British Journal of Pharmacology (1996) 118, 915-922

Antiproliferative and *c-myc* mRNA suppressive effects of tranilast on newborn human vascular smooth muscle cells in culture

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1 Newborn human vascular smooth muscle cells (VSMCs) proliferated faster and were more sensitive to platelet-derived growth factor-BB (PDGF-BB) than those from adults. In this study, we investigated mechanism of the inhibitory effect of tranilast on PDGF-BB-induced proliferation of VSMCs from newborns.

2 Tranilast (30-300 μM) concentration-dependently inhibited the VSMC proliferation in randomly growing cultures stimulated with PDGF-BB.

3 Tranilast (30-300 μM) concentration-dependently inhibited the [^{3}H]-thymidine incorporation into DNA in VSMCs that had been synchronized by 48 h serum depletion and then stimulated by addition of PDGF-BB. However, tranilast had little influence on unscheduled DNA synthesis in quiescent cells or on RNA and protein synthesis, unlike aphidicolin, actimomycin D, and cycloheximide.

4 In synchronized VSMC cultures, tranilast still inhibited the PDGF-BB-induced DNA synthesis even when added 18 h after stimulation of the quiescent cells. The mode of the antiproliferative action of tranilast was different from that of $NiCl_2$, genistein, or staurosporin. In addition, flow cytometry of synchronized VSMCs treated with tranilast revealed a blockade of PDGF-inducible cell-cycle progression at the G1/S checkpoint.

5 Northern blotting showed that tranilast (30-300 μM) concentration-dependently suppressed constitutive *c-myc* mRNA expression even when added 18 h after PDGF-BB-stimulation of quiescent VSMCs. Tranilast still had an inhibitory effect on the induction of *c-myc* mRNA when *de novo* protein synthesis was inhibited by cycloheximide and did not shorten the degradation of *c-myc* mRNA at the post-transcriptional level, demonstrating that tranilast directly inhibited *c-myc* mRNA expression at the transcriptional level.

6 These results suggest that the inhibitory effect of tranilast on PDGF-BB-induced proliferation is due to S-phase blockade and may be, at least in part, involved in the direct suppression of *c-myc* gene expression. Tranilast did not cause cell toxicity and may therefore hold promising potential for the prevention of vascular proliferative diseases.

Keywords: Tranilast; PDGF; vascular smooth muscle cells; proliferation; *c-myc*; restenosis; PTCA

Introduction

Percutaneous transluminal coronary angioplasty (PTCA) is an established therapy as a nonsurgical revascularization procedure for patients with coronary artery disease (Lange *et al.*, 1993). In contrast to its immediate and good success rate, the long-term efficacy of this procedure is limited by the occurrence of restenosis, the reclosure of coronary arteries, which happens within 3-6 months in approximately 30% to 50% of patients having undergone PTCA (Glagow, 1994). Excessive proliferation of vascular smooth muscle cells (VSMCs) is thought to be a key event in the development of restenosis after PTCA as well as in the formation of the advanced lesions of atherosclerosis (Clowes & Schwartz, 1985; Ip *et al.*, 1990). Various growth factors, vasoactive peptides, and cytokines are involved in the mechanism that causes restenosis (Ross, 1993). Among them, platelet-derived growth factor (PDGF) is considered to play a major role in formation of the neointima that appears after PTCA (Ross, 1993).

The role of the *c-myc* proto-oncogene in the abnormal proliferation of VSMCs in disease states after PTCA has recently received attention (Edelman *et al.*, 1995), because it was shown that the *c-myc* mRNA level in the vessel wall is enhanced after balloon injury (Miano *et al.*, 1993) and that antisense suppression of expression of *c-myc* has an inhibitory effect on both VSMC proliferation in culture and VSMC accumulation following balloon injury (Bennett *et al.*, 1994). The *c-myc* gene was identified as the cellular homologue of the *v-myc* oncogene found in several transforming retroviruses. In untransformed cells, the *c-myc* expression is linked closely to the growth state (Shichiri *et al.*, 1993; Hanson *et al.*, 1994). The *c-myc* gene is down-regulated in quiescent cells, but is rapidly induced by mitogenic growth factors (Kelly *et al.*, 1983). However, whereas most immediate early-response genes such as *c-fos* and *egr-1* are expressed only at the G0/G1 interface (Rothman *et al.*, 1994), *c-myc* is expressed at a constant level throughout the cell cycle in proliferating cells (Han *et al.*, 1985; Rabbitts *et al.*, 1985; Thompson *et al.*, 1985). This suggests that *c-myc* may have a continuous function throughout the cell cycle.

Tranilast, an anti-allergic drug, has been used clinically not only for patients with bronchial asthma, allergic rhinitis, and atopic dermatitis, but also those with keloids and hypertrophic scars (Azuma *et al.*, 1976; Suzawa *et al.*, 1992). Recently, a double-blind, large-scale, multicentre trial has shown the potent effect of tranilast (600 mg day^{-1} for three months) in preventing restenosis (restenosis rate: 14.7%, Tranilast 600 mg day^{-1}, $n=68$, $P<0.001$ vs. 46.5%, placebo, $n=71$) after PTCA (The TREAT study investigators, 1994). Also, we recently found tranilast to inhibit collagen synthesis as well as

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PDGF-induced migration and proliferation, in VSMCs derived from spontaneously hypertensive rats (SHR) (Miyazawa *et al.*, 1995). The VSMCs involved in intimal thickening induced by balloon injury display features indicating active synthesis (Simons *et al.*, 1993) such as occurs in foetal and newborn VSMCs. Also, newborn human VSMCs were shown to proliferate faster than those from adults (Dartsch *et al.*, 1990; Fujita *et al.*, 1993). Therefore, it is appropriate to examine the effect of tranilast on VSMCs from the newborn to explain the clinical efficacy of the drug on restenosis after PTCA.

