E, Image Gauge version 3.01 software; Fuji, Stockholm, Sweden). The sizes of the mRNAs were determined by reference to 18S and 28S rRNA, which were visualized by ethidium bromide staining. The membranes were stripped by boiling in 0.1% SDS before rehybridization.

The probes used for hybridization were cDNA fragments radiolabeled with [$\alpha$-$^{32}$P]dCTP using the Random Primers DNA Labeling System (Life Technologies). The probes NGAL (19), hCAP-18 (20), hBD-2 (21), and $\beta$-actin (20) have previously been described.

The probes for SLPI, hBD-1, and hBD-3 were amplified from cDNA from keratinocytes with the following primers: SLPI, 5′-ATGAAG TCCAGCGGCCTC-3′ and 5′-AAGAGAAATAGGCTCGTTTATTT-3′; hBD-1, 5′-GCTCAGCCTCCAAAGGAGC-3′ and 5′-AAAAGAATGCT TATAAAAAGTTCAT-3′; and hBD-3, 5′-GGAATCATAAACACATTA CAGAA-3′ and 5′-CGGGAATCATAAACACATTACAGAA-3′. The probe for hBD-4 was amplified from genomic DNA using the following primers: 5′-GCAGCCCCAGCATTATGCA-3′ and 5′-AAGCTACTGAG GTCCTACTTC-3′.

All PCR-amplified probes were cloned into plasmids and verified by DNA sequencing. The probes for labeling were liberated from the plasmids by restriction with suitable restriction enzymes. The digests were run on 1% agarose gels, and the probes were purified by gel extraction before labeling.

### *Quantitation of proteins*

Human CAP-18 and NGAL were measured by ELISA as described previously (15, 16). SLPI was measured by a sandwich ELISA using recombinant SLPI as standard.

### *Immunohistochemistry*

Following stimulation with growth factors, cytospins were prepared from trypsinated primary keratinocytes. The cytospins were fixed for 10 min in 10% formalin in PBS and subsequently washed with TBS. The slides were incubated with a 1/1000 dilution of rabbit polyclonal Abs against NGAL and hCAP-18 and a 1/666 dilution of rabbit polyclonal Abs against hBD-3. The Abs were diluted in TBS with 1% gelatin, 0.05% Tween 20 (Sigma-Aldrich), and 0.01% thimerasol, and the slides were incubated for 24 h at room temperature. After three 20-min washes in TBS with 0.05% Tween 20, the slides were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Pierce) diluted 1/1000 in the same buffer as the first Ab and incubated for another 24 h, followed by three 20-min washes. Color was developed with Fast Red chromogen (Sigma-Aldrich) in Tris buffer, and the slides were counterstained with Harris hematoxylin (EM Science, Gibbstown, NJ).

## Results

Keratinocytes were stimulated with the growth factors involved in wound healing (IGF-I, TGF-$\alpha$, TGF-$\beta$1, and bFGF) as well as with representative proinflammatory cytokines (IL-1$\beta$, IL-6, and TNF-$\alpha$). To avoid interference from growth factors already present in the medium, cells were grown in serum-free medium without insulin (insulin binds with low affinity to the IGF-I receptor) and with only 0.15 ng/ml EGF.

It has previously been noted that keratinocytes must reach a certain level of differentiation to express antimicrobial peptides (22). We chose a model in which primary keratinocytes were grown to confluence, then stimulated 24 later, since we found that this gave consistent expression of the antimicrobial peptides/polypeptides following stimulation with growth factors.

The cathelicidin hCAP-18 was up-regulated by IGF-I at both the protein level (ELISA) and the mRNA level (Fig. 1*A*). The long time course of induction was chosen to demonstrate the accumulation of hCAP-18 in the medium. The presence of hCAP-18 in stimulated keratinocytes was further verified by Western blot (Fig. 1*B*) and immunostaining with anti-hCAP-18 Abs of stimulated and unstimulated keratinocytes (Fig. 1*C*). The other growth factor and proinflammatory cytokines tested did not induce the expression of hCAP-18 in keratinocytes (data not shown).

