CXCL16 is a novel mediator of the innate immunity of epidermal keratinocytes

Mikiko Tohyama¹, Koji Sayama¹, Hitoshi Komatsuzawa², Yasushi Hanakawa¹, Yuji Shirakata¹, Xuju Dai¹, Lujuan Yang¹, Sho Tokumaru¹, Hiroshi Nagai¹, Satoshi Hirakawa¹, Motoyuki Sugai² and Koji Hashimoto¹

¹Department of Dermatology, Ehime University School of Medicine, Shitsukawa, Toon-city, Ehime 791-0295, Japan
²Department of Bacteriology, Hiroshima University Graduate School of Biomedicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8553, Japan

Keywords: anti-microbial activity, chemokine, CXCL16, innate immunity, keratinocyte

Abstract

The epidermis is constantly exposed to a variety of microbial pathogens and plays a vital role in resisting them. Soluble CXC chemokine ligand (CXCL) 16, which is one of the ELR⁺ CXC chemokines, acts as a mediator of innate immunity by attracting CXC chemokine receptor (CXCR) 6-expressing cells, such as activated T cells and NKT cells. However, the production of CXCL16 by non-immune cells remains unclear. We found that cultured keratinocytes produced a significant amount of CXCL16 (2–3 ng per 10⁶ cells per 24 h). Stimulation with tumor necrosis factor α, IL-1α, IFN-γ, peptidoglycan and polyninosinic-polycytidylic acid [poly(I:C)] enhanced CXCL16 production. The forms of CXCL16 in the culture supernatants had molecular weights of 14, 28 and 50 kDa. Immunohistochemical analysis revealed that the normal human epidermis expressed CXCL16. As several chemokines have anti-microbial activities, we studied the anti-microbial activity of CXCL16. The chemokine domain of CXCL16 at concentrations >5 μg ml⁻¹ had significant anti-microbial activity against Staphylococcus aureus and Escherichia coli. Killing activity was retained at the physiological salt concentration in the presence of carbonate. In conclusion, CXCL16 is a novel mediator of the innate immune reactivities of epidermal keratinocytes.

Introduction

The CXC chemokine ligand (CXCL) 16 was discovered as a ligand for CXC chemokine receptor (CXCR) 6. CXCL16 is a membrane-bound chemokine that consists of four distinct domains: the chemokine domain, mucin-like domain, transmembrane domain and ad cytoplasmic (1, 2). This structure is similar to that of fractalkine/CX3CL1, which is another membrane-bound chemokine. After cleavage, soluble CXCL16 acts as a chemoattractant for activated CD8 T cells, NKT cells and Th1-polarized T cells that express CXCR6 (1, 2). Cleavage is considered to be mediated by a disintegrin and metalloproteinase (ADAM) family protease, ADAM 10 (3, 4).

Shimaoka et al. (5) have reported a novel protein, designated SR-PSOX (scavenger receptor that binds phosphatidylserine and oxidized lipoprotein), which acts as a receptor for oxidized low-density lipoprotein (OxLDL). They have demonstrated that SR-PSOX is expressed by human macrophages and dendritic cells and that specifically binds OxLDL, leading to its internalization and degradation. In atherosclerotic lesions, macrophages express SR-PSOX. This finding suggests that SR-PSOX may play important roles in the formation of atherosclerotic lesions. Recently, this SR-PSOX has been found to be identical to CXCL16 (6).

In addition to these functions, Shimaoka et al. (6) have demonstrated that CXCL16 on macrophages and dendritic cells mediates the adhesion and phagocytosis of bacteria, such as Escherichia coli and Staphylococcus aureus, and bacterial recognition is mediated by the chemokine domain of CXCL16. These findings indicate that CXCL16 is not only a chemokine, but is also a multifunctional protein, which suggests that CXCL16 has some novel function.

