Tranilast Inhibits Cardiac Allograft Vasculopathy in Association With p21$^{\text{Waf1/Cip1}}$ Expression on Neointimal Cells in Murine Cardiac Transplantation Model

Atsushi Izawa, Jun-ichi Suzuki, Wataru Takahashi, Jun Amano, Mitsuaki Isobe

Abstract—Cardiac allograft vasculopathy is a major complication after cardiac transplantation, often limiting long-term recipient survival. N-(3,4-Dimethoxycinnamoyl)anthranilic acid (tranilast) inhibits cyclin-dependent kinase activity through p21$^{\text{Waf1/Cip1}}$ induction and arrests vascular smooth muscle cell proliferation in vitro. We tested a hypothesis that tranilast inhibits the vasculopathy characterized by diffuse intimal thickening in a murine heart transplantation model. Hearts from DBA/2 mice were heterotopically transplanted into B10.D2 mice as allografts. Oral administration of tranilast started 3 days before transplantation at doses of 550 or 1040 mg/kg per day until the animals were killed. Cardiac allograft vasculopathy was defined as luminal stenosis caused by neointimal formation. The percentage of luminal stenosis and cardiac rejection were analyzed 14 and 28 days after transplantation. Tranilast administration was associated with a marked reduction in luminal occlusion but with no significant effect on cardiac rejection. Immunohistochemical study of the tranilast-treated graft coronary arteries revealed enhancement of p21Waf1/Cip1 and decreased expression of proliferating cell nuclear antigen in the neointima. The significant reduction in allograft vasculopathy concomitant with the enhancement of p21$^{\text{Waf1/Cip1}}$ indicates that tranilast has an antiproliferative effect that could be applicable to clinical treatment of cardiac allograft vasculopathy. (Arterioscler Thromb Vasc Biol. 2001;21: 1172-1178.)

Key Words: transplantation ■ cardiac allograft vasculopathy ■ prevention ■ proliferating cell nuclear antigen ■ p21$^{\text{Waf1/Cip1}}$

Cardiac transplantation has evolved into a standard treatment for selected patients with end-stage heart failure. However, cardiac allograft vasculopathy (CAV) is a major posttransplantation complication, often limiting the long-term survival of allograft recipients. The initial CAV event is graft coronary endothelial injury, which is mediated by vascular adhesion molecules, inflammatory cells, and another immune system mediators.1,2 Endothelial damage elicits a repair response characterized by vascular smooth muscle cell proliferation and elaboration of connective tissue components. As a consequence, diffuse fibrous intimal hyperplasia develops. This injury and repair response is one aspect of the inflammatory response and is the generally accepted theory for the pathogenesis of CAV, which is a critical part of the chronic rejection process.1-5

N-(3,4-Dimethoxycinnamoyl)anthranilic acid (tranilast) has been effective in the treatment of allergic diseases, such as bronchial asthma, allergic rhinitis, and atopic dermatitis. This compound has been shown in hypertensive granulomatous inflammation to inhibit the growth of fibroblasts and suppress collagen accumulation,6 activities that are recognized as beneficial antiproliferative actions7 and are clinically effective against keloids and hypertrophic scars. Several recent clinical trials have shown the potent effect of tranilast in preventing restenosis after percutaneous transluminal coronary angioplasty (PTCA)8 and after directional coronary atherectomy.9 A further double-blind placebo-controlled multicenter trial is now testing whether tranilast can prevent restenosis after percutaneous transluminal coronary revascularization with or without stenting for single or multiple vessels.10 This agent inhibits collagen synthesis as well as migration and proliferation of cultured vascular smooth muscle cells.11 We are interested in the advantages of the antiproliferative effect of tranilast for preventing CAV after cardiac transplantation.

