Psoriasis is a chronic inflammatory dermatosis characterized by T helper cell 1 (Th1)/Th17/Th22-mediated inflammation and abnormal proliferation and differentiation of keratinocytes. Tumor necrosis factor-α (TNF-α) is the major pathogenic cytokine in psoriasis, and anti-TNF-α therapy greatly improves the symptoms of psoriasis. Epidermal keratinocytes in psoriatic lesions produce a variety of inflammatory mediators, including cytokine/chemokines and antimicrobial peptides, such as human β-defensin-2 (hBD-2), hBD-3, cathelicidin antimicrobial peptide (CAMP), and S100A7. LL-37, a CAMP, has broad antiviral, antibacterial, and antifungal effects and hBD-3 effectively kills Staphylococcus aureus, whereas hBD-2 and S100A7 show bactericidal effects against Gram-negative bacteria. In addition to their antimicrobial properties, these peptides exhibit a variety of immunomodulatory effects, and are involved in the pathogenesis of psoriasis. A recent study also highlighted the importance of the innate immune system in triggering psoriatic inflammation. Infectious or traumatic stimuli induce the release of LL-37 from keratinocytes.

Supported by a Shiseido Female Researcher Science grant (N.K.), a grant from the Lydia O’Leary Memorial Foundation (N.K.), and a grant-in-aid from the Japan Medical Association (N.K.).
with self-DNA released by damaged cells to form a complex that triggers Toll-like receptor 9 (TLR9) in plasmacytoid dendritic cells to produce type I interferons (IFNs), including IFN-α and IFN-β. These IFNs induce local maturation of myeloid dendritic cells to activate autoreactive Th1/Th17 cells, thereby triggering the psoriatic inflammation.3,10 Furthermore, incubation of keratinocytes with LL-37 greatly enhances their reactivity to TLR9 ligands to produce type I IFNs.9 hBD-2 and hBD-3 bind to C-C chemokine receptor 6 (CCK6) on immature dendritic cells or memory T cells, stimulating their recruitment to the sites of inflammation.11 LL-37, hBD-2, and hBD-3 act on keratinocytes and induce their migration, proliferation, and production of IL-6, C-X-C chemokine ligand 8 (CXCL8), CXCL10, and C-C chemokine ligand 20 (CCL20).12 S100A7 chemoattracts neutrophils or simulates their production of IL-6, CXCL8, and TNF-α.13

Patients with psoriasis are often obese and have metabolic disorders, including diabetes, hyperlipidemia, and hypertension.14 It was recently suggested that adipose tissue-derived cytokines, called adipokines, might link metabolic disorders with psoriasis.15 Visfatin is abundantly produced and secreted by adipocytes in visceral fat, and its circulating levels are increased in obesity and type 2 diabetes.16,17 Recent studies showed that serum visfatin levels are higher in psoriatic patients than in healthy individuals.18,19 Visfatin enhances the production of IL-6 and IL-1β in human monocytes16 and endothelial cells,20 and is considered to be an inflammatory adipokine. We recently reported that visfatin enhances the production of the chemokines CXCL8, CXCL10, and CCL20 together with TNF-α in human keratinocytes, and stimulates the expression of homologous chemokines in the murine epidermis.21 These effects of visfatin are mediated by NF-κB and STAT3.21 The results indicate that visfatin may promote the development of psoriasis by inducing chemokine expression. However, the effects of visfatin on the production of antimicrobial peptides have not been examined.

Our aim in this study was to determine whether visfatin stimulates human keratinocytes in vitro to enhance their production of the antimicrobial peptides CAMP, hBD-2, hBD-3, and S100A7, which are involved in the development of psoriasis. In addition, we examined the effects of intraperitoneal visfatin on the expression of homologous antimicrobial peptides in murine skin following topical administration of imiquimod, a TLR 7/8 agonist, which induces a psoriasis-like skin phenotype, similar to acanthosis and parakeratosis, as well as a mixed inflammatory infiltrate.22

Materials and Methods

Reagents

SB203580, PD98059, and hydroxy-2-naphthalenylmethylphosphonic acid Tris acetoxyethyl ester [HNMPA(AM)3] were purchased from Calbiochem (La Jolla, CA). Recombinant human TNF-α and mouse anti-human IL-1α, IL-1β, and IL-6 antibodies were purchased from R&D Systems (Minneapolis, MN). Rabbit anti–human TNF receptor 1 and 2 antibodies were from LifeSpan Biosciences (Seattle, WA). Recombinant human visfatin was purchased from Peprotech (Rocky Hill, NJ). Recombinant murine visfatin was purchased from Biovision Research Products (Mountain View, CA). FK866 HCl was purchased from Axon Medchem BV (Groningen, The Netherlands). Pam3Cys-Ser-(Lys)4·3HCl (Pam3CSK4) and polyinosinic-polycytidylic acid potassium salt [Poly(I:C)] were purchased from IMGENEX (San Diego, CA). Human genomic DNA from whole blood was purchased from Aviva Systems Biology (San Diego, CA). Mouse anti–human LL-37/CAMP antibody was from Osenses (Keswick, Australia). Synthetic LL-37 was from Peptide Institute (Osaka, Japan).

