Pitavastatin and CCR2 expression inTHP-1 cells

To elucidate the potential mechanism involved in the observed phenomenon, THP-1 cells were subjected to flow cytometry. As shown in Fig. 2A, the expression of CCR2 on the cell surface decreased after pitavastatin treatment, though not dramatically (10 μM, 48 hours). Further, semi-quantitative RT-PCR analysis revealed that CCR2 was not significantly altered at the message level following pitavastatin treatment (Fig. 2B).

Pitavastatin reduces MCP-1-induced activation of ERK1.2 and Rho AGTPase

It has been reported that ERK activation is involved in MCP-1-mediated THP-1 integrin activation (Ashida et al., 2001), therefore, we examined the effect of pitavastatin on MCP-1-mediated ERK activation. As shown in Fig. 3, MCP-1 induced phosphorylation of ERK protein in THP-1 cells.

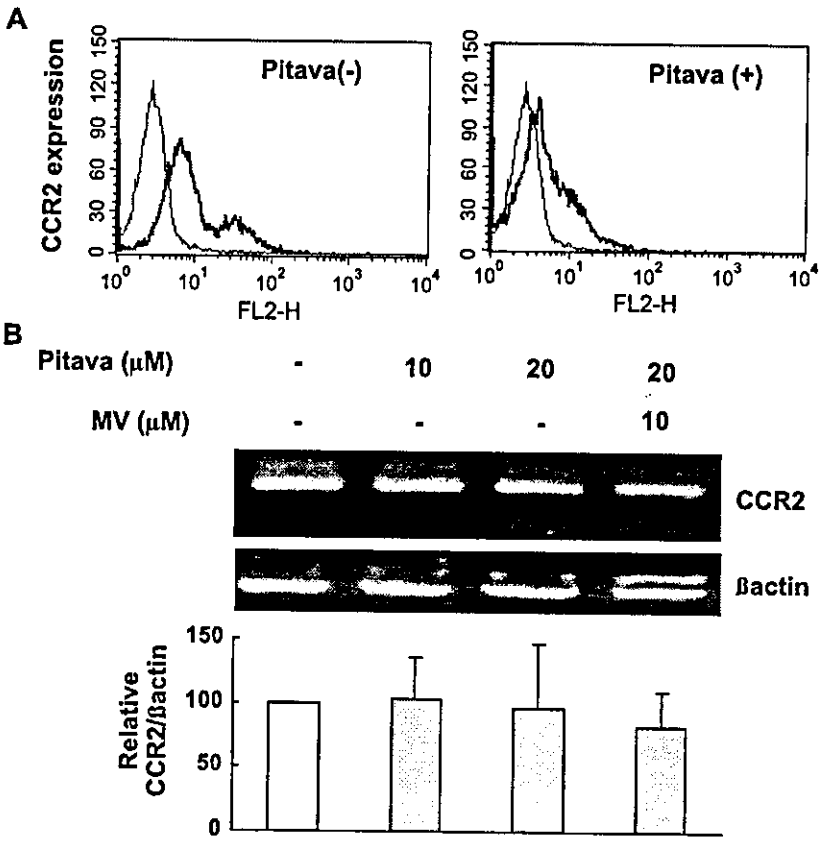

Fig. 2. Flow cytometric analysis of THP-1 cells before and after pitavastatin treatment. (A) THP-1 cells were pre-incubated in the absence (−) or presence (+) of 10 μM of pitavastatin (Pitava) for 48 hours, and then CCR2 expression (bold line) was detected using anti-CCR2 mAb followed by FITC-labeled anti-mouse polyclonal IgG and compared to non-binding IgG (thin line). (B) THP-1 cells were pre-incubated in the presence of the indicated amounts of pitavastatin (Pitava) for 48 hours, after which total RNA was recovered and cDNA was prepared. A semi-quantitative PCR assay was carried out using primers specific for MCP-1 and β actin, as described in Methods. PCR products were subjected to 1.5% agarose electrophoresis to detect cDNA. The intensity of each band was quantitated using an LAS1000 image analyzer and the results of 3 independent experiments are summarized as a Bar graph. There were no significant differences between the columns.

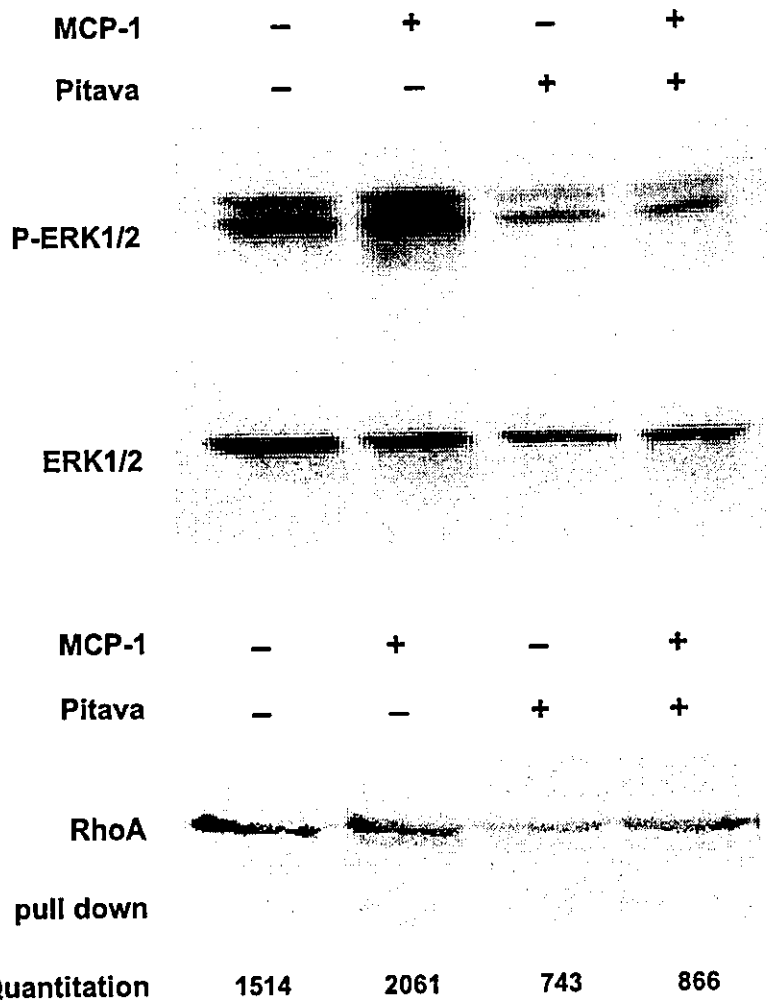

Fig. 3. Involvement of ERK1/2 and RhoA in MCP-1-induced THP-1-adhesion to vascular endothelium. THP-1 cells were incubated in the absence (−) or presence (+) of pitavastatin (Pitava, 10 μM, 48 hours) with or without stimulation by MCP-1 (50 ng/ml, 5–10 minutes). Total cell lysates were prepared and subjected to Western blot analysis, using antibodies against ERK1/2 and phopho-ERK1/2 antibodies, or captured with Rhotekin beads, and blotted using the anti-RhoA antibody, as described in Methods. The intensity of each band was quantitated using an LAS1000 image analyzer. Data shown are representative of 3 independent experiments.

However, when THP-1 cells were incubated in the presence of pitavastatin for 48 hours, the MCP-1-induced phosphorylation of ERK was significantly reduced (Fig. 3A). We also examined the involvement of RhoA GTPase in this phenomenon and found that not only resting, but also MCP-1 dependent activation of RhoA were reduced following pitavastatin treatment, based on Quantitative analysis of our pull-down assay results with Rhotekin beads (Fig. 3B).

Inhibition of RhoA GTPase, but not that of ERK1/2, reduced MCP-1-induced adhesion of THP-1 cells

To determine the potential role of Rho GTPase in the phenomenon, we treated THP-1 cells with clostridium botulinum C3 ADP-ribosyltransferase, a bacterial exoenzyme known to inactivate Rho

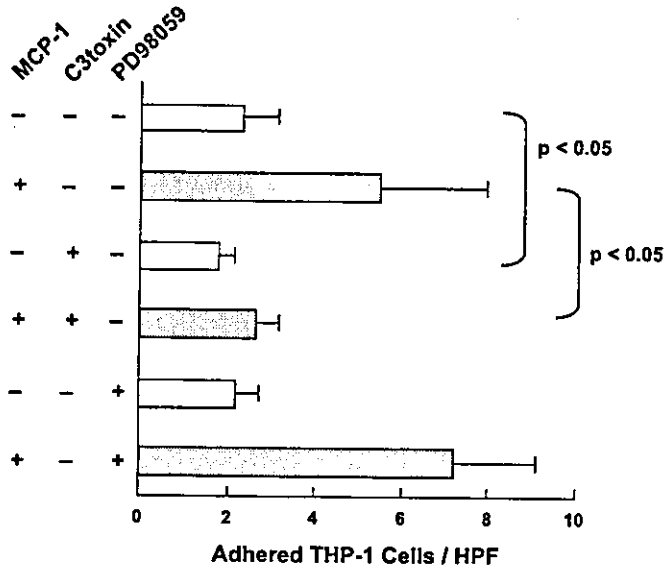

Fig. 4. Involvement of RhoA GTPase, but not of ERK1/2, in MCP-1-induced THP-1 adhesion to vascular endothelium. THP-1 cells were incubated in the presence of C3 exoenzyme (C3 toxin, 30 μg/ml, 48 hours) or PD98059 (10 μM, 4 hours) and an adhesion assay under flow was carried out in the presence or absence of MCP-1 (50 ng/ml), which was added just prior to the assay, as described in Methods. Data shown are representative of 4 independent experiments.

proteins in monocytic cells (Yoshida et al., 2001), for 48 hours at a concentration of 30 μg/ml. As shown in Fig. 4, treatment with the C3 exoenzyme for 48 hours resulted in a reduced number of adhered THP-1 cells following MCP-1 treatment ($p < 0.05$ vs. control THP-1). These effects of C3 toxin are in good agreement with our results from the RhoA pull-down assay shown in Fig. 3, thus confirming the importance of RhoA in MCP-1-dependent monocyte adhesion to endothelium. To further examine the role of ERK-dependent signalling in the MCP-1-induced enhanced adhesion of THP-1 cells, the cells were pre-treated with PD98059, a potent ERK kinase (MEK) inhibitor, prior to the adhesion assay. Interestingly, treatment with PD98059 failed to block the MCP-1-dependent THP-1 adhesion to HUVEC (Fig. 4).

Discussion

The accumulation of monocytes on vascular endothelium has been shown to be one of the earliest manifestations of atherosclerosis (Munro and Cotran, 1988). Previous pathological studies clearly demonstrated monocyte accumulation in aortic segments, even in the absence of lipid deposition (Cybulsky and Gimbrone, 1991). Further, a potential role for adhesion molecules and chemokines in atherosclerosis (Ashida et al., 2001) has been recently reported.

A previous study (Gerszten et al., 1999) used an in vitro adhesion assay performed under laminar flow conditions and revealed the dynamic role of MCP-1 in the transition from rolling to stable adhesion to vascular endothelium. Statins have been used to treat patients with hyperlipidemia, a major risk factor of atherosclerosis, because of their effects on cholesterol synthesis via the inhibition of the mevalonate pathway in the liver. However, recent observations have revealed that statins might exert lipid-independent effects in atherosclerosis. In addition, we recently demonstrated that treatment with statins

significantly reduced U937 cells adhesion, but not rolling, on activated HUVEC (Yoshida et al., 2001). In the present study, we found that treatment of THP-1 cells, from a monocytic cell line, with pitavastatin, a potent new HMG-CoA reductase inhibitor, significantly reduced the conversion from rolling to stable arrest on activated HUVEC under laminar flow conditions. This effect of pitavastatin on the transition of monocytes from rolling to adhesion was concentration-dependent (data not shown) and abrogated by simultaneous treatment with mevalonic acid.