In this study, tranilast was found to inhibit PDGF-induced proliferation of newborn human VSMCs in culture, and an attempt was made to characterize its mechanism of action. Furthermore, we examined *c-myc* mRNA expression as a potential target for tranilast in the VSMC.

Methods

Cell culture

Newborn and adult human aortic smooth muscle cells at the fourth passage culture were provided by Sanko-Junyaku (Tokyo). Confluent VSMCs were subcultured at a 1:5 split ratio in DMEM supplemented with 10% FBS. The cells were used within passages 5–10, and were characterized as smooth muscle by morphological criteria and by expression of smooth muscle α-actin. The cells were negative in mycoplasma assays.

Cell proliferation assay

We performed the cell proliferation assay by counting the cell number. First, we seeded VSMC at a density of 3×10^3 cells cm^{-2} in DMEM supplemented with 10% FBS in 25 cm^2 tissue culture flasks. The next day, the medium was discarded and fresh DMEM containing each of various concentrations of tranilast plus 50 ng ml^{-1} PDGF-BB was added to the cells. At 2 and 4 days after the addition of drugs, the number of cells was determined by use of a haemocytometer.

Measurement of DNA synthesis

Cells were grown to subconfluence in 96-well tissue culture dishes, and the growth was arrested by incubation for 48 h in a serum-free medium consisting of DMEM supplemented with 5 μg ml^{-1} insulin, 5 μg ml^{-1} transferrin, and 5 ng ml^{-1} selenium (ITS). The DMEM/ITS medium was employed to maintain the VSMC in a quiescent but not catabolic state, which condition resembles that of healthy cells in the normal arterial wall *in vivo* (Libby *et al.*, 1983). The DMEM/ITS medium was then removed, and fresh DMEM containing PDGF-BB was added to the quiescent cells. The cells were subsequently incubated for 20 h in the absence or presence of tranilast. The cells were then incubated with [^{3}H]-thymidine (46 kBq ml^{-1}) for 2 h in the absence or presence of tranilast. Next, ice-cold 10% trichloroacetic acid was added to each well, and the plates were kept at 4°C for 10 min. Trichloroacetic acid-insoluble materials were subsequently harvested onto Unifilter plates (GF/B 96, Packard) with a cell harvester. The extent of [^{3}H]-thymidine incorporation was determined by scintillation counting.

Measurement of macromolecule synthesis

Cells were made quiescent by DMEM/ITS as described above, and fresh DMEM containing tranilast, aphidicolin, actinomycin D, or cycloheximide was added to the cells. The cells were subsequently incubated for 4 h in the absence or presence of these drugs, and subsequently incubated with [^{3}H]-thymidine, [^{3}H]-uridine or [^{3}H]-leucine (46 kBq ml^{-1}) for 1 h in the absence or presence of the drugs. Trichloroacetic acid-insoluble materials were then harvested onto Unifilter plates (GF/B 96, Packard) with a cell harvester. The extent of [^{3}H]-thymidine, [^{3}H]-uridine, or [^{3}H]-leucine incorporation was determined by scintillation counting.

Flow cytometric analysis

To estimate the proportions of cells in different phases of the cell cycle, we measured cellular DNA contents by flow cytometry as described by March *et al.* (1993). Cellular DNA was stained with propidium iodide (40 μg ml^{-1}) for 16 h at 4°C. Cell cycle determination was performed with a Becton-Dickinson FACScan.

Northern blot analysis

Cells were grown to confluence in 175 cm^2 culture flasks, and the growth was then arrested by incubation for 48 h in DMEM/ITS medium. The cells were then stimulated with PDGF-BB (50 ng ml^{-1}). Total RNA was prepared from the cells by the method of Chomczynski & Sacchi (1987). Twenty micrograms of total RNA was loaded onto a 1.2% (w/v) agarose gel containing formaldehyde and, after electrophoresis, transferred onto Hybond N nylon membranes (Amersham, UK) by use of a VacuGene XL vacuum blotting system (Pharmacia/LKB, Piscataway, NJ, U.S.A.). Specific mRNAs were detected by hybridization with appropriate randomly primed ^{32}P-labelled cDNA probes. cDNA probes used were as follows: Human *c-myc* cDNA was a 0.47 kb fragment of human *c-myc* exon 2 (Takara, Tokyo). Human cdk2 cDNA was a 2 kb *Xho*I/*Xho*I fragment (Elledge *et al.*, 1992). Human GAPDH cDNA was a 0.8 kb *Pst*I/*Xba*I fragment (Tso *et al.*, 1985). Autoradiography of resulting Northern blots was quantified by scanning densitometry and integration of peak areas.

Lactate dehydrogenase assay

A lactate dehydrogenase assay (Wako, Osaka, Japan) was carried out on the cell culture supernatant of VSMCs treated with tranilast for 4 days to assess cytotoxic effects. This assay procedure was based on the conversion of NADH to NAD in the presence of pyruvate and LDH.

Materials

Tranilast, N-(3, 4-dimethoxycinnamoyl) anthranilic acid, was synthesized in our laboratories. Foetal bovine serum (FBS) was purchased from GIBCO Laboratories (Grand Island, NY, U.S.A.). DMEM (Dulbecco's Modified Eagle's medium) and PBS (phosphate-buffered saline) were purchased from Nissui (Tokyo). Human recombinant PDGF-BB was purchased from R&D Systems Inc. (Minneapolis, MN, U.S.A.). [^{3}H]-Thymidine, [^{3}H]-uridine, [^{3}H]-leucine, and [^{32}P]-dCTP were obtained from Du Pont-NEN (Daiichi-kaguku, Tokyo). Human *c-myc* probe was purchased from Takara (Tokyo). Human cyclin-dependent kinase 2 (cdk2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes were obtained from American Type Culture Collection (ATCC; Rockville, MD, U.S.A.).

