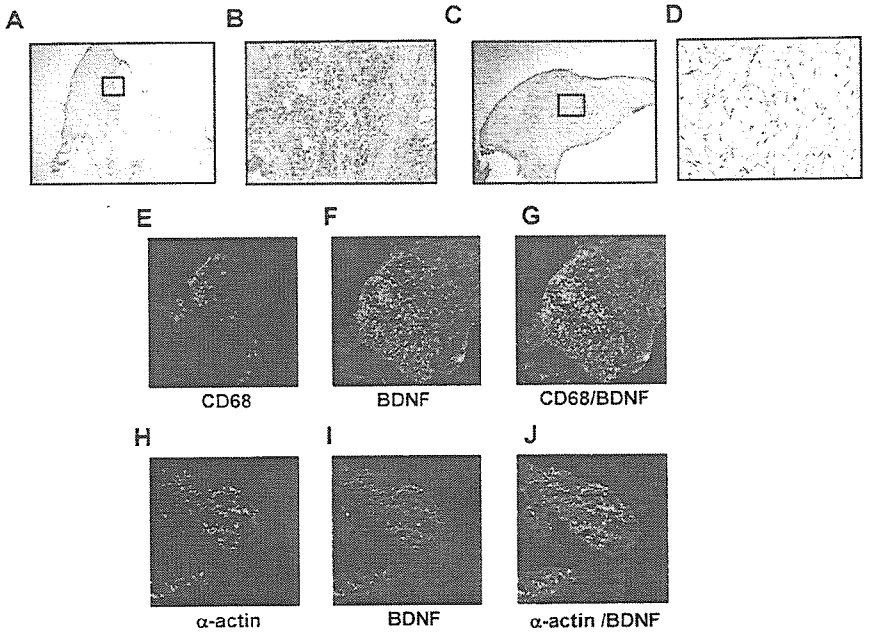

Figure 4. BDNF expression in DCA specimens. Low-power (A, C) and high-power (B, D) views of representative staining for BDNF of DCA specimens from angina patients. Immunohistochemical analysis revealed BDNF expression in inflammatory cells, smooth muscle cells, and extracellular matrix. E–J, Double immunofluorescence of BDNF with cell-specific markers. Anti-CD68 (E) and anti–α-actin (H) were used as markers of macrophage and smooth muscle cells, respectively. Red-labeled immunofluorescence indicates cell makers; green-labeled immunofluorescence indicates expression of BDNF (F, I). Colocalization of cell-specific makers and BDNF is shown by yellow-labeled immunofluorescence (G, J). Representative figure (n=5) is shown, and similar results were observed in all examinations.

cell types of BDNF-expressing cells, double immunofluorescence of BDNF and CD68 or α-actin, a specific marker of macrophages and smooth muscle cells, respectively, was carried out. As shown in Figure 4E through 4J, smooth muscle cells and macrophages expressed BDNF in coronary specimens of patients with angina pectoris.

Semiquantitative analysis demonstrated that BDNF expression in coronary arteries of UAP patients was more intense compared with SAP (Table 4). Representative cases of UAP and SAP are shown in Figure 5.

BDNF Enhanced NAD(P)H Oxidase Activity and Reactive Oxygen Species in Human CASMCs

The effects of BDNF on NAD(P)H oxidase activity were examined in human CASMCs. Stimulation with 100 ng/mL BDNF increased NADH- and NADPH-dependent oxidase activity ≈2.7- and 2.3-fold compared with that in control (nontreated) cells, respectively (Figure 6A). This oxidative activity was reduced by 10 μmol/L DPI, a selective inhibitor of NAD(P)H oxidase. In the presence of DPI, NADH- and NADPH-dependent oxidase activity in CASMCs was reduced by 74% and 45%, respectively. The effects of BDNF on NAD(P)H oxidase activity were dose and time dependent (Figure 6B). Furthermore, in experiments with dihydroethidium, an intracellular fluorescence probe, BDNF stimulation increased the generation of reactive oxygen species (Figure 6C).

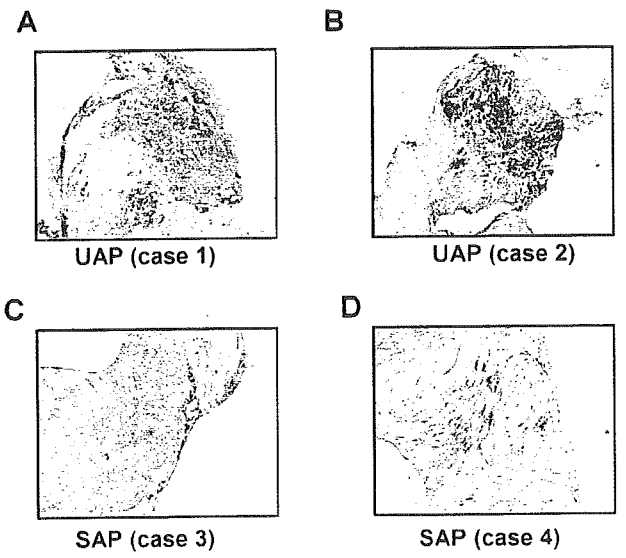

Figure 5. BDNF expression in coronary specimens of patients with angina pectoris. A B, Representative cases of UAP; C, D, representative cases of SAP. Semiquantitative analysis is shown in Table 4.

Discussion

The present study demonstrated a significantly greater Cs-Ao difference in plasma BDNF, but not NT-3, in the UAP group than in the SAP and non-CAD groups. Immunohistochemical analysis revealed that BDNF was expressed in atheromatous intima and adventitia in human coronary arteries. Intense BDNF immunoreactivity was observed in macrophages and smooth muscle cells in atherosclerotic coronary arteries. Semiquantitative analysis demonstrated that BDNF expression in UAP patients was more intense compared with SAP. Furthermore, BDNF enhanced NAD(P)H oxidase activity and superoxide production in cultured CASMCs, and its selective inhibitor suppressed the effect of BDNF. Thus, BDNF in the coronary vasculature might enhance oxidative stress via the activation of NAD(P)H oxidase.

BDNF has protective effects against injury or ischemia in both the central and peripheral nervous systems.[4–6] For example, Schabitz et al[19] demonstrated that intravenous BDNF injection reduces infarct size in rat model. On the other hand, several lines of evidence suggest that NTs potentiate neuronal death under some conditions such as serum or oxygen-glucose deprivation.[20,21] Kim et al[17] demonstrated that BDNF acts as a pronecrotic factor through activation of NAD(P)H oxidase in cortical cells, and other

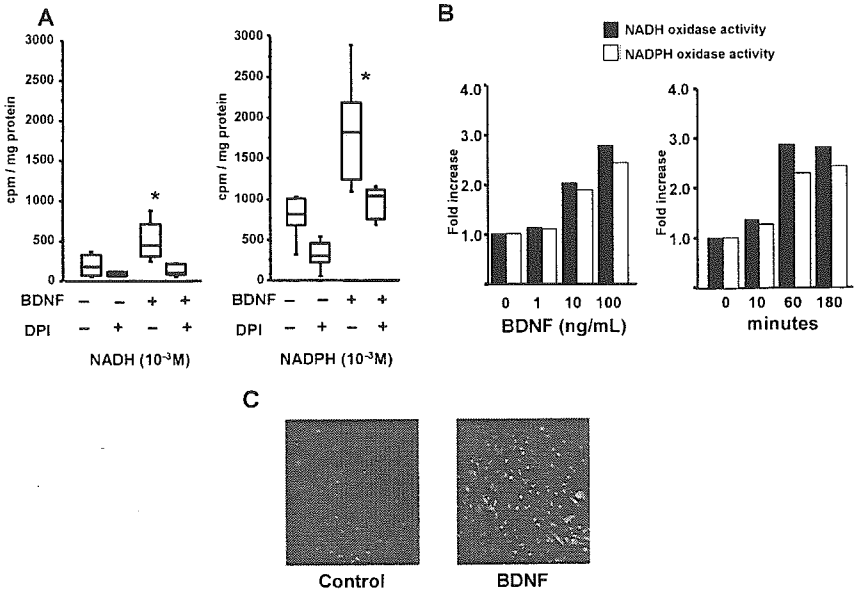

Figure 6. A, Effect of BDNF on NAD(P)H oxidase in cultured human CASMCs. Enzymatic activity of NAD(P)H oxidase in human CASMC homogenates was assessed by L-CL. NADH- (left) and NADPH- (right) dependent oxidase activity was enhanced by treatment with BDNF (100 ng/mL) for 1 hour. Treatment with DPI reduced the activity of NAD(P)H oxidase. The chemiluminescent signals are expressed as counts per minute per milligrams of protein. Data are expressed as medians, with 25th and 75th percentiles (boxes) and 10th and 90th percentiles (I bars). B, Dose response and time dependency of BDNF on NAD(P)H oxidase activity. In the dose-response study, CASMCs were stimulated with the indicated concentration of BDNF for 1 hour (left). In the time-course study, the cells were incubated for each period with 100 ng/mL BDNF (right). Graphs are representatives of 4 independent experiments. *$P<0.05$ for the chemiluminescent signals of CASMCs with and without BDNF preincubation. C, Effect of BDNF on reactive oxygen species generation in cultured human CASMCs. Generation of reactive oxygen species was assessed with the dihydroethidium method. Treatment with BDNF (100 ng/mL) increased superoxide generation vs control. Results are representative of 5 independent experiments.

studies indicated that NTs induce cell death in cerebral ischemia.[22] Thus, the intracellular signaling pathway mediated by NTs can act not only for survival but also as a proapoptotic or pronecrotic pathway in neuronal cells. Whether coronary BDNF induces cell necrosis or apoptosis of vascular cells warrants further investigation. BDNF enhanced the activity of NAD(P)H oxidase and the generation of superoxide in cultured smooth muscle cells. Because oxygen radicals activate matrix metalloproteinases,[23] the oxidative stress by BDNF might induce the instability of atherosclerotic plaques. On the other hand, BDNF has important roles in survival or as a development factor even in nonneuronal tissues such as endothelial cells.[24] BDNF in vasculature may work as a protective factor for endothelial cells in a counterregulatory mechanism. Further investigation is needed to clarify the precise mechanisms of BDNF in the pathogenesis of UAP.

