ELSEVIER

European Journal of Pharmacology 433 (2001) 163–168

ejp

www.elsevier.com/locate/ejphar

# Tranilast inhibits transplant-associated coronary arteriosclerosis in a murine model of cardiac transplantation

Akio Saiura [a], Masataka Sata [b,*], Yasunobu Hirata [b], Ryozo Nagai [b], Masatoshi Makuuchi [a]

[a] *Department of Surgery, University of Tokyo, Graduate School of Medicine, Tokyo 113-8655, Japan*
[b] *Department of Cardiovascular Medicine, University of Tokyo, Graduate School of Medicine, 7-3-1 Hongo, Bunkyo, Tokyo 113-8655, Japan*

Received 6 August 2001; received in revised form 25 October 2001; accepted 2 November 2001

**Abstract**

Accelerated coronary arteriosclerosis remains a major problem for the long-term survival of cardiac transplant recipients. However, the pathogenesis of graft vasculopathy is poorly understood and there is no effective therapy. Tranilast is a promising drug that may prevent post-angioplasty restenosis. Here, we investigated whether orally administered tranilast inhibits the development of intima hyperplasia in a mouse model of cardiac transplantation. Cardiac allografts from BALB/c mice were transplanted heterotopically into C3H/He mice. Mice were administered either vehicle or tranilast everyday by gavage. Morphometrical analysis of the cardiac allografts harvested at 2 months revealed that the administration of tranilast significantly reduced the development of coronary atherosclerosis. In the mice treated with tranilast, up-regulation of the cyclin-dependent kinase inhibitor p21 was observed in the allografts, accompanied by a reduced number of proliferating cells. Tranilast also suppressed transforming growth factor-β (TGF-β) expression. Tranilast may be effective in preventing transplant-associated arteriosclerosis through its anti-inflammatory and anti-proliferative effects. 

*Keywords:* Transplantation; Arteriosclerosis; Tranilast; Smooth muscle cell; Proliferation

## 1. Introduction

Although recent advances in immunosuppressive therapy have contributed to the dramatically enhanced early survival of cardiac transplant recipients (Billingham, 1987), accelerated coronary arteriosclerosis has emerged as the major problem facing long-term recipient survival. Histopathological examination of the arteriosclerotic lesions in the long-term cardiac allografts reveals the characteristic diffuse concentric lesions that are distinct from the usual coronary atherosclerotic lesions. The pathogenesis of graft vasculopathy is poorly understood and there is no effective therapy.

Tranilast, *N*-(3′ 4′ -dimethoxycinnamoyl)-anthranilic acid (*N*-5), is a drug that may prevent angiographic restenosis after percutaneous transluminal coronary revascularization, since the results of middle scale, randomized, double-blind, placebo-controlled trials have shown that tranilast significantly lowers the incidence of restenosis after conventional balloon-angioplasty (Tamai et al., 1999), directional coronary atherectomy (Kosuga et al., 1997) and coronary stenting (Hsu et al., 1996). A multicenter, double-blind, placebo-controlled trial is currently underway to evaluate the effects of tranilast on clinical, angiographic, and intravascular ultrasound findings of restenosis (Holmes et al., 2000).

Despite the clinical interest in this drug, the pharmacological actions of tranilast remain relatively unexplored, particularly with regard to its action on the vasculature. Tranilast was originally discovered as an anti-allergic drug that inhibits the release of various cytokines from mast cells and macrophages. Moreover, tranilast has been shown to interfere with the migration of vascular smooth muscle cells, a component of post-angioplasty restenosis, in vitro (Miyazawa et al., 1995). Recently, it was reported that tranilast inhibits vascular smooth muscle cell proliferation, another component of restenosis, and this activity was correlated with the up regulation of the cyclin-dependent kinase inhibitor $p21^{waf1}$ in vitro (Takahashi et al., 1999).

