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Tranilast suppresses intimal hyperplasia after photochemically induced endothelial injury in the rat

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Abstract

Intimal thickening in the femoral artery of spontaneously hypertensive rats (SHR) was initiated by endothelial damage induced by the photochemical reaction between green light and systemic rose bengal. This model represents a non-mechanical method of producing vessel wall denudation. Neointima formation was assessed by calculating the cross-sectional area of intima, media and lumen, using computer analysis. Tranilast (30, 100 and 300 mg/kg, p.o.), administered 2 days prior to endothelial injury, reduced intimal area by 29, 62 and 87%, respectively, compared to that of vehicle-treated controls. In cultured SHR-derived vascular smooth muscle cells, tranilast produced concentration-dependent inhibition of mitogenesis, whether stimulated by platelet-derived growth factor, basic fibroblast growth factor, insulin-like growth factor or fetal bovine serum. These results suggest that tranilast may be effective in preventing coronary restenosis.

Keywords: Tranilast; Photochemically induced thrombosis; Endothelial injury; Smooth muscle cell proliferation; Intimal thickening

1. Introduction

Percutaneous transluminal coronary angioplasty is a current and effective strategy for the treatment of coronary artery disease. However, its major drawback is a 30–40% rate of restenosis within the first 6 months after a successful operation (Serruys et al., 1988; Kaltenbach et al., 1985). Vascular restenosis may be due to migration of smooth muscle cells from media to intima within the arterial wall, proliferation of smooth muscle cells in the intima or excessive extracellular matrix production by smooth muscle cells as a response to endothelial denudation (Liu et al., 1989). The precise mechanisms underlying these processes are not yet fully understood, although a number of studies have indicated that certain neurohumoral factors may interact with smooth muscle cells in an autocrine and/or paracrine manner and thus contribute to the development of intimal hyperplasia. These factors include angiotensin II (Powell et al., 1989; Azuma et al., 1992), noradrenaline (Fingerle et al., 1991) and growth factors such as platelet-derived growth factor (Ferns et al., 1991) and basic fibroblast growth factor (Lindner and Reidy, 1991).

Already, several classes of drugs have been found to exert an inhibitory effect on intimal hyperplasia in animal models. These include antiplatelet drugs (Herbert et al., 1993), angiotensin-converting enzyme inhibitors (Powell et al., 1989) and Ca^{2+} channel antagonists (Arakawa et al., 1992). However, such actions may be indirect and none of the drugs is known to be effective against the restenosis that occurs following percutaneous transluminal coronary angioplasty procedures in humans (White et al., 1987; The MERCATOR Study Group, 1992; Whitworth et al., 1986). In the light of such discrepancies between basic and clinical studies, we badly need the development of new

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drugs which can effectively suppress intimal hyperplasia following percutaneous transluminal coronary angioplasty procedures.

Tranilast, *N*-(3,4-dimethoxycinnamoyl)anthranilic acid has been in clinical use for about a decade in Japan as an effective antiallergic drug (N-5′ Study Group in Children, 1979; Okuda et al., 1984). The antiallergic effect of tranilast is thought to result from an inhibition of the release of chemical mediators from mast cells and basophils (Koda et al., 1976; Komatsu et al., 1988). Recently, several studies have shown that tranilast may also prevent or reduce the formation of keloids and hypertrophic scars through its inhibition of abnormal fibroblast proliferation, collagen synthesis and the production of cytokines and oxygen-free radicals from activated macrophages and neutrophils (Suzawa et al., 1992a, b, c). More recently, it has been reported that tranilast markedly inhibits the proliferation and migration of smooth muscle cells as well as collagen synthesis by these cells (Tanaka et al., 1994). On the basis of these reports, we thought that this drug might be effective against the thickening of the intima which is observed after endothelial injury.