Data analysis

Statistical analysis was performed by ANOVA and Scheffe's *F* test on a Stat View 4.0 software program (Abacus Concepts, Inc., Berkeley, CA, U.S.A.). A P value of <0.05 was considered to be significant.

Results

Effect of tranilast on newborn human VSMC proliferation

Initial studies were designed to confirm the potential of adult and newborn human VSMC proliferation *in vitro*. The new-

born human VSMCs exhibited a markedly faster proliferation than adult human VSMCs (data not shown). Furthermore, the amount of [^{3}H]-thymidine incorporation into newborn human VSMC stimulated by PDGF-BB was significantly greater than that into the adult cells (data not shown).

Cell counting was used to evaluate the effect of tranilast on newborn human VSMC proliferation. As proliferating cells *in vivo* do not progress through the cell cycle in synchrony and are thus in different phases (G1, S, G2/M), we largely examined the effect of tranilast using randomly cycling VSMCs. Since PDGF-BB is a potent mitogen for VSMCs, an examination of the effect of tranilast was performed in the presence of 50 ng ml^{-1} PDGF-BB, a concentration producing submaximal stimulation. Tranilast (30–300 μM) concentration-dependently inhibited newborn human VSMC proliferation stimulated with 50 ng ml^{-1} PDGF-BB (Figure 1). This inhibition was not attributable to death of the cells, for the trypan blue dye exclusion test and LDH assay of cell supernatants demonstrated that tranilast was devoid of any cytotoxic effect on these cells (data not shown). In addition, tranilast did not induce any significant variation in cell detachment at the concentrations tested.

Effect of tranilast on the cell cycle in synchronized newborn human VSMCs

We examined DNA synthesis (S phase) by PDGF-BB [^{3}H]-thymidine corporation between 20 h and 22 h after PDGF-BB stimulation. PDGF-BB stimulated the DNA synthesis in a concentration-dependent manner with submaximal stimulation at 50 ng ml^{-1} in newborn human VSMCs (data not shown). Tranilast (30–300 μM) caused a significant concentration-dependent inhibitory effect on DNA synthesis in quiescent newborn human VSMCs that had been stimulated with 50 ng ml^{-1} PDGF-BB (Figure 2). To clarify the mechanism of the antiproliferative action of tranilast, we first examined the relationship between the time of addition of tranilast and its inhibitory action on PDGF-BB-induced DNA synthesis. As shown in Figure 3, full inhibition of PDGF-BB-induced DNA synthesis still occurred when tranilast was added at 18 h after the PDGF stimulation. In contrast, the inhibitory effects of $NiCl_2$, a blocker of the influx of extracellular Ca^{2+} (Kobayashi *et al.*, 1994); genistein, an inhibitor of tyrosine kinase (Akiyama *et al.*, 1987); and staurosporin, an inhibitor of protein kinase C (PKC) (Tamaoki *et al.*, 1986), were maximal when the drugs were added together with the PDGF-BB stimulation but were diminished if the drugs were added 18 h after the stimulation. Furthermore, experimental results obtained by flow cytometry in this study demonstrated that tranilast inhibited entry into the S phase of the cell cycle. After a 48 h exposure to DMEM/ITS medium, most of the VSMCs were arrested at the G0/G1 phase (G0/G1 = 94.0%, S = 0.6% and G2/M = 5.4%). After replacement of DMEM/ITS medium with DMEM containing 50 ng ml^{-1} PDGF-BB, the emergence of cells into and through the S phase was observed by flow cytometry using quantitative DNA staining with propidium iodide. The cycling cells were found to progress through the S phase approximately 22 h after PDGF-BB stimulation (G0/G1 = 79.9%, S = 17.4%, G2/M = 2.7%). The PDGF-BB-stimulated VSMCs with tranilast (100 and 300 μM) showed a block of cell-cycle progression occurring at G1 phase (100 μM tranilast: G0/G1 = 86.8%, S = 11.8%, G2/M = 1.3%; 300 μM tranilast: G0/G1 = 93.3%, S = 0.8%, G2/M = 5.9%).

Effect of tranilast on macromolecule syntheses in quiescent newborn human VSMCs

To examine whether tranilast has selective and cell-cycle phase non-specific inhibitory effects as do aphidicolin, actinomycin D, and cycloheximide, we examined the effects of tranilast on unscheduled DNA, RNA, and protein synthesis during the G0/G1 phase by measuring [^{3}H]-thymidine, [^{3}H]-uridine, and [^{3}H]-leucine incorporation into DNA, RNA, and protein, re-