The defensin hBD-1 is constitutively expressed in various epithelia (23). However, we found basal constitutive expression of hBD-1 in keratinocytes increasing over time relative to the housekeeping gene $\beta$-actin (a 3-fold increase was found at 24 h and a 7-fold increase at 48 h compared with the $\beta$-actin; Fig. 2*A*). However, expression was up-regulated ~50% compared with control at the mRNA level following 24- and 48-h stimulation with IL-6, which, however, was not followed by an increased level of hBD-1 in the medium (data not shown).

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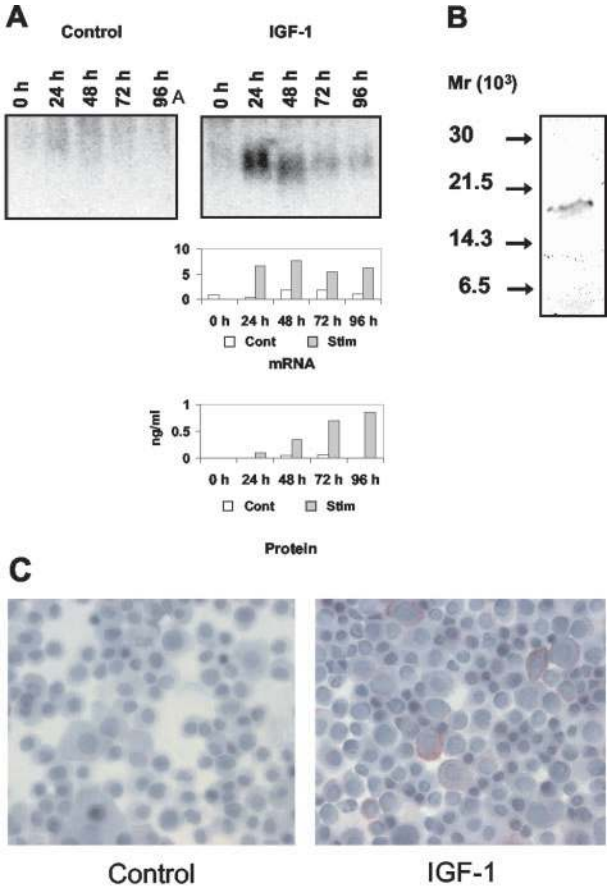


**FIGURE 1.** Expression of hCAP-18. *A*, Northern blot of total RNA from control cells and IGF-I-stimulated cells. The blot was hybridized with probes for hCAP-18 and $\beta$-actin ($\beta$-actin hybridization not shown). Below the Northern blot is a schematic presentation of the expression of hCAP-18 normalized to the expression of $\beta$-actin. Basal expression was given a value of 1. Human CAP-18 was measured by ELISA in the medium from control and stimulated keratinocytes. The concentration of hCAP-18 is shown in nanograms per milliliter. A schematic presentation of these measurements is shown below the Northern blot data. *B*, Material from stimulated keratinocytes was run on SDS-PAGE, followed by immunoblotting with anti-hCAP-18 Abs. A band of the appropriate molecular size was seen. *C*, Keratinocytes were either stimulated with IGF-I or left unstimulated. After 48 h of stimulation the cells were trypsinated, and cytospins were made, followed by immunostaining with anti-hCAP-18 Abs.