Here, we explored the therapeutic potential of tranilast to prevent neointimal hyperplasia occurring in the coronary arteries of cardiac allografts. Our findings demonstrate that tranilast, a widely prescribed drug without severe adverse

* Corresponding author. Tel.: +81-3-3815-5411; fax: +81-3-3814-0021.
*E-mail address:* sata-2im@h.u-tokyo.ac.jp (M. Sata).

0014-2999/01/$ - see front matter 
PII: S0014-2999(01)01501-1

effects, may serve as a prophylactic treatment of graft vasculopathy via its anti-inflammatory and anti-proliferative actions.

## 2. Materials and methods

### 2.1. Mice

BALB/c H-$2^d$ and C3H/He H-$2^k$ mice were purchased from Japan SLC (Shizuoka, Japan). Adult, male, 6- to 8-week-old mice were used throughout the study. All mice were kept in microisolator cages on a 12-h day/night cycle and fed on regular chow. All procedures involving experimental animals were carried out in accordance with the protocols approved in the local institutional guidelines for animal care of The University of Tokyo and complied with the *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 86-23, revised 1985).

### 2.2. Heterotopic cardiac transplantation

Cardiac transplantation was performed according to the method of Corry et al. (1973). In brief, donors and recipients were anesthetized intraperitoneally prior to surgery with 4% chloral hydrate at 0.01 ml/g body weight. Donor hearts from BALB/c mice were perfused with chilled, heparinized saline via the inferior vena cava. The aorta and pulmonary artery of the donor hearts were anastomosed to the abdominal aorta and inferior vena cava of the recipient C3H/He mice using a microsurgical technique. All recipients were treated with tacrolimus (FK506, Fujisawa Pharmaceutical, Osaka, Japan) intraperitoneally at a dose of 0.3 mg/kg daily to prevent the rejection of the cardiac allograft. The recipient mice were administered either vehicle ($n=6$) or tranilast (300 mg/kg/day, $n=6$) everyday by gavage.

### 2.3. Morphometric analysis

At 2 months, the cardiac allografts were harvested and embedded in paraffin. Serial sections (5 μm) were deparaffinized and stained with hematoxylin and eosin. Middle-sized coronary arteries were analyzed ($n=10$ arteries for each graft). The image was digitized using a Fujix Digital Camera (HC-300/OL, Fuji film, Tokyo) on a PROVIS AX80 microscope (Olympus, Tokyo). Morphometric analysis was performed using image analysis software (LIA32 for WINDOWS95 version 0.372, Kazukiyo Yamamoto, Nagoya, Japan).

### 2.4. Immunohistochemical analysis

Paraffin-embedded sections (5 μm thick) were stained with an anti-$p21^{waf1}$ antibody (sc-397, Santa Cruz) or an anti-transforming growth factor-β1 (TGF-β1) antibody (sc-146, Santa Cruz), followed by the avidin–biotin complex technique and Vector Red (Vector). The immunostaining for the proliferating cell nuclear antigen (PCNA) was performed using an anti-PCNA mouse monoclonal antibody (sc-56, Santa Cruz) and an M.O.M. immunodetection kit (Vector). The sections were counter-stained with hematoxylin.

### 2.5. Statistical analysis

All data were expressed as the mean value ± S.E.M. Mean values were statistically compared by the analysis

