Inhibition of Progression and Stabilization of Plaques by Postnatal Interferon-γ Function Blocking in ApoE-Knockout Mice

Mitsuhisa Koga, Hisashi Kai, Hideo Yasukawa, Tomoka Yamamoto, Yumiko Kawai, Seiya Kato, Ken Kusaba, Mamiko Kai, Kensuke Egashira, Yasufumi Kataoka, Tsutomu Imaizumi

Abstract—A role of interferon-γ is suggested in early development of atherosclerosis. However, the role of interferon-γ in progression and destabilization of advanced atherosclerotic plaques remains unknown. Thus, the aim of this study was to determine whether postnatal inhibition of interferon-γ signaling could inhibit progression of atherosclerotic plaques and stabilize the lipid- and macrophage-rich advanced plaques. Atherosclerotic plaques were induced in ApoE-knockout (KO) mice by feeding high-fat diet from 8 weeks old (w). Interferon-γ function was postnatally inhibited by repeated gene transfers of a soluble mutant of interferon-γ receptors (sIFNγR), an interferon-γ inhibitory protein, into the thigh muscle every 2 weeks. When sIFNγR treatment was started at 12 w (atherosclerotic stage), sIFNγR not only prevented plaque progression but also stabilized advanced plaques at 16 w: sIFNγR decreased accumulations of the lipid and macrophages and increased fibrotic area with more smooth muscle cells. Moreover, sIFNγR downregulated expressions of proinflammatory cytokines, chemokines, adhesion molecules, and matrix metalloproteinases but upregulated procollagen type I. sIFNγR did not affect serum cholesterol levels. In conclusion, postnatal blocking of interferon-γ function by sIFNγR treatment would be a new strategy to inhibit plaque progression and to stabilize advanced plaques through the antiinflammatory effects. (Circ Res. 2007;101:348-356.)

Key Words: atherosclerosis • inflammation • cytokine • gene therapy

There is increasing evidence that atherosclerosis is a kind of chronic inflammation characterized by continued recruitment and activation of monocytes and T-lymphocytes. Interferon-γ is highlighted as a key mediator of atherosclerosis. Interferon-γ is expressed at high levels in T-lymphocytes in atherosclerotic lesions. A variety of proatherogenic properties of interferon-γ have been shown in cultured cells, such as endothelial cells (ECs), vascular smooth muscle cells (SMCs), monocyte/macrophages, and T-lymphocytes. These include: differentiation of transmigrated monocytes to macrophages; activation of macrophages and T-lymphocytes; induction of proinflammatory cytokines (ie, interleukin [IL]-1β and IL-6); recruitment of monocyte/macrophages and T-lymphocytes through upregulation of chemokines (ie, monocyte chemoattractant protein [MCP]-1 and macrophage inflammatory protein [MIP]-1α) and adhesion molecules (vascular cell adhesion molecule [VCAM]-1 and intercellular adhesion molecule-1); and foam cell formation through upregulation of scavenger receptors. However, interferon-γ also has atheroprotective actions, such as induction of antiinflammatory cytokines, in a range of cell types. Thus, the complexity of the in vitro effects makes it difficult to elucidate the in vivo effects of interferon-γ on the development and progression of atherosclerosis.

Whitman et al demonstrated that chronic administration of recombinant interferon-γ enhanced atherosclerosis in ApoE-KO mice. In atherosclerotic mice, such as ApoE-KO mice and LDL receptor-KO mice, genetic ablation of interferon-γ or the interferon-γ receptors reduced atherosclerosis. These studies have suggested that interferon-γ promotes early development of atherosclerosis. However, when the therapeutic potential was considered, prophylactic inhibition of interferon-γ would not be feasible before the development of atherosclerosis. Accordingly, it must be investigated about the roles of interferon-γ on progression and destabilization of established advanced plaques. But, so far, no prior studies have addressed the effects of postnatal inhibition of interferon-γ signaling on the advanced plaques.

Thus, the aim of this study was to determine whether postnatal inhibition of interferon-γ signaling could inhibit progression of atherosclerotic plaques and stabilize the lipid- and macrophage-rich advanced plaques. It has been shown that overexpression of a soluble mutant of interferon-γ...
receptor (sIFNγR), an interferon-γ inhibitory protein, is a safe and effective tool to investigate the roles of interferon-γ in the pathogenesis of experimental models of inflammatory diseases.\(^{16,17}\) Therefore, in the present study, naked plasmid DNA encoding sIFNγR was injected repeatedly into the thigh muscle to produce sIFNγR, as a means to inhibit interferon-γ signaling in the advanced atherosclerotic stage of high-fat diet–fed ApoE-KO mice.

### Materials and Methods

The study protocol was reviewed and approved by the Animal Care and Treatment Committee of Kurume University. C57BL/6J ApoE-KO mice and C57BL/6J mice (wild-type) were purchased from Jackson Laboratory (Bar Harbor, Me) and housed under standard conditions of humidity, room temperature, and dark-light cycles in plenty of chow and water.

