Functional Inhibition of Ras by S-trans,trans-Farnesyl Thiosalicylic Acid Attenuates Atherosclerosis in Apolipoprotein E Knockout Mice

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Background—Atherosclerosis is a multifactorial disorder involving inflammatory processes. These responses are associated with robust activation of signaling cascades by diverse cell surface receptors in a variety of cell types. The processes that are involved in atherosclerosis would likely require intact Ras pathways, which play a key role in the control of cell growth, differentiation, and apoptosis.

Methods and Results—We examined whether the Ras inhibitor farnesyl thiosalicylic acid (FTS) can suppress atherogenesis in the apolipoprotein E–deficient mouse model. Mice were treated with FTS or a control regimen 3 times weekly for 6 weeks and fed a normal chow diet. Two additional groups included FTS-treated and control-treated mice that were fed a high-fat diet for 10 weeks. FTS reduced both fatty streaks and advanced lesions compared with the control treatment. Ras inhibition in vivo was evidenced by the reduced content of the active form of Ras (Ras-GTP) in aortas of FTS-treated mice. Splenocytes from the FTS-treated versus control mice exhibited reduced proliferation to oxidized LDL (OxLDL) but not to concanavalin A. IgG anti-OxLDL antibody levels were reduced in FTS-treated mice compared with controls. Whereas no effect of FTS was evident on plaque T lymphocyte and macrophage content, lesional vascular cell adhesion molecule-1 and nuclear factor-κB expression were considerably reduced compared with controls.

Conclusions—FTS suppressed atherosclerotic plaques in apolipoprotein E–deficient mice, providing a useful tool for research in atherosclerosis. (Circulation. 2002;105:2416-2422.)

Key Words: atherosclerosis ■ Ras ■ immune system ■ apolipoproteins

Atherosclerosis involves uninhibited accumulation of lipids in the vessel wall, which results in considerable morbidity and mortality.1 In recent years, a growing body of evidence has supported the participation of the immune system in the initiation and progression of atherosclerosis.1,2 This notion is based on the idea that atherosclerosis is a form of a chronic inflammatory state that involves interaction among endothelial cells, macrophages, T lymphocytes, and smooth muscle cells.1

Many of the diverse signals triggered by receptors involved in the atherosclerotic process would require intact Ras pathways, which play a key role in the control of cell growth and differentiation, in cell migration and adhesion, and in cell survival.3-5 Thus, overactivation of Ras caused by shear forces or oxidant stress could contribute to the atherosclerotic process in a manner analogous to that of human tumors that harbor activated ras genes or that overexpress receptors that activate Ras.

A number of experiments also support the direct involvement of active Ras in atherosclerosis. Shear forces resulting from turbulent flow and oxidative stress are contributory, and possibly essential, to atherogenesis and have been shown to activate Ras.6 Moreover, oxidized LDL (OxLDL), considered crucial in atherogenesis, has been shown to induce Ras activation in smooth muscle cells.7 These results, combined with the finding of instability at the H-ras minisatellite found in atherosclerotic plaques,8 suggest that Ras overactivation may be involved in the pathogenesis of the disorder. These data suggest that Ras is an appropriate interventional target in atherosclerosis; thus, we hypothesized that inhibition of Ras function would have a beneficial effect in attenuating the atherosclerotic process. Because cellular and humoral immune responses to OxLDL have been associated with atherosclerosis,1 Ras inhibition could also be contributory by attenuating the proliferative phenotype of lymphocytes, which have been shown to overexpress this protein.