Keratinocyte Culture

Human neonatal foreskin keratinocytes (Clonetics, Walkersville, MD) were cultured in serum-free keratinocyte growth medium (KGM; Clonetics) containing keratinocyte basal medium (KBM) supplemented with 0.5 μg/mL hydrocortisone, 5 ng/mL epidermal growth factor, 5 μg/mL insulin, and 0.5% bovine pituitary extract. Cells were used in the third passage.

Secretion of CAMP, hBD-2, hBD-3, and S100A7

A total of 5 × 10⁴ keratinocytes/well were seeded in triplicate wells on 24-well plates in 0.4 mL KGM, and allowed to adhere overnight. The cells were washed and then incubated in supplement-free KBM for 24 hours. The cells were washed and then incubated with the indicated concentrations of TNF-α, Pam3CSK4, Poly(I:C), and/or visfatin in 0.4 mL KBM for 48 hours. ELISAs were used to measure the culture supernatant levels of CAMP/LL-37 (Hycult Biotech, Plymouth Meeting, PA), hBD-2 (Phoenix Pharmaceuticals, Burlingame, CA), hBD-3 (α Diagnostic International, San Antonio, TX), and S100A7 (Abnova, Taipei, Taiwan), according to the manufacturers’ instructions.

Real-Time PCR

The keratinocytes were incubated for 8 hours as described above to analyze the mRNA levels of CAMP, hBD-2, hBD-3, and S100A7. Total cellular RNA was extracted using RNeasy Mini kits (SABiosciences, Frederick, MD), and cDNA was synthesized using SuperScript III First-Strand Synthesis kits (Invitrogen, Carlsbad, CA). Gene expression was quantified using TaqMan Gene Expression assays (Applied Biosystems, Warrington, UK). The mRNA expression levels of the antimicrobial peptides were normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and are represented as the fold change relative to control keratinocytes treated with KBM alone. In some experiments, keratinocytes were preincubated with 10 μg/mL anti–TNF receptor 1,
anti–TNF receptor 2, anti–IL-1z, anti–IL-1β, or anti–IL-6 antibodies for 30 minutes, before incubation with TNF-α and/or visfatin.

Biopsy specimens were taken from lesional skin of patients with psoriasis vulgaris (n = 8) and from normal skin of healthy individuals (n = 8) after obtaining the donors’ consent. The study was approved by the institutional review board of the University of Tokyo, Faculty of Medicine (695-(2)). Total RNA was extracted from skin samples and was used for real-time PCR as described above.

Responses to Genomic DNA

Human keratinocytes were incubated with 10 ng/mL visfatin and/or 1 ng/mL TNF-α, or with 0.1 μmol/L LL-37 in the presence or absence of 10 μg/mL anti–CAMP/LL-37 antibody for 48 hours. The cells were then washed and incubated with or without 10 μg/mL genomic DNA for 12 hours. The mRNA levels of IFNB1 were analyzed by real-time PCR.

Plasmids and Transfection

CCAAT/enhancer-binding protein (C/EBP) or activator protein-1 (AP-1)–responsive firefly luciferase vectors and constitutive Renilla luciferase vector (SABiosciences) were mixed with Fugene HD Transfection Reagent (Roche, Indianapolis, IN) and added to 3.0 × 10⁵ keratinocytes/well in 24-well plates. After 24 hours, the cells were washed and incubated with 0.4–μL KBM for 24 hours, and treated with 1 ng/mL TNF-α and/or 10 ng/mL visfatin. After 18 hours, the cells were lysed, and firefly and Renilla luciferase activities of the lysates were quantified using Dual-Luciferase Assay System (Promega, Madison, WI). The transcriptional activities of C/EBP and AP-1 are expressed as the ratio of firefly and Renilla luciferase activities.

Western Blotting

Western blotting was performed as previously described.23 Briefly, keratinocytes were incubated with 1 ng/mL TNF-α and/or 10 ng/mL visfatin for the indicated times and lysed in radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA) with 1 mmol/L phenylmethylsulfonyl fluoride. The lysates were then separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked and incubated with the primary antibodies: anti–phospho-c-Jun (Ser63), anti–phospho-C/EBPz (Thr222/226), anti–phospho p38 mitogen-activated protein kinase (MAPK) (Thr180/Tyr182), anti–pan c-Jun, anti–pan C/EBPz, anti–pan p38 MAPK, and anti–GAPDH (Cell Signaling Technology). The membranes were then incubated with appropriate horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and immunoreactivity was visualized with LumiGLO reagent (Cell Signaling Technology), according to the manufacturer’s instructions. The densities of the bands were measured using GeneTools analysis software version 4.02 (Syngene, Frederick, MD). In some experiments, keratinocytes were incubated with 50 μmol/L HNMPA(AM)₁ or 0.1 μmol/L FK866 for 30 minutes before incubation with visfatin and/or TNF-α.