2. Materials and methods

2.1. Intimal thickening model

Intimal thickening in the femoral artery of spontaneously hypertensive rats (SHR) was produced using the photochemically induced thrombosis model described previously (Hirata et al., 1994). In this study, we used SHR as the animal model of intimal hyperplasia, because it is assumed that hypertension is one of the risk factors in arteriosclerosis. This model represents a non-mechanical method of producing vessel wall denudation. Briefly, a total of 31 male SHR (12 weeks, 300–350 g) were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and a cannula was inserted into the jugular vein for rose bengal injection. The right femoral artery was carefully exposed and a pulse Doppler flow probe (PDV-20, Crystal Biotech America, USA) was attached to it for monitoring blood flow. Trans-illumination with green light (wavelength 540 nm) was achieved using a xenon light with both a heat-absorbing filter and a green filter (Hamamatsu Photonics, Hamamatsu, Japan). The irradiation was directed via an optic fibre positioned 5 mm away from a segment of intact femoral artery proximal to the flow probe. Irradiation, at a dose of 0.9 W/cm^2, was started when baseline blood flow was stable and, 5 min later, rose bengal (10 mg/kg) was injected. The femoral artery was considered to be occluded when the blood flow had completely stopped. The occlusive thrombus induced in this model is platelet-rich (Saniabadi, 1994). 30 min after thrombotic occlusion of the irradiated segment, the wound was closed. 24 h later, the rat was re-anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and spontaneous reflow was confirmed by monitoring the blood flow in the artery.

2.2. Drug administration

Tranilast was suspended in 0.5% carboxymethyl cellulose (CMC) and administered orally once a day in the stated dose, starting 2 days before the operation and continued until the animals were killed. Control animals received an equal volume of 0.5% CMC.

2.3. Assessment of intimal hyperplasia

The femoral artery was removed from each rat for histopathological examination 21 days after endothelial injury. Vessels were perfusion-fixed in situ at physiological pressure (160–180 mm Hg) with 1% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.4. Then, the femoral artery was removed and fixed further by overnight immersion in the same fixative. The specimens were sectioned transversely and stained with haematoxylin and eosin for light microscopy. The cross-sectional areas of the intima, media and lumen were calculated using a computerized apparatus (Videoplane, Germany). Mean blood pressure and heart rate were monitored under anaesthesia before the animals were killed.

2.4. Culture of SHR-derived vascular smooth muscle cell

SHR-derived vascular smooth muscle cells for use in our DNA synthesis study were isolated from the thoracic aorta of 12-week old SHR by the explant technique (Ross et al., 1974). Briefly, medial fragments of the thoracic aorta were aseptically sliced into fine pieces using ophthalmic scissors. Four or five slices were placed on a 35 mm plastic plate. The primary culture of smooth muscle cell was grown at 37°C in Dulbecco's modified Eagle's medium (DME) containing 20% heat-inactivated fetal bovine serum, penicillin (50 IU/ml), streptomycin (50 μg/ml) and kanamycin (100 μg/ml) under a humidified atmosphere of 5% CO_2 in air. The culture medium was renewed every 3–4 days. In this way, a confluent smooth muscle cell monolayer was obtained after about 3 weeks. Cells from the eighth passage in 10% fetal bovine serum/DME were used for the following DNA-synthesis study.

2.5. DNA synthesis

The effect of tranilast on cell proliferation was determined by a DNA-synthesis assay method using [^{3}H]thymidine incorporation. SHR-derived vascular smooth muscle cells (2×10^4 cells/well) were plated into 96-well plates in 10% fetal bovine serum/DME and grown until confluency (4 days). Then, the culture medium was aspirated, and the cells were washed with serum-free DME and made quiescent by incubation for 2 days in the same medium. The serum-free medium was then replaced by fresh serum-free medium containing one of four mitogens with tranilast and [^{3}H]thymidine (Dupont-New England Nuclear, Boston, NY, USA) at a final activity of 1.25 μCi/ml. After a 24 h incubation period, the incorporation of [^{3}H]thymidine into DNA was stopped and the cells were washed in ice-cold PBS and harvested using a cell harvester. The radioactivity was measured by liquid scintillation counting.