In the present investigation, increased Cs-Ao differences in BDNF were observed in the UAP patents, although there was no significant difference in the numbers of diseased vessels or the degree of coronary stenosis between the SAP and UAP groups. These findings indicate that BDNF in the coronary circulation seems to influence the disease state of angina pectoris rather than the degree of coronary atherosclerosis and plaque formation. Acute coronary syndrome usually occurs at sites with <70% stenosis, as determined by angiographic studies performed in patients before the onset of coronary events. Therefore, BDNF might be involved in the vulnerability of atherosclerotic plaques. There are several possible origins of BDNF in the coronary circulation, including vascular smooth muscle cells, accumulating inflammatory cells, adventitial fibroblasts, cardiac myocytes, and neural cells. Recently, it has been reported that platelets release BDNF; therefore, activated platelets are a potential origin of BDNF.[25] However, the intense immunoreactivity in patients with UAP suggests that BDNF in the coronary circulation likely comes from atherosclerotic plaques. Plaque rupture and erosion are key events in the pathogenesis of acute coronary syndrome, including unstable angina; therefore, it is possible that disrupted atherosclerotic plaques release BDNF. Some other factors may be involved in the increased Cs-Ao differences in BDNF. Thus, further investigations are necessary to establish the causal relationship between BDNF and plaque rupture.

Psychological stress produces significant increases in heart rate and blood pressure, which might lead to an increased myocardial oxygen demand. Kario et al[26] demonstrated that earthquake-induced stress increased not only blood pressure and blood viscosity determinants but also fibrin turnover with endothelial cell stimulation in a group of hypertensive elderly subjects, suggesting that acute stress might trigger cardiovascular events. On the other hand, psychological stress increases the secretion of NTs from central and peripheral nerves.[27–29] Neuropsychological studies demonstrate that psychological stress such as immobilization stress increases BDNF mRNA expression in the hypothalamus of experimental models.[29] There is no direct evidence that BDNF in the coronary vasculature is regulated by psychological stress in

humans; however, psychological stress might increase the production of BDNF in coronary beds, which in turn augments regional oxidative stress via NAD(P)H oxidase. Further investigation is needed to clarify the regulation of coronary BDNF and the direct effect of psychological stress on NT levels in the coronary vasculature.

Plasma BDNF levels are decreased in patients with psychological disorders such as depression, and the level recovers with antidepressant drug treatment.[27,30] In our study, we did not enroll patients with mental disorders, and no patients had taken antidepressant drugs or tranquilizers. Although the psychological states of the patients were not examined, we do not consider that the Cs-Ao differences in BDNF were influenced by these factors.

In conclusion, plasma BDNF, but not NT-3, was increased in the coronary circulation in patients with UAP, and BDNF expression was enhanced in coronary arteries of UAP patients. BDNF increased NAD(P)H oxidase activity and superoxide production in human CASMC culture. Our observations suggest that the enhanced oxidative stress induced by BDNF has an important role in plaque instability.

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Xenogenic smooth muscle cell immunization reduces neointimal formation in balloon-injured rabbit carotid arteries

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Abstract

Objective: Intimal hyperplasia plays an important role in a variety of types of vascular remodeling, particularly luminal narrowing after vascular injury. The vascular smooth muscle cells (VSMCs) in the neointimal area are a synthetic phenotype and have different epitopes from VSMCs in the normal media. The synthetic VSMCs in the neointima contain various possible antigens that can be targeted by the immune system. In this study, we tried to develop a new immunotherapy, which targets the synthetic VSMCs, for prevention of neointimal formation after angioplasty.

Method and results: Rabbits were repeatedly immunized with fixed xenogenic rat cultured VSMCs suspended in adjuvant as immunogens or injected with adjuvant and phosphate-buffered saline (PBS) or rat hepatocytes as controls every 2 weeks for 3 times. One week after the last immunization/injection, balloon injury of the left common carotid artery was performed. Four weeks after the injury, rabbits were euthanized and the neointimal lesion formation was assessed. The mean neointimal area of the PBS-injected, non-immunized group and the rat hepatocyte-immunized, control group was not statistically different (0.339 ± 0.036 and 0.350 ± 0.041 mm^2, P=NS). However, immunization with rat VSMCs significantly reduced the intimal lesion area (0.219 ± 0.0286 mm^2; $P<0.05$ vs. PBS-injected, non-immunized group and rat hepatocyte-immunized group.) PCNA-immunopositive proliferating VSMCs in the neointima were suppressed by the rat VSMC immunization ($1.34 \pm 0.49\%$ vs. $5.78 \pm 0.47\%$; $P<0.05$ vs. PBS-injected, non-immunized group). Rat VSMC immunization induced antibodies which had strong cross-reactivity against rabbit synthetic VSMCs. In experiments in vitro, proliferation and migration of rabbit VSMCs that were stimulated by serum, angiotensin (AT) II, platelet-derived growth factor (PDGF)-BB, fibroblast growth factor (FGF), and the phorbol ester PMA were significantly suppressed by treatment with immunoglobulin extracted from the VSMC-immunized rabbit plasma, implying that the immunoglobulin had some global effects on VSMCs. The rat VSMC-immunized rabbit immunoglobulin bound the rabbit AT1a receptor protein, which was expressed in COS7 cells by transfection of rabbit AT1a receptor pcDNA3. This binding to AT1a receptor may be one of mechanisms of the effects of VSMC-immunized immunoglobulin.

Conclusion: Xenogenic, synthetic rat VSMC immunization in rabbits induced auto-antibodies against synthetic rabbit VSMCs in a cross-reaction. The induced auto-antibodies against synthetic VSMCs may provide a possibility of new immunotherapy for vascular remodeling that forms neointimal lesions.

Keywords: Neointimal formation; Vascular remodeling; Vascular smooth muscle; Antibody; Immunotherpy

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This article is referred to in the Editorial by Thaunat et al. (pages 183–185) in this issue.

1. Introduction

Intimal hyperplasia plays an important role in a variety of types of vascular remodeling, particularly luminal narrowing after vascular injury such as that seen in restenosis following percutaneous coronary intervention. Proliferation and migration of vascular smooth muscle cells (VSMCs) from the media play a central role in neointimal formation, and various strategies have been developed to inhibit it, including inhibition of cell cycle of VSMCs, induction of apoptosis of VSMCs, and inhibition of intracellular signal transduction in VSMCs [1–4].

Recently immunological modulation has attracted attention as a possible therapeutic strategy of atherosclerosis [5–22]. Although only limited information is available, several studies have shown the possibility of immunotherapy against neointimal lesion formation after balloon injury. It seems that adequate induction of B-lymphocyte-related immunity has potential beneficial effects on neointimal formation. Nilsson et al. showed that immunization with homologous oxidized low density lipoprotein reduced neointimal formation after balloon injury in hypercholesterolemic rabbits [23]. In balloon-injured rat carotid arteries, it was demonstrated that treatment with an anti-P selectin monoclonal antibody reduced inflammation, in neointimal formation, and vascular remodeling [24]. However, until now, only few epitopes have been reported as appropriate targets of immunotherapy against neointimal formation.

It is possible that synthetic VSMCs in the neointima contain various possible antigens that can be targeted by the immune system. Since VSMCs play a central role in neointimal formation, we hypothesized that immune modulation targeting the synthetic VSMCs-associated antigens may inhibit neointimal formation after balloon injury. To prove this hypothesis, rabbits were immunized with the synthetic xenogenic rat VSMCs and then balloon-injured to induce neointimal formation.

2. Methods

2.1. Materials and animals

All drugs and culture media used in this study were purchased from Sigma Chemical Co. (MO) and WAKO (Japan). Male Japanese White Rabbits were purchased from a breeder (SLC, Hamamatsu, Japan) and kept under conventional conditions in our animal facility. Rabbits were fed normal chow (Oriental Yeast, Japan) and water ad libitum and maintained on a 12 h light/dark cycle. All animal experiments were conducted according to the "Guidelines for Animal Experiments at Kobe University Graduate School of Medicine", which complies NIH Guidelines.

2.2. Preparation for rat vascular smooth muscle cells and rat hepatocytes as immunogens

Rat VSMCs were prepared from thoracic aortas of male Sprague–Dawley rats by the collagenase digestion method and cultured as described [25]. For all experiments, rat aortic VSMCs from passage 5 to 8 were used. Rat hepatocytes (RLC-18, Cell No. RCB1484) were purchased from Rikken Cell Bank (Japan).

Rat VSMCs and rat hepatocytes were cultured in DMEM with 10% fetal bovine serum (FBS), and approximately 1×10^8 cells were collected and washed three times with phosphate buffered saline (PBS). The cells were fixed with 10% neutralized formaldehyde (WAKO, Japan) for 24 h at 4 °C. After being washed three times and incubated at 37 °C for 2 h to remove residual formaldehyde, the cells were washed again and re-suspended in PBS for use as immunogens.

2.3. Experimental protocol

Male Japanese White Rabbits (body weights were 2.0 kg) were divided into three groups. Injection of either rat VSMCs as immunogens (1×10^8 cells per rabbit), vehicle PBS for control non-immunized group or rat hepatocytes (1×10^8 cells per rabbit) for control immunized group was started. Those injections were subcutaneously performed in the rabbits' back with an equal volume of adjuvant every two weeks for three times. We used the Freund complete adjuvant for the first immunization, and the Freund incomplete adjuvant for the second and the third immunization.

