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TGF-α Regulates TLR Expression and Function on Epidermal Keratinocytes[1]

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The expression of TLRs on epithelial cells provides a first line of defense against invading pathogens. We investigated the regulated expression and function of TLR5 and TLR9 on human keratinocytes, because we found by immunohistochemistry that these TLRs are expressed in distinct layers of the epidermis. We found that TGF-α, a growth and differentiation factor that is present during wound healing and in psoriasis, increased the expression of both TLR5 and TLR9 on keratinocytes. In addition, TGF-α regulated the function of TLR5 and TLR9, because activation with their respective ligands enhanced the production of IL-8 and human β-defensins. These findings provide evidence that TGF-α up-regulates TLR expression and function, augmenting host defense mechanisms at epithelial surfaces. *The Journal of Immunology,* **2005, 174: 6137–6143.**

Epithelial cells are situated in an opportune position not only to act as a physical barrier, but also to serve as first responders against invading pathogens. Cytokines, chemokines, and antimicrobial peptides produced by epithelial cells enable them to participate in innate and acquired immune responses (1–6). In addition, epithelia express certain TLRs, which may contribute to their ability to sense and respond to invading pathogens (3, 7–21).

TLRs are pattern recognition receptors for conserved molecular patterns of pathogenic microorganisms. Toll receptors are found throughout evolution, from *Drosophila* to humans (22–24). In *Drosophila*, activation of the Toll pathway leads to production of antimicrobial peptides important in fending off bacterial and fungal pathogens (22–25). In humans, there are 10 TLRs (TLR1–10), each activated by a different microbial component, but triggering a common signaling cascade leading to the production of inflammatory cytokines (22–24). Some of the microbial components that activate TLRs have been characterized. TLR1 and TLR6 can interact with TLR2 and are activated by bacterial lipoproteins (23). TLR2 is also activated by bacterial peptidoglycan (22–24). TLR3 is activated by viral-derived dsRNA (22–24). TLR4 can be activated by Gram-negative bacterial endotoxin (LPS), and TLR5 is activated by bacterial flagellin (22–24). TLR7 and TLR8 are activated by ssRNA (26, 27) and antiviral compounds (23). TLR9 is activated by unmethylated DNA sequences (CpG dinucleotides) found in bacterial DNA (22–24). TLR1–5 and TLR9 are expressed in various epithelia (3, 12–19), including human keratinocytes (7–11, 20, 21).

The expression of TLRs on epithelial cells provides a mechanism for host defense against pathogens, yet the mechanism(s) that regulates their expression and function is not well understood. We previously reported that TGF-α, an important growth factor involved in wound healing and in the hyperproliferative skin disease psoriasis, can regulate the expression of antimicrobial peptides in human keratinocytes (5). This prompted us to examine the role of TGF-α in the regulation of TLR expression and function on human keratinocytes.

Materials and Methods

Patients and skin specimens

All studies performed were approved by the University of California, Los Angeles (UCLA) institutional review board. Patients were clinically diagnosed with psoriasis at the UCLA Division of Dermatology Clinic from January 2002 to January 2003. After informed consent was obtained, skin specimens were biopsied under local anesthesia (1% lidocaine with epinephrine) using a 4- to 6-mm punch biopsy (Acuderm). Three psoriatic plaque lesions and six normal skin specimens were obtained from a total of seven patients. Half of the specimen was fixed in formalin (10%) and embedded in paraffin, and the other half was embedded in Tissue-Tek OTC compound (Sakura Finetek USA) and placed into liquid nitrogen for frozen section. H&E staining was performed on paraffin sections (4 μm) by the Tissue Procurement and Histology Core Laboratory at UCLA according to guidelines used for clinical patient samples. Each slide was evaluated by a board-certified dermatopathologist at the UCLA Division of Dermatology to confirm the diagnosis of psoriasis.

Organotypic keratinocyte culture and stimulation

Primary epidermal cultures EPI-200–3S (MatTek) containing human epidermal keratinocytes were grown on collagen-coated Millicel CM membranes as previously described (5). Briefly, the cultures were placed in 12-well plates with medium supplied by the manufacturer. After 4 days of culture, the epidermal cultures were lifted to the air-liquid interface and then cultured for another 2 days according to the instructions of the manufacturer. Next, the medium was changed to medium without epidermal growth factor (EGF),[3] and cells were cultured for another 2 days. Medium was then replaced with medium containing recombinant human TGF-α (50

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[3] Abbreviations used in this paper: EGF, epidermal growth factor; hBD, human β-defensin.

