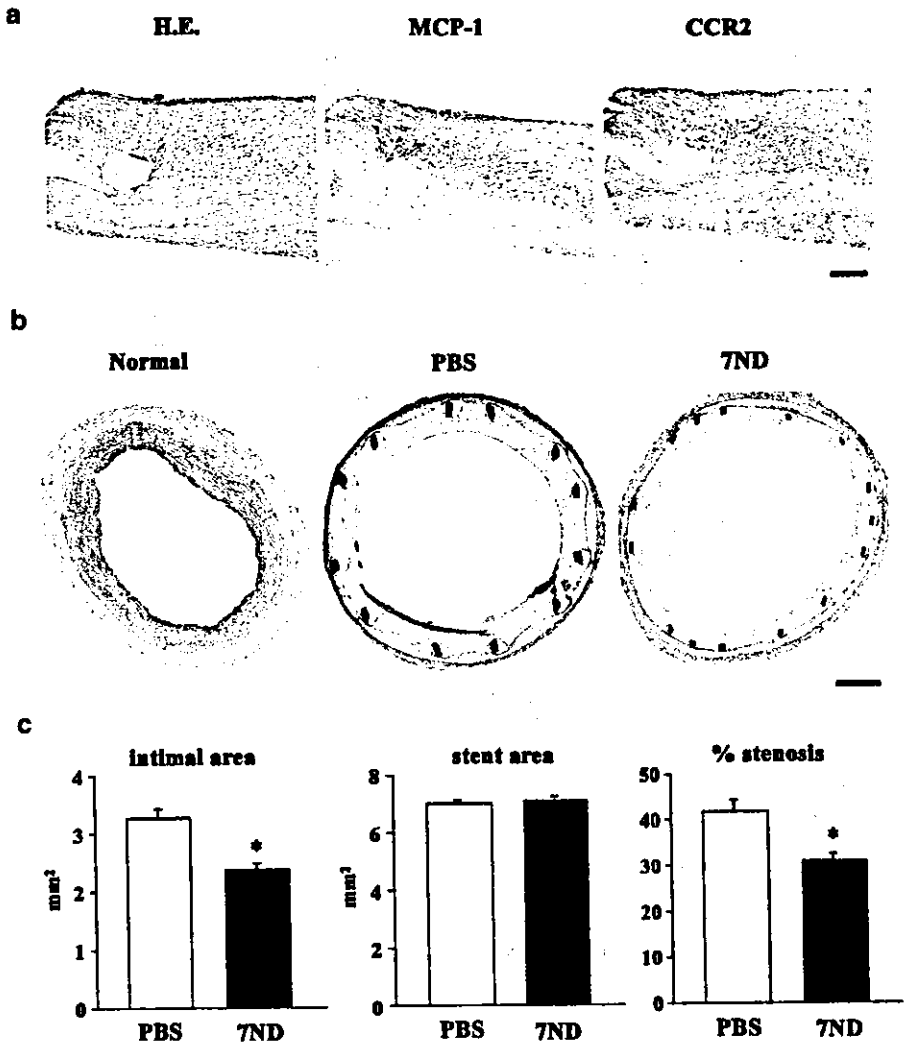

*Figure 4 Effects of 7ND gene transfer on in-stent neointimal hyperplasia in cynomolgus monkeys. (a) Arterial sections of stented artery from the PBS group stained with HE or immunohistochemically with the antibodies against MCP-1 and CCR2 28 days after stenting in monkeys. Bar = 100 μm. (b) Noninjured control artery section (left panel) and artery sections from the PBS-treated group (middle panel) and 7ND-transfected group (right panel) 28 days after stenting stained with elastic van-Gieson in monkeys. Bar = 500 μm. (c) Effect of 7ND gene transfer on stented area, intimal area, and % stenosis 28 days after stenting (n = 8 each). *P < 0.01 versus PBS.*

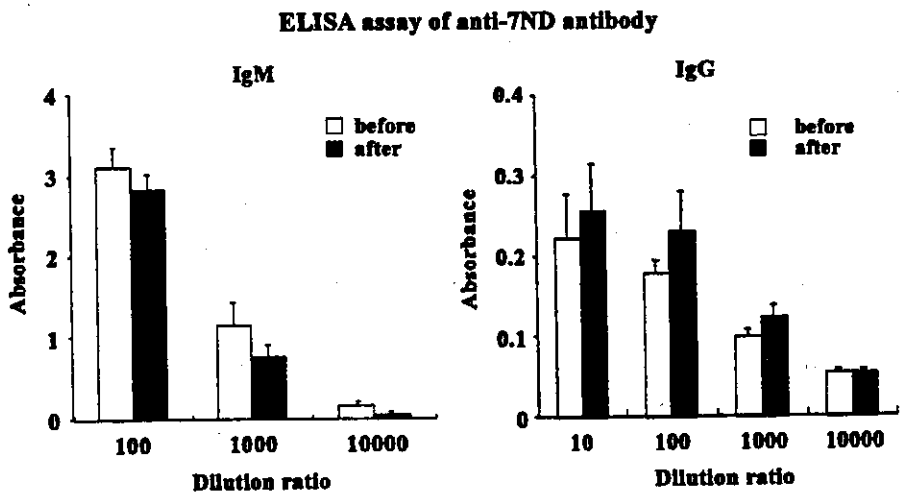

Figure 5 ELISA assay of anti-7ND antibody (IgM and IgG) in paired serum from 7ND-transfected monkeys.

Institute, Japan) as a template and inserted into the *Bam*H1 (5′) and Not1 (3′) sites of the pcDNA3 (Invitrogen) expression vector plasmid.[15]

Animal model of in-stent restenosis and gene transfer

The experiments were reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine, Japan, and were performed according to the Guidelines of American Physiologic Society. A part of this study was performed at the Kyushu University Station for Collaborative Research and the Morphology Core, Japan.

Male Japanese white rabbits (KBT Oriental, Tokyo, Japan) weighing 3.0–3.5 kg were fed a high cholesterol diet containing 1% cholesterol and 3% peanut oil for 2 weeks. They were then anesthetized by i.m. injection of xylazine (5 mg/kg) and ketamine (35 mg/kg). The right common carotid arteries were surgically exposed, and a 3F Fogarty balloon catheter was passed into one iliac artery under fluoroscopic guidance, and the balloon was then withdrawn and inflated over a 3-cm section of artery three times; then, a 15 mm-long Multilink stent (RX Multi-link plus, Guidant/ACS Inc.) mounted over the 3-mm balloon was implanted in the iliac artery (30-s inflation, 10 atm, stent-to-artery ratio of 1.2:1.0). When the stent was intended to be implanted bilaterally, the procedure was repeated in the contralateral iliac artery. The carotid artery was then ligated and the incision was closed. After the operation, all rabbits were fed the same high cholesterol diet.

At 3 days before stenting, rabbits were randomly divided into two groups: the PBS group (16 stents in 14 rabbits) was injected with PBS and the 7ND group (16 stents in 14 rabbits) was injected with the 7ND gene (500 μg/0.3 ml PBS) into their femoral muscle. To enhance transgene expression, all plasmid-injected animals received electroporation at the injection site immediately after injection with an electric pulse generator CUY21 (BTX, San Diego, CA, USA) as described previously.[17,19] All rabbits were killed with a lethal dose of anesthesia 7 or 28 days after stenting for biochemical, immunohistochemical, and morphometric analyses. IVUS study was also performed 28 days after stenting.

Male adult cynomolgus monkeys (5 years old) weighing 4–5 kg were purchased from Primate Ltd., Kumamoto, Japan and were fed laboratory diet containing 0.5% cholesterol starting 2 months before stent implantation. Monkeys were anesthetized with ketamine hydrochloride (10 mg/kg i.m.) and sodium pentobarbital (30 mg/kg i.v. to effect), and underwent placement of a 3×15 mm^2 stent as described above. Monkeys were also injected with PBS (eight stents in six monkeys) or 7ND plasmid (2.5 mg/500 μl PBS, eight stents in six monkeys) into their femoral muscle immediately after stenting. To enhance transgene expression, all monkeys were pretreated with i.m. injection of bupivacaine (0.25 mg/kg) at the injection site.[17] We reported the biologic efficacy of 7ND gene transfer using *in vivo* matrigel plug assay in monkeys.[17] In brief, MCP-1-induced inflammatory angiogenesis in the plugs was suppressed until 14 days after 7ND gene transfer. All monkeys were killed with a lethal dose of anesthesia 28 days after stenting for morphometric analysis. All animals received aspirin 81 mg/day and ticlopidine 100 mg/day until euthanasia.