One week after the last injection, rabbits were anesthetized with sodium pentothal. Blood sample was collected from the ear vein. Plasma immunoglobulin (IgG) levels were assessed using a commercially available kit (Bethyl Laboratories Inc. TX). A 2F Fogarty embolectomy catheter (Baxter, USA) was introduced through an aseptic neck incision produced in the facial branch of the external left carotid artery and positioned approximately at the origin of the common carotid artery. An acute balloon injury was performed by inflating the balloon with 0.1 mL saline solution and then gently pulling it back along the entire length of the common carotid artery with constant rotation as described before [26]. The catheter was then removed, the artery branch was ligated, and the surgical wound was closed. Two and four weeks after balloon injury, rabbits were euthanized, and the neointimal lesion formation was assessed.

2.4. Histological and immunohistochemical analysis of neointimal lesions

Serial equally spaced cross sections (5 μm thick) were obtained throughout the entire length of the carotid artery

for histological analysis (average of six sections per animal). All samples were routinely stained with hematoxyline and eosin or subjected to immunostaining with the anti-proliferating cell nuclear antigen (PCNA). For immunohistochemistry, slides were preincubated with 1% bovine serum to decrease nonspecific binding. Sections were incubated overnight at 4 °C with the mouse anti-PCNA antibody (DAKO, Denmark).

2.5. Quantification of neointimal lesions in sections of carotid arteries

Six equally spaced cross sections of the entire length of carotid arteries were used in all rabbits to quantify neointimal lesions. Using NIH imaging software, total cross-sectional neointimal area was measured between the endothelial cell monolayer and the internal elastic lamina. Total cross-sectional medial area was measured between the external and internal elastic lamina.

2.6. Quantification of proliferating neointimal cells

Serial cross sections (5 μm thickness) of carotid arteries were made. One section was used for HE-staining to calculate the total cell number in the neointimal area, and the next section was used for PCNA immunohistochemistry. The total PCNA-immunopositive neointimal cells and total neointimal cells in HE-staining in each serial section were counted. Then, the percentage of PCNA-immunopositive cells per total number of neointimal cells in each section was calculated, and the average of the six sections per animal was obtained for each animal.

2.7. Detection of apoptosis in the neointimal cells

Apoptosis in the neointimal cells was detected by TUNEL technique. Modified TUNEL assay was performed by use of DeadEnd colorimetric apoptosis detection system (Promega) according to the manufacturer's instructions. Briefly, tissue sections from the neointima of PBS injected non-immunized group and rat VSMC-immunized group were washed in PBS, and endogenous peroxidase was blocked by 0.3% hydrogen peroxide. Biotinylated nucleotide was incorporated at the 3′-OH DNA ends using terminal deoxynucleotidyl transferase (TdT). Streptavidin-HRP was then bound to these biotinylated nucleotides, which were detected using hydrogen peroxide and diaminobenzidine (DAB). Some of slides were treated with Dnase I for positive controls. Stained cells were counted under a light microscope.

2.8. Immunoglobulin purification from rabbit plasma

The immunoglobulin from the pooled plasma derived from the rabbits on day 7 after the third immunization or from control rabbits was purified with the use of a Protein G column (Sigma, MO).

2.9. Immunoblotting using rabbit plasma and protein analysis

Rabbit VSMCs were primarily cultured by the explant method and cultured in DMEM with 10% fetal bovine serum (FBS). Rabbit VSMCs were homogenized in a homogenizing buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM phenylmethyosulfonylfluoride, 10% glycerol, and 20 mM CHAPS. The whole protein (50 μg per lane) was resolved by SDS-PAGE (10% polyacrylamide), transferred to a PVDF membrane and probed with rabbit plasma obtained from PBS injected non-immunized control rabbits, rat hepatocyte-immunized control rabbits, or rat VSMC-immunized rabbits. Immunoreactive bands were visualized with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham, England; 1:3000 dilution) and an ECL detection kit (Amersham, England).

2.10. Counting of VSMC number in vitro

Cell proliferation was quantified by total cell number as previously described [27]. Rabbit VSMCs (5000 per well) were seeded in 96-well microtiter plates in 0.1 mL of DMEM–10% FBS. After 12 h incubation, the medium was replaced by DMEM with purified immunoglobulin from rat hepatocyte-immunized group and that from rat VSMC-immunized group (20 μg/mL, respectively). At 48 h after stimulation with agonists (10% FBS, 1 μM ATII, 10 ng/ml PDGF-BB, 10 ng/ml FGF, 1 μM PMA), cells were fixed by addition of 10 μL of glutaraldehyde and shaken for 15 min. After being washed 3 times with deionized water, plates were air-dried and stained for 20 min with 0.1% crystal violet solution in 200 mM MES, pH 6.0. After being washed 3 times with deionized water to remove excess dye, plates were air-dried before solubilization of the bound dye in 10% acetic acid. The optical density of dye extracts was measured at 595 nm by using a microplate reader (Bio-Rad). Values are means±SEM of 8 separate experiments in each group.

2.11. VSMC migration

Rabbit VSMCs were grown to confluence in 6-well culture plates. The monolayer-wounding cell migration assay was performed as described previously [28,29]. Cell layers were scraped with a sterile single edged razor blade and re-incubated in DMEM containing 5 mM hydroxyurea with purified immunoglobulin from rat hepatocyte-immunized rabbits or rat VSMC-immunized rabbits (20 μg/mL, respectively). Hydroxyurea was added to eliminate any confounding effects of cell proliferation. At 24 h after stimulation with agonists (10% FBS, 1 μM ATII, 10 ng/ml PDGF-BB, 10 ng/ml FGF, 1 μM PMA), cells were fixed and the maximum migration distance across the wound edge was analyzed.

2.12. VSMC viability assay

Rabbit VSMCs (5000 per well) were incubated in 96-well plastic plates with purified immunoglobulin from rat VSMC-immunized rabbits or rat hepatocyte-immunized rabbits (20 μg/mL, respectively). After 24 h, cell viability was assessed by measuring mitochondrial NADH-dependent dehydrogenase activity with a Cell Counting Kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) using sulfonated tetrazolium salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) [30]. Each measurement was done in triplicate and the results

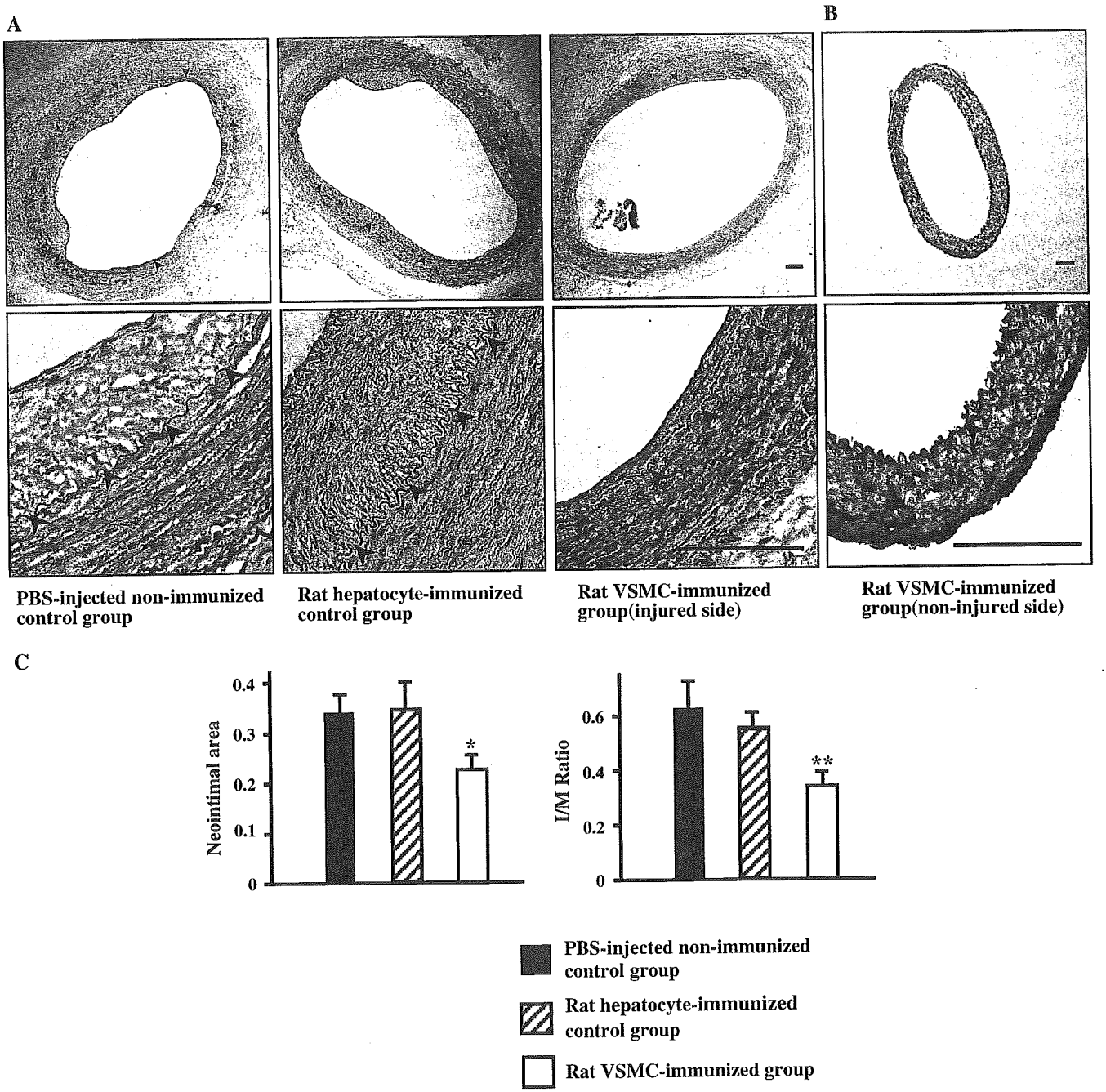

Fig. 1. (A) Representative light photomicrograph images showing injured carotid arteries from PBS-injected non-immunized control group, rat hepatocyte-immunized control group, and rat VSMC-immunized group at 4 weeks after balloon injury. Arrowhead indicates internal elastic lamina (IEL). Original magnifications are ×40 (upper) and ×200 (lower panels). Bars indicate 100 μm. (B) Representative light photomicrograph images showing the untouched contralateral common carotid artery from rat VSMC-immunized group. Arrowhead indicates internal elastic lamina (IEL). Original magnifications are ×40 (upper) and ×200 (lower panels). Bars indicate 100 μm. (C) Quantitative analysis of neointimal area and I/M ratio in rabbit carotid arteries at 4 weeks after injury in rat VSMC-immunized group (white bars), PBS-injected non-immunized control group (black bars), and rat hepatocyte-immunized control group (striped bars). Values are means±SEM in each group. *$P<0.05$ vs. PBS-injected non-immunized control group and rat hepatocyte-immunized control group. **$P<0.05$ vs. PBS-injected non-immunized control group.

were presented as a percentage of the value for rat hepatocyte-immunized and control-immunized groups.