CXCL16 expression has been studied mainly in macrophages and dendritic cells. CXCL16 production by non-immune cells remains controversial. Hofnagel et al. (7) reported the detection by reverse transcription (RT)-PCR of CXCL16 mRNA in cultured aortic smooth muscle cells and umbilical endothelial cells. However, another group did not detect CXCL16 mRNA by northern blot analysis (8). There are no reports describing the production of CXCL16 by keratinocytes. The epidermis is the primary barrier between the body and the outside environment. In addition to this physical
The epidermis functions as an innate immune barrier that resists microbial pathogens. Epidermal keratinocytes produce anti-microbial peptides, such as human β-defensins and hCAP18/LL-37, following differentiation or wounding (9–11). Furthermore, epidermal keratinocytes recognize bacteria and virus-associated, double-stranded RNA via Toll-like receptor (TLR)2 and TLR3, respectively, to produce anti-microbial peptides, cytokines and chemokines (12–15). Since CXCL16 is an important chemokine for host defense, it seems reasonable to assume that keratinocytes also produce CXCL16.

In this study, we report for the first time that epidermal keratinocytes constitutively produce CXCL16. Furthermore, we show that the chemokine domain of CXCL16 has anti-microbial activity. Thus, CXCL16 is a novel mediator of the innate immune reactivities of keratinocytes in the human epidermis.

Methods

Reagents and antibodies

Tumor necrosis factor α (TNF-α) and IL-1α were generous gifts from Dainippon Pharmaceutical Co., Ltd (Osaka, Japan). IFN-γ was a generous gift from Otsuka Pharmaceutical Co., Ltd (Tokyo, Japan). Recombinant CXCL16 (chemokine domain and extracellular domain (ECD)) and recombinant IFN-γ-inducible protein of 10 kDa (IP-10/CXCL10) were purchased from R&D Systems (Minneapolis, MN, USA). Peptidoglycan purified from *S. aureus* was purchased from Fluka (Buchs, Switzerland). Polynosinic-polycytidylic acid (poly(IC)] was purchased from Amersham (Piscataway, NJ, USA). The monoclonal antibodies against CD1a were purchased from Dako Japan (Kyoto, Japan). The antibodies against the chemokine domain (aa 51–68) and cytoplasmic domain (aa 248–260) of human CXCL16 (Fig. 4) were generated by immunization of rabbits with the synthetic peptides. The respective reactivities of the antibodies were confirmed by ELISA. IgG was affinity purified using the synthetic peptides.

Skin samples

Normal human skin was obtained from plastic surgery under a protocol approved by the Institutional Review Board of Ehime University School of Medicine.

Cell preparation and culture

Normal human keratinocytes, dermal fibroblasts and dermal microvascular endothelial cells (DMECs) were cultured as described previously (16–18). Keratinocytes were cultured in MCDB153 medium that was supplemented with insulin (5 μg ml⁻¹), hydrocortisone (0.5 μM), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM) and bovine hypothalamic extract (50 μg ml⁻¹). Cells that were passaged four times were used in the experiments. CD14⁺ monocytes were isolated from the buffy coats of healthy donors using the MACS cell isolation kit (Milteny Biotec, Bergisch Gladbach, Germany).

RNA preparation, RT–PCR, and real-time PCR

Total RNA was prepared from cells and epidermis using Iso-gen (Nippon Gene, Tokyo, Japan). The epidermis was separated from the dermis by treatment at 60°C for 1 min followed by immediate cooling in ice-cold PBS. RT–PCR was performed using RT-PCR High Plus (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. Twenty-five PCR cycles were used to amplify the CXCL16 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequences. The following primers were used: for CXCL16, 5'-GGCCCTTCA-TTTAAAACGGG-3' and 5'-GCCTGTCACATGGTGAAAC-3' and for GAPDH 5'-ACCAACGTCATGCCATCAC-3' and 5'-TCCACACCCTGGTGTGA-3'. The PCR products were visualized on 2% agarose gels that contained ethidium bromide and confirmed by size and direct DNA sequencing.