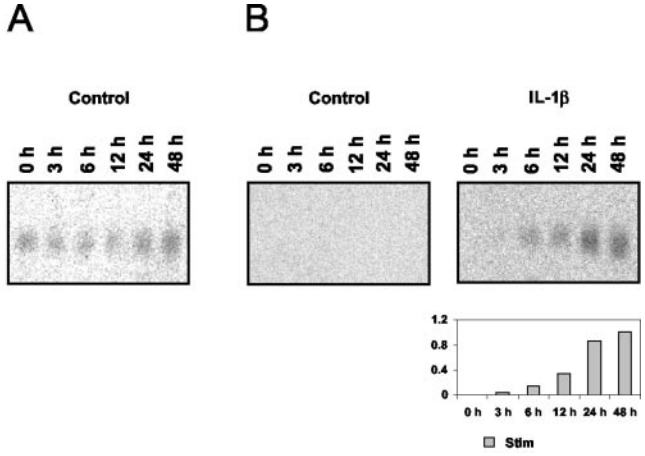


**FIGURE 2.** Northern blot of hBD-1 and hBD-2. *A*, Northern blot of total RNA from control cells. The blots were hybridized with probe for hBD-1 and $\beta$-actin ($\beta$-actin hybridization not shown). *B*, Northern blot of total RNA from control cells and IL-1$\beta$-stimulated cells. The blots were hybridized with probe for hBD-2 and $\beta$-actin ($\beta$-actin hybridization not shown). Since there is no basal expression of hBD-2, the maximal expression is given a value of 1 in the schematic representation of the hybridization intensity shown below the Northern blot.

Another defensin, hBD-2, was found to be up-regulated in keratinocytes by IL-1 in keratinocytes as previously described (22) (Fig. 2*B*). None of the other cytokines/growth factors induced the expression of hBD-2 (data not shown).

mRNA for hBD-3 was not detected in unstimulated keratinocytes, but was significantly induced by TGF-$\alpha$ (Fig. 3*A*). Even though IGF-I did not induce the expression of hBD-3, a 5-fold higher mRNA level was found in response to a combination of IGF-I and TGF-$\alpha$ (Fig. 3*A*) after 48 h of stimulation compared with stimulation with TGF-$\alpha$ alone. This is consistent with the finding that IGF-I causes *trans*-activation and *trans*-modulation of the EGF receptor (24, 25), and thus potentially augments the effect of TGF-$\alpha$, which binds to the EGF receptor. None of the proinflammatory cytokines or the other growth factors induced hBD-3 (data not shown). By immunoblot, hBD-3 was detected in the medium from keratinocytes stimulated with TGF-$\alpha$ and IGF-I/TGF-$\alpha$, but not in the medium from unstimulated cells (Fig. 3*B*), thus demonstrating the induction of hBD-3 at the protein level. Immunostains of primary keratinocytes with Abs against hBD-3 confirmed that hBD-3 peptide was induced to a greater extent by IGF-I/TGF-$\alpha$ than by TGF-$\alpha$ alone (Fig. 3*C*).

We were not able to detect any transcript for the recently described defensin hBD-4 (26) under any of these conditions by Northern blotting (data not shown).

There was a low basal expression of NGAL in the keratinocytes (Fig. 4*A*). This expression, however, was strongly induced by IL-1$\beta$ as described previously[4] and also by IGF-I and TGF-$\alpha$ (Fig. 4*A*). These growth factors furthermore had a synergistic/additive effect on the expression of NGAL. This was found at both the protein level (measured as protein secreted into the medium of the cells) and the mRNA level, where the expression of NGAL was induced faster and to a greater extent by the combination of IGF-I/TGF-$\alpha$ than by either growth factor alone.

The highest levels of NGAL (mRNA and protein) were induced by a combination of IGF-I/TGF-$\alpha$ after 48 h. The presence of NGAL in the medium from stimulated keratinocytes was detected by Western blot (Fig. 4*B*). Immunostains of primary keratinocytes with Abs against NGAL confirmed the induction of NGAL protein by growth factors (Fig. 4*C*).