**sIFNγR Plasmid**

The plasmid DNA encoding sIFNγR, the extracellular portion of the mouse interferon-γ receptor α-chain conjugated with mouse IgG1 Fc-segment, was a gift of Dr Prud’homme (McGill University, Montreal, Canada). The construction of the sIFNγR plasmid and the anti–interferon-γ effects ex vivo were described elsewhere.\(^{16,17}\) The plasmid was amplified, purified with an endotoxin-free purification kit (Quiagen) and stored at −20°C until use.

**sIFNγR Gene Transfer**

sIFNγR gene transfer was performed by using the naked DNA method, as described previously.\(^{16,18}\) Briefly, under ether anesthesia, 12.5 μg/g body weight of 0.5% bupivacaine (AstraZeneca) was injected into the thigh muscle to improve the efficiency of gene transfer.\(^{20}\) Twenty days after bupivacaine pretreatment, denoted doses of sIFNγR plasmid or the blank plasmid (mock) solved in 40 μL phosphate-buffered saline (PBS) were injected at the same site of the muscle as bupivacaine had been injected. To evaluate the efficacy of gene transfer, serum sIFNγR protein levels were examined after a single sIFNγR gene transfer in wild-type mice. sIFNγR plasmid (200 μg) was injected into the thigh muscle 3 days after bupivacaine treatment. At denoted days, blood was drawn from the right atrium and kept frozen at −80°C (n=10/group). Aliquots of the serum (300 μL) were subjected to immunoprecipitation followed by immunoblotting using an antibody against the extracellular portion of the mouse interferon-γ receptor α-chain (Santa Cruz Biotech).\(^{21}\) The signals were analyzed with the chemiluminescence detection system (Pierce Biotechnology).\(^{18}\)

**Inhibitory Effects of sIFNγR Gene Transfer on Interferon-γ-Induced Macrophage Mobilization**

In vivo anti–interferon-γ effect of sIFNγR gene transfer was determined, as follows. In wild-type mice, 10, 100, or 200 μg of sIFNγR plasmid or the mock (200 μg) was injected into the bupivacaine-pretreated thigh muscle (n=10/group). Three days after sIFNγR gene transfer, recombinant mouse Interferon-γ (8000 IU) dissolved in 50 μL of 25% pluronic F127 gel (Sigma Chemical Co) was injected intradermally onto the back. Twenty-four hours later, the injected dermis (5 mm×5 mm) was excised en-bloc, processed for the paraffinized sections, and subjected to immunohistostaining using monococyte/macrophage–specific monoclonal antibody (MOMA-2; Serotec) and a commercially available detection system (Vecstain Elite ABC kit; Vector Laboratories).\(^{22,23}\) The number of infiltrated MOMA-2–stained macrophages was counted in the whole sections of the skin (5 sections per animal). The averages of macrophage counts were expressed as the cell count/mm\(^2\).

**Experiment 1**

From 8 weeks old (w), ApoE-KO mice were fed high-fat diet containing 20% fat and 0.15% cholesterol (Oriental Yeast). Mice were randomized to sIFNγR-treated mice and mock-treated mice (n=40/group). sIFNγR or mock plasmid (200 μg) was injected into the bupivacaine-treated thigh muscle twice at 12 and 14 w. The right and left legs were used for the gene transfers, alternately. After 4-week treatment (16 w), mice were euthanized with an overdose of pentobarbital after collection of blood from the right atrium. Immediately after the mice were perfused with ice-cold PBS, the heart and aorta were excised and subjected to morphometric and histological analyses. Serum total cholesterol and high-density lipoprotein (HDL)-cholesterol were measured by a commercially available laboratory (SRL, Fukuo).

**En Face Plaque Area**

Immediately after mice were killed, the aorta (n=10/group) was excised and fixed in 10% buffered formalin for quantification of en face plaque area.\(^{24}\) Briefly, after the adventitial tissue was carefully removed, the aorta was opened longitudinally, stained with oil red O (Sigma), and pinned on a black wax surface. En face images were obtained by a stereomicroscope (SZX12, Olympus, Tokyo) equipped with a digital camera (Dxm1200, Nicon, Tokyo) and analyzed using Adobe Photoshop version 7.0 and Scion Image software. The percentage of the luminal surface area stained by oil red O was determined.\(^{24}\)

