Inflammatory Cytokine Tumor Necrosis Factor-α Enhances Nerve Growth Factor Production in Human Keratinocytes, HaCaT Cells

Koji Takaoka¹², Yasuhito Shirai¹*¹ and Naoaki Saito¹*²

¹Laboratory of Molecular Pharmacology, Biosignal Research Center, Kobe University, Rokkodai-cho 1-1, Nada-ku, Kobe, Hyogo 657-8501, Japan
²DSR Corporation, 1-1-15 Gomoudori, Nada-ku, Kobe, Hyogo 657-0814, Japan

Received May 10, 2009; Accepted October 7, 2009

Abstract. The skin lesions of inflammatory skin diseases (e.g., atopic dermatitis or psoriasis) accompany infiltration of inflammatory cells like macrophages, where abnormal sensory innervations and elevation of nerve growth factor (NGF) level are observed. It is thought that increased NGF mediates the abnormal innervations and this may cause the hypersensitivity of the skin. However, the mechanism of this increased NGF production in the skin is still unknown. Here, we show that tumor necrosis factor (TNF)-α, but not interferon-γ or interleukin-6, enhanced the NGF production in human keratinocytes. The enhanced NGF production was abolished by both Raf-1 kinase and MEK inhibitors, whereas specific inhibitors of p38 mitogen-activated protein kinase and c-Jun N-terminal kinase did not. The extracellular signal–regulated kinase (ERK) phosphorylation and expression of NGF mRNA were accelerated by TNF-α treatment. Furthermore, serum was necessary for the NGF production and epidermal growth factor could substitute for serum in the effect on NGF secretion. These results indicate that TNF-α enhances NGF production via the Raf-1 / MEK / ERK pathway in human keratinocytes, suggesting that regulating TNF-α is a therapeutic target to control NGF production and subsequent sensory innervations.

Keywords: nerve growth factor (NGF), tumor necrosis factor (TNF)-α, keratinocyte, extracellular signal–regulated kinase (ERK), signal transduction

Introduction

Atopic dermatitis and psoriasis are the two main inflammatory dermatoses. Atopic dermatitis is a chronic, relapsing, inflammatory skin disease that is characterized by highly pruritic, eczematous skin lesions. The skin lesions of atopic dermatitis are accompanied by an inflammatory infiltration of lymphocytes, monocytes /macrophages, eosinophiles, dermal dendritic cells, and epidermal dendritic cells. On the other hand, psoriasis is a chronic inflammatory skin disorder and the lesions exhibit proliferation of epidermal keratinocytes and infiltration of inflammatory cells.

Patients with the atopic dermatitis often complain of intense itching and excessive scratching, which are the major clinical symptoms in this disease (1). In the epidermis of lesional skin from patients with atopic dermatitis, cutaneous nerve fibers are present at high densities and their lengths are increased, reaching to the surface of the skin (2 – 4). This abnormal innervation is thought to be one of causes of the intense itching and scratching. In fact, the abnormal innervation is observed in the dry epidermis of acetone-treated mice (5) and in human allergic contact eczema (6), causing hypersensitivity in the lesional skin of patients with these skin diseases.

One of the major mediators inducing the nerve fiber expansion is thought to be nerve growth factor (NGF). NGF is released from keratinocytes in the skin and increases in the epidermis (7 – 11). On the other hand, an increased NGF content as well as an increased length of sensory nerve fibers are found in lesional skin from atopic dermatitis (12 – 14) and contact allergy (6).
Strong expression of NGF and its receptors are also observed throughout the entire epidermis in a psoriatic lesion (15 – 19). These findings suggest that enhanced keratinocyte NGF synthesis seems to stimulate abnormal innervation in inflammatory skin diseases (20), although keratinocyte-derived NGF physiologically plays an important role in the maintenance and regeneration of cutaneous nerves in the normal skin (7 – 11, 21).

