Tetracyclines Modulate Protease-Activated Receptor 2-Mediated Proinflammatory Reactions in Epidermal Keratinocytes[∇]

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In addition to their antibiotic effects, tetracyclines have anti-inflammatory action that is often beneficial in the control of inflammatory skin disorders. In this study, we examined the effects of tetracycline (TET) and two of its derivatives, doxycycline (DOX) and minocycline (MIN), on the production of interleukin-8 (IL-8) elicited by the activation of protease-activated receptor 2 (PAR2) in normal human epidermal keratinocytes (NHEK). In NHEK, the production of IL-8 stimulated by an agonist peptide of PAR2, SLIGKV-NH₂, at 100 μ M was significantly reduced by TET, DOX, or MIN at 5 and 10 μ M, concentrations that are noncytotoxic. The tumor necrosis factor alpha (TNF- α)-induced production of IL-8 was synergistically augmented by SLIGKV-NH₂, and that synergistic increase in the production of IL-8 was suppressed by 100 nM PAR2-specific small interfering RNA. It was also suppressed by TET, DOX, or MIN but not by the 14-membered-ring macrolide antibiotics erythromycin, roxithromycin, and clarithromycin, which also have anti-inflammatory activities, at 10 μ M. These results suggest that tetracyclines attenuate the PAR2–IL-8 axis in keratinocytes and thereby effectively modulate proinflammatory responses in the skin.

Tetracyclines are broad-spectrum antibiotics with a tetracyclic naphthacene carboxamide ring (18). In addition to their activities as antibiotics, tetracyclines show a variety of biological actions: anti-inflammatory activity, antiapoptosis activity, inhibition of proteolysis, and suppression of angiogenesis and tumor metastasis (21). Clinically, tetracyclines have been used to treat rheumatoid arthritis and various skin disorders, such as inflammatory acne, rosacea, bullous dermatoses, and neutrophilic dermatoses (12, 21). The anti-inflammatory activities of tetracyclines include the modulation of lymphocyte activation and neutrophil chemotaxis (6, 30), possibly by inhibiting matrix metalloproteinases (20), phospholipase A₂ (19), nitric oxide synthases (2), and/or caspase 1 (33). The ability of tetracyclines to bind Ca²⁺ and Mg²⁺ may account for some of those biological activities via the chelation of those cations and their transport into intracellular compartments (34). However, the exact molecular mechanism(s) of the immunomodulatory activities of tetracyclines in inflammatory skin disorders has not been fully delineated.

In response to various external stimuli and/or to endogenous proinflammatory cytokines, epidermal keratinocytes of the skin release chemokines such as interleukin-8 (IL-8), which recruit neutrophils and lymphocytes into the skin and exacerbate lesional inflammation (14). During inflammation of the skin, protease-activated receptor 2 (PAR2) (16) in keratinocytes is activated by serine proteases, such as leukocyte elastase (15) and mast cell tryptase (27), released from infiltrating immune cells. PAR2 activation amplifies the inflammation via the upregulation of intercellular cell adhesion molecule 1 (ICAM-1) expression (5) and the release of IL-8 from kerati-

nocytes (Fig. 1). Thus, the activation of PAR2 and/or its signaling pathways in the epidermis plays a pivotal role in inflammatory processes and their amplification in the skin.

We have recently shown that 14-membered-ring macrolides, which have immunomodulatory activities, suppress the IL-1 β -induced production of IL-8 that is synergized by the activation of PAR2, and we proposed the PAR2–IL-8 axis as a therapeutic target for the control of cutaneous inflammation (32). The aim of the present study was to determine whether tetracyclines, which have anti-inflammatory activities, also modulate the PAR2-mediated proinflammatory responses of keratinocytes.

MATERIALS AND METHODS

Materials. SLIGKV-NH₂ and its reverse peptide, VKGILS-NH₂, were from Bachem Inc. (Torrance, CA) and Sigma-Aldrich Corp. (St. Louis, MO), respectively. IL-1 β , tumor necrosis factor alpha (TNF- α), and gamma interferon (IFN- γ) were from Peprotech EC Ltd. (London, United Kingdom). Tetracycline HCl was purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and minocycline HCl and doxycycline HCl were from Sigma-Aldrich Corp.

Cell culture. Normal human epidermal keratinocytes (NHEK) (Cambrex Bio-Science Inc., Baltimore, MD) were seeded into T75 flasks (Corning Inc./Life Sciences, Acton, MA) at a density of 2×10^5 cells per flask in 10 ml KGM2 medium (Kurabo Industries Ltd., Osaka, Japan). The cells were incubated at 37°C under an atmosphere of 5% CO₂ in 95% air. When the cells became 75% confluent, they were trypsinized and subcultured into 12-well plates at an initial seeding density of 1×10^4 cells per cm².