SLPI was constitutively expressed in keratinocytes as previously described (27), and the expression increased over time (Fig. 5*A*). The basal expression was increased, however, by TNF-$\alpha$ and IL-$\beta$ as described in lung cell lines (28). The expression of SLPI was also increased by TGF-$\alpha$ and to a very minor extent by IGF-I. Again, the combination of IGF-I and TGF-$\alpha$ (Fig. 5A) resulted in the strongest induction of SLPI at both the protein and mRNA levels after 48 h. SLPI in the medium from stimulated keratinocytes was detected by Western blot (Fig. 5B).

The induction of antimicrobial peptides/polypeptides by growth factors was also tested in an organotypic epidermal culture previously used for demonstrating the induction of hBD-2 (22). In this system cells are grown in more defined medium without bovine pituitary extract. Insulin and EGF were removed from the medium

[4] J. B. Cowland, O. E. Sørensen, M. Sehested, and N. Borregaard. Neutrophil gelatinase-associated lipocalin (NGAL) is upregulated in human epithelial cells by IL-1$\beta$ but not by TNF-$\alpha$. *Submitted for publication.*

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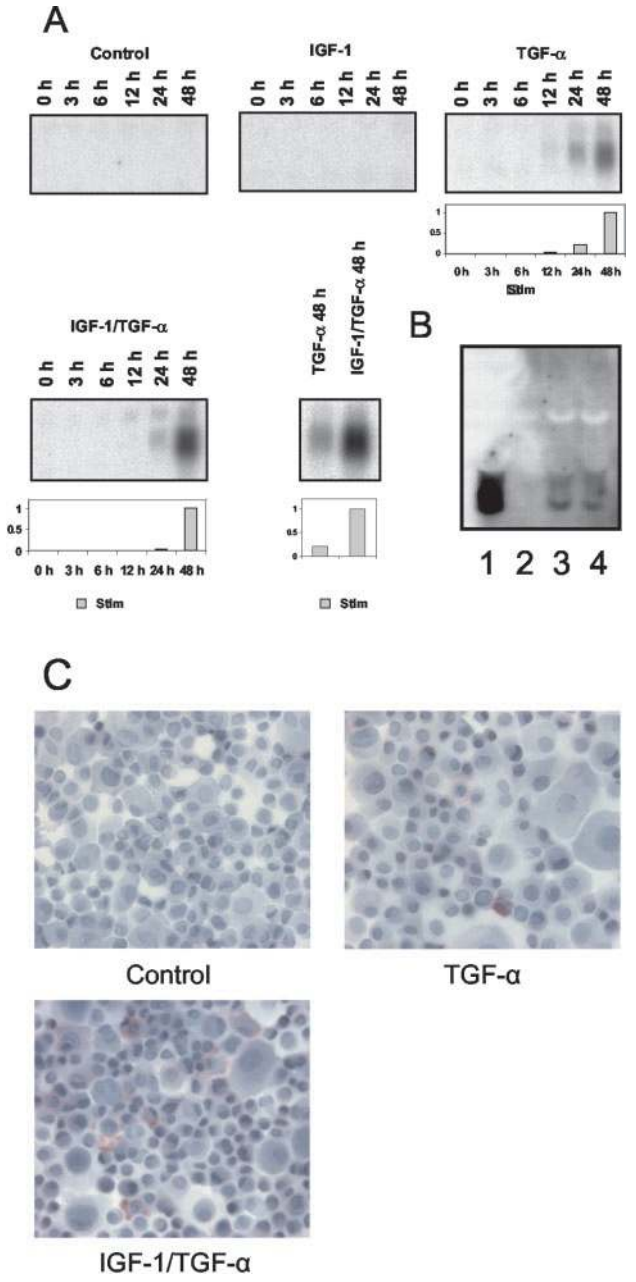