**Histology and Immunohistostaining**

After the mouse was euthanized and perfused with ice-cold PBS, the heart and the ascending aorta (n=10/group) were snap-frozen in OCT compound (Sakura FineTech) for histological and immunohistological analyses. Serial cryostat sections (6 μm in the thickness) of the aortic root were prepared as described previously.\(^{25}\) Briefly, atherosclerotic plaques were investigated at 5 independent section sets, each separated by 120 μm. Mallory–Azan staining and oil red O staining were performed to determine fibrotic tissue and lipid-rich core, respectively. The remaining sections were subjected to immunohistostaining using antibodies against monocyte/macrophage (MOMA-2), α-smooth muscle actin (α-SMA; DAKO Cytomation), phosphorylated form of signal transducer and activator of transcription-1 (STAT1; Assay BioTechnology), CD40 (BD Pharmingen), and CD40 ligand (CD40L; Santa Cruz). For quantitative estimation of the plaque contents, we analyzed oil red O–, MOMA-2–, Azan–, and α-SMA–stained areas by using Adobe Photoshop version 7.0 and Scion Image software. In each animal, the average for 5 independent sections was used for analysis. The histological plaque stability score was calculated as follows: the plaque stability score=(α-SMA–positive SMC area+Azan-stained collagen area)/(MOMA-2–stained macrophage area+oil red O-stained lipid area).\(^{25}\)

**Immunoblotting Analysis**

The aorta was immediately snap-frozen in dry-ice/acetone (n=10/group). After tissue homogenization, protein extract was separated by 10% SDS-PAGE and subjected to immunoblotting using antibodies against total STAT1 and phosphorylated STAT1 (Assay BioTechnology).\(^{22,26}\) The signals were detected and analyzed using the chemiluminescence detection system.\(^{18}\) Signal intensity of phosphorylated STAT1 was normalized by total STAT1 level in each sample.\(^{27}\)

**Real-Time RT-PCR Analysis**

The aorta was rapidly snap-frozen in dry-ice/acetone and stored at −80°C until use (n=10/group). Total RNA was extracted and subjected to real-time RT-PCR analysis for quantitative evaluation of the mRNA expression changes in IL-1β, IL-6, VCAM-1, MCP-1, MIP-1α, procollagen type 1, matrix metalloproteinase (MMP)-9, and MMP-13.\(^{28}\) Expression level of the target gene was normalized by GAPDH level in each sample. See the online supplement (available at http://circres.ahajournals.org) for details on procedures.

**Experiment 2**

ApoE-KO mice were fed high-fat diet from 8 w and randomized to the following 3 groups (n=10/group) at 12 w. The 8-week sIFNγR treatment group received sIFNγR gene transfer 4 times at 12, 14, 16, and 18 w; the 8-week mock treatment group received mock gene
was used throughout the following experiments.

Wild-Type Mice

Serum sIFN\(\gamma\)R was elevated for at least 3 to 7 days after gene transfer and being elevated for at least 2 weeks (Figure 1A).

During 4-week sIFN\(\gamma\)R treatment, all mice of sIFN\(\gamma\)R- and mock-treated groups were apparently healthy, and no mice were lost before the day of sacrifice. sIFN\(\gamma\)R treatment had no effects on body weight and blood pressure (Table). The 4-week sIFN\(\gamma\)R treatment did not affect serum total and HDL cholesterol levels. Peripheral blood counts of leukocytes and monocytes did not differ between sIFN\(\gamma\)R- and mock-treated mice (data not shown).

Effects on Plaque Area and Histological Aspects

Effects of postnatal inhibition of interferon-\(\gamma\) function on existing plaques were investigated at the atherosclerotic stage of ApoE-KO mice by starting sIFN\(\gamma\)R gene transfer at 12 w. Mock-treated ApoE-KO mice showed marked progression of atheromatous plaques at 16 w (Figure 2). sIFN\(\gamma\)R treatment for 4 weeks attenuated progression of the aortic plaques.

Postnatal interferon-\(\gamma\) function blocking not only reversed the plaque progression but also stabilized the advanced plaques. In mock-treated mice, the plaques consisted of massive oil red O–stained lipid core and bulky MOMA-2–stained macrophage accumulation, but had very thin Azan-stained fibrous cap with little \(\alpha\)-SMA–stained SMCs (Figure 2A).

Effects of Repeated sIFN\(\gamma\)R Gene Transfers on General Conditions in ApoE-KO Mice

In wild-type mice, temporal changes in serum sIFN\(\gamma\)R protein levels were evaluated after a single sIFN\(\gamma\)R gene transfer (200 \(\mu\)g) into the thigh muscle of wild-type mice. The macrophage infiltration was dose-dependently inhibited by sIFN\(\gamma\)R gene transfer into the thigh muscle dose-dependently inhibited sIFN\(\gamma\)R protein levels after a single sIFN\(\gamma\)R gene transfer (Figure 1B).

The macrophage infiltration was dose-dependently inhibited by sIFN\(\gamma\)R gene transfer under the same schedule; and the treatment cessation group received sIFN\(\gamma\)R gene transfer twice (at 12 and 14 w) and then mock gene transfer twice (at 16 and 18 w). At 20 w, mice were euthanized for the evaluation of the en face plaque area.

Statistical Analysis

Each quantitative analysis was performed by a single observer blinded to the experimental protocol. Data are expressed as mean\(\pm\)SD. Mann–Whitney U test was performed for the comparisons between 2 groups. Kruskal-Wallis test and Scheffé post-hoc analysis were used for the comparisons among 3 groups. A value \(P<0.05\) was considered to be statistically significant.