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Synthetic S-prenyl derivatives of rigid carboxylic acids resembling the carboxyl-terminal farnesylcysteine, common to all Ras protein acids, represent a group of functional Ras inhibitors. The most potent inhibitor in this group, \textit{S}-\textit{trans},\textit{trans}-farnesyl thiosalicylic acid (FTS), inhibits the growth of Ha-Ras-- N-Ras--, and K-Ras--transformed rodent fibroblasts by its direct effects on the mature membrane-anchored Ras.\textsuperscript{9-11} In Ras-transformed rodent cells and in human tumor cells, FTS displodes active Ras from its membrane anchorage sites, thereby inhibiting Ras-dependent cell transformation.\textsuperscript{11} Indeed, FTS has demonstrated efficacy with no adverse side effects or toxicity in a number of animal experimental tumor models.\textsuperscript{12-13} In a model of hepatic cirrhosis involving cellular proliferation, this agent was also beneficial in attenuating hepatocyte damage.\textsuperscript{14}

In the present study, we tested the hypothesis that functional inhibition of Ras could attenuate early and advanced atherosclerotic lesion formation in the apolipoprotein-E (apoE)--deficient mouse model.

\textbf{Methods}

\textbf{Animals}

ApoE-deficient mice on a C57BL/6 background\textsuperscript{15,16} were purchased from Jackson Laboratories (West Grove, Pa) and grown at the local animal house. Mice were fed either a normal chow diet containing 4.5% fat by weight (0.02% cholesterol) or a Western-type diet containing 42% calories from fat, 43% from carbohydrates, and 15% from fat (TD 96125, Harlan Teklad).

\textbf{Experimental Design}

In the first experiment, the effect of FTS on fatty streak formation (early atherosclerosis) was studied in apoE-deficient mice. For this purpose, 4-week-old male apoE-deficient mice received intraperitoneal injection with FTS (5 mg/kg body weight; n=7) or a control vehicle (n=8) 3 times a week for 6 weeks and were fed a normal chow diet. When the animals were killed, plasma was obtained for anti-OxLDL antibody level measurement, and hearts were removed for estimation of lesion size and plaque composition.

In the second study, the effect of FTS was studied on advanced atherosclerotic plaque development. For this purpose, 4-week-old apoE-deficient mice received intraperitoneal injection with FTS (n=11) or a control vehicle (n=9) according to the same protocol as described above except that they were fed a high-fat diet for 10 weeks. When these animals were killed, the same measurements were performed as in the first study, and spleen cells were obtained for assessment of proliferation in the presence of OxLDL.

\textbf{Lipid Profile}

Total plasma cholesterol and triglyceride levels were determined by an automated enzymatic technique (Boehringer Mannheim).

\textbf{Splenocyte Proliferation Assays}

Splenocytes (1×10^6 cells/mL) were incubated in triplicate in 0.2 mL of culture medium in microtiter wells in the presence or absence of 10 μg/mL mouse OxLDL for 72 hours. Proliferation was measured by the incorporation of [3H]thymidine into DNA during the final 12 hours of incubation, as described previously.\textsuperscript{17}

\textbf{Detection of Anti-OxLDL Antibodies and Isotypes by ELISA}

Ninety-six--well polystyrene plates (Nunc) were coated with either OxLDL, at a concentration of 10 μg/mL in PBS, or native LDL (both from humans) overnight at 4°C. The subsequent steps were performed as described previously.\textsuperscript{18} IgG isotypes were determined with an ELISA kit (Southern Biotechnology).