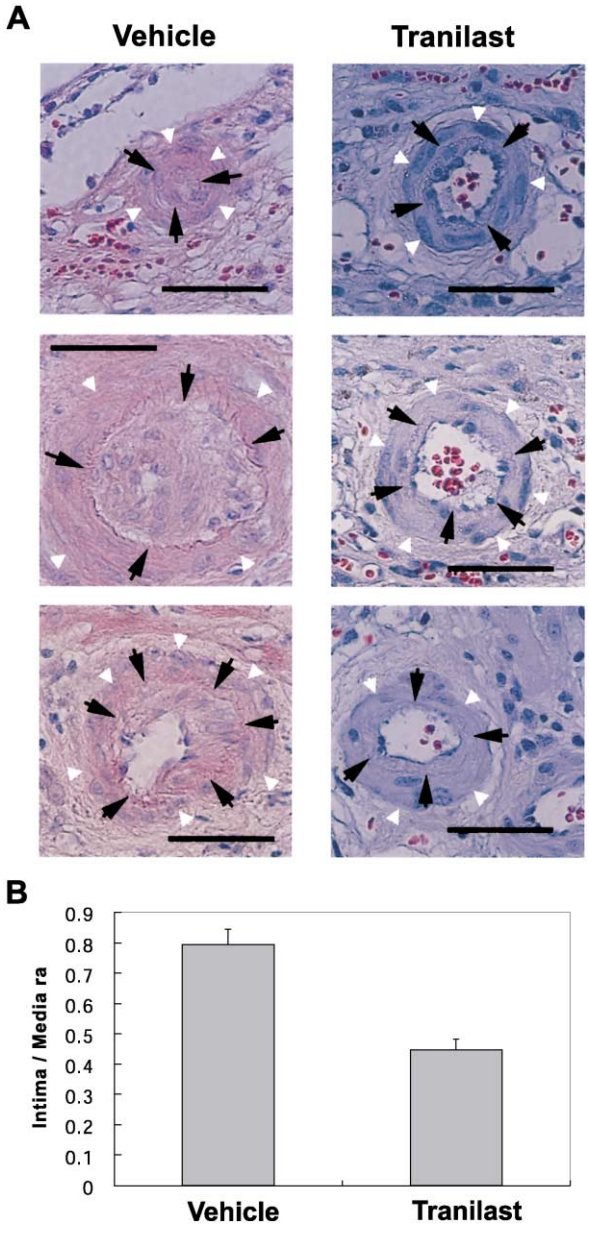


Fig. 1. Inhibitory effect of tranilast on neointimal hyperplasia in the graft coronary arteries. (A) Cross-sections harvested at 2 months were stained with hematoxylin and eosin ($n=6$ for each group). Black arrows and white arrow heads indicate the internal and external laminae, respectively. Bar, 50 μm. (B) The area occupied by the intima and media was measured in middle-sized arteries (10 arteries for each graft). The data are expressed as the mean value ± S.E.M.

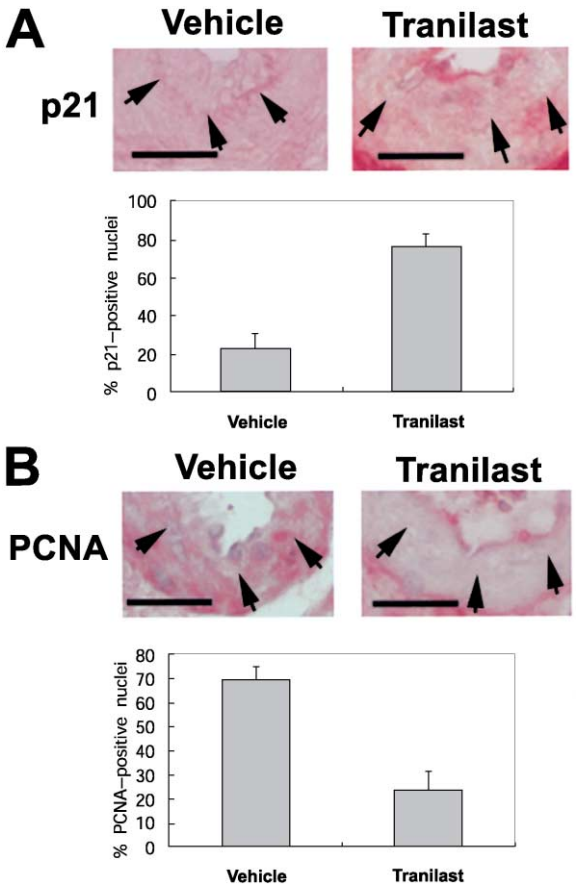


Fig. 2. Anti-proliferative effects of tranilast on murine cardiac allografts. (A) Expression of $p21^{waf1}$ on the sections harvested at 2 months. The proportion of $p21^{waf1}$-positive nuclei was counted and expressed as the mean value ± S.E.M. Arrows indicate internal elastic lamina. Bar, 25 μm. (B) Proliferating cells were identified by immunostaining for anti-proliferating cell nuclear antigen. The proportion of PCNA-positive cells was counted and expressed as the mean value ± S.E.M. Arrows indicate internal elastic lamina. Bar, 25 μm.

of variance (ANOVA) followed by Student's *t*-test. A *P* value of $< 0.05$ was considered to be significant ($n = 6$ for each group).

