Rho-Kinase Is Involved in Macrophage-Mediated Formation of Coronary Vascular Lesions in Pigs In Vivo

Kenji Miyata, Hiroaki Shimokawa, Tadashi Kandabashi, Taiki Higo, Kunio Morishige, Yasuhiro Eto, Kensuke Egashira, Kozo Kaibuchi, Akira Takeshita

Abstract—We have previously shown that long-term treatment with an inflammatory cytokine from the adventitia causes the development of coronary vascular lesions, with the accumulation of macrophages. Recent studies in vitro have suggested that small G-protein Rho and its effector, Rho-kinase/ROK/ROCK, may be the key molecules for various cellular functions, including cell adhesion and movement. In this study, we examined whether adventitia-derived macrophages cause the formation of coronary vascular lesions in vivo and, if so, whether Rho-kinase is involved in the process. Porcine coronary segments from the adventitia were chronically treated with monocyte chemoattractant protein-1 alone, oxidized low density lipoprotein alone, or both. Vascular lesion formation (neointimal formation and development of vascular remodeling) was mostly enhanced at the coronary segment cotreated with monocyte chemoattractant protein-1 and oxidized low density lipoprotein, where the phosphorylation of myosin binding subunit of myosin phosphatase was increased, indicating an increased activity of Rho-kinase in vivo. Histological examination demonstrated that macrophages were accumulated at the adventitia and thereafter migrated into the vascular wall. Long-term oral treatment with fasudil, which is metabolized to a specific Rho-kinase inhibitor (hydroxyfasudil) after oral absorption, markedly inhibited the myosin binding subunit phosphorylation, the macrophage accumulation and migration, and the coronary lesion formation in vivo. These results indicate that Rho-kinase is involved in macrophage-mediated formation of coronary vascular lesions in our porcine model in vivo. (Arterioscler Thromb Vasc Biol. 2000;20:2351-2358.)

Key Words: macrophages Rho-kinase adventitia arteriosclerosis oxidative stress

Studies in vitro have suggested that monocyte recruitment into the vessel wall promoted by chemokines, including CC-chemokines, such as monocyte chemoattractant protein (MCP)-1, is an early step in the process of arteriosclerosis. Indeed, in mice lacking the receptor for MCP-1 (CCR2), the formation of atherosclerotic lesions is significantly attenuated. We have previously demonstrated that long-term local treatment with interleukin (IL)-1β from the adventitia causes the development of coronary vascular lesions and vasospastic responses in pigs in vivo, with macrophage accumulation noted in the adventitia. Indeed, the importance of adventitial accumulation of inflammatory cells has been suggested for the pathogenesis of arteriosclerosis and acute coronary syndrome in general and for that of coronary lesion formation after coronary intervention in particular. However, it remains unknown whether adventitia-derived macrophages cause the formation of coronary lesions in vivo and, if so, what molecular mechanism(s) is involved in the process.

Recent studies in vitro have suggested that Rho family small G proteins, especially Rho and its effector, Rho-kinase/ROK/ROCK, may be the key molecules in various cellular functions, including vascular smooth muscle contraction and cell adhesion and movement. We also have recently demonstrated that the upregulation of Rho-kinase plays a key role in the pathogenesis of coronary artery spasm in our porcine model with IL-1β by inhibiting the myosin phosphatase and thereby enhancing the phosphorylations of myosin light chain in the coronary smooth muscle cells. Taken together, we hypothesized that the activation of Rho-kinase is involved not only in the vascular smooth muscle hypercontraction but also in the pathogenesis of arteriosclerosis.

Thus, the present study was designed to examine whether macrophages (accumulating in the adventitia) cause the coronary lesion formation in vivo and, if so, whether Rho-kinase activation is involved in the process. For this purpose, we used hydroxyfasudil, which we recently found is a specific inhibitor of Rho-kinase.

Methods

This experiment was reviewed by the Committee on Ethics in Animal Experiment of the Kyushu University and was carried out.
according to the Guidelines for Animal Experiments of the Kyushu University and of the Japanese Government.

