16. Kitamoto S,Nakano k, Hirouchi Y, Kohjimoto Y, Kitajima S, Usui M, Inoue S, Egashira K: Cholesterol-Lowering Independent Regression and Stabilization of Atherosclerotic Lesions by Pravastatin and by Antimonocyte Chemoattractant Protein-1 Therapy in Nonhuman Primates. ***Arterioscler Thromb Vasc Biol.*** 2004; 24:1522-1528.

17. Tsutsumi-Miyahara C, Sonoda KH, Egashira K, Ishibashi M, Oiao H, Oshima T, Murata T, Miyazaki M, Charo IF, Hamano S, Ishibashi T: The relative contributions of each subset of ocular infiltrated cells in experimental choroidal neovascularisation. ***Br J Ophthalmol.*** 2004; 88(9), 1217-1222.

18. Saiura A, Sata M, Hiasa K, Kitamoto S, Washida M, Egashira K, Nagai R, Makuuchi M: Antimonocyte Chemoattractant Protein –1 Gene Therapy Attenuates Graft Vasculopathy. ***Arterioscler Thromb Vasc Biol.*** 2004 ; 24: 1886-1890.

19. Ono H, Ichiki T, Fukuyama K, Iino N, Masuda S, Egashira K, Takeshita A: cAMP –Response Element-Binding Protein Mediates Tumor Necrosis Factor- α -induced Vascular Smooth Muscle Cell Migration. ***Arterioscler Thromb Vasc Biol.*** 2004; 24(9): 1634-1639.

20. Zhao Q, Ishibashi M, Hiasa K, Tan C, Takeshita A, Egashira K: Essential Role of Vascular Endothelial Growth Factor in Angiotensin II- Induced Vascular Inflammation and Remodeling. ***Hypertension*** 2004; 44(3): 264-270.

21. Ishibashi M, Egashira K, Zhao Q, Hiasa K, Ohtani K, Ihara Y, Charo IF, Kura S, Tsuzuki T, Takeshita A, Sunagawa K: Bone Marrow-Derived Monocyte Chemoattractant Protein-1 Receptor CCR2 Is Critical in Angiotensin II- Induced Acceleration of Atherosclerosis and Aneurysm Formation in Hypercholesterolemic Mice. ***Arterioscler Thromb Vasc Biol.*** 2004; 24: e174-e178.

22. Shimizu S, Nakashima H, Masutani K, Inoue K, Miyake K, Akahoshi M, Tanaka Y, Egashira K, Hirakata H, Otsuka T, Harada M: Anti-monocyte chemoattractant protein-1 gene therapy attenuates nephritis in MRL/lpr mice. ***Rheumatology*** 2004; 43(9): 1121-1128.

23. Niiyama H, Kai H, Yamamoto T, Shimada T, Sasaki K, Murohara T, Egashira K, Imaizumi T,: Roles of Endogenous Monocyte Chemoattractant Protein-1 in Ischemia-Induced Neovascularization. ***J Am Coll Cardiol*** 2004; 44(3): 661-666.

24. Ohtani K, Egashira K, Hiasa K, Zhao Q, Kitamoto S, Ishibashi M, Usui M, Inoue S, Yonemitsu Y, Sueishi K, Sata M, Shibuya M, Sunagawa K: Blockade of vascular endothelial growth factor suppresses experimental restenosis after intraluminal injury by inhibiting recruitment of monocyte lineage cells. ***Circulation*** 2004 ;110: 2444-2452.

25. Zhao Q, Egashira K, Hiasa K, Ishibashi M, Inoue S, Ohtani K, Tan C, Shibuya M, Takeshita A: Essential Role of Vascular Endothelial Growth Factor and Flt-1 Signals in Neointimal Formation After Periadventitial Injury. ***Arterioscler Thromb Vasc Biol.*** 2004; 24: 2284-2289.

26. Tsuruta S, Nakamuta M, Enjoji M, Katoh K, Hiasa K, Egashira K, Nawata H:

Anti-monocyte chemoattractant protein-1 gene therapy prevents dimethylnitrosamine-induced hepatic fibrosis in rats. *Int J Mol Med*.2004; 14(5): 837-842.

27. Kumai Y, Ooboshi H, Takada J, Kamouchi M, Kitazono T, Egashira K, Ibayashi S, Iida M: Anti-monocyte chemoattractant protein-1 gene therapy protects against focal brain ischemia in hypertensive rats. *J Cereb Blood Flow Metab*. 2004;24(12): 1359-1368.

(2) 口頭発表

＜国内学会＞

1. 江頭健輔：第 8 回日本適応医学会（平成 16 年 6 月 25 日、福島）シンポジウム「再狭窄・動脈硬化の分子機構における血管内皮増殖因子（VEGF）の役割に関する研究の新展開　VEGF は「内皮特異的血管保護因子」あるいは「多機能サイトカイン」か？」

2. 江頭健輔：第 36 回日本動脈硬化学会（日本血管生物医学会）（平成 16 年 7 月 23 日、福岡）動脈硬化病変の分子機構における血管内皮増殖因子（VEGF）の役割—VEGF は「内皮特異的血管保護因子」か—（ジョイントシンポジウム）

3. 江頭健輔、大谷規彰：第 36 回日本動脈硬化学会（平成 16 年 7 月 24 日、福岡）炎症を標的とした次世代遺伝子溶出型ステントの創製（シンポジウム）

4. 江頭健輔：第 27 回日本高血圧学会（平成 16 年 10 月 7 日、栃木）高血圧性血管傷害における白血球 MCP-1 受容体 CCR2 の重要性—炎症のニューパラダイム —（シンポジウム）

5. 江頭健輔：第 45 回日本脈管学会（平成 16 年 10 月 29 日、札幌）動脈硬化の最前線－基礎から臨床－「炎症制御による次世代遺伝子溶出型ステントの創製（シンポジウム）

6. 江頭健輔：第 24 回日本川崎病研究会（平成 16 年 11 月 12 日、京都）動脈硬化症疾患における炎症の役割　次世代医療としての血管内医療システムの創製（ランチョンセミナー）

7. 大谷規彰、江頭健輔：第 11 回九州血液血管研究会（平成 16 年 11 月 27 日、福岡）再狭窄・動脈硬化の分子機序における血管内皮増殖因子（VEGF）の役割の解明 VEGF は「内皮特異的血管保護因子」か、あるいは「多機能サイトカイン」か？

8. 江頭健輔：第 8 回日本心血管内分泌代謝学会ランチョンセミナー（平成 16 年 11 月 26 日、宮崎）　動脈硬化性疾患における炎症の役割—次世代医療としての炎症制御による血管内医療システムの創製—

9. 江頭健輔：福岡循環器学術講演会 2004（平成 16 年 12 月 3 日、福岡）ACS 治療のトピックス—PROVE IT の解釈と薬剤溶出型ステント新時代

10. 江頭健輔：第 23 回機能再建材料グループ研究会(平成 17 年 1 月 21 日、茨城)遺

伝子薬剤のコーティング技術について

11. 江頭健輔：第34回日本心脈管作動物質学会（平成17年2月4日、京都）遺伝子溶出型ステントによる次世代血管内治療システムの創製（シンポジウム）

12. 江頭健輔：第34回日本心脈管作動物質学会（平成17年2月4日、京都）心脈管の再生・遺伝子治療（シンポジウム：オーガナイザー）

13. 江頭健輔：第69回日本循環器学会総会（平成17年3月19日、神奈川）Local Gene Transfer via Gene-Eluting Stents for Prevention of Restenosis: An Anti-inflammation Strategy for Next-Generation Intravascular Therapy（シンポジウム）

14. 江頭健輔、石橋美奈子：第69回日本循環器学会総会（平成17年3月20日、神奈川）Bone marrow-derived monocyte chemoattractant protei-1 receptor CCR2 is critical in angiotensin II-induced vascular remodeling and atherosclerotic process（プレナリーセッション・シンポジウム）

＜国際学会＞

1. Egashira K: The Scientific meeting of Taiwan Society of Cardiology (May 8, 2004,Taipei) Anti-Monocyte Chemoattractant Protein-1 (MCP-1) Therapy Against (Plenary Speech)

2. Egashira K: Taiwan Society of Cardiology (May8 2004、Taipei) Hot Topics on Cholesterol Lowering Therapy on Atherosclerotic Vascular Disease (Satellite Symposium)

3. Egashira K: XVIII World Congress of the International Society for Heart Research (August 9 2004, Australaria) Anti-inflammation therapy targeting monocyte chemoattractant protein-1,as novel strategy to treat cardiovascular disease.(Symposium)

4. Egashira K: XVIII World Congress of the International Society for Heart Research (August 9 2004, Australaria) Symposium UAnti-inflammation therapy targeting monocyte chemoattractant protein-1,as novel strategy to treat cardiovascular disease.