2.6. Reagents

Tranilast was a gift from Kissei Pharmaceutical Company, Matsumoto, Japan. Recombinant platelet-derived growth factor-BB dimer (PDGF-BB), basic fibroblast growth factor (bFGF) and insulin-like growth factor (IGF-I) were purchased from Funakoshi Company, Tokyo, Japan. Fetal bovine serum was from GIBCO, Grand Island, NY, USA.

2.7. Statistics

Results are presented as means ± S.E.M. Statistical analysis was by analysis of variance (ANOVA) followed

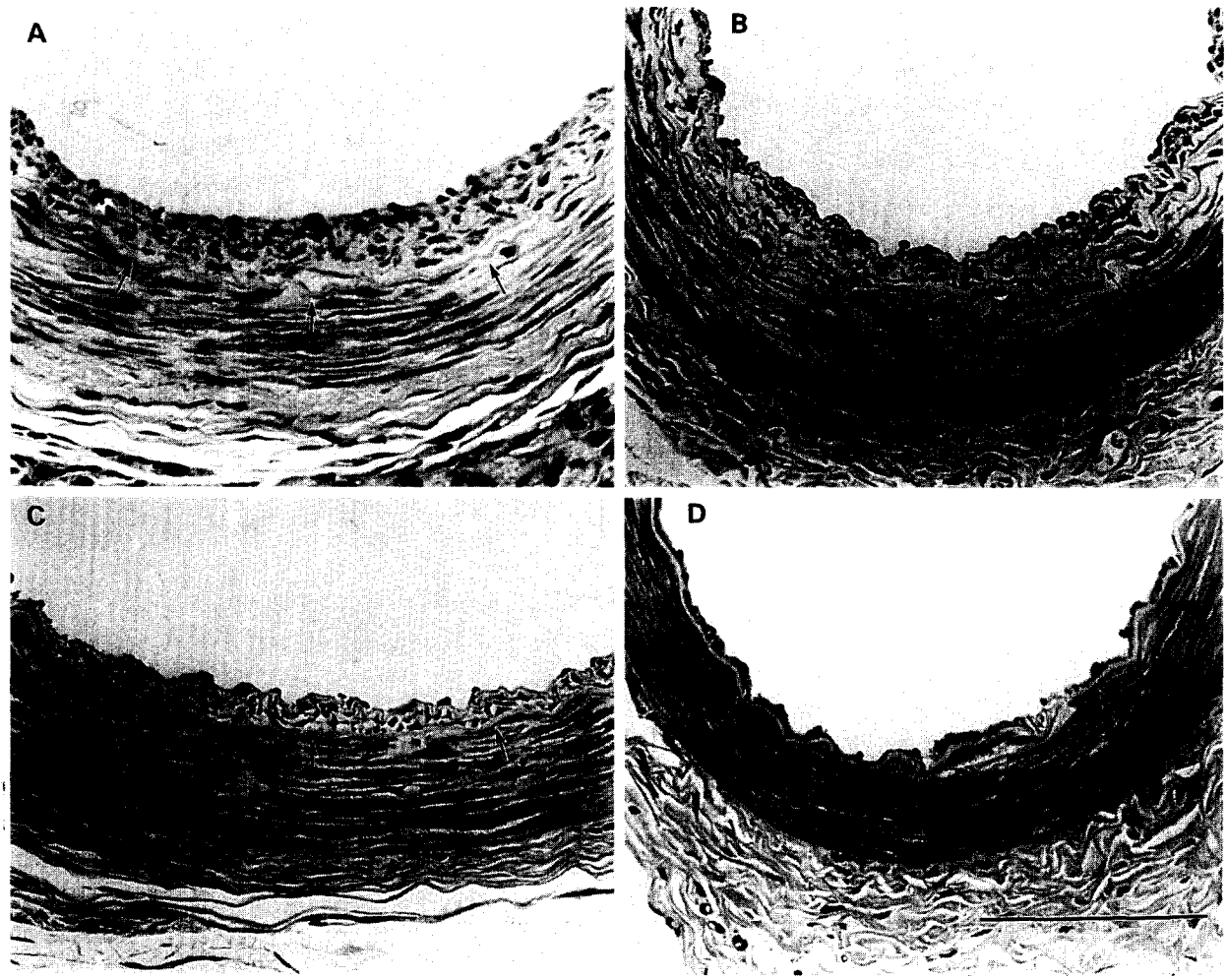

Fig. 1. Light photomicrographs of representative histological cross-sections from SHR femoral artery segments 3 weeks after photochemically induced endothelial injury. (A) Injured vessel treated with vehicle. (B), (C) and (D) injured vessels treated with tranilast (30 mg/kg, 100 mg/kg and 300 mg/kg, respectively). The internal elastic lamina is indicated by the arrows in (A) to (D). Haematoxylin-eosin stain (original magnification × 100; bar = 50 μm).

by a Bonferroni/Dunnett test. A P value < 0.05 was considered significant.

3. Results

3.1. Inhibition of intimal hyperplasia by tranilast

21 days after vessel denudation, a neointima had formed in the subendothelial layers in drug-untreated animals (Fig. 1A). Administration of tranilast, started 2 days before surgery and continued until the end of the experiment, produced a marked decrease in such intimal