2.13. Identification of a target protein which rat VSMC-immunized immunoglobulin recognizes

Rabbit AT1a receptor cDNA was a generous gift from Dr. Raymond C. Harris (Vanderbilt Medical Center, Vanderbilt University). The complete coding site was excited and cloned into the expression vector pcDNA3 (Invitrogen), and pcDNA3 without any expression insert was used as the control vector. COS7 cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured according to the manufacture's recommendations. COS7 cells were transfected by control pcDNA3 and rabbit AT1a receptor pcDNA3, respectively. The whole protein of these two groups of COS7 cells were resolved by SDS-PAGE, transferred to a PVDF membrane and probed with rabbit plasma obtained from PBS injected non-immunized control rabbits or rat hepatocyte-immunized control rabbits or rat VSMC-immunized rabbits respectively. Immunoreactive bands were visualized with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham, England; 1:3000 dilution) and an ECL detection kit (Amersham, England).

2.14. Statistical analysis

Values represent means±SEM. Differences between groups were compared using a one-way analysis of variance test followed by Fisher protected least significant difference. $P<0.05$ was accepted as statistically significant.

3. Results

3.1. Blood analysis and general appearance of rabbits

Rat VSMC-immunization induced no significant changes in liver function, renal function and lipid profiles. Mean body weight and behavior of rabbits did not change by the immunization. In comparison with the PBS injected non-immunized control group, rat VSMC-immunization did not affect plasma IgG levels (284±61 mg/dl in PBS injected group vs. 268±44 mg/dl in rat VSMC-immunized group; P=NS).

3.2. Effect of rat VSMC immunization on the neointimal lesion formation

All animals developed concentric intimal lesions in response to the balloon injury. At 4 weeks after balloon injury, the mean neointimal area of PBS injected non-immunized group and rat hepatocyte-immunized control group were 0.339±0.036 and 0.350±0.041 mm^2, respectively (P=NS). The intimal lesion area was not significantly reduced by the rat hepatocytes control immunization. However, immunization with rat VSMCs significantly reduced the intimal lesion area (0.219±0.0286 mm^2; $P<0.05$ vs. PBS injected non-immunized group and rat

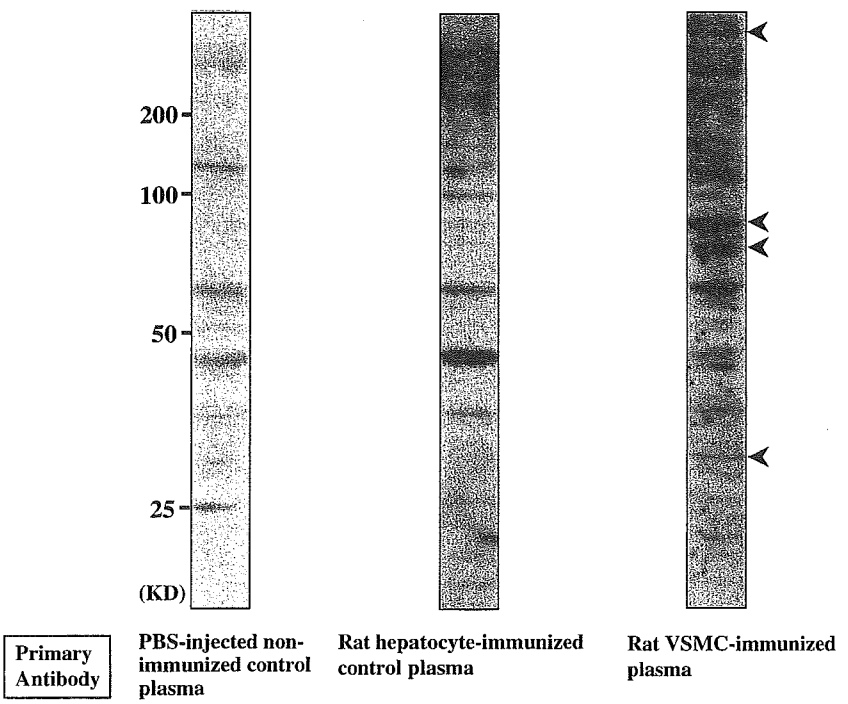

Fig. 2. Immunoblottings of proteins extracted from rabbit VSMCs using the plasma obtained from PBS injected non-immunized control rabbits (left), rat hepatocyte-immunized control rabbits (middle), or rat VSMC-immunized rabbits (right) as primary antibodies, respectively.

hepatocyte-immunized control group, Fig. 1A and C). The intimal/medial ratio was also significantly reduced in rat VSMC-immunized group (0.349±0.049) compared with PBS injected non-immunized group (0.662±0.114; $P<0.01$). There was no sign of infiltration of inflammatory cells at the contralateral common carotid artery (Fig. 1B). Immunization with rat VSMCs did not induce any pro-inflammatory effects on normal contractile VSMCs.

3.3. Rat VSMC immunization induced rabbit VSMC-reactive antibodies

To detect the antibodies, which were induced by xenogenic rat VSMC immunization, rabbit VSMCs proteins were resolved by SDS-PAGE, and immunoblotted with plasma obtained from the PBS injected non-immunized control rabbits, rat hepatocyte-immunized control rabbits ,

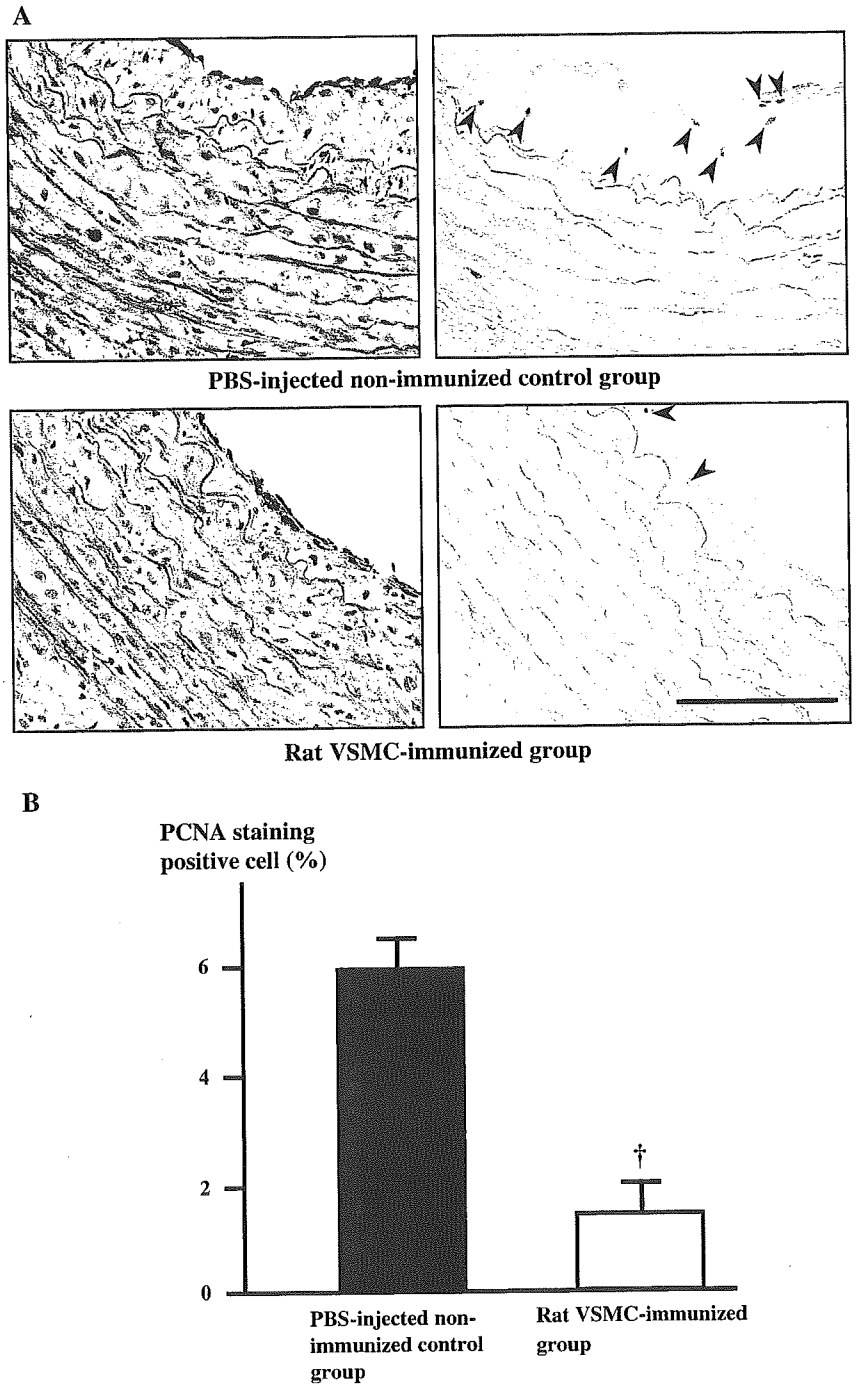

Fig. 3. (A) Photomicrographs of HE stainings (left panels) and PCNA immunostainings (right panels) in the neointima of injured carotid arteries at 2 weeks after balloon injury. Original magnification is ×200. A bar indicates 100 μm. (B) Quantitative analysis of PCNA positive proliferating cells in the neointima at 2 weeks after injury. Values are means±SEM of at least six rabbits in each group. †$P<0.01$ vs. PBS injected non-immunized control group.

or the VSMC-immunized rabbits. Only a few non-specific bands were detected when the plasma from the PBS injected non-immunized control rabbits was applied. Some bands were detected when the plasma from rat hepatocyte-immunized control rabbits was applied. In contrast, several new bands were clearly recognized in the immunoblot when we applied the plasma from rat VSMC-immunized rabbits (Fig. 2). It implies that the rat VSMC-immunized immunoglobulin recognized some new proteins of rabbit VSMCs that reacted with neither immunoglobulin from PBS injected non-immunized rabbits nor that from rat hepatocyte-immunized rabbits.