5. Egashira K: Special invited lecture at Shang-Hai medical school and Microport Inc.（October 14,2004、Shanghai,China）難治性心臓病に対する遺伝子溶出ステントとカテーテルの開発

(3) 出版物

＜著書＞

1. 北本史朗、江頭健輔：MCP-1　単行本「高脂血症ナビゲーター」（用語集）（第2章　成因・動態）「MCP-1」　2003年1月96-97　メディカルレビュー社

2. 北本史朗、江頭健輔：ケモカイン．NAVIGATOR 心臓ナビゲーター（浅田祐士

郎、江頭健輔、甲斐久史、古森公浩、佐田政隆、室原豊明　編）メディカルレビュー社　2004；94-95

＜総説＞

1. 江頭健輔：血管病における炎症のニューパラダイム（Overview）．　血管医学　2003；4(5)：7(461)-9(463)

2. 北本史朗、江頭健輔：動脈硬化・再狭窄と炎症　血管医学　2003；4(5)：27(481)-35(489)

3. 臼井　真、江頭健輔：糖尿病性虚血下肢・心臓病への再生医療．　Deabetes Frontier　メディカルレビュー社　2003；14(5)：623-628

4. 大谷規彰、江頭健輔：血管の形成と障害　　ケモカインと動脈硬化(MCP-1)．　Heart View（増刊号）2003; 7(12)：248-253

5. 北本史朗、江頭健輔：NOとインスリン抵抗性・血管病．　血管医学　2003；4(4)：43(369)-50(376)

6. 北本史朗、江頭健輔：将来可能な遺伝子治療．　分子心血管病　2003；4(6)：62(624)-68(630)

7. 北本史朗、江頭健輔：HMG-CoA 還元酵素阻害薬の抗炎症作用．　日本臨床　2003；62(1)：172-179

8. 江頭健輔：みえてきた再狭窄抑制のとき．　分子心血管病　2003；4(6)：1(563)-12(574)

9. 江頭健輔：抗炎症により動脈硬化を治療する新規アプローチーMCP1 阻害による動脈硬化の遺伝子治療ー　ACROSS　2003；3：8-11

10. 江頭健輔：動脈硬化性炎症のニューパラダイムー中心的役割を果たす主役 MCP-1ー　ACROSS　2003；3：12-13

11. 北本史朗、江頭健輔：血管内皮機能を改善する治療法．　Heart View2004; 8(2)：88(208)-92(212)

12. 北本史朗、江頭健輔：レニンーアンジオテンシン系と動脈硬化．　細胞　ニューサイエンス社 2004; 36(3)：12(90)-15(93)

13. 江頭健輔：血管医学の新展開ーニューパラダイムの構築から治療開発へー　羊土社　実験医学　2004；22(8)：84(1106)-88(1110)

14. 北本史朗、江頭健輔：動脈硬化・再狭窄の機序における MCP-1 の役割ー炎症仮説の証明　羊土社　実験医学　2004；22(8)：89(1111)-95(1117)

15. 大谷規彰、日浅謙一、江頭健輔：動脈硬化病変における VEGF 研究の新展開ー血管保護か血管病促進か？　羊土社　実験医学　2004;22(8):96(1118)-100(1122)

16. 日浅謙一、大谷規彰、江頭健輔：血管新生因子と細胞を用いた治療的血管新生療

法の現状と問題点　内科 2004; 94(2): 343-348

17. 大谷規彰、江頭健輔：病態と疾患からみた NO　動脈硬化症—NO and atherosclerosis—　日本臨床社　日本臨床増刊号　臨床分子内分泌学 1—心血管内分泌代謝系（上）—2004; 62(9): 544-547

18. 大谷規彰、江頭健輔、砂川賢二：動脈硬化病変の分子機序における VEGF の役割に関する研究の新展開—血管保護か血管病促進か？　2004；血管 27（4）：117-123

19. 北本史朗、江頭健輔：アンジオテンシン II と動脈硬化　CARDIAC PRACTICE 2005；16(1)：51-55

【研究成果の刊行物・別刷】

次のページ以降に論文別刷りを添付します。

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 thrombosis, 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 lesions[5,6] and injured arteries[7,8]; 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

Received September 19, 2002; first decision November 12, 2002; revision accepted November 26, 2002.

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Hypertension is available at http://www.hypertensionaha.org

DOI: 10.1161/01.HYP.0000051642.65283.36

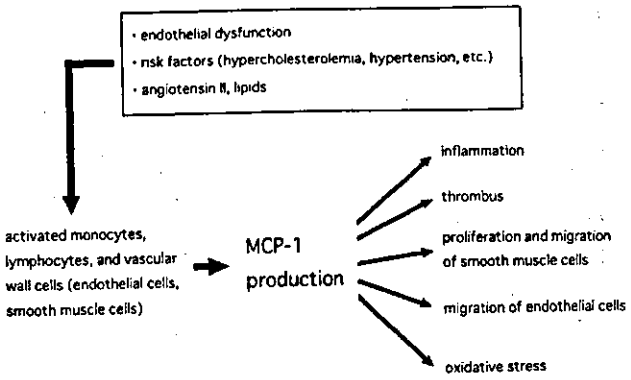


Figure 1. The role of the MCP-1 pathway in the pathogenesis of atherosclerosis, plaque destabilization, thrombosis, and restenosis.

diseases and introduce recent work that addresses the usefulness of anti–MCP-1 gene therapy. The study protocol was reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine, and the experiments were conducted according to the Guidelines of American Physiological Society. A part of this study was performed at the Kyushu University Station for Collaborative Research.

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.[25] Atheroma-forming cells (endothelial cells, smooth muscle cells, and macrophages)

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

		No. of Days After 7ND Transfection			
	Baseline	3	7	14	28
MCP-1, pg/mL	76±5	85±7	88±6	77±5	80±6
7ND, pg/mL	<20.0*	226±21	220±20	140±12	<20.0*

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.[46]

*Below detectable limits.

express MCP-1 and CCR2, and activity in this pathway is increased in atherosclerotic lesions.[26] 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,[27] proinflammatory cytokines,[27,28] chemokines, and matrix metalloproteinases[29] and thus accelerates atherosclerosis in hypercholesterolemic animals.[30,31] More importantly, MCP-1 induces tissue factor and inflammatory cytokines such as interleukin-6 in human arterial smooth muscle cells.[32] Abrogation of the MCP-1/CCR2 pathway inhibits the early development of atherosclerotic lesions in mice.[9,10] These findings suggest that MCP-1 contributes not only to vascular inflammation but also to the development of atherosclerosis, plaque

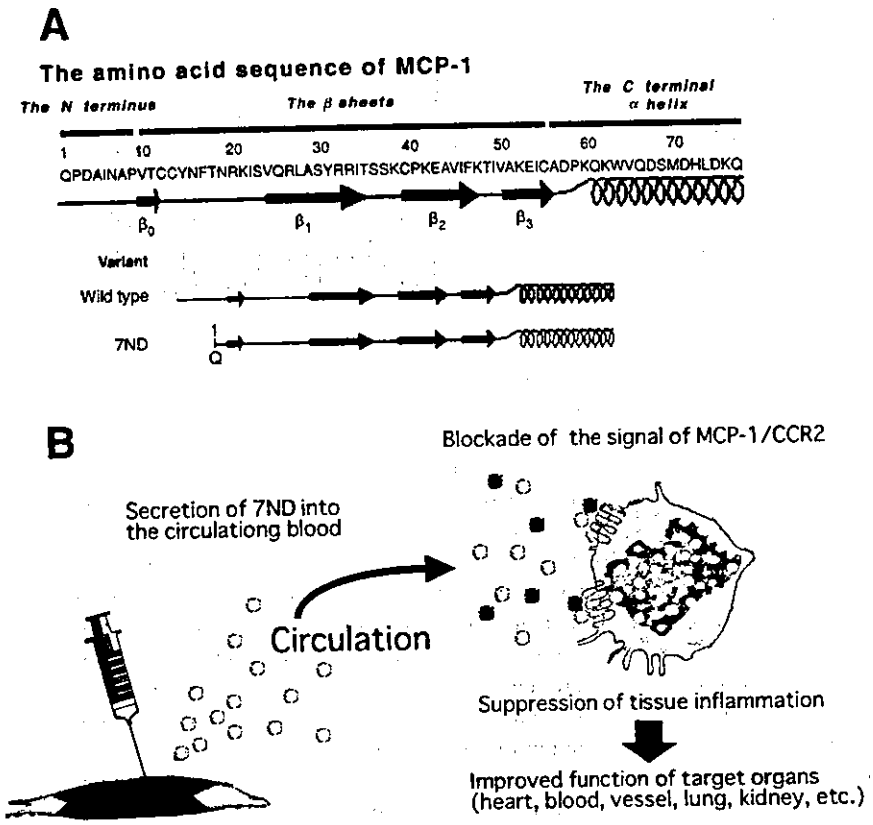
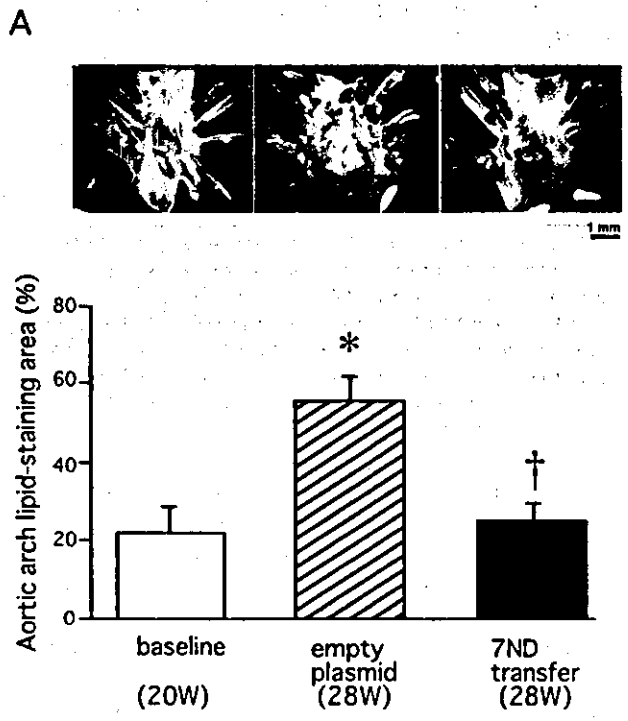
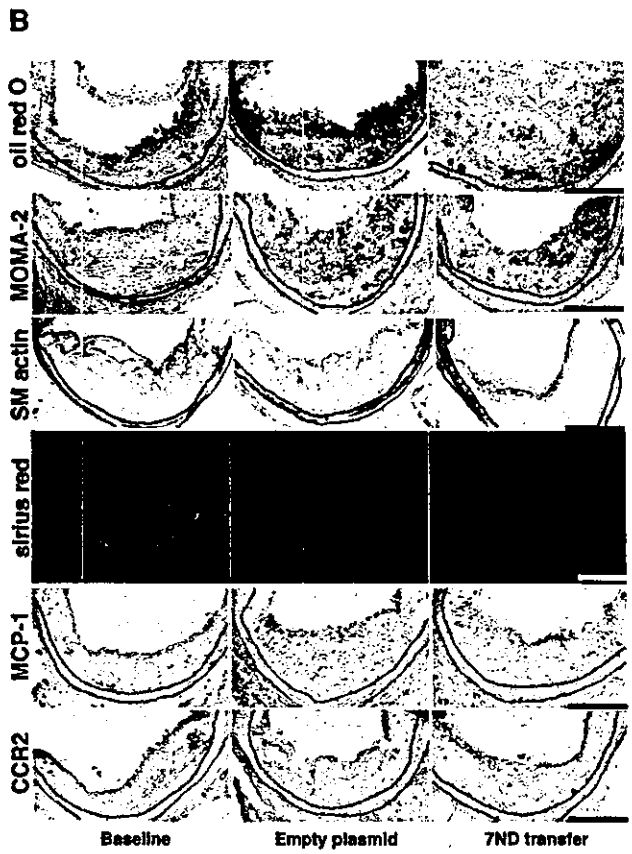


