Mouse Genetic Evidence That Tranilast Reduces Smooth Muscle Cell Hyperplasia via a p21WAF1-Dependent Pathway

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Objective—N-(3′,4′-Dimethoxycinnamoyl)-anthranilic acid (tranilast) is a drug that has been shown to reduce the incidence of restenosis after angioplasty in middle-scale clinical trials. Despite clinical interest in this drug, the pharmacological actions of tranilast remain relatively unexplored at a molecular level.

Methods and Results—We evaluated the effects of tranilast on vascular smooth muscle cell (VSMC) proliferation in wild-type mice and in mice lacking a cyclin-dependent kinase inhibitor, p21WAF1 (p21). Tranilast potently inhibited the proliferation of VSMC cultures derived from wild-type mice, but VSMCs derived from p21-deficient (p21−/−) mice were unaffected by this treatment. In a mouse femoral artery model of vascular injury, tranilast administration to wild-type mice led to an upregulation of p21 expression and a decrease in the number of proliferating VSMCs, as determined by immunostaining for proliferating cell nuclear antigen. In contrast, tranilast had no effect on the number of proliferating cell nuclear antigen–positive cells in the injured arteries of p21−/− mice. Administration of tranilast significantly reduced the neointimal VSMC hyperplasia in wild-type mice at 4 weeks but had no effect on lesion formation in p21−/− mice.

Conclusions—Our findings provide genetic evidence that tranilast inhibits intimal hyperplasia via a p21-dependent pathway, an activity that may contribute to its efficacy in the prophylactic treatment of postangioplasty restenosis. (Arterioscler Thromb Vasc Biol. 2002;22:1305-1309.)

Key Words: pharmacology ■ genetically altered mice ■ smooth muscle cells ■ restenosis ■ proliferation

Percutaneous transluminal angioplasty has been widely adopted as a treatment of atherosclerosis. However, in a significant number of cases, the procedure fails because of postangioplasty restenosis.1 Although the increasing use of new devices to dilate stenosed arteries has lowered the incidence of acute complications, restenosis still limits the long-term outcome of percutaneous interventions.2 Although much effort has been devoted to the development of strategies to prevent postangioplasty restenosis,3 as yet, no pharmacological treatment has been approved to reduce postangioplasty restenosis.

N-(3′,4′-Dimethoxycinnamoyl)-anthranilic acid (tranilast) is a drug that may be effective in preventing angiographic restenosis after percutaneous transluminal coronary revascularization, inasmuch as the results of middle-scale, randomized, double-blind, placebo-controlled trials have shown that oral administration of tranilast significantly lowers the incidence of restenosis after conventional balloon angioplasty,4 directional coronary atherectomy,5 and coronary stenting.6 A multicenter, phase-3, double-blind, placebo-controlled trial is currently under way to evaluate the effects of tranilast on clinical, angiographic, and intravascular ultrasound findings of restenosis.7 Despite the clinical interest in this drug, the pharmacological actions of tranilast remain relatively unexplored, particularly regarding its action in the vasculature. It has been shown to effectively inhibit lesion formation after vascular injury in rabbits fed a high-cholesterol diet.8 Tranilast has been shown to interfere with the migration of vascular smooth muscle cells (VSMCs), a component of postangioplasty restenosis, in vitro.9 Recently, it has been reported that tranilast inhibits VSMC proliferation, another component of restenosis, and that this activity is correlated with upregulation of the cyclin-dependent kinase (Cdk) inhibitor p21WAF1 (p21) in vitro and in vivo.10,11

To clarify the physiological significance of the p21 upregulation by tranilast in the prophylactic treatment of postangioplasty restenosis, we used a mouse model of acute vascular injury to examine the efficacy of tranilast in inhibiting intimal
hyperplasia in vivo in the wild-type (p21+/+) and p21-deficient (p21−/−) mouse strains. These data show that the induction of p21 expression is an essential feature of the antiproliferative actions of tranilast in vitro and in vivo.

Methods

Animals
Adult 30-week-old male p21+/+ mice (129/SvJ background) and p21−/− mice (129/SvJ background, a generous gift from Dr Phillip Leder, Department of Genetics, Harvard Medical School, Boston, Mass) were provided with regular food. All procedures involving experimental animals were performed in accordance with protocols approved by local institutional guidelines for animal care of The University of Tokyo.

Isolation of Mouse Aortic VSMCs and Western Blotting
VSMCs were isolated from outgrowths from explants of thoracic aortas of p21+/+ or p21−/− mice, as described. A replication-defective adenovirus expressing p21 (adeno-p21) has been described. Immunochemistry to detect p21 and α-smooth muscle actin was performed as described. Quiescent VSMCs were stimulated with 10% FBS for 20 hours. Western blotting was performed as described.

