(A)

(B)

Fig. 2. Effects of histamine (10^{-5} M) and IL-4 (1–100 ng/ml) on IL-6(A) and IL-8(B) production by HaCaT cells. Representative data of three experiments. *$P < 0.05$ compared with the spontaneous production (control). #$P < 0.05$ compared with the histamine-induced production.

Other investigators have already shown that T cell cytokines such as IFN-γ and IL-4 enhance the IL-6 production by keratinocytes [8,27,28]. The present study showed that histamine further augmented the IFN-γ- or IL-4-induced IL-6 production, suggesting that histamine may enhance the acanthotic process of various inflammatory skin diseases. On the other hand, we demonstrated that the histamine-induced IL-8 upregulation was completely abrogated by IFN-γ and IL-4. Various molecules, such as STAT family and SOCS family molecules, have recently been shown to mediate IFN-γ- and IL-4-dependent positive and negative

intracytoplasmic signaling pathways [29,30]. Further studies are needed to elucidate the molecular basis of differential regulation of IL-6 and IL-8 production. Interestingly, Sticherling et al. compared the immunolocalizations of IL-8 in sequential biopsies of psoriasis before and after treatment [23]. In active psoriasis, high IL-8 expression was found to be compartmentalized in the upper epidermis with a loss of immunoreactivity in the lower keratinocyte layers adjacent to the area of dermal mononuclear cell infiltration. During the course of therapy, homogeneous staining of IL-8 was gradually restored in the lower epi-

dermis with a decrease in inflammatory cell infiltration, suggesting that the cytokines from inflammatory cells inhibit the IL-8 production by keratinocytes. Since the infiltrated T cells produce IFN-γ in psoriasis [31], the secreted IFN-γ may reduce IL-8 production in the lower epidermis as has been demonstrated in the present study.

In conclusion, histamine may be an important modulator of epidermal milieu in combination with T cell cytokines.

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Propionibacterium acnes-induced IL-8 production may be mediated by NF-κB activation in human monocytes

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Abstract

Propionibacterium acnes (*P. acnes*) causes an inflammatory acne that is characterized by massive neutrophilic infiltration. IL-8 is thought to play an important role in the pathophysiology of *P. acnes*, although the mechanisms by which *P. acnes* up-regulates the release of IL-8, a neutrophilic chemokine, from target cells is not well understood. In this study, we investigated the mechanisms through which heat-killed *P. acnes* induces IL-8 production in THP-1 cells (a human monocytic cell line). We found that *P. acnes* is able to directly induce IL-8 production and IL-8 mRNA expression in human monocytic cells in a dose- and time-dependent manner through a mechanism requiring transcription factor NF-κB activation. Additionally, *P. acnes*-induced IL-8 secretion was inhibited by roxithromycin, a macrolide antibiotic, and its inhibitory effect seemed to be partially associated with the inhibition of *P. acnes*-induced NF-κB activation. This is the first study to show that NF-κB activation is involved in the IL-8 production of monocytic cells stimulated by *P. acnes*. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: IL-8; NF-κB; *P. acnes*

1. Introduction

Propionibacterium acnes (*P. acnes*) is one of the major organisms isolated from the surface of skin [1]. Adolescence and its accompanying seborrhea are associated with a significant increase in *P. acnes* [2,3]. Increased sebum production and duc-

tal hypercornification induce the proliferation of *P. acnes* in the sebaceous glands, resulting in acne vulgaris [4–7]. Histologically, the dermal infiltrates in acne vulgaris are mainly composed of lymphocytes and monocytes. When the sebaceous duct is ruptured, massive neutrophilic infiltration forms an abscess. In some cases, subsequent granulomatous reaction and perifollicular fibrosis cause the development of acne rosacea [8]. Even early acne lesions can reveal polymorphonuclear leukocyte accumulation at the periphery of pilosebaceous units, indicating that neutrophils can be

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found before the pilosebaceous unit is ruptured. It is known that *P. acnes* produces low molecular weight, neutrophil chemotactic factor(s) [9]. In addition, *P. acnes* has been shown activate complement via both the classic and the alternative pathways [10]. Therefore, once the pilosebaceous unit is ruptured, *P. acnes* activates complement vigorously, producing a neutrophil chemoattractant C5a [11]. The interaction between *P. acnes* and infiltrated monocytes and lymphocytes may also play an important role in the pathogenesis of inflammatory acne. *P. acnes* stimulates the production of the proinflammatory cytokines, interleukin-8 (IL-8) and tumor necrosis factor alfa by human monocytic THP-1 cells [12].

Interleukin-8 (IL-8) is a CXC-type chemokine that binds to the cellular seven transmembrane domain G protein-coupled receptors known as CXCR1 and CXCR2 [13,15]. IL-8 is a potent proinflammatory chemotactic factor that predominantly exerts its chemotactic effects on neutrophils [14–16]. In most cell types, transcriptional control of IL-8 expression appears to be the pivotal regulatory mechanism. NF-kB has been as the transcription factor that is involved in the positive regulation of the IL-8 genes [21–23]. In the treatment of acne, the beneficial effects of both systemic and topical antibiotics such as tetracycline and erythromycin are generally attributed to their suppressive effects on *P. acnes* [17]. However, there are some reports of these antibiotics having inhibitory effects on host inflammatory responses other than antibacterial activity [18–20].

This study was designed to determine whether heat-killed *P. acnes* can induce IL-8 production in human monocytic cells and whether a 14-member macrolide antibiotic, roxithromycin, can modulate *P. acnes*-induced IL-8 production. We found that *P. acnes* was able to directly up-regulate IL-8 production and IL-8 mRNA expression in human monocytic cells in a dose- and time-dependent manner through a mechanism requiring transcription factor NF-κB activation. We also found that *P. acnes*-induced IL-8 secretion and NF-κB activation was inhibited by roxithromycin.