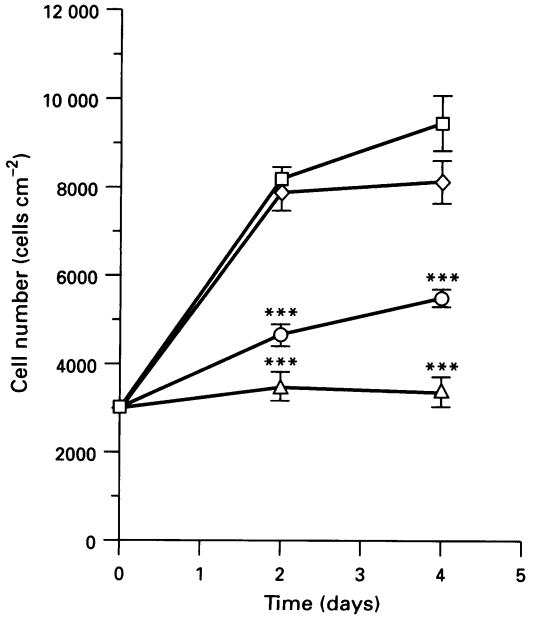

Figure 1 Time course of effect of tranilast on newborn human VSMC proliferation in DMEM supplemented with PDGF-BB. Cells were plated at a density of 3×10^3 cells cm^{-2} in DMEM supplemented with 10% FBS. The next day, the medium was discarded and fresh DMEM containing 50 ng ml^{-1} PDGF-BB plus vehicle (□, control) or 30 μM (◇), 100 μM (○), or 300 μM (△) of tranilast was added to the cells. At 2 and 4 days after the addition of the drugs, the number of cells was determined. Data are shown as means ± s.e. of 4 experiments. ***$P < 0.001$ as compared with the control.

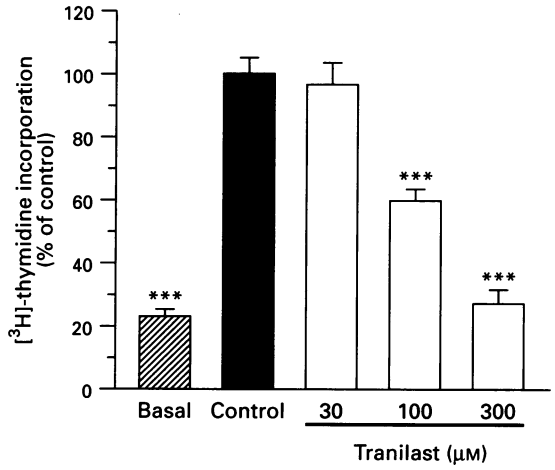

Figure 2 Effect of tranilast on PDGF-BB-induced DNA synthesis in quiescent newborn human VSMCs. Cells were first grown to subconfluence, and the growth was then arrested by incubation for 48 h in serum-free (DMEM/ITS) medium. The DMEM/ITS medium was then removed, and fresh DMEM with (control) or without (basal) 50 ng ml^{-1} PDGF-BB was added to the quiescent cells. The cells were subsequently incubated for 20 h in the absence (solid columns) or presence (open columns) of tranilast, and then incubated with [^{3}H]-thymidine (46 kBq ml^{-1}) for 2 h in the absence or presence of tranilast. Trichloroacetic acid-insoluble materials were harvested, and the extent of [^{3}H]-thymidine incorporation was determined by scintillation counting. Data are shown as means ± s.e. of 10 experiments. ***$P < 0.001$ as compared with the control.

spectively. Trichloroacetic acid-insoluble materials were then harvested onto Unifilter plates (GF/B 96, Packard) with a cell harvester. Tranilast did not have a great influence on the synthesis of these macromolecules in quiescent cells (Table 1).

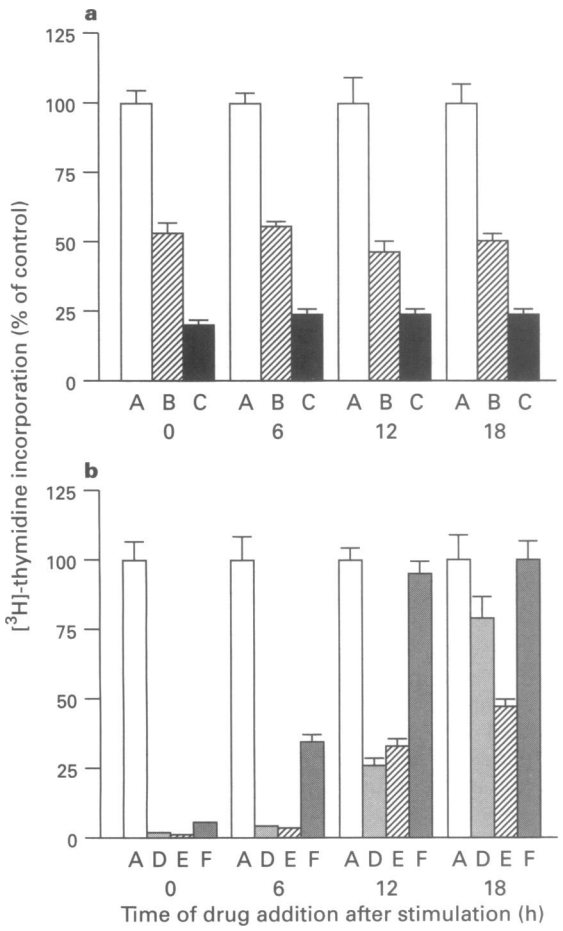

Figure 3 Effects of delayed addition of tranilast, $NiCl_2$, genistein, or staurosporin on PDGF-BB-induced DNA synthesis in newborn human VSMCs. Quiescent cells were stimulated at time zero by the addition of 50 ng ml^{-1} PDGF-BB. Vehicle (control) (A), or 100 μM tranilast (B), 300 μM tranilast (C), 1 mM $NiCl_2$ (D), 30 μM genistein (E), or 1 nM staurosporin (F) was added at the times indicated. The cells were pulse-labelled with [^{3}H]-thymidine (46 kBq ml^{-1}) for 2 h between 20 and 22 h after PDGF-BB stimulation. Trichloroacetic acid-insoluble materials were then harvested, and the extent of [^{3}H]-thymidine incorporation was determined by scintillation counting. Data are shown as means ± s.e. of 6 experiments.