A growing body of evidence indicates that cell cycle–regulatory genes and their products play an important part in the pathological conditions related to neointimal formation. The protein product of the Waf1/Cip1 gene, p21$^{\text{Waf1/Cip1}}$, is identified as a universal inhibitor for cyclin-dependent kinases,12-15 which are essential for cell progression through the G1-S check point. Gene transfer of p21$^{\text{Waf1/Cip1}}$ has been shown to inhibit intimal hyperplasia in arterial balloon injury models16,17 and vascular smooth muscle cell proliferation after vein grafting in rabbits.18

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TABLE 1. Allograft Myocardial Rejection Scores

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.25%</th>
<th>0.50%</th>
<th>Control</th>
<th>0.25%</th>
<th>0.50%</th>
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</thead>
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<tr>
<td><strong>Grafts, n</strong></td>
<td>7</td>
<td>5</td>
<td>5</td>
<td>7</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td><strong>Arteries, n</strong></td>
<td>44</td>
<td>25</td>
<td>28</td>
<td>39</td>
<td>43</td>
<td>36</td>
</tr>
<tr>
<td><strong>Rejection score (0–3)</strong></td>
<td>2.07±0.13</td>
<td>1.70±0.20</td>
<td>1.80±0.12</td>
<td>2.88±0.08</td>
<td>2.38±0.23</td>
<td>2.08±0.27</td>
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</tbody>
</table>

Values are mean±SEM. Rejection of heart grafts was graded as described in Methods. Tranilast treatment did not prevent allograft rejection.

The present study reveals that tranilast inhibits neointimal formation in a murine cardiac transplantation model. Immunohistochemical analysis suggests that tranilast induces the expression of p21\(^{\text{Waf1/Cip1}}\) on proliferative neointimal cells.

**Methods**

**Animals**
Male DBA/2 mice (age 4 to 6 weeks, 20 to 25 g) and male B10.D2 mice (aged 4 to 6 weeks, 20 to 25 g) were obtained from Japan Charles River Laboratories, Tokyo, Japan.

**Treatment**
Mice were fed a tranilast-compounded diet consisting of 0.25% or 0.50% tranilast. Prior unpublished studies have revealed these amounts to be the equivalents of 550 and 1040 mg/kg per day, respectively. The plasma concentration of the agent was at its plateau after 3 days of feeding; thus, treatment started 3 days before transplantation and continued until the animals were killed. Control mice received a standard diet.

**Heterotopic Cardiac Transplantation**
Abdominal heterotopic cardiac transplantation was performed in all mice by microsurgery, as previously described. Mice were anesthetized with intraperitoneal 4% chloral hydrate (0.01 mL/g). DBA/2 hearts were transplanted as allografts into B10.D2 recipients. Operation time averaged ~80 minutes. The overall success rate was >90%. The survival of cardiac allografts was assessed by daily palpation. Allografts were removed on postoperative days 7, 10, 14, and 28. The numbers of grafts and analyzed vessels are listed in Table 1.

**Histological Analyses**
Removed allografts were embedded in paraffin. Serial tissue sections (5 μm) were stained with elastica van Gieson to reveal the internal elastic lamina (IEL). From 5 distinct ventricular short-axis sections of each mouse, all of the identified coronary arteries were analyzed. Luminal occlusion of arteries was calculated as percent intimal thickening by two independent observers. The sections were photographed, blindly video-digitized, and stored in an image analysis system (NIH image). The area encompassed by the lumen and IEL was traced carefully, and the cross-sectional area luminal stenosis was calculated as follows: luminal occlusion=IEL area−luminal area)/IEL area×100 (%). Heart graft rejections were diagnosed and graded according to the criteria of the Working Formulation of the International Society for Heart and Lung Transplantation. Grading was from 0 to 3, depending on the amount of lymphocytic infiltration as follows: 0 indicates no infiltration; 1, mild infiltration; 2, moderate infiltration; and 3, severe, extensive infiltration, often with myocytic damage. Two observers in blind review examined slides, and mean scores derived from these independent reviewers were used.

