Inhibition of Intimal Thickening in the Rat Carotid Artery Injury Model by a Nontoxic Ras Inhibitor

Jacob George, Jessica Sack, Iris Barshack, Pnina Keren, Iris Goldberg, Roni Haklai, Galit Elad-Sfadia, Yoel Kloog, Gad Keren

Background—Neointimal formation with and without previous vascular injury is common after balloon dilation and in transplant arteriosclerosis. It involves proliferation and migration of medial smooth muscle cells and inflammation, processes that are regulated by Ras proteins and their down-stream effectors. Farnesylthiosalicylate (FTS) is a Ras inhibitor that interferes with Ras membrane anchorage and affects Ras proteins in their active state. In the present study, we tested the hypothesis that systemic administration of FTS will suppress intimal thickening in the rat carotid injury model.

Methods and Results—The effects of FTS on rat vascular smooth muscle cells (VSMC) and splenocytes proliferation were evaluated in vitro. The in vivo effects of FTS on the neointima of balloon-injured male Wistar rats, treated daily for 2 weeks with FTS (5 mg/kg weight, intraperitoneally) were evaluated by determination of Ras, Ras-GTP, and active ERK levels (3 days after injury), and by quantitative determination of the extent of intimal thickening and immunohisto-chemistry for Ras, iNOS, NFkB, and Ki-67 (2 weeks after injury). FTS inhibited VSMC and splenocyte proliferation as well as interferon-γ secretion by splenocytes in a dose-dependent manner. Compared with controls, FTS treatment resulted in a strong decrease in Ras-GTP and active ERK, and it significantly reduced intimal thickening after the injury. Ras expression appeared predominantly at areas of neointima regardless of the treatment group. NFkB and iNOS-positive cell numbers were reduced in sections of FTS treated rats.

Conclusion—FTS appears to act as a potent inhibitor of intimal thickening in a model of experimental arterial injury. (Arterioscler Thromb Vasc Biol. 2004;24:363-368.)

Key Words: intimal thickening ■ Ras ■ smooth muscle cell ■ proliferation

The occurrence of restenosis in ≈20% to 55% of patients after clinically successful revascularization remains the major limitation to percutaneous transluminal coronary angioplasty even in the era of stent placement. The formation of neointima after balloon denudation is thought to involve proliferation and migration of medial smooth muscle cells or modified adventitial fibroblasts. In recent years, evidence has also accumulated pointing toward the involvement of the immune system in atherosclerosis and restenosis. Recent studies indicate that drug-eluting stents reduce the occurrence of restenosis within the stents through inhibition of cell proliferation. However, the promising results reported so far do not guarantee long-term favorable results, which may be caused by the possible late weakening of the adventitial and medial support of the blood vessel. Nonetheless, it is interesting that rapamycin targets mammalian target of rapamycin, which itself is a down-stream effector of the Ras-Pi3-K pathway. Therefore, targeting Ras by Ras inhibitors could be expected to ameliorate restenosis to the extent of targeting mammalian target of rapamycin and possibly even others. This is because Ras proteins control, in addition to Pi3-K, a number of other key signaling enzymes that regulate cell growth, cell death, and cell migration.

Activating mutations in ras genes and receptor-mediated activation of wild-type Ras is common in many neoplastic human diseases and contributes to aberrant cell proliferation, migration, and survival. Active Ras is therefore considered as an important target for antineoplastic drugs. Many efforts have been made in an attempt to develop Ras inhibitors. These included the development of farnesyltrans-ferase inhibitors (FTI) in attempts to block Ras farnesylation, which is required for Ras functions, and antisense oligonucleotides against Ras to block Ras protein synthesis. Each of these groups of inhibitors has its own limitations. FTI, for example, block the functions of H-Ras and of other important cell regulators, such as RhoB, but do not block the functions...
of active K-Ras or N-Ras proteins. FTI and antisense oligonucleotides exhibit significant efficacy in animal models of cancer and of proliferative diseases, including experimental arterial injury, and are used with some success in clinical trials for cancer therapy.

An additional approach to inhibit Ras is the use of S-trans, trans-farnesylthiosalicylic acid (FTS), a synthetic compound that resembles the carboxyl-terminal farnesylcysteine of Ras and dislodges active Ras from its membrane anchorage domains. FTS inhibits growth of rodent and human cells expressing H-Ras, K-Ras, or N-Ras in vitro and inhibits tumor growth in animal models. In addition, FTS was shown to have beneficial effects in a number of animal models of proliferative diseases. We have recently reported that FTS was effective in reducing atherosclerosis in the apoE knockout mouse model, an effect that could be attributable to inhibition of inflammatory and proliferative processes that underlie the atherosclerotic progression.