It has been previously reported that treatment with statins reduces RhoA GTPase activation, thereby diminishing monocyte stable adhesion to vascular endothelium (Yoshida et al., 2001). The present findings extend the role of statin in modulation of the MCP-1-dependent conversion of THP-1 cells from rolling to stable adhesion, as well as subsequent signalling in THP-1 cells. Our results showed that slight reduction of CCR2 protein expression on the surface of THP-1 cells by pitavastatin, although its mRNA levels remained unchanged after pitavastatin treatment (Fig. 2A, B). These divergent effects of pitavastatin on mRNA and protein of CCR2 may require further investigation including potential changes in mRNA stability of CCR2. It was previously demonstrated that statin modulated the expression levels of adhesion molecules in monocytes following interferon γ-dependent signal transduction in an endothelial-like cell line (Chung et al., 2002), however, the observed reduction of CCR2 expression as shown in Fig. 2 does not fully explain the dramatic reduction of adhesion. Interestingly, in the present assay conditions, inhibition of RhoA, but not of ERK-1/2, reduced MCP-1-mediated THP-1 cell adhesion, suggesting that RhoA and ERK-1/2 independently regulate MCP-1-mediated signals in THP-1 cells, while RhoA GTPase plays a predominant role in the enhancement of adhesion induced by MCP-1. Although the importance of ERK-1/2 was stressed in a previous study (Ashida et al., 2001), we failed to find a relationship between ERK activation and MCP-1-dependent adhesion of THP-1 cells under our assay conditions. We utilized a well defined simulated flow chamber apparatus in an attempt to mimic monocyte recruitment that occurs in vivo, whereas that study individually observed cell adhesion under static conditions as well as chemotaxis using a Boyden chamber to determine specific intracellular signaling. These differences in experimental settings must be taken into account to fully understand the complex nature of monocyte recruitment to vascular endothelium in inflammation and atherosclerosis.

In conclusion, we demonstrated that treatment with statins dramatically reduced monocytic THP-1 cell conversion from rolling to adhesion induced by MCP-1 through the RhoA-dependent pathway. Our results suggest an anti-inflammatory role for pitavastatin.

Acknowledgements

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E-Selectin Blockade Decreases Adventitial Inflammation and Attenuates Intimal Hyperplasia in Rat Carotid Arteries After Balloon Injury

Ryo Gotoh, Jun-ichi Suzuki, Hisanori Kosuge, Tsunekazu Kakuta, Shinji Sakamoto, Masayuki Yoshida, Mitsuaki Isobe

Objective—Inflammation is one of the initial repair processes after vascular injury. E-selectin facilitates adherence of leukocytes to vascular endothelium at the site of inflammation. Because the role of E-selectin in this process is not fully understood, we studied the role of E-selectin in vascular injury with a flow chamber model and a rat model of carotid artery injury.

Methods and Results— We established a rat aortic endothelial cell (RAEC) culture system from the aortas of adult male rats. When rat myelomonocytes were suspended in a flow chamber, rolling and adhesion to lipopolysaccharide (LPS)-stimulated RAECs were observed. Cell rolling and adhesion were greatly reduced by addition of anti–E-selectin monoclonal antibody (mAb). We then induced balloon injury in the left carotid arteries of rats. E-selectin expression was enhanced in endothelial cells at adventitial small vessels 7 days after injury. Rats with balloon injury were injected intraperitoneally with anti–E-selectin mAb for 8 days. Inflammatory cell infiltration was reduced by anti–E-selectin mAb treatment at the adventitia at 7 days after injury. This reduction was associated with attenuation of intimal hyperplasia in the rats treated with the mAb.

Conclusions— These data suggest that E-selectin regulates adventitial inflammation through leukocyte adhesion and contributes to the process of intimal hyperplasia after balloon injury. (***Arterioscler Thromb Vasc Biol.* 2004; 24:2063-2068.)**

Key Words: adhesion molecules ■ adventitial inflammation ■ angioplasty ■ rat aortic endothelial cell ■ restenosis

Restenosis after percutaneous catheter intervention remains a serious clinical problem despite the development of new catheterization devices.[1–5] Neointima formation is caused by vascular smooth muscle cell proliferation. Various factors are involved in this process.[3–5] Among them, cell adhesion molecules are essential for the development of atherosclerosis.[6–8] A number of soluble factors are expressed by endothelial cells and recruited leukocytes.[7,9] P-selectin is one such cell adhesion molecule and is an established indicator of chronic and acute atherosclerotic events.[10–13] Elevated plasma levels of soluble P-selectin (sP-selectin) are associated with increased vascular injury in acute and chronic coronary artery disease (CAD).[12] We reported previously that anti–P-selectin monoclonal antibody (mAb) attenuates inflammatory responses and inhibits neointima formation in association with platelet accumulation after balloon injury of the carotid artery in rats.[13]

E-selectin assists in the rolling of leukocytes on activated endothelial cells and is expressed on the endothelium of atherosclerotic lesions.[7,9] Several studies have shown elevated plasma levels of soluble E-selectin (sE-selectin) in CAD. In one study, sE-selectin levels did not differ significantly between CAD patients and control subjects,[14] whereas other studies indicated that sE-selectin levels increased after angioplasty.[15,16] Examination of expression of cell adhesion molecule mRNAs in atherectomy specimens from patients with CAD revealed that expression of E-selectin mRNA was increased in restenosed coronary arteries in comparison with de novo lesions.[17] In peripheral arteries, vessel patency after angioplasty is associated with low levels of sE-selectin.[18] Despite these clinical findings, the role of E-selectin in acute vascular injury has not been investigated in animals. It is not known if blockade of E-selectin activity can attenuate intimal hyperplasia after vascular injury.

Thus, in the present study, we developed a new anti-rat E-selectin mAb and evaluated roles of E-selectin in the neointimal formation. We used an in vivo rat carotid artery balloon injury model and an in vitro flow chamber leukocyte

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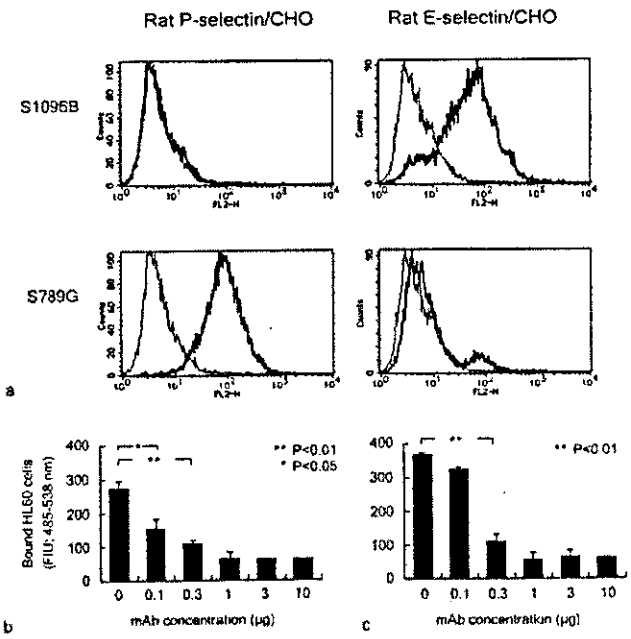

Figure 1. Characterization of mAb S1096B against E-selectin and mAb S789G against P-selectin. a, Binding specificity of S1096B (upper panel) and S789G (lower panel). Rat P-selectin–expressing CHO cells and rat E-selectin–expressing CHO cells were stained with mAbs (bold lines) or isotype control antibodies (thin lines). b, S1096B inhibited the binding of HL-60 cells to rat E-selectin–expressing CHO cells in a concentration-dependent manner. c, S789G inhibited the binding of HL-60 cells to rat P-selectin–expressing CHO cells in a concentration-dependent manner. Data are expressed as mean±SEM. FIU indicates fluorescence intensity unit; CHO, Chinese hamster ovary; mAb, monoclonal antibody.

adhesion model to test our hypothesis that expression of E-selectin can be a target for therapeutic intervention to reduce restenosis after angioplasty.

Methods

Animals

All animals were obtained from Japan SLC, Inc (Shizuoka, Japan) and were housed and handled as approved by the Institutional Animal Use and Care Committee of Tokyo Medical and Dental University. All experiments were conducted in conformity with the *Institutional Guidelines of Tokyo Medical and Dental University.*

Preparation of Anti-Rat E-Selectin MAb

Anti-rat E-selectin mAbs were generated by immunizing female BALB/c mice by footpad injection of 2 to 5×10^6 rat E-selectin–expressing Chinese hamster ovary (E-CHO). Popliteal lymph node cells were fused to mouse myeloma cells from the Japanese Collection of Research Bioresources (JCRB0113) with PEG4000 (Life Technologies, Inc, Grand Island, NY), and supernatants from the hybridomas were screened for the ability to bind to E-CHO cells. Cell binding assay was performed as described. The adhesion-blocking mAb was named S1096B. Anti-rat P-selectin mAb (S789G) was generated as previously described.[13,19] Specificities of these mAbs were confirmed by flow cytometry (Figure 1a). Flow cytometry was performed on a fluorescence-activated cell sorter Calibur (BD Bioscience) with these mAbs coupled to fluorescein isothiocyanate.

In Vitro Cell Binding Assay (Static)

E-CHO cells were plated at a concentration of 2×10^5 cells per well in 24-well multiplates and cultured overnight at 37°C under 5% CO_2. E-CHO monolayers were washed with phosphate-buffered saline and incubated with anti–E-selectin mAb at various concentrations (0.1, 0.3, 1, 3, 10 μg/mL) for 30 minutes at 4°C. Cultured HL-60 cells were suspended at a concentration of 1×10^7 cells/mL in RPMI1640 (Sigma, St. Louis, Mo) containing 10% fetal bovine serum (FBS) (Sigma). 3′-O-acetyl-2′,7′-bis (carboxyl-ethyl)-carboxyfluorescein diacetoxymethyl ester (BCECF-AM) was added to the cell suspension to a final concentration of 3 μmol/L. The cell suspension was incubated at 37°C for 30 minutes, and labeled cells were resuspended at a concentration of 2.5×10^5 cells/mL in RPMI1640 containing 1% FBS. Cell suspensions were plated at 200 μL/well, incubated for 30 minutes at 4°C, centrifuged at 120 rpm, and washed with phosphate-buffered saline containing 1% FBS. The attached cells were lysed with 1% Triton X-100 (Sigma). The fluorescence intensity unit of the cell lysates was measured with an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

Rat Aortic Endothelial Cell Culture

Three male Sprague–Dawley rats weighing 300 to 400 grams were killed by an overdose injection of pentobarbital sodium. The thoracic aorta was removed immediately and washed with RPMI1640 containing 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Inc). The aorta was filled with 1000 U/mL dispase (Godo Shusei, Tokyo, Japan) and 1% collagenase (Wako Pure Chemical Industries, Osaka, Japan) and incubated for 30 minutes at 37°C. The solution was then collected and centrifuged for 5 minutes at 1500 rpm. The supernatant was removed, and the cells were resuspended in RPMI1640 containing 10% FBS with 100 U/mL penicillin and 100 μg/mL streptomycin and 1 μg/mL amphotericin B (Sigma) and cultured on a rat collagen-I–coated dish (BD Biosciences). Rat aorta endothelial cells (RAECs) were identified by their characteristic cobblestone appearance, and identification was confirmed by the uptake of acetylated low-density lipoprotein labeled with 1,1′-dioctadecyl-1 to 3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI-Ac-LDL; Biomedical Technologies, Inc., Stoughton, Mass).[20] RAECs at passages 5 through 7 were used for experiments.