Histopathology and immunohistochemistry

Stented arterial segments were excised and fixed for 24 h with 6–10% paraformaldehyde or methacarn solution for several days. Each segment was divided into two parts at the center of the stent. The proximal part was embedded in methyl methacrylate mixed with *n*-butyl methacrylate to allow for sectioning through metal stent struts. Sections were stained with elastica van Gieson and hematoxylin–eosin (HE). To evaluate the thickening of the neointima, the areas encroached by the external elastic lamina, the internal elastic lamina, and the lumen area were measured. The distal part was used for immunohistochemical analysis. After stent struts were gently removed with microforceps, the tissue was dehydrated, embedded in paraffin, and cut into 5-μm thick slices. In rabbits, the sections were subjected to immunostaining using antibodies against rabbit monocytes/macrophages (RAM11, Dako), proliferating cell nuclear antigen (PCNA) (Dako), MCP-1 (R & D), endothelial cell (CD31, Dako), VEGF (Santa Cruz), or non-immune mouse IgG (Dako). The cells that underwent apoptosis were detected by using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining (*in situ* apoptosis detection kit, Takara). The number of apoptotic nuclei in arterial wall was counted, and the data were calculated and expressed as the percentage of apoptotic cells nuclei/total nuclei. In monkeys, the sections were immunohistochemically stained with antibodies against MCP-1 (R & D), CCR2 (Sigma Chemical), or non-immune mouse IgG (Dako). The slides were washed and incubated with biotinylated, affinity-purified goat IgG. Following avidin–biotin amplification, the slides were incubated with diaminobenzidine and counterstained with hematoxylin. The percentage of immunopositive cells per total cells in each section was calculated, and the average of five sections was reported for each animal.

Intravascular ultrasound procedure and analysis

High-resolution IVUS analysis was performed 28 days after stenting with an electron-scan IVUS system (Terumo TU-C100, Tokyo, Japan). A contour detection program was used for two-dimensional analysis. Quantitative IVUS measurements were performed at 10 cross-sections in each stent segment at 2-mm intervals from the distal stent end. Cross-sectional narrowing was calculated as the neointimal area divided by stent area. All the measurements were performed by a single sonographer who was unaware of the experimental protocol.

Plasma and tissue measurements

Plasma total cholesterol and LDL cholesterol were measured using a commercially available kit. To measure 7ND released by the transfected skeletal muscle, plasma and femoral skeletal muscle concentrations of 7ND were measured using the human MCP-1 enzyme-linked immunosorbent assay kit (Biosource) in rabbits.[19] As this human MCP-1 ELISA kit does not react with the rabbit MCP-1, we can measure plasma 7ND in rabbits.

Real-time reverse quantitative transcription-PCR

The normal and stented arteries were excised and washed with cold normal saline 7 days after stent placement. Stent was removed gently. The samples were

cleaned of excessive perivascular tissue, and frozen in liquid nitrogen. Total RNA was reverse transcribed, and the resultant cDNA was amplified by TaqMan Real-Time Reverse Transcription-PCR on the ABI Prism 7000 Sequence Detection as described previously.[18] The respective PCR primers and TaqMan probes were designed from GenBank databases using a software program (Applied Biosystems; Table 1). The results were analyzed using the Sequence Detection Software (Applied Biosystems) and expressed in arbitrary units and adjusted for GAPDH mRNA levels.

Measurement of antibody productions in 7ND-transfected animals

We examined whether anti-7ND IgG and IgM antibodies were produced in 7ND-transfected monkeys. In all, 96-well plates (ELISA PLATE HTYPE, SUMITOMO BAKELITE Co., Ltd.) were coated with 7ND protein (0.1 μg/ml). Paired serum before and 7 or 28 days after 7ND transfection was incubated on each coated well for 90 min at 37°C followed by incubation with HRP-conjugated goat antibodies against monkey IgG or IgM (Kirkegaard & Perry Laboratories) for 1–2 h at 37°C. TMB one solution (Promega) was used, and the absorbencies of each well were detected by using ELISA plate reader.

Statistical analysis

Data are expressed as the mean ± s.e. Statistical analysis of differences was compared by analysis of variance and Bonferroni's multiple comparison tests. A P-value of less than 0.05 was considered to be statistically significant.

Acknowledgements

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Rapid Communication

Bone Marrow–Derived Monocyte Chemoattractant Protein-1 Receptor CCR2 Is Critical in Angiotensin II–Induced Acceleration of Atherosclerosis and Aneurysm Formation in Hypercholesterolemic Mice

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Abstract—Angiotensin II (Ang II) is implicated in atherogenesis by activating inflammatory responses in arterial wall cells. Ang II accelerates the atherosclerotic process in hyperlipidemic apoE−/− mice by recruiting and activating monocytes. Monocyte chemoattractant protein-1 (MCP-1) controls monocyte-mediated inflammation through its receptor, CCR2. The roles of leukocyte-derived CCR2 in the Ang II-induced acceleration of the atherosclerotic process, however, are not known. We hypothesized that deficiency of leukocyte-derived CCR2 suppresses Ang II-induced atherosclerosis.

Methods and Results—A bone marrow transplantation technique (BMT) was used to develop apoE−/− mice with and without deficiency of CCR2 in leukocytes (BMT-apoE−/−CCR2+/+ and BMT-apoE−/−CCR2−/− mice). Compared with BMT-apoE−/−CCR2+/+ mice, Ang II-induced increases in atherosclerosis plaque size and abdominal aortic aneurysm formation were suppressed in BMT-apoE−/−CCR2−/− mice. This suppression was associated with a marked decrease in monocyte-mediated inflammation and inflammatory cytokine expression.

Conclusion—Leukocyte-derived CCR2 is critical in Ang II-induced atherosclerosis and abdominal aneurysm formation. The present data suggest that vascular inflammation mediated by CCR2 in leukocytes is a reasonable target of therapy for treatment of atherosclerosis. (*Arterioscler Thromb Vasc Biol.* 2003;24:e174-e178.)

Key Words: atherosclerosis ■ angiotensin II ■ inflammation ■ leukocytes

Chronic inflammatory processes have an important role in atherosclerotic plaque progression, destabilization, and subsequent rupture/thrombosis, resulting in acute coronary syndrome.[1,2] Therefore, identification of the critical inflammatory pathway involved in atherosclerotic plaque progression and destabilization might aid in the development of novel therapeutic strategies to reduce atherothrombotic events.

The renin-angiotensin system is now recognized as an important therapeutic target of atherosclerotic vascular disease.[3,4] Angiotensin II (Ang II) induces the production of reactive oxidative species and stimulates the expression of adhesion molecules (vascular cell adhesion molecule-1) and chemokines (monocyte chemoattractant protein-1 [MCP-1]).[3–5] Infusion of Ang II into hypercholesterolemic mice dramatically accelerates the atherosclerotic process, leading to the development of extensive atherosclerotic plaque formation and abdominal aortic aneurysm (AAA).[6,7] The Ang II-mediated acceleration of atherogenesis is characterized by the recruitment and activation of monocytes/macrophages and the degradation of elastin and collagen layers, suggesting that Ang II changes the lesion composition into a more destabilized phenotype. MCP-1 is a C-C chemokine that controls monocyte recruitment to the site of inflammation through its receptor, C-C chemokine receptor (CCR) 2.[8–10] We recently demonstrated that blockade of the MCP-1 pathway by transfection of mutant MCP-1 gene limits Ang II-induced progression and destabilization of atherosclerotic

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lesions in hyperlipidemic apoE−/− mice.[11] We and others also demonstrated that blockade or abrogation of MCP-1 or CCR2 attenuates hyperlipidemia-induced atherosclerosis in mice,[12–15] and that CCR2-deficient (CCR2−/−) mice display reduced neointimal formation after arterial injury.[16] Overall, these studies provide ample evidence for a decisive role of MCP-1/CCR2 in atherosclerosis formation, progression, and destabilization.

The role of MCP-1 and/or CCR2 in atherogenesis might be more complex. Ang II is thought to accelerate atherogenesis by stimulating MCP-1 expression and function in multiple cell types in atherosclerotic lesions such as endothelial cells, smooth muscle cells, and leukocytes. Because CCR2 is present in these cell types, activation of the MCP-1–CCR2 pathway mediates recruitment and activation of monocytes,[17] endothelial migration and angiogenesis,[18,19] and migration/proliferation of vascular smooth muscle cells.[20] It is impossible, however, to dissect the relative pathobiologic role of leukocytes versus nonleukocyte cells in the arterial wall using the systemic absence or blockade of MCP-1/CCR2. The aim of this study was to address the role of CCR2 on leukocytes in Ang II-mediated acceleration of the atherosclerotic process. We used bone marrow cell transplantation (BMT) techniques to create a murine model with a leukocyte-derived CCR2 deficiency and demonstrated the essential role of leukocyte-derived CCR2 in Ang II-induced acceleration of atherosclerotic processes.