Figure 2. Structure of MCP-1 and 7ND (A), and schema for strategy of anti–MCP-1 gene therapy by 7ND gene transfer (B). A, 7ND is an N-terminal deletion mutant of human MCP-1 that lacks the N-terminal amino acids 2 to 8 and acts as a dominant-negative inhibitor for MCP-1. Intramuscular transfection of 7ND gene therefore suppresses monocyte chemotaxis in remote organs by blocking the MCP-1/CCR2 signal pathway. B, To achieve effective blockade of the MCP-1/CCR2 signal pathway, we transfected the expression plasmid vector encoding 7ND gene into skeletal muscle. We reported that 7ND protein is secreted from the transfected skeletal muscle cells into the circulating blood, blocks the MCP-1/CCR2 signal pathway in remote target organs or tissues, and suppresses monocyte recruitment into the target organs or tissues





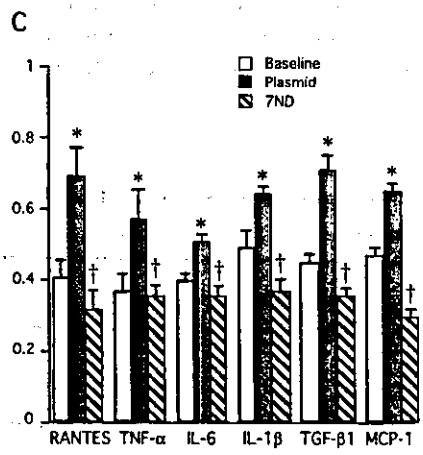


Figure 3. A, Transfection with the 7ND gene inhibits progression of established atherosclerotic lesions in the aortic arch of apoE-KO mice. Photomicrographs of the intraluminal surface of the aortic arch stained with oil red O. Quantitative comparison of atherosclerotic lesion size and lesions in baseline, 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 al[46]. 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 picro sirius 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-β 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]

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 al[24] 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 al[7] demonstrated that repeated injections of polyclonal antibodies against rat MCP-1 reduced neointimal formation in a rat model of carotid artery balloon injury. We[11] and others[12] 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

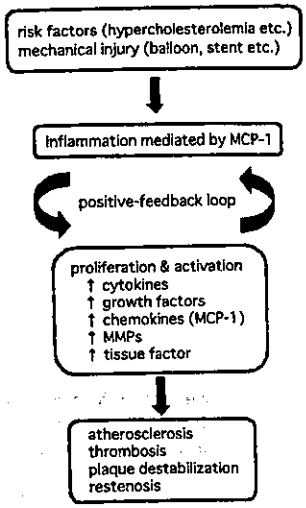


Figure 4. Schematic diagram of our hypothesis regarding the role of the MCP-1/CCR2 pathway in the development/progression of atherosclerosis and thrombosis, plaque destabilization, and restenosis. Because 7ND gene transfer suppressed expression of MCP-1 and the other chemokines and cytokines, it is likely that MCP-1–mediated inflammation creates a positive feedback loop (a vicious cycle) to enhance vascular inflammation and atherogenesis possibly through activating lesional monocytes. The beneficial effects of 7ND gene transfer on restenosis and established atherosclerotic lesions might be causedy mainly by suppression of monocyte recruitment and activation.

cellular proliferation with the immunosuppressant sirolimus might be an effective strategy to suppress in-stent restenosis.[39–42] Experimental data suggest that the beneficial effects of sirolimus-eluting stents are mediated at least in part by antiinflammatory effects.[39] Inhibition of the MCP-1 or CCR2 pathways attenuate in-stent neointimal hyperplasia in nonhuman primates.[43] These data suggest that MCP-1 and CCR2 have a pivotal role in the pathogenesis of restenosis after balloon injury or stent-induced injury.

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).[44] We therefore 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).[17] 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 arteriosclerosis in a rat model of chronic inhibition of NO synthesis.[17] 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 pathophysiologic 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,[9,10] whether postnatal blockade of MCP-1 could be a unique site-specific therapy against atherosclerosis was unknown. Apolipoprotein E–knockout (ApoE-KO) 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.[45] ApoE-KO 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.[1] Therefore,

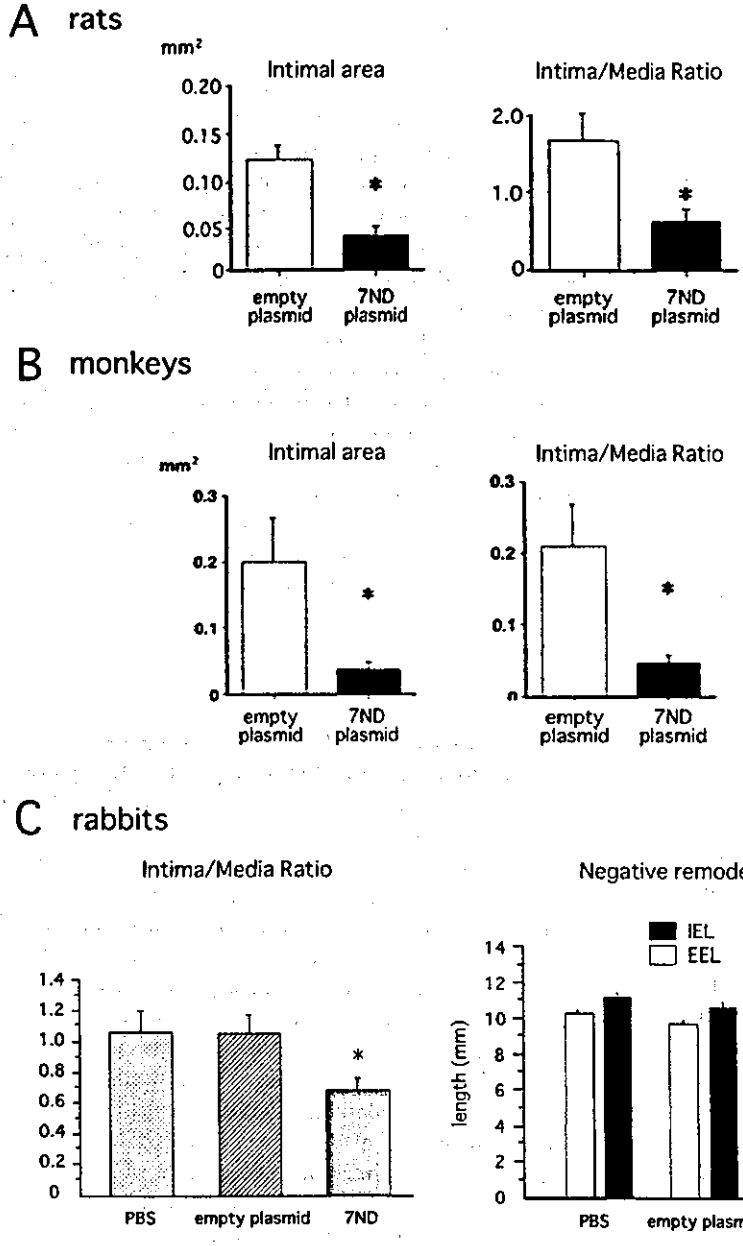


Figure 5. A, Effect of the intramuscular transfer of the 7ND gene on intimal area and intima/media ratio 28 days after balloon injury in rats transfected with empty plasmid or 7ND plasmid (n=8 each). *$P<0.01$ vs the empty plasmid treatment. Modified from Usui.[48] B, Effect of the intramuscular transfer of the 7ND gene on intimal area and intima/media ratio 28 days after balloon injury in monkeys (n=6). *$P<0.01$ vs the empty plasmid treatment. Modified from Usui.[48] C, Effect of intramuscular transfer of 7ND gene on neointimal formation (intima/media ratio) and negative remodeling on day 28 after balloon injury in rabbits. IEL indicates internal elastic lamina; EEL, external elastic lamina. *$P<0.05$, **$P<0.01$ vs PBS or empty plasmid. Modified from Mori.[49]

we tested the hypothesis that blockade of MCP-1 limits progression and destabilization of established lesions in ApoE-KO mice.[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.[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

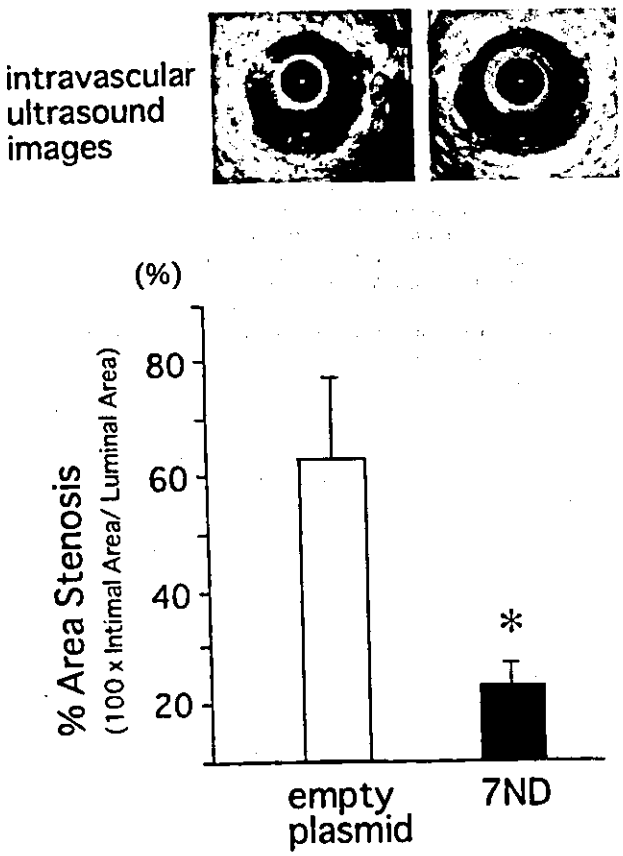


Figure 6. Effects of 7ND gene transfer on in-stent neointimal hyperplasia. Top, An intravascular ultrasound cross-section image in empty plasmid-transfected animal with large neointimal hyperplasia (left) and an intravascular ultrasound cross-sectional image in 7ND-transfected animal with small neointimal hyperplasia (right). Quantitative comparison of mean percent area stenosis within stent in the empty plasmid-transfected and 7ND-transfected rabbits as assessed by intravascular ultrasound. *$P<0.01$ vs empty plasmid.