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ng/ml; Sigma-Aldrich) with or without flagellin (1–100 ng/ml; from *Salmonella typhimurium*, gift from Dr. K. Smith, University of Washington, Seattle, WA) or CpG 1826 (sequence 5′-TCCATGACGTTCCTGACGTT-3′; phosphorothioate linked; 10–1000 nM; Invitrogen Life Technologies), and cells and supernatants were harvested after an additional 2 days of culture. In parallel experiments, the activity of CpG 1826 on human cells was confirmed by concentration-dependent induction of CD86 on $CD19^+$ B cells using techniques of flow cytometry according to methods previously described (28) (data not shown). The use of this preparation of CpG 1826 was previously reported (29), and the bacterial flagellin was prepared and purified from *S. typhimurium* as previously described (30, 31). In addition, the flagellin and CpG used in the present study were assessed for the presence of endotoxin by the *Limulus* amebocyte lysate test (BioWhittaker), and no detectable levels of endotoxin were found in the flagellin and CpG stocks (the limit of detection of the assay was 1 ng/ml stock solutions, which were used at dilutions of 1/100 to 1/10,000 in the cultures).

Preparation of human β-defensin-3 (hBD-3) mAb

Recombinant hBD-3 peptide (PeproTech) was analyzed for purity and concentration using mass spectroscopy and amino acid composition. Peptides were stored in 0.02% acetic acid at −80°C. BALB/c mice were immunized i.p. with 100 μg of hBD-3, using CpG (Coley Pharmaceutical Group) as an adjuvant, every 2 wk for a total of four injections. After hBD-3 Abs were detectable at >10,000 dilutions of immunized mouse serum, splenic B cells were fused with NS1 mouse myeloma cells according to procedures described previously (32). Hybridoma supernatants were initially screened by indirect ELISA. Briefly, Immulon-4 HBX 96-well plates (MTX Lab Systems) were coated overnight at 4°C with 0.2 μg/ml recombinant hBD-3 in carbonate buffer at pH 9.6 (BupH; Pierce). Wells were incubated with hybridoma fluid for 4 h at 37°C before binding with an anti-mouse IgG alkaline phosphatase-conjugated secondary Ab (Sigma-Aldrich) and were developed using *p*-nitrophenyl phosphate according to the manufacturer's recommendations (Sigma-Aldrich). Thirty candidates were further screened by Western blot. Briefly, recombinant hBD-3 peptide (20 ng/ml) in Novex Tricine SDS sample buffer (Invitrogen Life Technologies) was run on 16% Tricine gels (Invitrogen Life Technologies) using running buffer (Invitrogen Life Technologies) and transferred to polyvinylidene difluoride membranes using transfer buffer (Invitrogen Life Technologies) with 20% ethanol. Membranes were then incubated with hybridoma fluid diluted 1/100 overnight. After incubation with anti-mouse IgG alkaline phosphatase-conjugated secondary Ab (Sigma-Aldrich) for 1 h at room temperature, the blots were developed with 5-bromo-4-chloro-3-indolyl phosphate and NBT in carbonate buffer. The mAbs were isotyped using the IsoStrip Mouse mAb Isotyping Kit (Roche) according to the manufacturer's instructions, and protein abundance was determined with a Nephelometer 100 (Dade Behring). The anti-hBD-3 mAb (IgG2a isotype) used in the present study was tested for activity against native hBD-3 with synthetic hBD-3 (PeproTech) as a control, using methods of acid urea-PAGE and immunoblotting (Hoeffer) as previously described (5). Anti-hBD-3 mAb was used at a 1/1000 dilution of hybridoma supernatant for immunohistochemical studies (see below).

Immunohistochemistry

Detection of TLR5, TLR9, and hBD-3 on frozen cryostat sections of skin biopsy specimens or organotypic keratinocyte cultures were identified using mAbs by the immunoperoxidase method as previously described (33). Briefly, cryostat sections (4 μm) were fixed with acetone for 10 min and dried overnight at room temperature. Sections were blocked for 30 min with 10% horse serum (Vector Laboratories) before incubation with primary mAb against TLR5 (10 μg/ml; clone 19D759.2; Imgenex), TLR9 (10–20 μg/ml; clone 26C593; Imgenex), or hBD-3 (see above) and the corresponding isotype control Ab (Sigma-Aldrich) for 60 min. Sections were then incubated with secondary biotinylated horse anti-mouse IgG (Vector Laboratories) for 30 min and visualized using the ABC Elite system for 60 min (Vector Laboratories), followed by 3-amino-9-ethyl-carbazole peroxidase substrate for 10 min (Vector Laboratories). Slides were counterstained with Mayer's hematoxylin solution (0.1%; Sigma-Aldrich) and mounted in crystal mounting medium (Biomeda).

IL-8 ELISA

IL-8 in culture supernatants was quantified using a standard sandwich ELISA. Microtiter plates (Costar) were coated with an unconjugated anti-IL-8 capture Ab (clone G265-5; 1 μg/ml; BD Pharmingen) overnight (15 h), followed by incubation with blocking buffer for 1 h. Wells were then incubated for 2–3 h with culture supernatant samples or varying concentrations of recombinant human IL-8 (0–2500 pg/ml). Detection of IL-8 was achieved using a biotinylated anti-IL-8 Ab (clone G265-8; 1 μg/ml; BD Pharmingen). The plate was developed using Immunopure HRP-conjugated streptavidin (Pierce) and ABTS Microwell Peroxidase Substrate System (Kirkegaard & Perry Laboratories). The absorbance at 405 nm was read using a microtiter plate reader (Cambridge Technology), and concentrations of IL-8 were calculated from a standard curve of recombinant human IL-8 (BD Pharmingen).