The primer and probes used in the real-time PCR of CXCL16 mRNA were selected using the Primer Express software (Applied Biosystems, Norwalk, CT, USA) as follows: forward, 5'-AAGCCATTGAGACACACTG-3'; reverse, 5'-AC-TTGCTCTAGTCCCAGA-3' and 6FAM-ACGTACCGC-CGGAGCAT-TAMRA. cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems) following the manufacturer's suggested protocols. Real-time quantitative RT–PCR was performed with reagents recommended by the manufacturer (Applied Biosystems) and using the ABI PRISM 7700 Sequence Detection System. The levels of mRNA expression were normalized to that of GAPDH.

Protein preparation and western blot analysis

The supernatants of keratinocyte cultures were concentrated from 35 to 0.5 ml using Centricon-3 (Amicon, Beverly, MA). Twenty-microliter samples were separated on 12% SDS-PAGE, and transferred to a polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, Tokyo, Japan). After blocking with 1% non-fat dry milk, the membrane was treated with rabbit anti-chemokine domain antibodies of CXCL16. The antibody was detected with the Vistra ECF kit (Amersham). Fluorescence was observed on a Fluoromager (Molecular Dynamics, Sunnyvale, CA, USA).

Immunofluorescence microscopy

Frozen skin sections (5 μm) were fixed in cold acetone for 5 min and then reacted with the rabbit anti-CXCL16 antibody for 1 h at room temperature. After washing with PBS, the sections were incubated with fluorescence-labeled goat anti-rabbit IgG for 30 min at room temperature. For mouse anti-CD1a antibodies, anti-mouse rat antibodies labeled with Alexa Fluor 594 (Molecular Probes) was used as secondary antibodies. The fluorescence was observed under a fluorescence microscope.

ELISA

The ELISA development kit for CXCL16 was purchased from R&D Systems. The concentrations were measured according to the manufacturer's instruction. Optical density was measured with the Immuno Mini NJ-2300 microplate reader (Nalge Nunc International K.K, Tokyo, Japan).

Anti-bacterial assays

*Staphylococcus aureus* (209P) was grown in trypticase soy broth at 37°C. *Escherichia coli* strain HB101 was grown aerobically in Luria–Bertani broth. Overnight cultures of
S. aureus and E. coli were harvested, washed with PBS and suspended in 10 mM sodium phosphate buffer (NaPi; pH 6.8). The bacterial suspension was diluted to 10⁷ cells per ml with NaPi (pH 6.8), and 10 μl of the bacterial suspension (10⁵ cells) was inoculated into 200 μl of NaPi with or without various concentrations of chemokines and incubated for 2 h at 37°C. An appropriate dilution of the reaction mixture (100 μl volume) was plated on trypticase soy agar and incubated at 37°C overnight. The colony-forming units were assessed, and the anti-bacterial effect was calculated as the ratio of surviving cells to total cells. To demonstrate the effects of NaCl and NaHCO₃, NaCl (150 mM) or NaHCO₃ (50 mM) was added together with 10 mM NaPi (pH 6.8) in the anti-bacterial assay described above.

Results

CXCL16 production by cultured normal human keratinocytes

We analyzed, by RT–PCR, the levels of CXCL16 mRNA expression in cultured keratinocytes, normal human epidermis and CD14⁺ monocytes. Cultured keratinocytes and normal human epidermis, as well as CD14⁺ monocytes, expressed CXCL16 mRNA (Fig. 1A). We also evaluated, using real-time PCR, the levels of CXCL16 mRNA expression in keratinocytes, dermal fibroblasts, DMEC and CD14⁺ monocytes (Fig. 1B). The level of CXCL16 mRNA expressed by keratinocytes was one-sixth that of monocytes but was 5-fold higher than the levels expressed by dermal fibroblasts and DMEC.