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.[47] 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).[48] 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).[49] 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 contributed 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 antirestenosis and antiatherosclerosis paradigms.

References

1. Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. *Circulation.* 2001;104:365–372.
2. Ross R. Atherosclerosis: an inflammatory disease. *N Engl J Med.* 1999; 340:115–126.
3. Rollins BJ. Chemokines. *Blood.* 1997;90:909–928.
4. Mukaida N, Harada A, Matsushima K. Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions. *Cytokine Growth Factor Rev.* 1998;9:9–23.
5. Takeya M, Yoshimura T, Leonard EJ, Takahashi K. Detection of monocyte chemoattractant protein-1 in human atherosclerotic lesions by an anti-monocyte chemoattractant protein-1 monoclonal antibody. *Hum Pathol.* 1993;24:534–539.
6. Yla-Herttuala S, Lipton BA, Rosenfeld ME, Sarkioja T, Yoshimura T, Leonard EJ, Witztum JL, Steinberg D. Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. *Proc Natl Acad Sci U S A.* 1991;88:5252–5256.
7. Furukawa Y, Matsumori A, Ohashi N, Shioi T, Ono K, Harada A, Matsushima K, Sasayama S. Anti-monocyte chemoattractant protein-1/monocyte chemotactic and activating factor antibody inhibits neointimal hyperplasia in injured rat carotid arteries. *Circ Res.* 1999;84:306–3.
8. Taubman MB, Rollins BJ, Poon M, Marmur J, Green RS, Berk BC, Nadal-Ginard B. JE mRNA accumulates rapidly in aortic injury and in platelet-derived growth factor–stimulated vascular smooth muscle cells. *Circ Res.* 1992;70:314–325.
9. Boring L, Gosling J, Cleary M, Charo IF. Decreased lesion formation in $CCR2^{-/-}$ mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature.* 1998;394:894–897.
10. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low-density lipoprotein receptor–deficient mice. *Mol Cell.* 1998;2: 275–281.
11. Egashira K, Zhao Q, Kataoka C, Ohtani K, Usui M, Charo IF, Nishida K, Inoue S, Katoh M, Ichiki T, Takeshita A. Importance of monocyte chemoattractant protein-1 pathway in neointimal hyperplasia after periarterial injury in mice and monkeys. *Circ Res.* 2002;90:1167–1172.
12. Roque M, Kim WJ, Gazdoin M, Malik A, Reis ED, Fallon JT, Badimon JJ, Charo IF, Taubman MB. CCR2 deficiency decreases intimal hyperplasia after arterial injury. *Arterioscler Thromb Vasc Biol.* 2002;22: 554–559.
13. Takemoto M, Egashira K, Usui M, Numaguchi K, Tomita H, Tsutsui H, Shimokawa H, Sueishi K, Takeshita A. Important role of tissue angiotensin-converting enzyme activity in the pathogenesis of coronary vascular and myocardial structural changes induced by long-term blockade of nitric oxide synthesis in rats. *J Clin Invest.* 1997;99:278–287.
14. Usui M, Egashira K, Tomita H, Koyanagi M, Katoh M, Shimokawa H, Takeya M, Yoshimura T, Matsushima K, Takeshita A. Important role of local angiotensin II activity mediated via type 1 receptor in the pathogenesis of cardiovascular inflammatory changes induced by chronic blockade of nitric oxide synthesis in rats. *Circulation.* 2000;101:305–310.
15. Koyanagi M, Egashira K, Kitamoto S, Ni W, Shimokawa H, Takeya M, Yoshimura T, Takeshita A. Role of monocyte chemoattractant protein-1 in cardiovascular remodeling induced by chronic blockade of nitric oxide synthesis. *Circulation.* 2000;102:2243–2248.
16. Kitamoto S, Egashira K, Kataoka C, Koyanagi M, Katoh M, Shimokawa H, Morishita R, Kaneda Y, Sueishi K, Takeshita A. Increased activity of nuclear factor-κB participates in cardiovascular remodeling induced by chronic inhibition of nitric oxide synthesis in rats. *Circulation.* 2000;102: 806–812.
17. Egashira K, Koyanagi M, Kitamoto S, Ni W, Kataoka C, Morishita R, Kaneda Y, Akiyama C, Nishida KI, Sueishi K, Takeshita A. Antimonocyte chemoattractant protein-1 gene therapy inhibits vascular remodeling in rats: blockade of MCP-1 activity after intramuscular transfer of a mutant gene inhibits vascular remodeling induced by chronic blockade of NO synthesis. *Faseb J.* 2000;14:1974–1978.
18. Devaux B, Scholz D, Hirche A, Klovekorn WP, Schaper J. Upregulation of cell adhesion molecules and the presence of low-grade inflammation in human chronic heart failure. *Eur Heart J.* 1997;18:470–479.
19. Shioi T, Matsumori A, Kihara Y, Inoko M, Ono K, Iwanaga Y, Yamada T, Iwasaki A, Matsushima K, Sasayama S. Increased expression of interleukin-1β and monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 in the hypertrophied and failing heart with pressure overload. *Circ Res.* 1997;81:664–671.
20. Capers Qt, Alexander RW, Lou P, De Leon H, Wilcox JN, Ishizaka N, Howard AB, Taylor WR. Monocyte chemoattractant protein-1 expression in aortic tissues of hypertensive rats. *Hypertension.* 1997;30:1397–402.
21. Russell ME, Fujita M, Masek MA, Rowan RA, Billingham ME. Cardiac graft vascular disease: nonselective involvement of large and small vessels. *Transplantation.* 1993;56:1599–1601.
22. Nakamura R, Egashira K, Machida Y, Hayashidani S, Takeya M, Utsumi H, Tsutsui H, Takeshita A. Probucol attenuates left ventricular dysfunction and remodeling in tachycardia-induced heart failure: roles of oxidative stress and inflammation. *Circulation.* 2002;106:362–367.
23. Kolattukudy PE, Quach T, Bergese S, Breckenridge S, Hensley J, Altschuld R, Gordillo G, Klenotic S, Orosz C, Parker-Thornburg J. Myocarditis induced by targeted expression of the MCP-1 gene in murine cardiac muscle. *Am J Pathol.* 1998;152:101–111.
24. Cipollone F, Marini M, Fazia M, Pini B, Iezzi A, Reale M, Paloscia L, Materazzo G, D'Annunzio E, Conti P, Chiarelli F, Cuccurullo F, Mezzetti A. Elevated circulating levels of monocyte chemoattractant protein-1 in patients with restenosis after coronary angioplasty. *Arterioscler Thromb Vasc Biol.* 2001;21:327–334.
25. Moreno PR, Falk E, Palacios IF, Newell JB, Fuster V, Fallon JT. Macrophage infiltration in acute coronary syndromes: implications for plaque rupture. *Circulation.* 1994;90:775–778.
26. Peters W, Charo IF. Involvement of chemokine receptor 2 and its ligand, monocyte chemoattractant protein-1, in the development of atherosclerosis: lessons from knockout mice. *Curr Opin Lipidol.* 2001;12:175–180.
27. Jiang Y, Beller DI, Frendl G, Graves DT. Monocyte chemoattractant protein-1 regulates adhesion molecule expression and cytokine production in human monocytes. *J Immunol.* 1992;148:2423–2428.
28. Lu B, Rutledge BJ, Gu L, Fiorillo J, Lukacs NW, Kunkel SL, North R, Gerard C, Rollins BJ. Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1–deficient mice. *J Exp Med.* 1998;187:601–608.
29. Yamamoto T, Eckes B, Mauch C, Hartmann K, Krieg T. Monocyte chemoattractant protein-1 enhances gene expression and synthesis of matrix metalloproteinase-1 in human fibroblasts by an autocrine IL-1 α-loop. *J Immunol.* 2000;164:6174–6179.
30. Aiello RJ, Bourassa PA, Lindsey S, Weng W, Natoli E, Rollins BJ, Milos PM. Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E–deficient mice. *Arterioscler Thromb Vasc Biol.* 1999; 19:1518–1525.
31. Namiki M, Kawashima S, Yamashita T, Ozaki M, Hirase T, Ishida T, Inoue N, Hirata K, Matsukawa A, Morishita R, Kaneda Y, Yokoyama M. Local overexpression of monocyte chemoattractant protein-1 at vessel wall induces infiltration of macrophages and formation of atherosclerotic lesion: synergism with hypercholesterolemia. *Arterioscler Thromb Vasc Biol.* 2002;22:115–120.
32. Schecter AD, Rollins BJ, Zhang YJ, Charo IF, Fallon JT, Rossikhina M, Giesen PL, Nemerson Y, Taubman MB. Tissue factor is induced by monocyte chemoattractant protein-1 in human aortic smooth muscle and THP-1 cells. *J Biol Chem.* 1997;272:28568–28573.
33. Oshima S, Ogawa H, Hokimoto S, Nakamura S, Noda K, Saito T, Soejima H, Takazoe K, Ishibashi F, Yasue H. Plasma monocyte chemoattractant protein-1 antigen levels and the risk of restenosis after coronary stent implantation. *Jpn Circ J.* 2001;65:261–264.
34. Farb A, Weber DK, Kolodgie FD, Burke AP, Virmani R. Morphological predictors of restenosis after coronary stenting in humans. *Circulation.* 2002;105:2974–2980.
35. Sousa JE, Costa MA, Sousa AG. What is "the matter" with restenosis in 2002? *Circulation.* 2002;105:2932–2933.
36. Farb A, Sangiorgi G, Carter AJ, Walley VM, Edwards WD, Schwartz RS, Virmani R. Pathology of acute and chronic coronary stenting in humans. *Circulation.* 1999;99:44–52.
37. Grewe PH, Deneke T, Machraoui A, Barmeyer J, Muller KM. Acute and chronic tissue response to coronary stent implantation: pathologic findings in human specimen. *J Am Coll Cardiol.* 2000;35:157–163.