Preparation of recombinant hBD-2 peptide and hBD-2 Abs

The hBD-2 peptide was produced in recombinant baculovirus-infected insect cells as described previously (34). Briefly, BALB/c mice were immunized with OVA-conjugated hBD-2, their splenocytes were fused with X63-Ag8.653 myeloma cells, and cloned hybridomas were selected for ELISA reactivity with recombinant hBD-2. Mouse monoclonal hBD-2 was produced by the mouse ascites method and was a gift from Dr. L. Liu (UCLA). The rabbit anti-hBD-2 polyclonal Ab used in the present study was prepared as previously described (34, 35) and was tested for specificity for human hBD-2 in two separate Western blots on keratinocyte culture supernatants and on recombinant hBD-2 protein (35). In addition, using a Western blot, this Ab was also shown to be specific against recombinant hBD-2, but not hBD-1 or hBD-3 (36).

Human BD-2 ELISA

Human BD-2 in culture supernatants was quantified using a sandwich ELISA. Microtiter plates (Costar) were coated with an unconjugated monoclonal mouse anti-hBD-2 Ab overnight (15 h) and blocked with blocking buffer for 1 h. Wells were then incubated for 2–3 h with culture supernatant samples or varying concentrations of recombinant human hBD-2 (0–200 pg/ml). Detection of hBD-2 was achieved using a rabbit anti-hBD-2 polyclonal Ab, followed by a goat anti-rabbit peroxidase-labeled Ab, each for 1 h. The plate was developed, and absorbance was read as described above. Concentrations of hBD-2 were calculated from the standard curve of recombinant hBD-2.

RNA isolation

Total RNA was isolated from organotypic keratinocytes with TRIzol (Invitrogen Life Technologies) according to the 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 was assessed by running a sample on an agarose gel.

Real-time PCR

cDNA was synthesized using the iSCRIPT cDNA Synthesis Kit according to the manufacturer's recommendations (Bio-Rad). The primer and probe sequences for TLR5, TLR9, and the normalizer h36B4 were previously published (33, 37). Primers and probes were synthesized by Integrated DNA Technologies. Probes were synthesized with a 5′-FAM and 3′-Black Hole Quencher-1 (Integrated DNA Technologies). TaqMan reactions were conducted in TaqMan Universal PCR Master Mix (Applied Biosystems), preformed in duplicate on the MJR Opticon Continuous Fluorescence detector (MJ Research), and analyzed with Opticon Monitor software 1.08 (MJ Research). Results from duplicate wells were virtually identical. For hBD-2, the SYBR Green method was used, because no acceptable primer and probe sets for TaqMan could be generated. The following primers for hBD-2 were used: 5′-GGT GTT TTT GGT GGT ATA GGC G-3′ (forward) and 5′-AGG GCA AAA GAC TGG ATG ACA-3′ (reverse). SYBR Green reactions were conducted with the IQ SYBR Green mix under the recommended conditions and using the same instrumentation and software and the h36B4 as those described above. The relative quantities of mRNA per sample were calculated using the ΔΔC(T) formula, as previously described (38).

Results

Expression of TLR5 and TLR9 by keratinocytes in human skin

Human keratinocytes express TLR1–5 and TLR9, which facilitate recognition and responses to invading pathogens (7–11, 20, 21). We investigated the expression of TLR5 and TLR9 on keratinocytes, because our initial studies indicated that these receptors were expressed in distinct differentiation layers of normal human epidermis by immunohistochemistry (Fig. 1*A*, *top panels*). TLR5 expression was greatest in the basal keratinocytes, with labeling present in the bottom one to two layers. In contrast, TLR9 expression was found predominantly in keratinocytes in the upper layers of the epidermis, comprising the top two to three layers of cells.

