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Cilostazol reduces MCP-1-induced chemotaxis and adhesion of THP-1 monocytes by inhibiting CCR2 gene expression

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ABSTRACT

The chemotaxis and adhesion of monocytes to the injured endothelium in the early atherosclerosis is important. Cilostazol, a specific phosphodiesterase type III inhibitor, is known to exhibit anti-atherosclerotic effects mediated by different mechanisms. This study aimed to investigate the modulating effect of cilostazol on the MCP-1-induced chemotaxis and adhesion of monocytes. The gene expression of CCR2, the major receptor of MCP-1 in THP-1 monocytes, was also analyzed. The chemotaxis of monocytes toward MCP-1 was investigated using the transwell filter assay. Cilostazol dose-dependently inhibited the MCP-1-induced chemotaxis of monocytes which was shown to be cAMP-dependent. Using western blot analysis and flow cytometry method, we demonstrated the decrease of CCR2 protein at the cell membrane of monocytes by cilostazol treatment. Results from RT/real-time PCR confirmed the decrease of CCR2 mRNA expression by cilostazol which was also mediated by cAMP. Similar inhibition was also noted in human peripheral monocytes. The post-CCR2 signaling pathways including p44/42 and p38 MAPK were examined by western blot analysis. Result confirmed the inhibitory effect of cilostazol on the phosphorylation of p44/42 and p38 MAPK after MCP-1 stimulation. The activation of monocytes after MCP-1 treatment exhibited enhanced adhesion to vascular endothelial cells which was dose-dependently suppressed by cilostazol. Together, cilostazol was demonstrated, for the first time, to inhibit the CCR2 gene expression and MCP-1-induced chemotaxis and adhesion of monocytes which might therefore reduce the infiltration of monocytes during the early atherosclerosis. The present study provides an additional molecular mechanism underlying the anti-atherosclerotic effects of cilostazol.

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1. Introduction

Atherosclerosis is now recognized as a chronic inflammatory process. The excessive recruitment of monocytes to the sub-endothelial space is central to the pathology of atherosclerosis. The adhesion of circulating monocytes and consequent transmigration through the vascular endothelial layer are initiated from being attracted by chemokines released from injured endothelial cells which is a crucial step in the early atherosclerosis [1]. MCP-1, a well-known chemokine abundantly present in macrophage-rich atherosclerotic plaques in human, is responsible for the recruitment of monocytes to vascular intima space [2]. Induced expression of MCP-1 at sites of tissue injury and inflammation can lead to the increased recruitment of monocytes directly to those tissues, where activation and differentiation into macrophage occur [3]. The most prominent receptor for MCP-1 is CCR2, one of the 11 β-chemokine receptors characterized by seven-transmembrane domains and coupled to a GTP-binding protein. The binding of MCP-1 to CCR2 results in not only the chemotaxis of monocytes but also the following adhesion and spreading of monocytes [4,5]. CCR2(−/−) mice show defect in monocyte recruitment and decreased atherosclerotic lesions, indicating the important role of CCR2 in the development of atherosclerosis [6].

Cilostazol, chemically named 6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1H)-quinolinone, is a specific phosphodiesterase type III (PDE3) inhibitor. PDE3 inhibitors are well known to inhibit platelet aggregation and induce vasorelaxation by elevating intracellular cAMP [7,8]. Clinically, cilostazol is used to treat patients with intermittent claudication and restenosis after angioplasty [9–11]. In addition to its anti-platelet and vasodilatory effects, anti-inflammatory and anti-atherosclerotic effects have also been described [12,13]. Studies of the anti-atherosclerotic effects of cilostazol on vascular cells have revealed many potential underlying mechanisms. Cilostazol can inhibit leukocyte–endothelial cell interaction by increasing NO production and decreasing expressions of MCP-1 and VCAM-1 in vascular endothelial cells [14–16]. Cilostazol can also suppress proliferation of vascular smooth muscle cells by decreasing E2F and increasing p53 and p21 expressions [17,18]. Cilostazol has also been shown to inhibit the foam cell formation of

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macrophages by reducing the uptake of oxLDL [19]. However, the effects of cilostazol on the MCP-1-mediated chemotaxis and adhesion of monocyte have not yet been studied.

In the present study, we investigated the modulatory effect of cilostazol on the MCP-1-induced chemotaxis of monocytes and the CCR2 expression at both protein and mRNA levels. The post-CCR2 activation of monocytes including p44/42 and p38 MAPK signal pathways, and MCP-1-enhanced adhesion of monocytes toward endothelial cells was also examined after cilostazol treatment.

2. Materials and methods

2.1. Materials

Cilostazol was provided by Otsuka Pharmaceutical Co. (Tokushima, Japan). Monoclonal antibody against CCR2 was obtained from Epitomics Inc. (CA, USA) and monoclonal antibody against tubulin was obtained from Neomarker (Fremont, CA, USA). Dibutyryl cyclic AMP (dbcAMP) was purchased from Sigma (St. Louis, MO, USA). Rabbit anti-phospho-p38, rabbit anti-phospho-p44/42, rabbit anti-p38, rabbit anti-p44/p42, and goat anti-rabbit secondary antibodies conjugated with horseradish peroxidase were acquired from Cell Signaling Technology (Danvers, MA, USA). Recombinant MCP-1 was purchased from R&D Systems Inc. (Minneapolis, Minnesota, USA).

