Molecular Mechanisms Mediating Inflammation in Vascular Disease
Special Reference to Monocyte Chemoattractant Protein-1

Kensuke Egashira

Abstract—There are several clinical challenges for the treatment of intractable cardiovascular diseases, including restenosis, atherosclerotic complications resulting from plaque rupture, severe tissue ischemia, and heart failure. Emerging evidence suggests that an inflammatory process is involved in the pathogenesis of such intractable diseases. In particular, inflammatory responses to arterial injury, which cause continuous recruitment and activation of monocytes mainly through activation of the monocyte chemoattractant protein-1 (MCP-1) pathway, have a central role in restenosis and atherogenesis. We recently devised a new strategy for anti–MCP-1 therapy by transfecting an N-terminal deletion mutant of the MCP-1 gene into skeletal muscles. This mutant MCP-1 lacks the N-terminal amino acids 2 to 8, called 7ND, and works as a dominant-negative inhibitor of MCP-1. We demonstrated that 7ND gene transfer suppresses monocyte infiltration/activation after arterial injury and markedly inhibits experimental restenosis in animals after balloon injury or stent placement. Furthermore, 7ND gene transfer not only attenuated the development of early atherosclerotic lesions but also limited progression of preexisting atherosclerotic lesions and changed the lesion composition into a more stable phenotype in hypercholesterolemic mice. Vascular inflammation mediated by MCP-1 might create a positive feedback loop to enhance restenotic and atherosclerotic changes through activating lesional monocytes. Therefore, vascular inflammation mediated by MCP-1 has a central role in the development of experimental restenosis, atherosclerosis, and plaque destabilization, leading to acute coronary syndrome. This strategy for gene therapy might be useful against human restenosis, thereby opening a new therapeutic window for antirestenosis and antiatherosclerosis paradigms. (Hypertension. 2003;41[part 2]:834-841.)

Key Words: monocyte ▪ arteriosclerosis ▪ restenosis ▪ gene therapy ▪ inflammation

Inflammatory changes in the arterial wall have a central role in the development of restenosis and atherosclerosis.1,2 A considerable body of evidence supports the notion that various mediators such as adhesion molecules, cytokines, and chemokines are involved in the initiation and progression of atherosclerotic lesions.1,2 Monocyte chemoattractant protein-1 (MCP-1) is the most important chemokine that regulates migration and infiltration of monocytes/macrophages. MCP-1 belongs to a CC chemokine subfamily of chemokines. MCP-1 is the specific chemotactic factor for monocytes/macrophages and has an important role in the pathogenesis of chronic inflammatory disorders.3,4 The effects of MCP-1 are mediated mainly through CC chemokine receptor 2 (CCR2).3,4 MCP-1 causes chronic vascular inflammation and induces thrombus, proliferation and migration of vascular smooth muscle cells, angiogenesis, and oxidative stress (Figure 1). Previous studies indicate that (1) MCP-1 production from endothelial cells, smooth muscle cells, and lesional leukocytes increases in the presence of endothelial dysfunction and atherosclerotic risk factors (Figure 1); (2) MCP-1 expression is increased in atherosclerotic lesions5,6 and injured arteries7,8; and (3) eliminating MCP-1 function decreases neointimal hyperplasia after injury and atheroma formation in mice.9–12 We demonstrated that MCP-1 has an important role in coronary arteriosclerosis in a rat model of NO synthesis inhibition.13–17

Emerging evidence suggests that MCP-1–mediated inflammatory disorders are involved in restenosis and atherosclerosis, as well as in other treatment-intractable cardiovascular diseases, such as posttransplantation arteriosclerosis, vascular remodeling owing to hypertension, myocarditis cardiomyopathy, and cardiac dysfunction and remodeling after myocardial infarction.18–23 Therefore, therapeutic strategies targeting MCP-1 might become useful and practical treatments for cardiovascular diseases that are intractable with conventional therapies. In this regard, we devised a new strategy for anti–MCP-1 gene therapy by transfecting mutant MCP-1 gene.17,24 This strategy might be useful for clarifying the role of MCP-1 under pathophysiologic conditions in vivo. In this review, we describe the role of MCP-1 in cardiovascular...
Role of MCP-1 in Cardiovascular Disease