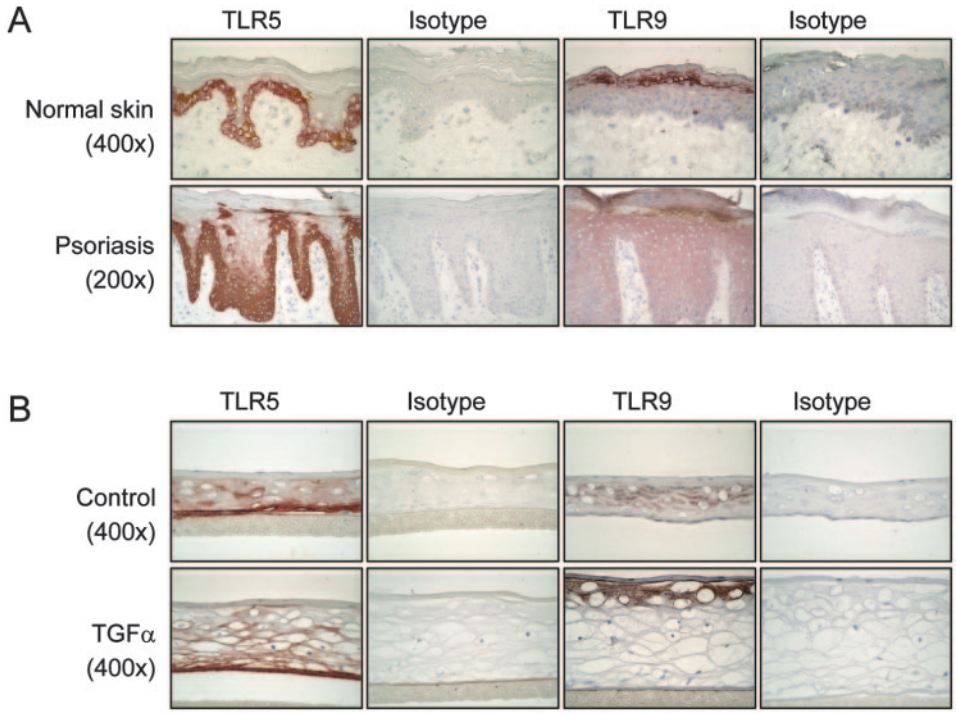

FIGURE 1. TLR5 and TLR9 are expressed in distinct differentiation layers of human keratinocytes. Representative photomicrographs of normal human skin and psoriasis (*A*) and of primary air-lifted organotypic keratinocyte cultures in the presence and the absence (control) of TGF-α (50 ng/ml; *B*). Skin specimens were frozen, sectioned, and labeled with anti-TLR5 mAb, anti-TLR9 mAb, or the appropriate isotype control mAb (isotype). Sections were visualized by the immunoperoxidase method and counterstained with hematoxylin. The data shown are representative of six specimens of normal human skin, three specimens of psoriasis, and three separate experiments with organotypic keratinocyte cultures, with similar results.

In the inflammatory skin disease psoriasis, the epidermis is thickened, representing a proliferation of keratinocytes. These keratinocytes have been previously shown to express TLR1, TLR2, and TLR5 (7, 8). We found that TLR5 expression was expressed predominantly in the bottom three to eight layers of cells (Fig. 1*A*, *bottom panels*). In contrast, TLR9 was expressed throughout the entire epidermis in psoriasis, with slightly enhanced labeling in the upper layers of the epidermis. These findings provide evidence that in psoriasis, where there is an increase in cell number, there is more diffuse expression of both TLR5 and TLR9. Based on the immunoperoxidase labeling for TLR5 and TLR9, we concluded that the level of expression of these TLRs may depend upon the differentiation stage of the keratinocytes as they mature from the basal layer in the epidermis. In addition, the more diffuse expression of TLR5 and TLR9 in psoriasis suggests that keratinocyte growth factors may play a role in regulating their expression.

Expression of TLR5 and TLR9 by primary air-lifted organotypic keratinocyte cultures

To determine the regulation and function of TLR5 and TLR9 expression in human keratinocytes, we used primary air-lifted organotypic keratinocyte cultures, which allow for keratinocyte differentiation into layers that resemble those seen in normal epidermis. We found by immunohistochemistry that TLR5 expression was greatest in the basal keratinocytes, and TLR9 expression was found predominantly in the upper layers of keratinocytes (Fig. 1*B*, *top panels*). Thus, the distinct pattern of TLR5 and TLR9 expression seen in normal human epidermis was preserved in the air-lifted organotypic keratinocyte cultures.

We previously demonstrated that the keratinocyte growth factor, TGF-α, which is found at high levels during wound healing and in psoriasis (39, 40), plays a role in host defense by increasing the production of antimicrobial peptides, including hBD-3, neutrophil gelatinase-associated lipocalin, and secretory leukocyte protease inhibitor, by human keratinocytes (5). These findings prompted us to evaluate whether TGF-α stimulation could also regulate the expression of TLR5 and TLR9 on keratinocytes, enhancing host defense mechanisms at epithelial surfaces. Stimulation of organotypic keratinocyte cultures with recombinant human TGF-α resulted in a proliferation of keratinocytes, resulting in more layers of keratinocytes in these organotypic cultures. In addition, there was a more diffuse expression of TLR5, with labeling seen throughout all layers of keratinocytes in these cultures, with the greatest labeling seen in the basal layers (Fig. 1*B*, *bottom panels*). TGF-α stimulation also resulted in labeling of TLR9, predominantly in the upper one to two layers of cells. These findings provide evidence that TGF-α-treated cultures show greater labeling for both TLR5 and TLR9, but also greater cell number.