## 3. Results

### 3.1. Inhibition of neointimal hyperplasia by tranilast

Cardiac allografts from BALB/c mice were transplanted into C3H/He mice heterotopically. Acute rejection was controlled by the administration of FK506 and the transplanted grafts continued to beat. At 2 months after the transplantation, the grafts were harvested for histopathological examination. The coronary arteries of the allografts developed severe arteriosclerosis (Fig. 1A). The graft atherosclerosis diffused through the entire artery rather than being focal. The internal proliferation of graft atherosclerosis occurred without significant damage to the internal elastic lamina. These pathological findings were consistent with those observed in human cardiac grafts (Billingham, 1987). Administration of tranilast (300 mg/kg/day) significantly inhibited the development of neointimal hyperplasia (intima/media ratio: $0.79 \pm 0.05$ vs. $0.45 \pm 0.04$; $n = 6$ for each group) (Fig. 1B).

### 3.2. Induction of $p21^{waf1}$ and inhibition of cell proliferation by tranilast

Next, we investigated the molecular mechanisms by which tranilast inhibited neointimal hyperplasia of the allografts. Previously, it was proposed that tranilast inhibited smooth muscle cells proliferation via a $p21^{waf1}$, a cyclin-dependent kinase inhibitor, -dependent pathway (Takahashi et al., 1999). We studied the effect of tranilast on the expression of $p21^{waf1}$ in the coronary arteries of the allografts (Fig. 2A). In the grafts from the recipient mice treated with vehicle, $p21^{waf1}$ expression was sparsely detected in the coronary arteries ($22.9 \pm 7.6\%$). Tranilast significantly increased the number of $p21^{waf1}$-positive cells in the coronary arteries ($75.9 \pm 6.4\%$). The up-regulation of $p21^{waf1}$ coincided with a decreased number of proliferating cells as determined by anti-PCNA immunostaining ($69.5 \pm 5.4\%$ vs. $23.5 \pm 7.8\%$, $p < 0.05$) (Fig. 2B).

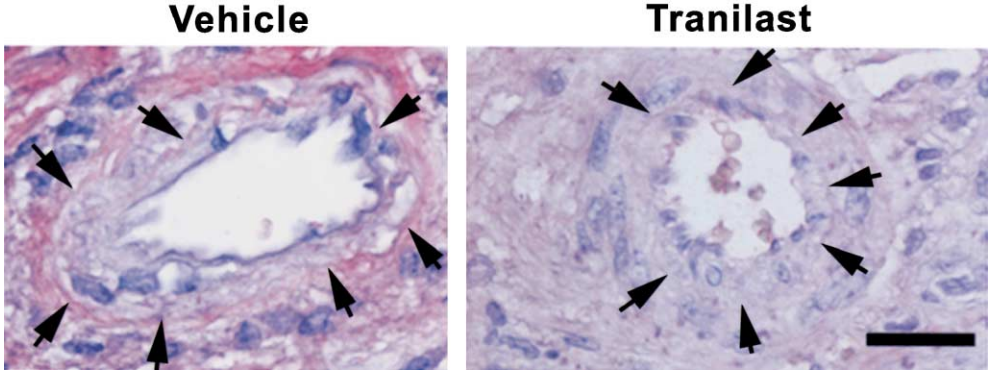


Fig. 3. Anti-inflammatory effects of tranilast on murine cardiac allografts. TGF-β1 expression was detected by immunohistochemistry on the cardiac allografts treated with vehicle or tranilast. Bar, 25 μm.