Fig. 1. CXCL16 mRNA expression. (A) CXCL16 mRNA expression by cultured keratinocytes, normal human epidermis and monocytes was analyzed by RT–PCR. GAPDH was used as an internal standard. (B) CXCL16 mRNA expression by monocytes, keratinocytes, dermal fibroblasts and DMEC was analyzed by real-time PCR. The expression levels were normalized to that of GAPDH. The level of mRNA expression by keratinocytes is expressed as one unit.

We also examined, by ELISA, the production of CXCL16 by cultured keratinocytes. CXCL16 was detected at 2–3 ng per 10⁶ cells per 24 h in the culture supernatant of unstimulated keratinocytes. Although cultured keratinocytes produce several chemokines, such as IL-8, MIP-3α and CTACK, in the absence of stimulation (19–21), the levels are significantly lower than that of CXCL16 (data not shown). CXCL16 mRNA expression (Fig. 2A) and production (Fig. 2B) increased following stimulation with inflammatory cytokines, which included TNF-α, IL-1α and IFN-γ, either alone or in combination.

CXCL16 production increased by ligands for TLR2 and TLR3

We studied whether TLR stimulation increased CXCL16 production. Peptidoglycan and poly(I:C), which are ligands for TLR2 and TLR3, respectively, increased CXCL16 mRNA expression and production in cultured keratinocytes, as shown by real-time PCR and ELISA, respectively (Fig. 3).

Generation of antibodies to the chemokine and cytoplasmic domains of CXCL16

We raised rabbit antibodies against the chemokine domain (Fig. 4A) and cytoplasmic domain (Fig. 4B) of CXCL16 to evaluate CXCL16 shedding. The anti-chemokine domain and anti-cytoplasmic domain antibodies were used for western blot (Fig. 5) and immunohistological (Fig. 6) analyses. The anti-cytoplasmic domain antibody was used for immunohistological analysis (Fig. 6).

Fig. 2. Increased production of CXCL16 induced by pro-inflammatory cytokines. (A) Keratinocytes were treated with IFN-γ (50 U ml⁻¹), TNF-α (10 ng ml⁻¹) and IL-1α (10 ng ml⁻¹). The levels of CXCL16 mRNA expression were analyzed at the indicated time point by real-time PCR. The expression levels were normalized to that of GAPDH as one unit. (B) Soluble CXCL16 in the culture supernatant was measured by ELISA. Culture supernatants were harvested after 48 h of treatment with IFN-γ (50 U ml⁻¹), TNF-α (10 ng ml⁻¹) and IL-1α (10 ng ml⁻¹), either alone or in combination. Data are presented as means ± SDs (n = 3). Statistical analysis was performed using the Student’s t-test with comparisons to the control. *P < 0.05 and **P < 0.005.
Shedding of CXCL16 by keratinocytes

The culture supernatants were concentrated and subjected to western blotting. The 55-kDa recombinant ECD and 10-kDa recombinant chemokine domain were used as positive controls (Fig. 5A and B). Proteins of 14, 28 and ~50 kDa were detected in the culture supernatant (Fig. 5A and B; arrow head). Antibodies treated with corresponding peptide of chemokine domain could not react with these proteins (data not shown). These results indicate that these proteins contain the chemokine domain.

CXCL16 expression in normal human epidermis

Next, we studied the expression of CXCL16 in normal human epidermis by immunohistological analyses using the anti-

Anti-microbial activity of CXCL16

Recently, non-ELR CXC chemokines, such as CXCL9, CXCL10 and CXCL11, have been shown to have β-defensin-like anti-bacterial activities (23). Since CXCL16 is a non-ELR CXC chemokine, we hypothesized that CXCL16 also had anti-bacterial activity.

We analyzed the anti-bacterial activities of CXCL10 and the chemokine domain and ECD of CXCL16 against E. coli and S. aureus (Fig. 8). The chemokine domain of CXCL16 showed anti-bacterial activity against E. coli (with about 60% cell survival) at concentrations >5 μg ml⁻¹. The killing
effect on *S. aureus* was more impressive. The chemokine domain of CXCL16 killed *S. aureus* at concentrations >1 \( \mu \text{g ml}^{-1} \), with only 30% of the bacteria surviving at 10 \( \mu \text{g ml}^{-1} \) of the peptide.