2. Materials and methods

2.1. Reagents

Heat-killed *P. acnes* (heated at 95 °C for 5 min) and roxithromycin were supplied from Eisai Pharmaceutical Company (Tokyo, Japan). Roxithromycin was dissolved in sterile PBS that did not contain calcium or magnesium chloride. FITC-conjugated anti-CD49d, CD54, HLA-DR and HLA-ABC mAbs and FITC-conjugated isotype matched IgG were purchased from Immunotech (France). TNF-α was obtained from Sigma (St. Louis, MO).

2.2. Cells and cell culture

The human monocytic THP-1 cell line was obtained from ATCC (TIB 202). The THP-1 cells were cultured in RPMI 1640 containing 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). The THP-1 cells were incubated with various concentrations (0–200 μg/ml) of *P. acnes* in 24 -well plates at 1×10^6 cells per ml per well for 24 h–72 h.

2.3. Measurement of cytokine production

Concentrations of IL-8 in the culture supernatant were measured using ELISA kits (Genzyme, Mineapolis, MN) according to the manufacturer's instructions.

2.4. Flow cytometry analysis

After 24 h–72 h of treatment with or without 100 μg/ml of *P. acnes* and 25–50 μg/ml of roxithromycin, the THP-1 cells were stained with FITC-conjugated anti-CD49d, CD54, HLA-DR and HLA-ABC mAbs and isotype control IgG for 30 min at 4 °C. Cytofluorometric analysis was carried out using a FACS Calibur cytometer (Beckman Coulter, Epics. XL, USA). Data were analyzed using CELL QUEST analysis software (Becton Dickinson, USA).

2.5. RNA isolation and quantitative real- time PCR

After treatment of the cells with the indicated stimuli, total RNA of the cells was extracted with TRIZOL reagent (Life Technologies, Gaithersburg, MD) and was reverse-transcribed using random primers and Superscript II reverse transcriptase (Life Technologies). The obtained cDNA was amplified using a quantitative real-time PCR system with a LightCycler Primer Set for the detection of human IL-8 and β-actin (Roche Diagnostics, Mannheim, Germany). Then ten microliters of cDNA were subjected to PCR reaction in a reaction mixture with a total volume of 20 µl containing 2 µl of 10 X PCR buffer including SYBR Green I dye, Taq DNA polymerase, deoxynucleotide triphosphate, (Roche Diagnostics, Mannheim, Germany), 2 µl of each forward and reverse primer and 4 mM $MgCl_2$. The PCR process consisted of an initial denaturation step of 10 min at 95 °C followed by 35 cycles (for β-actin) or 40 cycles (for IL-8) of rapid heating to 95 °C and then immediate cooling to an annealing temperature of 68 °C, which was held for 10 s, and an elongation step of product for 16 s at 72 °C. Fluorescence was measured at the end of the elongation step. Reactions without cDNA were always included as a negative control. Melting curves were generated to obtain melting temperatures for primer-dimers and the specific PCR product. The relative amounts of PCR products were analyzed by LIGHTCYCLER 3.3 software.

2.6. Transient cell transfection and luciferase reporter gene assay

THP-1 cells were transiently transfected with 2 µg of the firefly luciferase reporter gene under the control of NF-κB responsible elements (kindly provided by Dr Keiichi Nakayama, Kyushu University) and 0.2 µg of Renilla luciferase expression vector driven by thymidine kinase promoter (Promega) by lipofectamine 2000 reagent (Life Technologies, Gaithersburg, MD). The transfected cells were then transferred to 6-well plates and incubated for 24 h at a density of

8×10^5 cells per ml. After 24 h, the cells were further cultured in the presence or absence of *P. acnes* and roxithromycin for 5 h. Stimulation with TNF-α (10 ng/ml) served as a positive control. Luciferase activities were determined using a Dual Luciferase Assay System (Promega) and a MiniLumat (Berthold, Germany) model LB9506 luminometer and were expressed as a ratio of NF-κB-dependent firefly luciferase activity divided by control thymidine kinase Renilla luciferase activity (relative luciferase unit). Results were confirmed by three independent transfections.

2.7. Statistics

The statistical significance of the data was determined by Student's t-test. A $P < 0.05$ was taken as significant.

3. Results

3.1. P. acnes induced IL-8 production in human monocytic THP-1 cells

We first carried out experiments to determine whether *P. acnes* can induce IL-8 production in the human monocytic THP-1 cells. The THP-1 cells were incubated with 50 µg/ml of heat-killed *P. acnes* for 24–72 h, and the amounts of IL-8 production in the supernatants were measured. As shown in Fig. 1A, *P. acnes* induced IL-8 production in a time-dependent manner. We then incubated THP-1 cells with 0–200 µg /ml of *P. acnes* for 48 h. *P. acnes* dose-dependently augmented the IL-8 production in the THP-1 cells (Fig. 1B). In our experiments, supernatant of heat-killed *P. acnes* did not induce the IL-8 production from THP-1 cells (data not shown). We next examined the surface expressions of CD11b, CD49d, CD54, HLA-DR, and HLA-ABC on THP-1 cells in the presence or absence of *P. acnes*. *P. acnes* did not affect the expression of these surface molecules of THP-1 cells (data not shown).