**FIGURE 3.** Expression of hBD-3. *A*, Northern blot of total RNA from control cells and cells stimulated with IGF-I, TGF-$\alpha$, and the combination of IGF-I/TGF-$\alpha$. The blots were hybridized with probes for hBD-3 and $\beta$-actin ($\beta$-actin hybridization not shown). Since there is no basal expression of hBD-3, the maximal expression is given a value of 1 in the schematic presentation of the hybridization intensities shown below the Northern blots. No expression of hBD-3 was found in control cells. Even though IGF-I by itself did not induce the expression of hBD-3, the combination of IGF-I/TGF-$\alpha$ gave a 5-fold higher expression of hBD-3 than TGF-$\alpha$ alone. A direct comparison of the expression of hBD-3 following induction with TGF-$\alpha$ and the combination of IGF-I/TGF-$\alpha$ at 48 h was made by running the samples on the same blot and normalizing the intensity to $\beta$-actin. *B*, Western blot of hBD-3 in medium from keratinocytes. The medium from the keratinocytes after 48 h of stimulation was extracted with cation exchange beads, and the eluate from the beads was run on AU-PAGE, followed by immunoblotting with anti-hBD-3 Abs. *Lane 1*, 50 ng synthetic hBD-3; *lane 2*, extract from unstimulated keratinocyte medium; *lane 3*, extract from medium of TGF-$\alpha$-stimulated keratinocytes; *lane 4*, extract from medium of IGF-I/TGF-$\alpha$-stimulated keratinocytes. *C*, Keratinocytes were stimulated with TGF-$\alpha$ or IGF-I/TGF-$\alpha$ or were left unstimulated. After 48 h of stimulation the cells were trypsinated, and cytospins were made, followed by immunostaining with anti-hBD-3 Abs.