Results

Efficacy of a Single sIFN\(\gamma\)R Gene Transfer in Wild-Type Mice

In wild-type mice, temporal changes in serum sIFN\(\gamma\)R protein levels were evaluated after a single gene transfer of sIFN\(\gamma\)R plasmid (200 \(\mu\)g). Immunoblotting analysis showed that sIFN\(\gamma\)R protein was secreted into the serum, peaking at 3 to 7 days after gene transfer and being elevated for at least 2 weeks (Figure 1A).

Next, in vivo anti–interferon-\(\gamma\) effect of sIFN\(\gamma\)R gene transfer was determined in wild-type mice. In mock-treated mice, 24 hours after recombinant interferon-\(\gamma\) was injected intradermally onto the back, marked infiltration of MOMA-2–stained macrophages was noted at the interferon-\(\gamma\) injection site (Figure 1B). The macrophage infiltration was dose-dependently inhibited by sIFN\(\gamma\)R treatment, and the effect of 200 \(\mu\)g of sIFN\(\gamma\)R plasmid was maximal. Therefore, the dose was used throughout the following experiments.

Experiment 1

Effects of Repeated sIFN\(\gamma\)R Gene Transfers on General Conditions in ApoE-KO Mice

During 4-week sIFN\(\gamma\)R treatment, all mice of sIFN\(\gamma\)R- and mock-treated groups were apparently healthy, and no mice were lost before the day of sacrifice. sIFN\(\gamma\)R treatment had no effects on body weight and blood pressure (Table). The 4-week sIFN\(\gamma\)R treatment did not affect serum total and HDL cholesterol levels. Peripheral blood counts of leukocytes and monocytes did not differ between sIFN\(\gamma\)R- and mock-treated mice (data not shown).

Effects on Plaque Area and Histological Aspects

Effects of postnatal inhibition of interferon-\(\gamma\) function on existing plaques were investigated at the atherosclerotic stage of ApoE-KO mice by starting sIFN\(\gamma\)R gene transfer at 12 w. Mock-treated ApoE-KO mice showed marked progression of atheromatous plaques at 16 w (Figure 2). sIFN\(\gamma\)R treatment for 4 weeks attenuated progression of the aortic plaques.

Postnatal interferon-\(\gamma\) function blocking not only reversed the plaque progression but also stabilized the advanced plaques. In mock-treated mice, the plaques consisted of massive oil red O–stained lipid core and bulky MOMA-2–stained macrophage accumulation, but had very thin Azan-stained fibrous cap with little \(\alpha\)-SMA–stained SMCs (Figure 2A).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before Treatment</th>
<th>4-Week Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>sIFN(\gamma)R</td>
<td>Mock</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>20.6±0.7</td>
<td>21.3±0.6</td>
</tr>
<tr>
<td>Systolic pressure, mm Hg</td>
<td>131±2</td>
<td>132±3</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>...</td>
<td>2357±297</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>...</td>
<td>43±9</td>
</tr>
</tbody>
</table>

There were no significant differences in each parameter between sIFN\(\gamma\)R- and mock-treated ApoE-KO mice. Data are means\(\pm\)SD (n=11).

Effects of Repeated sIFN\(\gamma\)R Gene Transfers on General Conditions in ApoE-KO Mice

During 4-week sIFN\(\gamma\)R treatment, all mice of sIFN\(\gamma\)R- and mock-treated groups were apparently healthy, and no mice were lost before the day of sacrifice. sIFN\(\gamma\)R treatment had no effects on body weight and blood pressure (Table). The 4-week sIFN\(\gamma\)R treatment did not affect serum total and HDL cholesterol levels. Peripheral blood counts of leukocytes and monocytes did not differ between sIFN\(\gamma\)R- and mock-treated mice (data not shown).

Effects on Plaque Area and Histological Aspects

Effects of postnatal inhibition of interferon-\(\gamma\) function on existing plaques were investigated at the atherosclerotic stage of ApoE-KO mice by starting sIFN\(\gamma\)R gene transfer at 12 w. Mock-treated ApoE-KO mice showed marked progression of atheromatous plaques at 16 w (Figure 2). sIFN\(\gamma\)R treatment for 4 weeks attenuated progression of the aortic plaques.

Postnatal interferon-\(\gamma\) function blocking not only reversed the plaque progression but also stabilized the advanced plaques. In mock-treated mice, the plaques consisted of massive oil red O–stained lipid core and bulky MOMA-2–stained macrophage accumulation, but had very thin Azan-stained fibrous cap with little \(\alpha\)-SMA–stained SMCs (Figure 2A).
These findings suggested low plaque stability from the histological aspect. sIFNγ/H9253R treatment decreased the lipid and macrophage areas but increased fibrotic area and, to lesser extent, SMC area (Figure 3A and 3B). Accordingly, the histological plaque stability score was improved by sIFNγ/H9253R treatment (Figure 3C).