3.4. Effect of rat VSMC immunization on the proliferation and apoptosis of neointimal cells

To investigate whether the reduced neointimal lesion formation was due to the reduced proliferation of VSMCs in the neointimal lesion, the proliferation of neointimal VSMCs was investigated by the PCNA immunostaining on the day 14 after the injury (Fig. 3A). The percentage of PCNA-immunopositive cells was significantly reduced in VSMC-immunized group compared with in PBS injected control group (1.34%±0.49 vs. 5.78%±0.47, $P<0.01$, Fig. 3B).

Then to clarify the role of apoptosis in the reduction of neointimal formation, the TUNEL assay was carried out. As shown in Fig. 4, rat VSMC-immunization did not increase the number of TUNEL positive neointimal cells.

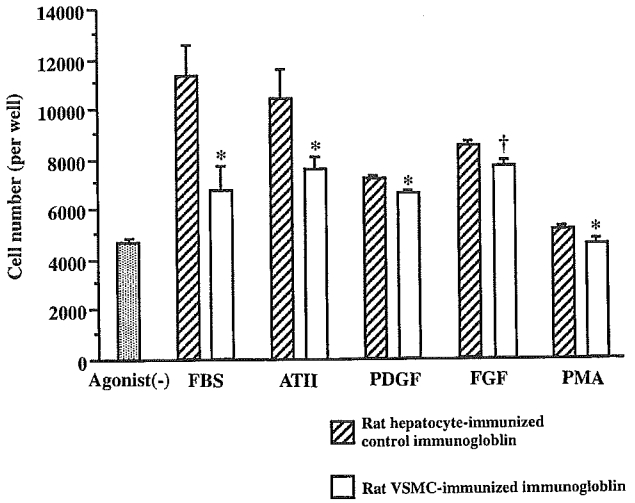

Fig. 5. Quantitative analysis of proliferation of cultured rabbit VSMCs treated with immunoglobulin obtained from rat hepatocyte-immunized control rabbits (striped bars) or rat VSMC-immunized rabbits (white bars). Values are means±SEM of separate six experiments in each group. †$P<0.01$ vs. the rat hepatocyte-immunized control group. *$P<0.05$ vs. PBS-injected non-immunized control group.

3.5. Effect of VSMC immunized immunoglobulins on rabbit VSMC proliferation and migration in vitro

The immunoglobulin (20 μg/ml) obtained from VSMC-immunized rabbits significantly suppressed rabbit VSMC cell numbers stimulated by FBS, ATII, PDGF, FGF, and PMA (Fig. 5) and rabbit VSMC migration stimulated by

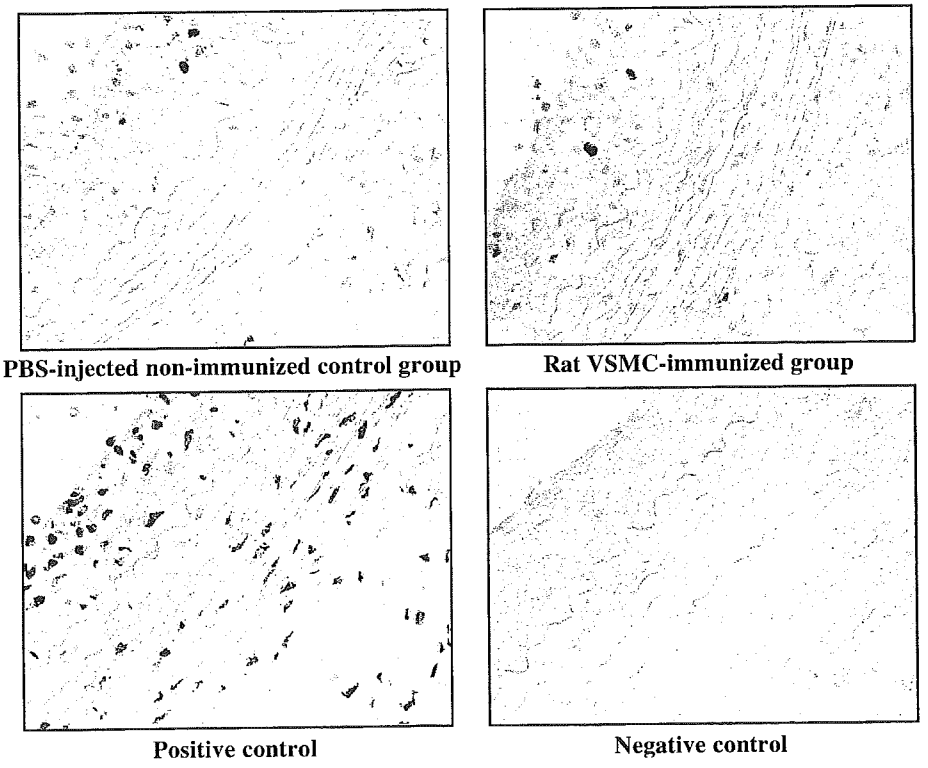

Fig. 4. Representative light photomicrograph images showing apoptosis in the neointimal cells using TUNEL technique.

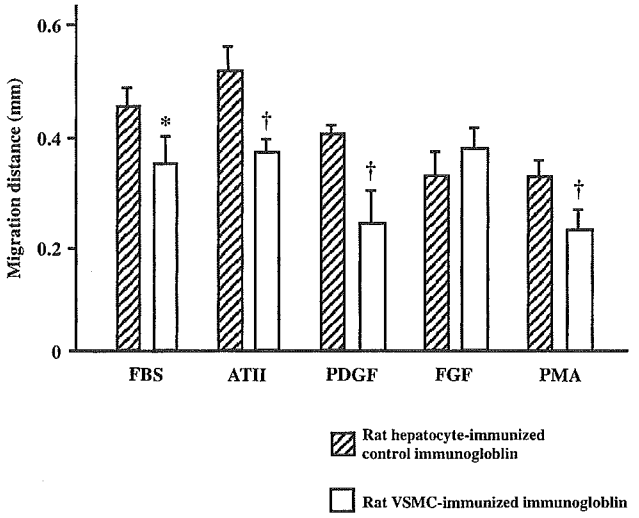

Fig. 6. Quantitative analysis of the migration distance evaluated by the maximally migrated point from the wounded edge in rat hepatocyte-immunized control group (striped bars) and in rat VSMC-immunized group (white bars), respectively. Values are means±SEM of five separate experiments in each group. †$P<0.01$ vs. the rat hepatocyte-immunized control group. *$P<0.05$ vs. PBS-injected non-immunized control group.

FBS, ATII, PDGF, and PMA (Fig. 6), compared with that from rat hepatocyte-immunized control rabbits.

3.6. Effect of immunized immunoglobulin on VSMC viability

The rat VSMC-immunized immunoglobulin used in the proliferation and the migration assays did not alter rabbit VSMC viability assessed by WST-1 assay (rat hepartocyte-immunized control group: 100% vs. rat VSMC-immunized group: 106±8.6%, P=NS).

3.7. AT1a receptor may be one of a target protein which rat VSMC-immunized immunoglobulin recognizes

As shown in Fig. 7, only rat VSMC-immunized rabbit plasma recognized the rabbit AT1a receptor protein which was expressed in COS7 cells by transfection of rabbit AT1a receptor pcDNA3 (molecular weight is about 50 kDa). No bands were detected by immunoglobulin from PBS-injected rabbits or that from hepatocyte-immunized rabbits.

4. Discussion

In the present study, we demonstrated for the first time that, immunization with xenogenic rat synthetic VSMCs reduced neointimal formation after balloon injury, in association with induction of auto-antibodies against rabbit VSMCs. The VSMCs which contribute to form neointimal lesions are different in phenotype from ones in the normal media [31]. VSMCs in the normal media consisted of a contractile phenotype, whereas VSMCs which contribute to form neointimal lesions show a synthetic phenotype [32]. And the epitopes of VSMCs also may be different between the contractile phenotype and the synthetic phenotype. We used cultured rat VSMCs as the xenogenic immunogens against rabbits. These rat VSMCs are the synthetic phenotype, and therefore immunization with rat VSMCs was likely to induce various antibodies against the synthetic VSMCs. And importantly, it seemed that certain antibodies cross-reacted between rat and rabbit VSMCs.