38. Feldman LJ, Aguirre L, Ziol M, Bridou JP, Nevo N, Michel JB, Steg PG. Interleukin-10 inhibits intimal hyperplasia after angioplasty or stent implantation in hypercholesterolemic rabbits. *Circulation*. 2000;101: 908–916.
39. Suzuki T, Kopia G, Hayashi S, Bailey LR, Llanos G, Wilensky R, Klugherz BD, Papandreou G, Narayan P, Leon MB, Yeung AC, Tio F, Tsao PS, Falotico R, Carter AJ. Stent-based delivery of sirolimus reduces neointimal formation in a porcine coronary model. *Circulation*. 2001; 104:1188–1193.
40. Sousa JE, Costa MA, Abizaid AC, Rensing BJ, Abizaid AS, Tanajura LF, Kozuma K, Van Langenhove G, Sousa AG, Falotico R, Jaeger J, Popma JJ, Serruys PW. Sustained suppression of neointimal proliferation by sirolimus-eluting stents: 1-year angiographic and intravascular ultrasound follow-up. *Circulation*. 2001;104:2007–2011.
41. Teirstein PS. Living the dream of no restenosis. *Circulation*. 2001;104: 1996–1998.
42. Morice MC, Serruys PW, Sousa JE, Fajadet J, Ban Hayashi E, Perin M, Colombo A, Schuler G, Barragan P, Guagliumi G, Molnar F, Falotico R. A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization. *N Engl J Med*. 2002;346:1773–1780.
43. Horvath C, Welt FG, Nedelman M, Rao P, Rogers C. Targeting CCR2 or CD18 inhibits experimental in-stent restenosis in primates: inhibitory potential depends on type of injury and leukocytes targeted. *Circ Res*. 2002;90:488–494.
44. Zhang Y, Rollins BJ. A dominant negative inhibitor indicates that monocyte chemoattractant protein 1 functions as a dimer. *Mol Cell Biol*. 1995;15:4851–4855.
45. Ni W, Egashira K, Kitamoto S, Kataoka C, Koyanagi M, Inoue S, Imaizumi K, Akiyama C, Nishida Ki K, Takeshita A. New anti-monocyte chemoattractant protein-1 gene therapy attenuates atherosclerosis in apolipoprotein E–knockout mice. *Circulation*. 2001;103:2096–2101.
46. Inoue S, Egashira K, Ni W, Kitamoto S, Usui M, Otani K, Ishibashi M, Hiasa K, Nishida K, Takeshita A. Anti-monocyte chemoattractant protein-1 gene therapy limits progression, and destabilization of established atherosclerosis in apolipoprotein E–knockout mice. *Circulation* 2002;106:2700–2706.
47. Hoffmann R, Mintz GS, Dussaillant GR, Popma JJ, Pichard AD, Satler LF, Kent KM, Griffin J, Leon MB. Patterns and mechanisms of in-stent restenosis: a serial intravascular ultrasound study. *Circulation*. 1996;94: 1247–1254.
48. Usui M, Egashira K, Ohtani K, Kataoka C, Ishibashi M, Hiasa K, Katoh M, Zhao Q, Kitamoto S, Takeshita A. Anti-monocyte chemoattractant protein-1 gene therapy inhibits restenotic changes (neointimal hyperplasia) after balloon injury in rats and monkeys. *Faseb J*. 2002;16: 1838–1840.
49. Mori E, Komori K, Yamaoka T, Tanii M, Kataoka C, Takeshita A, Usui M, Egashira K, Sugimachi K. Essential role of monocyte chemoattractant protein-1 in development of restenotic changes (neointimal hyperplasia and constrictive remodeling) after balloon angioplasty in hypercholesterolemic rabbits. *Circulation*. 2002;105:2905–2910.

Monocyte Chemoattractant Protein-1 Is an Essential Inflammatory Mediator in Angiotensin II-Induced Progression of Established Atherosclerosis in Hypercholesterolemic Mice

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Objective—Chronic inflammatory processes might be involved in the progression and destabilization of atherosclerotic plaques. Therefore, identification of the mechanism underlying arterial inflammatory function might lead to the development of novel therapeutic strategies. Angiotensin II (AngII) is implicated in atherogenesis by activating the vascular inflammation system, mainly through monocyte chemotaxis. Therefore, we hypothesized that AngII increases plaque size and promotes destabilization of established atheromas by activating the monocyte chemoattractant protein-1 (MCP-1) pathway.

Methods and Results—We report here that 4-week infusion of AngII not only increased plaque size but also induced a destabilization phenotype (ie, increased macrophages and lipids and decreased collagen and smooth muscle cells) of pre-existing atherosclerotic lesions of hypercholesterolemic mice. AngII also enhanced the gene expression of inflammatory cytokines (TNFα, IL-6, etc.) and chemokines (MCP-1, CCR2, etc). Blockade of MCP-1, by transfecting the deletion mutant of the human MCP-1 gene into the skeletal muscles, limited AngII-induced progression and destabilization of established atherosclerotic lesions and suppressed the induction of proinflammatory genes.

Conclusions—These data suggest that MCP-1 functions as a central inflammatory mediator in the AngII-induced progression and changes in plaque composition of established atheroma. (***Arterioscler Thromb Vasc Biol.* 2004; 24:534-539.**)

Key Words: atherosclerosis ■ hypercholesterolemia ■ cell adhesion molecules ■ inflammation ■ gene therapy

Atherosclerosis and its complications are the major cause of death in Western countries. Recent evidence suggests that chronic inflammatory processes have an important role in atherosclerotic plaque progression, destabilization, and subsequent rupture/thrombosis, resulting in acute coronary syndrome and stroke.[1] Therefore, identification of the critical inflammatory pathway involved in plaque progression and destabilization of pre-existing established atheromas might aid in the development of novel therapeutic strategies to reduce atherothrombotic complications. Angiotensin II (AngII) is implicated in atherogenesis beyond its hemodynamic effects.[2] Infusion of AngII into hypercholesterolemic mice dramatically accelerates the development and/or progression of atherosclerotic lesions and the effects of AngII occurred independent of changes in arterial pressure or plasma lipid concentration.[3,4] The mechanism of AngII-induced enhancement of atherogenesis is probably multifactorial, and includes hemodynamic effects, endothelial dysfunction and activation,[5] oxidative stress,[6] and inflammation.[7,8] AngII increases monocyte chemotaxis, activates nuclear factor-κB, and augments production of inflammatory cytokines and chemokines by arterial wall cells and monocytes.[9–12] AngII is very important in the pathogenesis of atherothrombotic complications, as evidenced by clinical benefits of angiotensin-converting enzyme inhibition[13] and AngII receptor blockers.[14] There are no reports, however, that address the mechanism of AngII-induced enhancement of atherogenesis and plaque destabilization under in vivo conditions.

Emerging evidence suggests that AngII activates cell inflammatory systems in arterial lesion.[1,2] Inflammatory changes in arterial lesions are characterized by the recruitment and activation of monocytes/macrophages, which are regulated by monocyte chemoattractant protein-1 (MCP-1).[15,16] Matrix metalloproteinases (MMP) and tissue factor,

Received December 2, 2003; accepted January 12, 2004.

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Consulting Editor for this article was Alan M. Fogelman, MD, Professor of Medicine and Executive Chair, Departments of Medicine and Cardiology, UCLA School of Medicine, Los Angeles, CA.

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Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org DOI: 10.1161/01.ATV.0000118275.60121.2b

which are produced mainly from lesional macrophages, are believed to greatly contribute to destabilization of human atherosclerotic lesions.[1] Recently, our group and others have also reported that MCP-1 has a vital role in the initiation and progression of atherosclerotic or arteriosclerotic lesions in experimental animals.[17–24] Essential roles of MCP-1 and its interactions with its receptor (CCR2) in AngII-induced arteriosclerosis are shown in CCR2-deficient mice.[25] Therefore, we hypothesized that AngII increases plaque size and promotes destabilization of established atherosclerotic lesions by activating MCP-1. To block the MCP-1/CCR2 signal pathway, an N-terminal deletion mutant of the MCP-1 gene (7ND), which lacks the N-terminal amino acids 2 to 8, was transfected into the skeletal muscle.[17,18] This mutant MCP-1 binds to its receptor CCR2 and blocks MCP-1–mediated monocyte chemotaxis. In previous studies, we demonstrated that 7ND protein was secreted from the transfected skeletal muscle cells into the circulating blood and subsequently blocked MCP-1–induced chemotaxis in remote organs. Here, we report that AngII infusion into hypercholesterolemic apolipoprotein-E knockout (apoE-KO) mice not only increased plaque size but also promoted atherosclerotic plaque transformation to a more destabilized phenotype, which had more lipid and macrophages and less collagen and fewer smooth muscle cells.[1] In addition to the morphological changes, AngII markedly induced gene expression of several important cytokines and chemokines. Blockade of the MCP-1 pathway limited AngII-induced lesion progression and destabilization and suppressed gene expression of proinflammatory factors.

Methods

Experimental Animals

C57BL/6J apoE-KO and wild-type mice, purchased from Jackson Laboratory (Bar Harbor, Me), were bred and maintained in the Laboratory of Animal Experiments at Kyushu University. The study protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments at 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 at Kyushu University School of Medical Sciences.

Expression Vector

7ND was constructed by recombinant polymerase chain reaction using a wild-type human MCP-1 cDNA as the template and cloned into *Bam*HI (5′) and *Not*I (3′) sites of the pcDNA3 expression vector (Invitrogen).[18]

Treatment

Male apoE-KO mice were fed a normal chow diet (Oriental Yeast) during the experiment. At age 30 weeks, mice were randomly assigned to 1 of 5 groups. The first group (sham group, n=10) received physiological saline via a subcutaneously implanted osmotic minipump (model 2004; Alzet). The second group (AngII+plasmid group, n=10) received AngII dissolved in saline through the minipump for 4 weeks and intramuscular injections of pcDNA3 plasmid DNA at biweekly intervals. The third group (AngII+7ND group, n=10) received AngII dissolved in saline through the minipump for 4 weeks and intramuscular injections of pcDNA3–7ND plasmid DNA (100 μg) into the femoral muscle at biweekly intervals. The fourth group (ARB group, n=10) received AngII dissolved in saline through the minipump for 4 weeks and AngII receptor blocker (ARB) (olmesartan 7.5 μg/g) in chow. This dose of olmesartan has no effect on AngII-induced increase in arterial pressure.[26] The fifth group (sham+7ND group, n=10) received physiological saline and intramuscular injections of pcDNA3–7ND plasmid DNA (100 μg).