Methods

Experimental Animals

Male apoE knockout mice were purchased from Jackson Laboratory (Bar Harbor, Me). apoE−/− CCR2−/− and apoE−/− CCR2+/+ mice with the same genetic background (C57BL/6J and 129/svjae hybrids) were supplied by Dr Charo.[12]

Experimental Protocol

The study protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences. A part of this study was performed at the Kyushu University Station for Collaborative Research and the Morphology Core Unit, Kyushu University Faculty of Medical Sciences.

To determine the specific role of CCR2 on leukocytes, we used the BMT technique to create mice with and without a leukocyte-selective CCR2 deficiency. At 8 weeks of age, BMT was performed as described previously.[21] Bone marrow cells were harvested from femurs and tibias of either test (apoE−/−CCR2−/−) or control (apoE−/−CCR2+/+) donor mice. The recipient apoE−/−CCR2+/+ mice received 1×10^7 bone marrow cells (0.3 mL) 4 hours after whole body irradiation with 7 Gy of X-rays (200-KVp, 20-mA, 0.3-mm Cu filter) at 1 Gy/min. These 2 groups of mice are referred to as BMT-apoE−/−CCR2−/− and BMT-apoE−/−CCR2+/+, respectively. At 14 weeks of age, BMT-apoE−/−CCR2−/− and BMT-apoE−/−CCR2+/+ mice were infused with Ang II (1.9 mg/kg per day) or phosphate-buffered saline via osmotic mini-pump (Alzet, Cupertino, Calif).[21]

In all experiments, mice were euthanized on day 7 or 28 of treatment for morphometric, immunohistochemical, and biochemical analysis. Peripheral arterial blood was collected immediately before the mice were euthanized. The aortas were isolated and either fixed in 10% buffered formalin for histological and immunohistochemical analysis or snap-frozen in liquid nitrogen (LN_2) and stored at −80°C for biochemical analysis. Systolic blood pressure was measured by the tail-cuff method before and 28 days after treatment.

Histology and Immunohistochemistry

To quantify the extent of the atherosclerotic lesions, the aortic arch and the thoracic aorta was opened longitudinally, stained with oil red O, and pinned out on a black wax surface. The percentage of the plaque area stained by oil red O to the total luminal surface area was determined.

To further quantify the atherosclerotic lesions in the aortic root, serial cryostat sections (6 μm) of the aortic root were prepared as described.[14] In brief, atherosclerotic lesions in the aortic root were examined at 5 locations, each separated by 120 μm, 4 to 5 serial sections were prepared from each location. Some of these sections were stained with Elastica van Gieson and oil red O (for lipid staining). Elastica van Gieson staining was used to delineate the internal elastic lamina for determination of the intimal area. The lipid composition of the lesion was determined by calculating the percent of the oil red O positive area to the total cross-sectional vessel wall area. The remaining sections were used for immunohistochemical analysis. Air-dried cryostat sections were fixed in acetone and stained with the respective antibody: antimouse macrophage antibodies (Mac-3; Serotec Inc, Raleigh, NC) and antihuman MCP-1 antibodies (Santa Cruz Biotechnology Inc, Santa Cruz, Calif). The sections were then counterstained with hematoxylin. Respective nonimmune IgGs (Dako) were used as negative controls. Similarly, the number of macrophage accumulated into the aortic root lesion was estimated.

A single observer blinded to the experiment protocol performed quantitative analysis of atherosclerotic lesions. All images were captured with a Nikon microscope equipped with a video camera and analyzed using Adobe Photoshop 6.0 and National Institutes of Health Image Software. In each case, the average value for 4 to 5 locations or sections for each animal was used for analysis.

Real-Time Reverse-Transcription Polymerase Chain Reaction Analysis

Real-time polymerase chain reaction amplification was performed with the mouse cDNA using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems).[21] The respective polymerase chain reaction primers and TaqMan probes were designed from GenBank databases using a software program (Table I, available online at http://atvb/ahajournals.org).

Flow Cytometry Analysis

Flow cytometry analysis was performed as described previously.[21] To determine CCR2 expression in monocytes, antibodies against phycoerythrin-conjugated antimouse monocyte (CD80) (Becton Dickinson Biosciences, San Jose, Calif), goat antimouse CCR2 (Santa Cruz Biotechnology Inc), and fluorescein isothiocyanate-conjugated mouse antigoat IgG (Santa Cruz Biotechnology Inc) were used. To determine CCR2 fluorescence intensity in lymphocytes and neutrophils, leukocytes were also stained using antibodies against phycoerythrin-conjugated antimouse CD11b (Mac-1), cy-chrome–conjugated antimouse T-cell receptor β chain monoclonal antibody (Becton Dickinson Biosciences). In control experiments, fluorescein isothiocyanate-conjugated nonspecific goat IgG was used to measure nonspecific binding. Stained cells were analyzed by fluorescence-activated cell sorter Calibur (Becton Dickinson Biosciences).

Plasma Measurements

Commercially available enzyme-linked immunosorbent assay kits (Biosource International, Camarillo, Calif) were used to measure plasma total cholesterol, triglyceride, low-density lipoprotein cholesterol, and mouse MCP-1 according to the manufacturer's instructions.

Statistical Analysis

Data were expressed as mean±SEM. Statistical analysis of differences was compared by analysis of variance using Bonferroni correction for multiple comparisons. $P<0.05$ was considered statistically significant.

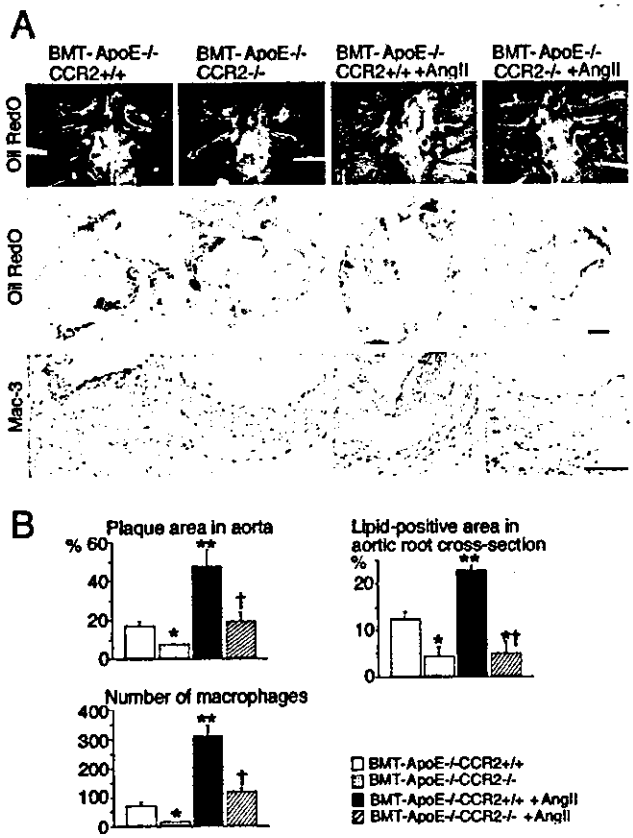

Figure 1. Suppressed Ang II-induced atherosclerotic lesions in BMT-apoE−/− CCR2−/− mice. A, Histopathologic and immunohistochemical pictures of atherosclerotic lesions. From the top to bottom panels, photomicrographs of the intraluminal surface of the aortic arch and thoracic aorta stained with oil red O, those of atherosclerotic lesions in the aortic root stained with oil red O, and those of atherosclerotic lesions in the aortic root immunostained with antimurine macrophage antibody (Mac-3). Bar indicates 100 μm. B, Quantitative comparison of atherosclerotic lesion size (% of oil red O stained area) in BMT-apoE−/−CCR2+/+,BMT-apoE−/−CCR2−/−, BMT-apoE−/−CCR2+/+ + Ang II, and BMT-apoE−/−CCR2−/− + Ang II groups. Data are reported as mean±SEM. N=6 to 8. *P<0.05, **P<0.01 versus BMT-apoE−/−CCR2+/+ group. †P<0.05, ††P<0.01 versus BMT-apoE−/−CCR2+/+ + Ang II group.