**Immunohistochemistry**
Paraffin-embedded serial sections were deparaffinized and rehydrated, and nonspecific antibody reactions were first blocked in methanol with 0.3% hydrogen peroxide for 30 minutes. After they were rinsed in PBS solution, sections were incubated at 4°C overnight in a humid atmosphere with polyclonal goat anti-mouse p21\(^{\text{Waf1/Cip1}}\) (sc-397-G, 1:500 dilution), polyclonal rabbit anti-mouse p53 (sc-6243, 1:500 dilution), and polyclonal rabbit anti-mouse proliferating cell nuclear antigen (PCNA) antibody (sc-7907, 1:500 dilution) to identify neointimal proliferative activity. Each of the primary antibodies was obtained from Santa Cruz Biotechnology, Inc. The sections were then treated with biotinylated rabbit anti-goat IgG (305-065-045, Jackson ImmunoResearch Laboratories, Inc) and donkey anti-rabbit IgG (711-065-152, Jackson ImmunoResearch Laboratories, Inc). Antibody-biotin conjugates were detected with an avidin-biotin peroxidase complex (Vectorstain ABC Kit, Vector Laboratories) according to the manufacturer’s instructions. Enzyme activity was detected with diaminobenzidine (0.5 mg/mL) with 0.05% NiCl in 50 mmol/L Tris buffer, pH 7.5, and sections were counterstained with Mayer’s hematoxylin. The specificities of the primary antibodies on paraffin-embedded sections were confirmed by immunostaining of normal murine intestine.

**Quantification of Immunostaining**
Immunohistochemically positive cells in the neointima of all coronary arteries were counted, and the p21\(^{\text{Waf1/Cip1}}\), p53-, or PCNA-positive cell ratios were determined as follows: p21\(^{\text{Waf1/Cip1}}\), p53-, or PCNA-positive cells/total neointimal cells×100 (%).

**Detection of Apoptotic Cells**
Apoptotic cells were detected in situ by a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) method with use of an Apoptosis In Situ Detection Kit Wako (Wako Pure Chemical Industries, Ltd). Tissue sections were deparaffinized, rehydrated, and incubated with protein digestion enzyme. After they were washed, the slides were immersed in TdT solution for 10 minutes at 37°C, followed by incubation with 0.3% hydrogen peroxide for 5 minutes. Sections were then treated with peroxidase-conjugated antibody (1:100 dilution). The diUTP incorporation at the site of DNA fragmentation was visualized with diaminobenzidine solution. Stained apoptotic cells in the neointima were counted.

**Analyses of Growth and Cell Proliferation**
Body weight and testis weight of control mice and 0.50% tranilast-treated mice were measured. Intestinal tissue of each mouse was harvested on day 14 (n=5) and on day 28 (n=5), and immunohistochemical staining for PCNA was performed to detect proliferative cells. Five intestinal glands in the lamina propria of each section were blindly selected and analyzed. A PCNA-positive cell ratio of each intestinal gland was determined as follows: PCNA-positive cells/total cells in the gland×100 (%). The cell differentiation and proliferation of bone marrow, spermatogenesis in testis, and germinal cell proliferation in hair follicles of murine skin were also analyzed on day 14 and on day 28, PCNA-positive cell ratios in seminiferous tubules and in hair follicles were calculated in a manner identical to that performed in the intestinal gland.

**Tranilast**
Tranilast was gift from Kissei Pharmaceuticals, Matsumoto, Japan. The drug-compounded chow was made by CLEA Japan Inc.
Statistical Analyses

All data are expressed as mean±SEM. Statistical differences between groups were determined by using 2-factor ANOVA followed by the Scheffé F test for multiple comparisons. A 2-sided value of $P<0.05$ was considered statistically significant. Correlation analyses were performed by the Pearson correlation test for luminal occlusion and PCNA-, p21Waf1/Cip1-, and p53-positive cell ratios.