The increased NGF levels in the lesional skin of the patients appear to be related to inflammation and inflammatory cell infiltration. The inflammatory cells, especially macrophages and T cells, infiltrate into the epidermis in inflamed skin and secrete inflammatory cytokines including tumor necrosis factor (TNF)-α, interferon (IFN)-γ, or interleukin (IL)-6 (22). TNF-α is a pivotal proinflammatory cytokine of the innate immune response and a key for skin inflammation (23). Plasma TNF-α concentration is increased in atopic dermatitis (24), and TNF-α and its receptors are upregulated in dermal blood vessels from patients with psoriasis (25). IFN-γ is also thought to be one of the important mediators in psoriatic inflammation. IFN-γ is up-regulated in mast cells of psoriatic skin (26). IL-6 is a major mediator of the host response to tissue injury and its level increases in psoriatic plaques and in the plasma of psoriasis patients (27). However, the direct relationship between inflammatory cytokines and NGF production in skin is not understood yet, although it has been shown that histamine enhances NGF production in human keratinocytes (28).

Thus, we hypothesized that some inflammatory cytokines secreted from infiltrated inflammatory cells stimulate NGF production in keratinocytes and investigated the stimulatory effects of cytokines on the NGF secretion. Here, we show an inflammatory cytokine TNF-α, but not INF-γ and IL-6, enhances NGF synthesis in cultured human keratinocytes, HaCaT cells. In addition, we revealed the pathway of TNF-α-enhanced NGF production because it is very important to prevent hyper-sensitivity in lesional skin of allergic diseases, which will improve the quality of life (QOL) of patients with these skin diseases.

Materials and Methods

Chemicals

Recombinant human TNF-α and IFN-γ were purchased from Sigma (St. Louis, MO, USA). Inhibitors of MEK (PD98059), p38 mitogen–activated protein kinase (MAPK) (SB203580), c-Jun N-terminal kinase (JNK) (SP600125), and Raf-1 kinase (GW5074) were purchased from Merck (Darmstadt, Germany). AG1478, a specific inhibitor of epidermal growth factor (EGF) receptor, was obtained from Wako (Osaka). Recombinant human keratinocyte growth factor (KGF), EGF, IL-4, IL-6, IL-13, colony-simulating factor (CSF), and platelet-derived growth factor (PDGF) were purchased from Peprothch (London, UK). All the other chemicals used were of analytical grade.

Cell culture

HaCaT cells, human keratinocytes were kindly provided by Dr. Oka (Kobe University). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Wako) with 0.45% glucose, 50 μg/ml streptomycin, and 50 units/ml penicillin (Gibco®; purchased from Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C with 5% CO₂.

Measurement of NGF

Using 6-well plates, 2 × 10⁵ HaCaT cells were cultured in 1.5 ml of DMEM (0.45% glucose) containing 50 μg/ml streptomycin, 50 units/ml penicillin, and 10% FBS (normal medium) for 48 h. After 12-h serum-free treatment, the cells were incubated with TNF-α, INF-γ, or IL-6 in 800 μl of the normal medium for 24 h. The final culture mediums were applied to the NGF Emax Immunoassay System (Promega, Southampton, UK), according to the manufacturer’s instructions in Nunc MaxiSorp 96-well microplates (Fisher Scientific, Loughborough, UK). To determine effects of inhibitors and cycloheximide on the NGF secretion, the reagents were added to the medium 15 min prior to the TNF-α treatment.

To see the effects of FBS or EGF, after 48-h culture in the normal medium and following 12-h pre-incubation in serum-free medium, HaCaT cells were incubated with or without TNF-α in 800 μl of DMEM in the presence or absence of FBS or EGF for 24 h.

