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Statins directly suppress cytokine production in murine intraepithelial lymphocytes

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ABSTRACT

Statins, inhibitors of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, are known not only as cholesterol-lowering agents but also as anti-inflammatory mediators. However, their regulatory effect on intestinal mucosal immunity remains unclear. The present study examined the possible direct effects of statin on intestinal intraepithelial lymphocytes (IELs), the front line cells of the intestinal mucosal immune system. Murine IELs were isolated from the small intestines of C57BL/6 mice. IELs activated with anti-CD3/CD28 monoclonal antibodies produced interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-2, and IL-4 in significant numbers; however, they did not produce IL-5. Both simvastatin and lovastatin suppressed IEL production of IFN-γ, TNF-α, IL-2, and IL-4 in a dose-dependent manner, whereas 48-h treatment with high concentrations (5×10^{-5} M) of simvastatin and lovastatin did not affect the number of IELs. The suppressive effect of the simvastatin was significantly restored by the addition of mevalonate, farnesyl pyrophosphate ammonium salt, and geranylgeranyl pyrophosphate ammonium salt, which are downstream metabolites of HMG-CoA. These findings suggest that statins have direct suppressive effects on the production of T helper 1-cytokines and IL-4 in IELs; these effects are associated with inhibition of the mevalonate pathway to some extent.

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1. Introduction

Statins are inhibitors of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, and they are the most effective agents for lowering cholesterol during the treatment of cardiovascular diseases in clinical practice [1]. Pleiotropic immunomodulatory effects of statins have been demonstrated in addition to their lipid-lowering properties [2]. As a result, much attention has been focused on their potential as therapeutic agents for the treatment of inflammatory and autoimmune diseases [3,4]. With regard to gastrointestinal diseases, several studies have shown that statins may be effective for the treatment of experimental colitis and that they could be potential therapeutic agents for inflammatory bowel diseases [5–8]. Statins may also be used as chemopreventive drugs for colitis-induced cancer [9]. Other studies demonstrated that statin-mediated effects on the immune system were pleiotropic; these effects included inhibition of T lymphocyte activation as well as proliferation, migration, and shifting of T lymphocytes [4,10–13]. However, the direct effects of statins on intestinal lymphocytes have not been examined to date.

Intraepithelial lymphocytes (IELs) are the front line cells of intestinal mucosal immunity. They are phenotypically and functionally distinct from T lymphocytes in the peripheral blood and spleen [14,15]. Suggested functions of IELs include mediation of inflammatory reaction, surveillance of the intestinal epithelium, and induction or maintenance of oral tolerance [16,17]. Previous studies have shown that IELs produce various cytokines such as interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), interleukin 2 (IL-2), IL-4, IL-5, IL-6, IL-10, IL-17, IL-15, transforming growth factor β1 (TGF-β1), and keratinocyte growth factor (KGF), all of

Abbreviations: HMG-CoA, enzyme 3-hydroxy-3-methylglutaryl coenzyme A; IBD, inflammatory bowel disease; IELs, intraepithelial lymphocytes; IFN-γ, interferon γ; TNF-α, tumor necrosis factor α; IL, interleukin; TGF-β1, transforming growth factor β 1; KGF, keratinocyte growth factor; GGPP, geranylgeranyl pyrophosphate ammonium salt; FPP, farnesyl pyrophosphate ammonium salt; HBSS, Hank's balanced salt solution; FBS, fetal bovine serum; CD, cluster of differentiation; PE, phycoerythrin; APC, allophycocyanin; TCR, T cell receptor; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; DMSO, dimethyl sulfoxide; Th, T helper; SEM, standard error of the mean; PBMCs, peripheral blood mononuclear cells; LFA-1, lymphocyte function-associated antigen 1.

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which contribute to defense against microbes, elimination of infected or transformed epithelial cells, and immunoregulatory function [18–20]. Recent studies have also investigated the direct regulation of IEL-derived cytokine production by several substances such as glutamine [21], histamine [22], and catecholamine [20].

The present study examined the possible direct effects of statins on IELs and clarified the mechanisms underlying IEL-mediated regulation of cytokine production by statin treatment.

2. Materials and methods

2.1. Mice

Male C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). Mice were housed in rooms in an animal facility. Temperature was maintained at 25 °C with 12-h light/dark cycles. Mice were fed standard chow and had access to bottled tap water unless specified otherwise. Mice weighing >22 g at 8–10 weeks of age were used for this study. The study was performed according to the guidelines and regulations for Laboratory Animal Care of Hamamatsu University School of Medicine.

2.2. Materials

Simvastatin, mevalonolactone (mevalonate), geranylgeranyl pyrophosphate ammonium salt (GGPP), farnesyl pyrophosphate ammonium salt (FPP), and penicillin–streptomycin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Lovastatin was purchased from WAKO (Osaka, Japan). Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), and antibiotic–antimycotic solution (penicillin, 10,000 IU/ml; streptomycin, 10 mg/ml) were purchased from Gibco (Grand Island, NY, USA). Percoll was purchased from GE Healthcare Bio-science AB (Uppsala, Sweden). Fluorescein isothiocyanate (FITC)-labeled anti-mouse CD3e, phycoerythrin (PE)-Cy7-labeled anti-mouse CD4, allophycocyanin (APC)-Cy7-labeled anti-mouse CD8α, PerCP-Cy5.5-labeled anti-mouse CD8β, PE-labeled anti-mouse CD19, APC-labeled anti-mouse T cell receptor (TCR)-β, and FITC-labeled anti-mouse TCRγδ monoclonal antibodies (mAbs) were purchased from BD PharMingen (San Diego, CA, USA). Dynabeads® Mouse T-Activator CD3/CD28 was purchased from Invitrogen (Oslo, Norway).

