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Inhibitory mechanism of tranilast in human coronary artery smooth muscle cells proliferation, due to blockade of PDGF-BB-receptors

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1 We have previously reported that tranilast, an anti-allergic drug, prevented the experimental intimal thickening in the rat and mouse femoral arteries and its effect may be exerted through the inhibition of vascular smooth muscle cell proliferation. However, its inhibitory mechanism has yet to be understood.

2 In this study, we investigated the inhibitory effect of tranilast on platelet-derived growth factor BB-homodimer (PDGF-BB) mediated signal transduction pathways in cultured human coronary artery smooth muscle cells (CASMCs).

3 Growth responses to PDGF-BB were measured by [^{3}H]-thymidine incorporation or cell counting. Activation of DNA synthesis and augmentation of cell proliferation stimulated by PDGF-BB in quiescent cultures of CASMCs were inhibited by tranilast in a concentration-dependent manner.

4 Western blot analysis of lysates from CASMCs with an anti-activated mitogen-activated protein (MAP) kinase antibody revealed that tranilast (10–300 μM) inhibited MAP kinase activation by PDGF-BB in a concentration-dependent manner. Tranilast also reduced PDGF-BB-stimulated tyrosine phosphorylation of a 180 kDa band, corresponding in mass to the PDGF β-receptor, as shown by immunoblots using an anti-phosphotyrosine antibody.

5 Receptor-binding study with [^{125}I]-PDGF-BB on CASMCs showed that tranilast (10–1000 μM) inhibited the specific binding of PDGF-BB to cell surface receptors in a concentration-dependent manner. Scatchard analysis revealed that pretreatment with 300 μM tranilast decreased the maximum binding capacity (B_{max}) from 27.6 to 18.0 fmol 10^6 cells^{-1} without affecting binding affinity ($K_d \approx 0.15$ nM), indicating a non-competitive inhibition of the receptor binding.

6 These results suggest that the suppression of human CASMC growth by tranilast might be at least partly due to blockade of PDGF-BB-receptor binding.

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Keywords: Tranilast; coronary artery smooth muscle cell; proliferation; platelet-derived growth factor; cell signal transduction

Abbreviations: BSA, bovine serum albumin; CASMCs, coronary artery smooth muscle cells; EDTA, ethylenediaminetetraacetic acid; ERK, extracellular signal-regulated kinase; FBS, foetal bovine serum; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MEK, MAP or ERK kinase; PBS, phosphate buffered saline; PBST, phosphate buffered saline containing 0.1% tween-20; PDGF, platelet derived growth factor; PTCA, percutaneous transluminal coronary angioplasty; PTK, protein tyrosine kinase; SAPK, stress-activated protein kinase; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SmBM, Smooth muscle Basal Medium; TCA, trichloroacetic acid; TP, tyrosine phosphorylation; VSMC, vascular smooth muscle cell

Introduction

Tranilast, *N*-(3,4-dimethoxycinnamoyl) anthranilic acid has been in clinical use in Japan as an effective anti-allergy and anti-keloid drug (Koda *et al*., 1976; Suzawa *et al*., 1992). We have previously reported that tranilast shows inhibitory effect in intimal thickening model induced by the photochemical reaction between green light and systemic rose bengal in the rat and mouse femoral arteries (Kikuchi *et al*., 1996; 1998). Tranilast also limited the extent of intimal hyperplasia in balloon injury models of rabbit (Fukuyama *et al*., 1996a) and dog (Shiota *et al*., 1999). In a recent randomized, double-blind TREAT study (Tamai *et al*., 1999), tranilast at a dose of 600 mg day^{-1} for 3 months has shown the potent effect in preventing restenosis after percutaneous transluminal coronary angioplasty (PTCA). It has also been reported that tranilast strongly prevented restenosis after directional coronary atherectomy (Kosuga *et al*., 1997).