Transient transfection and knockdown of PAR2. NHEK seeded into 12-well plates were incubated for 72 h, after which they were transfected with a PAR2-specific small interfering RNA (siRNA) (Qiagen, Inc., Hilden, Germany) by using the TransIT TKO transfection reagent (Mirus Bio Corp., Madison, WI) according to the manufacturer's instructions. After 24 h of incubation, the cells were treated with a cytokine and/or an agonist peptide, and 24 h later, the cells were harvested for RNA isolation.

qRT-PCR. Total RNAs were isolated from NHEK using the TRI reagent (Sigma-Aldrich Corp.), and cDNAs were synthesized using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). An ABI 7900HT sequence detection system (Applied Biosystems) was used for quantitative real-time PCR (qRT-PCR). An mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard for qRT-PCR. The product numbers of the primers and probes for PAR2 and GAPDH, which were

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FIG. 1. Activation and function of PAR2 in keratinocytes. Serine proteases cleave the N-terminal arm of PAR2, and the exposed new N-terminal peptide can activate the receptor. The activation of PAR2 accelerates the release of IL-8 from the cells and increases the expression of ICAM-1 and the uptake of melanin from melanocytes but suppresses the secretion of lipid lamellae, which is essential for skin barrier homeostasis.

obtained from Applied Biosystems Assays-on-Demand, are Hs 00608346 m1 and Hs 99999905 m1, respectively. The relative abundance of each target transcript was assessed with regard to internal controls according to the manufacturer's instructions. Experiments were repeated at least three times, and results are expressed as mean levels of induction of PAR2 transcripts $\pm$ standard deviations ($n = 3$).

ELISA for IL-8. NHEK seeded into 12-well plates were incubated for 72 h, and the medium was changed to KGM2 without additives. Cells were preincubated for 24 h in the presence or absence of each antibiotic, after which the agonist peptide SLIGKV-NH₂ or its reverse peptide, VKGILS-NH₂, and/or IL-1 β (100 ng/ml) or TNF- α (100 ng/ml) was added. Forty-eight hours later, culture media were collected and assayed for IL-8 by using a Quantikine human IL-8 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., Minneapolis, MN). Experiments were repeated at least three times, and results are expressed as means $\pm$ standard deviations for triplicate samples.

Cell viability assay. Cell viability was assessed using a modified 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay kit (Cell Counting kit 8; Dojindo Molecular Technologies, Inc., Gaithersburg, MD), according to the manufacturer's instructions. NHEK seeded into 24-well plates were incubated for 72 h, and the medium was changed to KGM2 without additives. The cells were then preincubated for 24 h in the presence or absence of each antibiotic. Forty-eight hours later, the kit reagent WST-8 was added directly to the culture medium, and the culture was incubated for an additional 1 h, after which the absorbance of formazan was measured at 450 nm by spectrophotometry.

Statistical analysis of data. Data are expressed as means $\pm$ standard deviations. One-way analysis of variance followed by Scheffe's test or Student's two-tailed t test was performed for statistical analysis of data. A P value of <0.05 was considered statistically significant.

RESULTS

Effects of tetracyclines on IL-8 production stimulated by the PAR2 agonist peptide. Proteolytic cleavage of PAR2 by serine proteases exposes a new NH₂ terminus domain that serves as a tethered ligand for the receptor itself (16) (Fig. 1). A synthetic receptor-activating peptide, SLIGKV-NH₂, which has a sequence identical to that of the human PAR2-tethered ligand domain, mimics the actions of the serine proteases (9). When NHEK were treated with 100 μ M SLIGKV-NH₂ for 48 h, the concentration of IL-8 in the medium increased (Fig. 2). Prior to examination of the effects of tetracycline (TET), doxycycline (DOX), or minocycline (MIN) on the PAR2-mediated production of IL-8, the possible cytotoxic effects of these drugs on NHEK were assessed using a modified MTT assay. NHEK were treated with TET, DOX, or MIN for 72 h, after which the

formazan absorbance in the culture medium was measured. Cell viability was drastically decreased at 100 μ M TET, DOX, or MIN but not at concentrations of 10 μ M or lower (data not shown). Therefore, we used 10 μ M and lower concentrations of these tetracyclines for further experiments.