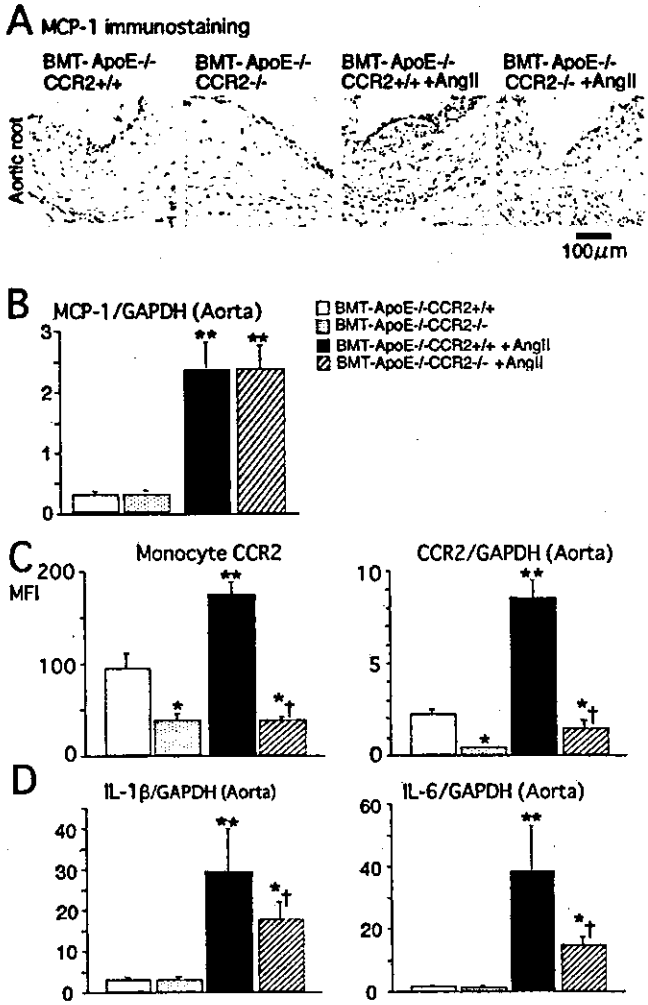

Figure 2. MCP-1, CCR2, and cytokine expression. A, Photomicrographs of cross-sections of the aortic root stained immunohistochemically for MCP-1 on day 28. Bar indicates 100 μm. B, MCP-1 gene expression by real-time reverse-transcription polymerase chain reaction in the aorta on day 7. Data are expressed as the ratio of MCP-1 mRNA to GAPDH mRNA. **P<0.01 versus BMT-apoE−/−CCR2+/+ group (n=6 each). C, CCR2 antigen expression on peripheral blood circulating monocytes by flow cytometry analysis. CCR2 gene expression of the aorta by real-time reverse-transcription polymerase chain reaction on day 7. Data are expressed as the ratio of MCP-1 mRNA to GAPDH mRNA. *P<0.05, **P<0.01 versus BMT-apoE−/−CCR2+/+ group; †P<0.05 versus BMT-apoE−/−CCR2+/+ + Ang II group (n=6 each). D, IL-1β and IL-6 gene expression by real-time reverse-transcription polymerase chain reaction in the aorta on day 7. Data are expressed as the ratio of MCP-1 mRNA to GAPDH mRNA. *P<0.05, **P<0.01 versus BMT-apoE−/−CCR2+/+ group; †P<0.05 versus BMT-apoE−/−CCR2+/+ + Ang II group (n=6 each).

Results

Bone Marrow-Derived CCR2 Is Critical for Ang II-Induced Acceleration of Atherosclerosis

To determine the role of BM-derived CCR2, Ang II was infused in BMT-apoE−/−CCR2+/+ and BMT-apoE−/−CCR2−/− mice. As reported previously,[11] Ang II infusion accelerates atherosclerotic process in BMT-apoE−/−CCR2+/+ mice. In contrast, Ang II-induced acceleration of atherosclerosis was suppressed in BMT-apoE−/−CCR2−/− mice (Figure 1A and 1B). In addition, Ang II-induced aortic inflammatory changes as well as lipid accumulation were markedly attenuated in BMT-apoE−/−CCR2−/− mice (Figure 1A and 1B).

Ang II-induced gene and protein expression of MCP-1 was examined 7 days after Ang II-infusion. Ang II infusion increased MCP-1 mRNA and immunoreactive MCP-1 levels in the aortic root, which were similar between BMT- apoE−/−CCR2+/+ and BMT-apoE−/−CCR2−/− mice (Figure 2A and 2B). Aortic CCR2 gene expression was also suppressed in BMT- apoE−/−CCR2−/− mice infused with and without Ang II (Figure 2C).

Ang II-induced changes in CCR2 antigen on circulating leukocytes were examined by flow cytometric analysis on day 7. Ang II infusion increased CCR2 antigen on monocytes in BMT-apoE−/−CCR2+/+ mice, which was blunted in BMT-apoE−/−C CR2−/− mice (Figure 2C). No CCR2 antigen was detected on lymphocytes or neutrophils in the presence or absence of Ang II infusion (data not shown).

These cytometric data indicate that CCR2 antigen was expressed mainly on circulating monocytes whether Ang II was infused.

In BMT- apoE−/−CCR2+/+ mice, Ang II infusion enhanced gene expression of IL-6 and IL-1β in the aorta on day 7 (Figure 2D). The Ang II-induced increases in IL-6 and IL-1β gene expression were reduced in BMT-apoE−/−CCR2−/− mice.

There were no significant differences in plasma MCP-1 levels between BMT-CCR2+/+ mice infused with or without Ang II on day 28 (Table I). There were no significant differences in Ang II-induced changes in systolic blood pressure or serum lipid levels (Table II, available online at http://atvb.ahajournals.org), suggesting that the observed effects of leukocyte-derived CCR2 deficiency cannot be explained by the effects on plasma lipids or Ang II-induced arterial hypertension. There were no significant differences in plasma MCP-1 levels between untreated BMT-apoE−/−CCR2+/+ and BMT-apoE−/−CCR2−/− mice on day 28 (Table II). In contrast, the plasma MCP-1 level dramatically increased in BMT-apoE−/−CCR2−/− mice infused with Ang II, compared with that in BMT-apoE−/−CCR2+/+ mice infused with Ang II.

Ang II-Induced AAA Formation Is Suppressed in BMT-ApoE−/−CCR2−/− Mice

As reported by others,[6,7] Ang II infusion induced AAA formation associated with the recruitment and activation of monocytes/macrophages into the adventitia and media and the degradation of elastin and collagen layers (Figure 3A). In separate experiments, to determine the effect of BM-derived CCR2 deficiency on Ang II-induced aneurysm formation, we quantified the incidence and measured the diameter of AAA. Mice that displayed 10% increase in abdominal aortic diameter were defined to have AAA. Compared with BMT-apoE−/−CCR2+/+ mice, BMT-apoE−/−CCR2−/− mice showed a significant reduction in the incidence of AAA formation (9 of 10 BMT-apoE−/−CCR2+/+ mice had AAAs versus only 1 of 10 for BMT-apoE−/−CCR2−/− mice; $P<0.01$). Furthermore, the Ang II-induced increase in maximum diameter of the abdominal aorta was not observed in BMT-apoE−/−CCR2−/− mice (Figure 3B).

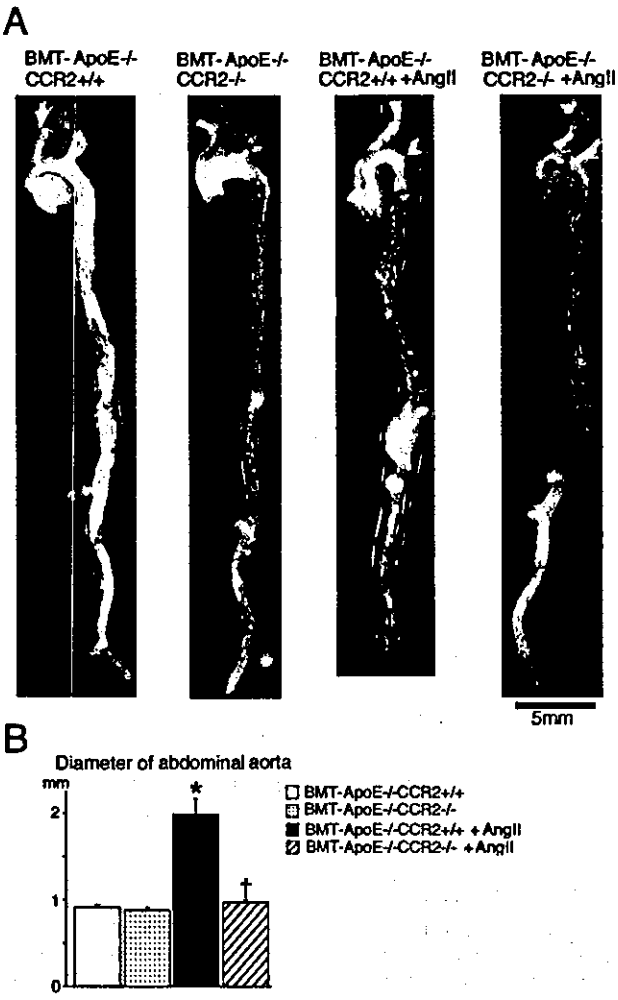

Figure 3. Suppression of Ang II-induced abdominal aortic aneurysm formation in BMT-apoE−/−CCR2−/− mice. A, Photomicrographs of the gross appearance of the ascending aorta to the abdominal aorta. There was significant abdominal aortic aneurysm formation in BMT-apoE−/−CCR2+/+ mice infused with Ang II, whereas there was no aneurysm formation in BMT-apoE−/−CCR2−/− mice. B, Diameters of the abdominal aorta. N=10 each. *$P<0.01$ versus BMT-apoE−/−CCR2+/+ mice with no Ang II infusion. †$P<0.01$ versus BMT-apoE−/−CCR2+/+ mice with Ang II infusion.