3.2. P. acnes increased the steady-state levels of IL-8 mRNA in human monocytic THP-1 cells

We used a quantitative real-time PCR assay to determine whether *P. acnes* increases the expression level of IL-8 mRNA in the THP-1 monocytic cells. As shown in Fig. 2, the expression levels of IL-8 mRNA were increased at 4 h–20 h after stimulation with *P. acnes* and reached a peak at 8 h. These data suggested that *P. acnes* up-regulated IL-8 production at the transcriptional level.
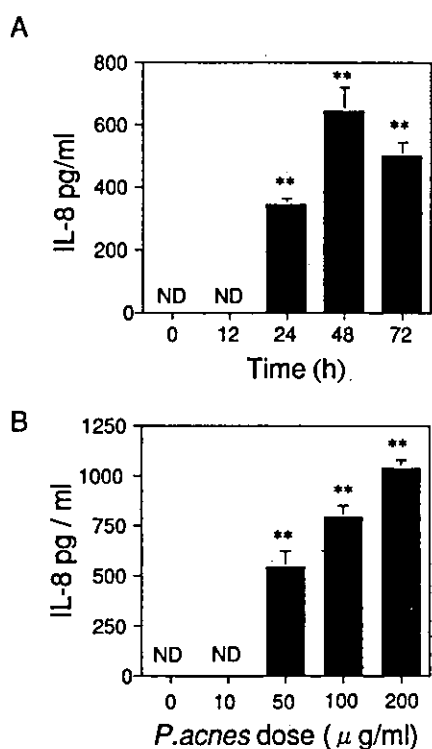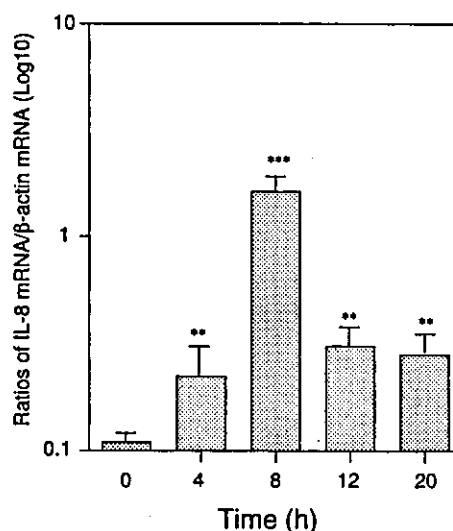

Fig. 2. Kinetics of IL-8 mRNA expression in THP-1 cells stimulated with *P. acnes*. Total RNA was extracted from THP-1 cells stimulated with *P. acnes* (100 µg/ml) for indicated periods of time, and a first-strand cDNA was synthesized from the RNA using reverse transcriptase. The expression level of IL-8 mRNA in cells was determined using quantitative real-time RT-PCR as described in Section 2. Data are shown as ratios of IL-8 mRNA to β-actin mRNA. Data are expressed as means ± S.D. **, $P < 0.01$, ***, $P < 0.001$ compared with controls. The results are representative of three separate experiments.

Fig. 1. *P. acnes*-induced IL-8 production by THP-1 cells. (A). Kinetics of IL-8 production by THP-1 cells. THP-1 cells (5×10^5) were incubated with *P. acnes* 50 µg/ml for indicated times, and then IL-8 concentrations in the culture supernatants were measured by ELISA. Data are expressed as means ± S.D. **, $P < 0.01$ compared with 0–12 h or without *P. acnes*. (B) Dose-dependent effect of *P. acnes* on IL-8 release. THP-1 cells (1×10^6) were stimulated with or without indicated dose of *P. acnes*, and the supernatants were harvested after 48 h for IL-8 measurement. Data are expressed as means ± S.D. **, $P < 0.01$ compared with controls. The results are representative of three separate experiments.

3.3. Roxithromycin decreased the IL-8 production of THP-1 cells stimulated with P. acnes

In order to investigate the effects of roxithromycin on the IL-8 production of THP-1 cells stimulated with *P. acnes*, THP-1 cells were incubated with various concentrations of roxithromycin in the presence or absence of *P. acnes*. Roxithromycin inhibited not only the IL-8 production but also the IL-8 mRNA expression of *P. acnes*-stimulated THP-1 cells in a concentration-dependent fashion (Fig. 3A and B). A trypan blue dye exclusion test showed that this inhibitory effect was not due to the cytotoxicity of roxithromycin (data not shown).

3.4. P. acnes induced activation of NF-κB in the THP-1 cells and its partial modulation by roxithromycin

NF-κB, a potent transcriptional activator, has been reported to be the main regulator of in

ducible expression of the IL-8 gene [17–19]. We, therefore, carried out experiments to determin whether *P. acnes* induces the activation of NF-κB in THP-1 cells and whether roxithromycin modulates its activation. The THP-1 cells were transfected with a luciferase reporter construct bearing a NF-κB site as described in Section 2. As shown in Fig. 4, we found that *P. acnes* actually induced

A

P.acnes (μ g/ml)	0	100	100	100	100
RXM (μ g/ml)	0	0	10	25	50

B

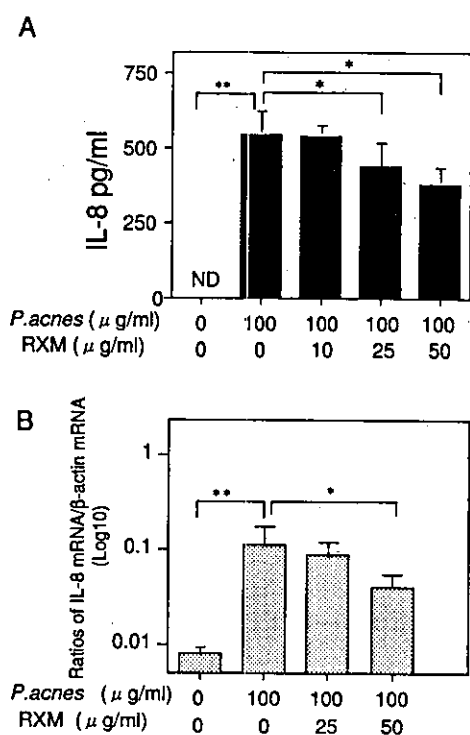| P.acnes (μ g/ml)	0	100	100	100
RXM (μ g/ml)	0	0	25	50

Fig. 3. Inhibition by Roxithromycin of IL-8 production in THP-1 cells stimulated with *P. acnes*. (A) Dose-dependent effect of roxithromycin (RXM) treatment on *P. acnes*-induced IL-8 release. THP-1 cells (1 × 10⁶) were stimulated with or without indicated dose of *P. acnes*, and the supernatants were harvested after 48 h for IL-8 measurement. Data are expressed as means ± S.D. *, $P < 0.05$ compared with controls. **, $P < 0.01$ compared with controls. (B) Roxithromycin inhibited the expression of IL-8 mRNA in THP-1 cells stimulated with *P. acnes*. Total RNA was extracted from THP-1 cells stimulated for 8 h with *P. acnes* in the presence or absence of roxithromycin, and then first-strand cDNA synthesis and expression of IL-8 mRNA were analyzed as described in the legend of Fig. 2. Data are shown as ratios of IL-8 mRNA to β-actin mRNA. Data are expressed as means ± S.D. *, $P < 0.05$ compared with controls. **, $P < 0.01$ compared with controls. The results are representative of three separate experiments.