**Effects on STAT1 Activation and CD40/CD40L Expression**

We investigated the effects of sIFNγR treatment on phosphorylation levels of STAT1, the major signaling molecule activated by interferon-γ. In wild-type mice, STAT1 expression was scarcely found in the aorta (Figure 4A). In mock-treated ApoE-KO mice, STAT1 was not only upregulated but also phosphorylated. Immunohistostaining revealed that phosphorylated STAT1 was observed mainly in the atherosclerotic plaques (Figure 4B). sIFNγR treatment remarkably reduced the STAT1 phosphorylation in the plaques.

Also, we examined the effects on CD40/CD40L signaling previously reported as a mediator of lesion progression. In mock-treated ApoE-KO mice, immunoreactive CD40 and CD40L were evident in the atherosclerotic plaques (Figure 4B). sIFNγR treatment remarkably attenuated the CD40 and CD40L inductions.

**Effects on the Gene Expression Changes**

To explore the mechanisms whereby sIFNγR treatment inhibits plaque progression and stabilizes the plaques, gene expression changes of the proinflammatory molecules and the molecules related to the extracellular matrix (ECM) metabolism were investigated in the aorta (Figure 5). In mock-treated ApoE-KO mice, IL-1β, IL-6, VCAM-1, MCP-1, and MIP-1α were markedly upregulated at 16 w. Postnatal inhibition of interferon-γ function substantially reduced the inductions of proinflammatory cytokines, adhesion molecule, and chemokines.

MMP-9 (gelatinase) and MMP-13 (interstitial collagenase), the enzymes that degrade the ECM of plaques, were upregulated in mock-treated ApoE-KO mice. sIFNγR treatment repressed the MMP expressions but upregulated procollagen type I, the major ECM component of plaques.

**Experiment 2**

Effects of the 8-week sIFNγR treatment were investigated for the long-term effectiveness under prolonged hypercholesterolemia. In the treatment cessation group, mice received 4-week sIFNγR treatment followed by 4-week mock treatment. There were no significant differences in body weight,
blood pressure, and serum lipid levels among the sIFNγR treatment group, the mock treatment group, and the treatment cessation group (data not shown). All mice in the 3 groups were apparently healthy during the observation period.

At 20 w, mock-treated mice showed further plaque progression (Figure 6). The 8-week sIFNγR treatment ameliorated the progression of the plaques, especially in the descending and abdominal aorta (Figure 6A). The en face plaque area was significantly smaller in sIFNγR-treated mice than in mock-treated mice (Figure 6B). In the treatment cessation group, lesion development was evident after sIFNγR treatment was stopped at 16 w, and the en face plaque area in the treatment cessation group was greater than in the 8-week sIFNγR treatment group but smaller than the mock treatment group at 20 w.

Discussion

Using sIFNγR gene transfer, the present study has demonstrated for the first time that postnatal blocking of interferon-γ function not only inhibits progression of atherosclerotic plaques but also stabilizes the lipid- and macrophage-rich advanced plaques in ApoE-KO mice. Moreover, sIFNγR treatment inhibited inductions of proinflammatory molecules and MMPs and increased procollagen type I expression.

ApoE-KO mouse is an appropriate model for investigating the role of interferon-γ in atherosclerotic lesions, because interferon-γ–producing T-lymphocytes were documented within the atherosclerotic lesions in this model.4 It has been shown that sIFNγR is an interferon-γ inhibiting protein, which competitively inhibits the binding of interferon-γ to the receptors on the target cells by binding interferon-γ in the extracellular space and/or in the circulation.16,17 To examine in vivo whether gene transfer of sIFNγR would increase sIFNγR production and inhibit inflammation, we injected the plasmid encoding sIFNγR into the thigh muscle by using the naked DNA method.29,30 As shown in Figure 1, sIFNγR gene transfer into the thigh muscle elevated serum sIFNγR protein levels and inhibited macrophage infiltration at the site of recombinant interferon-γ injection (dermis of the back).

Accordingly, it was suggested that the overexpressed sIFNγR proteins were secreted into the systemic circulation and turned inhibited the interferon-γ–induced inflammation in remote organs. Thus, in the present study, interferon-γ function was probably blocked in high-fat diet–fed ApoE-KO mice by overexpressing sIFNγR in the thigh muscle. We and others have shown that repeated gene transfers using the naked DNA method is a safe and reproducible approach to maintain the long-term transgene expression without adverse effects.18,29,30 Serum sIFNγR levels remained elevated for at least 2 weeks after a single gene transfer (Figure 1A). Accordingly, sIFNγR gene transfers were repeated twice at 2-week interval in the present study.

