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Roxithromycin inhibits chemokine-induced chemotaxis of Th1 and Th2 cells but regulatory T cells

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ABSTRACT

Background: Roxithromycin (RXM), a 14-member macrolide antibiotic, has a variety of bioregulatory functions such as anti-inflammatory effects, anti-oxidant effects, and modulation of immune responses.
Objectives: In this study, we analyzed the effect of RXM on chemokine-induced chemotaxis of Th1, Th2, and regulatory T (Treg) cells established from three normal human peripheral blood lymphocytes by the reported methods.
Methods and results: Incubation with 10 μM RXM for 18 h did not alter the expression profile of CXCR3 on Th1 cells and CCR4 on Th2 and Treg cells. However, upon RXM preincubation, the migration of Th1 cells to IP-10 and Th2 cells to TARC was partially suppressed, although RXM did not influence Treg cell migration. Erythromycin and clarithromycin at the same concentration did not exert such effects. F-actin polymerization and Ca^{++} influx induced by IP-10 and TARC in Th1 and Th2 cells, respectively, was down-regulated by RXM pretreatment.
Conclusion: These results imply that RXM exhibits bioregulatory function by influencing chemotaxis of Th1 and Th2 cells while leaving Treg cell migration unaffected.

1. Introduction

Macrolides were originally exploited as antibiotics against *Staphylococcus*, *Streptococcus pneumoniae*, *Moraxella*, and *Propionibacterium acnes* [1]. Some macrolides have a variety of bioregulatory functions such as anti-inflammatory and anti-oxidant effects, and modulation of immune responses including immunosuppression [2,3]. The 14-member macrolide roxithromycin (RXM) inhibits T cell proliferation responses, cytokine production by T cells and macrophages [4,5,6], and accessory functions of professional and non-professional antigen presenting cells [7,8]. In fact, RXM exerts beneficial effects on various skin diseases such as psoriasis [9,10], atopic dermatitis [7], prurigo, and eosinophilic pustule folliculitis [10] partly through these immunomodulatory activities.

The interaction between chemokines and chemokine receptors is crucial in cell trafficking such as steady-state circulation, inflammation, and tumor metastasis [11]. Among chemokines, IFN-γ-inducible protein 10 (IP-10/CXCL10) is a T helper (Th)1 chemokine with affinity to CXC chemokine receptor 3 (CXCR3) on Th1 cells [12]. On the other hand, thymus and activation-regulated chemokine (TARC/CCL17) is known as a Th 2 chemokine that binds to CC chemokine receptor 4 (CCR4) on Th2 cells. It has been reported that RXM influences production of chemokines and expression of chemokine receptors in relation to skin immunity [13,14]. In order to further clarify these issues, we assessed the effect of RXM on TARC and IP-10-induced chemotaxis of established Th1, Th2, and regulatory T (Treg) cells.

2. Materials and methods

2.1. Study participants

Two healthy males (age, 46 and 38 years) and a female (33 years) were enrolled in this study after written informed consent was obtained. The study was approved by the ethical committee of the Hamamatsu University School of Medicine, and conducted according to the Declaration of Helsinki principles.

2.2. Reagents and kits

RXM, erythromycin (EM), and clarithromycin (CAM) were obtained from Wako Pure Chemical Industries (Osaka, Japan); $CD4^+$ T Cell Isolation Kit II and CD45RO microbeads from Miltenyi Biotec (Auburn, CA); staphylococcal enterotoxin B (SEB), anti-human interleukin (IL)-4 monoclonal antibody (mAb), anti-human IL-12 mAb, recombinant (r)IL-2, phycoerythrin (PE)-labeled mouse IgG1 anti-human CD183 (CXCR3) mAb, PE-labeled mouse IgG1 anti-human CCR4 mAb, fluorescein isothiocyanate (FITC)-labeled mouse IgG1 anti-human CD4 mAb, FITC-labeled mouse IgG1 anti-human CD25 mAb, peridinin chlorophyll protein-labeled mouse

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IgG1 anti-human CD4 mAb, Cytoperm solution, and Perm/Wash buffer from BD Bioscience (San Jose, CA); FITC-conjugated anti-human interferon (IFN)-γ mAb, PE-conjugated anti-human IL-4 mAb, Goldiplug, and Cytofix/Cytoperm Plus Kit from BD Pharmingen (San Diego, CA); human rIL-12, and human rIL-4 from PeproTech (Rocky Hill, NJ); phytohemagglutinin (PHA), human rTGF-β thalidomide, dimethylsulfoxide (DMSO), FITC-conjugated phalloidin, and Fluo-3AM from Sigma–Aldrich (St. Louis, MO); 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) from Molecular Probes (Eugene, OR); and CD3/CD28 molecules from Dynal Biotec (Oslo, Norway).