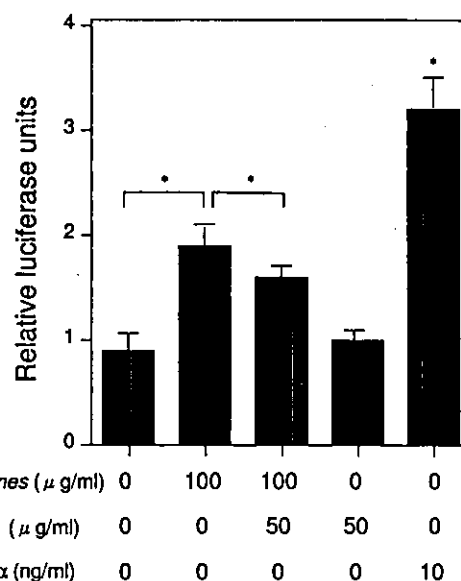| P. acnes (μ g/ml)	0	100	100	0	0
RXM (μ g/ml)	0	0	50	50	0
TNF-α (ng/ml)	0	0	0	0	10

Fig. 4. Effect of roxithromycin on *P. acnes*-induced NF-κB activity. THP-1 cells were transiently cotransfected with 2 μg of the firefly luciferase reporter gene under the control of NF-κB responsible elements and 0.2 μg of Renilla luciferase expression vector driven by thymidine kinase promoter by lipofectamine 2000 reagent as described in Section 2. After 24 h, cells were stimulated with 100 μg/ml *P. acnes* in the presence or absence of roxithromycin (RXM) for 5 h. As a control, THP-1 cells were stimulated with 10 ng/ml TNF-α. Luciferase activity is expressed as a ratio of NF-κB-dependent firefly luciferase activity divided by control thymidine kinase *Renilla* luciferase activity (relative luciferase unite). Data are expressed as means ± S.D. *, $P < 0.05$ compared with controls. Results were confirmed by three independent experiments.

the activation of NF-κB in THP-1 cells within 5 h after the stimulation. Activation of NF-κB was also observed after the stimulation with TNF-α as a positive control. In addition, roxithromycin partially inhibited the activation of NF-κB in *P. acnes*-stimulated THP-1 cells.

4. Discussion

In the present study, we first demonstrated that *P. acnes* induced IL-8 production and IL-8 mRNA accumulation in human monocytic THP-1 cells in a dose- and time-dependent manner. The enhancing effect of *P. acnes* stimulation on IL-8

synthesis is thought to be a relatively specific phenomenon because *P. acnes* did not affect the surface expression of CD11b, CD49d, CD54, HLA-DR, or HLA-ABC on the THP-1 cells. IL-8, a potent neutrophilic chemotic factor, is known to be released by various types of cells, such as monocytes, macrophages and epithelial cells [24–25]. Chemokines are the main proinflammatory mediators induced in monocytes by bacteria [26]. Interestingly, the IL-8 gene is the gene most highly induced by all bacterial stimulants out of 600 genes studied [26]. Thus, IL-8 seems to be a key proinflammatory mediator in innate immunity against bacteria by inducing a profound neutrophil chemoattraction. Mukaida et al. demonstrated that transcription of the IL-8 gene requires the coordinated activation of transcription factors, while NF-κB is the most crucial factor for IL-8 gene transcription [21–23]. The main cell wall components that stimulate monocytes in Gram-positive and Gram-negative bacteria are peptidoglycan and lipopolysaccharide, respectively, [26,27,29]. It has recently been demonstrated that both cell wall-attached and -free peptidoglycan and lipopolysaccharide bind to CD14 and specific Toll-like receptors of monocytes, resulting in activation of the transcription factor NF-κB and subsequent production of IL-8 [29]. It was also demonstrated in the present study that *P. acnes* actually induced the activation of NF-κB in human monocytes.

Several investigators have shown that roxithromycin inhibits IL-8 production by human bronchial epithelial cells [28]. Long-term administration of roxithromycin has been reported to be effective for the treatment of chronic airway diseases such as chronic sinusitis and diffuse panbronchiolitis [30]. Suzuki et al. showed that long-term administration of roxithromycin reduced the concentration of IL-8 in nasal discharge and the number of neutrophils in nasal smear in vivo as well as simultaneously improving paranasal sinus aeration [30–31]. Since large amounts of roxithromycin has been shown to accumulate in phagocytic cells and neutrophils [32–33], long-term therapy with a low-dose of

roxithromycin may have a potent in anti-inflammatory effect through inhibition of IL-8 production. In the present study, we demonstrated that roxithromycin partially inhibited IL-8 production, IL-8 mRNA accumulation and NF-κB activation in THP-1 cells stimulated with *P. acnes*. Although it is not known whether other macrolide antibiotics have effects similar to those of roxithromycin, these in vitro and in vivo findings should be useful for clinical trials of macrolides in the treatment of various disorders, such as acne rosacea and pyoderma, in which the activation of monocytes and neutrophils seems to be involved in the pathomechanisms.