In the sham+7ND, AngII+plasmid, and AngII+7ND groups, transgene expression was enhanced by local intramuscular electroporation at the injection site immediately after the injection. Six 100-V, 50-ms electronic pulses were applied to each injection site using an Electroporator CUY21 (BTX).[27] The dose of AngII (0.75 $mg \cdot kg^{-1} \cdot d^{-1}$) provides a serum level of AngII similar to that reported in patients with renovascular hypertension.[28] After 4 weeks of saline or AngII infusion, mice were euthanized after collection of blood from the vena cava.

Tissue Preparation

Tissue preparation was performed essentially as previously described.[18] Briefly, after the mice were euthanized, the heart and total aortic segments were rapidly removed after perfusion with phosphate-buffered saline. The total aortic segments (ascending thoracic aorta, arch, descending thoracic aorta, and abdominal aorta) were rapidly frozen in liquid nitrogen for later extraction of RNA. The heart, including the aortic root, was snap-frozen in OCT compound (Tissue-Tek) for histology and immunohistochemistry.

Histology and Immunohistochemistry

Serial cryostat sections (6 μm) of the aortic root were prepared as described.[18] In brief, atherosclerotic lesions in the aortic root were examined at 5 locations, each separated by 120 μm, and 5 to 6 serial sections were prepared from each location. Some of these sections were conventionally stained with orcein (for elastic fiber staining) and oil red O (for lipid staining). Interstitial collagens were stained by Picrosirius red (Sigma Chemical, St. Louis, Mo) and photographed under polarization microscopy as described previously.[29] The remaining sections were used for immunohistochemical analysis. Air-dried cryostat sections were fixed in acetone and stained with the respective antibody: anti-mouse monocyte/macrophage monoclonal antibodies (MOMA-2; Serotec), anti-alpha-SM actin monoclonal antibodies (alkaline phosphatase conjugated; Sigma Chemical), anti-human MCP-1, CCR2, MMP-13 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse MMP-9 polyclonal antibodies (Santa Cruz Biotechnology), and anti-rabbit tissue factor monoclonal antibody (American Diagnostica), as described previously.[18] Respective nonimmune IgGs (Dako) were used as negative controls. After incubation with the appropriate biotinylated affinity-purified secondary antibodies (Nichirei, Tokyo), the sections were incubated with alkaline phosphatase-labeled streptavidin solution (Nichirei) and visualized using a fast red substrate kit (Nichirei). The sections were then counterstained with Mayer hematoxylin.

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 Institute of Health Image Software. Orcein staining was used to delineate the internal elastic lamina for determination of the intimal area. The lipid composition of the lesion was evaluated by calculating the percent of the oil red O positive area versus the total cross-sectional vessel wall area. Similarly, the percent area of macrophage accumulation (MOMA-2–positive area), alpha-SM actin–positive area, and collagen deposition were estimated. In each case, the average value for 4 or 5 locations for each animal was used for analysis.

RNA Extraction and RNAse Protection Assay

Total RNA was extracted from the aorta using the acid guanidinium thiocyanate-phenol-chloroform method (Isogen, Nippon Gene). RNAse protection assays were performed with 20 μg of total RNA using a RiboQuant kit with a custom template set according to the manufacturer's protocol (PharMingen; San Diego, Calif). After RNase digestion, protected probes were resolved on denaturing polyacrylamide gels and quantified using a BASS-3000 system (Fuji

Film, Tokyo). The value of each hybridized probe was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) included in each template set as an internal control.

Serum Analysis and Systolic Blood Pressure Determination

Serum total and high-density lipoprotein cholesterol and triacylglycerol concentrations were determined using commercially available kits (Wako). Systolic blood pressure was measured weekly using a tail-cuff system with the mice in a conscious state.

Commercially available ELISA kits (Biosource) were used to measure human MCP-1 and mouse MCP-1 according to manufacturer's instructions. Plasma 7ND concentrations were measured by the use of this human ELISA kit.

Statistical Analysis

Data were expressed as mean±SEM. Differences between groups were determined using 2-way analysis of variance and a multiple comparison test; $P<0.05$ was considered to be statistically significant.

Results

7ND Gene Transfer Limits AngII-Induced Progression and Destabilization of Atherosclerotic Lesions

After a 4-week treatment, cross-sections of the aortic sinus region were examined. The sham group had established lesions characteristic of early fibrous plaques containing necrotic cores and a few foam cells covered by a well-formed fibrous cap (Figure 1A), as previously reported.[17,30,31] There was a marked increase in the intimal area in the AngII+plasmid group, compared with that observed in the sham and sham+7ND group (Figures 1A and 2). 7ND gene transfer prevented the AngII-induced increase in the intimal area. Treatment with ARB also prevented the AngII-induced enhancement of the intimal area.

We next examined the effects of 7ND gene transfer on AngII-induced changes in plaque composition using immunohistochemical analysis. In particular, lipid deposition, macrophages, smooth muscle cells, and interstitial collagen content were evaluated (Figures 1A and 2). AngII infusion enhanced lipid staining and macrophage staining and attenuated α-SM–positive and collagen–positive areas (Figures 1A and 2). 7ND gene transfer reduced the AngII-induced increases in lipid deposition and macrophage infiltration, and AngII-induced decrease in α-SM actin and collagen (Figures 1A and 2). Treatment with ARB also prevented the AngII-induced changes in lesion composition.

7ND Gene Transfer Attenuates AngII-Induced Upregulation of Cytokine and Chemokine Expression

To explore the mechanisms by which 7ND gene transfer limited progression and destabilization of pre-existing atheromas, we examined gene expression of a number of chemokines, chemokine receptors, and cytokines (Figure 3). RNase protection assay revealed AngII-induced increases in proinflammatory genes. 7ND gene transfer significantly attenuated the increased gene expression of inflammatory cytokines and chemokines (Figure 3).

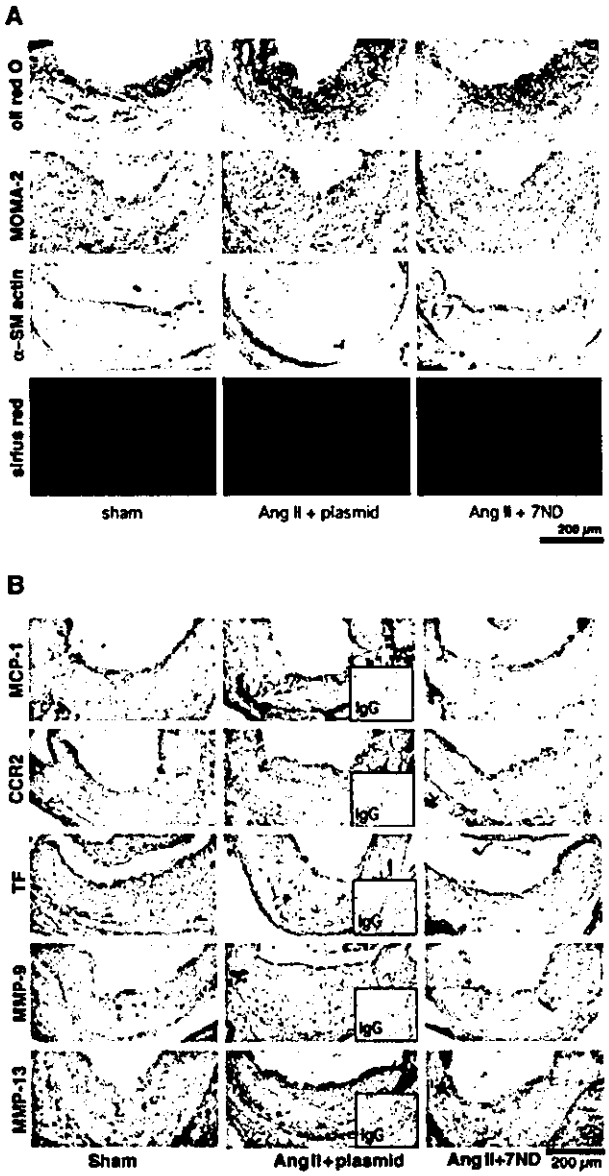

Figure 1. Histopathologic and immunohistochemical pictures of atherosclerotic lesions in the aortic root. A, 7ND gene transfer suppressed AngII-induced destabilization of the atheroma. From the top to the bottom panel, photomicrographs of atherosclerotic lesions stained with oil red O, immunostained with anti-murine macrophage antibody (MOMA-2) or anti-human α-SM actin antibody. Interstitial collagen was visualized using polarization microscopy after staining with Picrosirius red. Bar=200 μm. B, 7ND gene transfer suppressed the AngII-induced increase in immunoreactive MCP-1, CCR2, MMP-9, MMP-13, and tissue factor. Bar=200 μm.

Because 7ND gene transfer reduced MCP-1 gene expression, immunohistochemical staining for MCP-1 and its receptor (CCR2) was performed (Figure 1B). As expected, AngII infusion increased immunoreactive MCP-1 and CCR2 in the intimal lesions. Incomplete colocalization of MCP-1 and CCR2 suggests that CCR2 expression might increase not only in lesional monocytes but also in activated cells such as vascular smooth muscle cells. 7ND gene transfer reduced the

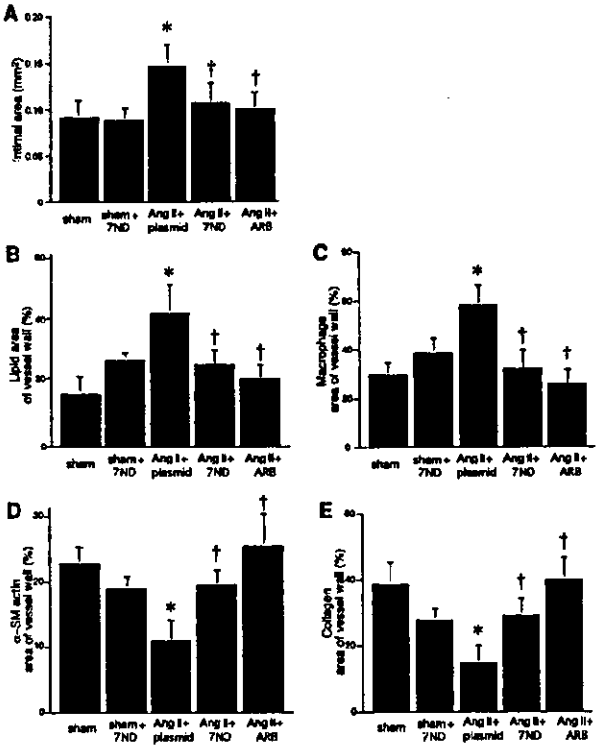

Figure 2. Quantitative comparison of atherosclerotic lesion size and lesion composition in sham-operation, sham+7ND, AngII+plasmid, AngII+7ND, and ARB groups. Data are reported as mean±SEM, n=8 to 10. *P<0.05 versus the sham group. †P<0.05 versus AngII+plasmid group.