Treatment with siRNAs

Keratinocytes were transfected with 50 nmol/L siRNA targeting NF-κB p65, STAT3, STAT1, STAT5 (Cell Signaling Technology), c-Jun, C/EBPz, or control siRNA (Santa Cruz Biotechnology) premixed with GeneSilencer Transfection Reagent (Genlantis, San Diego, CA) in KGM for 24 hours. The cells were washed and treated with KBM for 24 h, and were then incubated with 1 ng/mL TNF-α and/or 10 ng/mL visfatin for the indicated times. The protein expression of each target was assessed by Western blotting using specific antibodies (Cell Signaling Technology).

Mouse Experiments

The mouse experiments were approved by the animal research committee of the University of Tokyo. Female BALB/c mice age 6 to 8 weeks were maintained under specific pathogen-free conditions at the Department of Dermatology Animal Facility of the University of Tokyo. The mice were intraperitoneally injected with 10 μg of murine visfatin in 1 mL of PBS or PBS alone once daily for six consecutive days. Immediately after each injection, 62.5 mg of imiquimod cream (5%) (Beselna Cream; Mochida Pharmaceuticals, Tokyo, Japan) or Vaseline petroleum jelly (Unilever, London, UK) was applied to the shaved dorsal skin of the mice. The dose of visfatin was determined according to a previous study, which showed that intraperitoneal visfatin treatment increased serum IL-6 levels.16 The skin was excised 24 hours after the last application. Total RNA was extracted from the epidermal and dermal fractions, and reverse-transcribed for real-time PCR analysis. The mRNA expression levels of cathelin-related antimicrobial peptide (CRAMP), mDEFB4, mDEFB14, S100A7, TLR1, TLR2, TLR3, TLR9, and TNF-α were normalized to those of GAPDH, and are represented as the fold change relative to the control group treated with PBS plus Vaseline. The murine skin samples were also lysed in radioimmunoprecipitation assay buffer with 1 mmol/L phenylmethylsulfonyl fluoride, and protein levels of visfatin and β-actin in the lysates were determined by Western blotting using anti-visfatin (LifeSpan Biosciences) or anti–β-actin antibodies (Cell Signaling Technology).

Immunohistochemistry

Biopsy samples were taken from lesional and nonlesional skin of patients with psoriasis and from normal skin of healthy donors. The samples were formalin-fixed and embedded in paraffin, and sections (5 μm thick) were deparaffinized and rehydrated. The murine skin samples were
embedded in optimal cutting temperature compound and frozen. Sections (5 μm thick) were then fixed in acetone and washed with PBS. After blocking endogenous peroxidase and nonspecific binding, the slides were incubated overnight at 4°C with the primary antibodies (rabbit anti-human visfatin, rabbit anti-mouse CRAMP, and rabbit anti-mDEFB4 from LifeSpan Biosciences; rabbit anti-mouse S100A7 from Bioss (Woburn, MA) or isotype controls from Vector Laboratories, Burlingame, CA). After washing, the slides were incubated with biotin-conjugated secondary antibodies (Vector Laboratories) for 2 hours at room temperature. After washing, the samples were incubated with avidin–biotin peroxidase complex, developed with diaminobenzidine (brown staining), and counterstained with Mayer’s hematoxylin. The staining intensity of visfatin or antimicrobial peptides was analyzed by WinROOF imaging software version 7.0.0 (Mitani Corporations, Tokyo, Japan). The immunostained areas were identified using macroinstruction with algorithms for color extraction based on red/green/blue and hue/luminosity/saturation parameters. The staining intensity was measured in five randomly selected fields (200 × 200 μm) at a magnification of ×200. The mean intensity was calculated for each specimen.

Statistical Analysis

All statistical analyses were performed using Ekuseru-Toukei 2012 software (Social Survey Research Information Co., Tokyo, Japan). Values of \( P < 0.05 \) were considered statistically significant.

Results

Expression of Visfatin in Psoriatic Lesional and Nonlesional Skin and in Normal Skin

We first examined the expression of visfatin in lesional and nonlesional skin of psoriatic patients and in normal skin of healthy donors by immunohistochemistry. Visfatin expression was detected in epidermal keratinocytes in psoriatic lesional and nonlesional skin, and in normal skin, with similar staining intensities (Figure 1, A–C, and Supplemental Table S1). However, the staining intensity of visfatin was much greater in the dermal perivascular sites of psoriatic lesional skin, including endothelial cells and in some infiltrated cells, as compared with nonlesional and normal skin (Figure 1, D–F, and Supplemental Table S1). We then determined the mRNA expression of nicotinamide phosphoribosyltransferase (NAMPT, the visfatin gene) in psoriatic lesional skin and in normal skin from healthy donors. NAMPT mRNA expression, normalized to that of GAPDH, in psoriatic lesional skin (0.0208 ± 0.0088, \( n = 8 \)) was similar to that in normal skin (0.0221 ± 0.0084, \( n = 8 \); \( P = 0.6744 \), \( U \)-test). These results indicate that the expression of visfatin/NAMPT in psoriatic lesions is similar to that in normal skin.