The effects of tetracyclines on the PAR2-mediated stimulation of IL-8 production were assessed in NHEK that had been preincubated with TET, DOX, or MIN for 24 h prior to the addition of 100 μ M SLIGKV-NH₂. TET at a concentration of 5 or 10 μ M slightly but significantly decreased the concentration of IL-8 in the medium of SLIGKV-NH₂-treated NHEK (Fig. 2A). DOX also significantly decreased the IL-8 levels at these concentrations (Fig. 2B). MIN at a concentration of 5 or 10 μ M reduced the SLIGKV-NH₂-induced increase in the level of IL-8 (Fig. 2C). When each tetracycline was added simultaneously with SLIGKV-NH₂, the effect was similar to, or a little less than, that in cells treated 24 h before the addition of the agonist peptide. When these drugs were added after the addition of SLIGKV-NH₂, none of them affected the production of IL-8 (data not shown).

Because the production of IL-8 stimulated by PAR2 activation is dependent on PAR2 gene expression (32), these tetracyclines might decrease the expression of the PAR2 gene. This possibility was assessed by qRT-PCR, but when NHEK were treated with 10 μ M TET, DOX, or MIN for 48 h, the expression of the PAR2 gene was not reduced (Fig. 2D).

Effect of the PAR2 agonist peptide on the TNF- α - or IFN- γ -induced production of IL-8. We examined whether the activation of PAR2 affects the IL-8-inducing capability of the proinflammatory cytokines TNF- α and IFN- γ . When NHEK were treated with 100 ng/ml TNF- α or 100 μ M SLIGKV-NH₂ for 48 h, the concentration of IL-8 in the medium increased (Fig. 3A). Moreover, combined treatment with 100 ng/ml TNF- α and 100 μ M SLIGKV-NH₂ caused a synergistic increase in IL-8 levels that was greater than the sum of the separate effects of TNF- α and SLIGKV-NH₂ at the same doses. However, in NHEK transfected with 100 nM PAR2-specific siRNA, the basal level of IL-8 decreased and, regardless of the presence or absence of SLIGKV-NH₂, the effect of TNF- α was significantly suppressed. When the reverse control peptide VKGILS-NH₂ was used in place of SLIGKV-NH₂, IL-8 production by NHEK was not increased. Furthermore, VKGILS-NH₂ induced no synergistic increase in IL-8 levels, even in the presence of 100 ng/ml TNF- α (data not shown).

When NHEK were treated with 100 ng/ml IFN- γ , IL-8 levels increased and were similar to levels in NHEK treated with 100 μ M SLIGKV-NH₂ (Fig. 3B). In the presence of both IFN- γ and SLIGKV-NH₂, IL-8 levels were increased, but no synergistic increase was observed. IFN- γ -induced increases in IL-8 levels were significantly reduced in NHEK transfected with the PAR2-specific siRNA.

Effects of tetracyclines on the synergistic stimulation of IL-8 production induced by IL-1 β or TNF- α with the PAR2 agonist peptide. We then assessed whether tetracyclines modulate the PAR2-mediated synergistic increase in IL-8 production by NHEK. TET, DOX, or MIN (at a concentration of 10 μ M) was added to NHEK 24 h before treatment with 100 μ M SLIGKV-NH₂ and/or 100 ng/ml IL-1 β or TNF- α . In NHEK treated with IL-1 β , 10 μ M TET did not show any significant effect on the concentration of IL-8 in the medium, even in the presence of

FIG. 2. Effects of tetracyclines on the SLIGKV-NH₂-induced production of IL-8 by NHEK and on the expression of the *PAR2* gene. (A to C) In the presence or absence of the indicated concentration of TET (A), DOX (B), or MIN (C), NHEK were treated with 100 μM SLIGKV-NH₂ for 48 h, after which the concentration of IL-8 in the culture medium was measured by ELISA. Open bars, control; filled bars, SLIGKV-NH₂-treated cells. Analysis of variance followed by Scheffé's test was performed for statistical analysis of data. *, $P < 0.001$; ***, $P < 0.05$. (D) Effect of TET, DOX, or MIN on the expression of the *PAR2* gene in NHEK. NHEK were incubated without any drug (open bars) or with 10 μM TET, DOX, or MIN (filled bars) for 48 h, and the levels of PAR2 transcripts were determined by qRT-PCR.

SLIGKV-NH₂ (Fig. 4A). In NHEK treated with TNF-α, the synergistic increase in IL-8 production elicited by SLIGKV-NH₂ was slightly but significantly suppressed by treatment with TET (Fig. 4A). DOX also significantly decreased the agonist peptide-stimulated production of IL-8 in IL-1β- or TNF-α-treated NHEK (Fig. 4B). MIN substantially reduced the synergistic increases in IL-8 levels, although the reduced levels were not as low as those in control cells treated with SLIGKV-NH₂ (data not shown), IL-1β, or TNF-α alone (Fig. 4C).