\textbf{Measurement of Ras Expression}

Levels of Ras protein content were determined in homogenates of aortas obtained from FTS-treated mice or controls, with anti-Pan-Ras antibodies (PanRas Ab03, Santa Cruz) used as described previously.\textsuperscript{11} Aortas were homogenized (10% wt/vol) in cold homogenization buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.6), 1 mmol/L dithiothreitol, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 1 mmol/L benzamidine, 1 mmol/L PMSF, 5 U/mL aprotinin, and 20 mmol/L MgCl\textsubscript{2}. The nuclei and cell debris were then removed by a 10-minute 1000g spin. The resulting supernatant was used for assays. Samples containing 25 μg of proteins were used for determination of total Ras protein.\textsuperscript{11} Proteins were separated by 12.5% SDS--PAGE (mini-gel) and blotted onto nitrocellulose paper. The paper was blocked with 1.5% skim milk in Tris-buffered saline (pH 7.6) containing 0.05% Tween-20 and 150 mmol/L NaCl overnight, then incubated for 1.5 hours with 1:2000 dilution of Pan-Ras antibody in the same buffer. Immunoblots were then incubated for 1 hour with 1:7500 dilution of anti-mouse IgG horseradish-peroxidase conjugate and subjected to enhanced chemiluminescense assays. Bands were quantified by densitometry on a BioImaging System (202D, Dynco-Renium) with Tina 2.0 software (Ray tests).

\textbf{Determination of Active Ras in Aortic Samples From FTS-Treated and Control Mice}

The above-noted preparations of the aortas (500 μg of protein) were adjusted to 0.5% NP-40 and used for determination of levels of active GTP-bound Ras.\textsuperscript{19} The Ras binding domain of human c-Raf-1 (RBD) fused to glutathione-S-transferase (GST) in the expression vector pGEX-2T (a gift from J. Boss, Center for Biomedical Genetics, Utrecht, the Netherlands) was prepared in DH-10B \textit{Escherichia coli} cells. The bacterial lysate was rotated for 30 minutes at 4°C with glutathione-agarose beads (Sigma) in homogenization buffer containing NP-40. The beads were then washed with the same buffer and mixed with the above aorta preparation. Samples were rotated for 30 minutes at 4°C, after which the active Ras was precipitated and washed. SDS sample buffer was added to the precipitated Ras-GTP, and the apparent amount of Ras was determined by immunoblotting with the Pan-Ras antibody as detailed above.

\textbf{Assessment of Atherosclerosis}

Quantification of atherosclerotic fatty streak lesions was done by calculation of the lesion size in the aortic sinus as described previously.\textsuperscript{18} Lesion areas per section were counted with a grid by an observer unfamiliar with the tested specimen.

\textbf{Immunohistochemistry}

Immunohistochemical staining for T cells (CD3), T cell activation marker (interleukin-2 receptor), macrophages (Mac-1), nuclear factor-κB (NF-κB), and vascular cell adhesion molecule-1 (VCAM-1) were performed on 5-μm-thick frozen sections of aortic sinus.\textsuperscript{17} Positive cells were counted by 2 pathologists blinded to the study protocol and averaged. VCAM-1 was evaluated by morphometry as described previously.\textsuperscript{17} Briefly, image-analysis software was used to assess percent coverage of VCAM-1 from the total plaque area in ≥3 sections from 6 to 7 animals in each group. The highest levels were taken for comparative analysis among groups. The analysis was performed by 2 pathologists blinded to the tested section.

\textbf{Statistical Analysis}

All parameters were evaluated by the Student \textit{t} test. \(P<0.05\) was considered statistically significant. Results are expressed as mean±SEM unless otherwise specified.

\textbf{Results}

FTS treatment of chow-fed mice did not appear to influence total cholesterol levels (mean levels 258±61 mg/dL; \(P=\text{NS}\)).
Similarly, total cholesterol levels were not significantly different in the FTS-treated and control mice fed a Western diet (870 ± 252 versus 870 ± 185 mg/dL, respectively; P=NS). Triglyceride levels were similar in the FTS-treated and control chow-fed apoE-deficient mice (143 ± 28 versus 155 ± 31 mg/dL, respectively). No difference in triglyceride levels was evident when a high-fat diet was fed to the FTS-treated (188 ± 37 mg/dL) or control (184 ± 35 mg/dL) mice.

