The Macrolide Antibiotic, Roxithromycin Suppresses IFN- γ -Mediated Immunological Functions of Cultured Normal Human Keratinocytes

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The effects of the macrolide antibiotic, roxithromycin (RXM) on immunological functions of cultured normal human keratinocytes (NHK) were studied. RXM neither modulated the expression of intercellular adhesion molecule-1 (ICAM-1) and human histocompatibility leukocyte antigen (HLA)-DR nor mobilized intracellular free calcium in NHK that were cultured in medium alone. However, the ICAM-1 and HLA-DR expression induced by cultivation with interferon- γ (IFN- γ) was upregulated and suppressed, respectively, by pretreatment of NHK with RXM. Whereas ICAM-1 upregulation was observed with RXM at a concentration as high as 0.1 mM, the inhibition of HLA-DR expression by RXM was virtually dose-dependent at doses ranging from 100 nM to 0.1 mM. Accessory cell function of major histocompatibility complex (MHC) class II-bearing NHK in terms of the T-cell response to staphylococcal enterotoxin B was suppressed by pretreatment of NHK with RXM, indicating the functional consequence of RXM-induced reduction of MHC class II molecules. Furthermore, RXM inhibited IFN- γ -mediated upregulation of IL-1 α secretion by NHK. These results show that RXM suppresses immunological functions of keratinocytes triggered by IFN- γ and suggest the therapeutic relevance of RXM to T cell-mediated inflammatory skin diseases and T cell malignancies such as atopic dermatitis, psoriasis and cutaneous T cell lymphoma, which can be exacerbated by keratinocytes immunologically modulated by exposure to IFN- γ .

Key words roxithromycin; keratinocyte; ICAM-1; HLA-DR; IFN- γ ; IL-1 α

One of the characteristic immunological features of macrolides is the anti-inflammatory action. Erythromycin reduces the motility and oxygen-radical production of polymorphonuclear leukocytes.^{1,2} FK 506³ and rapamycin⁴ inhibits T-cell activation. Similarly, roxithromycin, a newly synthesized macrolide antibiotic, inhibits the T-cell response to mitogens and interleukin-2 (IL-2) production by lymphoid cells^{5,6} explaining in part the clinical relief of symptoms of asthma and atopic allergy.^{7–11}

Not only T cells and Langerhans cells, the “professionals” of immune reactions, but also epidermal keratinocytes play a critical role in the mechanism of various cutaneous inflammatory diseases such as atopic dermatitis and psoriasis.^{12,13} For example, keratinocytes produce cytokines such as IL-1, -6 and -8, and express major histocompatibility complex (MHC) class II molecules and adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) and B7/BB-1 antigen.^{14,15} Changes in the production and expression of these molecules have been reported to modulate immune reactions.

In the present study, we examined the immunomodulatory effect of roxithromycin (RXM) on cultured normal human keratinocytes (NHK). Our results showed that RXM suppresses immunological events in IFN- γ -treated keratinocytes, including expression of HLA-DR, secretion of IL-1 α and the ability to present superantigen. These results suggest the potential therapeutic effectiveness of RXM in several cutaneous diseases, mediated at least partially *via* stimulation of T cells by immunologically active keratinocytes.

MATERIALS AND METHODS

Reagents and Antibodies RXM was supplied by Roussel-Uclaf, Paris, France; staphylococcal enterotoxin

B (SEB) was purchased from Toxin Technology Inc., Sarasota, FL; fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (mAb) 84H10 (anti-human ICAM-1, mouse IgG1) was from Immunotech, Marseille, France; epidermal growth factor and whole bovine pituitary extract was from Gibco BRL, Gaithersburg, MD; recombinant human IFN- γ was from Genzyme Corp., Boston, MA; and FITC-conjugated mouse IgG1 isotype control antibody, phycoerythrin (PE)-conjugated anti-human histocompatibility leukocyte antigen (HLA)-DR mAb (L243) and PE-conjugated mouse IgG2 isotype control antibody were from Becton Dickinson, Mountain View, CA.

Cells NHK, derived from foreskins (Epipack, Clonetics Corporation, San Diego, CA) were grown in serum-free, keratinocyte-growth medium (KC-SFM, Gibco BRL), supplemented with 10 ng/ml epidermal growth factor and 60 ng/ml whole bovine pituitary extract in a humidified incubator with 5% CO₂ in air at 37°C.

Peripheral blood mononuclear cells (PBMC) were isolated from the heparinized venous blood of a healthy volunteer by means of Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) density gradient centrifugation. T cells were isolated by incubating PBMC in a prewarmed nylon wool column (Wako Pure Chemical Industries Ltd., Osaka, Japan) and contained 3% >B cells and 1% > monocytes, confirmed by flow cytometric analysis as previously described.¹⁶

RXM at concentrations less than 0.25 mM had no detectable cytotoxic effect on keratinocytes during the 168 h-incubation period, as assessed by the trypan blue exclusion test.