It is well-known that intimal thickening contributes to coronary restenosis following PTCA (Hamon *et al*., 1995). Intimal thickening may be due to migration of vascular smooth muscle cells (VSMCs) from media to intima within the arterial wall, unscheduled proliferation of VSMCs in the intima or excessive extracellular matrix production of VSMCs as a response to endothelial denudation (Liu *et al*., 1989; Ross, 1986; Schwartz *et al*., 1992). In these processes, cell growth factors such as PDGF released from activated platelets, VSMCs, and monocytes have been considered to play a key role (Ross, 1993). Therefore, it is anticipated that the blockade of cell signal transduction stimulated by these factors would be effective in preventing the initiation and progression of intimal thickening.

Recent advances in cellular and molecular biology have provided insight into the molecular mechanisms of growth

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factor-activated signal transduction in the cell growth control pathways. Cell proliferation is initiated by the transduction of mitogenic signals from cell surface receptor to nucleus *via* receptor protein tyrosine kinase, Ras, Raf1, MAP kinase kinase (MEK1), and MAP kinase (Marshall, 1994). Extracellular signal-regulated kinase (ERK) pathway is one of the three well-characterized MAP kinase pathways, namely JNK/SAPK, p38/MPK2, and ERK/MAPK (Hunter, 1997). ERK is a serine/threonine kinase, which is activated by a dual phosphorylation on threonine and tyrosine residues (Nishida & Gotoh, 1993). Phosphorylated and activated ERK migrates to the nucleus, where it phosphorylates several nuclear transcription factors, including ELK-1, ultimately leading to initiation of the gene transcription, the transition of cells from a quiescent to a proliferative state, DNA synthesis, and cell division (Janknecht & Hunter, 1997; Price *et al*., 1996). It has emerged that in membrane-to-nucleus signaling pathways, both p44 and p42 ERK isoforms (ERK1 and ERK2, respectively) play a critical role in the regulation of cell proliferation (Cowley *et al*., 1994; Hunter, 1995; Marshall, 1995).

Previously, Tanaka *et al*. (1994) demonstrated that tranilast has a marked inhibitory effect on cell proliferation induced by serum in cultured rat-derived VSMCs. Recently, it has been reported that tranilast also inhibits in VSMC proliferation induced by PDGF-BB, which is a powerful mitogenic isoform of PDGF (Fukuyama *et al*., 1996b; Miyazawa *et al*., 1995). We also obtained similar results from the *in vitro* studies with rat VSMCs (Kikuchi *et al*., 1996; Miyazawa *et al*., 1997). Multiple inhibitory actions of tranilast to PDGF-induced proliferation in VSMCs have been demonstrated, including the suppression of the early gene c-*myc* mRNA expression (Miyazawa *et al*., 1996), the restoration of cytokine-induced nitric oxide production (Hishikawa *et al*., 1996), the inhibition of calcium entry (Nie *et al*., 1996), and the suppression of transforming growth factor-β isoforms expression (Ward *et al*., 1998). However, the precise mechanisms underlying these inhibition have not yet been fully understood.

It is known that PDGF is released from activated platelets, which adhere to the sub-endothelial tissue at the site of endothelial injury, and PDGF may play a key role in VSMC proliferation. Taken together, it is suggested that the suppressive effect of tranilast on VSMC proliferation may be caused by the inhibition of PDGF-mediated events. Therefore, we hypothesized that the inhibition of PDGF-stimulated cell proliferation by tranilast would be due to the interference of PDGF receptor-mediated signal transduction pathway in cultured VSMCs. To substantiate this hypothesis, we designed this study to elucidate the molecular mechanisms of how tranilast exerts its antimitogenic effects in human CASMCs.

In the present study, we have examined the inhibitory effect of tranilast on PDGF-BB mitogenic action in cultured human CASMC and further investigated the mechanisms by which tranilast was able to suppress cell signal transducation events initiated by PDGF-BB. Our experiments demonstrated that tranilast markedly inhibited PDGF-BB-induced CASMC proliferation and decreased the MAP kinase activation.

Subsequently, to clarify whether this inhibition involves interference with the upstream events of MAP kinase cascade, we examined the effects of tranilast on receptor tyrosine phosphorylation and receptor binding at the cell surface. As a result, we found out that tranilast inhibited the binding of PDGF-BB to its receptor on CASMC membrane.