TGF-α increases mRNA levels of TLR5 and TLR9 by organotypic keratinocyte cultures

Because we found by immunohistochemistry that TLR5 and TLR9 were more diffusely expressed in cultures stimulated with TGF-α, we wanted to establish a mechanism for this up-regulation. mRNA for both TLR5 and TLR9 were detected by real-time PCR in these organotypic keratinocyte cultures. There was $\sim$1 log higher expression of TLR5 than TLR9 in unstimulated cultures (Fig. 2). In a concentration-dependent fashion, TGF-α induced mRNA levels of both TLR5 (4-fold at 50 ng/ml and 30-fold at 100 ng/ml) and TLR9 (almost 30-fold at 50 ng/ml and $>$400-fold at 100 ng/ml). The findings demonstrate that TGF-α induced the expression of TLR5 and TLR9 through transcriptional regulation or mRNA stability. The reason for the difference in the degree of up-regulation between TLR5 and TLR9 induced by TGF-α is unknown, but may be due to the higher baseline expression of TLR5 in these cultures.

TGF-α enhances TLR5- and TLR9-induced production of IL-8 by organotypic keratinocytes

To determine whether TGF-α mediates a functional change in TLR responsiveness by keratinocytes, we cultured organotypic keratinocytes simultaneously with TGF-α (50 ng/ml) in the presence of varying concentrations of the TLR5 and TLR9 ligands, bacterial flagellin (1–100 ng/ml) and unmethylated CpG motifs of bacterial DNA (CpG 1826) (10–1000 nM), respectively. After

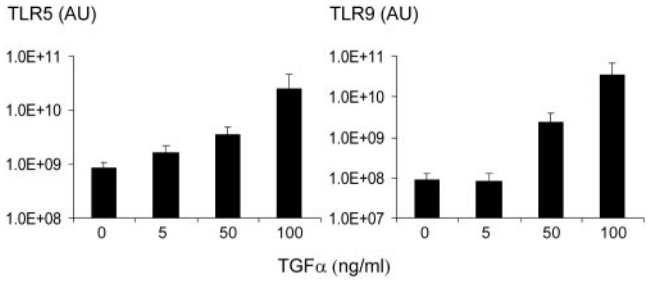

FIGURE 2. TGF-α increases the expression of TLR5 and TLR9 on organotypic keratinocyte cultures. Primary airlifted keratinocyte cultures were cultured in the presence of varying concentrations of TGF-α (5–100 ng/ml), and mRNA was harvested after 48 h. mRNA levels of TLR5 and TLR9 were determined using real-time PCR and normalized to the housekeeping gene *h36B4*. Data are presented on a logarithmic scale as the mean mRNA levels of TLR5 and TLR9 (arbitrary units (AU)) of three separate experiments ± SEM.

48 h of stimulation, we harvested cell culture supernatants and measured IL-8 production by ELISA. In the presence of TGF-α, we found that both flagellin and CpG DNA increased IL-8 production by keratinocytes (~7- to 8-fold; Fig. 3). In the absence of TGF-α, IL-8 was only minimally induced by either flagellin or CpG alone. These results suggest that TGF-α enhanced the TLR5- and TLR9-induced production of IL-8.

TGF-α enhances TLR5- and TLR9-induced production of hBD-2 by organotypic keratinocytes

The human β-defensins, hBD-2 and hBD-3, are small cationic peptides that are part of the defensin family of antimicrobial peptides and have been shown to play an important role in innate immune responses (1, 41). Both hBD-2 and hBD-3 show broad spectrum microbicidal activity against Gram-positive and -negative bacteria, such as *Escherichia coli* and *Staphylococcus aureus*, respectively (1, 41). They also have antimicrobial against fungi and enveloped viruses (1, 41). The antimicrobial activity of these peptides is thought to involve permeabilization of target cell membranes of microbes (1, 41).

Several previous reports have shown that various epithelial cells, including keratinocytes, that express TLR2 and TLR4 can produce hBD-2 after stimulation with bacterial peptidoglycan and LPS (3, 12). Given these findings, we sought to determine whether TGF-α can regulate TLR5 and TLR9 induction of hBD-2 by human keratinocyte organotypic cultures. We measured mRNA levels of hBD-2 after stimulation with bacterial flagellin, the TLR5 ligand, and CpG DNA, the TLR9 ligand, in the presence and the absence of TGF-α (Fig. 4*A*). We found that in the presence of TGF-α, all concentrations of flagellin tested (1–100 ng/ml) resulted in increased hBD-2 mRNA (~5- to 7-fold). In the presence of TGF-α, CpG stimulation also resulted in a concentration-dependent increase in hBD-2 mRNA (~7-fold). In the absence of TGF-α, no appreciable increases in hBD-2 mRNA were observed, except in the case of the highest concentration of flagellin (100 ng/ml), which resulted in increased hβD-2 mRNA (~6-fold) to a level similar to that observed in the presence of TGF-α.