Visfatin Enhances Basal or TNF-α–Induced CAMP, hBD-2, hBD-3, and S100A7 Production in Human Keratinocytes

We examined the effects of visfatin on basal or TNF-α–induced CAMP, hBD-2, hBD-3, and S100A7 production in human keratinocytes in vitro. Visfatin at 10 or 100 ng/mL enhanced basal and TNF-α–induced CAMP secretion (Figure 2A). Although visfatin alone did not affect hBD-2, hBD-3, or S100A7 secretion, it did enhance TNF-α–induced secretion of these proteins (Figure 2, B–D), and the stimulatory effects of visfatin were maximal at 10 ng/mL. These concentrations of visfatin with or without TNF-α did not reduce the viability of keratinocytes, as examined by the trypan blue dye exclusion test, with a viability of >95% compared with control cells treated with KBM alone. When the culture medium Ca\(^{2+}\) concentration was increased to 1 mmol/L, visfatin enhanced basal and TNF-α–induced secretion of these antimicrobial peptides (Supplemental Figure S1), similar to that in cells cultured in conventional KBM with a low Ca\(^{2+}\) concentration (<0.1 mmol/L).

We then sought to assess how the fixed concentration of visfatin (10 ng/mL) modulates the effects of various concentrations of TNF-α (0.1, 1, 10, and 100 ng/mL) (Figure 2, E–H). TNF-α alone increased CAMP, hBD-2, hBD-3, and S100A7 secretion in a concentration-dependent manner. Additive or synergistic effects of visfatin were observed when added
Visfatin induces antimicrobial peptides.

In combination with 0.1, 1, and 10 ng/mL TNF-α, however, at 100 ng/mL TNF-α, there were no significant differences in antimicrobial peptide secretion between cells treated with TNF-α alone and those treated with TNF-α plus visfatin. These results suggest that the additive or synergistic effects of visfatin were greatest at low concentrations of TNF-α (0.1, 1, or 10 ng/mL). Therefore, in subsequent experiments, we administered 1 ng/mL TNF-α together with 10 ng/mL visfatin.

In parallel with the increases in protein secretion, visfatin increased basal and TNF-α–induced CAMP mRNA expression (Figure 3A), as well as TNF-α–induced mRNA expression of hBD-2, hBD-3, and S100A7 after 8 hours of treatment, whereas visfatin alone did not elicit such changes (Figure 3B–D). Similar results were observed at 24 hours (data not shown). The addition of anti–TNF receptor 1 or anti–TNF receptor 2 antibodies suppressed the additive or synergistic effects of visfatin with TNF-α on the mRNA expression of antimicrobial peptides, indicating that TNF receptor 1 and 2 mediate these effects (Figure 3, E–H). TNF-α may induce the secretion of IL-1α, IL-1β, or IL-6 from keratinocytes, and these cytokines may act on keratinocytes in an autocrine or paracrine manner to enhance antimicrobial peptide production in synergy with visfatin. However, anti–IL-1α, anti–IL-1β, or anti–IL-6 antibodies did not suppress the additive or synergistic effects of visfatin with TNF-α on the mRNA expression of antimicrobial peptides (Figure 3, E–H), excluding the involvement of these cytokines. Similar results were obtained regarding the secretion of these antimicrobial peptides (data not shown).

Because infections induce the production of antimicrobial peptides in keratinocytes, we determined whether visfatin increases their production in synergy with the TLR1 and TLR2 ligand Pam3CSK4 or the TLR3 ligand Poly(I:C), mimicking pathogenic stimuli. Visfatin enhanced Pam3CSK4- and Poly(I:C)-induced secretion of CAMP, hBD-2, hBD-3, and S100A7 (Figure 4, A–D). Similar results were observed for the mRNA expression of these antimicrobial peptides (data not shown). Thus, it seems that visfatin enhances antimicrobial peptide production induced by infectious stimuli or by TNF-α.