Methods

Culture of human-derived coronary artery smooth muscle cells

Human-derived CASMCs were purchased from Clonetics (San Diego, CA, U.S.A.). Cells were cultivated at 37°C in smooth muscle cell basal medium (SmBM, Clonetics) containing 5% foetal bovine serum (FBS) supplemented with antibiotics gentamicin (50 μg ml^{-1}) and amphotericin-B (50 ng ml^{-1}) under a humidified atmosphere of 5% CO_2 in air. Culture medium was changed every 2 days and a confluent CASMC monolayer was obtained after about 5 days. Cells were routinely used from the fourth to eighth passages. Cell viability was tested by trypan blue exclusion.

Assay of cell proliferation

A direct assessment of cell proliferation was made by counting cells exposed to recombinant human PDGF-BB (R&D Systems Inc, Minneapolis, MN, U.S.A.) in the presence or absence of tranilast. Cells (3.5×10^4 cells dish^{-1}) were cultured in 35 mm dishes overnight in SmBM containing 5% FBS with antibiotics and achieved a density of $\approx4\times10^4$ cells dish^{-1} ($\approx$20% confluence). Cells were then washed and incubated overnight in SmBM containing 0.5% FBS and 0.1% BSA with antibiotics. On day 0 of the growth assay, cells were fed fresh SmBH containing 1% FBS and 0.1% BSA with or without 0.36 nM PDGF-BB (10 ng ml^{-1}) in the presence or absence of the indicated concentration of tranilast. The growth assay was carried out over a 4 day interval with fresh medium changes occurring every other day (both PDGF-BB and tranilast were replenished). On day 4, cells were washed in PBS, dissociated with trypsine/EDTA, and counted in triplicate with a hemocytometer (Erma, Tokyo, Japan).

Determination of DNA synthesis

The rate of DNA synthesis was assayed by measuring [^{3}H]-thymidine incorporated into DNA fragments of CASMCs. Cells were seeded into 24-well cluster culture plates at a density of 2×10^4 cells well^{-1} in SmBM containing 5% FBS and grown until confluency (4 days). Then, the cells were washed with serum-free SmBM containing 0.1% BSA and incubated in the same medium for 48 h to obtain quiescent non-dividing cells. The cultures were then incubated in the same medium containing 0.36 nM PDGF-BB in the absence or presence of tranilast. After 18 h incubation period, 0.5 μCi ml^{-1} of methyl-[^{3}H]-thymidine (Amersham, Buckinghamshire, U.K.) was added and incubated for an additional 6 h at 37°C. After incubation, the incorporation of [^{3}H]-thymidine into DNA was stopped by removal of the labelled medium. And then, the cells were washed three times with ice-cold phosphate buffered saline (PBS) and fixed for 10 min with ice-cold methanol. Subsequently, the cells were incubated twice with 10% trichloroacetic acid (TCA) for 10 min. Finally, the acid-insoluble fraction was lysed in 0.4 N NaOH (0.5 ml well^{-1}) and 4 ml of scintillation fluid (Hionic fluor, Packard, Meriden, CT, U.S.A.) was added. The radioactivity was counted in a liquid scintillation counter (LS 5000TS, Beckman, Fullerton, CA, U.S.A.). Finally, 50 μl of the residual solution was utilized for the determination of protein.