To confirm that increases in hBD-2 mRNA by TGF-α also resulted in increased protein levels of hBD-2, we developed an ELISA to detect the levels of hBD-2 in organotypic keratinocyte culture supernatants (Fig. 4*B*). Similar to results obtained with hBD-2 mRNA, we found that in the presence of TGF-α, both flagellin and CpG increased hBD-2 protein levels (~6- to 7-fold). In addition, in the presence of TGF-α, all concentrations of flagellin tested (1–100 ng/ml) resulted in a concentration-dependent increase in hBD-2, whereas only the lowest concentration of CpG tested (10 nM) resulted in the highest fold increase in hBD-2 (~3-fold). In the absence of TGF-α, no appreciable increases in hBD-2 were observed, except in the case of the highest concentration of flagellin (100 ng/ml), which resulted in increased hBD-2 to a level ~60% of that observed in the presence of TGF-α. These results provide evidence that that TGF-α can enhance the function of TLR5 and TLR9, because activation with their respective TLR ligands increased the production of hBD-2 mRNA and protein levels.

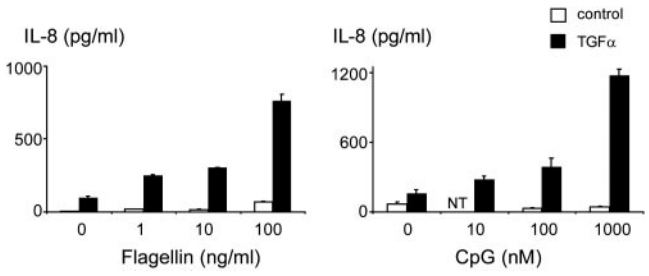

FIGURE 3. TGF-α enhances the TLR5- and TLR9-induced production of IL-8. In the presence (■) and the absence (control; □) of TGF-α (50 ng/ml), primary airlifted keratinocyte cultures were stimulated for 48 h with varying concentrations of the respective TLR5 and TLR9 agonists, bacterial flagellin (1–100 ng/ml) or CpG 1826 (10–1000 nM). Culture medium was collected and analyzed for IL-8 by sandwich ELISA. Data are expressed as levels of IL-8 (picograms per milliliter) ± SD. The data shown are representative of three experiments with similar results. NT, not tested.

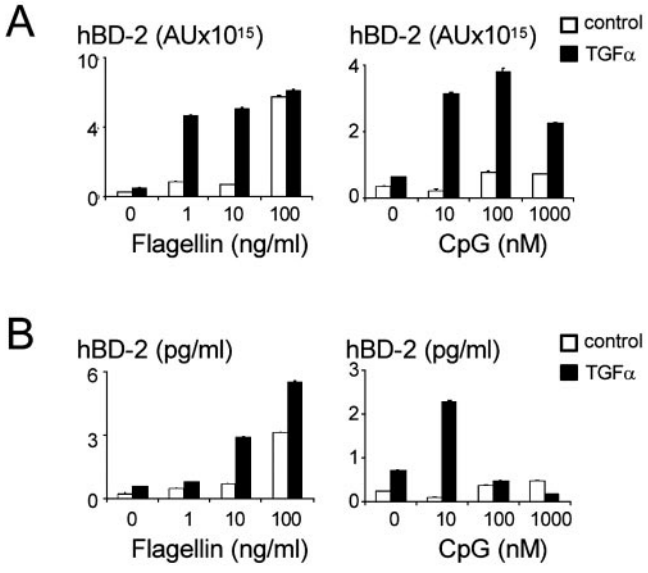

FIGURE 4. TGF-α enhances the TLR5- and TLR9-induced production of hBD-2. In the presence (■) and the absence (control; □) of TGF-α (50 ng/ml), primary airlifted keratinocyte cultures were stimulated for 48 h with varying concentrations of the respective TLR5 and TLR9 agonists, bacterial flagellin (1–100 ng/ml) or CpG 1826 (10–1000 nM). *A*, mRNA was harvested from the organotypic keratinocyte cultures, and levels of hBD-2 were quantified using real-time PCR and normalized to the housekeeping gene *h36B4*. The data are expressed as mRNA levels of hBD-2 (arbitrary units (AU)) ± SD. *B*, Culture supernatants were harvested from the organotypic keratinocyte cultures, and levels of hBD-2 were quantified by sandwich ELISA. The data are expressed as levels of hBD-2 (picograms per milliliter) ± SD and are representative of two experiments with similar results.

TGF-α enhances TLR5-induced production of hBD-3 by organotypic keratinocytes

We previously reported that TGF-α stimulation alone can induce the production of antimicrobial peptides by keratinocytes, including hBD-3 (5). Given these findings, we sought to determine whether TGF-α could further enhance TLR5- or TLR9-induced production of hBD-3 by human organotypic keratinocytes. We used an hBD-3 mAb and immunohistochemistry to detect the expression of hBD-3 in frozen sections of organotypic keratinocyte cultures (Fig. 5). In some samples there was nonspecific labeling at the edge between the organotypic keratinocytes and the collagen membrane on which these cells are grown, which is an artifact that is sometimes seen in immunohistochemistry. We found that organotypic keratinocytes in the presence of TGF-α had increased expression of hBD-3 compared with cultures in the absence of TGF-α (control), where expression was virtually undetectable. In the presence of TGF-α, flagellin (10 ng/ml) further increased the expression of hBD-3, whereas cultures treated with CpG (100 nM) contained similar levels of hBD-3 as those in cultures with TGF-α alone. These results provide evidence that TGF-α can regulate the TLR5-induced production of hBD-3 on keratinocytes.