To measure NGF levels secreted to extracellular medium and accumulated in keratinocyte cells, HaCaT cells were seeded on 10-cm dishes (8 × 10⁵ per dish) and cultured in the normal medium with or without 10 ng/ml of TNF-α for the indicated time. The medium were used to determine the secreted NGF. Cells were collected by centrifugation at 8,000 × g for 10 min and lysed by sonication in 100 μl of ice-cold 20 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose, 2 mM EDTA, 10 mM EGTA, 20 μg/ml leupeptin, 1 mM PMSF, and 0.25% Tween 20. After centrifugation at 12,000 × g for 10 min, the lysates were used for determination of intracellular NGF.

Western blotting

Cells grown on a 10-cm dish were washed with phosphate-buffered saline (PBS) on ice, scraped off
with 1 ml of PBS and collected by centrifugation at 8,000 × g for 10 min. After adding 0.1 ml of 20 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose, 2 mM EDTA, 10 mM EGTA, 20 μg/ml leupeptin, 1 mM PMSF, and 0.25% Tween 20, cells were lysed on ice by sonication and then centrifuged at 12,000 × g for 10 min to obtain the cell extracts. Protein concentrations were determined by using the BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). Approximately 70 μg of protein were subjected to 8% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Membranes were blocked in 5% nonfat milk in PBS-T (0.03% Triton X-100 in PBS) and then incubated with anti-ERK (p44/p42) rabbit antibody or anti-phospho-ERK (phospho-p44/p42) mouse monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA) as first antibody for 1 h. Membranes were then washed three times with PBS-T and incubated with the secondary antibody conjugated with anti-rabbit or anti-mouse horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h. Bands were visualized by using the enhanced chemiluminescent Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK). Phosphorylated ERK (phospho-ERK2) were expressed as phospho-ERK/ERK ratio because the amount of ERK was slightly different in each sample.

**Quantitative reverse transcription (RT)-PCR**

Total cellular RNA was extracted from HaCaT cells using commercial reagents (TRIzol Reagent, Invitrogen) according to the manufacturer’s instructions and quantified spectrophotometrically. Total RNA (1 μg) was reverse-transcribed into cDNA using the Super Script III First Strand Synthesis System for RT-PCR (Invitrogen) according to manufacturer’s instructions. Real-time PCR was performed in order to obtain quantitative values of NGF mRNA using an ABI Prism 7000 instrument (Applied Biosystems, Foster City, CA, USA). All samples were normalized to values of β-actin and results expressed as fold changes of threshold cycle (Ct) value relative to controls. Primers were synthesized commercially (Invitrogen), and the sequences were as follows: for NGF, the 5'-primer was 5'-TCA TCA TCC CAT CCC ATC TT-3' and the 3'-primer was 5'-CTT GAC AAA GGT GTG AGT CG-3'; for β-actin, the 5'-primer was 5'-TCT CCC AGC CTT CCT TCC TG-3' and the 3'-primer was 5'-CAA TGC CAG GGT ACA TGG TG-3'. Reverse transcription was performed according to supplier’s instructions, with 300 nM forward and reverse primers and 1 μl of cDNA in a 30-μl final reaction volume. Each sample was run in triplicate.

**Statistical analyses**

All experiments were performed in triplicate and repeated at least three times. For statistical analysis, one-way ANOVA (post-hoc test/Dunnet’s test or Bonferroni/Dunn correction) was used for comparison. Statistical significance is indicated by P values less than 0.05. Unless otherwise specified, all results are presented as the mean ± S.E.M.