thickening (Fig. 1B,C,D). Results quantified using morphometric analysis are shown in Fig. 2. The value obtained for the cross-sectional area of the intima in the injured femoral arteries of the group treated with tranilast (30, 100 and 300 mg/kg) was 0.495 ± 0.191, 0.262 ± 0.074 and 0.092 ± 0.030 ($\times 0.01$ mm^2) respectively. The last two values were significantly smaller than that for the vehicle-treated group, which was 0.696 ± 0.149 ($\times 0.01$ mm^2) (Fig. 2A). Expressed as percentages of this control value, the cross-sectional area of the intima was reduced by 28.8, 62.3 and 86.9%, respectively, in the tranilast-treated groups.

The area of the media in the tranilast-treated groups was almost the same as that of the control group (Fig. 2B). The values for the intima/media ratio in the tranilast (30, 100 and 300 mg/kg)-treated groups were 0.124 ± 0.05, 0.069 ± 0.019 and 0.022 ± 0.005, respectively, the last two values being significantly smaller than the intima/media ratio of the untreated group (0.169 ± 0.032; see Fig. 2C).

Pretreatment with tranilast, starting 2 days before the photochemical reaction was induced, had no effect on the time during which the femoral artery was occluded, the values being 14.1 ± 2.5 min for control and for tranilast, 18.2 ± 4.2 min (30 mg/kg), 14.0 ± 1.4 min (100 mg/kg) and 15.5 ± 2.4 min (300 mg/kg). Tranilast