A recent paper showed that keratinocytes exposed to LL-37, a CAMP, are more responsive to genomic DNA in terms of the production of type I IFNs, mimicking the priming of psoriatic inflammation. Therefore, we examined whether LL-37/CAMP induced mRNA expression in response to genomic DNA, which can be released from the epidermis during trauma, infection, or normal differentiation. IFNB1 mRNA expression in response to genomic DNA was increased in keratinocytes preincubated with visfatin and/or TNF-α compared with those preincubated with medium alone (Figure 5). The increases of IFNB1 mRNA levels were suppressed when cells were preincubated with an anti–LL-37/CAMP antibody together with visfatin and/or TNF-α (Figure 5), indicating a dependency on endogenous LL-37/CAMP released from keratinocytes. Therefore, endogenous LL-37/CAMP, induced by visfatin and/or TNF-α, appears to activate keratinocytes in an autocrine manner. IFNB1 mRNA expression in response to genomic DNA was markedly increased in keratinocytes preincubated with exogenous LL-37, and this increase was suppressed by preincubation with an anti–LL-37/CAMP antibody (Figure 5), supporting the specificity of the results.
C/EBPα Is Involved in the Stimulatory Effects of Visfatin on CAMP Production, and STAT3 Is Involved in the Stimulatory Effects of Visfatin on hBD-2, hBD-3, and S100A7 Production

It was reported that NF-κB and STAT3 promote the mRNA expression of hBD-2,23,24 and S100A7 genes,25,26 that AP-1 and STAT3 induce the expression of hBD-3,27 and that C/EBPα induces the expression of CAMP.28 Therefore, we examined whether these transcription factors were involved in visfatin-induced and/or TNF-α–induced CAMP, hBD-2, hBD-3, and S100A7 production by using siRNAs to knock down the expression of transcription factors. siRNAs targeting NF-κB p65, STAT3, AP-1 component c-Jun, and C/EBPα selectively reduced the corresponding protein levels (Supplemental Figure S2), siRNA targeting C/EBPα reduced visfatin-induced and/or TNF-α–induced CAMP secretion (Figure 6A), but other siRNAs did not. siRNA targeting NF-κB p65 reduced TNF-α–induced and visfatin plus TNF-α–induced hBD-2 secretion, whereas siRNA targeting STAT3 suppressed hBD-2 secretion induced by visfatin plus TNF-α, but not hBD-2 secretion induced by TNF-α alone (Figure 6B). As shown in Figure 6C, siRNA targeting c-Jun reduced TNF-α–induced and visfatin plus TNF-α–induced hBD-3 secretion, whereas siRNA targeting STAT3 suppressed hBD-3 secretion induced by visfatin plus...
Visfatin Induces Antimicrobial Peptides

Visfatin mediates its effects through TLR ligand. Visfatin increases TLR ligand—induced secretion of CAMP (A), hBD-2 (B), hBD-3 (C), and S100A7 (D). Keratinocytes were incubated with medium alone or with 10 ng/mL Pam3CSK4 or 10 μg/mL Poly(I:C) in the presence or absence of 10 ng/mL visfatin. The secretion of antimicrobial peptides was analyzed at 48 hours. Data are expressed as means ± SEM (n = 4). *P < 0.05 versus control cells; †P < 0.05 versus Poly(I:C) alone; ‡P < 0.05 versus Pam3CSK4 alone (one-way analysis of variance with Scheffe’s test). VIS, visfatin.

TNF-α, but not hBD-3 secretion induced by TNF-α alone. As shown in Figure 6D, siRNA targeting NF-κB p65 reduced TNF-α-induced and visfatin plus TNF-α—induced S100A7 secretion, whereas siRNA targeting STAT3 suppressed S100A7 secretion induced by visfatin plus TNF-α, but not S100A7 secretion induced by TNF-α alone. siRNAs targeting STAT1 and STAT5 did not reduce the secretion of antimicrobial peptides induced by visfatin and/or TNF-α (data not shown). Similar results were obtained for the mRNA expression of these antimicrobial peptides (data not shown). These results suggest that C/EBPα may be required for CAMP production induced by visfatin and/or TNF-α. NF-κB may be required for hBD-2 and S100A7 production induced by TNF-α alone and for the synergistic effects of visfatin on their production. AP-1 may be required for hBD-3 production induced by TNF-α alone and for the synergistic effects of visfatin on its production. Although STAT3 might not be required for TNF-α—induced production of hBD-2, hBD-3, and S100A7, it may be required for the synergistic effects of visfatin on their production.

Visfatin Alone or in Combination with TNF-α Enhances Transcriptional Activity and Phosphorylation of C/EBPα via p38 MAPK

We previously reported that visfatin alone enhanced the transcriptional activity of STAT3 via its tyrosine phosphorylation by Janus kinase 2.21 Furthermore, visfatin only marginally enhanced NF-κB activity via inhibitory κB kinase, but synergistically enhanced TNF-α—induced NF-κB activity.21