**Results**

**Inflammatory cytokine TNF-α, but not IFN-γ or IL-6, enhanced NGF secretion from human keratinocytes**

First, we examined whether inflammatory cytokines enhance NGF secretion from human keratinocytes. Keratinocytes constitutively secreted a low amount of NGF without any stimulation; the NGF secretion induced by FBS alone was defined as basal secretion. The secretion was dose-dependently increased by TNF-α; the stimulatory effect of TNF-α was manifested at 5 ng/ml and maximized at 10 ng/ml, which is 3.3-fold the secretion of basal level (Fig. 1A). In contrast to TNF-α, IFN-γ did not enhance NGF secretion, but rather showed inhibitory effects (Fig. 1C). IL-6 did not affect NGF secretion even at high concentrations (Fig. 1D). These experiments were performed in the presence of serum but most experiments of NGF secretion in the previous reports were performed under serum-free conditions (28 – 30). We, therefore, checked whether FBS was required for the NGF secretion from keratinocytes. The cells cultured without serum could hardly secrete of NGF and additional TNF-α could not induce a sufficient level of secretion in the absence of FBS (Fig. 1B), indicating that serum is necessary for the basal and TNF-α–enhanced NGF secretion from keratinocytes. The following experiments were performed in the presence of serum.

**MEK-ERK pathway is involved in the TNF-α–enhanced NGF secretion**

To identify the signal transduction pathway of the TNF-α–enhanced NGF secretion, we examined the effect of typical MAPK inhibitors, PD98059, SB203580, and SP600125. PD98059, which selectively blocks ERK activity through the inhibition of ERK1/2 phosphorylation by MEK1/2, reduced the TNF-α–enhanced NGF secretion to the basal level at the concentration of 2.0 μM and completely inhibited it at 2.0 μM (Fig. 2A). On the other hand, SB203580, a specific inhibitor of p38 MAPK, did not inhibit the TNF-α–enhanced NGF secretion (Fig. 2B). SP600125, a specific inhibitor of JNK, inhibited NGF secretion weakly but not significantly (Fig. 2C).
We then tried to confirm that TNF-\(\alpha\) signaling could induce the phosphorylation of ERK. Keratinocytes were pre-incubated for 12 h in serum-free medium, and then the medium was replaced by fresh medium containing FBS (10%) and/or TNF-\(\alpha\) (10 ng/ml). FBS alone stimulated ERK phosphorylation at 30 min, and the ERK phosphorylation was significantly enhanced by TNF-\(\alpha\) (Fig. 3: A and B). The TNF-\(\alpha\)-enhanced ERK phosphorylation peaked at 30 min, while the phosphorylation induced by FBS alone was maximized around 30 – 60 min (Fig. 3B). Each phosphorylation level decreased at 120 min (Fig. 3B) and reached to the basal level at 6 h (data not shown). On the other hand, TNF-\(\alpha\) alone could not induce the phosphorylation. These results indicate that the MEK-ERK pathway is involved in the basal and TNF-\(\alpha\)-enhanced NGF secretions, and TNF-\(\alpha\) treatment enhanced and hastened the ERK phosphorylation.

**Raf-1 kinase is involved in the TNF-\(\alpha\)-enhanced NGF secretion**

It is well known that Raf-1 is involved in the upstream portion of the MEK-ERK pathway. We thus examined whether Raf-1 kinase is also involved in the TNF-\(\alpha\)-induced enhancement of NGF secretion. A Raf-1 kinase inhibitor, GW5074, inhibited the TNF-\(\alpha\)-enhanced NGF secretion, but not the basal secretion (Fig. 4); 10 nM GW5074 suppressed the TNF-\(\alpha\)-enhanced NGF secretion by 33%, corresponding to that 64% of NGF additionally secreted by TNF-\(\alpha\) treatment being suppressed. However, 100 nM GW5074 did not affect the basal NGF secretion (Fig. 4, open column). These results indicate that Raf-1 is involved in the signaling pathway of the TNF-\(\alpha\)-enhanced NGF secretion, but not in the basal secretion.
TNF-$\alpha$ enhanced and hastened mRNA expression and protein synthesis of NGF

To determine the effect of TNF-$\alpha$ on NGF mRNA expression, we next quantified relative mRNA levels in keratinocytes. Keratinocytes were pre-incubated in serum-free medium for 12 h and then cultured with TNF-$\alpha$ (10 ng/ml) for 24 h in the presence of the inhibitors. The culture medium was assayed for NGF secretion. Open columns show basal NGF secretion from non-treated cells, corresponding to 5.22 ± 1.76 (A), 5.30 ± 1.28 (B), and 7.08 ± 1.28 pg/ml (C), respectively. Values are each the mean ± S.E.M. of triplicate cultures of three independent experiments. *P<0.05 vs. control cultures without inhibitors (+TNF-$\alpha$) by one-way ANOVA with Dunnett’s test.