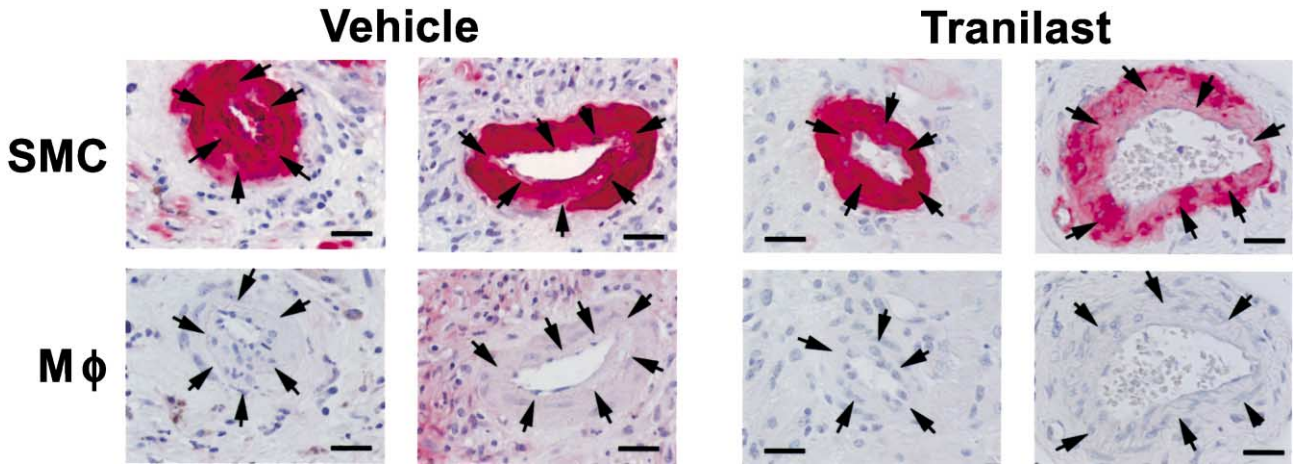


Fig. 4. Cellular composition of the intimal lesions. Two months after transplantation, the cardiac grafts treated with vehicle or tranilast were harvested. The paraffin-embedded sections were stained with antibodies against α-smooth muscle actin, F4/80, and CD3ε to detect smooth muscle cells, macrophages and T cells, respectively. Bar, 25 μm. SMC, smooth muscle cell; Mφ, macrophage.

### *3.3. Suppression of TGF-β expression by tranilast*

Tranilast was initially identified as an anti-atopic agent. Besides its anti-proliferative effects, inhibition of cytokine release from mast cells and macrophages is supposed to contribute to the inhibitory effects of tranilast in the animal models of post-angioplasty restenosis (Ward et al., 1998). Thus, we studied the effects of tranilast on the expression of TGF-β, which has been shown to be a major cytokine accounting for the pathogenesis of various vascular lesions (Clark et al., 2001; Morrell et al., 2001; Nabel et al., 1993; Waltenberger et al., 1996). Consistent with previous in vitro data (Capper et al., 2000), TGF-β expression was suppressed in the graft coronary arteries, when the recipients were treated with tranilast (Fig. 3).

### *3.4. Effects of tranilast on the cellular composition of the intimal lesions*

We investigated the effects of tranilast on the cellular composition of the graft vasculopathy. Two months after transplantation, we harvested the cardiac grafts treated with vehicle or tranilast. In both groups, the neointima was exclusively composed of smooth muscle cells (Fig. 4). We seldom detected macrophages or T cells in the vascular lesions as determined by immunostaining for F4/80 (Fig. 4) or CD3ε (data not shown), respectively. These results indicate that tranilast has little effect on the cellular composition of the intimal lesions.