Simvastatin was dissolved in dimethyl sulfoxide (DMSO) and diluted in Milli Q; lovastatin was dissolved in ethanol; and mevalonate, FPP, and GGPP were dissolved in Milli Q. Final concentrations of DMSO and ethanol were less than 0.3% and 1%, respectively.

2.3. Isolation of IELs and preparation of splenocytes

IELs were isolated from mice according to a method described previously, albeit with minor modifications [22,23]. Briefly, an inverted small intestine was cut into four segments, which were transferred to a 50-ml conical tube containing 45 ml of HBSS supplemented with 5% FBS and 1% penicillin/streptomycin. The tube was shaken in an orbital shaker at 160 rpm in the horizontal position for 45 min at 37 °C. Cell suspensions were passed through a glass wool column. The cells were suspended in a 30% Percoll solution and centrifuged for 20 min at 400*g*. After the initial centrifugation, cells at the bottom of the solution were subjected to Percoll discontinuous-gradient centrifugation, and IELs were recovered at the interphase of 44% and 70% Percoll solutions. The obtained cells were washed and resuspended in the culture medium (RPMI-1640 supplemented with 10% FBS and 1% antibiotic solution). The cell purity of the IEL compartment, as assessed by flow cytometry according to forward and side scatter characteristics, was 84.7 ± 1.1%. The viability of IELs, as determined by trypan blue exclusion, was always ⩾98%.

Splenocytes were isolated from mice according to a method described previously [22]. Briefly, the spleen was removed from sacrificed mice and cut into several pieces. The cell suspensions were then centrifuged, and the cell pellet was resuspended in Dulbecco's phosphate-buffered saline. The red blood cells were lysed in lysing buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2 ethylene diamine tetraacetic acid). Splenocytes were washed twice and resuspended in the culture medium (RPMI-1640 supplemented with 10% FBS and 1% antibiotic solution). Viability of the splenocytes, as determined by trypan blue exclusion, was always ⩾98%.

2.4. Analysis of IEL subtypes by flow cytometry

IELs were adjusted to a concentration of 1×10^6 cells/ml. Cells were incubated on ice for 30 min by the following antibodies, which were used for flow cytometry: FITC-labeled anti-mouse CD3e, PE-labeled anti-mouse CD3e, PE-Cy7-labeled anti-mouse CD4, APC-Cy7-labeled anti-mouse CD8a, PerCP-Cy5.5-labeled anti-mouse CD8b, PE-labeled anti-mouse CD19, APC-labeled anti-mouse TCRβ, and FITC-labeled anti-mouse TCRγδ mAbs. The surface phenotypes of IELs were analyzed using fluorescence-labeled anti-mouse mAbs for 30 min on ice. The stained cells were washed and examined using a FACSAria cell sorter (BD Bio-sciences, San Jose, CA). Data were analyzed using the FlowJo 7.6.1 software (Treestar, Inc., San Carlos, CA).

2.5. Effects of statins on cytokine production determined by cytometric bead array

IELs were adjusted to a concentration of 1×10^6 cells/ml in culture media; pretreated for 15 min with or without simvastatin/lovastatin at final concentrations of 5×10^{-7}, 5×10^{-6}, and 5×10^{-5} M; and incubated for 48 h at 37 °C in a humidified atmosphere of 5% CO_2 in air with or without anti-CD3/CD28 mAb. The supernatants were collected 48 h after incubation in IELs and splenocytes. IFN-γ, TNF-α, IL-2, IL-4, and IL-5 were detected using the BD Cytometric Bead Array Mouse T helper (Th1)/Th2 Cytokine Kit according to the manufacturer's instructions (BD Bio-sciences).

2.6. Effects of simvastatin and mevalonate metabolites on cytokine production determined by cytometric bead array

IELs were adjusted to a concentration of 1×10^6 cells/ml in culture media and pretreated with mevalonate, FPP, or GGPP for 15 min at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 15 min, simvastatin at a final concentration of 10 μM was added in addition to anti-CD3/CD28 mAb. The supernatants were collected 48 h after incubation in IELs, and IFN-γ, TNF-α, IL-2, IL-4, and IL-5 were detected using the BD Cytometric Bead Array Mouse Th1/Th2 Cytokine Kit according to the manufacturer's instructions (BD Bio-sciences).

2.7. Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Student's unpaired two-tailed t-test was used for comparisons between two groups. One-way analysis of variance, followed by Fisher's protected least significant difference test, was used for comparisons between more than two groups. $p < 0.05$ was considered statistically significant. Statistical analysis was performed using StatView 5.0 (SAS Institute Inc., Cary, NC, USA).

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