Immunoblotting of MAP kinase phosphorylation

MAP kinase activation (phosphorylation) was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and Western immunoblotting procedure using a phospho-specific MAP kinase antibody® which recognizes only the phosphorylation status of p44 and p42 MAP kinases (New England Biolabs, Beverly, MA, U.S.A.). This antibody allowed us to evaluate the activation of p44/p42 MAP kinases. Cells were seeded into 24-well cluster culture plates at a density of 5×10^4 cells well^{-1} in SmBM containing 5% FBS with antibiotics and grown until confluency (4 days). After 48 h incubation with serum-free SmBM containing 0.1% BSA and antibiotics to obtain quiescent non-dividing cells, the cells were incubated with PDGF-BB (0.36 nM) in the presence or absence of tranilast in 1 ml of SmBM containing 25 mM HEPES buffer and 0.1% BSA at 37°C. After 5 min, the cells were lysed by adding 100 μl of boiling electrophoresis sample buffer [125 mM Tris-HCl (pH 6.8), 2% SDS, 5% glycerol, 0.003% bromophenol blue, 1% β-mercaptoethanol, and 1 mM sodium orthovanadate]. And then, the cell lysates were immediately scraped off the plates, transferred to a microfuge tube, and sonicated. After boiling for 5 min, total protein in the lysate was measured by the Lowry method (Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer's protocol, and equivalent quantities of protein (10 μg of protein per lane) were separated on linear gradient SDS-polyacrylamide gels (Daiichi Pure Chemicals, Tokyo, Japan) and the proteins were electrotransferred onto a polyvinylidene difluoride membrane (Immobilon PVDF Transfer Membrane, Nihon Millipore, Tokyo, Japan) with a semidry transfer unit (AE-6675, Atto, Tokyo, Japan). Nonspecific binding sites were blocked overnight by incubation in PBS containing 0.1% Tween-20 (Bio-Rad), 3% BSA, and 3% skim milk (DIFCO, Detroit, MI, U.S.A.) at 4°C. Subsequently, the membranes were immunoblotted with 2 μl ml^{-1} of phospho-specific MAP kinase antibody or p44/42 MAPK antibody for 1 h at room temperature with constant shaking, washed, and then incubated with 0.5 μl ml^{-1} horseradish peroxidase (HRP)-linked anti-rabbit Ig antibody (Amersham). After 1 h at room temperature, bound immunoglobulins were visualized by a chemiluminescence method with an ECL Western blotting analysis system (Amersham). The ECL detected blots were exposed to Polaroid film (612, Polaroid, Cambridge, MA, U.S.A.). The signal intensity (integral volume) of the appropriate bands on the film was analysed using an Imaging Densitometer (Model GS-700, Bio-Rad) and the Molecular Analyst software package (Bio-Rad).

Immunoblotting of receptor tyrosine phosphorylation

Protein tyrosine phosphorylation was also analysed by SDS–PAGE followed by Western immunoblotting procedure using an anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A.). The procedure was performed as described above, except for 1 min of incubation time by PDGF-BB. For PDGF receptor identification, the membranes were stripped of phosphotyrosine antibody followed by reprobing with anti-human PDGF β-receptor antibody (Upstate Biotechnology). Stripping the antibodies from the membranes were carried out according to the following procedure. Briefly, after the film exposure, the membranes were washed four times with PBST for 5 min. And then, the membranes were incubated for 30 min at 50°C in the stripping buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM β-mercaptoethanol]. Finally, the membranes were washed six times with PBST for 5 min.

Radioligand binding assay

Hosang & Rouge have provided the evidence that human VSMCs are equipped with specific cell surface receptors for PDGF (Hosang & Rouge, 1989). Binding assays were performed according to the method described previously (Inui *et al*., 1993), with slight modification. Cells were seeded into 24-well cluster culture plates at a density of 5×10^4 cells well^{-1} in SmBM containing 5% FBS with antibiotics. After reaching confluence, cells were transfered to the SmBM containing 0.1% BSA with antibiotics, cultured for an additional 2 days, and used to assay. Monolayer cultures were washed three times with ice-cold PBS and incubated with 0.05 nM [^{125}I]-PDGF-BB (44 TBq mmol^{-1}, Amersham) in the presence or absence of tranilast in 1 ml of SmBM containing 25 mM HEPES-NaOH buffer (pH 7.4) and 0.1% BSA at 4°C for 2 h. After washing four times with the medium at 4°C, the cells were lysed in 25 mM HEPES-NaOH buffer (pH 7.4) containing 1% Triton X-100 and 10% glycerol, and cell-associated radioactivity was measured in a gamma counter (ARC-2000, Aloka, Tokyo, Japan). Nonspecific binding was determined in the presence of 36.3 nM unlabelled PDGF-BB. Specific binding was determined by subtracting the nonspecific binding from total binding.

For determination of the effects of tranilast on saturation binding, monolayer cultures were incubated with increased amounts (0.01–0.3 nM) of [^{125}I]-PDGF-BB. Bound radioactivity, in the presence or absence of 300 μM tranilast, was determined as described above. The dissociation constant (K_d) and maximum binding (B_{max}) for [^{125}I]-PDGF-BB were estimated by Scatchard analysis. Cell numbers were determined in parallel cultures (triplicates) by trypsinization and counting of detached cells in a hemocytometer.