Dynamic Flow Assay

Two hundred microliters of RAEC suspension was put on a rat collagen-I–coated coverslip (BD Biosciences) and placed over a 6-well plate at a seeding density of 3×10^5 cells/mL. Three days later, RAECs were stimulated by 1 μg/mL lipopolysaccharide (LPS) (from *Escherichia coli* serotype O127:B8; Sigma) for 4 hours. Cells were washed twice with RPMI1640 containing 1% FBS and incubated with or without antibody (10 μg/mL) S1096B or MOPC-21 (non-immunized mouse IgG1; PharMingen, San Diego, Calif). The flow chamber was constructed as described previously.[21] A plastic heating plate (Tokai Hit Co) was mounted on the stage of an inverted microscope (IX50; Olympus) to maintain the temperature of the chamber at 37°C. The coverslip was attached to the chamber placed on the microscope stage. The system was then filled with 1×10^6 cells/mL of rat myelomonocytic cell (c-WRT-7-LR, Health Science Research Resources Bank, JCRB0168)[22,23] suspended in adhesion media (Dulbecco modified phosphate-buffered saline + 0.9 mmol/L $CaCl_2$ + 0.2% human albumin). This suspension was drawn off at controlled flow rates with a syringe pump (Model 44; Harvard Apparatus) connected to the outlet flow chamber to generate calculated wall shear stresses of 1.0 dyne/cm^2 for 10 minutes. The perfusion period was videotaped with a digital video recorder containing a time generator. The captured images were transferred to a PC computer for image analysis to determine the number of rolling and adherent c-WRT-7-LR cells in 5 to 10 randomly selected 200× microscopic fields for each experiment. Cells were considered adherent after 10 seconds of stable contact with the monolayer. Rolling leukocytes were easily recognized because their velocities were much slower (up to 80 μm/s) than those of free-flowing cells.

Vascular Injury Model

Male Sprague–Dawley rats were anesthetized by intraperitoneal administration of pentobarbital sodium (50 mg/kg). The rat carotid

arteries were dilated and denuded of endothelium with a 2-French Fogarty balloon embolectomy catheter (Baxter Health Care) introduced into the left common carotid artery through the external carotid artery.[24,25] The catheter was retracted 3 times. After the catheter was removed, the external carotid artery was ligated.

MAb Treatment Study

Rats were assigned randomly to 1 of 4 treatment groups: MOPC-21 (nonimmunized IgG) treatment (group C), S1096B (anti–E-selectin mAb) treatment (group E), S789G (anti–P-selectin mAb) treatment (group P), and both S1096B and S789G treatment (group E+P). MAbs were administered intraperitoneally to rats at a dosage of 4 mg/kg 30 minutes before arterial injury and once daily for 7 consecutive days after injury as previously reported.[13] Carotid arteries were harvested 14 days (n=8, each group) and 56 days (n=8, each group) after balloon injury.

Histology and Morphometry

Treated rats were euthanized by overdose injection of pentobarbital, and the injured carotid artery was then perfusion-fixed at 100 mm Hg and embedded in paraffin. Each artery was stained with van Gieson elastin stain and subjected to blinded morphometric examination under a video microscope (HC-300I; Nikon) equipped with a computerized digital image analysis system (SCION Image, public domain software). The areas of the external elastic lamina (EEL), the internal elastic lamina (IEL), and the lumen were measured. Medial and neointimal areas were calculated as follows: medial area=EEL area − IEL area; neointimal area=IEL area − lumen area; neointima/media (I/M) ratio=neointimal area/medial area. The circumferences (lengths) of the EEL and IEL were also measured to determine vascular shrinking.

Immunohistochemical Study

Harvested carotid arteries were immediately embedded in optimal cutting temperature compound and frozen at −20°C. Immunohistochemical analysis was performed on the frozen sections. A Vectastain Elite ABC kit (Vector Laboratories) was used on the sections with S1096B (anti–E-selectin), S789G (anti–P-selectin), anti-CD31 antibody (PharMingen), anti-CD45 antibody (PharMingen), anti–ED-1 antibody (Cosmo Bio, Tokyo, Japan), and MOPC-21 (nonimmunized IgG) as primary antibodies. Sections were incubated with diaminobenzidine (Vector Laboratories) at 100 mg/mL for 5 minutes, counterstained with hematoxylin, and then mounted permanently with coverslips.

Statistical Analysis

All data are presented as mean±SEM. Experimental groups were compared with 1-way analysis of variance and a post hoc test (Fisher protected least significant difference) for multiple comparisons. Values derived from the dynamic flow assay were compared by Mann–Whitney U test. $P<0.05$ were considered statistically significant.

Results

Characterization of Anti–E-Selectin Monoclonal Antibody (S1096B)

S1096B is a mouse IgG1 antibody. The specificity of this anti–E-selectin mAb was confirmed by flow cytometry by binding to E-CHO cells. S1096B bound to E-CHO cells but not to P-selectin–expressing CHO cells (P-CHO). S789G bound to P-CHO cells, but not to E-CHO cells (Figure 1a). HL-60 cells adhered to E-CHO cells untreated with antibody. S1096B blocked adhesion of HL-60 cells to E-CHO cells in a concentration-dependent manner (Figure 1b). S789G blocked adhesion of HL-60 cells to P-CHO cells in a concentration-dependent manner (Figure 1c).

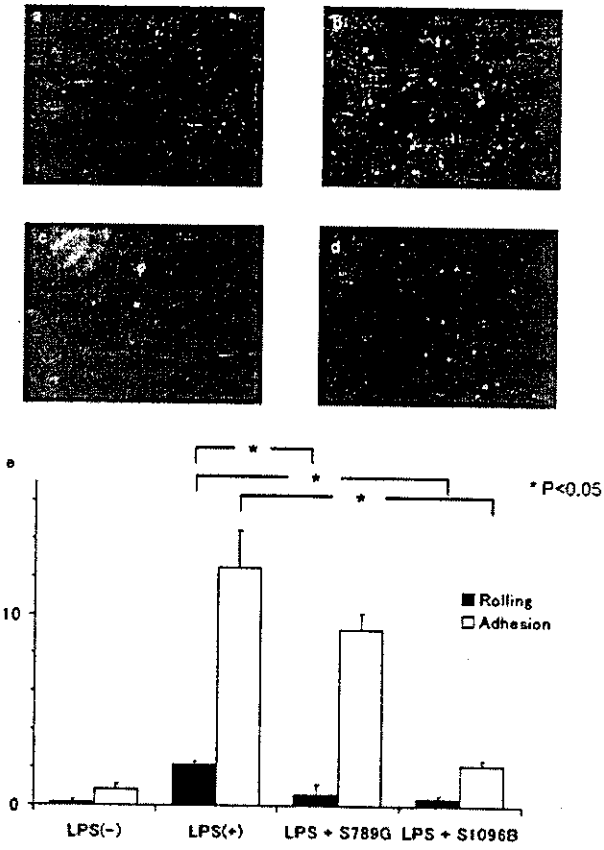

Figure 2. Effect of mAb S1096B against E-selectin and mAb S789G against P-selectin under physiological flow conditions. Rat myelomonocytic cells (c-WRT-7-LR) were suspended in a flow chamber with a rat aorta endothelial cell (RAEC)-cultured coverslip. a, The cells did not adhere to unstimulated RAECs. b, The cells did adhere to RAECs stimulated with LPS. c, S1096B inhibited this adhesion. d, S789G less inhibited this adhesion. e, Number of c-WRT-7-LR cells adhered to stimulated RAECs. The number of rolling and adhering cells was reduced by treatment with S1096B. Addition of S789G reduced the number of rolling cells but did not reduce the number of adherent cells. Data are expressed as mean±SEM. Bar=10 μm.

S1096B Blocks E-Selectin–Dependent Rolling and Adhesion Under Flow Conditions

Under flow conditions, c-WRT-7-LR cells did not roll or adhere to unstimulated RAECs (0.86±0.26 cells/field) (Figure 2a). When RAECs were stimulated with LPS, adhesion of c-WRT-7-LR cells was detected (data not shown). This adhesion was not changed after incubation with MOPC-21 (nonimmune IgG) (12.50±1.97 cells/field) (Figure 2b and 2e) but was reduced significantly by addition of S1096B (2.13±0.30 cells/field; $P<0.01$) (Figure 2c and 2e). Addition of S789G did not significantly reduce adhesion (Figure 2d and 2e). Rolling of c-WRT-7-LR cells was observed after RAECs stimulated with LPS were incubated with MOPC-21 (2.13±0.72 cells/field). Rolling was significantly reduced by treatment with S1096B or S789G (0.38±0.18 cells/field, 0.60±0.51 cells/field; $P<0.05$) (Figure 2e).

Expression of E-Selectin After Vascular Injury In Vivo

Representative photomicrographs of arteries harvested and sectioned 7 days after balloon injury and stained with

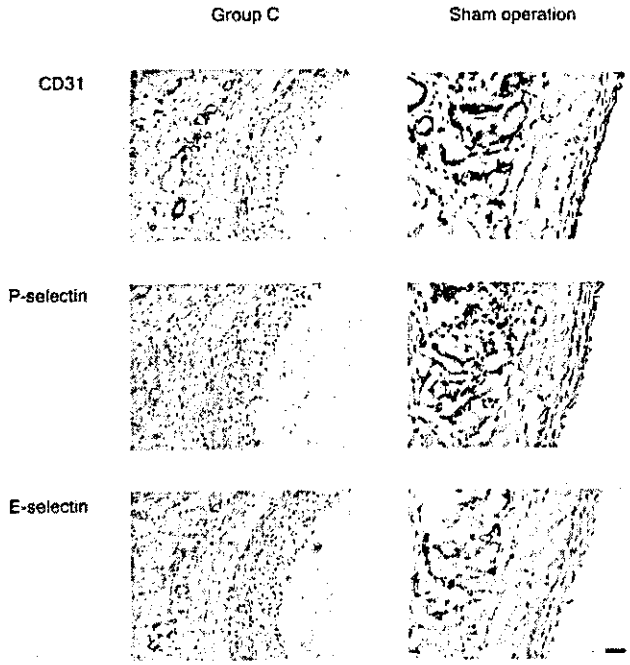

Figure 3. Representative frozen sections of rat carotid arteries at 7 days after balloon injury treated with control IgG (group C) and those of uninjured artery (sham operation). Immunostaining with anti-CD31 antibody, anti-P-selectin antibody, and anti-E-selectin antibody. In group C, E-selectin and P-selectin staining was enhanced in adventitial endothelial cells in the *vasa vasorum*. In the sham operation group, faint staining of both selectins was noted. Bar=10 μm.

antibody to E-selectin or P-selectin are shown in Figure 3. Positive staining was not observed in sections stained with control IgG (data not shown). We used anti-CD31 antibody to visualize endothelial cells. Most CD31-positive cells were present in the adventitia, indicating that the injured lumina had not yet re-endothelialized at 7 days after injury. E-selectin and P-selectin staining of adventitial endothelial cells was increased. In the sham operation group, faint staining of both selectins was detected.