## 4. Discussion

We examined the effects of tranilast on graft-arteriosclerosis in a murine cardiac transplant model. We found that orally administered tranilast strongly inhibited the development of graft neointimal hyperplasia, which was mainly composed of smooth muscle cells. Tranilast upregulated the expression of cyclin-dependent kinase inhibitor $p21^{waf1}$, coinciding with a decreased number of proliferating smooth muscle cells. TGF-β1 expression in the allografts was also suppressed by tranilast. Taken together, the strong inhibition of graft vasculopathy by tranilast was likely due to its cumulative anti-proliferative and anti-inflammatory effects.

The precise pathogenesis of transplant arteriosclerosis is largely unknown, but like common atherosclerosis, it is believed to result in great part from a localized inflammatory-fibroproliferative response (Ross, 1996). It has been observed that monocytes and T lymphocytes adhere to the endothelium at earlier time points (Hruban et al., 1990; Russel et al., 1994). These activated inflammatory cells secrete various cytokines that induce the migration and proliferation of smooth muscle cells, resulting in an increase of connective tissue. Therefore, various molecular strategies that target the inflammatory response to the graft (Allan et al., 1997; Larsen et al., 1996; Sata et al., 2001) and the subsequent proliferation of smooth muscle cells (Kawauchi et al., 2000; Suzuki et al., 1997) have been tested in animal models of transplantation. However, given the more complex pathology of human graft vasculopathy, no treatment has been established to prevent clinical transplant-associated arteriosclerosis. Consistent with this notion is the clinical evidence that immunosuppresants, such as cyclosporin A or FK506, are known to paradoxically accelerate graft vasculopathy (Billingham, 1987; Calne et al., 1989; Demetris et al., 1985; Meiser et al., 1991; Sommer et al., 1985), probably stimulating the expression of mitogens for vascular smooth muscle cells such as endothelin (Bunchman and Brookshire, 1991) and TGF-β (Hojo et al., 1999). Therefore, a pharmacological strategy targeting both inflammatory response and proliferation have been desired. Our data, along with previously published results, indicate that tranilast may be an effective therapy for preventing graft-

vasculopathy via both anti-inflammatory and anti-proliferative effects.

Up to now, it was thought that medial smooth muscle cells migrated into the subendothelial layer, where they proliferated (Ross, 1996). However, we and the others recently reported that the lesions developed in the allograft coronary arteries were composed of the cells from the recipient, and we proposed the existence of putative smooth muscle progenitor cells that attach to the graft endothelia, proliferate and contribute to graft vasculopathy (Saiura et al., 2001; Shimizu et al., 2001). It is plausible that tranilast affects homing, differentiation and proliferation of putative smooth muscle progenitor cells derived from the recipient.

In conclusion, our results reveal a new therapeutic action of tranilast, i.e., the prevention of graft vasculopathy. Tranilast has been widely used as an anti-allergic medication to treat asthma, hives, atopic dermatitis, rhinitis and conjunctivitis, with a relatively low incidence of adverse effects. Tranilast is an anti-atopic/allergic drug, for oral use, and known to be safe in humans without significant immunosuppressive effects. While the safety and efficacy of gene therapy is still controversial (Friedman, 2000), pharmacological intervention for patients with cardiac allograft-arteriosclerosis seems to be more feasible. Thus, tranilast may be administered as a prophylactic treatment for transplant-associated arteriosclerosis along with conventional immunosuppresive drugs, when clinical studies prove its efficacy.

## Acknowledgements

This study was supported in part by grants from the Japan Heart Foundation, the Tokyo Biochemical Society, the Sankyo Foundation of Life Science, the Japan Foundation of Cardiovascular Research, the Naito Foundation, the Yamanouchi Foundation for Research on Metabolic Disorders, the Japan Research Foundation for Clinical Pharmacology, the NOVARTIS Foundation for the Promotion of Science, the Shionogi Foundation, and the Asahi Glass Foundation (Dr. Sata).

## References

Allan, J.S., Choo, J.K., Vesga, L., Arn, J.S., Pins, M.R., Sachs, D.H., Madsen, J.C., 1997. Cardiac allograft vasculopathy is abrogated by anti-CD8 monoclonal antibody therapy. Ann. Thorac. Surg. 64, 1019.