As demonstrated in Fig. 2, when we applied rat VSMC-immunized plasma as the primary antibody, several new proteins of rabbit VSMCs were detected by the Western blot analysis, which were not detected when PBS injected non-immunized control plasma or rat hepatocyte-immunized control plasma was applied It implies that the rabbit plasma obtained from rat VSMC-immunized rabbits had an antibody response distinct from the one induced by PBS or rat hepatocytes. Importantly, as demonstrated in Fig. 1, only rat VSMC-immunization reduced the neointimal lesion formation after balloon injury. These results might implicate that the induced antibodies reacting with the synthetic VSMCs but not others might play critical roles in the reduction of neointimal area in this study.

Apoptosis of VSMCs is implicated in the formation of neointimal lesions [33]. In the aortic allograft model in rats, Plissonnier et al. reported the induction of apoptosis by alloantisera [34]. Therefore, we investigated apoptosis of VSMCs in both the neointimal lesion and the normal media by TUNEL staining, but we did not find significant differences between rat VSMC-immunized group and PBS injected non-immunized group. On the other hand, the proliferative capability of VSMCs estimated by PCNA staining in neointimal lesions was significantly suppressed

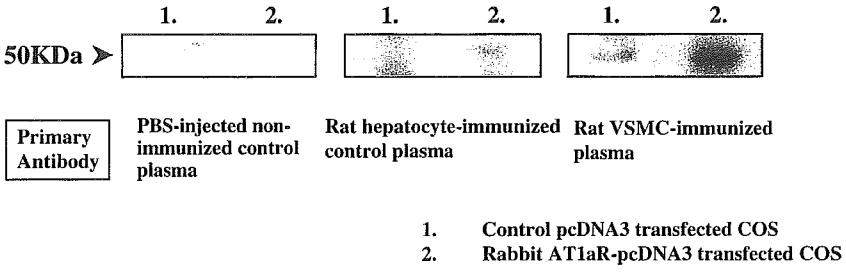

Fig. 7. The rabbit AT1a receptor protein expressed in COS7 cells by transfection of the rabbit AT1a receptor pcDNA3 was immunoblotted with PBS injected non-immunized control rabbit plasma (left panel), rat hapatocyte-immunized rabbit plasma (middle panel), or rat VSMC-immunized rabbit plasma (right panel), respectively. pcDNA3 without any expression inserts was used as the control transfection.

by rat VSMC-immunization (Fig. 3A and B), whereas no differences were detected in the media, at 2 weeks after balloon injury. Thus, proliferation of VSMCs in the neointima was suppressed by rat VSMC-immunization. This may be related to that neointimal lesions themselves may be more antigenic than the normal media. Plissonnier et al. reported that normal media was a "privileged" immunological site as compared to neointimal lesions in the vascular chronic rejection process in allotransplantation/immunization [35].

To further clarify the effects of the immunization on VSMC function, we performed in vitro assay. For in vitro assay, we purified immunoglobulin from plasma using a protein G column, because we needed to avoid the effects of many cytokines and growth factors which were contained in the immunized plasma. Therefore, in the study in vitro, we could investigate the direct effect of induced immunoglobulins. At first we compared viability of rabbit VSMCs between rat hepatocyte-immunized immunoglobulin treated control group and rat VSMC-immunized immunoglobulin treated group by use of WST-1 assay, and confirmed that viability of VSMC did not differ between two groups. Therefore VSMC-immunized immunoglobulin didn't have severe cell toxicity against VSMCs. The VSMC-immunized rabbits immunoglobulin significantly suppressed rabbit VSMC cell numbers stimulated by FBS, ATII, PDGF, FGF, and PMA (Fig. 5) and rabbit VSMC migration stimulated by FBS, ATII, PDGF, and PMA (Fig. 6). We applied immunoglobulins to the culture media, which bind only to the cell surface proteins. Therefore we speculate that rat VSMC-immunized immunoglobulin might bind to some surface proteins which globally regulate the proliferation and migration of rabbit VSMCs. As one of candidates for such surface proteins, we focused on AT1a receptor, since the inhibitory effects of VSMC-immunized rabbits immunoglobulin were prominent in ATII-induced proliferation and migration. As shown in Fig. 7, rat VSMC-immunized immunoglobulin was immunoreactive against the AT1a receptor protein which was expressed in COS7 cells by the rabbit AT1a receptor pcDNA3 transfection, whereas no bands were detected by immunoglobulin from PBS-injected rabbits or that from hepatocyte-immunized rabbits. Therefore, it is possible that rabbit AT1a receptor protein is one of target proteins which rat VSMC-immunized immunoglobulin recognizes and that binding of rat VSMC-immunized immunoglobulin to rabbit AT1a receptors might have inhibited the ATII signaling. Not only ATII signaling but almost all stimuli tested, however, were affected by the adjunction of rat VSMC-immunized immunoglobulin. Therefore it is likely that a much more global effect than the blocking effect on these receptors was involved. At the present time, we cannot show more detailed molecular mechanisms of the inhibitory effects of xenogenic VSMC immunization, and further investigation is needed.

This study demonstrated for the first time that xenogenic VSMC immunization significantly reduced neointimal formation in the balloon-injured carotid arteries. Our data indicate the possibility of immunotherapy for neointimal formation with xenogenic VSMCs by breaking immune tolerance against autologous VSMCs in a cross-reaction between the xenogenic homologs and self molecules, similar to the case of immunotherapy for tumor angiogenesis [36].

In the present study, rat VSMC-immunization didn't cause any serious side effects. The body weight and behavior of rabbits did not change by the immunization, and also blood analysis didn't show any significant changes in liver functions, renal functions, and lipid profiles. However, at the present time we cannot show the target protein(s) of VSMC-immunized immunoglobulin that regulates the functions of rabbit VSMC globally. Although further investigation on the target protein(s) and the possibility of immunological regulation is needed, immunological regulation shown in our study may be a new immunotherapy for vascular remodeling which forms neointimal lesion.

Acknowledgment

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Original Article

Interaction between Amlodipine and Simvastatin in Patients with Hypercholesterolemia and Hypertension

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3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors are often prescribed in association with antihypertensive agents, including calcium antagonists. Simvastatin is an HMG-CoA reductase inhibitor that is metabolized by the cytochrome P450 (CYP) 3A4. The calcium antagonist amlodipine is also metabolized by CYP3A4. The purpose of this study was to investigate drug interactions between amlodipine and simvastatin. Eight patients with hypercholesterolemia and hypertension were enrolled. They were given 4 weeks of oral simvastatin (5 mg/day), followed by 4 weeks of oral amlodipine (5 mg/day) co-administered with simvastatin (5 mg/day). Combined treatment with simvastatin and amlodipine increased the peak concentration (C_{max}) of HMG-CoA reductase inhibitors from 9.6±3.7 ng/ml to 13.7±4.7 ng/ml ($p<0.05$) and the area under the concentration-time curve (AUC) from 34.3±16.5 ng h/ml to 43.9±16.6 ng h/ml ($p<0.05$) without affecting the cholesterol-lowering effect of simvastatin. This study is the first to determine prospectively the pharmacokinetic and pharmacodynamic interaction between amlodipine and simvastatin. (*Hypertens Res* 2005; 28: 223–227)

***Key Words*: drug interaction, simvastatin, amlodipine, hypercholesterolemia**

Introduction

Control of hypercholesterolemia is important for the prevention of coronary artery disease (CAD) (*1–5*). Currently, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors are the first-choice therapeutic agents for patients with hypercholesterolemia (*6–8*). The HMG-CoA reductase inhibitor simvastatin is widely used and has been shown to reduce morbidity and mortality from CAD (*9*). Simvastatin is an inactive lactone pro-drug that is hydrolyzed by esterases to simvastatin acid, the active competitive inhibitor of HMG-CoA reductase (*10–12*). Simvastatin and simvastatin acid are mainly metabolized by the cytochrome P450 (CYP) 3A4 to 3′,5′-dihydrodiol, 3′-hydroxy and 6′-exomethylene (*10–12*). The pharmacokinetics of simvastatin has been reported to be affected by potent CYP3A4 inhibitors such as itraconazole (*13*), erythromycin (*14*), verapamil (*14*) and nelfinavir (*15*). Moreover, we have previously reported that diltiazem, which is a selective inhibitor of CYP3A4 (*16*, *17*), caused a 2-fold increase of the area under the concentration-time curve (AUC) of HMG-CoA reductase inhibitors (*18*).

Hypercholesterolemia is often accompanied by hypertension, an associated risk factor for CAD (*19–21*). Calcium antagonists have been widely used in the treatment of hypertension and/or angina pectoris (*22–26*), and are often prescribed in association with a lipid-lowering agent such as simvastatin. Amlodipine is one of the 1,4-dihydropyridine calcium antagonists with a long elimination half-life (*27–29*). Amlodipine undergoes the oxidative metabolism of dihydro-

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Table 1. Patient Demographics and Basic Medical Data

Age (years old)	64.1±6.0
Sex (male/female)	5/3
Body weight (kg)	61.5±5.9
Total cholesterol (mg/dl)	253±31
LDL-cholesterol (mg/dl)	164±26
HDL-cholesterol (mg/dl)	54±9
Triglyceride (mg/dl)	179±95

Values are mean±SD. LDL, low-density lipoprotein; HDL, high-density lipoprotein.

pyridine to a pyridine analogue by CYP3A4 (*30*). In an *in-vitro* study, amlodipine was shown to have strong inhibitory effects on CYP1A1, CYP2B6 and CYP2C9, and a weak inhibitory effect on CYP3A4 when using microsomes from human B-lymphoblast cells expressing CYP (*31*). Although amlodipine is one of the most frequently used calcium antagonists, the drug interaction between amlodipine and substrate drugs for CYP3A4 has not been clinically investigated. In this study we prospectively studied the pharmacokinetic and pharmacodynamic drug interaction between amlodipine and simvastatin in patients with hypercholesterolemia and hypertension.