Although the anti-microbial activities of peptides are often lower at physiological salt concentrations (24), Dorschner *et al.* (25) found that carbonate, which is naturally present in the bodily fluids of mammalian tissues, preserves the activities of anti-microbial peptides at physiological salt concentrations. As expected, CXCL16 lost the ability to kill *S. aureus* following the addition of 150 mM NaCl and, indeed, the bacteria grew better under this condition (Fig. 9). However, the addition of 50 mM NaHCO3 clearly restored the killing activity of CXCL16. The anti-microbial activity of CXCL16 at 10 \( \mu \text{g ml}^{-1} \) was almost equal to that of human \( \beta \)-defensin 2 at 1 \( \mu \text{g ml}^{-1} \).

**Discussion**

In the present study, we demonstrate for the first time that epidermal keratinocytes produce CXCL16. In addition, we show that the chemokine domain of CXCL16 has anti-microbial activity. Since the epidermis is part of the innate immune defense system, CXCL16 represents a novel mediator of epidermal innate immunity.

CXCL16 is one of the most important chemokines that recruit NKT cells (26). Microbial pathogens activate NKT cells via CD1d on activated dendritic cells during bacterial infection (reviewed in 27). These cells become active very early in the immune response against microbial pathogens (reviewed in 28). In the present study, we show that CXCL16 production by keratinocytes increases following stimulation with peptidoglycan. Therefore, keratinocytes may play an important role in the recruitment of NKT cells during bacterial infection, although it remains unclear whether NKT cells contribute to the immune response to gram-positive bacteria. NKT cells are also important in the defense against viral infections (28). Herpes simplex virus type 1 infection leads to the development of larger skin lesions in CD1d\(^{-/-}\) mice than in control mice, which suggests that the immune response of CD1d-restricted NKT cells is impaired (29). In the present study, we show that poly(I:C) enhances the production of CXCL16 by keratinocytes, which suggests that epidermal keratinocytes are able to recruit NKT cells during infection.

---

**Fig. 6.** Expression of CXCL16 in normal human epidermis. Frozen sections of normal human skin were stained with antibodies against the chemokine domain (A and B) and cytoplasmic domain (D and E) of CXCL16. (B) and (E) are higher magnifications of (A) and (B), respectively. Pre-immune sera of anti-chemokine domain (C) and anti-cytoplasmic domain (F) could not stain normal human skin. Immunofluorescence was observed under the fluorescence microscope. The dashed lines indicate the dermo-epidermal junctions. Scale bar: 50 \( \mu \text{m} \).
viral infection. Therefore, CXCL16 production by keratinocytes is an important step in the initiation of host defenses against bacterial and viral infections.

Moreover, the constitutive expression of CXCL16 by keratinocytes may participate in the recruitment of resident T cells to the skin, so-called skin-homing T cells, which exist under the resting condition and can initiate immune reactions in the absence of T-cell recruitment from the circulation (30). These cells are characterized by the expression of cutaneous lymphocyte-associated antigen (CLA). A recent report has demonstrated that 50% of CLA+ skin-resident T cells express CXCR6 (31). In contrast, only 2% of circulating CLA+ T cells express CXCR6. Constitutive production of CXCL16 by keratinocytes may contribute to the residence status of T cells and may play a role in cutaneous immune surveillance.

Another important innate immune system exists in the skin. Human epidermal keratinocytes produce anti-microbial peptides, such as β-defensins and CAP18/LL-37 (9–12). Recently, several chemokines have been shown to have similar anti-microbial activities (23, 32, 33). CXC chemokines that lack the ELR (Glu-Leu-Arg) motif, which include CXCL9, CXCL10 and CXCL11, have anti-microbial peptides due to a highly positive charge at the C-terminus (23). CXCL16 belongs to the ELR+ CXC chemokine family, and it also contains positively charged amino acids. In the present study, we show that the chemokine domain of CXCL16 exerts potent anti-microbial activities against E. coli and S. aureus. Therefore, CXCL16 can act as an anti-microbial peptide in the epidermis. Although keratinocytes produce CXCL9, CXCL10 and CXCL11 (34, 35), production is limited to those keratinocytes that are stimulated by cytokines, such as IFN-γ. In contrast, keratinocytes constitutively produce CXCL16, which indicates that CXCL16 is one of the first lines of innate immune defense before the start of inflammation.