Here, we examined whether visfatin alone or together with TNF-α affects the transcriptional activities of C/EBP and AP-1 in keratinocytes. Visfatin or TNF-α alone increased the transcriptional activity of C/EBP, whereas additive effects were observed in cells treated with visfatin plus TNF-α (Figure 7A). These stimulatory effects were suppressed by the p38 MAPK inhibitor SB203580, but not by the mitogen-activated protein kinase inhibitor PD98059 (Figure 7A). Visfatin and/or TNF-α induced C/EBPα phosphorylation at Thr222/226, which was suppressed by SB203580, but not by PD98059 (Figure 7B). In addition, the increase of CAMP secretion induced by visfatin and/or TNF-α was suppressed by SB203580, but not by PD98059 (Figure 7C). Visfatin and/or TNF-α induced p38 MAPK phosphorylation, which was suppressed by SB203580, but not by PD98059 (Figure 8). These results suggest that visfatin and/or TNF-α enhance the transcriptional activity and phosphorylation of C/EBPα by activating p38 MAPK. By contrast, TNF-α increased AP-1 activity (Supplemental Figure S3A) and phosphorylation of the AP-1 component c-Jun (Supplemental Figure S3B). Visfatin did not affect basal or TNF-α—induced AP-1 activity or c-Jun phosphorylation.

NAMPT Activity and Insulin Receptors Are Not Involved in Visfatin-Induced Expression of Antimicrobial Peptides

It was reported that visfatin can exert its actions by promoting NAMPT enzymatic activity.29—31 It was also suggested that...
Figure 6  Effects of siRNA knock down of transcription factors on CAMP, hBD-2, hBD-3, and S100A7 secretion. Keratinocytes transfected with siRNAs targeting NF-κB p65, STAT3, c-Jun, C/EBPα, or control siRNA were treated with 1 ng/mL TNF-α and/or 10 ng/mL visfatin. CAMP (A), hBD-2 (B), hBD-3 (C), and S100A7 (D) secretion was analyzed at 48 hours. *P < 0.05 versus control cells; †P < 0.05 versus visfatin alone; ‡P < 0.05 versus TNF-α alone; ‡P < 0.05 versus TNF-α plus visfatin (one-way analysis of variance with Scheffe's test). Data are expressed as means ± SEM (n = 4). VIS, visfatin.
insulin receptor signaling pathways may be involved in the effects of visfatin although the original study showing that visfatin can bind to insulin receptors was retracted. Therefore, we examined the potential roles of NAMPT activity and/or insulin receptors in visfatin-induced expression of antimicrobial peptides and the transcriptional activities of C/EBP family members in keratinocytes by treating cells with 0.1 μmol/L inhibitors of NAMPT (FK866) and 50 μmol/L insulin receptor tyrosine kinase (HNMPA(AM))3. We previously reported that these concentrations of FK866 and HNMPA(AM)3 blocked NAMPT activity and insulin receptor signaling, respectively, in keratinocytes. However, neither FK866 nor HNMPA(AM)3 suppressed visfatin-induced secretion of CAMP, hBD-2, hBD-3, and S100A7; C/EBP activity (Supplemental Table S2); or the phosphorylation of C/EBPz or p38 MAPK (Supplemental Figure S4). Similar results were obtained for the mRNA expression of these antimicrobial peptides (data not shown). These results indicate that NAMPT activity and insulin receptors are not involved in the effects of visfatin on antimicrobial peptide production in keratinocytes.

**Figure 7** p38MAPK inhibition suppresses visfatin functions. Visfatin enhances basal or TNF-α–induced C/EBP transcriptional activity (A), phosphorylation of C/EBPz (B), and CAMP secretion (C), and these effects are suppressed by p38 MAPK inhibition. Keratinocytes transfected with firefly/Renilla luciferase vectors (A) and untransfected keratinocytes (B and C) were pretreated with 1 μmol/L SB203580 or 10 μmol/L PD98059 for 30 minutes, and were then incubated with 10 ng/mL visfatin and/or 1 ng/mL TNF-α. A: Transcriptional activity measured at 18 hours. B: CAMP secretion measured at 48 hours. *P < 0.05 versus control cells; †P < 0.05 versus visfatin alone; ‡P < 0.05 versus TNF-α alone; §P < 0.05 versus TNF-α plus visfatin (one-way analysis of variance with Scheffé’s test). Data are expressed as means ± SEM (n = 4). B: Western blotting of phosphorylated C/EBPz at 30 minutes. The band densities of phosphorylated C/EBPz, normalized to those of total C/EBPz, are represented as the fold change relative to control keratinocytes treated with KBM alone. The results represent data from four separate experiments.

To examine the effects of visfatin in vivo in a murine skin model of psoriasis, we intraperitoneally injected BALB/c mice with recombinant murine visfatin and measured the mRNA expression of CAMP, mDEFB4, mDEFB14, and S100A7 in murine imiquimod-treated skin.