S1096B Blocks E-Selectin–Dependent Leukocyte Accumulation

We examined the effect of S1096B on leukocyte accumulation after vascular injury by immunohistochemistry. We used anti-CD45 antibody to detect leukocytes and anti-ED-1 antibody to detect macrophages (Figure 4a) and analyzed percentages of CD45-positive and ED-1–positive cells over time (Figure 4b). At days 1 and 3 after injury, the intima and media could not be clearly differentiated. In the early period after vascular injury, CD45-positive and ED-1–positive cells accumulated predominantly in the adventitia. With the progression of intimal hyperplasia, these cells transmigrated into the intima. The accumulation of CD45-positive and ED-1–positive cells in the adventitia was inhibited by treatment with S1096B.

Inhibition of E-Selectin–Dependent Adhesion Attenuates Injury-Induced Intimal Hyperplasia and Vascular Remodeling

There was no significant difference in body weight between groups of rats. Morphometric analysis is shown in Figure 5a.

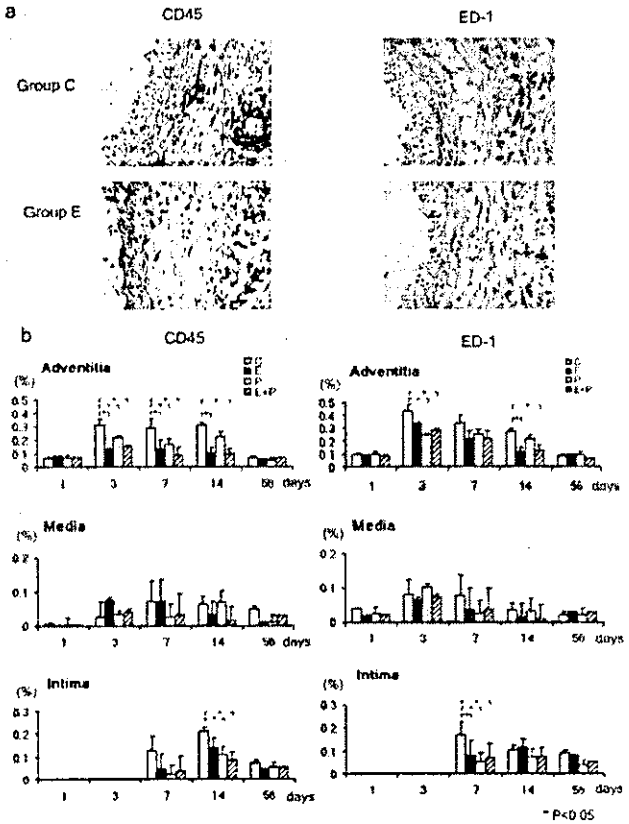

Figure 4. a, Representative frozen sections of rat carotid arteries at 7 days after balloon injury treated with control IgG (group C) and anti-E-selectin mAb (group E). b, Analysis of CD45-positive and ED-1–positive cells in the 4 groups over time. Rats were treated with control IgG (group C), anti-E-selectin mAb (group E), anti-P-selectin mAb (group P), or anti-E-selectin and anti-P-selectin mAbs (group E+P). Left, Percentages of CD45-positive cells in the adventitial (upper), medial (middle), and intimal (lower) layers over time. Right, Percentages of ED-1–positive cells in the adventitial (upper), medial (middle), and intimal (lower) layers over time. Data are expressed as mean±SEM.

The I/M ratio of group E was significantly less than that of group C at 8 weeks after injury (1.49±0.20 versus 2.07±0.14; $P<0.05$). The ratios at 2 weeks after injury did not differ statistically (1.40±0.11 versus 1.62±0.15) (Figure 5a, left). The I/M ratio of group P and that of group E+P were significantly lower than that of group C at 2 weeks after injury (1.16±0.17 and 1.10±0.09, respectively, versus 1.62±0.15, $P<0.05$). The I/M ratio of group P was not different from that of group E+P at 2 and 8 weeks after injury. Thus, a synergistic effect of dual blockade of E-selectin and P-selectin was not identified. The neointimal areas in group E and group P were reduced compared with that in group C at 8 weeks after injury. The medial area in each of the 4 groups did not differ significantly at 8 weeks after injury (Figure 5a, right).

Morphometric analysis showed that IEL and EEL lengths are significantly greater in the mAb treatment groups (group E, group P, and group E+P) than group C at 8 weeks after injury indicating suppression of negative remodeling (Figure 5b). A synergistic effect of dual blockade of E-selectin and P-selectin was not identified in this analysis. Representative

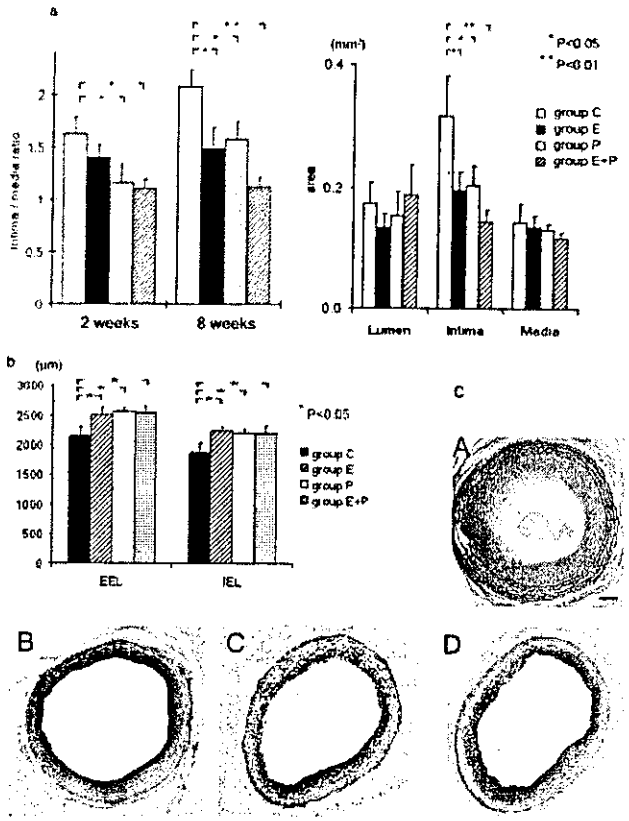

Figure 5. a, Morphological analysis of rat carotid arteries after balloon injury. Rats were treated with control IgG (group C), anti–E-selectin mAb (group E), anti–P-selectin mAb (group P), or anti–E-selectin and anti–P-selectin mAbs (group E+P). Left panel shows analysis of intima/media (I/M) ratio at 2 weeks and 8 weeks after injury. Right panel shows the neointimal area and the medial area in each group at 8 weeks after injury. b, Morphometric analysis of internal elastic lamina circumference length (IEL) and external elastic lamina circumference length (EEL) at 8 weeks after injury. These parameters in group E, group P, or group E+P are significantly greater than that in group C. c, Photomicrographs of representative cross-sections of rat left common carotid arteries in each group at 8 weeks after injury. A indicates group C; B, group E; C, group P; D, group E+P. Data are expressed as mean±SEM. Bar=100 μm; van Gieson elastin stain.

images of left common carotid artery sections in the 4 groups at 8 weeks after injury are shown in Figure 5c.

Discussion

In the present study, we found that anti–E-selectin mAb S1096B blocked rolling and adhesion of myelomonocytes on aortic endothelial cells in an in vitro flow chamber model. Our in vivo study showed that anti–E-selectin mAb attenuated intimal hyperplasia after balloon injury, with significantly reduced infiltration of leukocytes in the adventitia.

E-selectin mediates rolling of leukocytes at the site of inflammation,[7,9] and expression of E-selectin is limited to endothelial cells in inflamed tissue.[9] E-selectin cannot play a role in the earliest phases of acute inflammation because de novo gene transcription is necessary for expression.[9] Our immunohistochemical studies showed that normal vessel walls do not express E-selectin. Similarly, myelomonocytes did not adhere to unstimulated RAECs. We found that injured lumina had not re-endothelialized at 7 days after balloon injury and that E-selectin was not expressed on the luminal side but was expressed on endothelial cells in the adventitia. Most inflammatory cells were identified in the adventitia, particularly in the *vasa vasorum*, and anti–E-selectin mAb reduced the infiltration of inflammatory cells in this region at this stage. Thus, E-selectin plays a role in leukocyte accumulation in the adventitia in our model of vascular injury.

Mechanical injury of arteries leads to the infiltration of inflammatory cells;[24] release of chemokines,[26] cytokines,[27] and other chemical mediators;[28] transmigration of monocytes and macrophages;[6] and proliferation of vascular smooth muscle cells.[6,7,24,26,27] Our findings indicate that suppression of inflammatory cell infiltration into the adventitia by anti–E-selectin mAb is associated with reduced intimal hyperplasia after balloon injury. Thus, inflammation in the adventitia could be an important factor in the development of intimal hyperplasia and vascular shrinkage.

The mechanism of adventitial inflammation in intimal hyperplasia is unknown. The intensity of the adventitial inflammatory response correlates with the severity of atherosclerosis and restenosis after balloon angioplasty.[29,30] Although we did not determine the role of E-selectin in these processes in the present study, there are several possible mechanisms. For example, inflammatory cells that infiltrate in response to enhanced E-selectin expression in the adventitia may release a variety of chemical mediators, including cytokines, chemokines, and growth factors, which promote transmigration and proliferation of vascular smooth muscle cells in the media and lead to hyperplasia of the intima. In addition, our data show improvement of vascular shrinkage after mAb treatment, indicating that inflammation of the adventitia leads to negative remodeling of the injured vessels. This remodeling could be reduced by anti-selectin treatment.

Our previous study showed that an anti–P-selectin mAb reduces the accumulation of leukocytes in the adventitia and neointima and prevents neointimal formation at 2 weeks after balloon injury.[13] Similar results were obtained in the present study. According to our previous study,[13] expression of P-selectin on platelets is important for suppression of inflammation and for reduction of intimal formation. In the present study, anti–E-selectin mAb significantly attenuated intimal hyperplasia at 8 weeks after injury. However, it did not attenuate intimal hyperplasia at 2 weeks after injury. Unlike E-selectin, P-selectin expression by endothelial cells and platelets does not require de novo gene transcription. Therefore, P-selectin responds immediately to acute inflammation that is caused by arterial injury. These differences between the 2 selectins may account for their different roles in intimal thickening. We suggest that adventitial inflammation mediates late-stage intimal hyperplasia via expression of E-selectin. This is supported by our analysis of inflammatory cell infiltration overtime, as shown in Figure 4. Cell infiltration in the adventitia was observed initially at 3 days after induction of injury. This was followed by inflammation in the intima at 7 to 14 days after injury and by intimal hyperplasia at later stages. Anti–E-selectin mAb suppressed early-stage adventitial infiltration. Therefore, it is reasonable to speculate that inhibition of adventitial inflammation by blockade of

E-selectin–dependent cell adhesion leads to the attenuation of intimal hyperplasia observed at 8 weeks after injury.

Because P-selectin and E-selectin play different roles in the recruitment of leukocytes into the site of inflammation, a synergistic action of dual blockade of the 2 selectins was expected. However, we did not observe such synergism in the present study. Tendencies toward decreased I/M ratios and intimal areas but without statistical significance were detected. Also, the IEL and EEL lengths of group P and group E were not different from those of group E+P at 8 weeks after injury. Possible synergism of these selectins will be studied in future experiments.