Billingham, M.E., 1987. Cardiac transplant atherosclerosis. Transplant. Proc. 19, 19.

Bunchman, T.E., Brookshire, C., 1991. Cyclosporine-induced synthesis of endothelin by cultured human endothelial cells. J. Clin. Invest. 88, 310.

Calne, R.Y., Collier, D.S.J., Lim, S., Pollard, S.G., Samaan, A., White, D.J.G., Thiru, S., 1989. Rapamycin for immunosuppression in organ allografting. Lancet 8656, 227.

Capper, E.A., Roshak, A.K., Bolognese, B.J., Podolin, P.L., Smith, T., Dewitt, D.L., Anderson, K.M., Marshall, L.A., 2000. Modulation of human monocyte activities by tranilast, SB 252218, a compound demonstrating efficacy in restenosis. J. Pharmacol. Exp. Ther. 295, 1061.

Clark, K.J., Cary, N.R., Grace, A.A., Metcalfe, J.C., 2001. Microsatellite mutation of type II transforming growth factor-beta receptor is rare in atherosclerotic plaques. Arterioscler., Thromb., Vasc. Biol. 21, 555.

Corry, R.J., Winn, H.J., Russell, P.S., 1973. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. Transplantation 16, 343.

Demetris, A.J., Lasky, S., Theil, D.H., Starzl, T.E., Dekker, A., 1985. Pathology of hepatic transplantation. A review of 62 adult allograft recipients immunosuppressed with a cyclosporine/steroid combination. Am. J. Pathol. 118, 151.

Friedman, T., 2000. Principles for human gene therapy studies. Science 287, 2163.

Hojo, M., Morimoto, T., Maluccio, M., Asano, T., Morimoto, K., Lagman, M., Shimbo, T., Suthanthiran, M., 1999. Cyclosporine induces cancer progression by a cell-autonomous mechanism. Nature 397, 530.

Holmes, D., Fitzgerald, P., Goldberg, S., LaBlanche, J.M., Lincoff, A.M., Savage, M., Serruys, P.W., Willerson, J., Granett, J.R., Chan, R., Shusterman, N.H., Poland, M., 2000. The PRESTO (Prevention of restenosis with tranilast and its outcomes) protocol: a double-blind, placebo-controlled trial. Am. Heart J. 139, 23.

Hruban, R.H., Beschorner, W.B., Baumgartner, W.A., Augustine, S.M., Ren, H., Reitz, B.A., Hutchins, G.M., 1990. Accelerated arteriosclerosis in heart transplant recipients is associated with a T-lymphocyte-mediated endothelialitis. Am. J. Pathol. 137, 871.

Hsu, T.-S., Tamai, H., Ueda, K., 1996. Efficacy of tranilast on restenosis after coronary stenting (abstract). Circulation 94 (Suppl. I), I.

Kawauchi, M., Suzuki, J., Morishita, R., Wada, Y., Izawa, A., Tomita, N., Amano, J., Kaneda, Y., Ogihara, T., Takamoto, S., Isobe, M., 2000. Gene therapy for attenuating cardiac allograft arteriopathy using ex vivo E2F decoy transfection by HVJ-AVE-liposome method in mice and nonhuman primates. Circ. Res. 87, 1063.

Kosuga, K., Tamai, H., Ueda, K., Hsu, Y.S., Ono, S., Tanaka, S., Doi, T., Myou-U, W., Motohara, S., Uehata, H., 1997. Effectiveness of tranilast on restenosis after directional coronary atherectomy. Am. Heart J. 134, 712.

Larsen, C.P., Elwood, E.T., Alexander, D.Z., Ritchie, S.C., Hendrix, R., Tucker-Burden, C., Cho, H.R., Aruffo, A., Hollenbaugh, D., Linsley, P.S., Winn, K.J., Pearson, T.C., 1996. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. Nature 381, 434.

Meiser, B.M., Billingham, M.E., Morris, R.E., 1991. Effects of cyclosporin, FK506, and rapamycin on graft-vessel disease. Lancet 338, 1297.