Results

Graft Survival

All allografts kept beating for the duration of observation. The strength of beat in the tranilast treatment mice was comparable to that in the control mice.

Histological Findings

There was extensive evidence of rejection that was not significantly ameliorated by tranilast (Table 1). Characteristic coronary artery vasculopathies were observed in the nontreated control mice as shown in Figure 1A and 1D. Grafts treated with 0.50% tranilast (Figure 1C and 1F) show inhibitory effects on luminal stenosis. Statistical significance was seen in mice treated with 0.50% tranilast on luminal occlusion (70.3±1.5% [n=7] versus 45.5±5.3% [n=5] on day 14 and 73.3±3.1% [n=7] versus 47.7±4.7% [n=6] on day 28 for control mice versus treated mice, respectively, $P<0.05$; Figure 2). Mice treated with 0.25% tranilast demonstrated no significant effect.

Immunohistochemistry

p21Waf1/Cip1, p53, and PCNA immunostaining was not detectable in normal murine coronary arteries. Immunoreactivity of the primary antibodies was found in the nuclei of neointimal cells. PCNA immunostaining of the grafts is shown in Figure 3A through 3F, and control group sections demonstrate considerable PCNA-positive cells with luminal stenosis (Figure 3A and 3D). Treatment groups showed a marked reduction in PCNA-positive cells: 18.0±1.5% (n=7) versus 6.1±0.9% (n=5) on day 14 (P<0.01) and 17.3±2.3% (n=7) versus 6.6±2.1% (n=6) on day 28 (P<0.01) for control mice versus 0.50% tranilast-treated mice, respectively (Figure 4).

p21Waf1/Cip1 expression in tranilast-treated and control mice is shown in Figure 3G through 3L. In tranilast-treated allografts, p21Waf1/Cip1-positive cells were found in the neointima (Figure 3I and 3L). The enhanced expression of p21Waf1/Cip1 was found: 1.2±0.4% (n=7) versus 5.1±0.4% (n=6) on day 14 (P<0.05) and 1.7±0.4% (n=7) versus 7.3±1.1% (n=6) on day 28 (P<0.01) for control versus 0.50% tranilast-treated mice, respectively (Figure 5).

p53-positive cells were not found among neointimal cells of either control mice or tranilast-treated mice.

A positive correlation was found between luminal occlusion and the PCNA-positive cell ratio ($r=0.914$, $P=0.01$), and a negative correlation was found between luminal occlusion and the p21Waf1/Cip1-positive cell ratio ($r=-0.826$, $P=0.04$).

Detection of Apoptotic Cells

No apoptotic cells were found in the neointima of either control mice or tranilast-treated mice.

Growth and Complications

Body weight and testis weight showed no statistical difference between control mice and 0.50% tranilast-
treated mice (Table 2). No complications occurred as a result of the tranilast treatment. Serum levels of transaminase, alkaline phosphatase, total bilirubin, and cholinesterase showed no aggravation of liver function (data not shown). The bone marrow showed normal blood cell formation. We carefully thought about the tranilast effect on spermatogenesis in testis, proliferation of intestinal cells, and germinative cells in hair follicle of murine skin. These physiologically normal cell proliferations were not inhibited by tranilast treatment (Table 2).

**Discussion**

CAV, characterized by accelerated coronary artery intimal thickening, remains a major cause of late death in cardiac transplant recipients. New immunosuppressive agents are being investigated, and FTY720 and rapamycin have been recently reported to reduce CAV in an experimental model of heart transplantation. Vasculopathy is an immune-mediated chronic inflammation of graft coronary arteries, and it induces migration and proliferation of vascular smooth muscle cells and subsequent intimal thickening.