In the first experiment, early atherosclerotic lesions were significantly attenuated (52% reduction) by treatment with FTS (mean aortic lesion size 37 000 ± 4300 μm$^2$) compared with controls (77 000 ± 17 000 μm$^2$; P<0.01; Figures 1A, 2A, and 2B). Although less pronounced, FTS reduced the more advanced plaques induced by high-fat diet feeding (mean lesion size 285 000 ± 15 300 μm$^2$) compared with the control group (348 000 ± 25 000 μm$^2$; P<0.05; Figures 1B, 2C, and 2D). This was equivalent to a reduction of 28%.

To determine whether Ras expression was influenced by FTS treatment, we evaluated Ras protein content in aortas from FTS-treated mice compared with controls in the first experiment. Ras protein content did not differ in atheromatous aortas from FTS-treated mice compared with nontreated mice (Figure 3). To evaluate whether active Ras...
was diminished as a result of the treatment, we evaluated Ras-GTP content and found it reduced by 40% in aortas of mice treated with FTS for 6 weeks compared with controls \((P<0.05; \text{Figure 3B})\).

Next, we explored the effect of anti-Ras treatment on the cellular and humoral immune response to OxLDL, which is known to prevail in atherosclerotic apoE-deficient mice. Basal proliferative response of splenocytes from FTS-treated mice (second experiment) did not differ from that of control animals (Figure 4A). However, when primed with OxLDL, no significant reactivity was evident with FTS treatment (second experiment), whereas a 24% \((P<0.05)\) increase in thymidine uptake was obtained in control mice (Figure 4A). Splenocytes from FTS-treated or control mice did not differ with regard to their concanavalin A-induced proliferation (data not shown).

Similar to the effects on cellular immunity, IgG anti-OxLDL antibody levels were reduced in mice treated with FTS (second experiment) compared with controls \((\text{mean OD } 0.22\pm0.08 \text{ versus } 0.76\pm0.3; P<0.05; \text{Figure 4B})\). No differences were evident among groups with respect to IgG isotype distribution (data not shown).

To investigate whether the reduction in atherosclerotic lesion size induced by FTS altered plaque composition, we evaluated the relative density of macrophages, total lymphocytes, and activated (interleukin-2 receptor positive) lymphocytes. FTS treatment in both experiments did not appear to influence the relative numbers of macrophages and lymphocytes in either experiment (Figure 5).

We then evaluated, by morphometry, the relative expression of a key adhesion molecule (VCAM-1) in plaques after FTS treatment. Administration of FTS considerably reduced VCAM-1 expression in fatty streaks of mice from the 6-week treatment schedule compared with controls.
A similar, although less pronounced, effect of FTS was evident on VCAM-1 expression in more advanced plaques (43% reduction by FTS; Figures 6 and 7).

Because NF-κB is a transcription factor known to trigger VCAM-1 expression, we sought to evaluate its relative abundance in the plaques. We found that NF-κB–positive cells were reduced in FTS-treated lesions (second experiment) compared with controls (Figures 6B, 7E, and 7F). The numbers of NF-κB–expressing cells were too low to obtain a meaningful count in the first experiment.

Discussion

In the present study, we provide evidence for the efficacy of functional Ras inhibition by FTS in murine atherosclerosis. The effect was obtained in early fatty streaks and in more advanced plaques induced by high-fat diet supplementation.

There are several theoretical mechanisms whereby Ras could influence atherosclerotic plaque initiation and progression. It has been suggested that ras mutations are present in samples from human atherosclerotic plaques. Ras has been shown to activate NF-κB, a transcription factor known to trigger production and expression of adhesion molecules, which are essential to the initiation of atherosclerotic plaques. It was recently found that NF-κB is expressed within atherosclerotic plaques. We have shown here that VCAM-1 expression within the plaques is reduced after anti-Ras treatment. Moreover, NF-κB–positive cells were reduced in lesions after FTS treatment, which supports the contention that interference with Ras activation and signaling could result in attenuation of NF-κB–mediated induction of adhesion molecules. Interestingly, VCAM-1 plaque coverage was reduced to a larger extent by FTS in early plaques than in more advanced plaques. These findings are consistent with the relatively more important role of VCAM-1 in early atherosclerotic lesions.