Effect of tranilast on c-myc *mRNA expression in synchronized newborn human VSMC*

The *c-myc* gene is a nuclear proto-oncogene that is deemed to play a role in VSMC proliferation. To address whether the inhibition of DNA synthesis by tranilast was accompanied by modulation of the growth-related oncogene *c-myc*, we examined PDGF-BB-induced *c-myc* expression throughout the cell cycle. As expected (Kelly *et al.*, 1983), the *c-myc* mRNA level was very low in quiescent cells, rose 2–5 fold within 30 min of stimulation, and was constitutively expressed throughout the cell cycle (data not shown). Addition of tranilast (30–300 μM) to the cycling VSMC at 18 h after PDGF-BB stimulation attenuated the accumulation of *c-myc* mRNA in a concentration-dependent manner within 2 h (Figures 4a, 5a). In contrast, the cdk2 mRNA expression was very low in quiescent cells, and after PDGF-BB stimulation it began to increase slowly and was maintained at a constant level from 2 h (data not shown). Tranilast did not inhibit the cdk2 mRNA expression concentration-dependently at 18 h after PDGF-BB stimulation (Figures 4a, 5a). On the other hand, treatments of the VSMCs with $NiCl_2$, genistein, or staurosporin for 2 h did not block the *c-myc* mRNA expression (Figures 4b, 5b).

To elucidate the mechanism of action of the *c-myc* suppressive effect of tranilast, we first investigated whether protein synthesis was required for the tranilast attenuation of *c-myc* expression. The VSMC 18 h after PDGF-BB stimulation were treated with either tranilast, cycloheximide, or the combination of these agents for 30, 60, or 120 min. Then total RNA was isolated, and the level of *c-myc* mRNA in each sample was determined (Figure 6). As expected (Wisdom *et al.*, 1991), cycloheximide alone induced *c-myc* expression; whereas tranilast alone caused a substantial decrease in the mRNA level, and in the presence of both drugs the elimination of mRNA by tranilast was not inhibited completely. Secondly, to examine whether repression of *c-myc* expression by tranilast occurred at a transcriptional or post-transcriptional level, we examined the effects of tranilast on the half-life of *c-myc* transcripts. Quiescent VSMCs were stimulated with PDGF-BB for 18 h to elevate the levels of *c-myc* mRNA. Next, either 10 μg ml^{-1} actinomycin D or 10 μg ml^{-1} actinomycin D and 300 μM tranilast were added, and cells were harvested at several time points between 0 and 120 min. Total RNA was extracted, and the levels of *c-myc* mRNA remaining at the different time points were quantified by densitometry of Northern blots. No difference in the *c-myc* mRNA half-life (22 min) was detected for the VSMC treated with PDGF-BB in comparison with VSMC treated with PDGF-BB plus tranilast (Figure 7). However, a short lag (about 30 min) was seen before message levels declined in tranilast-treated cells. The reason for this lag is unknown, but the lag may reflect the time that tranilast delays actinomycin D incorporation into the cells.

Table 1 Effects of tranilast, aphidicolin, actinomycin D, and cycloheximide on DNA, RNA and protein synthesis in quiescent VSMCs from newborn human subjects

	Incorporation (% of control)		
Treatment	*[^{3}H]-thymidine*	*[^{3}H]-uridine*	*[^{3}H]-leucine*
Control	100.0	100.0	100.0
Tranilast (30 μM)	84.9 ± 15.9	96.3 ± 6.2	98.3 ± 13.1
Tranilast (100 μM)	85.6 ± 7.4	103.3 ± 9.0	87.6 ± 4.7
Tranilast (300 μM)	65.3 ± 10.1	60.8 ± 8.0	77.7 ± 7.5
Aphidicolin (100 μM)	18.2 ± 1.5***	99.2 ± 10.5	83.3 ± 5.8
Actinomycin D (0.1 μM)	37.8 ± 3.9*	28.3 ± 3.1***	88.0 ± 8.8
Cycloheximide (10 μM)	25.8 ± 3.3**	47.1 ± 5.9**	21.5 ± 3.1***

Quiescent cells were incubated with vehicle (control), tranilast (30–300 μM), aphidicolin (100 μM), actinomycin D (0.1 μM), or cycloheximide (10 μM) for 4 h. The cells were then incubated with [^{3}H]-thymidine, [^{3}H]-uridine, or [^{3}H]-leucine (46 kBq ml^{-1}) for 1 h. Trichloroacetic acid-insoluble materials were subsequently harvested, and the extent of [^{3}H]-thymidine, [^{3}H]-uridine, or [^{3}H]-leucine incorporation was determined by scintillation counting. *$P<0.05$, **$P<0.01$, ***$P<0.001$ as compared with the control.