Fig. 2. The effects of typical MAPK inhibitors on NGF secretion. Keratinocytes were pre-incubated with the indicated concentrations of PD98059 (A), SB203580 (B), and SP600125 (C) for 15 min and then cultured with TNF-$\alpha$ (10 ng/ml) for 24 h in the presence of the inhibitors. The culture medium was assayed for NGF secretion.

Fig. 3. The effects of TNF-$\alpha$ on ERK phosphorylation. A: Typical Western immunoblotting of ERK phosphorylation. Keratinocytes were pre-incubated for 12 h in serum-free medium and then treated with 10 ng/ml TNF-$\alpha$ (TNF), 10% FBS (FBS), or FBS + TNF-$\alpha$ (FBS + TNF) for the indicated periods of time. The levels of phosphorylated ERK2 (upper panel) and total ERK2 (lower panel) were analyzed by Western immunoblotting. Similar results were obtained in the other two independent experiments. B: Quantification of ERK phosphorylation by NIH image software. Open columns show the phosphorylated ERK levels by treatment with TNF-$\alpha$ alone (TNF). Shadowed columns and closed columns show the levels of phospho-ERK in keratinocytes treated with FBS alone (FBS) and FBS + TNF-$\alpha$ (FBS + TNF), respectively. Data are expressed as phospho-ERK/ERK ratio. Values each represent the mean ± S.E.M. of three independent experiments. **P<0.01 vs. values of TNF-$\alpha$ alone, ††P<0.005 vs. values of 0 time (one-way ANOVA with Bonferroni/Dunn correction).
extracellular medium and accumulated in keratinocyte cells at several time points (Fig. 5C). Intracellular NGF protein levels were continuously low in the TNF-α-treated and –untreated keratinocytes, while secreted NGF remarkably increased. Extracellular NGF protein secreted from TNF-α-treated keratinocytes was already detected at 6 h (Fig. 5C, inset) and maximized at 24 h. On the other hand, the secretion from TNF-α-untreated cells was detected after 9 h, consistent with the delay of mRNA fluctuation. The NGF secreted from keratinocytes treated with TNF-α for 24 h was increased 2.35-fold compared with that from untreated keratinocytes. These results indicate that TNF-α accelerated and enhanced NGF synthesis and almost all NGF protein synthesized in keratinocytes was secreted to the medium continuously.

Fig. 4. Raf-1 kinase is involved in the TNF-α-enhanced NGF secretion. Keratinocytes were pre-incubated with the indicated concentrations of Raf-1 kinase inhibitor (GW5074) for 15 min and then cultured with or without TNF-α (10 ng/ml) for 24 h. The culture medium was subjected to the assay for NGF secretion. Open columns show basal NGF secretion from non-treated cells (corresponding to 6.72 ± 1.34 pg/ml) and the effect of GW5074 on the basal NGF production. Values are each the mean ± S.E.M. of triplicate cultures of three independent experiments.