Tranilast is widely used in the treatment of allergic diseases. Its safety has been established in 15 years of clinical use. It has received recent attention in the field of cardiovascular medicine as an antiproliferative agent; potent inhibitory effects on proliferation and migration of rat and human aortic vascular smooth muscle cells.
have been demonstrated. This antiproliferative action has been investigated in several animal models of arterial injury for the prevention of vascular intimal thickening.\(^{12-35}\)

\(p21^{Waf1/Cip1}\) has been reported to play an important regulatory role in the control of cell proliferation. It is an intrinsic inhibitor of cyclin/cyclin-dependent kinase complexes\(^{12-15}\) and can limit cell progression through G\(_1\) to S phase.\(^{14}\) PCNA has proved to have an essential role in DNA replication and cell proliferation.\(^{37}\) \(p21^{Waf1/Cip1}\) can bind to PCNA and inhibit PCNA function, resulting in both G\(_1\) and G\(_2\) arrest.\(^{38}\) Furthermore, adenovirus-mediated overexpression of \(p21^{Waf1/Cip1}\) has been shown to arrest vascular endothelial and smooth muscle cell proliferation and to inhibit neointimal formation in balloon angioplasty arterial injury models.\(^{16,17}\)

Tranilast has been shown in vitro to arrest the proliferation of human coronary smooth muscle cells at the G\(_1\) phase of the cell cycle via induction of \(p21^{Waf1/Cip1}\) and p53.\(^{39,40}\) Moreover, it has been shown to suppress the neointima/media area ratio by increased expression of \(p21^{Waf1/Cip1}\) in a rat carotid balloon-injury model.\(^{40}\) \(p21^{Waf1/Cip1}\) induction may play an important antiproliferative role in blocking the cell cycle progression of smooth muscle cells.

In a double-blind large-scale multicenter clinical trial, oral tranilast administration at dose of 600 mg/d TID significantly reduced restenosis after PTCA; the restenosis rate was 46.5% with placebo versus 14.7% with tranilast.\(^{5}\) The maximum plasma concentration of tranilast after a single oral administration of 200 mg was 94.1 ± 20.2 \(\mu\text{mol/L}\), and the half-life was 7.4 ± 1.5 hours; the plasma concentrations of the agent after oral doses of 600 mg/d TID were 50 to 200 \(\mu\text{mol/L}\) (Y. Abe, unpublished data, 1995). Mean murine plasma concentrations of orally administered tranilast were 52 \(\mu\text{mol/L}\) (at 550 mg/kg per day) and 148 \(\mu\text{mol/L}\) (at 1040 mg/kg per day) in our preliminary study. The latter concentration was comparable to that observed with clinically administered oral doses of 600 mg/d and matched concentrations that inhibited proliferation of human vascular smooth muscle cells in vitro.\(^{39}\)

This is the first report of the inhibitory effect of tranilast on CAV in a murine cardiac transplantation model. The therapeutic effect was not accompanied with marked side effects. The analyses of growth and cell proliferation showed no difference between control mice and 0.50% tranilast-treated mice. It is an interesting finding that the antiproliferative effect of tranilast was selective for cell proliferation on graft neointimal cells; however, the mechanism of the specificity remains to be investigated.

The reduction of neointimal formation was correlated with enhanced \(p21^{Waf1/Cip1}\) expression and with decreased PCNA in the neointimal cells. Our results are consistent with recent findings that tranilast prevents luminal stenosis in arterial injury models through induction of \(p21^{Waf1/Cip1}\) on proliferating neointimal cells.\(^{40}\) It is important to note, however, that the pathogenesis of CAV is different from that of intimal thickening after balloon injury in the presence of consecutive immune-mediated intimal proliferation. PCNA expression in a pig model of balloon-injured arteries was maximal after 7 days and returned to baseline after 28 days.\(^{41}\) PCNA expression in control nontreated mice showed a mild decrease between day 14 and day 28 but was high on postoperative day 28 (Figure 4), suggesting that graft coronary neointimal cells proliferate constantly over 28 days after transplantation.