AngII-induced increase in immunostaining for MCP-1 and CCR2.

Immunohistochemical staining for MMP and tissue factor were also performed (Figure 1B). AngII infusion increased immunoreactive MMP-9, MMP-13, and tissue factor. 7ND gene transfer reduced the AngII-induced increase in MMP and tissue factor.

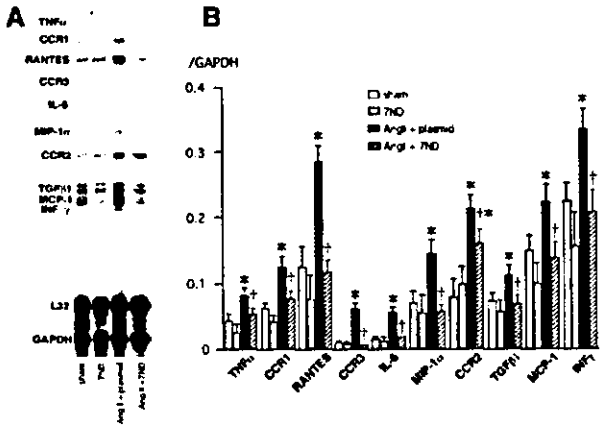

Figure 3. Effects of 7ND gene transfer on the AngII-induced gene expression of several chemokines (RANTES and MCP-1), chemokine receptors (CCR1, CCR2, and CCR3), and cytokine (TNF-α, IL-6, IL-1β, TGF-β1) in the aorta. A, Representative autoradiograph of RNase protection assay. B, Summary of densitometric analysis. Data are expressed as the ratio of each mRNA to the corresponding GAPDH mRNA. *P<0.05 versus the sham group. †P<0.05 versus AngII+plasmid group; n=6 to 7.

Beneficial Effects of 7ND Gene Transfection Had No Relation With Serum Lipid Levels or Systolic Blood Pressure

There were no statistically significant differences in serum total cholesterol levels among the groups (sham group, 480±21; AngII+plasmid group, 503±18; AngII +7ND, 488±19; ARB group, 478±22 mg/dL). 7ND gene transfer did not affect AngII-induced increases in systolic blood pressure (sham group, 102±3; AngII+plasmid group, 153±8; AngII +7ND, 157±6; ARB group, 110±8 mm Hg at 4 weeks of treatment).

7ND Gene Transfer Increases Plasma Concentrations of 7ND

Concentrations of 7ND and MCP-1 in plasma and transfected muscle were measured. In wild-type mice transfected with 7ND without AngII infusion, 7ND was detected in transfected muscle and plasma 3, 7, and 14 days after transfection (Table). Plasma MCP-1 concentrations did not change during the course of experiments.

Discussion

We demonstrated that blockade of MCP-1×7ND gene transfer limited AngII-induced progression of pre-existing advanced atherosclerotic lesions in hypercholesterolemic mice. Because 7ND gene transfer did not affect serum lipid or blood pressure levels, AngII-induced enhancement of atherogenesis must be a direct effect of AngII on cells in the atherosclerotic vascular wall. These data suggest that the essential role of MCP-1 in AngII increases plaque size of the pre-existing atheroma.

A clinically significant finding of the present study is that AngII increased markers of plaque instability. Qualitative changes in plaque are more likely than a decrease in plaque size or the degree of stenosis to contribute greatly to a reduction in cardiovascular events caused by atherothrombotic complications. Lesional macrophages might be a major source of cytokines, MMP, and tissue factor. Increased degradation of interstitial collagen by MMP and decreased cytokine synthesis have been demonstrated in human atherosclerotic lesions prone to rupture.[1] Resultant decreases in interstitial collagen might weaken the plaque's biomechanical strength, which in turn increases the likelihood of plaque rupture.[1] Tissue factor regulates plaque's thrombogenicity.[32] MCP-1 increases tissue factor expression and activity in human vascular smooth muscle cells.[33] We recently reported that 7ND gene transfer inhibited progression and destabilization of advanced atheroma by reducing inflammation in apoE-KO mice that were not received AngII.[24] We demonstrate the preservation of interstitial collagen in the atherosclerotic plaque associated with decreased expression of MMP and tissue factor by 7ND gene transfer. Therefore, MCP-1-induced inflammation appears to be an essential step not only in AngII-induced progression but also in AngII-induced changes in composition of established atheroma.

Plasma Concentrations of MCP-1 and 7ND After 7ND Transfection in Wild-Type Mice

		Days After 7ND Transfection			
	Baseline	3	7	14	28
Plasma MCP-1, pg/mL	88±5	92±6	94±6	90±6	86±5
Plasma 7ND, pg/mL	<20.0 (below detectable limit)	124±14	120±11	66±9	<20.0 (below detectable limit)
Muscular tissue 7ND, pg/mg protein	<20.0 (below detectable limit)	362±28	260±22	105±20	<20.0 (below detectable limit)

Values are mean±SE, n=6 to 8.

AngII causes endothelial dysfunction by decreasing nitric oxide synthase activity, mainly through oxidative stress, which in turn results in inflammation in the arterial wall.[1,2] We previously demonstrated an increase in tissue Ang-II activity after blockade of nitric oxide synthesis.[34] In the present study, 7ND gene transfer suppressed AngII-induced increase in MCP-1, CCR2, and other cytokine expression. Because recent data[1] suggest that lesional inflammatory cells release enzymes such as angiotensin-converting enzyme that generate AngII,[2,35,36] local AngII levels increase as macrophages becomes activated by oxidized LDL.[35] Our data strongly support the notion that an increased concentration and/or activity of AngII creates a positive-feedback mechanism for further increases in AngII generation, atherogenesis, and atherothrombotic events.

In conclusion, the present data demonstrate the essential role of MCP-1–mediated inflammation in AngII- induced progression of established atheroma in hypercholesterolemic mice. Because the activity and formation of AngII is enhanced at the inflamed human atherosclerotic sites that are prone to rupture, it is reasonable to propose that increased local action of AngII contributes greatly to the process of plaque rupture and subsequent cardiovascular ischemic complications by acting as an inflammatory mediator. Thus, AngII might augment inflammatory functions of atherosclerotic lesions in the presence of risk factors such as hypercholesterolemia. The clinical benefit of angiotensin-converting enzyme inhibitors or AngII receptor blockers might be caused by anti-inflammatory activity.

Acknowledgments

This study was supported by grants-in-aid for Scientific Research (14657172, 14207036) from the Ministry of Education, Science, and Culture, Tokyo, Japan, by Health Science Research Grants (Comprehensive Research on Aging and Health, and Research on Translational Research) from the Ministry of Health Labor and Welfare, Tokyo, Japan, and by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research, Tokyo, Japan.

References

1. Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. *Circulation.* 2001;104:365–372.
2. Dzau VJ. Theodore Cooper Lecture: Tissue angiotensin and pathobiology of vascular disease: a unifying hypothesis. *Hypertension.* 2001;37: 1047–1052.
3. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. *J Clin Invest.* 2000;105:1605–1612.
4. Weiss D, Kools JJ, Taylor WR. Angiotensin II-induced hypertension accelerates the development of atherosclerosis in apoE-deficient mice. *Circulation.* 2001;103:448–454.
5. Laursen JB, Rajagopalan S, Galis Z, Tarpey M, Freeman BA, Harrison DG. Role of superoxide in angiotensin II-induced but not catecholamine-induced hypertension. *Circulation.* 1997;95:588–593.
6. Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK, Harrison DG. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest.* 1996;97:1916–1923.
7. Kim JA, Berliner JA, Nadler JL. Angiotensin II increases monocyte binding to endothelial cells. *Biochem Biophys Res Commun.* 1996;226: 862–868.
8. Chen XL, Tummala PE, Olbrych MT, Alexander RW, Medford RM. Angiotensin II induces monocyte chemoattractant protein-1 gene expression in rat vascular smooth muscle cells. *Circ Res.* 1998;83: 952–959.
9. Ruiz-Ortega M, Bustos C, Hernandez-Presa MA, Lorenzo O, Plaza JJ, Egido J. Angiotensin II participates in mononuclear cell recruitment in experimental immune complex nephritis through nuclear factor-kappa B activation and monocyte chemoattractant protein-1 synthesis. *J Immunol.* 1998;161:430–439.
10. Hahn AW, Jonas U, Buhler FR, Resink TJ. Activation of human peripheral monocytes by angiotensin II. *FEBS Lett.* 1994;347:178–180.
11. Hernandez-Presa M, Bustos C, Ortego M, Tunon J, Renedo G, Ruiz-Ortega M, Egido J. Angiotensin-converting enzyme inhibition prevents arterial nuclear factor-kappa B activation, monocyte chemoattractant protein-1 expression, and macrophage infiltration in a rabbit model of early accelerated atherosclerosis. *Circulation.* 1997;95:1532–1541.
12. Kim S, Izumi Y, Yano M, Hamaguchi A, Miura K, Yamanaka S, Miyazaki H, Iwao H. Angiotensin blockade inhibits activation of mitogen-activated protein kinases in rat balloon-injured artery. *Circulation.* 1998;97:1731–1737.
13. Yusuf S, Sleight P, Pogue J, Bosch J, Davies R, Dagenais G. Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med.* 2000;342:145–153.
14. Dahlof B, Devereux RB, Kjeldsen SE, Julius S, Beevers G, Faire U, Fyhrquist F, Ibsen H, Kristiansson K, Lederballe-Pedersen O, Lindholm LH, Nieminen MS, Omvik P, Oparil S, Wedel H. Cardiovascular morbidity and mortality in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. *Lancet.* 2002;359:995–1003.
15. Gerard C, Rollins BJ. Chemokines and disease. *Nat Immunol.* 2001;2: 108–115.
16. Mukaida N, Harada A, Matsushima K. Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions. *Cytokine Growth Factor Rev.* 1998;9:9–23.
17. Egashira K, Koyanagi M, Kitamoto S, Ni W, Kataoka C, Morishita R, Kaneda Y, Akiyama C, Nishida KI, Sueishi K, Takeshita A. Anti-monocyte chemoattractant protein-1 gene therapy inhibits vascular remodeling in rats: blockade of MCP-1 activity after intramuscular transfer of a mutant gene inhibits vascular remodeling induced by chronic blockade of NO synthesis. *FASEB J.* 2000;14:1974–1978.
18. Ni W, Egashira K, Kitamoto S, Kataoka C, Koyanagi M, Inoue S, Imaizumi K, Akiyama C, Nishida Ki K, Takeshita A. New Anti-Monocyte Chemoattractant Protein-1 Gene Therapy Attenuates Atherosclerosis in Apolipoprotein E-Knockout Mice. *Circulation.* 2001;103: 2096–2101.
19. Boring L, Gosling J, Cleary M, Charo IF. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature.* 1998;394:894–897.

20. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low-density lipoprotein receptor-deficient mice. *Mol Cell.* 1998;2: 275–281.
21. Mori E, Komori K, Yamaoka T, Tanii M, Kataoka C, Takeshita A, Usui M, Egashira K, Sugimachi K. Essential role of monocyte chemoattractant protein-1 in development of restenotic changes (neointimal hyperplasia and constrictive remodeling) after balloon angioplasty in hypercholesterolemic rabbits. *Circulation.* 2002;105:2905–2910.
22. Egashira K, Zhao Q, Kataoka C, Ohtani K, Usui M, Charo IF, Nishida K, Inoue S, Katoh M, Ichiki T, Takeshita A. Importance of monocyte chemoattractant protein-1 pathway in neointimal hyperplasia after periarterial injury in mice and monkeys. *Circ Res.* 2002;90:1167–1172.
23. Usui M, Egashira K, Ohtani K, Kataoka C, Ishibashi M, Hiasa K, Katoh M, Zhao Q, Kitamoto S, Takeshita A. Anti-monocyte chemoattractant protein-1 gene therapy inhibits restenotic changes (neointimal hyperplasia) after balloon injury in rats and monkeys. *FASEB J.* 2002;16: 1838–1840.
24. Inoue S, Egashira K, Ni W, Kitamoto S, Usui M, Otani K, Ishibashi M, Hiasa K, Nishida K, Takeshita A. Anti-monocyte chemoattractant protein-1 gene therapy limits progression and destabilization of established atherosclerosis in apolipoprotein E-knockout mice. *Circulation.* 2002;106:2700–2706.
25. Bush E, Maeda N, Kuziel WA, Dawson TC, Wilcox JN, DeLeon H, Taylor WR. CC chemokine receptor 2 is required for macrophage infiltration and vascular hypertrophy in angiotensin II-induced hypertension. *Hypertension.* 2000;36:360–363.
26. Usui M, Egashira K, Tomita H, Koyanagi M, Katoh M, Shimokawa H, Takeya M, Yoshimura T, Matsushima K, Takeshita A. Important role of local angiotensin II activity mediated via type 1 receptor in the pathogenesis of cardiovascular inflammatory changes induced by chronic blockade of nitric oxide synthesis in rats. *Circulation.* 2000;101:305–310.
27. Zhao Q, Egashira K, Inoue S, Usui M, Kitamoto S, Ni W, Ishibashi M, Hiasa Ki K, Ichiki T, Shibuya M, Takeshita A. Vascular endothelial growth factor is necessary in the development of arteriosclerosis by recruiting/activating monocytes in a rat model of long-term inhibition of nitric oxide synthesis. *Circulation.* 2002;105:1110–1115.
28. Capers Qt, Alexander RW, Lou P, De Leon H, Wilcox JN, Ishizaka N, Howard AB, Taylor WR. Monocyte chemoattractant protein-1 expression in aortic tissues of hypertensive rats. *Hypertension.* 1997;30:1397–1402.
29. Junqueira LC, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem J.* 1979;11:447–455.
30. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis.* 1987;68: 231–240.
31. Nakashima Y, Plump AS, Raines EW, Breslow JL, Ross R. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler Thromb.* 1994;14:133–140.
32. Aikawa M, Voglic SJ, Sugiyama S, Rabkin E, Taubman MB, Fallon JT, Libby P. Dietary lipid lowering reduces tissue factor expression in rabbit atheroma. *Circulation.* 1999;100:1215–1222.
33. Schecter AD, Rollins BJ, Zhang YJ, Charo IF, Fallon JT, Rossikhina M, Giesen PL, Nemerson Y, Taubman MB. Tissue factor is induced by monocyte chemoattractant protein-1 in human aortic smooth muscle and THP-1 cells. *J Biol Chem.* 1997;272:28568–28573.
34. Takemoto M, Egashira K, Usui M, Numaguchi K, Tomita H, Tsutsui H, Shimokawa H, Sueishi K, Takeshita A. Important role of tissue angiotensin-converting enzyme activity in the pathogenesis of coronary vascular and myocardial structural changes induced by long-term blockade of nitric oxide synthesis in rats. *J Clin Invest.* 1997;99:278–287.
35. Diet F, Pratt RE, Berry GJ, Momose N, Gibbons GH, Dzau VJ. Increased accumulation of tissue ACE in human atherosclerotic coronary artery disease. *Circulation.* 1996;94:2756–2767.
36. Kitazono T, Padgett RC, Armstrong ML, Tompkins PK, Heistad DD. Evidence that angiotensin II is present in human monocytes. *Circulation.* 1995;91:1129–1134.

Gene Therapy (2004) 11, 126–132
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RESEARCH ARTICLE

Inhibition of neointimal hyperplasia after balloon injury by cis-element 'decoy' of early growth response gene-1 in hypercholesterolemic rabbits

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Early growth response factor-1 (Egr-1) is a transcription factor that is rapidly activated after vascular injury and thus might contribute to vascular proliferation and inflammation. We hypothesized that Egr-1 might therefore be a therapeutic target against restenosis. Hypercholesterolemic rabbits were intraluminally administered synthetic DNA as a 'decoy' against Egr-1 immediately after carotid artery balloon injury. Efficient transfection was confirmed by the delivery of a fluorescence-labeled decoy. Gel mobility-shift assay showed increased Egr-1 activity after balloon injury and its prevention by Egr-1 decoy transfection in vivo. Egr-1 decoy transfection attenuated early inflammation and proliferation and later neointimal hyperplasia. In addition, Egr-1 decoy transfection reduced gene expression and protein production of Egr-1-dependent genes such as platelet-derived growth factor-B, transforming growth factor-β1, and monocyte chemoattractant protein-1. The Egr-1 pathway has an essential role in the pathogenesis of neointimal hyperplasia after balloon injury in hypercholesterolemic rabbits. This decoy strategy is a potential practical form of therapy for human restenosis.
Gene Therapy (2004) **11**, 126–132. doi:10.1038/sj.gt.3302153

Keywords: *neointimal hyperplasia; inflammation; growth factor*

Introduction

Atherosclerosis and restenosis following percutaneous coronary intervention are now recognized to result from proliferative-inflammatory responses to injury in the arterial wall.[1–3] Both balloon angioplasty and stent implantation are useful treatments for dilating atherothrombotic stenosis.[4,5] The overall benefits of this intervention, however, are hampered by local arterial renarrowing (restenosis), which occurs in 30–50% of cases within 6 months.[4,5] No pharmacologic strategies have demonstrated definite benefits to reduce the rate of restenosis. Although silorimus-eluting stents might suppress restenosis,[6,7] its benefits have not yet been completely established.

Experimentally, injury to the arterial wall by balloon or stents rapidly activates intracellular signals, such as mitogen-activated protein kinases, and various transcription factors, such as early growth response factor-1 (Egr-1).[8,9] Egr-1 is an immediate-early gene product and a zinc-finger transcription factor that regulates a number of pathophysiologically relevant genes.[8,10] Egr-1 is induced and activated after injury in vascular smooth muscle cells, endothelial cells, and leukocytes. A number of Egr-1-dependent genes are upregulated in human and experimental atherosclerotic lesions.[11,12] Among a variety of transcription factors that are activated after arterial injury, Egr-1 might be a practical and ideal target for therapeutic intervention to prevent restenosis, because (1) Egr-1 is expressed at low or undetectable levels in the normal artery, and is rapidly upregulated by mechanical injury; (2) Egr-1 is activated by multiple stimuli that might contribute to the development of restenotic and atherosclerotic lesions; and (3) Egr-1 controls the expression of various genes whose products are related to the development of vascular lesion – factors that induce proliferation/migration (platelet-derived growth factor (PDGF), transforming growth factor-β1 (TGF-β1), matrix metalloproteinases), inflammation (monocyte chemoattractant protein (MCP)-1), and thrombosis (tissue factor).[8,10] Khachigian *et al*[13] recently generated a DNAzyme that specifically cleaves Egr-1 mRNA and reported that adventitial delivery of the DNA enzyme with pluronic gel attenuated neointimal hyperplasia in balloon-injured rat carotid artery model, and that intraluminal delivery of the DNAzyme with the transport catheter inhibited in-stent neointimal hyperplasia of coronary arteries in pig model.[14] They did not, however, address the mechanism by which blockade of Egr-1 attenuated neointimal hyperplasia or causative factor(s) that might contribute to the inhibitory effects on neointimal hyperplasia under *in vivo* conditions. The delivery of anti-Egr-1 agents intraluminally might provide a reasonable starting point for clinical application of anti-Egr-1

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Received 09 April 2003; accepted 11 August 2003