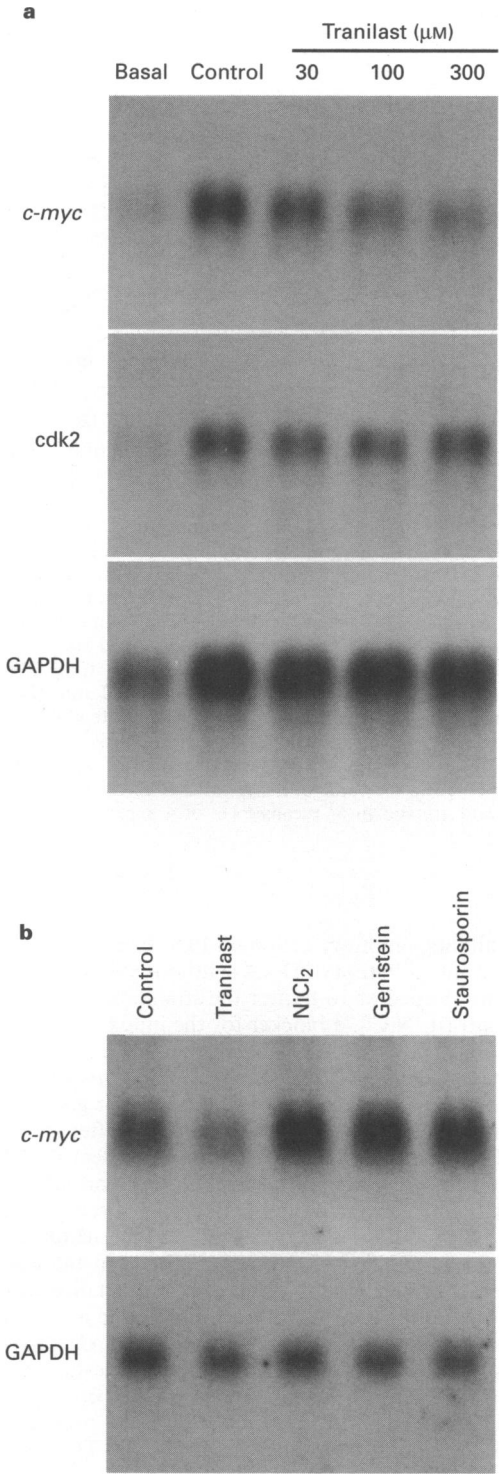

Figure 4 Typical autoradiograms of Northern blot analysis of human VSMC mRNA for *c-myc*, cdk2, and GAPDH in human VSMCs. Fresh DMEM with (control) or without (basal) 50 ng ml^{-1} PDGF-BB was added to the quiescent cells. (a) The cells were incubated for 18 h and then treated with vehicle (control) or 30 μM, 100 μM, or 300 μM tranilast for 2 h. (b) The cells were incubated for 18 h and then treated with vehicle (control) or 300 μM tranilast, 1 mM $NiCl_2$, 30 μM genistein, or 1 nM staurosporin for 2 h. The cells were harvested; and then 20 μg of total RNA was fractionated on a 1.2% (w/v) agarose gel, transferred onto Hybond N nylon membranes, and hybridized to the appropriate cDNA probes.

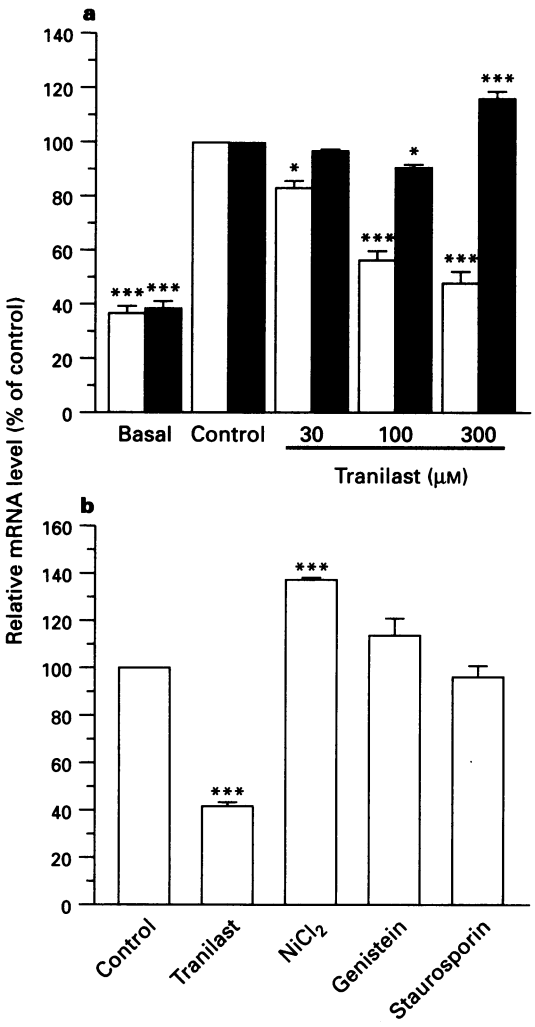

Figure 5 Effects of tranilast, $NiCl_2$, genistein, and staurosporin on PDGF-BB-induced *c-myc* (open columns) or cdk2 (solid columns) mRNA expression. Fresh DMEM with (control) or without (basal) 50 ng ml^{-1} PDGF-BB was added to the quiescent cells. (a) The cells were incubated for 18 h and then treated with vehicle (control) or 30 μM, 100 μM, or 300 μM tranilast for 2 h. (b) The cells were incubated for 18 h and then treated with vehicle (control) or 300 μM tranilast, 1 mM $NiCl_2$, 30 μM genistein, or 1 nM staurosporin for 2 h. Autoradiograms of the Northern blots shown in Figure 4 were quantified by scanning densitometry and integration of peak areas. Relative mRNA level shows the value of *c-myc* or cdk2 mRNA corrected for GAPDH mRNA. Data are shown as means ± s.e. of 4 experiments. $^{*}P<0.05$, $^{***}P<0.001$ as compared with the control.