Fig. 5. TNF-α enhanced and hastened mRNA expression and protein synthesis of NGF. A: Keratinocytes were pre-incubated in serum-free medium for 12 h, and then FBS (10%) with or without TNF-α (10 ng/ml) were added to the cultures. Cells were incubated for the indicated time and collected. Total cellular RNA was extracted and levels of NGF mRNA were determined by quantitative RT-PCR as described in Materials and Methods. Values were normalized to that of β-actin and expressed as fold changes of Ct value relative to controls. Each data point indicates the mean ± S.E.M. of three separate experiments. B: Keratinocytes were pre-incubated with cycloheximide (500 nM) for 15 min, and subsequently TNF-α was added to the cultures followed by 24-h incubation and the assay for NGF secretion. Values are each the mean ± S.E.M. of triplicate cultures of three independent experiments. The NGF amount in the basal secretion was 5.57 ± 2.44 pg/ml. ****P<0.001 vs. basal secretion (−TNF) and ††P<0.005 vs. “without cycloheximide” (one-way ANOVA with Bonferroni/Dunn correction). C: Keratinocytes were cultured for 48 h in conditioned medium and then TNF-α (10 ng/ml) was added to the culture. At the indicated time, the NGF secreted to the medium and that in the cell were measured. Values are each the mean ± S.D. of triplicate determinations. *P<0.05, **P<0.01, and ****P<0.001 vs. values of “without TNF” (Student’s t-test).
EGF is one of factors necessary for the basal and TNF-α–enhanced NGF secretion

We demonstrated that FBS was required for NGF production (Fig. 1B), indicating that FBS contains some factor(s) necessary for NGF production. To determine the factor(s), we tried heat-treated serum. Heat treatment of serum at 56°C for 30 min decreased NGF secretion from the TNF-α–untreated or –treated keratinocytes by 34.1% and 33.8%, respectively. The effect of serum on the basal and TNF-α–enhanced NGF secretion disappeared completely by heat treatment at 80°C for 10 min (Fig. 6A), indicating that the factor(s) is heat-unstable (e.g., protein). To identify the factor(s), we tested effects of KGF, CSF, PDGF, IL-4, IL-13, and EGF instead of serum on the NGF secretion. However, KGF, CSF, PDGF, IL-4, and IL-13 could not substitute for serum (data not shown). Finally, we found that EGF dose-dependently induced NGF secretion from keratinocytes and the EGF-induced NGF secretion was enhanced by TNF-α (Fig. 6B). The NGF secretion induced by TNF-α + EGF or EGF alone was almost completely inhibited by AG1478, a specific inhibitor of EGF receptor (Fig. 6C), confirming the indispensable effect of EGF on these NGF secretions. Moreover, the NGF secretion induced by TNF-α + EGF was inhibited by PD98059 and GW5074 (Fig. 6C), similarly to the NGF secretion induced by TNF-α + FBS (Figs. 2A and 4). These results suggested that EGF might be the one of the factors in FBS necessary for the NGF secretion.

Discussion

NGF is a chemotactic factor for nerve cells and keratinocyte-derived NGF is thought to play an important role in normal maintenance, proliferation, and differentiation of not only cutaneous nerves but also keratinocytes, melanocytes, and other cells in the skin (31–33). Specifically, NGF is very important for normal innervation of nerve fibers in the skin during wound healing. However, abnormal elongation of nerve fibers with increased level of NGF is observed in lesions of patients with atopic dermatitis (12–14), psoriasis (15, 17), and other inflammatory skin diseases (6). These observations suggest that NGF could be the key factor in the pathogenesis of these conditions.
substance to play a central role in abnormal innervation in the skin. The abnormal elongation of nerve fibers raises sensitivity in the lesional skin of the patients and the hypersensitivity decreases the QOL of patients. Therefore, understanding the mechanism of cutaneous abnormal production of NGF is very important for preventing hypersensitivity in the lesional skin and for improving the symptoms as well as QOL of patients.