2.2. Cell culture

The human monocytic leukemia cell line THP-1 was obtained from ATCC and grown in suspension culture of RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and antibiotics. Cells were subcultured by diluting the medium with fresh growth medium in a 1:4 ratio, and grown at 37 °C in a humidified atmosphere with 5% CO_2/95% air. Human monocytes were isolated from the buffy coats of normal blood bank donors with Ficoll-Hypaque density gradient centrifugation followed by adherence to the culture dish for one hour and cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and 10% (v/v) pooled human serum. Human vascular endothelial cells (HUVECs) were isolated from the vein of human umbilical cords and grown in EGM provided by Clonetics (MD, USA). Cells were maintained in a humidified atmosphere with 5% CO_2/95% air at 37 °C. HUVECs were passaged 3–5 times prior to use in experiments. Cell viability was analyzed by LDH assay (Promega Co., WI, USA) following manufactorer's procedure.

2.3. Transwell filter migration assay

Microporous membrane (pore size, 8 μm) transwell inserts (Costar, Cambridge, MA) were used for the chemotaxis assay. THP-1 cells treated with cilostazol (0, 10, 25, 50, and 100 μM) or dbcAMP (250 μM) for 24 h were washed once with PBS, and 2×10^5 cells in 200 μl RPMI were added to the upper chamber, with 400 μl RPMI containing 20 ng/ml MCP-1 in the lower chamber. THP-1 cells were allowed to migrate for 1 h at 37 °C in an atmosphere of 5% CO_2/95% air and then the inserts were fixed and stained with Liu's stain. The non-migratory cells were removed before the membrane was mounted and the number of migratory cells was observed and counted under a microscope.

2.4. Western blot analysis

Cell extract was prepared by processing cells in lysis buffer containing Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM DTT, 2 mM PMSF and 1% Triton X-100 with three times of freeze–thaw cycles and centrifugation. The 1st supernatant was used as cytosolic protein extract. The pellet was re-dissolved in lysis buffer, sonicated until the solution became clear and after centrifuging again, the 2nd supernatant was used as the membrane protein extract. The protein concentration of the cell extracts was determined by Bradford assay (Bio-Rad Laboratories, CA, USA). Extracts with the same amount of proteins were separated by 10% SDS–PAGE either for coomassie blue stain or transferred onto a PVDF membrane. Membrane was incubated at 4 °C in blocking solution containing 5% bovine serum albumin (BSA) in TBST for 1 h, followed by 2 h incubation in blocking solution containing appropriate dilution of primary antibody (anti-CCR2: 1/200, anti-phospho-p38, anti-phospho-p44/42, anti-p38, and anti-p44/42: 1/1000, and anti-tubulin: 1/800). After washing three times in TBST, the membrane was then incubated in TBST containing secondary antibody conjugated with horseradish peroxidase for 1 h. Membranes were washed three times in TBST and positive signals were developed with enhanced chemiluminescence (Amershan Pharmacia Biotech, Little Chalfont Buckinghamshire, England).

2.5. Flow cytometry

THP-1 cells treated with or without 100 μM cilostazol for 24 h were collected by centrifugation and subsequently the cells were washed with PBS containing 0.5% BSA, and incubated in blocking solution containing 5% BSA in PBS for 30 min at room temperature. Cells were then incubated with the addition of primary antibody (anti-CCR2, 1/25) in blocking solution for 2 h at room temperature. After washing three times with PBS containing 0.5% BSA, cells were incubation with FITC-conjugated secondary antibody (Rabbit-FITC 1:150) for 30 min at 4 °C. Cells were washed three times with PBS containing 0.5% BSA and processed for analysis using a fluorescence-activated cell sorter and Cell Quest software (FACScan, Becton Dickinson).

2.6. RNA isolation and RT/real-time PCR

Total cellular RNA was isolated by lysis in a guanidinium isothiocyanate buffer, followed by a single step of phenol–chloroform–isoamyl alcohol extraction. In brief, 5×10^6 cells were lysed in 0.5 ml solution D containing 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sodium sarcosine, and 0.1 M β-mercaptoethanol, with vigorous vortexing. Sequentially, 50 μl of 2 M sodium acetate (pH 4.0), 0.5 ml phenol, and 100 μl chloroform–isoamyl alcohol (49:1, v:v) were added to the homogenate. After vortexing for 1 min, the solution was centrifuged at 12,000 rpm for 20 min at 4 °C. The RNA was precipitated by the addition of 0.5 ml isopropanol and kept at −80 °C for 1 h. RNA was pelleted by centrifuging the solution at 12,000 rpm for 20 min at 4 °C. After the RNA pellet was rinsed in ice-cold 75% ethanol, the dry RNA was dissolved in DEPC-treated ddH_2O. The cDNA was synthesized from total RNA using M-MLV reverse transcriptase (USB Corporation, USA). Quantitative real time PCR was performed with universal cycling conditions (15 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 1 min at 55 °C, and 30 s at 72 °C). Cycle threshold (CT) values were determined by automated threshold analysis with Mx-Pro Mx3005P v4.00 software. PCR primers used were as follows: CCR2 forward primer, 5′-ATGCTGTCCACATCTCGTTCTCG -3′ and reverse primer, 5′-TTATAAACCAGCCGAGACTTCCTGC-3′; and GAPDH forward primer, 5′-GACCTGACCTGCCGTCTA-3′ and reverse primer, 5′-AGGAGTGGGTGTCGCTGT-3′.

2.7. Cell adhesion assay

THP-1 cells with or without 100 μM cilostazol treatment were activated by 20 ng/ml MCP-1 for 24 h. THP-1 cells (2×10^5) were

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