Effects on the Plaque Progression and Stability

The present study has provided novel evidence that postnatal inhibition of intrinsic interferon-γ inhibits plaque progression of ApoE-KO mice in the atherosclerotic stage (Figure 3). This is in line with previous studies showing that genetic ablation of interferon-γ or the interferon-γ receptors prevented the early development of atherosclerotic plaques in ApoE-KO mice and LDL receptor-KO mice.5,14,15 The present study suggested that sIFNγR treatment not merely delayed but inhibited plaque progression in the atherosclerotic stage as long as sIFNγR therapy was continued (Figure 6). Moreover, the fact that the lesion development caught up to the mock-treated group after the cessation of sIFNγR treatment may indicate that interferon-γ is a key molecule for progression of advanced plaques under prolonged hypercholesterolemia. Our observations not only support the notion that interferon-γ plays a substantial role in atherosclerotic plaque formation but also raise the possibility that blocking of interferon-γ–mediated pathway would be a new target for prevention and treatment of atherosclerosis.

Another novel finding was that postnatal inhibition of interferon-γ reduced lipid and macrophage accumulations and thickened fibrous cap by increasing fibrous tissue and SMCs, leading to stabilization of the lipid- and macrophage-rich advanced plaques with thin fibrous cap (Figure 3). These findings suggest that targeting interferon-γ signaling is a
potential strategy for regression and destabilization of advanced atherosclerotic lesions, particularly vulnerable plaques prone to rupture.

The present study is different from our previous one which showed that sIFNγR treatment prevented the formation of atherosclerotic plaque in ApoE-KO mice when sIFNγR gene therapy was started from the preatherosclerotic stage. Whereas in this study, sIFNγR treatment was performed in ApoE-KO mice with established, advanced plaques. sIFNγR treatment not only inhibited the progression of the plaques but also stabilized them. Thus, the present study suggests that interferon-γ pathway inhibition would have the therapeutic potential for atherosclerotic diseases.

Possible Mechanisms of Actions
Because sIFNγR treatment did not change serum cholesterol levels, it is plausible that the observed effects are independent of cholesterol levels. The examinations on the tissue gene expressions delineated several possible sites of action of sIFNγR on the plaque progression and histological stability. First, the antiinflammatory action would be mostly considerable. In atherosclerotic lesions, interferon-γ is produced by T-lymphocytes and activates macrophages and T-lymphocytes. The activated macrophages are not only transformed to the lipid-laden foam cells but also produce proinflammatory cytokines, which in turn stimulate production of chemokines in the vascular wall cells and adhesion molecules on the ECs and SMCs. Therefore, interferon-γ creates the self-amplifying cycle of activation of plaque inflammation and recruitment of inflammatory cells, leading to further plaque progression and destabilization. STAT1 phosphorylation was inhibited in atherosclerotic plaques of sIFNγR-treated ApoE-KO mice (Figure 4), suggesting that overexpressed sIFNγR blocked the intrinsic interferon-γ-induced signaling in the atherosclerotic lesions. In this study, sIFNγR treatment attenuated the augmented expressions of proinflammatory cytokines (IL-1β and IL-6), chemokines (MCP-1 and MIP-1α), and VCAM-1 in ApoE-KO mice in the atherosclerotic stage (Figure 5).
Thus, it was likely that sIFNγR abolished the interferon-γ–induced vicious cycle, being the mechanisms for inhibition of plaque progression and destabilization.

Another important site of action of sIFNγR would be the ECM homeostasis. sIFNγR treatment increased fibrous tissue and SMCs in the plaques (Figure 3), associated with procollagen type I induction and MMP-13 and MMP-9 downregulations (Figure 5). These findings were consistent with earlier studies demonstrating that interferon-γ inhibited proliferation and procollagen expression of cultured SMCs and stimulated MMP production of cultured macrophages and SMCs. However, it was suggested that interferon-γ induced SMC proliferation and intimal matrix deposition in animal models of proliferative vascular disorders, such as neointima formation after vascular injury and neointima formation in isolated artery segment. The discrepancy may be explained by the differences in the underlying pathophysiology, the animal species studied, and the interactions of SMCs to the surrounding cells and other humoral factors among these disease models. In the present study, we not only confirmed previous findings in vitro but also showed novel evidence that interferon-γ may participate in destabilization of the advanced plaques by degrading the ECM and by inhibiting intimal SMC proliferation.

The immune mediator CD40L and its receptor CD40 are crucial for initiation of plaque formation and for progression and destabilization of advanced plaques. The CD40L signal induces production of cytokines and MMPs. As shown in Figure 4B, sIFNγR treatment reduced the CD40/CD40L expressions in the plaques, suggesting that interferon-γ promotes production of cytokines and MMPs in the plaques through the CD40/CD40L pathway. Several mechanisms for this inhibition of CD40/CD40L system are considered. MCP-1 plays a role in CD40/CD40L inductions in the plaques in ApoE-KO mice. In this study, sIFNγR treatment decreased MCP-1 expression in ApoE-KO mice (Figure 5). Thus, sIFNγR treatment would have indirectly attenuated the CD40/CD40L expressions by decreasing MCP-1 induction.