The anti-microbial activities of chemokines and anti-microbial peptides are salt sensitive (24, 36, 37). Therefore, there is a question as to whether the anti-microbial activities of these chemokines function in vivo at the physiological salt concentration. Dorschner et al. (25) have clearly shown that carbonate enhances anti-microbial activity at the physiological salt concentration. The present study also shows that CXCL16 retains anti-microbial activity at the physiological salt concentration in the presence of carbonate. This result suggests that CXCL16 is active in vivo as an anti-microbial peptide.

ADAM 10 causes the shedding of CXCL16 from the membranes of macrophages (3, 4). Since keratinocytes express ADAM 10 (38), it is conceivable that ADAM 10 also mediates the shedding of CXCL16 from keratinocytes. In a previous report, human CXCL16-transfected COS-7 cells were shown

![Fig. 7. Expression of CXCL16 on Langerhans cells. Frozen sections of normal human skin were double stained with antibodies against the CXCL16 chemokine domain (green) and CD1a (red) or CXCL16 cytoplasmic domain (green) and CD1a (red).](https://academic.oup.com/intimm/article-abstract/19/9/1095/665654)
Data are presented as means ± SDs (n = 3). Statistical analysis was performed using the Student’s t-test with comparisons to the control. *P < 0.05 and **P < 0.005.

CXCL16 production by epidermal keratinocytes

Fig. 8. Anti-microbial activities of CXCL16. The anti-microbial activities of CXCL10 and the chemokine domain (CD) and ECD of CXCL16 were studied. The anti-bacterial effect was calculated as the ratio of surviving cells to total cells. The activities for Escherichia coli (A) and Staphylococcus aureus (B) were examined in 10 mM NaPi. Data are presented as means ± SDs (n = 3). Statistical analysis was performed using the Student’s t-test with comparisons to the control. *P < 0.05 and **P < 0.005.

Fig. 9. Recovery of the anti-microbial activity of CXCL16 in high-salt medium by carbonate. The anti-microbial activities of the chemokine domain of CXCL16 and human β-defensin 2 against Staphylococcus aureus were examined in media that contained 150 mM NaCl without NaHCO₃ (gray bars) or with 50 mM NaHCO₃ (black bars). Data are presented as means ± SDs (n = 3). Statistical analysis was performed using the Student’s t-test. *P < 0.05 and **P < 0.005.

to secrete a 32-kDa protein into the culture supernatant, the level of which was decreased by the addition of an ADAM 10 inhibitor (4). However, the CXCL16 produced from keratinocytes in the present study appeared as 14, 28 and 50 kDa proteins. The mechanism for proteolytic cleavage of CXCL16 in keratinocytes may differ from that in macrophages.

In conclusion, CXCL16 is a mediator of the innate immune reactivities of epidermal keratinocytes.

Acknowledgements
We thank Teruko Tsuda and Eriko Tan for their excellent technical assistance.

Funding

Abbreviations
ADAM disintegrin and metalloproteinase
CLA cutaneous lymphocyte-associated antigen
CXCL CXC chemokine ligand
CXCR CXC chemokine receptor
DMEC dermal microvascular endothelial cell
ECD extracellular domain
GAPDH glyceraldehyde-3-phosphate dehydrogenase
OxLDL oxidized low-density lipoprotein
poly(I:C) polyinosinic-polycytidylic acid
RT reverse transcription
TLR Toll-like receptor

References
CXCL16 production by epidermal keratinocytes