Collectively, these observations led us to consider the possibility that inhibition of Ras by FTS could be an effective treatment for restenosis. The apparent selectivity of FTS toward active (GTP-bound) Ras and lack of toxic or adverse side effects in animals support this possibility. It is also important to note that an optimal strategy for ameliorating restenosis involves inhibition of smooth muscle cell proliferation and migration without interference with efficient re-endothelialization after the procedure. In this regard, use of FTS seems to be an appropriate potential strategy, because it does not influence endothelial proliferation and it was shown to inhibit cell migration in vitro. We therefore investigated the effect of FTS on intimal hyperplasia in a model of experimental arterial injury.

Methods

Smooth Muscle Cell Culture and Proliferation Assays
Vascular smooth muscle cells (VSMC) were obtained from the carotid arteries of Wistar rats by use of the collagenase and elastase digestion method, and cell proliferation was assayed by 3H-thymidine incorporation as described.20

Splenocyte Cell Proliferation and Interferon-γ Secretion
Total splenocytes were obtained from untreated rats and single-cell suspension was made. Spleen cells (2 × 10^7 well) were incubated in triplicates (in 96-well flat-bottom plates) for 72 hours in 0.2 mL complete culture medium (RPMI 1640+0.5% inactivated mouse sera) in the presence or absence of FTS and Con A. Proliferation was measured by the incorporation of 3H-thymidine into DNA during the final 12 hours of incubation. Interferon-γ (IFN-γ) was determined in the medium 24 hours after serum stimulation in the absence or in the presence of FTS using ELISA kit (PharMingen) according to the manufacturer’s instructions.

Rat Carotid Injury Model
Endothelial denudation and vascular injury was performed in the left common carotid artery of male Wistar rats, as described.21 Rats received daily intraperitoneal injections of FTS (5 mg/kg) or control vehicle starting after the injury. Animals were euthanized after 3 or 14 days, and the right and left carotid arteries were taken out and either homogenized or fixed in 4% paraformaldehyde until embedding in paraffin. The arteries were cut in 10-μm sections and stained with hematoxylin and eosin (H&E), and computer-assisted morphometric analyses were performed.

Determination of Ras, Ras-GTP, and Phospho-ERK
Carotid arteries from FTS-treated rats or controls, 3 days after injury, were homogenized (10% weight/volume) in cold homogenization buffer as detailed elsewhere. Samples containing 25 μg protein were used for the determination of total Ras and phospho-ERK by Western immunoblotting using, respectively, pan-anti-Ras Ab (Ab903; Santa Cruz, CA) and anti-phospho-ERK Ab (Sigma), and enhanced chemiluminescence assays. Bands were quantified by densitometry. These noted preparations of the aortas (500 μg protein) were adjusted to 0.5% NP-40 and used for the determination of Ras-GTP by the glutathione S-transferase-RBD pull-down assay followed by Western immunoblotting with pan-anti-Ras Ab as detailed previously.

Immunohistochemistry
Paraffin-fixed sections (10 μm) of carotid arteries obtained from animals after being euthanized were stained with pan-Ras antibody anti-Ki-67 and anti-NF-kB antibodies as described. Proliferative index was determined as the number of cells positive for Ki67total cell nuclei contained within the internal elastic lamina.

Statistical Analysis
Results of all parameters were computed using 2-tailed Student t test. Results are presented as mean±SEM. P<0.05 was considered significant.

Results

FTS Inhibits VSMC Proliferation and IFN-γ Secretion by Lymphocytes In Vitro
As VSMC appear to constitute a predominant portion of the neointima in injured rats, we first evaluated the effect of FTS in vitro on proliferation of carotid artery cultured VSMC. We found that serum-stimulated thymidine incorporation into DNA was significantly reduced in VSMC cultured in the presence of FTS in a dose-dependent manner. The observed degree of inhibition of thymidine uptake by the VSMC at 1 μmol/L, 10 μmol/L, and 100 μmol/L FTS was 19%, 49%, and 93%, respectively (Figure 1A). The concentration for 50% inhibition (IC50) was 10 μmol/L. Under the same conditions, FTS inhibited the Ras-dependent activation of ERK in VSMC, as evident by the observed decrease in phospho-ERK with an estimated IC50 of 10 to 15 μmol/L (Figure 1B).