Induction of ICAM-1 and HLA-DR NHK at 90% confluency in 6-well flat-bottomed culture plates (Nunc, Nunc, Denmark) were fed with 1 ml KC-SFM without epidermal growth factor or whole bovine pituitary extract

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to inhibit proliferation, treated with varying concentrations of RXM in the same medium for 48 h, and then incubated for an additional 48 h with fresh KC-SFM containing 200 U/ml IFN- γ and RXM at the same concentration as in the previous incubation.

Flow Cytometry Single cell suspensions were prepared by treatment of NHK with 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS: 10 mM sodium phosphate, 133 mM sodium chloride, pH 7.4). After washing in PBS, 10^5 cells were incubated with 5 μ l FITC-conjugated anti-ICAM-1 mAb and 5 μ l PE-conjugated anti-HLA-DR mAb or FITC- and PE-conjugated mouse isotype control antibodies for 30 min on ice. The stained cells were analyzed in a FACScan apparatus (Becton Dickinson). Mean fluorescence intensity was calculated on a log scale.

Measurement of Intracellular Calcium Concentration NHK on a coverglass were incubated with 20 mM Fura-2 acetoxymethylester (Dojin Laboratories, Kumamoto, Japan) in phenyl red-free keratinocyte growth medium (Clonetics Corporation) in the dark for 1 h at 37°C. Following thorough washing, 0.1 mM RXM was added to the cells in the same medium. Fluorescence intensity was measured using a digital image processing system, Argus-100/CA, designed by Hamamatsu Photonics Co., Ltd. (Hamamatsu, Japan).

Proliferation Assay for T-Cell Response to SEB in the Presence of IFN- γ -Treated NHK Subconfluent NHK cultured in 96-well flat-bottom microtiter plates (Corning) at 37°C under 5% CO₂ were incubated with 0.1 mM RXM in minimal essential medium (MEM; Nissui Co., Tokyo, Japan) for 48 h. Cells were fed with fresh medium containing 200 U/ml IFN- γ and 0.1 mM RXM for an additional 48 h. T cells purified using a nylon column (10^5 cells/well) were added to the treated NHK and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/l L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 units/ml penicillin, and 100 μ g/ml streptomycin with SEB at 1, 10 or 100 ng/ml and 1 μ g/ml of indomethacin (Sigma). Triplicate cultures (total 150 μ l/well) were maintained for 72 h. One μ Ci/well methyl tritiated thymidine (³H-TdR; Amersham Intl., Arlington, IL) was added 16 h before harvesting. Cells were collected on glass-fiber filters using an automated cell harvester (Cambridge Technologies, Watertown, MA) and ³H-TdR uptake was measured using a scintillation counter.

ELISA for IL-1 α in Culture Supernatants from Human Keratinocyte Semiconfluent NHK were incubated in 24-well plates (1 ml culture medium/well) for 48 h with or without 0.1 mM RXM in KC-SFM and then incubated with or without 200 U/ml IFN- γ for an additional 48 h. Commercially available ELISA kits (Otsuka Pharmaceutical Co., Tokushima, Japan) were used for measuring human IL-1 α in the supernatants. The data were expressed as the mean of triplicates.

Statistical Analysis Data from three independent experiments were analyzed using Student's *t*-test.

RESULTS

RXM Modulation of ICAM-1 and HLA-DR Expression on NHK Induced by IFN- γ Incubation of NHK with RXM up to 0.1 mM for 48 h did not induce either ICAM-1 or HLA-DR molecules on their surface (Fig. 1a,b). Next, cells were incubated with RXM for 48 h and then treated with IFN- γ for an additional 48 h. Although ICAM-1 expression was not altered by the pretreatment with RXM up to 0.01 mM, 0.1 mM RXM significantly upregulated IFN- γ -induced ICAM-1 expression (Fig. 1a). In contrast, IFN- γ -induced HLA-DR expression was suppressed by RXM at 100 nM to 0.1 mM in a dose-dependent manner (Fig. 1b).

Effects of RXM on Intracellular Calcium Concentration We examined the effect of RXM on the mobilization of intracellular calcium in NHK, since erythromycin increases the intracellular calcium concentration in gastric zymogen cells¹⁷⁾ and the level of intracellular free calcium affects the expression of ICAM-1 on NHK.¹⁸⁾ However, levels of cytoplasmic free calcium in NHK were not altered by RXM up to 0.2 mM (data not shown). This suggests that RXM modulates the response of keratinocyte to IFN- γ without mobilizing intracellular free calcium. In addition, IFN- γ itself did not mobilize intracellular free calcium.

RXM Inhibits the Ability of IFN- γ -Treated NHK to Stimulate T Cells in Response to SEB Nylon wool column-purified T cells from a healthy volunteer and SEB at 1, 10 or 100 ng/ml were added to NHK pretreated sequentially with RXM and IFN- γ , and the proliferative response of the T cells was measured (Fig. 2). Compared with the background response of T cells in the presence of untreated NHK, SEB significantly stimulated T cells with IFN- γ -treated NHK in a dose-dependent manner. At all SEB concentrations used, the T cell response was significantly inhibited by preincubation of NHK with 0.1 mM RXM.