Reagents

Tranilast, N-(3′,4′-dimethoxycinnamoyl) anthranilic acid, was a gift from Kissei Pharmaceutical Company (Matsumoto, Japan). Foetal bovine serum was purchased from Gibco (Grand Island, NY, U.S.A.). Other chemicals were of the highest grade commercially available.

Data analysis

Values are expressed as mean ± s.e.mean, of at least three separate experiments. Statistical analysis was made with ANOVA followed by a Bonferroni-Dunnett test for comparisons between groups. Differences with a P value of less than 0.05 were considered statistically significant.

Results

Inhibition of cell proliferation by tranilast

We assessed the effects of tranilast on the cell proliferation stimulated by PDGF-BB by a direct cell count of trypsinized cells. As shown in Figure 1, incubation with PDGF-BB (0.36 nM) resulted in a 3.8 fold increase in cell number compared with basal value. Tranilast (10–300 μM) reduced PDGF-BB-stimulated cell proliferation in a concentration-dependent manner with significant inhibition observed at 100 μM (51.3 ± 2.3%, $P < 0.001$), and at 300 μM (64.1 ± 1.8%, $P < 0.001$). The IC_{50} value for the inhibitory effect of tranilast was 118.3 μM (basal values were subtracted on calculation).

To rule out the possibility that the proliferation inhibition was due to cytotoxicity, two methods were used to assess cell viability. First, incubation of CASMCs with tranilast (300 μM) for 4 days did not result in morphological changes obvious by microscopic examination. Second, we evaluated the effect of tranilast on membrane permeability by measuring the ability of tranilast-treated cells to exclude trypan blue. Incubation of CASMCs with tranilast (300 μM) for 4 days did not result in greater uptake of trypan blue than that of untreated cells (data not shown). Therefore, tranilast appears to inhibit cell proliferation stimulated by PDGF-BB without affecting cell viability.

Inhibition of DNA synthesis by tranilast

As shown in Figure 2, exposure of quiescent cells to PDGF-BB (0.36 nM) caused a 12.7 fold increase in [^{3}H]-thymidine incorporation compared with basal. Tranilast (10–300 μM) inhibited PDGF-BB-stimulated [^{3}H]-thymidine incorporation in a concentration-dependent manner with significant inhibition observed at 100 μM (67.6$\pm$6.3%, $P<0.01$), and a maximal inhibition observed at 300 μM (91.7$\pm$0.3%, $P<0.001$). The IC_{50} value for the inhibitory effect of tranilast was 81.2 μM.

Inhibition of MAP kinase phosphorylation by tranilast

In the present study, we confirmed that PDGF-BB stimulation of the human CASMCs leads to a transient increase in the activity of both p44 and p42 MAP kinases (ERK1 and ERK2) reaching a maximum at 5 min followed by a rapid return towards basal levels (data not shown). Therefore, this time point was chosen for the experiments. As shown in Figure 3B (lane 2), after stimulation with PDGF-BB (0.36 nM), one major band of 42 kDa and one minor band of 44 kDa were detected by immunoblotting using a polyclonal antibody specific for the phosphorylated p44/p42 MAP kinases. The predominant MAP kinases peptide species possessed a molecular weight of 42 kDa and most probably represents ERK2. A less intense band of 44 kDa is consistent with ERK1. In all experiments, basal phosphorylation of p44 and p42 proteins was undetectable (Figure 3B, lane 1). The preincubation of the cells with tranilast (100 or 300 μM) resulted in a significant inhibition of PDGF-BB-stimulated activation for ERKs in a concentration-dependent manner (Figure 3B). Densitometric analysis showed that the relative intensity in the sum of activities of p44 and p42 proteins in the presence of 100 and 300 μM tranilast was 69.9$\pm$6.0 and 39.3$\pm$2.4 (per cent as compared with control), respectively (Figure 3C). The IC_{50} value for the inhibitory effect of tranilast was 203.4 μM. In addition, immunoblotting experiments using a specific anti-MAP kinase antibody demonstrated that p44 or p42 MAP kinase proteins (phosphorylation-state independent) levels in the same samples were not altered by tranilast treatment (Figure 3A). These results suggest that tranilast may inhibit the cell proliferation by modulating the activation of MAP kinase.