Methods

Subjects

Eight patients with mild hypertension and hypercholesterolemia who had been treated with simvastatin (5 mg/day) and the angiotensin-converting enzyme inhibitor enalapril (5 mg/day) for more than 3 months were enrolled. Before the start of any antihypertensive therapy, the mean systolic and diastolic blood pressure levels (SBP/DBP) were 151±29 mmHg and 88±11 mmHg, respectively. The patient demographics and basic medical data are shown in Table 1. Patients had no history of hepatic or renal disease. The study protocol was approved by the Ethical Committee of Hamamatsu University School of Medicine. All subjects gave written informed consent before participating in the study.

Study Design

This was a two-phase fixed-order design study. In the first period, patients were administered oral simvastatin (5 mg/day) alone for 4 weeks. In the second period, patients were co-administered amlodipine (5 mg/day) and simvastatin (5 mg/day) for 4 weeks. No drug other than simvastatin and amlodipine was taken during the study period.

Blood Sampling

Blood samples were obtained on the last day of each of the trial periods. After an overnight fast, a pre-dosing venous blood sample was taken, which was used to measure serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglyceride (TG) enzymatically, and the low-density lipoprotein cholesterol (LDL-C) concentration was calculated according to the Friedewald formula method (*32*). All patients drank a glass of water after swallowing the tablets. Blood samples were then taken 2, 3, 4 and 6 h after simvastatin administration. A standardized breakfast and lunch were served 2 and 4 h after drug intake. Plasma was separated within 30 min and stored at −70°C until analysis.

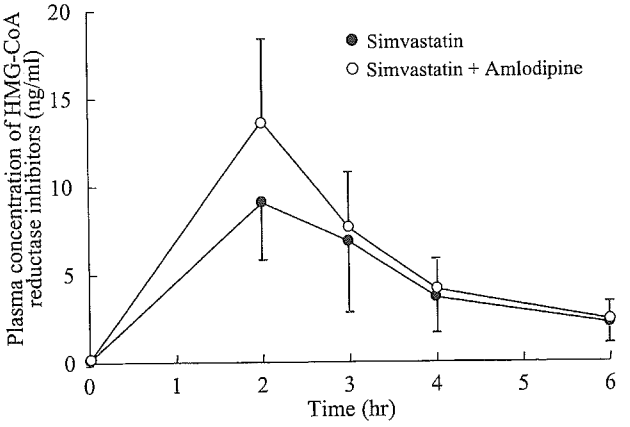

Fig. 1. *Time profiles of the mean plasma concentrations of HMG-CoA reductase inhibitors on the last day of 4 weeks of treatment with simvastatin (5 mg/day) or combined treatment with simvastatin (5 mg/day) and amlodipine (5 mg/day). Each point represents the mean±SD.*

Determination of Simvastatin HMG-CoA Reductase Inhibitor Concentrations

Plasma concentrations of HMG-CoA reductase inhibitors were determined as previously described (*33*). An equal volume of methanol was added to the plasma samples and the mixtures were vortexed thoroughly, kept on ice for 10 min, and centrifuged. Fifty microliters of the supernatants were dried in an evaporator (SpeedVac; Savant Instruments, Farmingdale, USA). The reaction mixture (96 μl) was added directly to the dried residues to make a final volume of 100 μl containing 0.1 mol/l KPO_4 (pH 7.4), 10 mmol/l 1,4-dithiothreitol (DTT), 0.2 mmol/l $NADH^+$ (made fresh daily), 5 mmol/l glucose-6-phosphate, 1.4 U/ml glucose-6-phosphate dehydrogenase and 1 mg/ml bovine serum albumin. The reaction mixture was incubated for 5 min at 37°C, and soluble rat liver HMG-CoA reductase was added to 2 μl buffer A: 0.04 mol/l KPO_4 (pH 7.4), 0.05 mol/l KCl, 0.1 mol/l sucrose, 0.03 mol/l ethylenediaminetetraacetic acid (EDTA) and 0.01 mol/l DTT (added immediately before use). The mixture was incubated at 37°C for 5 min in the presence of the inhibitor-con-

Table 2. Pharmacokinetic Parameters of Simvastatin HMG-CoA Reductase Inhibitor Concentrations

	C_{max} (ng/ml)	$t_{1/2}$ (h)	AUC(0–∞) (ng h/ml)
Simvastatin	9.6±3.7	2.08±0.59	34.3±16.5
Simvastatin+amlodipine	13.7±4.7*	1.97±0.61	43.9±16.6*

Values are mean±SD. C_{max}, maximal measured concentration; $t_{1/2}$, the elimination half-life; AUC(0–∞), area under the concentration-time curve. *$p<0.05$ *vs.* simvastatin monotherapy.

taining plasma sample. The reaction was started with 2 μl of 1.25 mg/ml HMG-CoA containing 17.5 μCi/ml glutaryl-3-[^{14}C]HMG-CoA. After an additional 6-min incubation at 37°C, 20 μl of 5 mol/l HCl was added to lactonize the mevalonic acid formed. After 15 min, 3.5 ml of a 1:1 suspension of BioRad AG 1 × 8 resin (200–400 mesh) was added and the tubes (13 × 100) were thoroughly vortexed. [^{14}C]Mevalonolactone was filtered from the resin suspension through polystyrene filters (pore size 70 μm; EverGreen, Los Angeles, USA) into scintillation vials containing 15 ml of Aquasol-2 (New England Nuclear, Newton, USA) and counted on a scintillation counter. The percentage of inhibition was converted to the inhibitor concentration using a standard curve constructed by extracting from the control plasma containing known amounts of L-654, 969, the free acid form of simvastatin. The results were expressed as nanograms of inhibitor per milliliter of plasma. The intra- and inter-day coefficients of variation for the HMG-CoA reductase activity assay were less than 6%.

Data Analysis

The pharmacokinetics of simvastatin was characterized by the peak concentration (C_{max}), the time to C_{max} (T_{max}), the elimination half-life ($t_{1/2}$) and the area under the plasma concentration-time curve from 0 to infinity [AUC(0–∞)]. The C_{max} and T_{max} were obtained directly from the original data. The terminal rate constant (k_e) used for the extrapolation was determined by regression analysis of the log-linear part of the concentration-time curve for each subject. The $t_{1/2}$ was determined by $0.693/k_e$. The AUC(0–∞) was calculated by the trapezoidal rule for the observed values and subsequent extrapolation to infinity. Data are represented as the mean±SD. Data were analyzed by a paired *t*-test or Wilcoxon signed-rank test where appropriate. Differences with p values <0.05 were considered statistically significant.

Results

No subjects reported a serious clinical, laboratory or other adverse effect, and no subjects were discontinued.

Pharmacokinetics of Simvastatin HMG-CoA Reductase Inhibitor Concentrations

Plasma concentrations of HMG-CoA reductase inhibitors after oral simvastatin dosing with or without amlodipine are shown in Fig. 1, and pharmacokinetic parameters of simvastatin are shown in Table 2. Co-administration of amlodipine with simvastatin significantly increased the C_{max} and AUC(0–∞) of HMG-CoA reductase inhibitors to 1.4- and 1.3-fold, respectively, in simvastatin monotherapy, but did not affect the $t_{1/2}$ and T_{max} of HMG-CoA reductase inhibitors.

Pharmacodynamics

Lipid profile, including TC, LDL-C, HDL-C, and TG during simvastatin monotherapy and combined treatment with simvastatin and amlodipine, are shown in Fig. 2. There were no significant differences in lipid profiles between the two periods.

The SBP and DBP values are shown in Table 3. Both measures were significantly higher during simvastatin monotherapy than during the pretrial control period with enalapril. After administration of amlodipine, both SBP and DBP tended to decline ($p=0.06$ and $p=0.08$, respectively). The blood pressure values during combined treatment with simvastatin and amlodipine did not differ from those during the pretrial control period with enalapril.

Discussion

Calcium antagonists and HMG-CoA reductase inhibitors are often prescribed together for the treatment of hypertension and/or angina pectoris in patients with hypercholesterolemia (*1*, *6*, *7*). Amlodipine is used with many drugs, such as oral hypoglycemic drugs, β-blockers, angiotensin-converting enzyme inhibitors, and so on. However, there have been no reports on the interaction between amlodipine and any other drug, with the exception that the interaction of amlodipine with grapefruit juice was shown to increase the AUC of amlodipine (*34*). This study is the first to report that amlodipine affected the plasma concentrations of HMG-CoA reductase inhibitors.

Simvastatin is hydrolyzed by esterases to simvastatin acid, which is an active inhibitor of HMG-CoA reductase (*10–12*). Simvastatin is extensively metabolized to several oxidative products by CYP3A4 (*10–12*). Some of the hydroxyl acid forms of these products also inhibit HMG-CoA reductase (*10*, *11*). In this study, we measured the total HMG-CoA reductase inhibitory activity resulting from simvastatin acid and all other active acid metabolites of simvastatin, since this level is

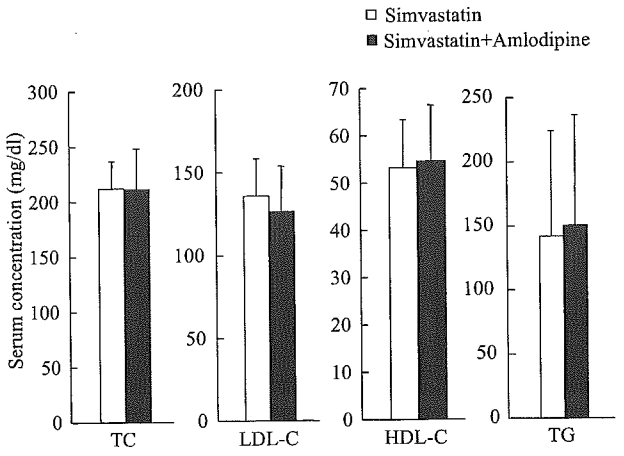

Fig. 2. *Mean levels of serum lipid parameters on the last day of 4 weeks of treatment with simvastatin (5 mg/day) or combined treatment with simvastatin (5 mg/day) and amlodipine (5 mg/day). TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides. Each column represents the mean±SD.*

believed to be relevant to the systemic adverse effects for this class of agents (*35*).