Acknowledgements

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ELSEVIER

Differential regulation of thymus- and activation-regulated chemokine induced by IL-4, IL-13, TNF-α and IFN-γ in human keratinocyte and fibroblast

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Abstract

The CC chemokine thymus- and activation-regulated chemokine (TARC/CCL17) acts on CC chemokine receptor 4 (CCR4), which is known to be selectively expressed in Th2 cells. In order to compare the regulatory profiles of TARC production by tumor necrosis factor-α (TNF-α), IFN-γ, interleukin-4 (IL-4) and IL-13 in keratinocytes and fibroblasts, HaCaT cells, a human keratinocyte cell line, and NG1RGB cells, a human skin fibroblast cell line, were used. The expression of TARC protein was measured using enzyme-linked immunosorbent assay (ELISA), and the mRNA level was detected by reverse transcriptase polymerase chain reaction (RT-PCR). The spontaneous expression of TARC protein and mRNA levels were augmented by TNF-α and IFN-γ and were inhibited by IL-4 and IL-13 in the keratinocytes. The fibroblasts expressed the TARC protein and mRNA only in the presence of IL-4+TNF-α or IL-13+TNF-α stimulation. IFN-γ further enhanced the IL-4+TNF-α or IL-13+TNF-α-induced TARC production in the fibroblasts. Thus, TNF-α and IFN-γ -induced TARC production was differentially regulated by IL-4 and IL-13 in human keratinocytes and fibroblasts. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Keratinocytes; Fibroblasts; Thymus-and activation-regulated chemokine; TNF-α; IFN-γ; IL-4; IL-13

1. Introduction

Chemokines, a family of low-molecular-weight proteins that induce specific types of leukocyte chemotaxis, play essential roles in regulating extravasation and tissue accumulation of lymphocytes during immune and inflammatory responses [1,2]. Thymus-and activation-regulated chemokine (TARC/CCL17) was identified by cloning the

D3A gene from peripheral blood mononuclear cells (PBMCs) after stimulation with PHA [3]. TARC is a basic protein with a predicted molecular weight of 8 kDa, and is produced by dendritic cells [3–5], keratinocytes [6,7] and bronchial epithelial cells [8]. TARC acts as a specific ligand for CC chemokine receptor 4 (CCR4), which is predominantly expressed on Th2-type CD4+T cells [2,3]. Thus, TARC selectively chemoattracts Th2 cells into inflammatory sites. In a murine study, a monoclonal antibody against TARC inhibited Th2-mediated bacteria-induced fulminant hepatic failure [9].

Predominant infiltration of Th2 cells has been found in allergic disorders such as atopic dermatitis and asthma [10–12]. TARC has been shown to be overproduced in keratinocytes of NC/Nga mice exhibiting atopic dermatitis-like lesions [4]. Lymphocytes that infiltrate acute or early lesional skin of atopic dermatitis are mainly Th2 cells, which produce interleukin-4 (IL-4), IL-5, IL-10 and IL-13. However, it has been reported that Th1 cells, which produce IFN-γ, increase in number in chronic lesions [13,14]. Keratinocytes also secrete various kinds of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) [15]. Thus, it would be interesting to know how these cytokines regulate the production of TARC. In this study, we examined the production of TARC in keratinocytes and fibroblasts in the presence of IL-4, IL-13 or TNF-α, IFN-γ, and we found that IL-4 and IL-13 reciprocally regulated the production of TARC in keratinocytes and in fibroblasts.

2. Materials and methods

2.1. Reagents

Dulbecco's modified eagle's medium (DMEM), penicillin–streptomycin and trypsin–EDTA were purchased from Gibco-BRL (Grand Island, NY). RITC 80-7 medium was purchased from IWAKI Glass (Chiba, Japan). Fetal bovine serum (FBS) was obtained from HyClone (Logan, USA). Recombinant human IL-4 and IL-13 were purchased from PeproTech (Rocky Hill, NJ). Recombinant human TNF-α and IFN-γ, and agarose were

purchased from Sigma (Life Science Research, USA). Human TARC was obtained from R&D Systems (Minneapolis, MN). An RNA isolation reagent, Isogen, and ethidium bromide were purchased from Nippon Gene (Tokyo, Japan). Superscript™ II RNase reverse transcriptase was purchased from Gibco-BRL.

2.2. Cell culture

HaCaT cells, a spontaneously immortalized, nontumorigenic human skin keratinocyte cell line (kindly provided by Dr N.E. Fusenig, DKFZ Heidelberg), and NG1RGB cells, a normal human skin fibroblast cell line (kindly provided by Dr H. Nagamune, Tokushima University, Japan), were used for the experiments. HaCaT cells were maintained in DMEM with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml). NG1RGB cells were maintained in RITC 80-7 with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml). HaCaT cells (4×10^5 cells per well) were plated into 24-well plates, and NG1RGB cells (2×10^6 cells per well) were plated into six-well plates, respectively. They were then cultured for 24 h. Before stimulation, all of the culture medium in each well was replaced by identical formation containing 0.1% BSA in place of FBS. The cells were treated with the medium only or with various concentrations (0.1–100 ng/ml) of TNF-α, IFN-γ, IL-4 and IL-13. After incubation for the indicated periods of time, the supernatant was collected from each culture dish and centrifuged at 15 000 rpm for 1 min and then stored at −80 °C until further analysis.

2.3. TARC enzyme-linked immunosorbent assay (ELISA)

Concentrations of TARC in the supernatant were measured with an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's instructions using an ELISA reader, Labsystems Multiskan MS (DAI-NIPPON, Tokyo). This ELISA method enabled detection of TARC concentrations of greater than 15.6 pg/ml.