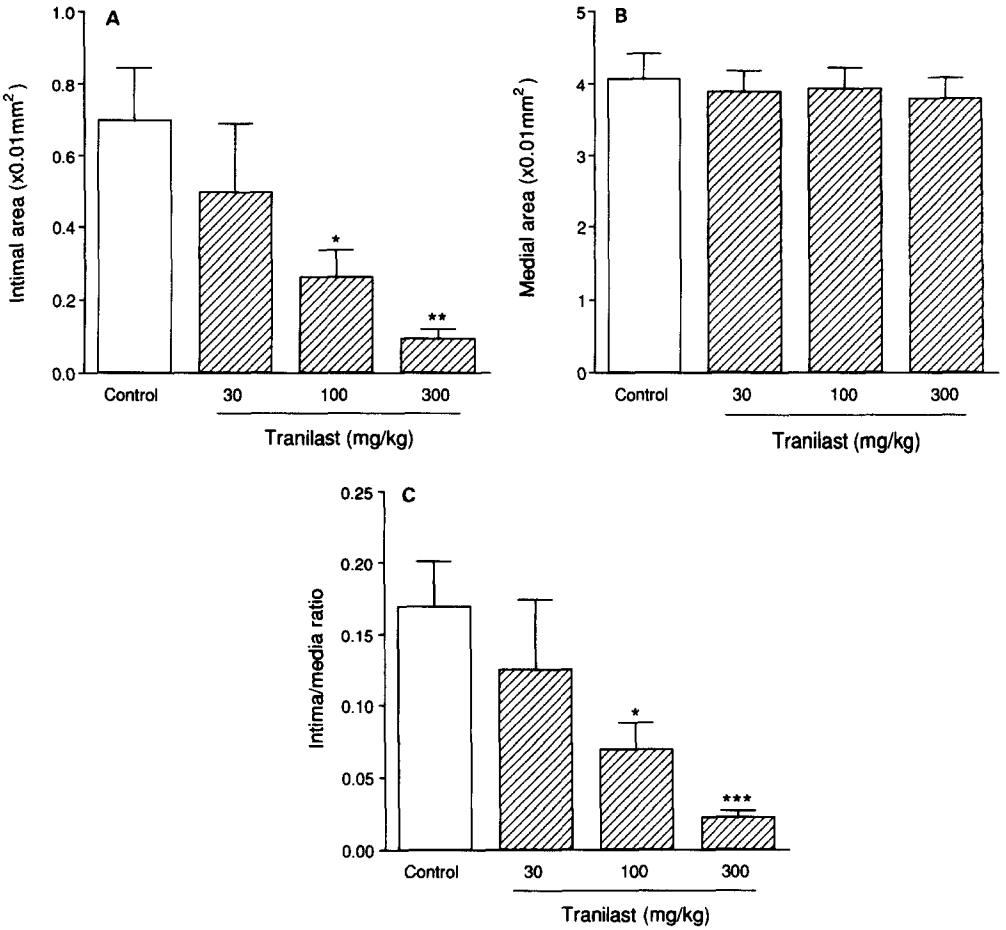

Fig. 2. Bar graph showing the effect of tranilast on (A) intimal area, (B) medial area and (C) intima/media ratio after endothelial denudation in SHR femoral artery. Intimal area and intima/media ratio, but not medial area, were dose dependently reduced by tranilast. Results are presented as means ± S.E.M. ($n = 6$–9). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control value (from vehicle-treated rats).