Discussion

The important and novel finding of this study was suppressed Ang II-induced acceleration of atherosclerotic process in BMT-apoE−/−CCR2−/− mice. The present study, therefore, represents the first direct evidence for the critical role of CCR2 on leukocytes, especially on monocytes, in Ang II-induced acceleration of atherosclerosis.

BMT from apoE−/−CCR2−/− to apoE−/−CCR2+/+ mice was associated with blunted expression of CCR2 antigen on circulating leukocytes, especially on circulating monocytes. This BMT-apoE−/−CCR2−/− mice displayed suppressed monocyte/macrophage infiltration and lipid accumulation into the atherosclerotic lesions induced by Ang II infusion. To elucidate the mechanism of suppressed Ang II-induced inflammation in BMT-apoE−/−CCR2−/− mice, we examined local and systemic expression of MCP-1. The lack of a detectable difference in Ang II-induced local MCP-1 expression between BMT-apoE−/−CCR2+/+ and BMT-apoE−/−CCR2−/− mice suggests that suppression of Ang II-induced inflammation might result from the lack of CCR2 on monocytes, but not the result of reduced expression of MCP-1. The increase in plasma MCP-1 concentrations in BMT-apoE−/−CCR2+/+ and BMT-apoE−/−CCR2−/− mice infused with Ang II might reflect compensatory local overproduction of MCP-1 in any tissue, including vascular tissues. The present data of attenuated gene expression of inflammatory cytokines in the aorta from BMT-apoE−/−CCR2−/− mice support the notion that activated lesional leukocytes might produce inflammatory and growth-promoting signals, which in turn lead to further acceleration of atherosclerosis. Our present data suggest that anti-inflammation caused by blockade of CCR2-mediated mono-

cyte infiltration can interrupt the positive feedback cycle between inflammation and atherogenesis.

We previously reported that CCR2 expression and function are enhanced in circulating monocytes in hypertensive animals and humans through an AT1 receptor-mediated mechanism; increased CCR2 on monocytes is an important predictor of the presence of hypertension; and monocyte CCR2 is critical for monocyte-mediated inflammation and remodeling in Ang II-induced hypertension in mice.[21] Our present data extend our previous study by showing the decisive role of CCR2 on monocyte in Ang II-induced acceleration of atherosclerosis. Overall, these findings rule out the possibility that expression of CCR2 by resident arterial cells is involved primarily in the mechanism of Ang II-induced vascular remodeling and atherosclerosis. However, Sata[22] and other investigators have reported that BM-derived progenitor cells can migrate to atherosclerotic lesions, differentiate into vascular wall cells, and thus contribute to the development of vascular remodeling and atherosclerosis. Although the degrees to which BM-derived progenitors contribute to the mechanism of vascular disease remain unclear, we do not completely exclude the possibility that expression of CCR2 by BM-derived progenitor cells other than circulating monocytes contributed to the present results.

In conclusion, the present study provides first evidence that CCR2 expressed on monocytes has a critical role in Ang II-induced acceleration of the atherosclerotic process. This finding might also apply to the vascular pathology of atherosclerosis caused by other stimuli such as hypercholesterolemia and/or hypertension, because enhanced CCR2 expression on circulating monocytes has been demonstrated in animals and humans with hypercholesterolemia[23] or hypertension.[21]

Acknowledgments

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抗炎症により動脈硬化を治療する新規アプローチ
—MCP-1阻害による動脈硬化の遺伝子治療

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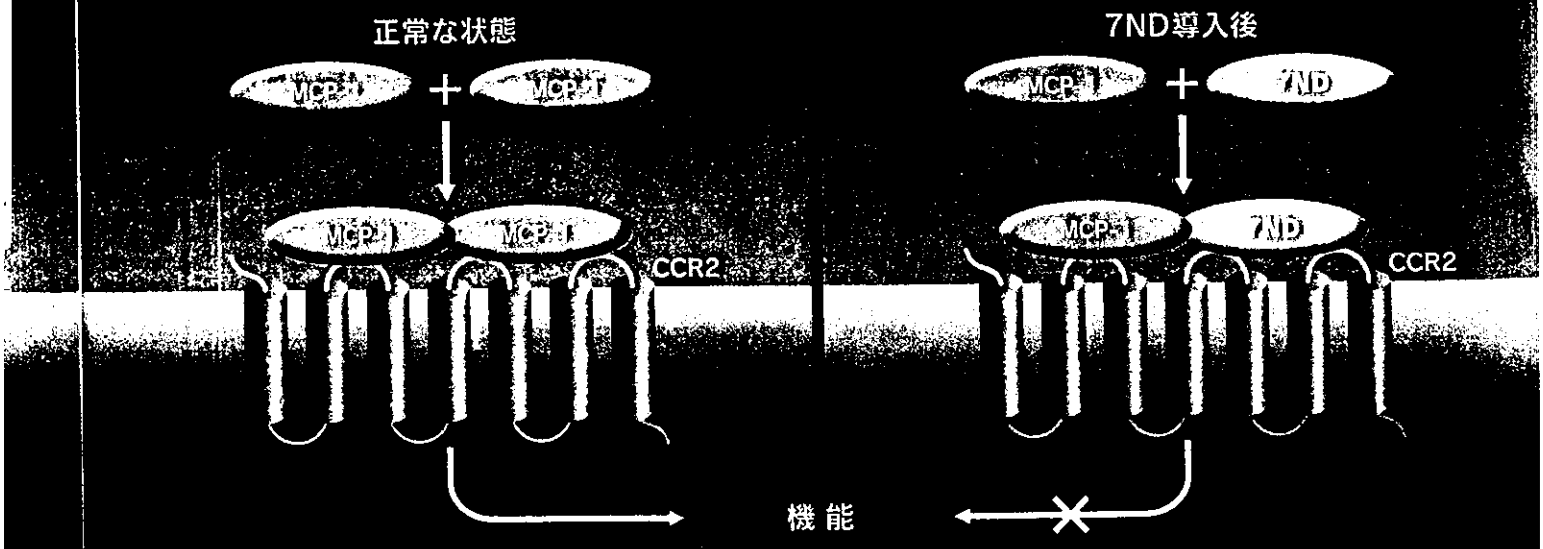
単球遊走促進因子(MCP-1；monocyte chemoattractant protein-1)の阻害による
動脈硬化の遺伝子治療が始まろうとしている。
変異型MCP-1(7ND)の遺伝子を組み込んだプラスミドを骨格筋に注射し，産生される7NDによって
単球の血管壁侵入を促すMCP-1の働きを阻害し，炎症を阻止して動脈硬化の治療をしようとする新しいアプローチで
2003年5月の厚生労働省への再申請を経て承認へ向かう見込みである。
すでに霊長類(カニクイザル)による有用性と安全性の成績がまとまっている。
今後の可能性を含め，実施を計画している江頭健輔氏に，その実際について解説いただいた。

図1 7NDによるMCP-1受容体(CCR2)の阻害



単球の遊走，血管平滑筋細胞の移動，組織因子(TF)産生，
血管内皮細胞の移動，O_2^-の産生

動脈硬化性炎症を水際で阻止する

動脈硬化に炎症が関与していることは，基礎と臨床の両面からさまざまな研究が行われ，確立されてきた。生活習慣病などの危険因子があると，血管内皮障害が惹起されNO活性の低下をきたし，炎症を招く。それがプラーク形成につながり，その不安定化と破裂によって，動脈硬化性疾患を引き起こす。動脈硬化の「炎症仮説」といわれるものである。

炎症の過程では，単球の血管壁への接着と侵入，マクロファージ化した単球の酸化LDLの貪食，泡沫細胞化が起こり，プラーク形成へと進んでいく。このうち，単球侵入後の病的プロセスについては多くの研究が重ねられ，分子機序が詳細に解明されてきた。それに比べ，炎症の引き金となる単球の接着・侵入についての機序解明の研究は比較的新しく，そのため抑制法の開発研究は立ち後れていた。われわれはそのプロセスこそ動脈硬化性炎症を水際で阻止しうるポイントと考え，探索研究を続けてきた。

MCP-1と変異型MCP-1(7ND)