\(p21^{Waf1/Cip1}\) has been shown to be regulated via a p53-dependent or p53-independent pathway.\(^{42}\) Recent studies have suggested the tranilast effect on \(p21^{Waf1/Cip1}\) enhancement is through a p53-dependent pathway.\(^{39,40}\) It remains to be determined. The growth-inhibitory effect of \(p21^{Waf1/Cip1}\) was also shown in p53-deficient cells with their ability to bind PCNA.\(^{38}\) In the present study, p53 was not expressed in tranilast-treated neointimal cells, and enhancement of \(p21^{Waf1/Cip1}\) appeared to be achieved through a p53-independent pathway. A possible hypothesis is that the \(p21^{Waf1/Cip1}\) inhibition of PCNA function could reduce neointimal formation. Further analyses will be necessary to elucidate the mechanisms of the \(p21^{Waf1/Cip1}\) enhancement and cell cycle arrest by tranilast.

It has been proposed that \(p21^{Waf1/Cip1}\) induction leads to apoptosis of human epidermoid carcinoma cells\(^{43}\) and vascular smooth muscle cells\(^{44}\) in vitro. Our TUNEL analysis showed no positive cell in the tranilast-treated neointima, suggesting that apoptosis did not play a part in the inhibitory effect of tranilast on CAV.

Tranilast showed no apparent immunosuppressive effect, on the basis of our myocardial rejection scores. This suggests that the therapeutic efficacy of tranilast may be dependent not on an immunologic effect but on its antiproliferative action with the induction of \(p21^{Waf1/Cip1}\). It is possible to use tranilast for inhibition of graft vasculopathy in addition to conventional immunosuppressive agents in the clinical setting because of the absence of immunosuppressive effects. However, there is a need to consider whether the antiallergic effect and the anti-inflammatory effect\(^{e}\) are related to the antiproliferative effect of tranilast on CAV.

### TABLE 2. Analyses of Growth and Cell Proliferation

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<tr>
<th></th>
<th>Day 14</th>
<th>Day 28</th>
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<tr>
<td></td>
<td>Control</td>
<td>0.50% Tranilast</td>
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<td>Body weight, g</td>
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<tr>
<td>5</td>
<td>31.5±0.9</td>
<td>32.6±0.3*</td>
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<tr>
<td>Testis weight, mg</td>
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<tr>
<td>5</td>
<td>77.7±7.9</td>
<td>78.0±7.1*</td>
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<td>PCNA on intestinal gland, %</td>
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<td>5</td>
<td>89.6±1.2</td>
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<tr>
<td>PCNA on seminiferous tubule, %</td>
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<tr>
<td>5</td>
<td>32.5±1.5</td>
<td>33.7±1.1*</td>
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<tr>
<td>PCNA on hair follicle, %</td>
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<tr>
<td>5</td>
<td>67.1±2.5</td>
<td>66.2±1.9*</td>
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</tbody>
</table>

Values are mean±SEM. Percentage of PCNA-positive cells was calculated as described in Methods.

*No statistical difference compared with control.
Although the inhibitory effect of tranilast on CAV was statistically significant, luminal occlusion in the 0.50% tranilast-treated group was still as much as 47.7±4.7% on postoperative day 28 (Figure 2). Thus, there is a study limitation in that we could not demonstrate whether the effect of tranilast provides satisfactory improvement for both long-term coronary perfusion and recipient survival. It is our hope that the present study will open a way for improved drug strategies for prevention of CAV after cardiac transplantation.

In conclusion, we confirm that the antiproliferative effect of tranilast can be useful for preventing intimal thickening of allograft coronary arteries, which is a cardinal feature of CAV. Understanding p21 induction should contribute to our ability to apply the antiproliferative effect of tranilast clinically. Further experiments are needed to address whether clinical administration of tranilast and/or conventional immunosuppressants would enhance allograft survival by preventing CAV.

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References


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