**Animal Preparation**

Domestic male pigs (2 to 3 months old and weighing 25 to 30 kg, Nihon Crea, Tokyo, Japan) were used. The animals were housed individually under a controlled room temperature. They were sedated with ketamine hydrochloride (12.5 mg/kg IM) and anesthetized with sodium pentobarbital (20 mg/kg IV). They were then intubated and mechanically ventilated with a respirator. Under aseptic conditions, a left thoracotomy was performed, and the proximal segments of the left anterior descending and left circumflex coronary arteries were carefully dissected.

In the first series of experiments, 3 coronary segments were dissected and were gently wrapped with cotton mesh absorbing either recombinant human MCP-1 bound to microspheres (50 ng, Sigma Chemical Co) alone, oxidized LDL (Ox-LDL) alone (100 μg), or both (n=6). In other 6 animals, the dissected 3 coronary segments were treated with either MCP-1 alone, native LDL (100 μg) alone, or both. Animals were maintained on a regular diet for 4 weeks. We have previously confirmed that the treatment with control microspheres alone causes no significant lesions of the porcine wall.

The 3 coronary segments were randomly used for hematoxylin and eosin staining, immunostaining for macrophages and MCP-1 (n=6) 3, 7, and 14 days after the adventitial treatment with MCP-1 and Ox-LDL.

In the third series of experiments, an additional 12 animals received the adventitial treatment with MCP-1 and Ox-LDL at the 3 segments, and they were randomly divided into 2 groups: one group was treated with regular diet alone (control group, n=6), and another group was treated with oral administration of fasudil (5 mg/kg per day, Asahi Chemical Industry), starting 1 day before the operation and continuing for 2 weeks (fasudil group, n=6). The end point of 2 weeks was chosen because we noted in the first protocol series that the vascular lesion was already formed at 2 weeks after the surgery. The 3 coronary segments were randomly used for hematoxylin and eosin staining, immunostaining for macrophages (frozen section), and Western blot for phosphorylated myosin binding subunit (MBS). We recently found that fasudil is metabolized to hydroxyfasudil after oral administration to human.5 Addition of hydroxyfasudil to MCP-1 (50 μg, Sigma; n=3) or nonimmune mouse IgG (50 μg, n=3), together with MCP-1 (50 ng), to confirm the in vivo effect of microsphere-bound MCP-1.

In the second series of experiments, as mentioned in detail in the following sections, histological examination was performed for the movement of adventitia-derived macrophages (n=6) and for immunostaining for macrophages and MCP-1 (n=6) 3, 7, and 14 days after the adventitial treatment with MCP-1 and Ox-LDL.

Microspheres (colored microspheres, E-Z Trac; 200 000 in number of 0.1 mm in diameter, which bind to the amino residues of proteins, including cytokines) were added to 50 mL of 1 mmol/L HCl solution and centrifuged 4 times at 1200 rpm for 5 minutes. The microspheres were then resuspended in 20 mL of NaHCO3/NaCl solution with 10 μg of recombinant human MCP-1. The microspheres were allowed to bind with MCP-1 at room temperature for 1 hour and then at 4°C overnight. After centrifugation at 1200 rpm for 5 minutes, the supernatant was separated. The MCP-1-bound microspheres in the pellet were resuspended with Tris-HCl buffer solution for 1 hour to block any remaining active sites. The MCP-1-bound microspheres were finally washed and resuspended. The final concentration of MCP-1 was 1 μg/mL, and 0.05 mL of the suspension (50 ng of MCP-1) was applied to the adventitia of the porcine coronary artery. All of the above procedures were performed under sterile conditions. With our method, MCP-1 is firmly bound to microspheres by a covalent bond at the amino residues of the protein and does not detach from the spheres, allowing the detection of endogenous MCP-1 by immunostaining in the coronary vessel wall.