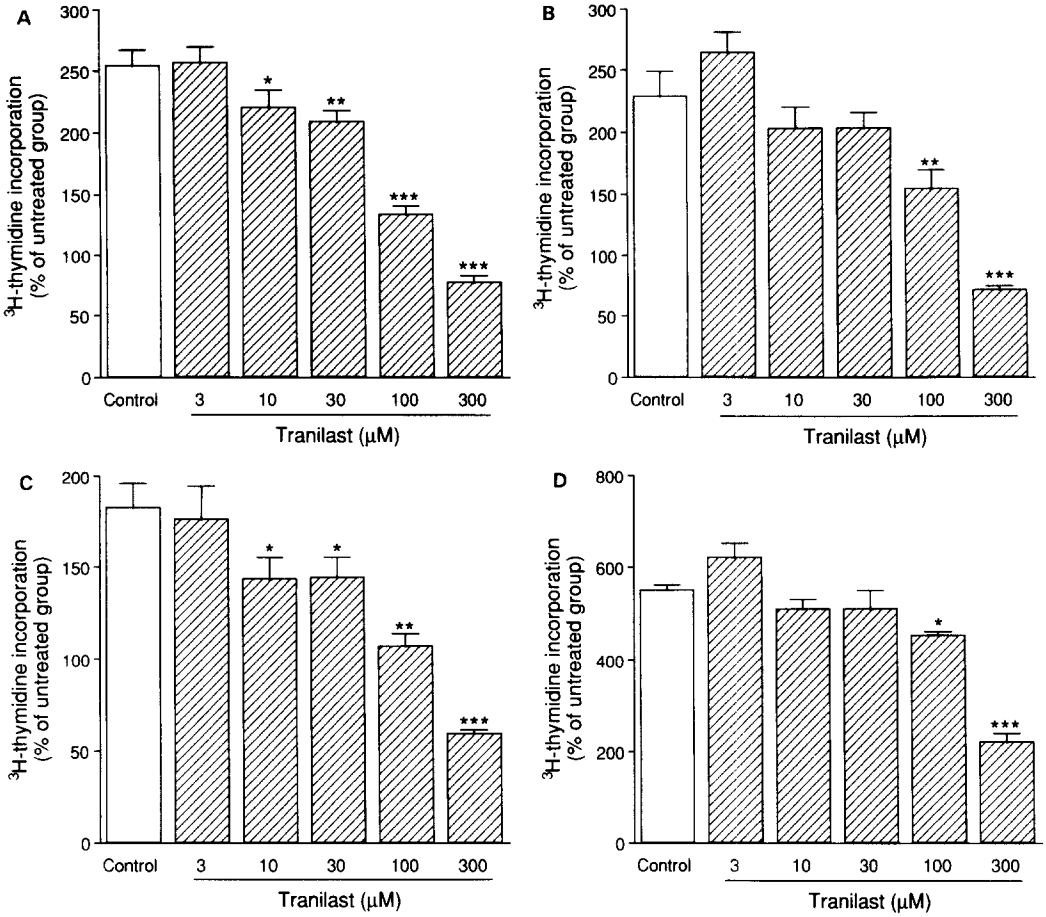

Fig. 3. Bar graph showing the effect of tranilast on the incorporation of [^{3}H]thymidine into SHR-derived vascular smooth muscle cells stimulated by (A) PDGF-BB (0.3 ng/ml), (B) bFGF (10 ng/ml), (C) IGF-I (30 ng/ml) and (D) fetal bovine serum (1%). Growth-arrested smooth muscle cells were stimulated with the indicated mitogen in the absence or presence of the indicated dose of tranilast for 24 h, then 1.25 μCi/ml [^{3}H]thymidine was added concomitantly to the medium. Each column shows the increase in [^{3}H]thymidine incorporation expressed relative to the mean [^{3}H]count (100%) in the absence of any mitogen. Results are presented as means ± S.E.M. ($n = 4$–6). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control value (from vehicle-treated, mitogen-stimulated cells).

did not inhibit the platelet aggregation induced by collagen or ADP in vitro (data not shown). Under our experimental conditions, reflow occurred spontaneously and completely within 24 h after the thrombotic occlusion, with or without drug treatment.

In rats given the highest dose of tranilast (300 mg/kg), mean blood pressure and heart rate (202.5 ± 4.4 mm Hg and 379.3 ± 11.8 beats per min, respectively) were not significantly different from those of untreated controls (200.3 ± 5.4 mm Hg and 389.6 ± 6.9 beats per min, respectively), indicating that the observed suppression of intimal thickening was not secondary to any change in haemodynamics.

3.2. Inhibition of mitogenesis in SHR-derived vascular smooth muscle cells by tranilast

The effect of tranilast on [^{3}H]thymidine uptake by smooth muscle cells after a 24 h incubation period is shown in Fig. 3. Tranilast (3–300 μM) produced a concentration-dependent inhibition of mitogenesis in cultured SHR-derived smooth muscle cells, irrespective of whether mitogenesis was stimulated by PDGF-BB (0.3 ng/ml), bFGF (10 ng/ml), IGF-I (30 ng/ml) or fetal bovine serum (1%).

4. Discussion

In this study, a thrombotic occlusion of the rat femoral artery was induced via the photochemical reaction between transluminal green light and systemically administered rose bengal. Rose bengal is known to be one of the most efficient photosensitizer dyes and its photo-excitation by green light results in the generation of singlet molecular oxygen ($^1O_2$) by a 'Type II photodynamic' energy transfer. Singlet oxygen causes endothelial injury which is followed by platelet adhe-

sion and aggregation and the formation of a platelet-rich thrombus at the site of the photochemical reaction (Saniabadi, 1994). This model represents a non-mechanical method of achieving vessel denudation and experimental intimal thickening (Hirata et al., 1994; Nakashima and Nishiyama, 1994). Following the spontaneous thrombolysis which occurred within 24 h after occlusion, numerous platelets remained adhered to the denuded vessel wall (data not shown). Since adherent platelets may release growth factors such as PDGF and Interleukin-1β which promote smooth muscle cell migration and proliferation (Liu et al., 1989), such growth factors, derived from platelets and injured endothelial cells, may well have contributed to the neointimal accumulation seen in our model. Hence, the initial response in our model, despite there being no mechanical damage to the media, is similar to that seen following balloon angioplasty in humans (Ip et al., 1991). Therefore, this model may be appropriate for investigating the suppressive effect of drugs on post-angioplasty restenosis.