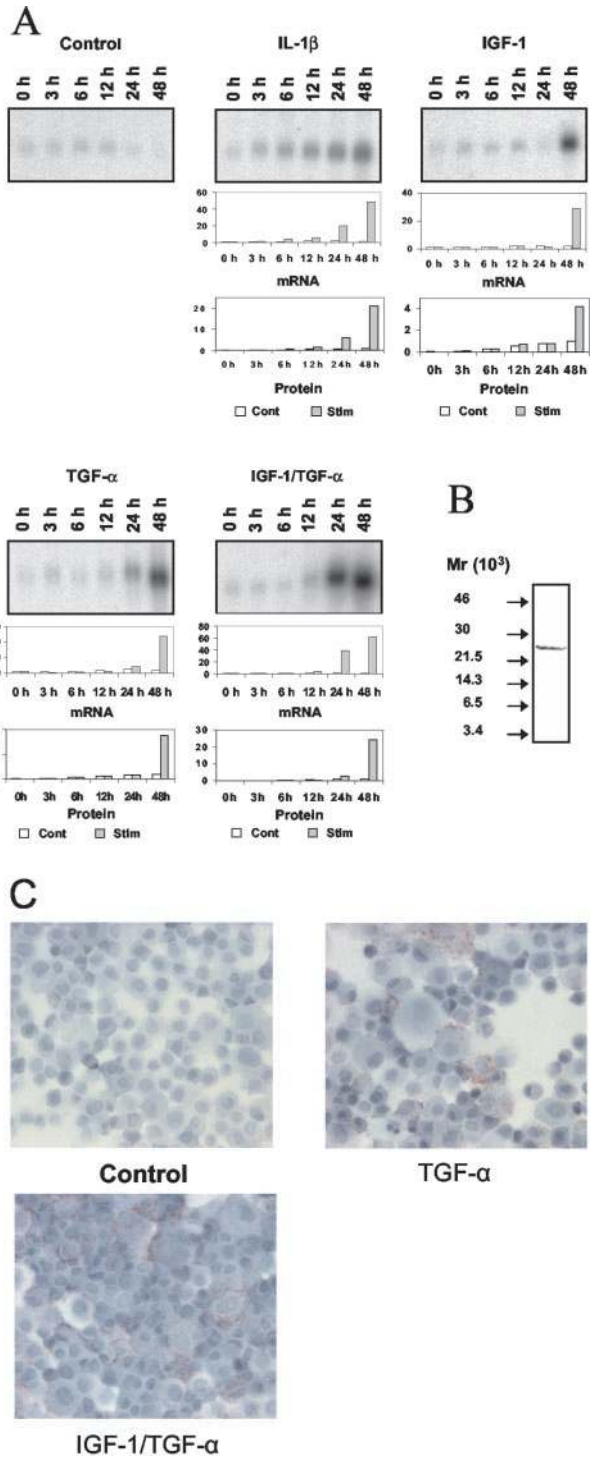


**FIGURE 4.** Expression of NGAL. *A*, Northern blot of total RNA from control cells, and cells stimulated with IGF-I, TGF-$\alpha$, the combination of IGF-I/TGF-$\alpha$, and IL-$\beta$. The blots were hybridized with probes for NGAL and $\beta$-actin ($\beta$-actin hybridization not shown). The basal expression of NGAL at 0 h is given the value 1 in the schematic representation of the hybridization intensities shown below the Northern blots. NGAL was measured by ELISA in the medium from control and stimulated keratinocytes. The concentration of NGAL is shown in nanograms per millliliter. A schematic presentation of these measurements is shown below the schematic representation of the Northern blot data. *B*, Medium from stimulated keratinocytes was run on SDS-PAGE, followed by immunoblotting with anti-NGAL Abs. A band of the appropriate molecular size was seen. *C*, Keratinocytes were either stimulated with TGF-$\alpha$ or IGF-I/TGF-$\alpha$ or was left unstimulated. After 48 h of stimulation the cells were trypsinated, and cytospins were made, followed by immunostaining with anti-NGAL Abs.

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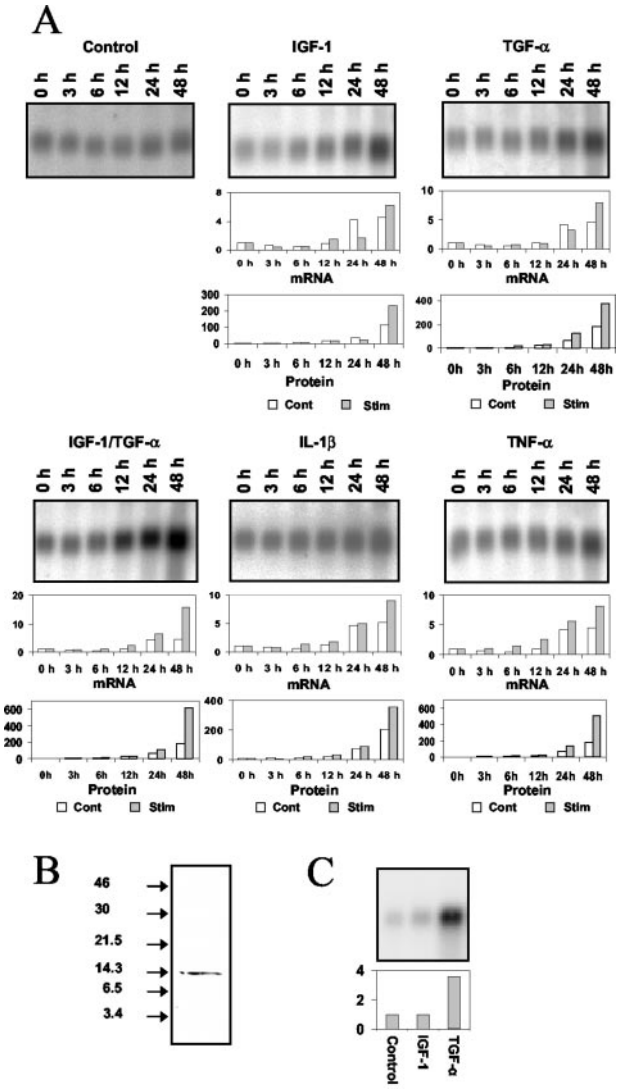