In animal and human atherosclerotic lesions, ≈80% of leukocytes are monocytes/macrophages, and 10% to 20% of them are memory T-lymphocytes. Atheroma-forming cells (endothelial cells, smooth muscle cells, and macrophages) express MCP-1 and CCR2, and activity in this pathway is increased in atherosclerotic lesions. Oxidative stress, oxidized inflammatory lipids, and redox-sensitive transcription factors (NF-κB, AP-1, etc) reportedly contribute to increased expression of MCP-1. Furthermore, activation of the MCP-1/CCR2 pathway induces adhesion molecules, proinflammatory cytokines, chemokines, and matrix metalloproteinases and thus accelerates atherosclerosis in hypercholesterolemic animals. More importantly, MCP-1 induces tissue factor and inflammatory cytokines such as interleukin-6 in human arterial smooth muscle cells. Abrogation of the MCP-1/CCR2 pathway inhibits the early development of atherosclerotic lesions in mice. These findings suggest that MCP-1 contributes not only to vascular inflammation but also to the development of atherosclerosis, plaque

Plasma Concentrations of MCP-1 and 7ND after 7ND Transfection in Mice

<table>
<thead>
<tr>
<th>No. of Days After 7ND Transfection</th>
<th>Baseline</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1, pg/mL</td>
<td>76 ± 5</td>
<td>85 ± 7</td>
<td>88 ± 6</td>
<td>77 ± 5</td>
<td>80 ± 6</td>
</tr>
<tr>
<td>7ND, pg/mL</td>
<td>&lt;20.0*</td>
<td>226 ± 21</td>
<td>220 ± 20</td>
<td>140 ± 12</td>
<td>&lt;20.0*</td>
</tr>
</tbody>
</table>

Values are mean ± SE, n = 6 to 8. Plasma concentrations of 7ND released by the transfected skeletal muscle were measured by the use of human MCP-1 ELISA kit (Biosource). Plasma MCP-1 concentrations were measured with murine MCP-1 ELISA kit (Biosource). Wild-type mice (C57BL/6J) were transfected with intramuscular injections of pcDNA3–7ND plasmid DNA (100 μg) into the femoral muscle. Transgene expression was enhanced by intramuscular electroporation at the injection site immediately after injection.* Below detectable limits.
destabilization, and thrombosis (Figure 1), which results in acute coronary syndrome.

Inflammation also contributes to the development of restenotic changes after balloon injury or stenting. Inflammatory and proliferative cells in the injured artery are shown to express MCP-1 after injury. Interestingly, a rapid and prolonged production of MCP-1 is reported in patients who present with restenosis after balloon angioplasty.24,33 Cipollone et al24 demonstrated that patients with restenosis have a prolonged increase in plasma MCP-1, whereas nonrestenotic patients have only a transient increase in plasma MCP-1. Thus, human arteries with underlying hypercholesterolemia and/or atherosclerosis are likely to represent prolonged production of MCP-1 after arterial injury. Therefore, elucidating the underlying mechanism of prolonged production of MCP-1 after vascular injury would open the way to identify molecular mechanisms of restenosis. Furukawa et al7 demonstrated that repeated injections of polyclonal antibodies against rat MCP-1 reduced neointimal formation in a rat model of carotid artery balloon injury. We11 and others12 demonstrated that mice lacking CCR2 displayed diminished neointimal hyperplasia formation after femoral arterial injury. There might be important differences between injury associated with balloon dilatation and that associated with stent implantation. In addition to mechanical injury, a foreign body response to stent prosthesis induces intense inflammation in the arterial wall, with ensuing production of cytokines and growth factors that subsequently induce proliferation and migration of vascular smooth muscle cells.34–37 As a result, neointimal hyperplasia is more than 2-fold greater after stent implantation than after balloon angioplasty.36,38 Inhibition of