Effects of 14-membered-ring macrolides on the synergistic stimulation of IL-8 production by TNF-α and the PAR2 agonist peptide. The 14-membered-ring macrolides erythromycin (ERY), roxithromycin (RXM), and clarithromycin (CLR) attenuate the IL-1β-induced production of IL-8 in NHEK, which can be synergized by treatment with SLIGKV-NH₂ (32). Hence, we examined whether these macrolides would affect the production of IL-8 induced by the PAR2 agonist peptide and TNF-α. However, in contrast to the effects of these macrolides on IL-1β-treated NHEK, treatment with 10 μM ERY (Fig. 5A), RXM (Fig. 5B), or CLR (Fig. 5C) did not decrease the concentration of IL-8 in the medium of NHEK treated with both 100 μM SLIGKV-NH₂ and 100 ng/ml TNF-α.

DISCUSSION

PAR2 is a member of the unique G-protein-coupled receptor subfamily with seven transmembrane domains and is expressed abundantly in various organs, including the skin. Recent findings suggest that PAR2 is involved in various aspects of skin pathophysiology (Fig. 1), including melanin transfer (23), itch generation due to mast cell tryptase (27), and epidermal barrier homeostasis via the suppressed secretion of lipid lamellae from keratinocytes (8). PAR2 binding to G proteins has been shown in the epidermis, especially in the stratum granulosum (26) and in skin affected by the inflammatory process. The suppression of allergic and toxic contact dermatitis in PAR2 knockout mice suggests an important role for PAR2 in cutaneous inflammation (22). We have focused on the proinflammatory role of PAR2 in keratinocytes as a novel target for the treatment of cutaneous inflammation.

Tetracyclines are useful as options for treating a variety of inflammatory skin disorders and can be administered orally (21). In the present study, we show a novel biological activity of TET and two of its derivatives, MIN and DOX, that attenuates the PAR2-mediated production of IL-8 by keratinocytes. These tetracyclines decrease the production of IL-8 at concen-

FIG. 3. Effects of SLIGKV-NH₂ and a *PAR2*-specific siRNA on TNF- α - or IFN- γ -induced production of IL-8 by NHEK. NHEK were transfected with 100 nM *PAR2*-specific siRNA for 24 h as indicated and were then treated with 100 ng/ml TNF- α (A) or IFN- γ (B) with or without 100 μ M SLIGKV-NH₂. After 24 h of incubation, the concentration of IL-8 in the culture medium was measured by ELISA. Student's two-tailed *t* test was performed for statistical analysis of data. *, *P* < 0.001. Open bars, control; filled bars, cells transfected with the *PAR2*-specific siRNA.

trations of 10 μ M (Fig. 2A to C), which approach the therapeutic concentrations of those drugs in serum (1) and are not cytotoxic to keratinocytes in culture. The PAR2-mediated production of IL-8 depends on the expression of the *PAR2* gene (32), which is not reduced by the concentrations of tetracyclines used (Fig. 2D). Therefore, it is unlikely that the effects of these tetracyclines on the production of IL-8 are due to decreased expression of the *PAR2* gene.

Proinflammatory cytokines, matrix metalloproteinases, and antimicrobial peptides are upregulated in the inflammatory stage of acne (31), which can be effectively treated with tetracyclines. One reason for the favorable effects of tetracyclines on cutaneous inflammation may be a reduction of neutrophil chemotaxis (6, 7). The present study suggests that the therapeutic effects of tetracyclines may be due, at least in part, to modulation of the PAR2-mediated production of IL-8, which recruits neutrophils to the inflammatory lesions. Tetracyclines are also effective for the treatment of rosacea (24). In skin with rosacea, PAR2 is widely expressed by keratinocytes, in which endogenous proteases such as kallikrein-related peptidases that activate PAR2 are upregulated (25). Therefore, the effectiveness of tetracyclines for treating rosacea might also be PAR2 mediated, although alternative mechanisms involving angiogenesis have been postulated (21).