In Vitro Proliferation Assay
VSMCs were synchronized in 1% FBS for 48 hours. The cells were then stimulated to proliferate with 10% FBS, 20 μmol/L 5-bromo-2′-deoxyuridine (BrdU), and tranilast (Kissei Pharmaceutical Co) at the indicated concentrations for 21 hours. Anti-BrdU staining was confirmed that the VSMCs derived from p21−/− mice.

Results

Upregulation of p21 and Inhibition of Retinoblastoma Gene Product Phosphorylation by Tranilast
To investigate the molecular mechanism by which tranilast inhibits VSMC growth, we isolated VSMCs from aortas of p21+/+ and p21−/− mice. We verified the purity of the culture by an immunofluorescence study with the use of an anti–α-smooth muscle actin antibody (see online data supplement, available at http://atvb.ahajournals.org). Immunostaining confirmed that the VSMCs derived from p21−/− mice are deficient in p21 and that adenovirus-mediated gene transfer of p21 restored the expression of p21 (see online data supplement).

We investigated the effect of tranilast on p21 expression in wild-type VSMCs stimulated by FBS (Figure 1A). Quiescent VSMCs expressed an appreciable amount of p21 protein, which was slightly upregulated by stimulation with FBS. Quiescent VSMCs were stimulated to proliferate for 20 hours in the absence or presence of tranilast. Cell lysates were subjected to Western blotting by using antibodies against p21 (Santa Cruz Biotechnology), phosphorylated Rb (p-Rb, Santa Cruz Biotechnology), and p27 (Santa Cruz Biotechnology). B. Mouse VSMCs were isolated from aortas of p21+/+ and p21−/− mice were cultured in 1% FBS for 48 hours. The VSMCs were then stimulated to proliferate by 10% FBS in the presence of 20 μmol/L BrdU for 21 hours. Proliferating cells were identified by incorporation of BrdU into DNA. The proportion of BrdU-positive nuclei was counted and expressed as mean ± SEM. *P<0.05 vs control (0 μmol/L tranilast).
was detected in VSMCs derived from p21−/− mice. A moderate dose of tranilast (100 μmol/L) failed to inhibit Rb phosphorylation in p21−/− VSMCs induced by FBS. We also found that tranilast had no effect on expression of p27^{kip1}, another Cdk inhibitor, in wild-type VSMCs and p21−/− VSMCs. Taken together, these results indicate that tranilast inhibits Cdk activity by upregulating p21.

**p21-Mediated Inhibition of VSMC Proliferation by Tranilast In Vitro**

Next, we studied the impact of genetic ablation of p21 on the pharmacological effects of tranilast. Ten to 100 μmol/L tranilast induced p21 expression in wild-type VSMCs in 10% FBS (Figure 1B). Administration of physiological levels of tranilast (10 to 100 μmol/L) po	ently inhibited the proliferation of wild-type VSMCs in a dose-dependent manner, as determined by incorporation of BrdU, an analogue of thymidine, into DNA. Tranilast had no effect on p21−/− cells at any dose examined.

**p21-Dependent Inhibition of VSMC Proliferation by Tranilast In Vivo**

We evaluated the effects of tranilast in p21+/+ and p21−/− mice by using a mouse model of vascular injury that may resemble acute balloon injury. This injury resulted in the rapid onset of medial smooth muscle cell apoptosis and marked enlargement of the lumen, followed by robust VSMC proliferation and the formation of an intimal lesion. At 2 weeks, p21-positive cells were sparsely detected in the neointima as well as in the media (5.9±2.3%) of the injured femoral artery in the p21+/+ mice treated with the vehicle alone (Figure 2A). Administration of tranilast markedly increased the number of p21-positive cells (48.1±6.9%) in the growing neointima as well as in the media. The upregulation of p21 coincided with a decreased number of PCNA-positive cells (85.8±4.7% versus 82.5±4.7%, Figure 2C). In contrast, no p21-positive cells were detected in the injured artery of p21−/− mice. Correspondingly, administration of tranilast did not affect the number of PCNA-positive cells (85.8±4.7% versus 82.5±3.9%). Although p27 expression was expressed in regenerated endothelial cells, we seldom detected p27 in the glowing neointima in wild-type and p21−/− mice, at least at this time point (Figure 2B). There was no significant difference in PCNA expression between the p21+/+ and p21−/− mice treated with the vehicle alone.