In conclusion, these data suggest that E-selectin controls adventitial inflammation through leukocyte adhesion and contributes to the process of intimal hyperplasia in the late stage after balloon injury. Blockade of E-selectin may be a new strategy to control restenosis after coronary balloon angioplasty.

Acknowledgments

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Pitavastatin Inhibits Remnant Lipoprotein-Induced Macrophage Foam Cell Formation Through ApoB48 Receptor–Dependent Mechanism

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Objective—Atherogenic remnant lipoproteins (RLPs) are known to induce foam cell formation in macrophages in vitro and in vivo. We examined the involvement of apoB48 receptor (apoB48R), a novel receptor for RLPs, in that process in vitro and its potential regulation by pitavastatin.

Methods and Results—THP-1 macrophages were incubated in the presence of RLPs (20 mg cholesterol/dL, 24 hours) isolated from hypertriglyceridemic subjects. RLPs significantly increased intracellular cholesterol ester (CE) and triglyceride (TG) contents (4.8-fold and 5.8-fold, respectively) in the macrophages. Transfection of THP-1 macrophages with short interfering RNA (siRNA) against apoB48R significantly inhibited RLP-induced TG accumulation by 44%. When THP-1 macrophages were pretreated with pitavastatin (5 μmol/L, 24 hours), the expression of apoB48R was significantly decreased and RLP-induced TG accumulation was reduced by 56%. ApoB48R siRNA also inhibited TG accumulation in THP-1 macrophage induced by β–very-low-density lipoprotein derived from apoE−/− mice by 58%, supporting the notion that apoB48R recognizes and takes-up RLPs in an apoE-independent manner.

Conclusions—RLPs induce macrophage foam cell formation via apoB48R. Pitavastatin inhibits RLP-induced macrophage foam cell formation. The underlying mechanism involves, at least in part, inhibition of apoB48R-dependent mechanism. Our findings indicate a potential role of apoB48R in atherosclerosis. (***Arterioscler Thromb Vasc Biol.* 2005; 25:424-429.**)

Key Words: remnant lipoproteins ■ foam cell formation ■ apoB48 receptor ■ statin ■ atherosclerosis

Clinical studies have revealed that remnant lipoproteins (RLPs), which are produced by hydrolysis of chylomicrons (CMs) and very-low-density lipoproteins (VLDL), are closely related to atherosclerosis, independent of high-density lipoprotein and low-density lipoprotein (LDL).[1,2] There is also increasing evidence that RLPs play a causative role in atherogenesis, and we recently reported that they induced monocyte-endothelial interaction and vascular smooth muscle cell proliferation.[3,4] However, the effect(s) of RLPs on cellular mechanism(s) during atherogenesis have not been fully elucidated.

Atherogenesis involves the appearance of lipid-loaded foam cells derived from macrophages in the arterial intima. RLPs from hypertriglyceridemic VLDL and CMs cause rapid lipid accumulation, and induce foam cell formation in macrophages, whereas normal VLDL and LDL do not.[5] Thus, inhibition of macrophage foam cell formation induced by RLPs may contribute to the prevention of atherosclerosis. It has been reported that mechanisms independent of apolipoprotein (apo) E and LDL receptor family are involved in lipid accumulation in macrophages.[6] Recently, apoB48 receptor (apoB48R) was shown to be involved in the uptake of triglyceride-rich lipoproteins (TRLs) and contribute to atherogenesis,[7] although the role of apoB48R in this process remains unclear. Herein, we report for the first time to our knowledge the dominant role of apoB48R in RLP-induced foam cell formation by selective downregulation of apoB48R with short interfering RNA (siRNA).

Recently, 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase inhibitor, or statin, has been suggested to have beneficial effects for the prevention of atherosclerosis, independent of its LDL cholesterol-lowering effect.[8] We found that statins reduced monocyte adhesion to endothelial cells via inhibition of Rho GTPase pathway.[3,9] In the present study, treatment with pitavastatin lowered RLP-induced macrophage foam cell via, at least in part, inhibition of the expression of apoB48R in THP-1 macrophages. Our results suggest that apoB48R pathway might be a novel therapeutic

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target for atherogenesis, particularly in hypertriglyceridemic patients. Further, pitavastatin may exert a beneficial effect on atherogenesis by suppressing this pathway.

Methods

Reagents and Cell Culture

Pitavastatin was kindly provided by Kowa Pharmaceutical Company, Tokyo, Japan. Phorbol-12-myristate-13-acetate (PMA), C3 exoenzyme, farnesyl-pyrophosphate (FPP), and geranylgeranyl-pyrophosphate (GGPP) were purchased from Wako. RPMI 1640, DMEM, and FBS were obtained from GIBCO BRL. Antibodies used in the present study were as follows: mouse anti-RhoA monoclonal antibody (Santa Cruz Biotechnology), rabbit anti-GAPDH antibody (Sigma), HRP-conjugated goat anti-mouse IgG, and HRP-conjugated goat anti-rabbit IgG (Cal-tag). Rabbit anti-human apoB48R antibody was a generous gift from Dr Sandra H. Gianturco and Dr William A. Bradley, The University of Alabama at Birmingham.

Human monocytic THP-1 cells were obtained from American Type Culture Collection and maintained in RPMI 1640 medium supplemented with 10% FCS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mmol/L L-glutamine in a humidified atmosphere of 5% CO_2 at 37°C. To obtain THP-1 macrophages, THP-1 cells were plated in 35-mm dishes (1×10^7 cells/dish) and incubated with PMA at a concentration of 200 nmol/L for 4 days. Human peripheral blood monocytes were isolated from healthy volunteers using Ficoll-Paque (Pharmacia Biotech) and a monocyte-negative isolation kit (Miltenyi Biotech). To examine cell viability, cells were stained with 4′, 6-Diamidino-2-phenylindole dihydrochloride (DAPI; 200 ng/mL) (Dojindo), and viable cells were counted at the required time point using a hemacytometer.

Lipoprotein Preparation

EDTA plasma was obtained from 24 patients with hypertriglyceridemia who showed an elevated RLP concentration (>7.5 mg cholesterol/dL) 4 hours after eating breakfast. They had no cardiovascular diseases or diabetes and had not taken cardiovascular medicine or antioxidants. The protocol of this study complied with the guidelines for the conduct of research involving human subjects by the Committee on Human Research at Tokyo Medical and Dental University. RLPs were isolated from plasma samples using an RLP-C Kit, as described previously,[10] then dialyzed overnight against 5 L of PBS containing 50 μmol/L EDTA (pH 7.4) and sterilized using a 0.22-μm filter unit (Millipore). The prepared RLPs were analyzed by SDS-PAGE in a 5% to 20% linear gradient gel (Funakoshi) and visualized with a silver stain reagent (Daiichi). Densitometry showed that apoB48 comprised only 5% to 7% of total apoB in RLPs, as we and others reported previously.[3,11] β-VLDL (density<1.006 g/mL) was isolated from freshly prepared plasma taken from apoE−/− mice (obtained from Jackson Laboratory, Bar Harbor, Me, and fed a normal diet) at 8 weeks of age by ultracentrifugation.

Oil Red O Staining

THP-1 macrophages were seeded into multi-well slides (Nunc) at a concentration of 1×10^6 cells per well. The cells were washed 3 times with phosphate-buffered saline, fixed with formaldehyde, and stained with oil red O. Lipid accumulation was observed under a microscope.

Cellular Lipid Analysis

To determine intracellular lipid contents, THP-1 macrophages were removed from the culture plates and washed twice with phosphate-buffered saline. Then, intracellular lipids were extracted using isopropanol/hexane. Cholesterol ester, triglyceride (TG), and protein mass were determined enzymatically.

Immunoblotting

Cells were disrupted in a lysis buffer and total cell lysates prepared. An equal amount of protein (10 μg) was subjected to SDS-PAGE, after which immunoblotting was performed using the indicated antibodies. Immunoreactive proteins were detected using an enhanced chemiluminescence advance (Amersham Pharmacia Biotech). For a RhoA translocation assay, cell lysates from the membranes and cytosol fractions were prepared as described previously.[9]

Transfection of siRNA Against ApoB48R

siRNA was designed to target the coding sequence of human apoB48R cDNA. The target sequences were directed to the single-strand region according to the predicted secondary RNA structure and sequences of the form $(AA/CA)N_{19}$ with GC contents of <70% were selected from this region.[12] Nineteen nucleotide RNAs followed by TT/TG were selected, then chemically synthesized and gel-purified. Sequences corresponding to the siRNA were nucleotides 1060 to 1079 for apoB48R coding region (GenBank accession number AF141332). Nonrelevant 19 RNAs were used to generate the control siRNA. Double-stranded siRNAs were generated and transfected into THP-1 macrophages, as described previously.[13] Transfection efficiency was evaluated using BLOCK-IT Fluorescent dsRNA (Invitrogen);[14] 64.3±13.8% of THP-1 macrophages were positive for fluorescein isothiocyanate 48 hours after transfection.

RhoA Pull-Down Assay

RhoA pull-down assay was performed using Rho activation kit (Upstate) following the manufacturer's protocol.[15]

Statistical Analysis

Results are presented as the mean±SD. Data were analyzed using analysis of variance (ANOVA), with a value of $P<0.05$ considered significant.

Results

RLPs Induce Foam Cell Formation in THP-1 Macrophages

We investigated whether RLPs could induce macrophage foam cell formation. First, THP-1 cells were incubated in the presence of PMA at a concentration of 200 nmol/L for 4 days for full differentiation. After the medium was replaced with fresh medium with or without RLPs, THP-1 cells were incubated for an additional 24 hours. Oil red O staining showed that treatment with RLPs induced foam cell formation in PMA-treated THP-1 macrophages (Figure 1). Intracellular TG and cholesterol ester contents in the cells were also significantly increased 24 hours after the addition of RLPs in a dose-dependent manner (Figure 1).