そのなかで注目したのがMCP-1である。単球遊走促進因子として，白血球から日本の研究者がクローニングし，機能解析を行った蛋白で，動脈硬化における炎症発生，すなわち単球が血管壁に接着・侵入する過程になくてはならないケモカインである。最近ではさらに，血栓，平滑筋の増殖・遊走，内皮の遊走，酸化ストレスなどにも関係する多彩な機能が明らかにされている。

その受容体(CCR2)は単球だけでなく，血管平滑筋細胞や内皮細胞にも存在し，単球や平滑筋細胞から分泌されたMCP-1が結合すると，さまざまな炎症因子や増殖因子，組織因子が放出され，上述のような動脈硬化の発生・進行に関わる多彩な機能を発揮する。

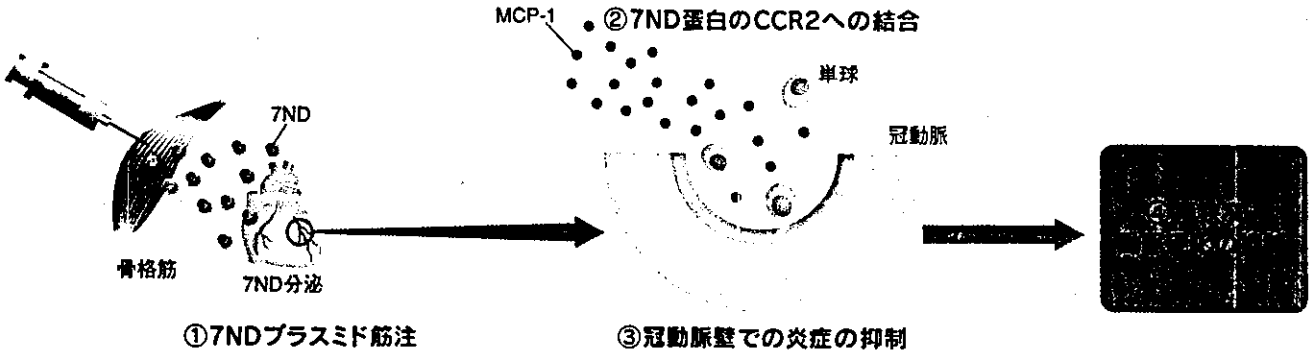
動脈硬化を防ぐには，1つの方法として，このMCP-1とCCR2の結合を阻害し，炎症を阻止してやればよい。そのために，われわれは7NDと呼ばれる変異型MCP-1を作製し，これを用いることにした。MCP-1のN末端の2番目から8番目までのアミノ酸が欠損したもので，正常型と同様，2量体を形成してCCR2に結合する。しかし，受容体シグナルは発生せず，MCP-1の機能が発揮できない。すなわち，CCR2に対して，dominant negative inhibitorとして働くのである(**図1**)。

遺伝子導入による抗MCP-1治療 ―冠動脈形成術後の再狭窄を対象に

治療法として，この7NDをコードする遺伝子をプラスミドに組み込み，骨格筋に注射する方法をとった。骨格筋細胞で7ND遺伝子を発現させて7NDを分泌させ，血流に乗せて冠動脈に送り込むのである(**図2**)。7ND蛋白を直接静脈内に投与することも治療法としての選択肢ではあるが，蛋白での投与となるために，高用量を持続投与することが必要である。

さらに，他の医薬品と同様，有効性と安全性を確認するステップに時間を要する。そのため，臨床で用いるまでには多大な時間を要することになるだろう。そこで，遺伝子治療実施の動向をふまえ，臨床応用をより

図2 7ND遺伝子導入による抗MCP-1遺伝子治療戦略



早く実現するため，プラスミドによる遺伝子導入法を選んだのである。

現時点での治療対象は，冠動脈形成術後の再狭窄予防である。冠動脈形成術は，日本で年間15万例，世界では150万例が実施され，その7～8割をステントが占める。今後，その需要は世界的にますます増加すると予測されている。しかし，その再狭窄率はステント拡張後で20～30%，バルーン拡張後では30～40%にも達し，大きな課題となっている。しかも，再狭窄発生の機序が十分に解明されていないため，予防法が確立していない。近年，米国でラパマイシンコーティングステントが開発され，注目されているが，わが国独自の，すなわち，ジャパン・オリジナルの医学的対策が急務となっている。

サルでステント再狭窄の抑制を確認

われわれは，前述のような独自に開発した7ND遺伝子導入による抗MCP-1療法を用い，再狭窄や動脈硬化に対する有効性を実験レベルで明らかにしてきた。ラットに7NDプラスミドを筋注すると，7ND遺伝子が発現し，循環血中に7NDの分泌が認められた。さらに，それが遠隔部位におけるMCP-1による単球浸潤を抑制することも示した[1]。

実験的再狭窄については，7ND遺伝子導入によって，カフによる外膜傷害マウスモデルで70～80%，バルーン傷害モデルではラット・サルで60～70%，ウサギで40%という抑制が認められている[2,3,4]。ステントモデルでもウサギとサルで再狭窄が抑制された。**図3**は，高コレステロール食を負荷したカニクイザルの実験的ステント再狭窄モデルによる成績で，ステント留置28日後，7ND遺伝子導入群では対照群に比べて，内膜肥厚の度合いが低く，再狭窄が抑制されていることがわかる[5]。

ウサギによる検討では，7ND遺伝子導入により，IL-6とIL-1β，そしてVEGFが抑制されていることが明らかになった。ラットによる検討では興味深いことに，MCP-1発現そのものも抑制されていることが観察されており，これは7NDによるMCP-1の抑制によって単球の遊走が抑えられ，局所に集まってくる単球が減少したことによる結果として，サイトカインやMCP-1の放出がさらに抑制されるという，単球遊走による雪だるま式の炎症・増殖が抑制されることによると考えられた(**図4**)。

動脈硬化に関しても，apo EおよびMCP-1(あるいはその受容体)のダブル欠損マウスや7ND遺伝子導入において初期病変が抑制されることが示されており[6]，われわれはさらに7ND遺伝子導入によってプラークの発生だけでなく進展を止めることができることを報告している[7]。

図3 7ND遺伝子導入によるステント再狭窄(新生内膜形成)の抑制

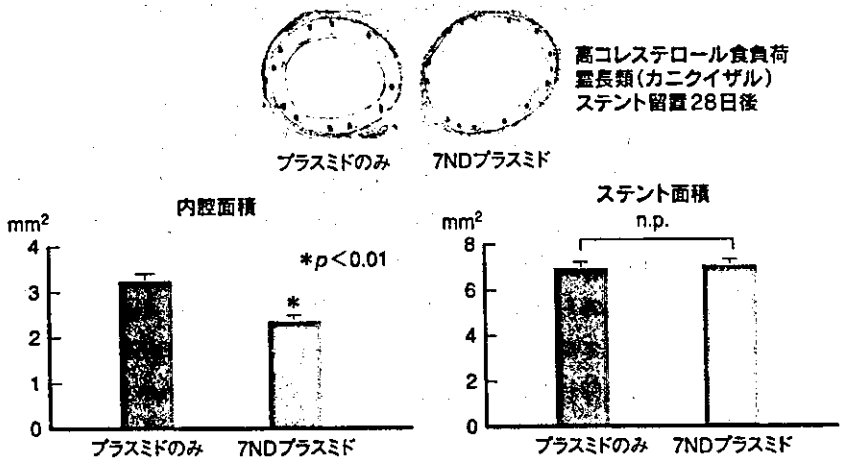
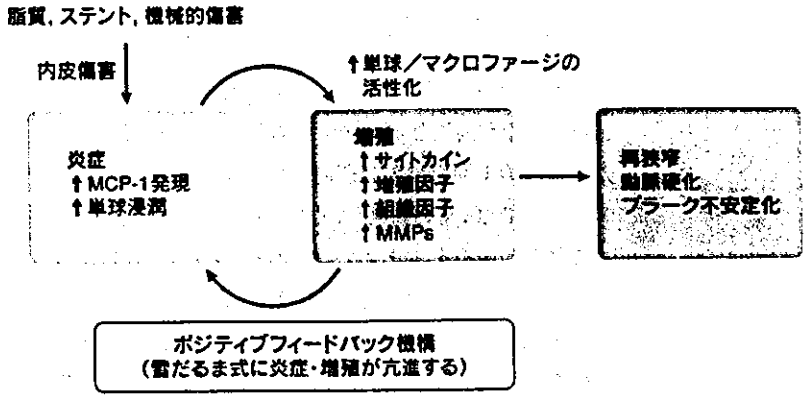


図4 再狭窄・動脈硬化の成因におけるMCP-1の役割(炎症仮説)



3年に及ぶサルの毒性試験で安全性を示す

遺伝子治療において最も懸念されることは，その治療法の安全性である。一般にプラスミドによる遺伝子導入はウイルスベクターを用いないことから，安全性が高いと考えられている。