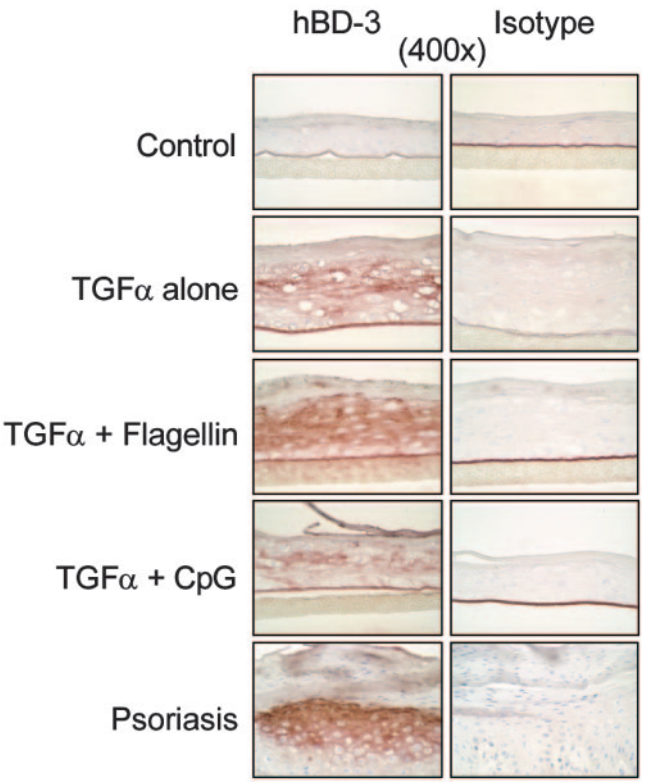

FIGURE 5. TGF-α enhances the TLR5-induced expression of hBD-3. In the presence and the absence of TGF-α (50 ng/ml), primary airlifted keratinocyte cultures were stimulated for 48 h with the respective TLR5 and TLR9 agonists, bacterial flagellin (1–100 ng/ml) or CpG 1826 (10–1000 nM). Representative photomicrographs of frozen sections of organotypic keratinocyte cultures in the absence (control) and the presence of TGF-α with or without flagellin (10 ng/ml) or CpG (100 nM) and psoriatic skin lesions (used as a positive control for hBD3 detection) labeled with hBD-3 mAb or IgG2a isotype control mAb (isotype). Sections were visualized by the immunoperoxidase method and counterstained with hematoxylin. The data shown are representative of two experiments with similar results.

Discussion

TLRs on epithelial cells play an important role in recognizing invading pathogens and initiating host immune responses. Activation of TLR2, TLR4, and TLR5 expressed by various epithelia can contribute to host defense by induction of cytokines (9, 10, 13, 14, 19, 20) and antimicrobial peptides (3, 12). In this study we examined the regulated expression and function of TLR5 and TLR9 on keratinocytes, given that our preliminary studies found that TLR5 and TLR9 were expressed in distinct microanatomic locations of the epidermis. We report that the growth and differentiation factor, TGF-α, which is important during wound healing and is found at increased levels in psoriasis, regulates the expression and function of TLR5 and TLR9 on human keratinocytes.

To study the regulation and function of TLR5 and TLR9 expression by keratinocytes, we used primary air-lifted organotypic keratinocyte cultures as a model system for epithelia. These organotypic cultures allow for keratinocyte differentiation into layers that resemble those seen in normal epidermis. A key finding was that TGF-α up-regulated the expression of TLR5 and TLR9 mRNA and protein. The effect on the expression of TLR9 was more pronounced, which may be due to the growth and differentiation properties of TGF-α, which promotes maturation of keratinocytes into the upper and most differentiated layers of the epidermis where TLR9 is almost exclusively expressed.

Another important finding was that TGF-α greatly enhanced the function of these TLRs on keratinocytes. In the presence of TGF-α, activation of keratinocytes with the respective TLR5 and TLR9 ligands, bacterial flagellin and CpG DNA, resulted in increased production of the proinflammatory cytokine, IL-8, and the antimicrobial peptide, hBD-2. In addition, in the presence of TGF-α, stimulation with the TLR5 ligand, bacterial flagellin, resulted in increased expression of hBD-3. These effects were well beyond those of TGF-α stimulation alone. IL-8 is a neutrophil chemoattractant, and recruitment of neutrophils to the site of an invading pathogen at an epithelial interface may contribute to effective clearance of the infection. Furthermore, hBD-2 and hBD-3 are members of a class of antimicrobial peptides called defensins that have broad-spectrum antimicrobial activity against bacterial, fungal, and viral pathogens (1). This antimicrobial activity for both hBD-2 and hBD-3 against bacterial and fungal infections, in particular, has been well documented (1, 2, 6). Furthermore, hBD-2 has chemoattractant properties for immature dendritic cells and T cells to a site of infection, which may facilitate the development of adaptive immune responses at the site of infection (42). Overall, these data indicate that growth and differentiation factors such as TGF-α can augment innate and acquired immune responses at epithelial surfaces.