**Fluorescent Microscopic Examination**

Adventitia-derived macrophages were labeled in vivo according to the method of Melnicoff et al. Briefly, 0.05 mL of fluorescent dye solution (1 μmol/L, PKH26 fluorescent staining kit, Zynaxis Cell Sciences) was simultaneously applied at the adventitia with MCP-1 and Ox-LDL, and the coronary artery was obtained 3, 7, and 14 days after the procedure from different animals (n=2 each). Fluorescent images were obtained by fluorescence microscopy (LSM-GB200, Olympus) at an excitation wavelength of 551 nm and an emission wavelength of 567 nm. In a preliminary study, we confirmed that adventitia-derived macrophages absorb the dye and become positive (by fluorescence examination) together with elastic fibers (especially internal and external elastic lamina [IEL and EEL, respectively]) and that circulating blood cells never become positive by this method. Therefore, this method allows the serial observation of the movement of adventitia-derived macrophages in vivo.

**Histopathology and Immunohistochemistry**

The animals were killed with a lethal dose of sodium pentobarbital, and then the hearts were removed. Left coronary arteries were perfused via a constant-pressure perfusion system (120 cm H2O) with saline (500 mL) and subsequently with 5% formaldehyde (1000 mL). After the fixation, the left anterior descending and left circumflex coronary arteries were cut transversely, dehydrated, embedded in paraffin, and cut into 5-μm-thick slices. These sections were stained with hematoxylin and eosin and van Gieson’s elastic stain for photomicroscopy. The degree of intimal thickening was analyzed quantitatively with a computer-assisted picture system (Genlock System, Sony).

Three vessel areas were measured, including the luminal area and an area encircled by the IEL and EEL. The intimal area was determined as AI=AIe−AIa, where AI is the intimal area, and AIe and AIa are the areas within the IEL and the luminal area, respectively. The degree of neointimal formation was expressed by the following 3 parameters: intimal area (ie, AI [mm2]), maximal intimal thickness (mm) measured with a caliper, and percent intima calculated by the following equation: AI/Al×100 (%). The degree of vascular remodeling was expressed by the change in the vessel areas by the following equation: (AS−ASC)/ASC×100, where AS and ASC are vessel areas of the coronary segments at the treated and the normal control site, respectively. We used the average of 3 consecutive sections from a single arterial segment. The variability of the 3 sections was small.

In some experiments, these segments were embedded in OCT compound (Sakura Fine Technical Co) without being embedded in paraffin, cut into 5-μm-thick slices, and stained with oil red O. For immunostaining, these segments were immediately embedded in OCT compound without fixation, frozen, and cut into 5-μm-thick slices. Serial cryosections were stained with an antibody to human macrophages (PM-2K) or to rat MCP-1 (clone C4) or nonimmune mouse IgG (Zymed Laboratory). In a preliminary study, we confirmed that those antibodies cross-react well with porcine macrophages and MCP-1, respectively.
Measurements of Rho-Kinase Activity
To measure Rho-kinase activity, the extent of phosphorylated MBS of myosin phosphatase, one of the substrates of Rho-kinase, was measured by SDS-PAGE, followed by electrophoretic transfer of the proteins to a nitrocellulose membrane. The amounts of phosphorylated MBS (20 μg protein in each sample) were quantified by immunoblot procedures. β-Actin was used as an internal control.

Data Analysis
All results are expressed as mean±SEM. Multiple comparisons were made by ANOVA for repeated examinations, followed by the Fisher post hoc test. Paired data were analyzed by Student t test. A value of P<0.05 was considered to be statistically significant.

Results
Neointimal Formation and Geometric Remodeling of the Coronary Artery
At the coronary segment treated with MCP-1 alone or Ox-LDL alone, neointimal formation was noted, the extent of which was enhanced at the segment cotreated with MCP-1 and Ox-LDL (Figures 1A and 1B). Similarly, the vascular cross-sectional areas were reduced by those treatments compared with the adjacent normal coronary segment (vascular remodeling), and the extent of the remodeling was the highest at the segment cotreated with MCP-1 and Ox-LDL (Figure 1C). No such augmenting effect was noted with native LDL (n=6, data not shown). These vascular effects of MCP-1 were abolished with the simultaneous treatment with a neutralizing antibody against MCP-1 (n=3) but not with a nonimmune IgG (n=3) (data not shown).