安全性についてわれわれは，よりヒトに近い動物として霊長類(カニクイザル)を用い，すべて外部委託により，毒性試験を行ってきた。使用量の最大3倍の7NDプラスミドを筋注し，1，3，6ヵ月，1年，最大3年まで経過観察しており，体重，尿，便，血液学，血液生化学，炎症(CRP，サイトカイン)について検討したうえで，最終的には全身の重要臓器を解剖して，異常(毒性)がないことを確認している。

7NDの免疫原性・抗原性についても検討しており，カニクイザルでは7ND遺伝子導入後に抗体価の上昇がみられないことを確認している。

本治療法では，7NDは血流に乗って全身に回るため，全身性にMCP-1を抑制することによる副作用も懸念される。この点については，MCP-1欠損マウスでは正常な発育や妊娠・分娩がみられ，風邪をひくといった免疫反応の低下により引き起こされる病態の増加もみられないことがわかっている。MCP-1が抑制されて単球が遊走しなくなっても，通常の感染防御には支障がないものと考えられる。ただし，結核やリステリア症のように，単球がないと防御が成立しない感染症や抗原に対しては影響を与える可能性が考えられ，まれとは思われるが，これらは治療対象から除外すべきであろう。

他の免疫性疾患に応用の可能性も

サルにおいて，0.5mg/kg/日の投与量で以上述べてきたような有効性と安全性が認められている。さらに，この用量の1回投与で2週間，効果が持続することがサルだけでなく他の動物(マウス，ラット)でも示された。

これらの成績をふまえて，2002年2月，「冠動脈形成術後再狭窄に対する抗MCP-1遺伝子治療」として，厚生労働省に臨床試験の実施申請を行った。その後，主に安全性に関する検討の追加を指示されたため，それ以前から行っていたサルでの安全性試験結果をまとめ，2003年5月に再申請を行った。

将来的には，局所投与という観点から，7NDコーティングステントの開発も考えられる。臨床で安全に使用できるウイルスベクターが開発されれば，それを用いた局所遺伝子導入も考えたい。また，MCP-1は再狭窄や動脈硬化だけでなく，臓器移植後の拒絶反応や心不全，腎硬化症，肝線維症・肝硬変，肺線維症・肺高血圧，さらには関節リウマチ，炎症性腸疾患，病的(癌)血管新生などにも関係していることがわかっている。これらは，現在までのところ根本的な治療法がなく，ステロイドや免疫抑制薬しか治療法がない「難病」である。再狭窄予防に関してわれわれの抗MCP-1遺伝子治療の有効性と安全性が示されれば，これらの難治性疾患への応用の道も開かれるのではないかと期待している。

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概論

血管医学の新展開
—ニューパラダイムの構築から治療開発へ

江頭健輔

臨床医学である「血管医学」の重要性は，血管の形態と機能制御が臓器ならびに生命の維持に不可欠であること，虚血性心疾患などの血管病の増加が著しいこと，血管医学によって血管病のメカニズム解明と治療法開発につながること，などから明らかである．血管医学に関する研究成果を基盤にして，医学から医療に移行するプロジェクトがはじまっている．「血管を創る」治療法として血管新生の基礎・臨床研究が世界的に進んでいる．また，「血管を護る」対策についても多くの研究が進んでいる．血管医学からの生命科学が開拓され，その成果が臨床医学へ還元されるときを迎えつつある．

はじめに

血管病の分子メカニズムに関する研究は2000年以降を振り返ってみるだけでもめざましいものがある．いくつもの新しいパラダイムが構築され，その多くは動脈硬化性疾患の治療開発へ応用されつつある．また，血管医学の研究成果が悪性腫瘍や炎症性疾患などの難治性疾患の治療開発研究に応用されはじめている．

今回，本書「血管研究の最先端と治療への展開」の第2章「血管病態の分子メカニズム」の分担編集を担当させていただく機会を得て9本の特集を取りあげた．いずれもこのテーマに関する最新の話題であり，その多くはニューパラダイムの構築に貢献したものであり，今後その成果を基盤にして治療法あるいは診断法の開発研究に移行しようとしている．本稿では，これらの研究のバックボーンともいえる「血管医学」の新展開について述べる．

1. 血管に視点をおいた「血管医学」の重要性

血管に視点をおいた臨床医学である「血管医学」の重要性は以下のように要約される．

第1に，血管の形態と機能がシステムとして正常に作動することが臓器ならびに生命の維持に不可欠であることはいうまでもない．血管系のシステム欠損によって重要臓器の形成不全・機能不全が生じることがわかっているし，内皮細胞機能障害が心血管イベントの独立し

[キーワード&略語]
血管医学，炎症，血管新生，トランスレーショナルリサーチ
VEGF：vascular endothelial growth factor（血管内皮増殖因子）
HGF：hepatocyte growth factor（肝細胞増殖因子）

Frontier of vascular medicine
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た予測因子となることも明らかにされている．次世代医療として期待されている血管新生療法や再生療法も健全な血管系の発生/分化を伴わなければ成立しない．健全な血管では血管壁構成細胞群（内皮，平滑筋，線維芽細胞）が相互に機能しあって，循環，細胞遊走/増殖，凝固/線溶などが正常に機能するように働いている（第2章-4）．さらに，炎症・免疫疾患，造血異常，腫瘍などの病態形成にも血管系の存在は必須である．

第2に，高齢者の増加によって血管医学の臨床的重要性はかってないほどに高まっている．虚血性心疾患だけでなく，脳血管疾患・腎硬化症・網膜疾患・末梢血管疾患・大動脈瘤などの血管病の増加が著しく，これら血管疾患の国民死亡率と全医療費に占める割合はきわめて大きい．心不全は血管疾患と同様に代表的循環器疾患であるが，その多くは冠動脈疾患を基盤として発症するようになった．したがって，血管疾患の診断と治療に関する知識は循環器専門医だけでなく一般臨床医にとってもきわめて重要になってきたわけである．また，これらの血管疾患の診療研究に携わる医師・研究者は内科系だけでなく多くの診療科（脳内科，血管外科，眼科，腫瘍内科・外科）と基礎講座に所属しているので，血管疾患のメカニズム解明と治療開発には講座・診療科の垣根を越えた横断的共同研究体制の構築も必要であろう．

第3に，血管疾患は国民病ともいえる生活習慣病と密接に関連する．高コレステロール血症，高血圧症，糖尿病では合併症としての血管障害が臨床的に重要であり，生活習慣病の治療は単に脂質，血圧，血糖の値をコントロールするだけでなく，「血管保護」を認識して進めるよう推奨されている（第2章-6）．また，「ヒトは血管とともに老いる」といわれるように老化と血管病は密接にかかわっている．したがって，血管を視点においた血管医学を推進することにより生活習慣病や老化による血管病のメカニズム解明と治療法開発につながるであろう（第2章-8）．さらに，老化によって生じる難治性疾患の治療開発のきっかけが得られる可能性もある．

最後に，血管病は全身性疾患であるので，血管医学に関する診療/治療は内科学の基本の1つとなり臨床医学としての重要性もきわめて大きい．

2. 血管病を治療する対策の創製

生活習慣病は血管障害を生じさせることによって臨床的に重要な病態となる．大規模臨床試験で心血管病の予後を改善することが示されているACE阻害薬，スタチン，アンジオテンシン受容体拮抗薬などは共通に血管内皮機能を改善させることも血管障害の臨床的重要性をいっそう高めるものである（第2章-4，第3章-5）．したがって，血管内皮を保護する，あるいは，血管再生を誘導する治療法の開発は「血管医学」の大きなテーマである．血管障害因子刺激の上流あるいは下流にある新しい遺伝子/タンパク質を同定し，疾患発症リスクの階層化や疾患の診断/治療に応用することによって，血管病の予防法・治療法が創製されるであろう．また，内皮細胞障害や動脈硬化の感受性遺伝子多型を同定できれば，リスクの階層化や治療開発研究が進むであろう．

「血管を創る」治療法としての血管新生因子による血管新生（第2章-3，第3章-1），自己骨髄系細胞（第3章-2）やES細胞（第2章-7）を用いた血管新生，医用工学技術を駆使したハイブリッド人工血管（第3章-3），などの開発研究が進んでいる．血管新生/再生の研究は生命科学上の意義があるだけでなく，臨床医学全体に大きく貢献するものである．