In agreement with previous results, we found that FTS inhibited lymphocytes proliferation in vitro. Spontaneous and Con A-induced proliferation of rat splenocytes were significantly diminished in the presence of increasing concentrations of FTS (see online Figure I, available at http://atvb.ahajournals.org). Next, we examined the possibility that FTS affects secretion of IFN-γ, a proinflammatory cytokine known to be involved in vascular proliferation. IFN-γ concentrations in the culture media of FTS-treated splenocytes was significantly decreased in FTS-treated cells (Table 1). It should be emphasized that IFN-γ with or without FTS treatment was determined 24 hours after plating, whereas thymidine incorporation was assayed 3 days after plating. Therefore, the observed reduction in the amounts of IFN-γ in the media of the treated cells reflects a true suppression.
FTS Inhibits Ras and ERK Activation in Balloon-Injured Carotid Arteries

Immunohistochemical analysis of carotid arteries obtained from balloon-injured rats (2 weeks after balloon dilatation) demonstrated an abundantly expressed Ras in the neointimal cells, and only low expression was evident in the media and adventitia (Figure 2). We then examined whether FTS can inhibit Ras in the balloon-injured arteries. Rats received 5 mg/kg FTS (intraperitoneally) or vehicle after balloon injury and daily thereafter. Arteries were removed 3 days after the injury and subjected to the determination of total Ras protein and Ras-GTP as an indication of Ras activation and of phospho-ERK as a readout of active Ras signaling. Compared with the vehicle controls, FTS treatment caused a small reduction in total Ras (25%) and a strong reduction in Ras-GTP (70%) and in phospho-ERK (95%) (Figure 3A and B). Quantitative densitometry indicated that the decrease in Ras-GTP and phospho-ERK, not in total Ras, were statistically significant (Figure 3B). Together, these results indicate that FTS affected active Ras in vivo, and this manifested in the reduction of signals downstream of Ras.

FTS Inhibits Neointima Formation

Next, we examined the effect of FTS on the neointima after balloon injury of the carotid. In agreement with other experiments in FTS-treated rats, this treatment did not result in any change in the general health of the rats or in their weight throughout the 2-week treatment (data not shown). The carotids were then removed for the determination of the effect of the treatment on neointimal formation. A first experiment, in which a total of 8 rats (mean age: 14 weeks) were studied, a 76% reduction was evident in the neointimal area in...
FTS-treated rats as compared with control animals (mean area of 38 000 \( \mu \)m\(^2\) in FTS-treated rats as compared with 161 000 \( \mu \)m\(^2\) in the controls; \( P=0.02 \)). Figure 4 depicts representative sections of H&E-stained carotid arteries of noninjured, control, and FTS-treated rats. We then performed a second experiment using 8 controls and 10 FTS-treated rats (mean age: 20 weeks). In this experiment, we found that intimal area was significantly reduced (by 68%) in rats treated with FTS (68 300±22 800 \( \mu \)m\(^2\)) in comparison with control-treated animals (211 600±56 900 \( \mu \)m\(^2\); \( P=0.02 \); Figure 5A). FTS did not significantly influence medial area (240 000±22 000 \( \mu \)m\(^2\)) in the FTS as compared with the control group (220 000±10 600 \( \mu \)m\(^2\), Figure 5B). Intimal to medial ratio (I/M) was significantly reduced in FTS-treated rats (0.3±0.11) as compared with controls (1.0±0.26; \( P=0.02 \); Figure 5C). Taken together, these results demonstrate that FTS selectively inhibited neointima formation without damage to the medial area.

We also conducted immunohistochemical studies on arteries obtained at the time of euthanizing the rats to determine whether the FTS treatment had affected inflammatory (iNOS and NF-kB) and proliferative (Ki-67) cell markers. A quantitative determination of sections from each of the rats was performed accordingly (Figure I). The number of Ki-67-positive cells/total nuclei (proliferative index) in the internal elastic lamina did not differ between FTS-treated animals and controls 2 weeks after injury (data not shown). However, when the effect was tested after 7 days, proliferative index was reduced in FTS-treated as compared with controls (Figure IC).

**Discussion**

The involvement of Ras in vascular proliferative and inflammatory processes has prompted us to evaluate the effects of the Ras inhibitor FTS on smooth muscle cell and lymphocytes in vitro and on neointimal size of carotid balloon-injured rats. A large body of evidence exists to support the contention that dedifferentiated smooth muscle cells form the major cell type within the neointima after arterial injury. Inhibiting VSMC proliferation at the stages that immediately follow arterial damage is expected to result in reduction of neointimal size. We observed that FTS-inhibited thymidine incorporation in cultured VSMC in a dose-dependent manner, attesting for a significant inhibitory effect on proliferation. These results support the notion that the observed reduction in neointimal size in the FTS-treated rats (Figures 4 and 5) is largely caused by the inhibition of Ras and a consequent inhibition of VSMC proliferation.