Expression of Remnant Receptors During Differentiation in THP-1 Macrophages

To elucidate the mechanism(s) by which RLPs induce macrophage foam cell formation, we examined the expression levels of representative remnant receptors and related proteins with immunoblotting.[16] During the differentiation process, immunoreactive LDL receptors nearly disappeared. In contrast, CD36, a scavenger receptor not present in THP-1 monocytes, appeared after PMA treatment, indicating that THP-1 monocytes differentiated into macrophages (Figure 2A). LDL receptor–related protein (LRP) was not detected throughout the differentiation process (data not shown). Interestingly, the expression level of apo48R, which is present in monocytes, remained unchanged throughout the

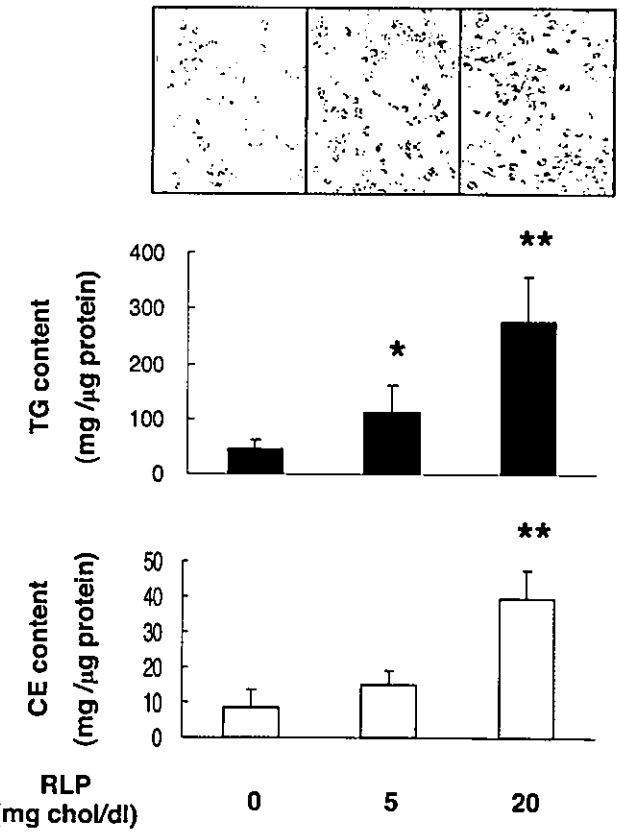

Figure 1. RLPs induce foam cell formation in THP-1 macrophages. THP-1 macrophages were incubated in the presence of RLPs at the indicated concentrations for 24 hours. Photos are representative oil red O staining results of 3 separate experiments. Bar graphs show cellular lipid contents in THP-1 macrophages (n=4). *$P<0.05$. **$P<0.01$ vs 0 mg cholesterol/dL.

differentiation process (Figure 2A) and was not downregulated by incubation with RLPs (Figure 2B).

siRNA Against ApoB48R Decreases RLP-Induced Foam Cell Formation in THP-1 Macrophages

To elucidate the specific role of apoB48R in RLP-induced foam cell formation, we introduced siRNA against apoB48R into THP-1 macrophages. Transfection of THP-1 macrophages with apoB48R siRNA reduced the expression of apoB48R 48 hours after transfection in an apoB48R siRNA dose-dependent manner. However, apoB48R siRNA did not affect the expression of GAPDH (Figure 3A) or that of CD36 (data not shown). When THP-1 macrophages were transfected with apoB48R siRNA, RLP-induced foam cell formation was significantly reduced (Figure 3B). Next, THP-1 macrophages were incubated with β-VLDL derived from apoE−/− mice, a model ligand of apoE-devoid remnant lipoproteins, because it has been reported that apoB48R takes-up TRLs in an apoE-independent fashion.[7,17] ApoB48R siRNA also reduced macrophage foam cell formation induced by β-VLDL. ApoB48R siRNA was more effective compared with the case of RLP-induced foam cell formation (Figure 3C). These results indicate that apoB48R plays a role, at least in part, in the taking up of RLPs, and that the suppression of apoB48R expression may reduce RLP-induced foam cell formation.

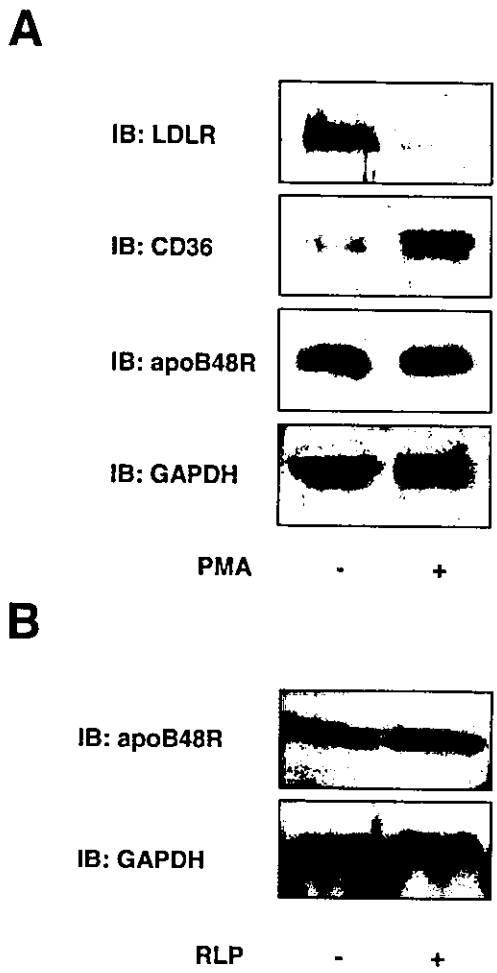

Figure 2. Expression of remnant receptors on THP-1 monocytes and THP-1 macrophages. A, The expression of remnant receptors and related proteins on THP-1 monocytes (PMA−) and THP-1 macrophages (PMA+) were detected with immunoblotting (blots are representative of 3 separate experiments). B, The expression of apoB48R on THP-1 macrophages was detected with immunoblotting before (−) and after (+) RLP treatment (20 mg cholesterol/dL, 24 hours) (blots are representative of 3 separate experiments).

Pitavastatin Inhibits RLP-Induced Foam Cell Formation in THP-1 Macrophages

To examine the effect of pitavastatin on RLP-induced foam cell formation, THP-1 macrophages were incubated with or without pitavastatin for 24 hours. THP-1 lipid contents, very low at baseline, were not significantly affected by treatment with pitavastatin (data not shown). Further, when THP-1 macrophages were pretreated with pitavastatin before RLP treatment, foam cell formation, detected by oil red O staining, was significantly inhibited, and the increments of intracellular TG and cholesterol ester content by RLPs were also significantly reduced by pretreatment with pitavastatin in a dose-dependent manner (Figure 4A).

Pitavastatin Suppresses ApoB48R Expression in THP-1 Macrophages Through RhoA-Dependent Mechanism

Next, we examined whether pitavastatin modulates the expression levels of apoB48R in the THP-1 macrophages. The

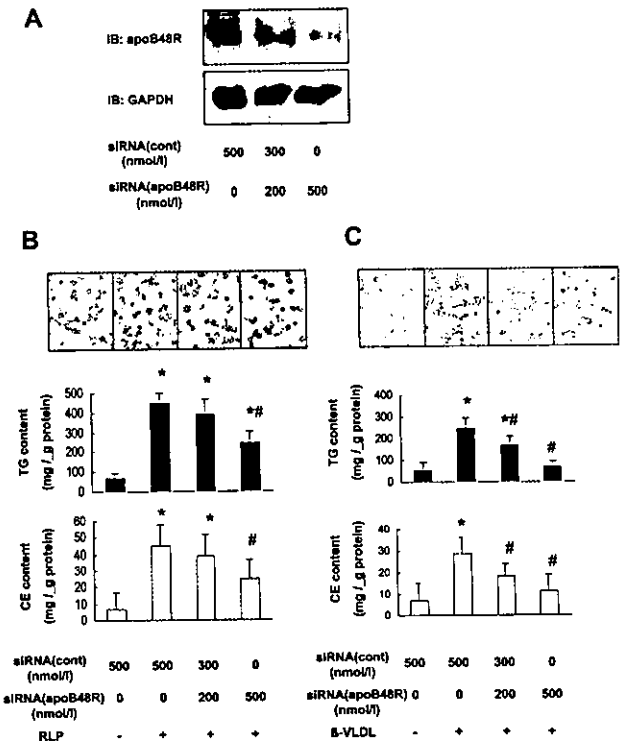

Figure 3. siRNA against apoB48R significantly decreases RLP-induced foam cell formation in THP-1 macrophages. A, THP-1 macrophages were transfected with apoB48R siRNA or control siRNA at the indicated final concentrations, as described in Methods. Forty-eight hours after transfection, the expression of apoB48R was detected with immunoblotting (blots are representative of 3 separate experiments). B, THP-1 macrophages were transfected as in A, and 48 hours after transfection, they were incubated with (+) or without (−) RLP (20 mg chol/dl, 24 hours). Photos are representative oil red O staining results of 3 separate experiments. Bar graphs show cellular lipid contents in THP-1 macrophages (n=4). *P<0.05 vs siRNA (cont) 500 nmol/l, siRNA (apoB48R) 0 nmol/l, RLP (−); #P<0.05 vs siRNA (cont) 500 nmol/l, siRNA (apoB48R) 0 nmol/l, RLP (+). C, THP-1 macrophages were transfected as in A, and 48 hours after transfection, they were incubated with (+) or without (−) β-VLDL (20 mg chol/dl, 24 hours). Photos are representative oil red O staining results of 3 separate experiments. Bar graphs show cellular lipid contents in THP-1 macrophages (n=4). *P<0.05 vs siRNA (cont) 500 nmol/l, siRNA(apoB48R) 0 nmol/l, β-VLDL (−); #P<0.05 vs siRNA (cont) 500 nmol/l, siRNA (apoB48R) 0 nmol/l, β-VLDL (+).

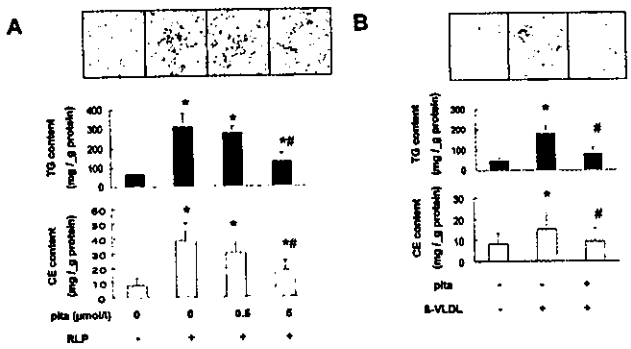

Figure 4. Pitavastatin inhibits RLP-induced foam cell formation in THP-1 macrophages. A, THP-1 macrophages were preincubated in the presence of pitavastatin (pita) at the indicated concentrations for 24 hours and then incubated with (+) or without (−) RLP (20 mg chol/dl, 24 hours). Photos are representative oil red O staining results of 3 separate experiments. Bar graphs show cellular lipid contents in THP-1 macrophages (n=4). *P<0.01 vs pita 0 μmol/l, RLP (−); #P<0.05 vs pita 0 μmol/l, RLP (+). B, THP-1 macrophages were preincubated in the presence (+) or absence (−) of pitavastatin (pita, 5 μmol/l, 24 hours) and then incubated with (+) or without (−) β-VLDL (20 mg chol/dl, 24 hours). Photos are representative oil red O staining results of 3 separate experiments. Bar graphs show cellular lipid contents in THP-1 macrophages (n=4). *P<0.05 vs pita (−), β-VLDL (−); #P<0.05 vs pita (−), β-VLDL (+).

expression level of apoB48R was significantly decreased after 24 hours of incubation with pitavastatin in a dose-dependent manner (Figure 5A). Statins are known to inhibit the mevalonate pathway, resulting in inhibition of the activity of RhoA, a Rho GTPase family member. Many studies have reported that statins exert their bioactive effects via inhibition of RhoA, independent of a lipid-lowering effect. Thus, we examined whether RhoA is involved in the reduction of apoB48R expression by pitavastatin. When THP-1 macrophages were pretreated with GGPP before pitavastatin treatment, reduction of apoB48R expression by pitavastatin was almost restored. In contrast, FPP was not so effective in recovering apoB48R expression compared with GGPP (Figure 5B). To examine whether pitavastatin treatment had an effect on RhoA activity, the GTP-binding capacity of RhoA was measured in THP-1 macrophages. As shown in Figure 5C, the amount of GTPγS-bound RhoA was reduced when THP-1 macrophages were treated with pitavastatin. Pitavastatin also decreased membrane translocation of RhoA in THP-1 macrophages (data not shown). In addition, C3 exoenzyme, a specific RhoA inhibitor, reduced apoB48R expression in THP-1 macrophages (Figure 5D) and inhibited RLP-induced foam cell formation (Figure 5E). Pitavastatin treatment did not affect the expression of LDL receptor and LRP in THP-1 macrophages (data not shown). Although pitavastatin suppressed CD36 expression by 15% at 5 μmol/L (data not shown), the reduction rate was much lower than that of apoB48R.