Figure 3. A, Transfection with the 7ND gene inhibits progression of established atherosclerotic lesions in the aortic arch of apoE-KO mice. Photomicrographs of the intraluminal surface of the aortic arch stained with oil red O. Quantitative comparison of atherosclerotic lesion size and lesions in baseline, empty plasmid, and 7ND-transfected ApoE-KO mice are also shown. Data are reported as mean±SEM, n=9 to 10. *P<0.05 vs the baseline; †P<0.05 vs empty plasmid group. Modified from Inoue et al46. B, 7ND transfection changes the composition of the atheroma such that it is more stable. From the top to bottom, photomicrographs of atherosclerotic lesions stained with oil red O or immunostained with anti-murine macrophage antibody (MOMA-2), anti-human α-SM actin antibody, anti-MCP-1 antibody, and anti-CCR2 antibody. Interstitial collagen was visualized by using polarization microscopy after staining with picrosirius red. Internal and external elastic layers are highlighted with blue and black lines, respectively. Bar, 200 μm. Modified from Inoue et al.46 C, Effect of 7ND transfection on chemokine (RANTES and MCP-1) and cytokine TNF-α, IL-6, IL-1β, TGF-β gene expression in the abdominal aorta. Data are expressed as the ratio of each mRNA to the corresponding GAPDH mRNA. *P<0.05 vs the baseline and 7ND-transfected group. Modified from Inoue et al.46
cardiovascular inflammation, the beneficial effects of 7ND gene transfer on restenosis and established atherosclerotic lesions might be caused mainly by suppression of monocyte recruitment and activation.

Anti–MCP-1 Gene Therapy by Intramuscular Transfection of Mutant MCP-1 Gene

Because MCP-1–mediated inflammation appears to have a central role in the pathogenesis of cardiovascular inflammation and its disease process, we sought a new therapeutic strategy to target the MCP-1/CCR2 pathway. An N-terminal deletion mutant of MCP-1, called 7ND, which lacks the N-terminal amino acids 2 to 8, forms inactive heterodimers with wild-type MCP-1 and exerts its inhibitory activity as a dominant-negative inhibitor under in vitro conditions (Figure 2A). Therefore, we evaluated the use of gene therapy to block MCP-1 activity in vivo by using intramuscular transfection of this mutant MCP-1 gene. The use of skeletal muscle as a biofactory to produce a secreted protein has been reported previously. From a clinical point of view, this strategy (the delivery of plasmid DNA by intramuscular injection) is simple and shown to be nontoxic. No gene delivery systems of clinical use with acceptable safety for local gene delivery to coronary artery lesions are available at the present time. We demonstrated that (1) intramuscular transfection of plasmids encoding the human 7ND gene into skeletal muscle resulted in secretion of 7ND protein into the circulating blood, and (2) the 7ND protein binds to the MCP-1 receptor on monocytes or target cells and, thus, (3) achieved an effective and sufficient blockade of MCP-1 activity in remote organs (Figure 2B). The therapeutic effects of this strategy may depend on the protein secreted into circulation by the transgene. To confirm the efficacy of transgene, we measured plasma MCP-1 and 7ND concentrations in mice after intramuscular transfection of 7ND gene (Table). Plasma MCP-1 concentrations did not change during the course of experiments, whereas 7ND was detected in plasma 3, 7, and 14 days after transfection.

This strategy also suppressed monocyte recruitment into the coronary vessels and the development of coronary atherosclerosis in a rat model of chronic inhibition of NO synthesis. Furthermore, there were no apparent side effects during the period of the study. On the basis of these pioneering studies, this strategy might be a useful and feasible form of gene therapy against inflammation and related diseases mediated by MCP-1 in humans. This strategy might also be useful for clarifying the role of MCP-1 under pathophysiological conditions in vivo, especially in organs into which direct gene transfer is difficult.