Miyazawa, K., Kikuchi, S., Fukuyama, J., Hamano, S., Ujiie, A., 1995. Inhibition of PDGF- and TGF-beta 1-induced collagen synthesis, migration and proliferation by tranilast in vascular smooth muscle cells from spontaneously hypertensive rats. Atherosclerosis 118, 213.

Morrell, N.W., Yang, X., Upton, P.D., Jourdan, K.B., Morgan, N., Sheares, K.K., Trembath, R.C., 2001. Altered growth responses of pulmonary artery smooth muscle cells from patients with primary pulmonary hypertension to transforming growth factor-beta(1) and bone morphogenetic proteins. Circulation 104, 790.

Nabel, E.G., Shum, L., Pompili, V.J., Yang, Z.Y., San, H., Shu, H.B., Liptay, S., Gold, L., Gordon, D., Derynck, R., Nabel, G.J., 1993. Direct transfer of transforming growth factor beta 1 gene into arteries stimulates fibrocellular hyperplasia. Proc. Natl. Acad. Sci. U. S. A. 90, 10759.

Ross, R., 1996. Genetically modified mice as models of transplant atherosclerosis. Nat. Med. 2, 527.

Russel, P.S., Chase, C.M., Winn, H.J., Colvin, R.B., 1994. Coronary atherosclerosis in transplanted mouse hearts. Am. J. Pathol. 144, 260.

Saiura, A., Sata, M., Hirata, Y., Nagai, R., Makuuchi, M., 2001. Circulating smooth muscle progenitor cells contribute to atherosclerosis. Nat. Med. 7, 382.

Sata, M., Luo, Z., Walsh, K., 2001. Fas ligand overexpression on allograft endothelium inhibits inflammatory cell infiltration and transplant-associated intimal hyperplasia. J. Immunol. 66, 6964.

Shimizu, K., Sugiyama, S., Aikawa, M., Fukumoto, Y., Rabkin, E., Libby, P., Mitchell, R.N., 2001. Host bone-marrow cells are a source of donor

intimal smooth-muscle-like cells in murine aortic transplant arteriopathy. Nat. Med. 7, 738.

Sommer, B.G., Innes, J.T., Whitehurst, R.M., Sharma, H.M., Ferguson, R.M., 1985. Cyclosporine-associated renal arteriopathy resulting in loss of allograft function. Am. J. Surg. 149, 759.

Suzuki, J.-I., Isobe, M., Morishita, R., Aoki, M., Horie, S., Okubo, Y., Kaneda, Y., Sawa, Y., Matsuda, H., Ogihara, T., Sekiguchi, M., 1997. Prevention of graft coronary arteriosclerosis by antisense cdk2 kinase oligonucleotide. Nat. Med. 3, 900.

Takahashi, A., Taniguchi, T., Ishikawa, Y., Yokoyama, M., 1999. Tranilast inhibits vascular smooth muscle cell growth and intimal hyperplasia by induction of $p21^{waf1/cip1/sdi1}$ and p53. Circ. Res. 84, 543.

Tamai, H., Katoh, O., Suzuki, S., Fujii, K., Aizawa, T., Takase, S., Kurogane, H., Nishikawa, H., Sone, T., Sakai, K., Suzuki, T., 1999. Impact of tranilast on restenosis after coronary angioplasty: tranilast restenosis following angioplasty trial (TREAT). Am. Heart J. 138, 968.

Waltenberger, J., Akyurek, M.L., Aurivillius, M., Wanders, A., Larsson, E., Fellstrom, B., Funa, K., 1996. Ischemia-induced transplant arteriosclerosis in the rat. Induction of peptide growth factor expression. Arterioscler., Thromb., Vasc. Biol. 16, 1516.

Ward, M.R., Sasahara, T., Agrotis, A., Dilley, R.J., Jennings, G.L., Bobik, A., 1998. Inhibitory effects of tranilast on expression of transforming growth factor-beta isoforms and receptors in injured arteries. Atherosclerosis 137, 267.