**FIGURE 5.** Expression of SLPI. *A*, Northern blot of total RNA from control cells and cells stimulated with IGF-I, TGF-$\alpha$, the combination of IGF-I/TGF-$\alpha$, IL-1$\beta$, and TNF-$\alpha$. The blots were hybridized with probes for SLPI and $\beta$-actin ($\beta$-actin hybridization not shown). The basal expression of SLPI at 0 h is given a value of 1 in the schematic representation of the hybridization intensities shown below the Northern blots. *B*, SLPI was measured by ELISA in the medium from control and stimulated keratinocytes. The concentration of SLPI is shown in nanograms per milliliter. A schematic presentation of these measurements is shown below the schematic representation of the Northern blot data. *C*, Northern blot of total RNA from keratinocytes in organotypic culture. The cultures were stimulated with IGF-I, TGF-$\alpha$, or the combination of IGF-I/TGF for 48 h. The blots were hybridized with probe for SLPI and $\beta$-actin ($\beta$-actin hybridization not shown). The basal expression of SLPI at is given a value of 1 in the schematic representation of the hybridization intensity shown below the Northern blot.

48 h before stimulation. In this system TGF-$\alpha$ induced a 4-fold increase in SLPI expression at the mRNA level (Fig. 5*C*). Stimulation with TGF-$\alpha$ induced NGAL and hBD-3 to the same extent as in the confluent cultures of primary keratinocytes (data not shown). In this more defined medium used for the organotypic culture it was not possible, however, to leave out the insulin long enough to demonstrate an effect of IGF-I. The greater induction of SLPI by TGF-$\alpha$ in the organotypic culture is probably due to a higher degree of differentiation of the cells in this system. In normal (differentiated) skin, basal expression of SLPI is also found (10).

Table I. *Expression of antimicrobial peptides/polypeptides in human keratinocytes in response to growth factors and proinflammatory cytokines*[a]

| | NGAL | SLPI | hCAP-18 | hBD-1 | hBD-2 | hBD-3 | hBD-4 |
|---|---|---|---|---|---|---|---|
| IGF-I | + | + | + | − | | | |
| TGF-$\alpha$ | + | + | | − | | + | |
| TGF-$\beta$ | − | − | | − | | | |
| bFGF | − | − | | − | | | |
| IL-1$\beta$ | + | + | | − | + | | |
| IL-6 | − | − | | − | | | |
| TNF-$\alpha$ | − | + | | − | | | |

[a] +, induced/increased expression of a peptide/protein in response to the factor/cytokine; −, no induction or increase in the expression. A blank space denotes no detectable expression.

EGF, like TGF-$\alpha$, binds to the EGF receptor, but is much less potent. When keratinocytes were stimulated for 48 h with 100 ng/ml EGF, a small increase in NGAL mRNA was noted. When EGF was given in combination with IGF-I, a small increase in hBD-3 and SLPI mRNA was observed along with the increase in NGAL mRNA (data not shown). The amount of EGF present in our cell medium (0.15 ng/ml) to maintain keratinocyte growth is therefore unlikely to influence our results.

TGF-$\beta$1 and bFGF did not induce/increase the expression of any of the peptides/polypeptides tested.

Immunostains of keratinocytes (Figs. 1*C*, 3*C*, and 4*C*) showed an uneven expression of antimicrobial peptides/polypeptides after stimulation, suggesting that keratinocyte differentiation affects the antimicrobial response. This was previously shown for hBD-2 (22). The granular staining, especially of NGAL (Fig. 4*C*), suggests a granular (lamellar body) localization in the keratinocytes as was found for hBD-2 (29).

The results of the induction experiments are summarized in Table I. The summary demonstrates that the expression of antimicrobial peptides/polypeptides in human keratinocytes was individually regulated in response to different stimuli, so that none of the antimicrobial peptides/proteins examined was regulated in the same manner. Furthermore, each tested growth factor and proinflammatory cytokine induced its own profile of antimicrobial peptides/polypeptides in human keratinocytes.