The proinflammatory cytokine IL-1 strongly induces the production of IL-8 by NHEK, and the effect of IL-1 is synergized by the stimulation of PAR2 (9, 10, 32). We show evidence that the activation of PAR2 markedly enhances the production of IL-8 induced by TNF- α as well as that induced

FIG. 4. Effects of tetracyclines on the synergistic production of IL-8 induced by SLIGKV-NH₂ in IL-1 β - or TNF- α -treated NHEK. In the presence or absence of 10 μ M TET (A), DOX (B), or MIN (C), NHEK were treated with 100 ng/ml IL-1 β or TNF- α and/or 100 μ M SLIGKV-NH₂ for 48 h. The concentration of IL-8 in the culture medium was then measured by ELISA. Open bars, control; filled bars, cells treated with TET, DOX, or MIN. Student's two-tailed *t* test was performed for statistical analysis of data. *, *P* < 0.001; **, *P* < 0.01; ns, not significant.

by IL-1 in NHEK, whereas the stimulation of PAR2 does not potentiate the effect of IFN- γ (Fig. 3). Thus, the signals from PAR2 interact with those from some cytokines to amplify their responses. Such a booster effect has been observed in keratinocytes when TNF- α is administered in combination with IFN- γ (3, 29), but it is not clear whether PAR2 uses the same signaling system as IFN- γ for the induction of IL-8 in keratinocytes.

We show in this study that tetracyclines suppress the PAR2-mediated synergistic enhancement of IL-8 production induced

FIG. 5. Effects of the 14-membered-ring macrolides ERY, RXM, and CLR on the synergistic production of IL-8 induced by SLIGKV-NH₂ in TNF- α -treated NHEK. In the presence or absence of 10 μ M ERY (A), RXM (B), or CLR (C), NHEK were treated with 100 ng/ml TNF- α and/or 100 μ M SLIGKV-NH₂ for 48 h. The concentration of IL-8 in the culture medium was then measured by ELISA. Open bars, control; filled bars, cells treated with ERY, RXM, or CLR. Student's two-tailed *t* test was performed for statistical analysis of data. ns, not significant.

by IL-1 and TNF- α in NHEK (Fig. 4). The anti-inflammatory effects of tetracyclines on skin disorders might be due, at least in part, to suppression of the synergized production of IL-8 by keratinocytes. The effect of MIN is greater than that of DOX; in contrast, TET is less effective (Fig. 4). The different efficacies among tetracyclines have also been reported with regard to other biological processes, such as nitric oxide synthesis (2) and inactivation of glial cells (35). MIN and DOX are more lipophilic than TET, and the lipid solubility of MIN is almost twice that of DOX (4, 11). The different lipophilicities of these drugs might affect their passage across the plasma membrane into cells. The activation of PAR2 transiently increases intracellular Ca²⁺ levels in keratinocytes, which trigger the downstream binding of NF- κ B to DNA (17, 25). The chelation of Ca²⁺ by MIN or DOX might suppress the downstream signal transduction for the enhanced production of IL-8 (18). The possibility that these tetracyclines bind SLIGKV-NH₂ or otherwise interfere with its binding to PAR2 has not been examined in the present study.

We have recently shown that 14-membered-ring macrolides, such as ERY and RXM, suppress the IL-1 β -induced production of IL-8 synergized by the activation of PAR2 (32). Interestingly, however, these macrolides do not affect TNF- α -induced synergism, as shown in this study (Fig. 5). This suggests that these macrolides modulate the synergistic production of IL-8 in different ways from MIN and DOX. Komine et al. have shown that RXM suppresses p38 phosphorylation and NF- κ B-driven transcription independently of the inhibition of I κ B degradation in HaCaT cells, a keratinocyte cell line (13). Takahashi et al. (28) have also reported that RXM reduces the transcriptional activities of both AP-1 and NF- κ B in NHEK. Whether MIN and DOX interfere with these particular signaling molecules and/or transcription factors in keratinocytes has not been determined, but common signaling systems might be affected by MIN and DOX and by these macrolides, at least in the PAR2-mediated synergistic production of IL-8 induced by IL-1 β .

During the course of cutaneous inflammation, keratinocytes release abundant amounts of IL-8 in response to proinflammatory cytokines such as IL-1 and TNF- α , which are amplified

by the activation of PAR2, possibly triggered by upregulated kallikrein-related peptidases from lesional keratinocytes (25) and/or by other serine proteases from infiltrating inflammatory cells. PAR2 in the epidermis plays a pivotal role as a sensor, the activation of which leads to the exacerbation of skin inflammation. Tetracycline derivatives exhibit a novel activity, distinct from their antibiotic effects, that modulates the PAR2-IL-8 axis and attenuates the proinflammatory process in epidermal keratinocytes. The modulatory effects of those tetracyclines might explain their effectiveness in treating various inflammatory skin disorders.

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