Here we, for the first time, showed that TNF-α, but not INF-γ and IL-6, enhanced NGF secretion from keratinocytes. For example, the 24-h treatment with TNF-α increased NGF secretion from 32.8 to 77.0 pg/ml, corresponding to 1.19 and 2.79 pg/mg wet weights of keratinocytes (Fig. 5C). The increased amount of NGF by TNF-α seems to be small, but it is physiologically significant because TNF-α–induced NGF secretion was about 2.3-fold the basal level. This is similar to the values reported by Kinkelin et al. (6) who indicated that the NGF content in normal human skin was 2.9 ± 0.5 pg/mg wet weight and the amount of NGF in contact aczema skin, in which increased length of nerve fibers was observed, was 4.2 ± 0.6 pg/mg wet weight (6). The fact that TNF-α induces NGF secretion from keratinocytes suggests that regulation of cytokines secreted from infiltrated inflammatory cells is very important to control NGF production in the skin. This hypothesis is supported by following reports showing that TNF-α regulates NGF production and secretion from different cell types and tissues. Exogenous and autocrined TNF-α enhances NGF production in astrocytes (34). In fibroblasts, TNF-α has been shown to induce NGF production (29, 30, 35), whereas IFN-γ or IL-1β was not effective (35). In adipocytes, TNF-α also induces NGF production, whereas IL-6 shows an inhibitory effect (36). Moreover, mast cell-derived TNF-α can promote nerve fiber elongation in the skin during contact hypersensitivity in mice (37). These findings implicate that inflammation and related cytokines, especially TNF-α, can regulate NGF production. However, in most cases, signal transduction to produce NGF has not been fully understood.

In this study, we revealed that the TNF-α–enhanced NGF production is mediated via the MEK/ERK pathway. Based on our findings and previous reports, a possible scheme for the TNF-α–enhanced NGF production in keratinocytes is shown in Fig. 7. Generally, the response to TNF-α is triggered by binding to one of two distinct receptors, designated TNFR1 (55-kD TNFR) and TNFR2 (75-kD TNFR), which are differentially regulated in various cell types in normal and diseased tissue. In normal and psoriatic skin, epidermal cells express TNFR1 but not TNFR2 (25) and we also confirmed the TNFR1 expression in keratinocytes using quantitative RT-PCR (data not shown), indicating that the TNF-α–enhanced NGF secretion was mediated by TNFR1. However, there was no difference in TNFR1 mRNA expression levels between the TNF-α–treated and –untreated keratinocytes (data not shown). Subsequently, the TNF-α signal is transduced to Raf-1 and the MEK/ERK pathway, although how TNF-α signaling activates Raf-1 is still unknown (38). Surprisingly, Raf-1 seems not to be involved in the serum-induced basal NGF production because Raf-1–kinase inhibitor could not suppress basal NGF production (Figs. 4 and 6). The results suggest that A-Raf or B-Raf is involved in the serum-induced basal secretion from keratinocytes. It is noteworthy that TNF-α enhances NGF production by accelerating and up-regulating the ERK phosphorylation and following production of NGF mRNA. The up-regulation of NGF mRNA seemed to be mediated by AP-1 because the NGF gene contains an AP-1 site that has been shown to be functionally required for NGF transcription (39–41). In general, the Fos and Jun
families of transcription factors are known to bind to the AP-1 sequences. The former is phosphorylated by ERK and the latter by JNK and p38 MAPK. However, p38 MAPK inhibitor was not effective on the NGF production and JNK inhibitor showed weak inhibition, although inhibition of ERK phosphorylation was very effective. The results suggest that JNK and p38 MAPK may complementarily activate the Jun family, while c-Fos expression may be predominantly mediated by ERK. The involvement and importance of c-Fos in the NGF production was supported by the report describing that c-Fos is involved in lesion-increases in NGF expression in mouse fibroblasts (42). Finally, the TNF-α–enhanced NGF is completed by protein synthesis and immediate secretion. In the meanwhile, it is well known that regulation of the transcription factor NF-κB is a key component of TNF-α signal transduction (43), but NF-κB is not likely the major mediator of the TNF-α–enhanced NGF production from keratinocytes because inhibitors of IκB kinase, BAY 11-7082 (Merck) at 20 μM or a IκB kinase–inhibitor peptide (Merck) at 50 μg/ml, inhibit it only by 7.7% and 26.8%, respectively (data not shown).