Pitavastatin Suppresses ApoE-Deficient β-VLDL Uptake by THP-1 Macrophages

To further confirm whether the inhibition of RLP-induced foam cell formation by pitavastatin is dependent on apoB48R, we examined the effect of pitavastatin on the uptake of β-VLDL derived from apoE−/− mice. THP-1 macrophages were incubated with apoE-deficient β-VLDL in the presence or absence of pitavastatin, and then lipid accumulation was evaluated. These β-VLDL induced macrophage foam cell formation, although to a lesser extent when compared with native RLPs. Consistent with the effect of pitavastatin on apoB48R expression, pretreatment with pitavastatin reduced lipid accumulation in THP-1 macrophages (Figure 4B), supporting the notion that this compound may serve to reduce lipid accumulation even in the absence of apoE-dependent pathway.

Pitavastatin Suppresses ApoB48R Expression in Human Peripheral Blood Macrophages

To demonstrate the involvement of apoB48R in RLP uptake and its potential regulation by pitavastatin in more pathophysiological conditions, we conducted experiments using human peripheral blood macrophages. Western blot analysis showed that pitavastatin treatment for 24 hours significantly reduced

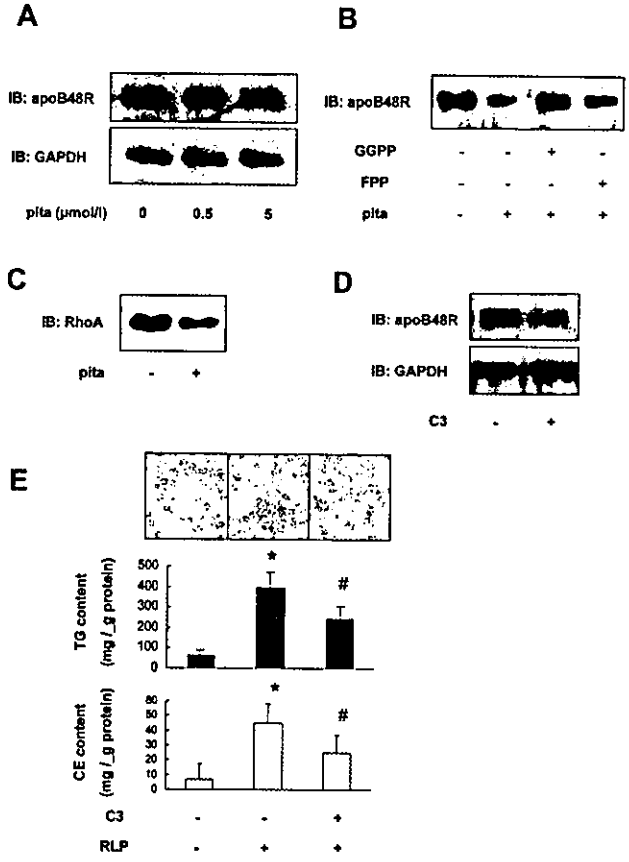

Figure 5. Pitavastatin suppresses apoB48R expression in THP-1 macrophages via RhoA-dependent mechanism. A, THP-1 macrophages were preincubated in the presence of pitavastatin (pita) at the indicated concentrations for 24 hours, then the expression of apoB48R on THP-1 macrophages was detected with immunoblotting (blots are representative of 4 separate experiments). B, THP-1 macrophages were preincubated in the absence (−) or presence (+) of GGPP (10 μmol/l, 24 hours) or FPP (10 μmol/l, 24 hours), then incubated in the absence (−) or presence (+) of pitavastatin (pita, 5 μmol/l, 24 hours) prior to immunoblotting (blots are representative of 3 separate experiments). C, THP-1 macrophages were preincubated in the presence (+) or absence (−) of pitavastatin (pita, 5 μmol/L, 24 hours), then RhoA activity in THP-1 macrophages was determined using RhoA pull-down assay, as described in Methods (blots are representative of 3 separate experiments). D, THP-1 macrophages were incubated in the presence (+) or absence (−) of C3 exoenzyme (30 μg/ml, 48 hours), then the expression of apoB48R on THP-1 macrophages was detected with immunoblotting (blots are representative of 3 separate experiments). E, THP-1 macrophages were preincubated as in (D) and then incubated with (+) or without (−) RLP (20 mg chol/dl, 24 hours). Photos are representative oil red O staining results of 3 separate experiments. Bar graphs show cellular lipid contents in THP-1 macrophages (n=3). *$P<0.05$ vs C3 (−), RLP (−); #$P<0.05$ vs C3(−), RLP (+).

apoB48R expression and lipid accumulation induced by RLPs in human peripheral blood macrophages (Figure I, available online at http://atvb.ahajournals.org), which were similar to the effects seen in THP-1 macrophages.

Discussion

It is known that excessive RLPs enter vascular walls, where they are taken-up by macrophages and induce foam cell formation in those cells. TRL remnants are found in atherosclerotic plaques;[18] however, the mechanisms by which RLPs induce foam cell formation have not been fully elucidated. The LDL receptor, known to bind RLPs, nearly disappear during the differentiation of monocytes into macrophages, whereas apoB48R is constantly present during the differentiation process, in striking contrast to LDL receptor families. Because apoB48R is detected in the reticulo-endothelial system, but not in hepatic cells, we focused on this unique remnant receptor and examined its role in RLP-induced macrophage foam cell formation. ApoB48R is found in human atherosclerotic plaques,[7] suggesting its involvement in the uptake of TRLs by macrophages. ApoB48R binds the apoB48 of dietary TRL (CM and CM remnant) or the apoB48-equivalent domain of apoB100 in hypertriglyceridemic VLDL.[17] Dietary TRLs, which lack the C-terminal domain of apoB100 that binds to the LDL receptor, cannot bind to the LDL receptor via apoB48, the major apoB species formed in the intestine. Thus, apoB48R may account for, at least in part, the observed direct macrophage uptake of TRLs in vivo and for foam cell formation seen in humans with elevated TRLs.[19,20]

The present results showed that gene silencing of apoB48R by siRNA directly leads to a successful inhibition of RLP-induced foam cell formation. Because apoB48R is an apoE-independent receptor in human and murine monocyte-macrophages,[21,22] we conducted experiments using β-VLDL devoid of apoE, which were also able to induce macrophage foam cell formation. Interestingly, the effect of apoB48R siRNA was more prominent in the case of β-VLDL treatment. Taken together, our findings indicate that apoB48R is involved in the uptake of RLP and foam cell formation, independent of an apoE-mediated pathway. Further, because the expression levels of apoB48R do not diminish after the cells accumulate extensive lipids, macrophages may allow further uptake of these lipoproteins by this pathway, as is the case with scavenger receptors. The relatively low affinity of nascent VLDL for apoB48R may account for the fact that they do not induce monocyte foam cell formation.

We also showed that pitavastatin inhibits RLP-induced foam cell formation. The expression levels of remnant receptors in THP-1 macrophages in the presence of pitavastatin were examined. Neither LDL receptor nor LRP were detected in those macrophages after treatment, indicating that pitavastatin does not affect the differentiation process once THP-1 cells have fully differentiated into macrophages. In the present study, the expression level of apoB48R was significantly reduced by treatment with pitavastatin and it also significantly reduced foam cell formation induced by RLP or β-VLDL loading.

Further, the effect of pitavastatin on other receptors related to RLP uptake was also examined. Pitavastatin did not affect LDL receptor and LRP. CD36, a scavenger receptor considered to be involved in atherogenic lipoprotein uptake, is also reported to be modulated by pitavastatin.[23] However, the effect of pitavastatin on CD36 was relatively small at the concentrations used in the present study, compared with its effect on apoB48R. It has been reported that statins have no effect on acyl CoA: cholesterol acyltransferase (ACAT) activity[24] and downregulate rather than upregulate ABCA1 in macrophages in vitro.[25] Considering that the gene silencing of apoB48R by siRNA significantly reduced RLP-induced foam cell formation (Figure 3), we believe that apoB48R is involved in the uptake of RLPs and that pitavastatin

decreased RLP uptake by macrophages at least in part by modulating apoB48R-dependent mechanism, although yet-unknown mechanism(s) may be involved in this process.

It is known that statins downregulate GGPP, an isoprenoid intermediate. Isoprenoids are important for post-translational modification of proteins, such as the small GTP-binding proteins. It has been reported that many of the cholesterol-independent effects of statins may be mediated by the inhibition of Rho GTPase. Thus, in the present study, we investigated whether apoB48R expression is modulated by RhoA-dependent pathway. The pitavastatin-mediated reduction of apoB48R expression was restored in the presence of GGPP, which bypass the HMG-CoA reductase-RhoA pathway. Moreover, C3 exoenzyme, a specific RhoA inhibitor, reduced apoB48R expression. Therefore, it may be possible that RhoA plays a role as a positive regulator of apoB48R expression and that pitavastatin decreases apoB48R expression by inhibiting the geranylgeranylation of RhoA.

The regulation of apoB48R expression remains unclear, although peroxisome proliferator-activated receptors were recently reported to inhibit apoB48R expression.[26] As shown in Figure 2, apoB48R was not regulated by sterol content or the state of differentiation of human and murine monocyte-macrophages. Our results are the first to show that pitavastatin suppresses apoB48R via a RhoA-dependent mechanism, independent of sterol content or state of differentiation. Further, we found that pitavastatin decreased the expression of apoB48R in human peripheral blood macrophages and RLP-induced foam cell formation. Based on these findings, we considered that this mechanism also potentially occurs in vivo.

In conclusion, our data indicate that foam cell formation induced by RLP is, at least in part, mediated by an apoB48R-dependent pathway. Therefore, apoB48R may be a novel therapeutic target for atherogenesis, particularly in hypertriglyceridemic patients.

Acknowledgments

The authors gratefully acknowledge support (10178102) and special coordination funds from the Ministry of Education, Science, Technology, and Culture of Japan. We also thank Dr Gianturco and Dr Bradley for providing the rabbit anti-human apoB48R antibody. The authors express their sincere appreciation to Naoko Tomie, Keiko Takahashi, and Noriko Nitta for their technical assistance.