**Intraperitoneal Visfatin Increases mRNA and Protein Expression of CRAMP, mDEFB4, mDEFB14, and S100A7 in Murine Imiquimod-Treated Skin in Vivo**

Visfatin induces antimicrobial peptides in the skin, topical administration of imiquimod increased their expression, and intraperitoneal visfatin enhanced imiquimod-induced mRNA expression of these peptides (Figure 9, A–D). The results indicate that visfatin acts synergistically with imiquimod to increase mRNA expression of these antimicrobial peptides. Imiquimod also increased TNF-α mRNA expression in the skin, whereas visfatin did neither, whether in the presence or absence of imiquimod (Figure 9E). Visfatin increased mRNA expression of TLR1, TLR2, TLR3, and TLR9 in imiquimod-treated skin (Figure 9, F–I), whereas visfatin alone did not.

We then examined the effects of intraperitoneal visfatin on the protein expression of these peptides by immunohistochemical staining using specific antibodies. There are currently no antibodies specific for mDEFB4. We detected weak staining for CRAMP, mDEFB4, and S100A7 in epidermal keratinocytes of control skin (Figure 10, A–C).
Intraperitoneal administration of visfatin alone did not increase the staining intensity of these proteins (Figure 10, F–H, and Supplemental Table S3). Imiquimod increased the epidermal thickness and increased the staining intensity of these antimicrobial peptides (Figure 10, K–M, and Supplemental Table S3), which was further enhanced by intraperitoneal administration of visfatin (Figure 10, P–R, and Supplemental Table S3). These results indicate that visfatin might enhance the expression of antimicrobial peptides in murine imiquimod-treated skin in vivo. Intraperitoneal administration of visfatin increased the staining intensity of visfatin in control and imiquimod-treated skin (Figure 10, D, I, N, and S, and Supplemental Table S3). Western blotting confirmed that intraperitoneal administration of visfatin increased the expression of visfatin in the lysates of control and imiquimod-treated skin (Supplemental Figure S5). These results indicate that intraperitoneally injected visfatin may reach the skin, possibly via the circulation.

Discussion
Visfatin in combination with TNF-α enhanced the production of the antimicrobial peptides CAMP, hBD-2, hBD-3, and S100A7 in human keratinocytes in vitro. Visfatin also stimulated mRNA and protein expression of the homologous antimicrobial peptides in murine imiquimod-treated skin, in vivo, as a model of psoriasis. Because imiquimod increased TNF-α levels in the skin (Figure 9E), visfatin may enhance the expression of antimicrobial peptides synergistically with imiquimod-induced TNF-α. This is the first study showing that visfatin increases the expression of antimicrobial peptides. Visfatin concentrations in the skin were not examined in earlier studies, and the reported serum visfatin levels vary considerably between studies, with median values of 62.2 and 21.3 ng/mL19 or 4.41 and 2.57 ng/mL18 in patients with psoriasis and healthy donors, respectively. Thus, the effective visfatin concentrations (10 or 100 ng/mL) in vitro may be at the boundary between physiological and pathological levels. Interestingly, serum TNF-α levels also varied between studies, with median values of 19.6 and 10.1 pg/mL35 or 17.8 and 3.9 pg/mL36 in patients with psoriasis and healthy donors, respectively. Additionally, the median TNF-α levels in suction blister fluids of psoriatic lesional skin were reported to be 145 pg/mL (range, 38 to 463 pg/mL)37 or 194.5 pg/mL (range, 92 to 491 pg/mL)38 compared with 8.5 pg/mL (range, 0 to 35 pg/mL) in normal skin.37 The TNF-α levels may be further elevated in

Figure 9  Intraperitoneal visfatin increases mRNA expression of antimicrobial peptides and TLRs in murine imiquimod-treated skin. BALB/c mice were intraperitoneally injected with 10 μg of visfatin or PBS for six consecutive days. Immediately after each injection, shaved dorsal skin was treated with imiquimod cream (IMQ) or Vaseline as a control. At 24 hours after the last treatment, total RNA was extracted from skin samples for real-time PCR. The mRNA expression levels of antimicrobial peptides, TNF-α, and TLRs were normalized to those of GAPDH, and are expressed as the fold change relative to the PBS+Vaseline group. CRAMP (A), mDEFB4 (B), mDEFB14 (C), S100A7 (D), TNF-α (E), TLR1 (F), TLR2 (G), TLR3 (H), and TLR9 (I). Data are expressed as means ± SEM (n = 4 per group). *P < 0.05 versus PBS+Vaseline; †P < 0.05 versus PBS+IMQ (one-way analysis of variance with Scheffé’s test). IMQ, imiquimod.
the microenvironment surrounding epidermal keratinocytes. Thus, the TNF-α levels (0.1 to 10 ng/mL) that were responsible for the synergistic effects with visfatin in vitro may be pathological, similar to that in psoriatic lesions. Our results indicate that increases in visfatin levels within the range of 10 to 100 ng/mL may potentiate the effects of pathological levels of TNF-α (0.1 to 10 ng/mL), as in psoriatic lesions, to induce antimicrobial peptide expression (Figure 2). However, visfatin did not potentiate the effects of the highest TNF-α level tested here (100 ng/mL) (Figure 2), probably because of saturation of the signaling pathway responsible for inducing antimicrobial peptide expression in keratinocytes.