2.4. Detection of TARC by reverse transcriptase polymerase chain reaction (RT-PCR)

Human keratinocyte HaCaT cells and fibroblast NG1RGB cells were cultured and stimulated as described above, and then the cells were washed with PBS and total RNA was extracted. Extracted RNA was subjected to reverse transcription using a superscript™ II RNase reverse transcriptase kit. Transcripts of the constitutively expressed gene for β-actin served as control in each sample. The sequences of the PCR primers were as follows: TARC sense, 5'-ATGGCCCCACTGAAGAT-GCT-3'; TARC antisense, 5'-TGAACAC-CAACGGTGGAGGT-3'; and β-actin sense, 5'-CCTCGCCTTTGCCGATCCGC-3'; β-actin antisense, 5'-AGGTAGTCAGTCAGGTCCCG-3'. These primers yielded PCR products of the expected sizes of 332 bp for TARC mRNA and 598 bp for β-actin mRNA. The initial stage of the PCR protocol consisted of denaturation at 94 °C for 7 min, annealing at 58 °C for 2 min and elongation at 72 °C for 3 min. The second stage, followed by 34 cycles of TARC cDNA amplification, consisted of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and elongation at 72 °C for 2 min. For the amplification of β-actin cDNA, the cycles included one step of denaturation at 94 °C for 7 min, annealing at 56 °C for 2 min and elongation at 72 °C for 3 min, and then 29–34 cycles of the second step by the same procedure as that of the second stage of TARC cDNA PCR. The results were visualized on a 2% agarose gel, which was then stained with ethidium bromide (1 μg/ml) and examined with Print-graphFX (ATTO, Tokyo) consisting of a charge-coupled device camera, an ultraviolet transillumi-nator, and an analysis program.

2.5. Statistical analysis

Data are expressed as means ±S.E.M. Statistical significance of differences was analyzed using an analysis of variance (ANOVA) method ANOVA. A P-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Production of TARC protein and mRNA expression were augmented by TNF-α and IFN-γ but were inhibited by IL-4 and IL-13 in human keratinocytes

We first studied the effects of TNF-α, IFN-γ, IL-4 and IL-13 on the production of TARC by human keratinocytes. HaCaT cells were cultured with medium only or with 0.1–100 ng/ml of TNF-α, IFN-γ, IL-4 and IL-13 for 72 h, and the concentrations of TARC in the supernatants were quantified by ELISA. TNF-α (Fig. 1A) and IFN-γ Fig. 1B) significantly augmented the spon-taneous production of TARC from HaCaT cells in a dose-dependent manner. In contrast, IL-4 (Fig. 1C) and IL-13 (Fig. 1D) dose-dependently inhib-ited the TARC production. Time-course experi-ments showed that the up- or down-regulatory effects of these cytokines were time-dependent for 72 h (Fig. 2). To confirm the results, we next examined the expression levels of TARC mRNA in HaCaT cells incubated with medium only, IL-4, IL-13, TNF-α or IFN-γ for 24 h. In accordance with the protein levels, the TARC mRNA expres-sion was enhanced by TNF-α and IFN-γ and inhibited by IL-4 and IL-13 (Fig. 3). In each experiment, the inhibitory effects of IL-4 and IL-13 on TARC production by keratinocytes were similar, but the enhancing effect of IFN-γ was stronger than that of TNF-α.

In order to determine whether similar regulatory mechanisms operate in fibroblasts, we examined the TARC production by NG1RGB cells, a hu-man fibroblast cell line. However, NG1RGB cells did not produce detectable amounts of TARC even in the presence of TNF-α, IFN-γ, IL-4 and IL-13 (Table 1).

3.2. Reciprocal regulation by IL-4 and IL-13 of TARC production by keratinocytes and fibloblasts in the presence of TNF-α

To further elucidate the regulatory effects, we next examined the co-stimulatory response of TARC production in the keratinocytes and the fibroblasts under various combinations of these
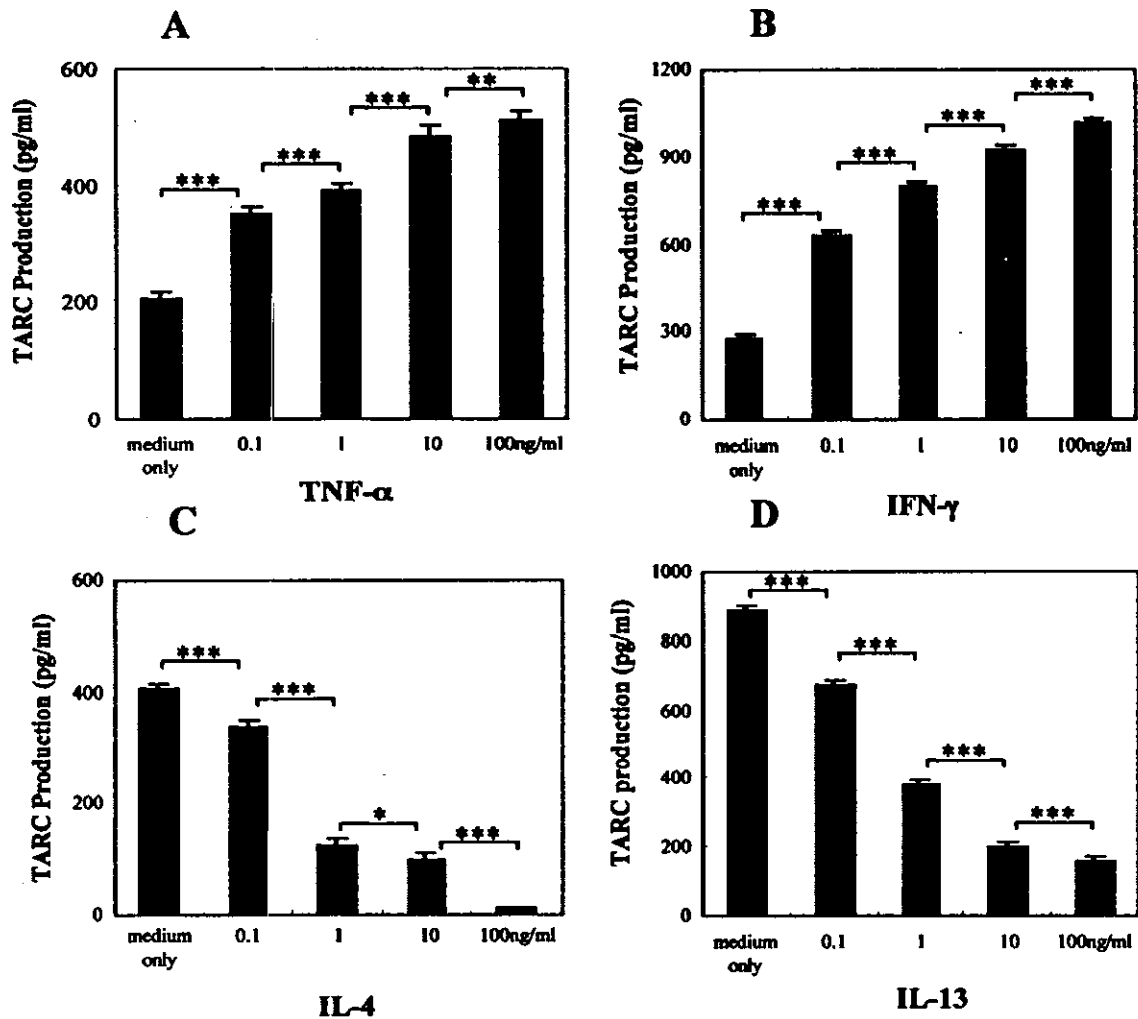
Fig. 1. Production of TARC by HaCaT keratinocytes stimulated with various concentrations of TNF-α (0–100 ng/ml), IFN-γ (0–100 ng/ml), IL-4 (0–100 ng/ml) or IL-13 (0–100 ng/ml). HaCaT cells were cultured for 72 h with the indicated concentrations of cytokines. Data are presented as means±S.E.M. Representative data are shown ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