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1 章 RNAi法の基礎知識－原理とメカニズム

1 RNAiのメカニズム

明石英雄　宮岸 真　川崎広明　多比良和誠

はじめに：RNAiとは何か

1998年，最初に線虫で報告されたRNA interference（RNAi，RNA干渉）は，二本鎖RNA（double-stranded RNA：dsRNA）によってその配列特異的にmRNAが分解され，その結果遺伝子の発現が抑制される現象である[1]（図1）．その後，RNAiは，線虫を含め，昆虫，植物，菌類などのさまざまな生物種間で保存されている現象であることが示され，生物共通の核酸レベルの防御システムであることが示唆されている[2]．RNAiを利用したノックダウン法は，簡便であり，しかも遺伝子発現の抑制に大きな効果が期待できるため，非常に話題を呼んでおり，近年，おびただしい数の文献が発表されてきている．当初，哺乳動物細胞への応用は難しいとされてきたが，最近，哺乳動物細胞に対する応用方法も確立されつつあり，医療分野にも大きく期待されるものとなってきた[3]．本項では，このRNAiと呼ばれる興味深い現象について，その原理と生物学的役割について概説したい．

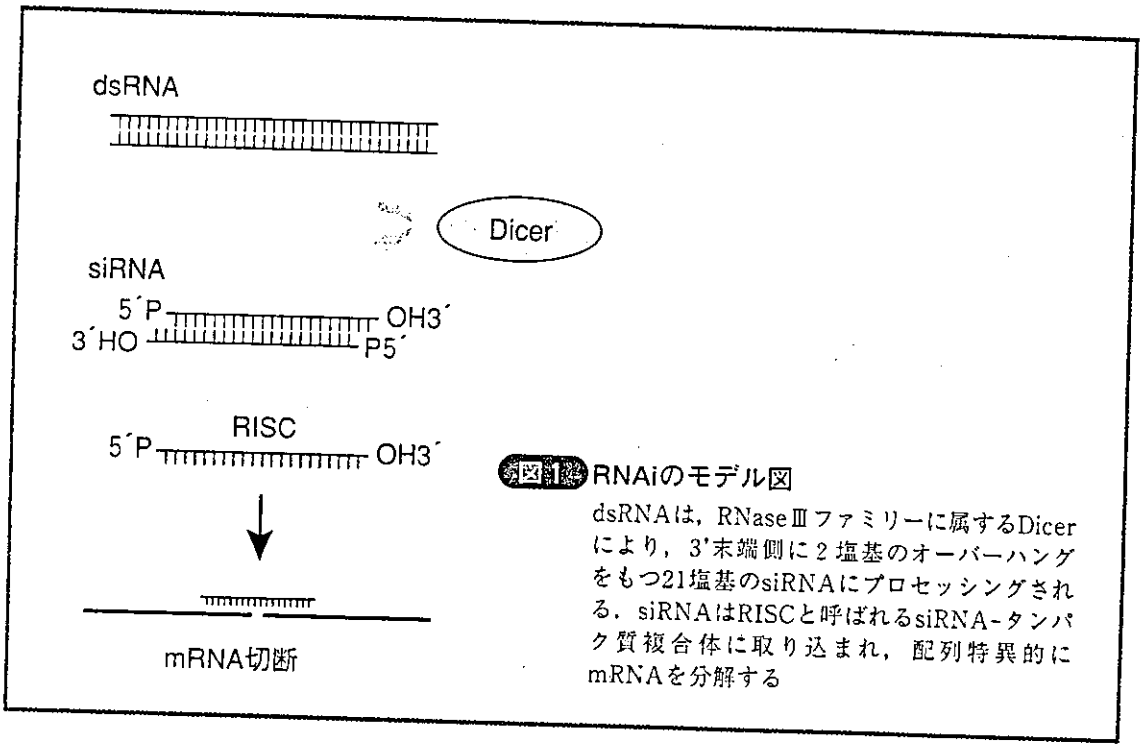

図1 RNAiのモデル図
dsRNAは，RNaseⅢファミリーに属するDicerにより，3'末端側に2塩基のオーバーハングをもつ21塩基のsiRNAにプロセッシングされる．siRNAはRISCと呼ばれるsiRNA-タンパク質複合体に取り込まれ，配列特異的にmRNAを分解する

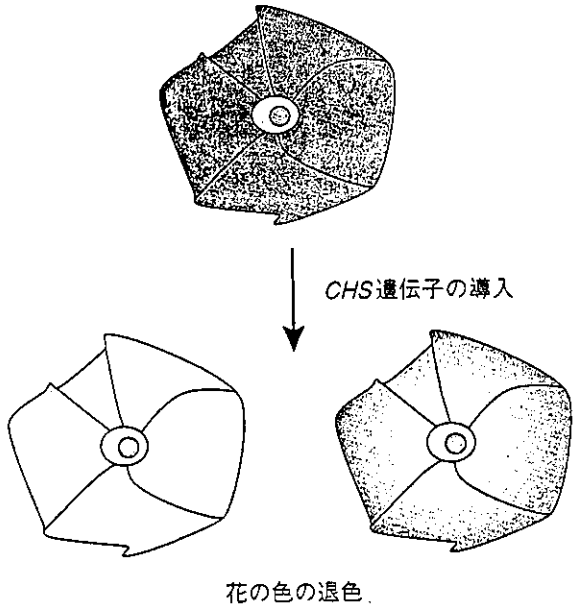

図2 *CHS*遺伝子のコサプレッションによる花の色の退色
センス*CHS*遺伝子を導入すると，その外来*CHS*遺伝子が働かないばかりか，内在の*CHS*遺伝子もコサプレッションにより抑制され，花の色の退色が起こる．不思議なことに，*CHS*遺伝子の転写は通常通り行われていたが，mRNAの蓄積は見られなくなった．そのため，外来遺伝子に対応するmRNAのみが，転写後何らかの機構で選択的に分解されていることが示唆された

RNAを介したサイレンシング

遺伝子の発現が何らかの形で抑制されることを，遺伝子のサイレンシングという．サイレンシングは，転写時サイレンシング（Transcriptional gene silencing：**TGS**）と転写後サイレンシング（Post-transcriptional gene silencing：**PTGS**）の大きく2種類に分けられる．TGSは，DNAからmRNAが転写されなくなることによってサイレンシングを起こす現象で，遺伝子のメチル化やクロマチンの構造変化などが関わっているといわれている．PTGSは，転写は通常通り行われるが，mRNAが何らかの原因で分解されるか，あるいは翻訳が阻害されることにより，遺伝子の発現が抑えられる現象である．

遺伝子のサイレンシングは，その研究背景の違い，生物種によって異なった呼び名で呼ばれてきた．植物，線虫，ショウジョウバエで，内在の遺伝子と相同性のある遺伝子またはその遺伝子の一部を導入し，その発現が抑えられることは，コサプレッション（Co-suppression：共抑圧という意味）と呼ばれる．コサプレッションは，その相同配列がプロモーターあるいは転写調節配列である時はTGS，転写領域である時はPTGSレベルで起こる．ケリング（Quelling：鎮圧という意味）は，菌類の同様な現象で，PTGSレベルで起こる．

RNAを介したサイレンシングの現象として最初に報告されたのは，1990年，植物のカルコン合成酵素（*CHS*）遺伝子のコサプレッションであると思われる（図2）[4)5)]．カルコン合成酵素は，花の色の主要な成分であるアントシアニンを合成するために，重要な働きをする酵素として知られている．濃い花の色を作り出すために導入した*CHS*遺伝子によって，内在の*CHS*遺伝子の発現が抑制され，逆にまだらに色が抜けたり，白い花ができるというこの不思議な現象は，長い間その機構はわからなかった．どうやってサイレンシングされる遺伝子とサイレンシングされない遺伝子を見分けているのであろうか？

その導入した遺伝子を詳細に解析した結果，**回文配列**〔ある同じ配列がお互いに逆向きにつながっているもの（Inverted repeat）〕が関係していること[6)]，**遺伝子のメチル化**をしばしば伴っていること[7)]，*CHS* mRNA内部の二重鎖を形成する部分の分解物が関与していること[8)]などが明らかにされてきた．これらの現象の解明に重要な鍵を与えたのが，線虫における二重鎖RNA（dsRNA）によるRNAiの報告[1)]，および1999年のBaulcombeのグループによる，コサプレッションを起こしている植物にだけ25塩基前後の小さなセンスおよびアンチセンスRNAが含まれているとい

う報告[9]であった．コサプレッションとして報告されているものには，RNAiに見られる現象と非常に類似したものが多く観察されており，また共通の因子が関与していることが明らかになってきている．

RNAiの発見

RNAiは，dsRNAによってその配列特異的にmRNAが分解され，その結果遺伝子の発現が抑制される現象である．dsRNAによって遺伝子のサイレンシングができることが最初にわかった発端は，線虫におけるアンチセンスを用いた研究からであった．1995年，GuoとKemphuesは*par-1*と呼ばれる遺伝子をアンチセンスRNAで抑制する実験を行った[10]．アンチセンスRNAをインジェクションすると，予想通り*par-1*の発現を抑制したが，驚いたことに，コントロールとして用いたセンスRNAも同様に*par-1*の発現を抑制し，*par-1*変異株の表現形を示した．多くの研究者がこの矛盾に関心を示した．

1998年に，FireとMelloらによってその謎が解き明らかされた[1]．アンチセンスRNAとセンスRNAを，それぞれRNAポリメラーゼを用いて試験管内で合成する時，ほんの少しであるが非特異的に逆向きのRNAができてしまう．そのコンタミネーションによってできるdsRNAが遺伝子サイレンシングの本体であったのである．Fireらはゲル電気泳動で精製したアンチセンスRNAおよびセンスRNAは遺伝子の発現を抑制できないこと，また，アンチセンスRNAとセンスRNAをアニールさせたdsRNAが非常に効率よく遺伝子の発現を抑制できることを明らかにした．

その後，RNAiは線虫をはじめ，トリパノソーマ，ヒドラ，プラナリア，菌類，ショウジョウバエ，植物などのさまざまな生物細胞種において効果的な遺伝子発現ノックアウト法として用いられてきており，また詳細な機構もここ1，2年でしだいに明らかになりつつある[11]．これまで観察されたRNAiの一般的な性質として，遺伝子の抑制効果はその二重鎖領域の長さに依存する，ということがある[12]．100塩基対（base pair：bp）以下のdsRNAは効果が低く，500 bp程度以上あれば90%程度標的遺伝子の発現を抑える．また，配列特異性はほぼ厳密であるが[13]，相同性が高く同じ配列をもったmRNAはもちろん分解される．

RNAiのメカニズム解明に向けて —RNAiの配列特異性を担うsiRNA

Tuschlらのグループは，ショウジョウバエの抽出物を用いた*in vitro*の系を構築し[14]，RNAiのメカニズム解明に関連する非常に重要な一連の実験を行った．長いdsRNAが21～23塩基のRNAに分解されることを観察し，それらの分解されたRNAによりターゲットmRNAが21～23塩基の間隔で切断されることを示した[15]．このRNAの長さは，18～24塩基にわたっていたが，ほぼ80%が21～23塩基であった．このことから，21～23塩基のRNAがターゲット配列認識のためのガイドRNAとして働いていることが示唆され，それは実際に，化学合成した21～22 bpのdsRNAを用いて，ターゲットmRNAの切断が行われることにより示された[14]．またそのガイドRNAは，Tuschlらにより**siRNA**（short interfering RNA）と名付けられた．

siRNAの配列特異性は非常に高く，21塩基中わずか数個の配列のミスマッチにより，効果はほとんど見られなくなった．siRNAの長さは21塩基，オーバーハングは2塩基のものが最も効果が高く，4塩基以上のオーバーハングは効果がほとんど見られなかった．標的mRNAの切断サイトは，siRNAのガイドRNA側の5'末端側から正確に10～11塩基のところであり，siRNAにより厳密に制御されていた[16]．

生成したsiRNAには，5'末端側にモノリン酸と3'末端側に水酸基が存在した．化学合成したsiRNAは，通常5'末端，3'末端とも水酸基である．この5'末端水酸基のsiRNAでも有効であり，5'末端リン酸基のsiRNAの方が，若干効果が高いか同程度であった[16][17]．詳細に検討すると，合成siRNAの5'末端水酸基は，胚抽出物中で速やかにリン酸化された．リン酸化を防ぐため，5'末端をメトキシ基（CH_3O）にすると，RNAi効果は完全