The decreased CD40/CD40L signals might simply reflect the reduction of infiltrating cells in the plaques, such as macro-

---

**Figure 6.** Effects of 8-week sIFNγR treatment on atherosclerotic lesions. A, Representative en face photographs showing the oil red O–stained atherosclerotic plaques of the aorta at 20 w. B, Pooled data of the effects on the en face plaque area at 20 w. Bar=1×SD (n=10). *P<0.01 vs mock-treated ApoE-KO mice. **P<0.01 vs sIFNγR-treated ApoE-KO mice.
phages and lymphocytes, which express CD40 or CD40L independently of interferon-γ. However, we observed that sIFNγR treatment not only reduced the mobilization of peritoneal macrophages but also attenuated the CD40/CD40L expressions of macrophages by the direct action in ApoE-KO mice (supplemental Figure I). Thus, the decreased CD40/CD40L signals in plaques may not simply reflect the reduction of infiltrating cells but may be caused by the direct inhibition of the CD40/CD40L pathway by sIFNγR treatment.

Lastly, interferon-γ impedes reverse cholesterol transport and promotes the expression of scavenger receptors and the transformation of macrophages and SMCs to foam cells.40,41 Although we did not address this issue in this study, sIFNγR treatment might have reduced the lipid-rich core through the improvement of reverse cholesterol transport and through the inhibition of foam cell formation.

Potential Merits of Targeting Interferon-γ

Using double knockout mice, recent studies have demonstrated that genetic ablation of proinflammatory molecules, such as IL-1β,42 MCP-1,43 and ICAM-1,44 reduces early development of atherosclerotic lesions in ApoE-KO mice. Also, postnatal inhibition of MCP-1 function by overexpressing a dominant negative mutant of MCP-1, 7ND, has been shown to prevent the growth and destabilization of advanced plaques in ApoE-KO mice.24 As compared with the strategy focusing on each of the molecules, targeting interferon-γ may have an advantage such as a wide-range inhibition of the downstream events, because interferon-γ regulates the multiple steps of development, progression, and destabilization of the plaques,8,9,12 as shown in Figure 5.

Limitations of This Study

Although the long-term effectiveness of sIFNγR treatment was achieved by repeating sIFNγR gene transfers, the efficacy of sIFNγR treatment was partial and the treatment cessation resumed plaque progression. This raises concerns about the potential limitation of this approach. In this regard, the development of recombinant sIFNγR protein or synthetic peptides containing the pharmacologically active domain of sIFNγR protein would be a desirable approach, which may be feasible for long-term treatment. However, considering chronic nature of atherosclerosis, prolonged sIFNγR therapy may not be practical for the prevention of atherosclerosis in humans. Nevertheless, we still think that our approach may have a therapeutic potential because it is well known that cardiovascular events occur because of the rupture of unstable plaques. Our approach did stabilize the lipid- and macrophage-rich advanced plaques (Figure 3). Thus, the stabilization of the unstable plaques would be the potential therapeutic target of postnatal interferon-γ function inhibition during limited treatment period. Although no apparent side effects were found during the period of this study, careful observation is warranted over longer period before the consideration of clinical application because the long-term systemic inhibition of interferon-γ may result in impaired tumor immunosurveillance and susceptibility to microbial infections. To avoid the possible systemic adverse effects, drug-eluting stent or the local delivery of recombinant sIFNγR protein using nano-particles, which target atherosclerotic plaques, may be desirable.

In conclusion, the present study is the first report showing that postnatal inhibition of interferon-γ prevents progression of existing atherosclerotic plaques and stabilizes the lipid- and macrophage-rich advanced plaques. These findings raised the possibility that the inhibition of interferon-γ pathway is a potential therapeutic target for stabilizing the vulnerable plaques prone to rupture and subsequently for preventing cardiovascular events.

Acknowledgments

We thank Kaoru Moriyama, Yayoi Yoshida, Reiko Fujyoshi, Miyuki Ouchida, Mibo Kogure, and Kimako Kimura for their skillful technical assistance.

Sources of Funding

This study was supported in part by a grant for Science Frontier Research Promotion Centers and Grants-In-Aid for Scientific Research (to H.K. and T.I.) from the Ministry of Education, Science, Sports and Culture, Japan, and by research grants from Kimura Memorial Heart Foundation (to T.I.).

Disclosures

None.

References

15. Buono C, Come CE, Stavrakis G, Maguire GF, Connelly PW, Lichtman AH. Influence of interferon-gamma on the extent and phenotype of


Inhibition of Progression and Stabilization of Plaques by Postnatal Interferon-γ Function Blocking in ApoE-Knockout Mice

Mitsuhisa Koga, Hisashi Kai, Hideo Yasukawa, Tomoka Yamamoto, Yumiko Kawai, Seiya Kato, Ken Kusaba, Mamiko Kai, Kensuke Egashira, Yasufumi Kataoka and Tsutomu Imaizumi

*Circ Res.* 2007;101:348-356; originally published online May 10, 2007;
doi: 10.1161/CIRCRESAHA.106.147256

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/101/4/348

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2007/07/05/CIRCRESAHA.106.147256.DC1

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org/subscriptions/
ONLINE SUPPLEMENTAL INFORMATION

Expanded Methods

RNA extraction and real-time reverse transcription (RT)-polymerase chain reaction (PCR) analysis.