Effect of 7ND Gene Transfer on Atherosclerosis and Plaque Destabilization

Although mice lacking MCP-1 or CCR2 display reduced initial atheroma formation, whether postnatal blockade of MCP-1 could be a unique site-specific therapy against atherosclerosis was unknown. Apolipoprotein E–knockout (Apoe−/−) mice develop hypercholesterolemia and atherosclerotic lesions similar to those observed in humans and are widely used for studying the pathogenesis of atherosclerosis. Therefore, we tested the effectiveness of 7ND gene transfer on the development of atherosclerosis in this model. Apoe−/− mice (7 or 8 weeks of age) were fed a Western-type diet and randomized into 2 groups. The mice were injected with PBS or 7ND gene (5 μg 7ND vector plasmid encapsulated in hemagglutinating virus of Japan-liposome) into the femoral muscles at weeks 0 and 3 after the start of a Western-type diet. After 6 weeks on the diet, the control group mice had typical fatty atherosclerotic lesions in the aortic root. Macrophage infiltration and MCP-1 immunostaining were observed in atherosclerotic lesions. The 7ND gene transfer effectively blocked MCP-1 activity and inhibited the formation of atherosclerotic lesions, but had no effect on serum lipid concentrations. Furthermore, this strategy increased the lesional extracellular matrix content, suggesting that blockade of MCP-1 reduced markers of plaque destabilization. These data suggest that MCP-1 can be a novel therapeutic target for atherosclerosis.

Investigation of molecular mechanisms underlying later complications of atherosclerosis is clinically very important, because atherosclerotic complications such as acute myocardial infarctions and stroke develop during the later stages of atherosclerosis. Lesion composition rather than size or degree of the stenosis of the lesion is believed to determine the likelihood of plaque rupture and subsequent thrombotic complications such as acute coronary syndrome. Therefore,
we tested the hypothesis that blockade of MCP-1 limits progression and destabilization of established lesions in ApoE-KO mice.\textsuperscript{46} ApoE-KO mice were fed a normal chow diet during the experiment. At 20 weeks of age, the baseline group of mice was killed to determine the extent of baseline established lesions. Other mice were randomly assigned into 2 groups. The 7ND-transfected group received intramuscular injections of naked pcDNA3 to 7ND plasmid DNA (100 μg) into the femoral muscle at biweekly intervals for up to 8 weeks. Plasma MCP-1 concentrations did not change during the course of experiments, whereas 7ND was detected in plasma up to 2 weeks after transfection. Blockade of MCP-1 by 7ND gene transfer limited progression of preexisting atherosclerotic lesions independent of serum cholesterol levels (Figure 3A). In addition, blockade of MCP-1 changed the lesion composition into a more stable phenotype, ie, containing fewer macrophages and lymphocytes, less lipid, and more smooth muscle cells and collagen. This finding warrants clinical attention because interstitial collagen in the shoulder region is considered to be a critical determinant of fibrous cap integrity.\textsuperscript{1} This strategy decreased expression of CD40, the CD40 ligand, tissue factor, and matrix metalloproteinases-9 and -13 in the atherosclerotic plaque (Figure 3B), and normalized the increased chemokine (RANTES and MCP-1) and cytokine (TNFα, IL-6, IL-1β, and TGFβ-1) gene expression (Figure 3C). Suppression of the expression of MCP-1 and the other chemokines and cytokines by 7ND gene transfer implies that MCP-1–mediated inflammation creates a positive feedback loop to enhance vascular inflammation and atherogenesis, possibly through activating lesional monocytes (Figure 4). The beneficial effects of 7ND gene transfer on established atherosclerotic lesions might be owing mainly to the suppression of monocyte recruitment and activation. These data suggest that anti–MCP-1 therapy not only limits
progression of established preexisting atheroma but also limits transformation from destabilized plaques to stable plaques, suggesting that blockade of the MCP-1/CCR2 pathway might lead to reductions in atherosclerotic complications.