Discussion

Excessive VSMC proliferation is considered to be a hallmark of restenosis after PTCA and atherosclerotic disease (Macleod *et al.*, 1994). The removal of endothelium appears to be the critical initiating event which is followed by platelet deposition and thrombus formation that lead to the initially predominant process of VSMC proliferation (Epstein *et al.*, 1994). In this context, PDGF has been shown to be involved in the accumulation of VSMCs after balloon injury (Ferns *et al.*, 1991; Jawien *et al.*, 1992), and is present in developing lesions seen in restenosis and atherosclerosis (Wilcox *et al.*, 1988; Majesky *et al.*, 1990). Furthermore, PDGF-BB has been demonstrated to be a potent mitogen and chemoattractant for VSMCs (Grotendorst *et al.*, 1982). These observations validate the importance of the therapeutic aim of inhibiting PDGF-induced VSMC proliferation. Tranilast, which inhibited human VSMC proliferation in this study, as it did SHR-VSMC proliferation in a recent study (Miyazawa *et al.*, 1995), might therefore be expected to prevent or inhibit the progress of vascular diseases. In addition, the inhibitory effect of tranilast occurred at concentrations that are attainable in plasma during therapeutic dosing by oral administration of 600 mg day^{-1} tranilast,

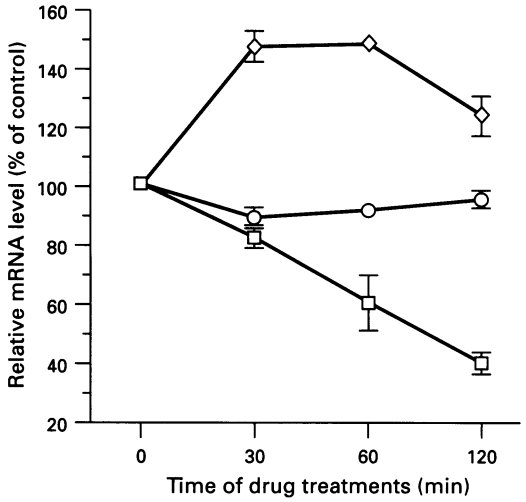

Figure 6 Effects of tranilast and cycloheximide on PDGF-BB-induced *c-myc* expression at early S phase in newborn human VSMC. Fresh DMEM with 50 ng ml^{-1} PDGF-BB was added to the quiescent cells. The cells were incubated for 18 h and then treated for the indicated times (0–120 min) with either 300 μM tranilast (□), 10 μM cycloheximide (◇), or the combination of these drugs (○). The cells were harvested; and then 20 μg of total RNA was fractionated on a 1.2% (w/v) agarose gel, transferred onto Hybond N nylon membranes, and hybridized to the appropriate cDNA probes. Autoradiography of resulting Northern blots was quantified by scanning densitometry and integration of peak areas. Relative mRNA level shows the value of *c-myc* mRNA corrected for GAPDH mRNA. Data are shown as means ± s.e. of 4 experiments.

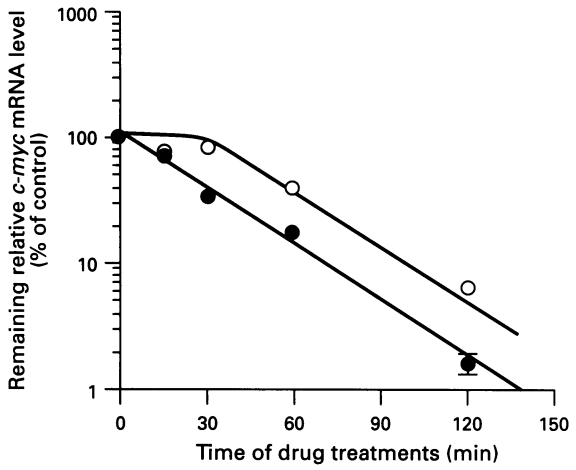

Figure 7 Effects of tranilast and actinomycin D on PDGF-BB-induced *c-myc* mRNA at early S phase in newborn human VSMCs. Fresh DMEM containing 50 ng ml^{-1} PDGF-BB was added to the quiescent cells. The cells were incubated for 18 h and then treated for the indicated times (0–120 min) with 10 μg ml^{-1} actinomycin D (●), or the combination of 10 μg ml^{-1} actinomycin D and 300 μM tranilast (○). After the cells had been harvested, 20 μM of total RNA was fractionated on a 1.2% (w/v) agarose gel, transferred onto Hybond N nylon membranes, and hybridized to the appropriate cDNA probes. Autoradiography of resulting Northern blots was quantified by scanning densitometry and integration of peak areas. Relative mRNA level shows the value of *c-myc* mRNA corrected for GAPDH mRNA. Data are shown as means ± s.e. of 4 experiments.

which concentrations are similar to those having inhibitory effects on extracellular matrix mRNA expression (K. Miyazawa *et al.*, unpublished observation) and angiotensin II-induced activity (Miyazawa *et al.*, 1996). These properties would also contribute towards the vascular protective effect of tranilast.

VSMCs derived from neointima are considered to proliferate faster *in vitro* than those from media (Majesky *et al.*, 1988; Dartsch *et al.*, 1990; Li *et al.*, 1994). In this study, we compared the proliferation between VSMCs from newborn and adult human VSMC in culture. Our data indicated that newborn human VSMCs proliferate faster than adult human ones, thus confirming several earlier reports (Dartsch *et al.*, 1990; Fujita *et al.*, 1993). In addition in terms of reactivity to PDGF-BB, newborn human VSMCs showed a greater concentration-dependent increase in proliferation than adult VSMCs. More recently, studies performed on myosin isoform expression indicated an up-regulation of myosin heavy chain B of the non-muscle type in restenotic arteries and in the foetus and newborn (Leclerc *et al.*, 1992; Aikawa *et al.*, 1993). These characteristics of newborn human VSMCs seem to be similar to those of neointimal VSMCs after PTCA. Thus, newborn human VSMCs are more suitable cells than the adult VSMCs to assess the effect of tranilast on VSMC proliferation, if the goal is to reduce abnormal proliferative activity such as neointimal VSMC after PTCA.