## Discussion

We hypothesized that disruptions of the physical barrier may generate a signal to the innate immune system and initiate responses that would prevent an impending invasion from surrounding microbes. Indeed, increased expression of cathelicidins and SLPI were previously demonstrated following cutaneous injury (8, 10). We reasoned that growth factors are logical candidates as inducers of antimicrobial peptides, since growth factors are produced to stimulate the regeneration of wounded tissue after injury (30). The growth factor response ceases after regeneration of the tissue, when the physical barrier protecting against microbial infections is re-established.

Our study demonstrates that growth factors of major importance in wound healing, IGF-I and TGF-$\alpha$, also induce or enhance the expression of the antimicrobial peptides/polypeptides hCAP-18, hBD-3, NGAL, and SLPI in human keratinocytes. Furthermore, these growth factors had a synergistic/additive effect in inducing expression of some of these antimicrobial peptides/polypeptides. These growth factors are present in saliva (31, 32) and have long been thought to support the proliferation of cells in wounds when animals lick their wounds. Our findings indicate that these growth factors may also aid in the prevention of infections in the wound.

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TGF-$\alpha$ is a central factor in wound healing, but it also has possible immunological functions (33). Indeed, we found that TGF-$\alpha$ induced the expression of the same number of antimicrobial peptides/polypeptides as the proinflammatory cytokine IL-1.

The induction of antimicrobial peptides/polypeptides by growth factors may explain the presence of these proteins/peptides in psoriasis and cutaneous injury. The peptides/polypeptides hCAP-18, SLPI, NGAL, and hBD-3 have been detected in psoriatic lesions (6, 7, 10, 11), and hCAP-18 and SLPI are increased in wounds (cutaneous injury) (8, 10). The IGF-I receptor and TGF-$\alpha$ are increased in the psoriatic epidermis (34, 35), and both IGF-I and TGF-$\alpha$ are expressed in wounds (36, 37).

In the last few years many studies have attempted to understand how in the course of infection bacteria and bacterial products induce the expression of antimicrobial peptides in epithelial cells. The generation of growth factors in inflamed lesions may contribute to this response. It is noteworthy that TGF-$\alpha$ is present and reportedly released from neutrophils, monocytes, and eosinophils (38, 39) recruited to the epithelia in the course of inflammation. Furthermore, the synthesis of TGF-$\alpha$ is induced in macrophages following exposure to LPS (40).

Although antimicrobial peptides/polypeptides typically have a broad spectrum of antimicrobial activity, there are differences in their specificity (1). We found that the spectrum of antimicrobial peptides/proteins induced in human keratinocytes depends on the agonist (growth factor, cytokine) present. As a result, keratinocytes may respond to different pathological stimuli by different patterns of expression of antimicrobial effector molecules. This was true even for the structurally and genetically closely related $\beta$-defensins. Because of the different antimicrobial specificities of the peptides/polypeptides, the ability to vary the defensive repertoire may be of functional importance. Their ability to generate a differentiated immune response also underscores the importance of the keratinocyte as an immunocompetent cell in the innate immune system.

Induction of antimicrobial proteins/peptides has been most thoroughly described in insects (*Drosophila*) (1), where the principal inducers of antimicrobial peptide expression were molecules previously known to regulate growth and development (41). In higher animals both IGF-I and TGF-$\alpha$ have this as their major function (42, 43). Conversely, the proinflammatory cytokines known to induce the expression of antimicrobial peptides, IL-1 and IL-6, have also been found to stimulate the growth of human keratinocytes (44) and thus may also be considered growth factors. Thus, from insects to man the processes of growth and expression of antimicrobial peptides appear to be intertwined.

From the clinical point of view, identification of the role of growth factors as mediators of induced expression of antimicrobial peptides/polypeptides in human keratinocytes raises the possibility that these factors could be manipulated to increase the resistance of skin grafts to infection (45).

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