**p21-Dependent Reduction of Neointimal Formation by Tranilast**

In this model, the dilated lumen gradually narrows because of neointimal formation that is exclusively composed of α-smooth muscle actin-positive cells. The neointima continues to grow until 3 weeks, after which no significant change in the size of the neointima is observed. There was no significant difference in neointima thickness between the p21+/+ and p21−/− mice treated with the vehicle alone (Figure 3). Consistent with the decrease in the number of proliferating cells at 2 weeks, administration of tranilast significantly reduced the neointima thickening in p21+/+ mice at 4 weeks (intima/media ratio 2.6±0.3 versus 1.5±0.2, P<0.05; Figure 3). In contrast, tranilast had no effect on lesion formation in p21−/− mice (2.3±0.2 versus 2.6±0.3, P<0.05 vs vehicle).
Tranilast partially inhibits smooth muscle cell hyperplasia in p21+/+ mice but not in p21−/− mice. A, A spring wire was inserted into the femoral artery of p21+/+ and p21−/− mice, after which the blood flow of the femoral artery was restored. Mice were administered either the vehicle or tranilast (300 mg/kg per day PO) every day. The injured arteries were harvested at 4 weeks. B, Cross sections harvested at 4 weeks were stained with hematoxylin and eosin (top). Black and white arrows indicate the internal and external elastic laminae, respectively. Bar=50 μm. NI indicates neointima; M, media. The digitized images were analyzed morphometrically by using image analysis software (bottom). All data are expressed as mean±SEM (n=11 for each group). *P<0.05 vs vehicle.

P=NS). Taken together, these findings demonstrate that tranilast significantly reduces neointima hyperplasia via a p21-dependent inhibition of VSMC proliferation.

Discussion

In the present study, we demonstrate that tranilast upregulates the Cdk inhibitor, p21, and that this is accompanied by inhibition of VSMC proliferation in vitro and in vivo in the wild-type mice. Tranilast failed to inhibit VSMC proliferation when endogenous p21 was genetically ablated. These results indicate that tranilast pharmacologically modifies the cell cycle in proliferating VSMCs and reduces neointimal hyperplasia.

Tranilast was originally discovered as an antiallergic drug that inhibits the release and/or production of chemical mediators, cytokines, and active oxygen in inflammatory cells. It has been assumed that tranilast inhibits vascular lesion formation through the anti-inflammatory effects. Our findings provide evidence that inhibition of VSMC proliferation via a p21-dependent pathway may contribute to efficacy of tranilast in the prophylactic treatment of postangioplasty restenosis.

It has been reported that exuberant VSMC proliferation plays a pivotal role in restenosis after balloon angioplasty and stent implantation. Cell-cycle entry and progression, the final common pathway of cell-growth response, depends on the carefully regulated expression and activation of Cdns and their regulatory subunits, the cyclins. The kinase activity of the cyclin/Cdk holoenzyme is also negatively regulated by the Cdk inhibitors. Among the Cdk inhibitors, p21 has a broad specificity for Cdns, and the induction of endogenous p21 in VSMCs has been shown to be crucial in the termination of vascular lesion formation. Furthermore, p21 gene transfer is reported to cause G1 cell-cycle arrest of VSMCs and functions to inhibit neointimal hyperplasia after vascular injury. Thus, p21 represents a potential molecular target for the prevention of intimal hyperplasia that is associated with postangioplasty restenosis. Tranilast has been widely used as an antiallergy medication to treat asthma, hives, atopic dermatitis, rhinitis, and conjunctivitis with a relatively low incidence of adverse effects. Because the safety and efficacy of gene therapy is still controversial, the pharmacological stimulation of endogenous p21 expression by tranilast appears to be more clinically feasible than the transfer of an exogenous p21 gene.

It has been reported that p21 is induced in vascular lesions in pigs and humans, and it has been proposed that p21 plays a role in limiting arterial cell proliferation in vivo. Surprisingly, in the present study, there was no significant difference in PCNA expression or neointima thickness between vehicle-treated p21+/+ and p21−/− mice. These results suggest that other Cdk inhibitors, such as p27KIP1 and p57KIP2, may substitute the function of p21 in the absence of p21 in controlling the proliferation of VSMCs in vascular lesions. Consistent with this notion is the observation that mice lacking p21 develop normally, whereas mice lacking p27KIP1 display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Future studies will clarify temporal and spatial regulation of p27KIP1 or p57KIP2 expression after vascular injury in p21+/+ and p21−/− mice.

One should be cautious in extrapolating our findings to the prophylactic effect of tranilast in patients. We do not know whether p21-dependent reduction of VSMC proliferation by tranilast can be observed in humans as it has in mice, although tranilast has been shown to inhibit human coronary smooth muscle cell proliferation with the upregulation of p21. Furthermore, the pathology of human restenotic lesions after angioplasty and stent implantation is more complex than that of the lesions produced by animal models of vascular injury. Smooth muscle cell accumulation is only one component of restenosis. It is conceivable that tranilast inhibits restenosis because of the cumulative effects of its antiproliferative, immunomodulatory, and antiangiogenic actions.

In conclusion, our findings clarify one of the potential molecular mechanisms by which tranilast inhibits postangioplasty restenosis and suggest that the pharmacological modification of VSMC cell-cycle activity can be considered as an effective strategy in the prophylactic treatment of vascular proliferative diseases.

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