ELISA Analysis of IL-1 α in Supernatants from NHK IL-1 α is known to be a major keratinocyte-derived cytokine responsible for activation of thymocytes and D10 cells.¹⁹⁾ We thus performed ELISA for IL-1 α in the supernatants from NHK. As shown in Fig. 3, RXM itself did not modify IL-1 α production by keratinocytes, compared with the "no addition" group, while IFN- γ significantly enhanced the production of IL-1 α . This promoting effect of IFN- γ was completely inhibited by RXM.

DISCUSSION

Our study demonstrated that RXM suppressed the immunological functions of keratinocytes induced by IFN- γ , exemplified by inhibition of HLA-DR expression, interference with accessory cell ability to induce the T-cell response to SEB, and decreased production of IL-1 α ; the only exception was the augmentation of ICAM-1. Interference of the accessory ability of keratinocytes seems to result from downregulation by RXM of HLA-DR induction by IFN- γ , since HLA-DR molecules expressed on keratinocytes are essential for the presentation of SEB

Fig. 1. Effects of RXM on the IFN- γ -Induced Expression of ICAM-1 (a) and HLA-DR (b) in NHK, Determined by Flow Cytometric Analysis

Cells were first incubated with RXM at concentrations ranging from 10 nM to 0.1 mM or medium alone, washed, and then incubated with IFN- γ (200 U/ml) for an additional 48 h. Mean fluorescence intensity was expressed as the mean value $\pm$ standard deviation of three independent experiments. * Significantly different from that of the control at $p < 0.01$.

Fig. 2. Effects of RXM on the Accessory Cell Ability of IFN- γ -Treated NHK for the T Cell Response to SEB

NHK were cultured for 48 h, with or without 0.1 mM RXM, followed by incubation, with or without 200 U/ml IFN- γ for an additional 48 h, in the presence of RXM. Then, NHK were cocultured with nylon wool-treated T cells and SEB for 72 h. Wells were pulsed with ^3H -TdR for the last 16 h prior to harvesting. Values represent means $\pm$ standard deviation of triplicate cultures. * Significantly different from that of the control at $p < 0.05$ or less.

to T cells.²⁰⁾ Although ICAM-1 transduces a costimulatory signal to T cells in the interaction between antigen presenting cells and T cells, RXM-upregulated ICAM-1 expression on keratinocytes could not augment the T-cell response to SEB. This suggests that IFN- γ -induced ICAM-1 seems to be sufficient for the induction of the T cell response to SEB presented by keratinocytes.

In certain inflammatory skin diseases such as atopic dermatitis, psoriasis and cutaneous T cell lymphoma, the lesioned skin is frequently colonized by *Staphylococcus aureus* and antibiotics are occasionally used in the treatment of these diseases.²¹⁻²⁴⁾ One of the possible mechanisms of exacerbation of these skin diseases by superficial bacterial infection is the activation and proliferation of T cells by superantigenic exotoxins released from bacteria. In this case, not only professional antigen presenting cells in the skin, Langerhans cells,^{25,26)} but also MHC class II-bearing epidermal keratinocytes^{20,26,27)} are capable of presenting the superantigens to and activating the T cells. Since epidermal keratinocytes do not constitutively express MHC class II molecules on their surface and, as far as we know, IFN- γ is the only cytokine that induces MHC class II molecules on

Fig. 3. Effects of IFN- γ and RXM on IL-1 α Production by NHK

Semiconfluent NHK was initially incubated, with or without 0.1 mM RXM for 48 h, followed by incubation, with or without 200 U/ml IFN- γ for 48 h, in 24-well plates (2 ml medium/culture). After cultivating for 2 d, the supernatants were collected and the IL-1 α concentration was assayed by ELISA. The data represent means $\pm$ standard deviation of triplicate assays.

keratinocytes, exposure to IFN- γ is a prerequisite for keratinocytes to function as the antigen presenting cell. Thus, recruitment of IFN- γ -secreting activated T cells into the skin environment stimulates keratinocytes to act as an amplifier of immune reactions in the epidermis when superantigens are present.

IL-1 α has been shown to be a pleiotropic inflammatory mediator with numerous biologic effects on many different cell types.¹⁹⁾ For example, it enhances the proliferation and IL-2 production of certain populations of T cells and the chemotaxis and growth of keratinocytes, thus promoting cutaneous inflammation. The present study showed that the IL-1 α secretion of cultured human keratinocytes was upregulated by IFN- γ , consistent with the previous finding of IFN- γ augmentation of IL-1 release from various cells.²⁸⁾ This result suggests that this antibiotic suppresses inflammatory skin damage by decreasing IL-1 production.

In conclusion, it is probable that bacterial superantigen and IL-1-mediated exacerbation of skin inflammation is effectively inhibited by RXM via both its antibiotic and immunosuppressive activities. Thus, the therapeutic effectiveness of RXM against atopic dermatitis, psoriasis and cutaneous T cell lymphoma is thought to be promising.

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