four cytokines. The augmenting effects of TNF-α and IFN-γ were neutralized by IL-4 and IL-13 in the keratinocytes. IFN-γ did not further enhance the up-regulatory effect of TNF-α in the keratinocytes (Table 1).

In sharp contrast, IL-4, IL-13, TNF-α and IFN-γ did not induce the TARC production in the fibroblasts. The significant production of TARC was observed only in the dual presence of IL-4 and TNF-α or IL-13 and TNF-α (Table 1). Interest-

ingly, IFN-γ synergistically enhanced the TARC production induced by TNF-α+IL-4 or TNF-α+IL-13 in the fibroblasts (Table 1).

To confirm the effects of TNF-α+IL-4 or TNF-α+IL-13 on TARC production by the fibroblasts, we next examined the mRNA levels of TARC in the fibroblasts stimulated with various combinations of cytokines. As shown in Fig. 4, TNF-α induced detectable mRNA accumulation for TARC only in the presence of IL-4 or IL-13. In
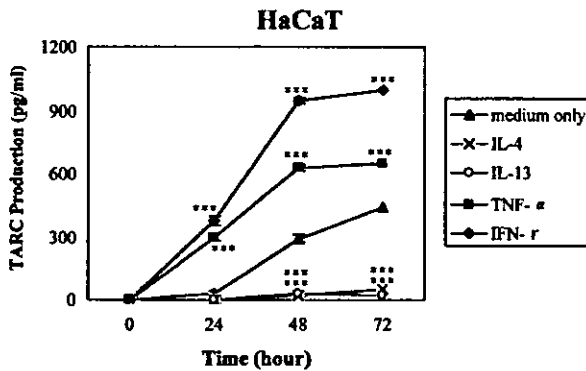
Fig. 2. Time courses of TARC production from HaCaT keratinocytes sitmulated with TNF-α (50 ng/ml), IFN-γ (100 ng/ml), IL-4 (10 ng/ml) or IL-13 (50 ng/ml) for 24, 48 or 72 h. Data are presented as means ± S.E.M. Representative data are shown ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with representative medium only.
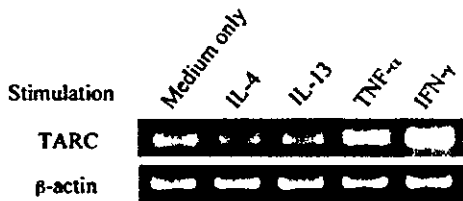

Fig. 3. Comparison of TARC mRNA levels by HaCaT keratinocytes incubated with IL-4 (10 ng/ml), IL-13 (50 ng/ml), TNF-α (50 ng/ml) or IFN-γ (100 ng/ml) for 24 h. Representative data of two independent experiments with similar results are shown.

addition, IFN-γ further enhanced the accumulation of TARC mRNA induced by TNF-α+IL-4 or TNF-α+IL-13 stimulation in the fibroblasts.

4. Discussion

We examined the production of TARC by keratinocytes and fibroblasts, two major cell populations of skin, in a mixture of proinflammatory (TNF-α), Th1 (IFN-γ) and Th2 (IL-4 and IL-13) cytokines. In keratinocytes, the synthesis of TARC was strongly enhanced by TNF-α and IFN-γ but was inhibited by IL-4 and IL-13 both in protein and in mRNA levels. In contrast, IL-4 and IL-13 induced the production of TARC protein and mRNA by fibroblasts in the presence

Table 1
Levels of TARC protein expression by human kerationcyte HaCaT cells and fibroblast NG1RGB cells

Stimulation	TARC Production (pg/ml)	
	HaCaT cells	NG1RGB cells
Medium only	291.45	0
IL-4	25.05[a]	0
IL-13	27.10[a]	0
TNF-α	628.02[a]	0
IFN-γ	946.54[a]	0
TNF-α+IFN-γ	865.19[a]	0
IL-4+TNF-α	154.06[b]	286.22[b]
IL-13+TNF-α	128.56[b]	246.45[b]
IL-4+IFN-γ	377.83[c]	0
IL-13+IFN-γ	442.24[c]	0
IL-4+TNF-α+IFN-γ	682.20[d]	797.75[d]
IL-13+TNF-α+IFN-γ	625.60[d]	755.28[d]