Macrophage Migration Into the Coronary Artery
In Vivo
The histological examination with fluorescent dye (Figure 2A) and with an antibody to macrophages (Figure 2B) demonstrated that a number of macrophages were accumu-
lated at the adventitia 3 days after the operation and thereafter gradually migrated into the vascular wall. There was no leakage of non–cell-associated dye in the intima or the media 3 and 7 days after the procedure (Figure 2A). We confirmed that fluorescence-positive cells were macrophages by immunostaining for the cells in an adjacent section (Figure 2B). This migration of macrophages was probably due in part to the expression of endogenous MCP-1 in the media and the intima, as evidenced by the immunostaining for the protein (Figure 2C). The specificity of the immunostainings was confirmed by the negative staining with nonimmune IgG (data not shown). Staining with oil red O demonstrated that some of these adventitia-derived macrophages caused foam cell lesions in the intima near the IEL (data not shown).

Rho-Kinase Activation
Immunoblot analysis demonstrated that the Rho-kinase activity, as expressed by the phosphorylation of MBS of myosin phosphatase after normalization to β-actin, was significantly increased at the segment cotreated with MCP-1 and Ox-LDL by ≈2.5 fold in the control group (Figure 3).

Inhibitory Effect of Fasudil/Hydroxyfasudil In Vivo
Long-term oral treatment with fasudil inhibited the MBS phosphorylation at the segment cotreated with MCP-1 and Ox-LDL to the basal levels seen in the normal coronary artery in the fasudil group, confirming its inhibitory effect on Rho-kinase (Figure 3). In the control group, the macrophage

Figure 2. Fluorescence microscopic examination for macrophages (A) and immunostaining for macrophages (B) and for endogenous MCP-1 (C) at the coronary segment cotreated with MCP-1 and Ox-LDL 3, 7, and 14 days after treatment (frozen sections). Macrophages are shown by white arrows (A) and by black arrowheads (B). Macrophages first appeared at the adventitia in response to MCP-1 and Ox-LDL 3 days after treatment; they moved toward the intima 7 and 14 days after treatment. The expression of endogenous MCP-1 may contribute, at least in part, to this movement.
accumulation was noted in the media and the adventitia 2 weeks after the adventitial treatment with MCP-1 and Ox-LDL; this accumulation was significantly inhibited in the fasudil group (Figure 4). Finally, the long-term treatment with fasudil significantly inhibited the neointimal formation and the development of vascular remodeling in vivo (Figure 5).

Discussion

The novel findings of the present study were that (1) long-term adventitial treatment with MCP-1 induced neointimal formation and geometric remodeling of the coronary artery in vivo, (2) the formation of the coronary vascular lesions was enhanced by oxidative stress with Ox-LDL, (3) adventitia-derived macrophages played an important role in the creation of coronary vascular lesions in vivo, and (4) Rho-kinase was involved in the molecular mechanism for the formation of coronary vascular lesions in our porcine model in vivo. To the best of our knowledge, this is the first study that demonstrates the involvement of Rho-kinase in the macrophage-mediated formation of coronary vascular lesions in vivo.

Role of Adventitia-Derived Macrophages

Although previous studies have suggested the potential importance of the adventitia, its role in vascular lesion formation has been largely ignored. We have previously shown that long-term adventitial treatment with inflammatory cytokines causes the development of vascular remodeling, neointimal formation, and vasospastic responses in porcine coronary arteries in vivo. Because adventitial accumulation of macrophages was noted in our models, we aimed in the present study to examine the potential role of adventitia-derived macrophages in vascular lesion formation in vivo. To stimulate the adventitial accumulation of macrophages, we chronically treated the adventitia of the porcine coronary artery with MCP-1, a well-known CC-chemokine for macrophages. Indeed, this treatment caused a development of the vascular lesions similar to that found by us with inflammatory cytokines. Furthermore, the vascular lesion formation caused by MCP-1 was significantly enhanced by cotreatment with Ox-LDL but not with native LDL, indicating an enhancing effect of oxidative stress by Ox-LDL. It is conceivable that macrophages were activated by Ox-LDL and that they released cytokines and chemokines in the adventitia, forming a vicious cycle toward a vascular lesion formation in vivo. Although the number of macrophages in the intima was relatively small even at 2 weeks after the application of MCP-1 and Ox-LDL, we consider that the recruitment and activation of macrophages at the adventitia per se initiated the cytokine network in the media, resulting in the neointimal formation and geometric remodeling in our porcine model.