2.3. Cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by centrifugation with Ficoll-Paque PLUS in LeucoSep (Greiner Bio-One, Frickenhausen, Germany) from three volunteers. $CD4^+$ T cells were prepared from PBMC with a $CD4^+$ T Cell Isolation Kit II by negative selection. The remaining $CD4^-$ cells were used as antigen-presenting cells (APC) to establish Th1, Th2, and Treg cells. $CD45RA^+CD4^+$ cells were purified from $CD4^+$ T cells with the CD45RO microbeads.

2.4. Cell culture

Culture medium was RPMI supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptethanol, 10^{-5} M sodium pyruvate, 25 mM HEPES, 1% non-essential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin from Gibco-BRL (Carlsbad, CA); human TARC and IP-10 from R&D systems (Minneapolis, MN). PBMC (5×10^6 cells/well) in culture medium were incubated in 12-well tissue culture plates at 37 °C in 5% CO_2 in air.

2.5. Generation of Th1, Th2 and Treg cells

All type of cells was established in serum-free medium in the presence of APC and various stimulants according to the reported methods [15–17]. Each cell type was come from PBMCs of three volunteers. In brief, SEB, an anti-human IL-4 antibody, rIL-12, and rIL-2 were employed to establish Th1 cells [15]. For establishment of Th2 cells, PHA, thalidomide, anti-IL-12 antibody, rIL-4, and rIL-2 were used [16]. Treg cell were established with SEB and human rTGF-β [17].

2.6. Immunofluorescence staining and flow cytometric analysis

For cell surface staining, cells were doubly stained with PE-labeled and FITC-labeled mAb in PBS for 30 min at room temperature. For intracytoplasmic cytokine staining, 1 μl of Goldiplug was added to each well during the last 6 h of culture according to the manufacturer's protocol. The cells were then reacted with 100 μl of CytoFix for 15 min at 4 °C, washed with phosphate-buffered saline containing 0.1% saponin, and stained with FITC-conjugated anti-human IFN-γ mAb and PE-conjugated anti-human IL-4 mAb for 30 min, followed by reaction with peridinin chlorophyll protein-labeled mouse IgG1 anti-human CD4 mAb. After washing in PBS, 10,000 cells were analyzed on a FACSCalibur (Becton Dickinson). Mean fluorescence intensity (MFI) was calculated on a log scale. Cells stained with isotype-matched mAbs served as the control.

2.7. Cell proliferation assay

$CD4^+$ T cells were labeled with 5 μM CFSE in DMSO for 15 min at 37 °C as previously described [18], stimulated with CD3/CD28 for 4 days, and analyzed by flow cytometry [18]. Cells once divided showed half the CSFE intensity of parental cells shown as the multipeak histogram of several divisions. In order to examine the suppressive effect of Treg cells on lymphocytic proliferation, autologous Treg cells were added to $CD4^+$ T cells ranging from 0.05 to 1.0 for 24 h, and the cell mixtures were subjected to CFSE analysis. Control culture continued with Treg cells.

2.8. Incubation of cells with macrolide

Cells were cultured for 8 h in the presence or absence of either 10 μM RXM, EM, or CAM. Cell viability after incubation with macrolides was >90% as judged by dye exclusion.