In the present study, we investigated the mechanism of action of tranilast on the VSMC proliferation. PDGF-BB mediates its effects via type α and β tyrosine kinase receptors (Koyama *et al.*, 1994). Upon ligand binding, the intrinsic kinase activities of these receptors are increased, thereby initiating a cascade of events such as elevated phospholipid metabolism and Ca^{2+}-influx, which lead to subsequent DNA synthesis (Ronnstrand *et al.*, 1992; Pazin *et al.*, 1992). By measuring PDGF-BB-induced [^{3}H]-thymidine incorporation into the VSMC, we demonstrated that tranilast concentration-dependently reduced the DNA synthesis. Furthermore, we examined the relationship between the time of the addition of tranilast and its inhibitory action on PDGF-BB-induced DNA synthesis. Full inhibition of DNA synthesis still occurred when tranilast was added at 18 h after the stimulation with PDGF-BB. In contrast, $NiCl_2$, a blocker for the influx of extracellular Ca^{2+} (Kobayashi *et al.*, 1994), genistein, an inhibitor of tyrosine kinase (Akiyama *et al.*, 1987), and staurosporin, an inhibitor of PKC (Tamaoki *et al.*, 1986), did not give complete inhibition when the drugs were added at 18 h after stimulation of PDGF-BB. In addition, tranilast did not affect PDGF-BB-induced Ca^{2+}-influx, or inositol triphosphate formation in the VSMCs (K. Miyazawa, unpublished data). Moreover, tranilast did not have any significant effect on unscheduled DNA synthesis or on RNA and protein synthesis in the quiescent cells. These results suggest that tranilast may act directly on the S phase and inhibit DNA synthesis, unlike the actions of the above inhibitors on the influx of extracellular Ca^{2+}, tyrosine kinase, and PKC. In agreement with this suggestion, flow cytometry of VSMCs synchronized by serum depletion revealed a blockade of PDGF-BB-inducible, cell-cycle progression at the G1/S checkpoint in tranilast-treated cells. Therefore, tranilast may act on the S phase.

VSMCs exhibited tight regulation of *c-myc* expression in response to factors affecting their proliferation (Dean *et al.*, 1986; Bennett *et al.*, 1994). For example, it is known that *c-myc* is down-regulated in response to mitogen withdrawal or to the action of antiproliferative cytokine interferon-γ (IFN-γ) or of growth inhibitors like heparin, cyclic AMP, and cyclic GMP analogues (Pukac *et al.*, 1990; Bennett *et al.*, 1994). Also, antisense oligodeoxynucleotides targeting *c-myc* mRNA were shown to inhibit VSMC proliferation as well as their migration (Biro *et al.*, 1993; Bennett *et al.*, 1994). These findings suggest that *c-myc* suppression may be an important part of the VSMC growth-arrest programme. Since tranilast inhibited DNA synthesis even when added at early S phase, we examined whether tranilast suppresses *c-myc* mRNA expression at this phase. Interestingly, tranilast rapidly down-regulated the *c-myc* expression, but not cdk2 expression, at early S phase, suggesting that the *c-myc* suppressive effect of tranilast may be

a prerequisite event for inhibition of the DNA synthesis. It has been reported that *c-myc* protein has been detected in several cell types in high-molecular-weight complexes containing DNA polymerase alpha and several other enzymes necessary for DNA replication (Studzinski *et al.*, 1991). These observations might suggest a functional role of tranilast for *c-myc* repression during the DNA replicative phase.

Several experiments have been performed to try to elucidate the mechanism by which tranilast inhibits *c-myc* gene expression. First, the *c-myc* suppressive effect of tranilast was shown to be independent of enhancement of intracellular cyclic nucleotide concentrations (Komatsu *et al.*, 1988; K. Miyazawa, personal communication). Second, tranilast blocked elevation of the *c-myc* mRNA levels in the presence of cycloheximide plus PDGF-BB; and tranilast did not induce protein synthesis, as judged from the result on [^{3}H]-leucine incorporation, demonstrating that tranilast did not require synthesis of a protein product but directly suppressed *c-myc* mRNA induction. Finally, actinomycin D experiments provided evidence that tranilast does not affect the stability of *c-myc* mRNA but rather acts to inhibit its transcription. Therefore, tranilast is suggested to have a direct inhibitory effect on *c-myc* transcription at the level of initiation or elongation as indicated for other antiproliferative agents (Kimchi, 1992). However, further experimentation will be required to define the complete relationship between the direct block of *c-myc* transcription and the inhibition of DNA synthesis by tranilast.

Current experimental studies using *c-myc* antisense oligonucleotides in animal models suggest that the repression of *c-myc* beyond the immediate postinjury period is effective and that, in the future, the antisense oligonucleotide may be useful as a therapeutic modality of significance for restenosis after PTCA (Bennett *et al.*, 1994; Edelman *et al.*, 1995). However, the requirement for continued therapy against *c-myc* beyond the initial dosing of antisense oligonucleotide is clinically disturbing as well as scientifically intriguing. Also, it is difficult to store the antisense oligonucleotides in the appropriate restenotic lesions for long periods. On the other hand, tranilast can be absorbed immediately after oral administration and can be administered clinically for long periods of time.

In conclusion, this study has demonstrated that tranilast inhibits PDGF-induced proliferation of newborn human VSMCs, which proliferate more rapidly than adult human VSMCs, by inhibiting S phase of the cell cycle. Furthermore, this inhibitory effect of tranilast may be associated with its *c-myc* mRNA suppressive effect at the transcriptional level. Since tranilast did not cause cell toxicity, it may be a useful drug for prevention of restenosis after PTCA.

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(Received October 4, 1995
Revised February 11, 1996
Accepted March 4, 1996)