The pharmacokinetics of simvastatin has been shown to be affected by potent CYP3A4 inhibitors (*13–15*, *18*). Amlodipine, which is metabolized by CYP3A4, has been reported to show inhibitory effects on CYP3A4 *in vitro* (*31*). However, the influence of amlodipine on the substrate drugs of CYP3A4 has not been clarified yet. In this study, amlodipine significantly increases the AUC of HMG-CoA reductase inhibitors after co-administration of simvastatin by 30%. It has been reported that the AUC of HMG-CoA reductase inhibitors was increased 4-fold with itraconazole (*13*), which is known to be a potent inhibitor of CYP3A4. Some studies have show adverse effects, including rhabdomyolysis, in patients treated with simvastatin and CYP3A4 inhibitors such as itraconazole and ketoconazole (*8*). These reports suggested that the co-administration of simvastatin with these inhibitors enhanced the risk of adverse effects, because of the dose-dependent toxicity of HMG-CoA reductase inhibitors. In our previous study, diltiazem increased the AUC of HMG-CoA reductase inhibitors 2-fold (*18*). On the other hand, amlodipine increased the AUC of HMG-CoA reductase inhibitors by only 30% in this study. In addition, it has been reported that the CYP3A4 inhibitory effect of diltiazem was higher than that of amlodipine after therapeutic doses (*36*). Therefore, the difference of the impact on the plasma concentrations of HMG-CoA reductase inhibitors may depend on the difference of the CYP3A4 inhibitory potency between amlodipine and diltiazem.

It has been reported that an increase in the plasma concentrations of HMG-CoA reductase inhibitors following co-administration of simvastatin and diltiazem resulted in a reduction of TC and LDL-C levels (*18*). However, we did not observe such a reduction of TC and LDL-C levels, despite the fact that amlodipine increased the plasma concentrations of HMG-CoA reductase inhibitors. The pharmacokinetic interactions observed in the present study, such as the 30% increase in the AUC of HMG-CoA reductase inhibitors, may not have been sufficient to alter the pharmacodynamic response. Moreover, we cannot exclude the possibility that the number of patients was not sufficient to detect the pharmacodynamic differences. Further investigations will be needed to clarify the pharmacodynamic impact of simvastatin with amlodipine on TC and LDL-C.

In conclusion, this study is the first report of the drug interaction between simvastatin and amlodipine after a long-term treatment. Although amlodipine increases the plasma concentrations of HMG-CoA reductase inhibitors, the impact of amlodipine on simvastatin is smaller than that of diltiazem. Since these drugs are often used concomitantly for patients with hypertension and hypercholesterolemia, amlodipine could be used more safely with simvastatin than diltiazem.

Table 3. Systolic BP and Diastolic BP during Pretrial Control Period with Enalapril, Simvastatin Monotherapy and Combined Treatment with Simvastatin and Amlodipine

	Systolic BP (mmHg)	Diastolic BP (mmHg)
Simvastatin+enalapril (pretrial control period)	135±19	78±13
Simvastatin	152±22*	89±13*
Simvastatin+amlodipine	140±17	81±11

Values are mean±SD. BP, blood pressure. *$p<0.05$ *vs.* simvastatin+enalapril.

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The authors are grateful to Dr. Takashi Ishizaki (Teikyo-Heisei University) for his helpful insights and to H. Kobayashi for technical assistance.

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HMG-CoA 還元酵素阻害薬 Pravastatin 服用患者におけるリスクファクターと血清脂質値に関する調査

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Risk Factors and Serum Cholesterol Concentrations in the Patients Given HMG-CoA Reductase Inhibitor, Pravastatin

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Purpose : HMG-CoA reductase inhibitors (statins) have been widely used in the treatment of hypercholesteremia in Japan as well as in Western countries. Although statins have been shown to be effective in the prevention of coronary heart disease (CHD) in high-risk patients, the potential benefit of statins on the overall mortality has not been proven in subjects at lower risk for CHD. In this study, we investigated the risk factors and serum cholesterol concentrations in patients given pravastatin.

Methods : Patients who were given pravastatin during the period from June 2002 until May 2003 in the Hamamatsu University Hospital were studied. Data for height, body weight, age, gender, smoking and history of diabetes mellitus, hypertension and CHD in the patients were collected from their case records. Serum cholesterol concentrations were determined before and after the treatment with pravastatin. The ethics committee in the Hamamatsu University approved this study.

Results : There were 213 male (37.4%) and 356 female (62.6%) patients given pravastatin. The mean age of the patients was 63.9 yrs, and % of the patients aged under 50 yrs was 10.7%. Seventy-seven % of the patients had no history of CHD. Female patients without smoking, diabetes mellitus, hypertension and CHD constituted 17% of all patients. Total and LDL cholesterol levels in all groups were significantly decreased by 17.6% and 25.5%, respectively, after the administration of pravastatin. Treatment with pravastatin was started at the lower total cholesterol levels in male patients or patients with CHD than in female patients or patients without CHD.

Conclusion : Our results suggest that significant numbers of patients with a low risk for CHD were prescribed the statins, and that placebo-controlled large-scale trials should be conducted to demonstrate the benefit and safety of statin treatment on overall mortality in Japan.

Key words : HMG-CoA reductase inhibitors, statins, pravastatin, hypercholesteremia, risk factor

緒　論

近年，わが国においてもライフスタイルの欧米化などにより動脈硬化性疾患が増加し，死因統計で癌と並ぶ大きな位置を占めるようになった．国内外の多くの研究から血清コレステロール値が上昇するに従い，男

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Table 1 Demographic characteristics of the patients treated with pravastatin at the point of the survey

	Male	Female	Total
Number of patients	213 (37.4%)	356 (62.6%)	569 (100%)
Age [years]	63.2±11.6	64.2±12.2	63.9±12.0
Height [cm]	164.3±6.2	151.8±6.1	156.5±6.1
Weight [kg]	63.2±10.0	52.0±8.9	56.2±9.3
Periods for the treatment with pravastatin [month]	48.9±40.4	59.5±46.5	55.5±44.6
Smoking	63 (11.1%)	31 (5.4%)	94 (16.5%)
Risk factors			
Coronary heart disease	80 (14.1%)	52 (9.1%)	132 (23.2%)
Diabetes mellitus	73 (12.8%)	126 (22.1%)	199 (34.9%)
Hypertension	141 (24.8%)	206 (36.2%)	347 (61.0%)

Values are numbers of patients (% of all patients (n=569)), or mean ± SD.

女を問わず虚血性心疾患発症リスクは増加することが示され[1~3]，高コレステロール血症治療の重要性がますます高まっている．高コレステロール血症に対する薬物療法の選択肢はいくつかあるが，なかでも3-hydroxy-3-methylglutaryl-coenzyme A（HMG-CoA）還元酵素阻害薬（スタチン）は強力なLDLコレステロール（LDL-C）低下作用を有することから，現在第一選択薬として用いられている．欧米諸国を中心に行われた多くの大規模臨床試験では，虚血性心疾患患者を対象とした二次予防試験だけでなく，虚血性心疾患既往歴のない一次予防の場合においても，スタチンによるLDL-Cの低下が心血管イベントの発生率や虚血性心疾患死亡率，さらに総死亡率を低下させることが示されている[4~6]．

一方，わが国では虚血性心疾患の発生率が欧米諸国の1/4から1/10と低いことが知られている[7]．さらに遺伝的素因やライフスタイルも欧米諸国のそれらと異なることから，欧米諸国における大規模試験の結果を日本人にそのまま適応できるかどうか疑問視する意見もある[8]．

わが国においては1989年にpravastatinが発売されて以来，数種のスタチンが臨床適用され，多くの患者に投与されている．しかしわが国においてスタチンがどのような背景を持つ患者に使用されているかを実態調査した報告はほとんどない．スタチンの適正使用を推進するためにも，スタチン使用の実態を把握することは重要である．本研究では，浜松医科大学附属病院においてpravastatinを投与されている患者を対象とし，リスクファクター（年齢，性，喫煙習慣，糖尿病，高血圧，虚血性心疾患の既往）およびpravastatin服用前後の血清脂質値を調査した．

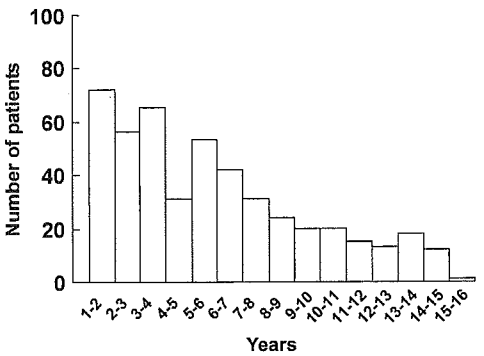

Fig. 1 Periods treated with pravastatin

方　法

浜松医科大学附属病院において2002年6月から2003年5月の間にpravastatin（メバロチン®）を投与された全患者(581例)中，カルテおよび病院オーダリングシステムを調査しえた569例を対象とした．調査期間（2003年6月～2003年8月）中のpravastatin最終投与日における対象患者の身長，体重，年齢と喫煙歴ならびに虚血性心疾患，糖尿病および高血圧の既往の有無について調査した．さらにpravastatin服用前と調査時における血清脂質値が調査可能であった478例において総コレステロール（TC），HDLコレステロール（HDL-C），LDLコレステロール（LDL-C）およびトリグリセリド（TG）を調査した．Pravastatin服用前かつ調査時の臨床検査値をカルテないしオーダリングシステム上から調査することが可能であった症例においては，アスパラギンアミノトランスフェラーゼ（AST），アラニンアミノトランスフェラーゼ（ALT），クレアチンキナーゼ（CK），血清クレアチニン（s-Cre），血液尿素窒素（BUN），随時血