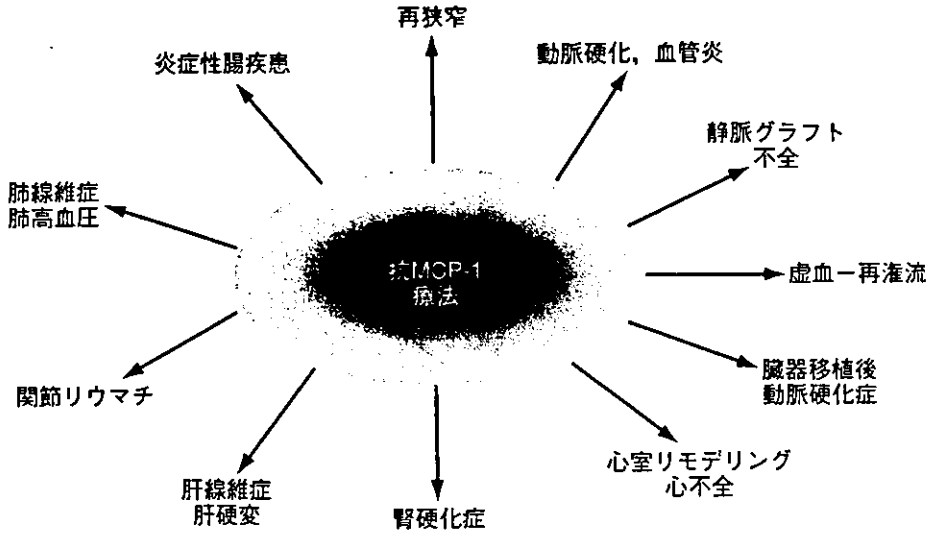

図1　抗MCP-1療法の幅広い臨床応用の可能性

血管新生/再生は臓器の発生/再生に必須であり，癌も血管なしには成長できない．内皮細胞増殖因子VEGFや肝細胞増殖因子HGFによる血管新生治療は重症虚血肢の新しい治療法として臨床研究が進んでいる（第3章-1）．自己骨髄細胞を用いた血管新生療法はわが国の研究者らが世界に先駆けて開発し，その臨床的有効性を報告した．最近では世界的レベルで重症下肢虚血ならびに虚血性心疾患患者に対する自己細胞療法が実施されている．しかし，血管新生療法の陰の部分も指摘されはじめた．たとえば，VEGFの臨床試験で少数例を対象とした第Ⅰ/Ⅱ相試験では有効と報告されたが，無作為化試験あるいは多数例を対象とした大規模試験では有効性が証明されていない．さらに，VEGF投与が実験的動脈硬化を促進する，VEGF抑制によって動脈硬化病変が減少する，などの報告があり，VEGFによる血管新生療法の成績は注意して解釈する必要がでてきた（第2章-2）．骨髄細胞移植についても，投与した細胞のほとんどは血管形成に貢献しない，細胞融合に起因する，などの批判がある．将来，より強力な血管新生能を付与した細胞，ES細胞あるいはケモカインを用いた血管新生療法などの新しい対策が必要になるであろう（第2章-8）．

「血管を護る」対策も進んでいる（第2章-9）．われわれはmonocyte chemoattractant protein-1（MCP-1）抑制による抗炎症療法の探索研究（トランスレーショナルリサーチ）を進めている．MCP-1のN末端欠失体（7ND）がMCP-1の強力な抑制因子として作用することを利用して7NDプラスミド遺伝子導入による抗MCP-1療法を開発した（第2章-1）．この戦略によって，動脈硬化の発生・進行の抑制とプラーク安定化がもたらされること，退縮が生じること，再狭窄反応（傷害後内膜肥厚）が抑制されること，を霊長類モデルを含む実験動物を用いて明らかにした．これらの研究によって，動脈硬化性疾患のメカニズムにおける新しいパラダイム（炎症仮説の証明）が構築できたと考えている．また，MCP-1の機能は多彩であり，平滑筋遊走増殖・線維化・血管新生に必須である．MCP-1機能阻止によって高血圧性心疾患・心不全，重症腎疾患，脳血管障害，網膜疾患，病的血管新生，肺高血圧症，線維症，臓器移植後動脈硬化，などの難治性炎症性疾患にも有用であることを示す実験成果が集積されつつある（第2章-5）（図1）．

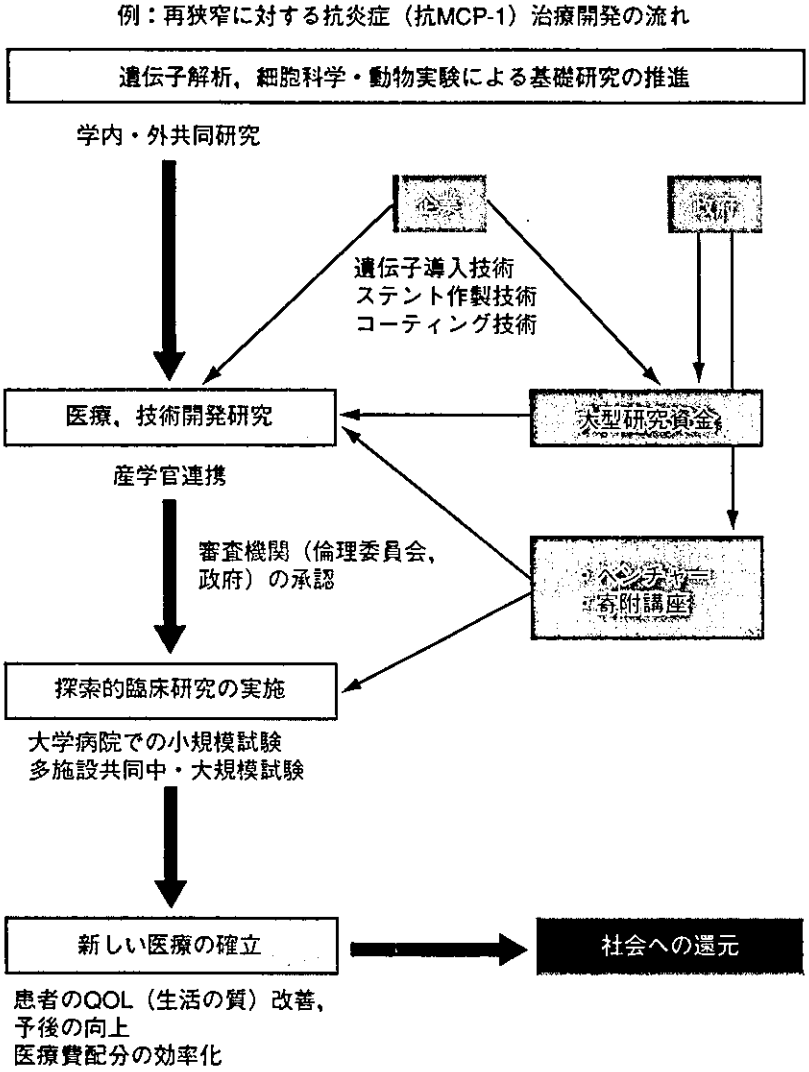

図2　トランスレーショナルリサーチの流れ

動脈硬化による狭窄を解除する方法としてカテーテルによるインターベンションが血管疾患の治療として確立している．しかし，冠動脈インターベンション後再狭窄が30％程度に生じることが問題である．最近，薬物溶出型ステントが登場し再狭窄に対して画期的臨床成績を示したことから，再狭窄克服に向けての開発研究の第一幕がスタートした．われわれは，7NDの成果を駆使して遺伝子溶出型ステントなどを開発し，画期的血管内治療システムを創製することをめざしている（第2章-1）（図2）．

このような新しい治療開発研究が実際に応用されるには，治療的遺伝子，ペプチド，タンパク質を標的臓器や組織の局所に送達するDDS（ドラッグデリバリーシステム）の開発もきわめて重要である．遺伝子導入用ベクター，生分解性材料，ナノ技術，などによる画期的DDSの開発も重要な研究テーマである（第2章-9）．

3. 血管医学からの生命科学の開拓と臨床医学への還元

わが国の血管医学研究のレベルは，本書の内容から自明なように世界的レベルに達しつつ

ある．これは，1990年代後半から大型研究費が血管生物学分野ならびに循環器疾患の臨床研究分野に配分されるようになったことの反映でもある．したがって，血管医学研究に携わるわが国の研究者の使命は，独自性・独創性のある研究成果を世界へ発信することであろう．

血管を対象としたこのような生命科学研究は単に循環器疾患だけでなく，広く臨床医学全体にフィードバックできることが期待されることから，血管医学は生命科学としても重要な分野である．

おわりに

質の高い研究・教育の遂行と研究成果の臨床医学・社会への還元は21世紀の大学に課せられた使命である．したがって，研究成果を臨床応用し，社会に還元することを目標にした臨床・基礎研究を実施することがわれわれの使命である．その目標達成のためには，臨床に視点をおいた高いレベルの研究・教育が必須であり，高い診断・治療技術を有する臨床チームを組織しなくてはならない．高水準の循環器診療・研究の環境が構築されれば，自然と次世代を担う多くの人材が育成されるであろう．