In the assessment of intimal hyperplasia using this model, it is important to determine whether or not the drugs used affect the function of platelets, because an antiplatelet action might alter the extent of endothelial cell damage. Pretreatment with tranilast had no effect on the thrombotic occlusion time of the femoral artery in our model, nor did tranilast inhibit platelet aggregation in vitro. These results indicate that the endothelial cell damage following the photochemical reaction and the burden still imposed by the thrombus following endogenous thrombolysis were probably similar in the control and tranilast-treated groups.

Tranilast produced a dose-dependent inhibition of intimal thickening in SHR femoral arteries. The dosages of tranilast used in this model are about the same as the effective dosage used in the experimental allergic studies (Koda et al., 1976). It also suppressed, in SHR-derived smooth muscle cell, the mitogenesis stimulated of several mitogens, namely PDGF-BB, bFGF, IGF-I and fetal bovine serum. A similar suppressive effect was also produced by tranilast on the proliferation of human-derived vascular smooth muscle cells (data not shown), indicating that its antiproliferative effect is not exerted only in the rat. The concentration of tranilast (100 μM) which inhibits cell proliferation is comparable to the maximum plasma level when tranilast, at a dose of 100 mg/kg, was administered to rats orally (data not shown). A fundamental question arises as to the mechanism(s) underlying the antiproliferative effect of tranilast. It would seem reasonable to assume that a common mechanism by which cell proliferation is stimulated by growth factors is suppressed by tranilast. It is believed that free cytosolic Ca^{2+} serves as a key intracellular signalling mechanism in cell proliferation (Williamson and Monck, 1989). It is in line with this notion that PDGF-BB, bFGF and IGF-I have been reported to increase intracellular Ca^{2+} levels in various cell types through L-type voltage-dependent or -independent Ca^{2+} channels in association with their proliferative effect (Mogami and Kojima, 1993; Kojima et al., 1988; Tsuda et al., 1985). As the proliferative effects of all these stimuli were suppressed by tranilast in the present study, it is a resonable hypothesis that their inhibitory effect on free intracellular Ca^{2+} is the target for tranilast. Further support for this idea may be provided by studies showing that the inhibition of antigen-induced histamine release from mast cells by tranilast occurs via inhibition of Ca^{2+} influx (Komatsu et al., 1988).

A review of the literature on tranilast indicates that this drug has a broad spectrum of pharmacological actions which might lead to a suppression of intimal hyperplasia. These include inhibition of interleukin-1β and release of superoxide radical from activated leukocytes (Suzawa et al., 1992c), chemotactic migration of medial smooth muscle cells into the intima (Tanaka et al., 1994) and extracellular matrix deposition (Suzawa et al., 1992c). These actions of tranilast might well contribute to the prevention of intimal thickening seen in this study.

In a recent clinical trial, tranilast, at a dose of 600 mg/day for 3 months, reduced the rate of post-percutaneous transluminal coronary angioplasty restenosis (Ueda et al., 1993). Further, a double-blind, large-scale multicentre trial which was aimed at evaluating the ability of tranilast to prevent restenosis has shown that this drug, again at a dose of 600 mg/day for 3 months, produced beneficial effects as compared with placebo-control (with tranilast, restenosis rate was reduced to 14.7%, with placebo-control, to 46.5%; The TREAT Study Investigators, 1994). Moreover, no serious adverse reactions were reported in these studies. We conclude that our results, seen in this light, suggest that tranilast may prove to be a valuable agent for the prevention of coronary restenosis.

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