The aorta was rapidly snap-frozen in dry-ice/acetone and stored at -80°C until use (n=10/group). After the frozen samples were homogenized in TRIzol (Invitrogen, Tokyo) using FastPrep homogenizer (Thermosavant, Holbroool, NY), the total RNA was extracted and reverse-transcribed by using 1st-strand reaction mix beads (Amersham Biosciences, Tokyo)\(^1\). For quantitative analysis of the target mRNA expression, aliquots of the RT products were amplified and analyzed in triplicate using the TaqMan One-Step RT-PCR Master Mix Reagents and a Sequence Detection System model 7700 (Applied Biosystems, NJ), as previously described \(^1\). Primer pairs and TaqMan probes for interleukin-1\(\beta\), interleukin-6, vascular cell adhesion molecule-1, monocyte chemoattractant protein-1, macrophage inflammatory protein-1\(\alpha\), procollagen type 1, matrix metalloproteinase (MMP)-9, and MMP-13 were obtained from Applied Biosystems. The TaqMan Rodent GAPDH Control Reagents were used to detect mouse GAPDH as the internal standard. Expression level of the target gene was normalized by GAPDH level in each sample.
Online Supplemental Data

Effects of sIFNγR treatment on the expressions of CD40 and CD40L on peritoneal macrophages

Method

Macrophage preparation

From 8-week old (w), Apo E-knockout (ApoE-KO) mice were fed high fat diet containing 20% fat and 0.15% cholesterol (Oriental Yeast, Tokyo). Mice received 200 µg of plasmid DNA encoding the extracellular portion of the mouse interferon-γ receptor α-chain conjugated with mouse IgG1 Fc-segment (sIFNγR) or the mock plasmid at 12 w (n=10/group). Macrophage preparation was performed according to the method described by Miles et al. Three days after gene transfer, the mice were injected intraperitoneally with 2 mL of 4% thioglycollate (Sigma CO, St. Lewis, MO) to elicit macrophage migration to the peritoneal cavity. After mice were euthanized with an overdose of pentobarbital, the peritoneal cells were harvested by washing the peritoneal cavity with 5 mL of phosphate-buffered solution containing 1% bovine serum albumin (BSA/PBS). The cells were washed with BSA/PBS twice and re-suspended in BSA/PBS with the final concentration at 1.5x10^7 cells/mL. The number of total peritoneal cells (cells/mL) was determined using an automated cell counter.

Flow cytometric analysis

Immediately after the peritoneal cells were harvested, aliquots of the cells (1.5x10^6 cells/500 mL BSA/PBS) were pre-incubated with anti-murine CD16/32 antibody (Becton Dickinson, San Jose, CA) for 10 min at 4°C to block the Fc...
receptors. After wash with BSA/PBS, aliquots of the cells (1.5x10^6 cells/500 mL) were incubated with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse F4/80 antibody (monocytes/macrophages) (Serotec, Tokyo) and phycoerythrin (PE)-conjugated rat anti-mouse CD40 (Serotec) or with FITC-conjugated F4/80 antibody and PE-conjugated rat anti-mouse CD154 (CD40L, Serotec) for 1 hour at 4°C. After wash with BSA/PBS and fixation with 1% paraformaldehyde, the stained cells were analyzed with a FACS Caliber flow cytometer (Becton Dickinson). The number of peritoneal macrophages was calculated from the peritoneal cell count multiplied by the proportion of F4/80-positive cells, as determined by flow cytometric analysis.

**Results**

sIFNγR treatment significantly reduced the number of peritoneal macrophages by 24% in thioglycollate-treated ApoE-KO mice (Figure I A). sIFNγR treatment significantly reduced the proportion of CD40-positive and CD40L-positive subsets in the peritoneal macrophages by 30% and 37% relative to mock-treated mice, respectively (Figure I B). Thus, the reductions of CD40- and CD40L-positive subsets in macrophages were greater than that of the decrease in macrophage number. These findings suggest that sIFNγR treatment not only reduced the mobilization of macrophages to the peritoneal cavity but also attenuated the CD40/CD40L expressions of peritoneal macrophages.

**Figure Legend of Supplemental Figure I.**

*Effects of sIFNγR treatment on the CD40 and CD40L expressions of peritoneal macrophages in ApoE-KO mice*
Three days after peritoneal thioglycollate injection, peritoneal macrophages were harvested and subjected to flow cytometric analysis. A. sIFNγR treatment reduced the number of peritoneal macrophages in ApoE-KO mice. B. sIFNγR treatment decreased the proportions of CD40-positive (a) and CD40L-positive (b) subsets of peritoneal macrophages. Bar=1xSD (n=10). *p<0.05 vs. mock-treated ApoE-KO mice.

References


Supplemental Figure I