We found that FTS treatment reduced Ras-GTP content by ≈40% in control mice aortas. Because homogenates from the aortas contain a heterogenous population of cells, only part of which are actively proliferating, the effect of FTS is only apparently incomplete. Nevertheless, it is likely that Ras inhibition in active Ras-expressing cells within the atheroma would be significantly more pronounced. Total Ras protein did not differ between FTS-treated and control aortas. This finding can also be explained by the mixed population of cells within the plaques, not all of which express Ras to a similar level. Thus, although FTS treatment was sufficient to inhibit active Ras in the aortas, protein content was not influenced. The important role of Ras in cellular growth, migration, and differentiation implies that its inhibition may have potentially hazardous effects. The partial effect on total Ras protein may explain the lack of side effects that would be expected to result from inhibition of such a key signaling protein.

Atherogenesis is a process in which the immune system appears to be an active participant. Accordingly, activated lymphocytes have been detected in human and murine plaques, sometimes even preceding the infiltrating lipid-laden macrophages. Ras protein expression has
been shown to be involved in regulating lymphocyte activation (reviewed in Genot and Cantrell). Thus, interference with lymphocyte activation by anti-Ras treatment could be a beneficial antiatherogenic mechanism. In the present study, we did not observe any differences in lymphocyte density within atherosclerotic plaques between FTS-treated and control mice, nor did we find evidence that activation markers were reduced as a result of anti-Ras therapy. However, systemic immune responses toward OxLDL, which have been associated with development of atherogenesis, were indeed altered. Accordingly, the proliferative response of splenocytes from FTS-treated mice to OxLDL was reduced compared with responses recorded from control lymphocytes. Moreover, antibodies to OxLDL were also diminished by FTS treatment. These findings imply that attenuation of immune reactions that result from oxidative stress may have been mediated by interference with Ras activation, and this could have contributed to the antiatherogenic effect.

Function and dysfunction of endothelial cells play a pivotal role in atherogenesis. Strong in vivo support for this notion has recently been provided by Celletti et al., who showed that vascular endothelial growth factor enhances atherosclerosis in apoE/ apoB100 mice. Because angiogenesis promoters are known to transmit signals through the Ras system, we expect that interference with Ras could have induced suppression of atherosclerotic lesions. Studies that used gene delivery of dominant negative that involves, apart from lymphocytes and macrophages, smooth muscle cells, which occupy lesions that are relatively more advanced. Approaches designed to block Ras-mediated smooth muscle cell proliferation and migration have been successful in several in vitro studies. Moreover, studies that used gene delivery of dominant negative forms of ras have shown them to be effective in suppressing neointimal formation after experimental carotid injury. It is thus possible that by altering the proliferative phenotype of atherosclerotic lesions, FTS could have induced suppression of atherosclerotic lesions. Additionally, FTS inhibits cell migration, a property that may be relevant to atherosclerosis suppression, which involves the recruitment of monocytes and lymphocytes followed by smooth muscle cells and fibroblasts.

In the present study, we used a relatively novel Ras inhibitor that has some advantages over other inhibitors designed to inhibit Ras. To the best of our knowledge, FTS is the only single compound that was shown to affect all isoforms of activated Ras, namely, activated H-, K-, and N-Ras. Other compounds, such as farnesyl transferase inhibitors, for example, are good inhibitors of H-Ras but not of other Ras isoforms. This, together with the lack of FTS toxicity in animal models, makes FTS an acceptable potential drug for the study of atherosclerosis.

In conclusion, FTS, a functional Ras inhibitor, has been shown to be particularly effective in suppressing early (and to a lesser extent, more advanced) atherosclerosis. These effects brought about by FTS, although probably multifactorial, may prove to be an interesting tool in the study of atherosclerosis.

References


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