CAMP expression was induced by visfatin or TNF-α alone, whereas the combination of visfatin plus TNF-α had an additive effect on its expression. CAMP expression was dependent on C/EBPα, and visfatin and/or TNF-α induced phosphorylation of C/EBPα at Thr222/226 by activating p38 MAPK, and ultimately enhanced its transcriptional activity. The CAMP gene contains a C/EBP element, and a recent study showed that the pharmacological stressor thapsigargin enhances C/EBP-dependent expression of CAMP in human HaCaT keratinocytes and the expression of its murine ortholog by p38 MAPK-mediated phosphorylation of C/EBPα at Thr222/226. Thus, the effects of visfatin and/or TNF-α mimic those of thapsigargin.

By contrast, hBD-2 and S100A7 expression was not induced by visfatin alone, but visfatin synergistically enhanced TNF-α−induced expression of these antimicrobial peptides. The induction of hBD-2 or S100A7 by TNF-α alone was mediated by NF-κB, whereas their induction by TNF-α plus visfatin was mediated by the cooperation of NF-κB and STAT3. It appears that hBD-2 or S100A7 expression may be dependent on NF-κB, whereas STAT3 does not induce their expression directly, but instead promotes NF-κB-driven expression. DNA-binding activities and/or interaction with transcriptional co-activators of STAT3 on the promoters of hBD-2 and S100A7 may be less potent than those of NF-κB. In our previous study, visfatin alone greatly enhanced STAT3 activity but only marginally increased NF-κB activity, although it synergistically enhanced TNF-α−induced NF-κB activity in keratinocytes.

Visfatin alone did not induce hBD-3 expression but did enhance TNF-α−induced expression. The expression of hBD-3 induced by TNF-α alone was mediated by AP-1, whereas its expression induced by TNF-α plus visfatin was mediated by the cooperation of AP-1 and STAT3. TNF-α alone enhanced AP-1 activity by phosphorylating its component c-Jun, but visfatin did not. Thus, AP-1 may be essential for the expression of hBD-3. Visfatin activates STAT3, which does not directly induce hBD-3 expression, but may enhance AP-1−mediated expression.

It was reported that visfatin induces pathophysiological effects via insulin receptor signaling pathways, or via its NAMPT activity to convert nicotinamide to nicotinamide mononucleotide. The results of our inhibitor studies suggest that insulin receptors and NAMPT activity are not involved in visfatin-induced activation of p38 MAPK and the resultant activation of C/EBPα. Thus, visfatin may bind to a currently unknown receptor on keratinocytes and generate intracellular signals leading to p38 MAPK activation, independently of
insulin receptors or NAMPT activity. These putative visfatin receptors should be identified in future studies.

Our in vitro and in vivo results suggest that visfatin may promote the expression of the antimicrobial peptides CAMP, hBD-2, hBD-3, and S100A7 by keratinocytes in human skin in vivo. Because visfatin enhanced the expression of antimicrobial peptides in murine imiquimod-treated skin, but not in untreated skin, some infectious or traumatic co-stimuli may be required to elicit the effects of visfatin in vivo. Visfatin also increased TLR1, TLR2, TLR3, and TLR9 levels in imiquimod-treated skin (Figure 9), indicating that visfatin may enhance cellular responsiveness to pathogen- or damage-associated molecular patterns to induce antimicrobial peptide expression. Therefore, visfatin may potentiate the cutaneous innate immune systems to trigger psoriatic inflammation via antimicrobial peptides and TLRs. Visfatin protein expression in epidermal keratinocytes is similar in psoriatic skin lesions and normal skin. Although visfatin protein expression in dermal perivascular sites appeared to be increased in psoriatic lesion, total NAMPT mRNA expression was not increased relative to that in normal skin, indicating that dermal visfatin/NAMPT has a negligible effect. It was reported that serum visfatin levels are higher in patients with psoriasis than in healthy donors. Thus, the increase of serum visfatin levels in psoriasis may be caused by its increased expression in extracutaneous organs. Patients with psoriasis associated with metabolic disorders, visceral fat–derived visfatin may contribute to the increased serum levels of visfatin. It was hypothesized that visfatin present in the bloodstream may stimulate the mRNA and protein expression of antimicrobial peptides in epidermal keratinocytes of psoriatic lesions. Therefore, visfatin may exacerbate psoriatic skin lesions by inducing the expression of antimicrobial peptides and chemokines.21 Taken together, these findings suggest that visfatin may be an accelerator for psoriasis, and could provide a potential therapeutic target. By regulating systemic visfatin levels, it may be possible to control the exaggerated production of antimicrobial peptides in psoriatic skin lesions and alleviate its symptoms.

Acknowledgments

We thank Hiroko Sato for maintaining the keratinocytes and Yoshiko Ito and Tamami Kaga for performing immunohistochemistry.

Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2013.01.044.

References


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