Consistent with the importance of Ras in VSMC proliferation, we found that Ras expression was robust and localized to the neointimal regions (Figure 2). Moreover, FTS treatment not only decreased neointimal size but also caused a strong reduction in the levels of active Ras-GTP (70%) and active phospho-ERK (95%) in the injured arteries. The small decrease in total Ras (25%) did not gain statistical significance. These results are in accord with the known mechanism of action of the Ras inhibitor, which affects the active GTP-bound forms of Ras proteins. Unlike in tumors that express mutated constitutively active Ras, in normal cells expressing wild-type Ras, Ras-GTP is formed through growth factors and cytokine stimulation of GDP for GTP exchange. Only a small fraction of the total Ras protein is converted into the GTP-bound state, even in the case of robust receptor stimulation. This and the FTS-induced mislocalization of Ras-GTP, not Ras-GDP, explains the stronger and significant reduction in arterial Ras-GTP as compared with total Ras observed in the arteries of the FTS-treated rats (Figure 3). The possibility that FTS affects farnesylated proteins other than Ras seems unlikely, because FTS does not act as a farnesyl transferase inhibitor. In intact cells, it has no effect on prenylated proteins such as the heterotrimeric G proteins or the Rac and Rho proteins. The inhibition of the activated Ras by FTS also explains the lack of toxic or adverse side effects of the compound as shown in previous studies. In these studies, it was found that FTS treatment in rats and mice models had no effect on white and red blood cell counts or on platelets and had no significant effects on body or organ weights.

Inflammatory processes have gained recent interest as mediators of arterial injury. Lymphocytes represent important candidate effector cells that could play a role in restenosis and atherosclerosis, and active Ras is involved in lymphocytes proliferation and differentiation. Systemic administration of FTS is thus expected to inhibit vessel wall infiltration by inflammatory cells. Indeed, we found that similar to VSMC, splenocytes also exhibited a dose-dependent FTS inhibition of proliferation. Moreover, lymphocytes are known to secrete the proinflammatory cytokine, IFN-\( \gamma \), and we observed that this capacity is significantly reduced by treatment of lymphocytes with FTS. IFN-\( \gamma \) has...
recently received interest because it has been demonstrated to have an important role in neointimal formation; therefore, inhibition of IFN-γ activity or reduction in its secretion is expected to contribute to a diminished restenotic process. Supporting the role of inhibitory effect of FTS on inflammation as a causal mechanism was the finding of lower numbers of NF-κB and iNOS-positive cells in the arterial wall of FTS treated rats. The proliferative index assayed 7 days after induction of arterial injury showed that FTS exhibited a suppressive effect on early intimal hyperplasia. However, when assessed at 14 days, no differences were evident between the groups, perhaps because of “escape” of the suppressive effect.

Approaches designed to block Ras-mediated smooth muscle cell proliferation and migration have been successful employing in vitro studies. Moreover, studies using gene delivery of dominant negative forms of ras have been shown effective in suppressing neointimal formation after experimental carotid injury. These studies, together with the present results, point toward Ras protein as an attractive therapeutic target in intimal thickening. However, the described delivery modes harbor potential limitations because of the nonselectivity of ras inhibition and use of the yet unproven safety gene delivery techniques. We believe that FTS may be a fair alternative, not only because of its potential anti-VSMC proliferative capacity but also because of its relative specificity and lack of interference with endothelial proliferation required for an adequate response to injury. In addition, the effect of FTS on active Ras provide a reasonable premise that it will affect mostly the highly active Ras-dependent cells involved in the disease and not other cells and tissues that depend less on active Ras. Indeed, no toxic or adverse side effects have been recorded in animals treated with FTS at the effective doses of the Ras inhibitor. These properties of FTS have proven useful in several experimental tumor models and also in delaying hepatic fibrosis in a cirrhosis model.
Thus, inhibition of active Ras may represent a novel method of reducing restenosis and additional disorders involving neointimal hyperplasia (ie, transplant arteriosclerosis, primary pulmonary hypertension). As current trends favor use of drug-eluting stents for treatment of restenosis, FTS should be tested in local delivery systems. However, recent data suggest that even rapamycin, which is the most widely used drug on stent coatings, is being tested by systemic administration because of the beneficial virtues of this mode of delivery. In any case, Ras inhibition may have particular benefit in stent restenosis, because vascular injury in this context appears to harbor dominant proinflammatory characteristics.

References
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