2.9. Real-time horizontal chemotaxis assay

Time-lapse images of cell migration during chemotaxis were observed directly with an optically accessible horizontal chemotaxis apparatus TAXIScan *via* a CCD camera (EZ-TAXIScan; GE Healthcare, Tokyo, Japan) as described [19]. The apparatus consisted of front and back chambers containing cells and a chemoattractant, respectively, which were connected by a microchannel. A 1-μl suspension of cells (5×10^6 cells/ml) was placed in one compartment, and 1 μl of either TARC at 25 mg/ml or IP-10 at 10 mg/ml was injected into the other compartment to initiate chemotaxis under the concentration gradient in the channel. Data were analyzed using the Image J software (NIH, Bethesda, MD) and the Manual Tracking plug-in produced by FP Cordelieres (Institut Curie, Orsay, France; http://rsb.info.nih.gov/ij/plugins/manual-tracking.html).

2.10. F-actin polymerization

Phalloidin has been found to bind only to polymeric and oligomeric forms of actin and not to monomeric actin. Therefore, the level of polymerized actin was determined by staining cells with phalloidin as described before. Cells were permeabilized in a Cytofix/Cytoperm solution for 20 min, washed in Perm/Wash buffer for 10 min, and incubated with 5 mg/ml FITC-conjugated phalloidin for 30 min. All procedures were done at 4 °C. Cells were analyzed on a FACSCalibur, and the level of actin-polymerization was expressed by MFI. Because MFI varied among each cell type, the percentage of fluctuation was calculated by the ratio before and after treatment with chemokines as follows: %MFI = MFI of treated cells/MFI of control, non-treated cells.

2.11. Calcium influx

Cells were incubated with 0.8 mM Fluo-3AM/DMSO in RPMI supplemented with 5% FCS for 30 min at 37 °C as described [20]. Fluorescence intensity was continuously measured on a FACSCalibur for 50 s after incubation with Fluo-3AM/DMSO. Chemokine was added into cell suspensions at 10 s after starting the measurement of Ca^{++} concentrations. For quantitative evaluation, the variation of intracellular Ca^{++} concentrations was calculated according to the following equation: $(F - F_{min})/(F_{max} - F) \times 100$, in which F was MFI of chemokine-treated cells, F_{min} was MFI of cells incubated with 6 mM EGTA to chelate Ca^{++}, and F_{max} was MFI of cells incubated with 700 mM ionophore in DMSO [21].

2.12. Statistical analysis

All values were expressed as means ± standard deviation (SD). Data were analyzed with Student's *t*-test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Immunological characteristics of established cells

More than 80% of Th1 cells expressed CD4 and CXCR3 and produced intracellular IFN-γ (Fig. 1A and B), while 75% of Th2 cells were positive for CD4 and CCR4, and 12% synthesized IL-4 (Fig. 1C and D). Since Th2 cells established according to the reported method [16] express relatively low levels of IL -4 and no IFN-γ, we used this cell line. Around 90% of Treg cells expressed CD4, CCR4, and CD25 (Fig. 1E and F), and none produced intracellular IFN-γ or IL-4 (Fig. 1G). The CSFE assay revealed that Treg cells suppressed proliferation of autologous $CD4^+$ T cells in response to immobilized CD3/CD28 in a dose-dependent manner (Fig. 2).

3.2. RXM down-regulates the migration of Th1 and Th2 cells, but not of Treg cells, toward IP-10 and TARC without influencing chemokine receptor expression

The migration of Th1, Th2, and Treg cells toward IP-10 or TARC was observed on a TAXIScan for 60 min. Non-treated control Th1 and Th2 cells ran toward IP-10 and TARC, respectively (Fig. 3). On the other hand, RXM-treated cells did not migrate toward the corresponding chemokine. Both non-treated control and RXM-treated Treg cells exhibited comparable chemotaxis to TARC. RXM did not affect chemokine receptor expression such as CXCR3 on Th1 cells, CCR4 on Th2 cells (Fig. 4A–D), or CCR4 on Treg cells (data not shown) as revealed by flow cytometry. Preincubation with CAM and EM did not influence the migration pattern of Th1, Th2, or Treg cells toward corresponding chemokines (data not shown).

3.3. F-actin polymerization to chemokines in Th1, Th2, and Treg cells

Actin polymerization is an early event that controls cell migration and reorganization of the actin cytoskeleton [22]. In Th1 cells, the baseline MFI of phalloidin was 503.7 ± 13.4. MFI increased by 106.2 ± 0.46% with IP-10 compared to control ($p < 0.05$). MFI significantly decreased by 92.23 ± 2.6% with RXM