In the hBD-2 real-time PCR and ELISA experiments, we found that the lower concentrations of CpG in the presence of TGF-α resulted in increased levels of hBD-2 mRNA and protein, respectively. The reason for this is unknown, but it may be due to either feedback inhibition by the high levels of hBD-2 and/or CpG or to mild toxicity of CpG at these higher concentrations on these organotypic keratinocyte cultures. We favor the idea that the down-regulation of hBD-2 at the higher concentrations of CpG is most likely a feedback inhibition inherent in hBD-2 production, because this phenomenon did not occur with production of IL-8 in these same cultures.

The mechanism by which TGF-α regulates activation of TLR5 and TLR9 is unknown, but may be due to up-regulation of TLR5 and TLR9 expression on keratinocytes and/or by cross-talk between signaling pathways of TGF-α and TLRs. We have demonstrated that TGF-α up-regulates both TLR5 and TLR9 mRNA

(Fig. 2). However, the latter possibility, that TGF-α and TLR signaling synergize, is more likely, because we found that keratinocytes are unresponsive to TLR5 and TLR9 ligands in the absence of TGF-α (Figs. 3–5). TGF-α is an autocrine and paracrine keratinocyte growth and stimulating factor that activates keratinocytes by binding to the EGF receptor (EGF-R) on the surface of these cells. EGF-R activates several different signaling molecules, including Src, Ras, STAT3, phospholipase C-γ, PI3K, and members of the MAPK family (43–45). There is evidence demonstrating that TLR5 and TLR9 activate some common signaling cascades with the EGF-R. For example, studies have demonstrated that MAPK is important in TLR5-mediated epithelial IL-8 expression (46), and PI3K is important in TLR9 activation (47).

In addition, we have found that keratinocytes in normal skin and in organotypic cultures under control conditions (in the absence of TGF-α) express TLR5 and TLR9 (Fig. 1), but are apparently unresponsive to TLR5 and TLR9 ligands (Figs. 3 and 4). The reason for this is unknown, but it could be explained by potential cross-talk between the signaling pathways of TGF-α stimulation and TLR stimulation. One could speculate that the epithelial cells of the skin, which are in constant contact with the outside environment, must have a mechanism(s) to down-regulate inflammatory immune responses in normal skin when the epithelial barrier is intact and is not under attack by invading pathogens. Conversely, there also must be mechanism(s) to up-regulate the protective immune responses when they are needed, such as when the epithelial barrier is breached. This may occur in the context of a wound, when the host becomes more susceptible to invading pathogens (40). During wound healing, growth factors such as TGF-α are produced that stimulate the proliferation of epithelial cells to help re-establish the physical barrier (5, 39, 40). Thus, when the skin is injured and the epithelial barrier is breached, growth factors such as TGF-α may provide appropriate cross-talk to TLR signaling mechanisms to regulate host defense responses.

In the disease psoriasis, a condition in which keratinocytes are hyperproliferative, and TGF-α is known to be increased (39), we found that the expression of TLR5 and TLR9 was also up-regulated. Interestingly, skin lesions of psoriasis possess high levels of antimicrobial peptides, including hBD-2 and hBD-3 (1, 2, 6, 41, 48, 49), and rarely get superinfected by bacteria (49, 50). Indeed, hBD-2 and hBD-3 were first discovered in psoriatic scales (2, 6). Therefore, our findings not only define a role for TGF-α in host defense, but also offer an explanation for the increased levels of TLRs and antimicrobial peptides observed in psoriasis. It is intriguing that human cationic antimicrobial protein hCAP-18/LL-37 (cathelicidin) is expressed at increased levels in keratinocytes in psoriasis (49, 51). Future investigations will assess the pattern of this and other antimicrobial peptides induced in keratinocytes by growth factors in combination with TLR activation.

In the present study we found that TLR5 and TLR9 were expressed in distinct keratinocyte layers in both human epidermis and organotypic keratinocyte cultures. TLR5 expression was found mostly in the basal keratinocytes, whereas TLR9 expression was found mostly in the upper, more differentiated, keratinocytes. The reason for this specific distribution of these TLRs in human epidermal keratinocytes is unknown. However, one may speculate that their different expression patterns depend upon the differentiation stage of keratinocytes as they mature in the epidermis.

Ultimately, identification of the role of TGF-α as an important regulator of TLR expression and function in human keratinocytes suggests that TLR-mediated responses can be up-regulated at epithelial surfaces. This raises the possibility that the expression of TLRs could be manipulated to augment host defense mechanisms and increase the threshold to infection at epithelia surfaces. This function may be of particular importance, because epithelia are in constant interaction with pathogens from the surrounding environment, and their ability to recognize and respond to these pathogens through TLRs may be an important first-line host defense mechanism.

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Disclosures

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