Effects of 7ND Gene Transfer on Experimental Restenosis

The benefits of percutaneous coronary interventions are hampered by triggering local arterial renarrowing (restenosis). As mentioned above, vascular injury owing to balloon dilatation or stent implantation induces inflammatory responses that accelerate the recruitment and activation of monocytes. Anatomically, in-stent restenosis results exclusively from neointimal hyperplasia, whereas restenosis after balloon angioplasty results from neointimal hyperplasia and negative remodeling of the arterial wall. We hypothesized that MCP-1–mediated inflammation is essential in the development of restenotic changes after balloon injury or stent implantation in rats, rabbits, and monkeys. We demonstrated that blockade of MCP-1 by 7ND gene transfer suppressed monocyte infiltration/activation at the injured site and markedly inhibited restenotic changes (neointimal hyperplasia) after balloon injury of the carotid artery in rats and monkeys (Figure 5A and 5B). This strategy also suppressed the local production of MCP-1 and inflammatory cytokines. In hypercholesterolemic rabbits in which neointimal formation and negative remodeling developed after balloon injury, 7ND gene transfer attenuated such changes (Figure 5C). In hypercholesterolemic rabbits and monkeys, 7ND gene transfer inhibited monocyte infiltration/activation in the stented arterial wall and thus reduced the development of in-stent restenosis (Figure 6, K. Egashira, unpublished data, 2002).

Our data, therefore, indicate that locally produced MCP-1 not only induces the recruitment of monocytes but also activates lesional monocytes and vascular smooth muscle cells to produce the inflammatory cytokines, which might then cause experimental restenosis. Thus, MCP-1–mediated inflammation in the arterial wall is likely to create a positive-feedback mechanism to enhance inflammation and proliferation of the injured arterial wall (Figure 4). It is also possible that MCP-1 activated adventitial myofibroblasts, which may in turn contribute to the development of restenosis after injury. Our finding in nonhuman primates is meaningful because many therapeutic strategies that have proven effective in reducing restenosis in nonprimate animal models have failed to demonstrate substantial effect on human restenosis. Therefore, monocyte infiltration and activation mediated by MCP-1 are essential in the development of experimental restenosis.

Conclusion

In conclusion, the inflammatory changes mediated by MCP-1 are essential and important in mediating chronic inflammation in cardiovascular disease, especially in experimental restenosis as well as atherosclerosis and plaque destabilization. Future studies are needed to address the role of hematopoietic stem cells in the effect seen on atherosclerosis and restenosis. Our findings support the hypothesis that (1) MCP-1 is a novel therapeutic target against cardiovascular inflammation and related diseases, and (2) anti–MCP-1 gene therapy with mutant MCP-1 transfection might be a useful and practical form of therapy against human restenosis after coronary intervention. Because of the potential pathogenetic role of MCP-1 in other treatment-intractable inflammatory disorders, our strategy might have broader clinical applications.

Perspectives

From a clinical point of view, the potential side effects of anti-MCP-1 gene therapy merit mentioning. We assume that blockade of MCP-1 with our strategy does not cause serious local or systemic side effects, because (1) mice lacking MCP-1 or CCR2 display no serious health problems, (2) the delivery of plasmid DNA by intramuscular injection is now in clinical stages and is proven to be safe, and (3) intramuscular transfer of 7ND gene is nontoxic and safe in nonhuman primates. We have not yet investigated whether long-term inhibition of MCP-1 function affects the systemic immunoprotective ability in humans. Future studies will require careful observation over a long period of time to establish the
true risk/benefit ratio. We are planning to apply this strategy to clinical restenosis after percutaneous coronary intervention, and this clinical protocol is now under deliberation by the Gene Therapy Committee of Ministry of Health, Labor and Welfare of the Japanese government. Future clinical study would open a new therapeutic window for anti-restenosis and antiatherosclerosis paradigms.

References


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