In addition, we showed that FBS was necessary for the basal and TNF-α–enhanced NGF secretion (Fig. 1B), suggesting proliferative conditions are important. This is very unique to keratinocytes compared with the TNF-α–induced NGF secretion from other cells (31–33) but consistent with previous reports that NGF is secreted from proliferating and pre-confluent keratinocytes rather than more differentiated, stratified cells (17, 31) and that NGF is expressed in the epidermal basal layer (15, 18). To identify the factors in the serum, we tested some cytokines and growth factors including EGF, KGF, CSF, PDGF, IL-4, and IL-13 because the effect of serum on NGF production is heat-labile (Fig. 6A). However, IL-4, IL-13, CSF, and PDGF did not show any effects on NGF production; and KGF induced NGF secretion at quite low levels (data not shown). Although KGF activates p90RSK through ERK activation (44–46), probably this signaling goes down a different pathway from NGF production. On the other hand, the effect of EGF was similar to that of FBS; EGF alone induced basal NGF secretion and additional TNF-α enhanced the NGF secretion. Both of the NGF secretions were inhibited by MEK inhibitor (Fig. 6B), and EGF alone also induced the ERK phosphorylation (data not shown). These results suggest that EGF is one of the factors necessary for the FBS effect on NGF secretion. However, AG1478, a specific inhibitor of EGF receptor, showed about 20% inhibition of the NGF secretion induced by TNF-α + FBS (data not shown), indicating that there are additional factors in the serum necessary for MEK activation and following NGF production. For example, complements are a possible candidate because heat treatment of FBS at 56°C for 30 min reduced NGF production by 34% (Fig. 1B), and the complement system has been emerging as an important aspect in skin inflammation (47–49).

As described above, regulation of NGF is one of the most effective strategies to reduce itching and scratching to prevent the aggravation of skin lesions and improve QOL for patients. In fact, high-affinity NGF-receptor inhibitors, AG879 and K252a, are effective in improving the established dermatitis (50) and scratching behavior in the NC/Nga atopic dermatitis model mouse (51), and anti-NGF antibody is also effective in inhibiting epidermal innervation and scratching in NC/Nga mouse (52). However, complete interception of NGF signaling may cause some side effects because autocrined NGF is thought to be involved in keratinocyte proliferation and important for normal elongation of neuronal fibers. Thus, to prevent abnormal excess of NGF production is more important as a therapeutic target. Our findings suggest that TNF-α is a new potential target for preventing hypersensitivity of the skin. Indeed, anti–TNF-α biological agents are highly effective for preventing hypersensitivity of a large population of patients (53). In addition, two TNF-α–blocking agents, etanercept and infliximab, have been recently used for treatment of psoriasis, rheumatoid arthritis, and other inflammatory diseases and showed a significant improvement (54). However, the effects of these agents on atopic dermatitis and the abnormal innervation are still unknown. We expect further trials of these agents to improve the hypersensitivity of atopic dermatitis, psoriasis, and other inflammatory skin diseases with improving QOL of patients. Furthermore, our findings suggest possibility of Raf-1 as a therapeutic target to control abnormal excess of NGF production, although further experiments will be necessary.

In conclusion, our study demonstrated that the inflammatory cytokine TNF-α enhances NGF production via the Raf-1 / MEK / ERK pathway in human keratinocytes. These findings suggest that inflammation and infiltration of inflammatory cells into epidermis are involved in the NGF-mediated abnormal innervations resulting in the hypersensitivity of inflammatory skin diseases.

Acknowledgments

This work was supported by a Grants-in-Aid for Scientific Research from the Global Center of Excellence (COE) Program of the Ministry of Education, Culture, Sports, Science, and Technology of Japan and grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, Hyogo COE program from Hyogo prefecture, Japan, and the Takeda Science Foundation. We thank
Dr. Oka, Kobe University, for providing HaCaT cells.

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