HaCaT cells and NG1RGB cells were incubated for 48 h with IL-4 (10 ng/ml), IL-13 (50 ng/ml), TNF-α (50 ng/ml), IFN-γ (100 ng/ml) or various combinations of these cytokines. Representative data are shown ($n = 3$). [a], $P < 0.001$ in comparison with Medium only; [b], $P < 0.001$ with TNF-α; [c], $P < 0.001$ with IFN-γ; [d], $P < 0.001$ with TNF-α+IFN-γ.

of TNF-α. IFN-γ per se did not stimulate the fibroblasts to synthesize TARC protein but it synergistically augmented the IL-4 +TNF-α- or IL-13+TNF-α-induced TARC synthesis in the fibroblasts.

Various types of inflammatory cells and their cyto-chemokines play important roles in the pathomechanisms of atopic dermatitis. Many investigators have demonstrated a predominant emergence of Th2 cells both in peripheral blood and in skin lesions of patients with atopic dermatitis [16–18]. The infiltration of Th2 cells in patients with atopic dermatitis is clearly demonstrable in vivo, particularly in atopic patch test sites, with allergens. Yamada et al. reported that infiltration of IL-4-producing lymphoid cells was observed in the perivascular area of the dermis even at 2 h after application of mite allergen [19]. However, in later stages of the disease, Th1-type IFN-γ ?is produced in the lesions of atopic dermatitis [13,14]. TNF-α has been reported to be up-regulated in mast cells in the lesional skin of atopic dermatitis [20]. In the present study, we demonstrated that both human skin keratinocytes and fibroblasts are potent producers of TARC in a
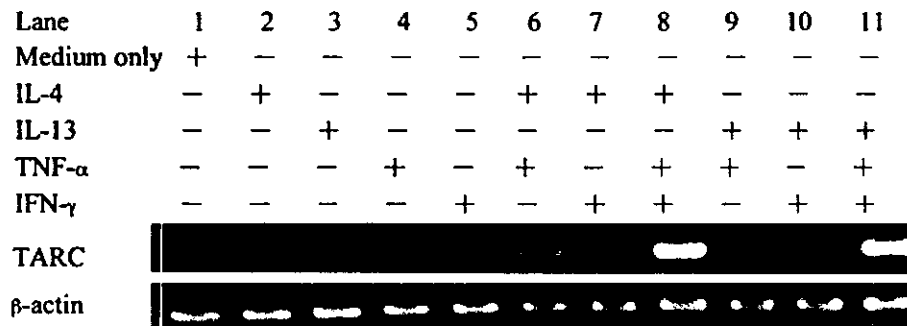
Fig. 4. Levels of TARC mRNA expression by human fibroblast NG1RGB cells incubated for 24 h with IL-4 (10 ng/ml), IL-13 (50 ng/ml), TNF-α (50 ng/ml), IFN-γ (100 ng/ml) or various combinations of these cytokines. Representative data of two independent experiments with similar results are shown.

certain combination with TNF-α, IFN-γ, IL-4 and IL-13. Vestergaard et al. reported that TARC was highly expressed in the basal epidermis of lesional skin of NC/Nga atopic dermatitis model mice [6]. Kakinuma et al. recently reported serum TARC levels of patients with atopic dermatitis were significantly higher than those of healthy control subjects and patients with psoriasis. The serum TARC levels were significantly correlated with clinical severity scores and with levels of eosinophils and serum soluble E-selectin. Kakinuma et al. also immunohistologically detected the expression of TARC in the lesional epidermis of atopic dermatitis [21].

Since TARC is a specific chemoattractant for Th2 cells, it would be interesting to know the differential regulation of TARC synthesis by keratinocytes and fibroblasts. PAM 212 cells, a murine keratinocyte cell line, produced TARC after stimulation with TNF-α or IFN-γ [6]. In the previous study, IL-10 greatly augmented the TNF-α+IFN-γ-induced TARC production by human keratinocyte HaCaT cells [22]. An obvious difference between keratinocytes and fibroblasts is their responses to IL-4 and IL-13. In our experiments, IL-4 and IL-13 inhibited the production of TARC by HaCaT cells but synergistically induced the de novo synthesis of TARC by NG1RGB fibroblasts only in the presence of TNF-α. Human corneal fibroblasts have also been shown to produce TARC only in the presence of both IL-4

and TNF-α [23]. These results indicate (1) that fibroblasts require dual stimulation with IL-4 and TNF-α for the production of TARC, (2) that TNF-α and IFN-γ act as enhancers of TARC production by keratinocytes and fibroblasts and (3) that IL-4 and IL-13 act as inhibitors of TARC production by keratinocytes but as enhancers of TARC production by fibroblasts. Sekiya et al. have recently reported the regulation of TARC production by bronchial epithelial cells. Two bronchial cell lines, A549 and BEAS-2B, reacted to produce TARC protein by dual stimulation with TNF-α+IL-4 [8]. Moreover, IFN-γ synergistically up-regulated the TNF-α+IL-4-induced TARC production, manifesting a similar regulatory pattern to that observed in the NG1RGB skin fibroblasts in the present study.

Campbell et al. reported that CCR4 and TARC were important in the recognition of skin vasculature by circulating T cells and in directing lymphocytes that were involved in systemic immunity to their target tissues [24]. It is known that the much higher level of mononuclear cell infiltration is observed in the dermis than in the epidermis of a chronic lesion of atopic dermatitis [25].

Although the precise molecular mechanisms remain unclear, the differential regulation by IL-4 and IL-13 in the TARC production by keratinocytes and fibroblasts is a very interesting evidence.

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