In the present study, we demonstrated the progressive migration of macrophages from the adventitia toward the intima. Although the exact mechanism of this macrophage migration remains to be clarified, the expression of endogenous chemokines (including MCP-1) may be involved because we showed the expression of endogenous MCP-1 in the intima and the media. It is also interesting to note that in the coronary segment cotreated with MCP-1 and Ox-LDL, foam cell lesions were developed in the intima in normolipemic animals. The interactions between the adventitia and the media/intima remain to be elucidated in future studies.
Involvement of Rho-Kinase Activation in Coronary Lesion Formation

Rho-kinase has been implicated in the molecular mechanisms of not only vascular smooth muscle contraction but also any phenomenon that requires actomyosin interaction, including chemotaxis of inflammatory cells. Furthermore, recent studies demonstrated that Rho/Rho-kinase is substantially involved in the proliferation, differentiation, adhesion, and migration of various types of cells, including vascular smooth muscle cells and inflammatory cells. Thus, it is highly possible that Rho-kinase is involved in the molecular mechanism of arteriosclerosis. Indeed, in the present study, we demonstrated that Rho-kinase activity was significantly increased at the coronary segment cotreated with MCP-1 and Ox-LDL. Furthermore, hydroxyfasudil, which we recently found is a specific inhibitor of Rho-kinase, markedly suppressed the Rho-kinase activity (as evidenced by the Western blotting for phosphorylated MBS) and inhibited the macrophage accumulation in the adventitia, the migration into the media, and the subsequent vascular lesion formation.

Figure 5. A, Representative photographs (hematoxylin and eosin staining) of the coronary artery segments 2 weeks after cotreatment with MCP-1 and Ox-LDL in a pig without treatment (control group) or with long-term oral treatment with fasudil (fasudil group). B and C, Quantitative analysis of the inhibitory effect of the long-term treatment with fasudil on the development of neointimal formation (B) and vascular remodeling (C). Neointimal formation and the development of vascular remodeling noted in the control group were markedly inhibited in the fasudil group.
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Limitations of the Study
Several limitations of the present study can be raised. First, it has been generally accepted that adhesion and migration of monocytes/macrophages in the intima, rather than in the adventitia, play an important role in the initiation of vascular lesion formation. Although we also appreciate the importance of the inflammatory changes in the intima, we consider that adventitial inflammation may also play an important role in the vascular lesion formation as discussed above. Furthermore, because we found that focal and long-term treatment with MCP-1 is applicable at the adventitia but not at the intima, we used the present adventitial approach. Second, the doses of MCP-1 and of Ox-LDL that were applied to the adventitia should have a clinical implication. Indeed, the dose of MCP-1 corresponds to that found in the artery after balloon injury. The dose of Ox-LDL was chosen on the basis of a previous study that demonstrated the content of Ox-LDL in atherosclerotic blood vessels. Third, the localization of Rho-kinase in the coronary artery was not examined in the present study. Although this point remains to be examined in a future study, we consider from the data of macrophage migration in vivo that one of the main sites for the Rho-kinase expression was macrophages and, to a lesser extent, vascular smooth muscle cells that showed hypercontractive response to serotonin. Fourth, endothelial function was not examined in the present study. However, we have recently demonstrated that endothelial vasodilator function is fairly preserved in a porcine model with adventitial application of IL-1β, an original for the present model. Fifth, a detailed mechanism for macrophage movement from the adventitia toward the intima remains to be examined in future studies. Sixth, although we previously confirmed that hydroxyfasudil has a specific inhibitory effect on Rho-kinase, other effects of this compound might be involved. This point remains to be examined in a future study in which selective inhibition of Rho-kinase can be achieved by in vivo gene transfer of dominant-negative Rho-kinase.

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