Inhibition of PDGF-receptor tyrosine phosphorylation by tranilast

Subsequent to PDGF binding, the PDGF receptor protein tyrosine kinase (PTK) is activated, resulting in autophosphorylation of the cytoplasmic domain. To confirm that the observed inhibition by tranilast on MAP kinase activation was caused by decreasing the activity of receptor PTK, an upstream regulator of MAP kinase cascade, the Western blot analysis was carried out with receptor tyrosine phosphorylation (TP). It is known that treatment of VSMCs with PDGF causes a rapid and transient increase in protein TP that precedes cell mitogenesis. In a preliminary examination by immunoblotting using anti-phosphotyrosine antibody in CASMCs, we also confirmed that the PDGF-BB-stimulated increase in receptor TP is transient, with peaking at the first 1 min after growth factor addition and then returning rapidly towards basal levels (data not shown). Therefore, this time point was chosen for the experiments. As shown in Figure 4A (lane 2), after stimulation with PDGF-BB (0.36 nM), an apparent molecular mass of 180 kDa was detected by

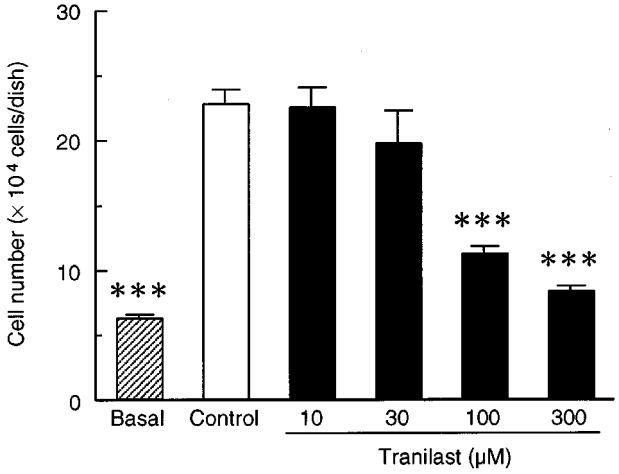

Figure 1 Effect of tranilast on cell proliferation stimulated by PDGF-BB. Sub-confluent (20%) cells were continuously cultured with PDGF-BB (0.36 nM) in the absence or presence of the indicated concentration of tranilast. After 4 days in culture, the cells in triplicate dishes were trypsinized and counted as described in Methods. Data are expressed as mean$\pm$s.e.mean ($n=4$). Basal: PDGF-BB was not added to the medium. Control: PDGF-BB alone was added to the medium. ***$P<0.001$ as compared with the control.

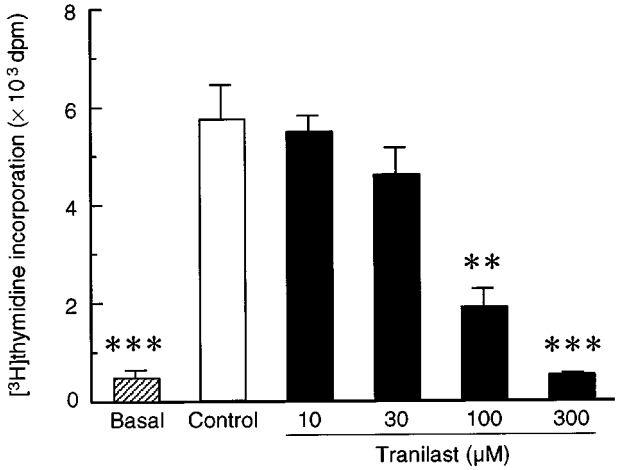

Figure 2 Effect of tranilast on DNA synthesis stimulated by PDGF-BB. Confluent monolayers of serum-starved cells were incubated with PDGF-BB (0.36 nM) alone or with tranilast for 18 h at 37°C. Tranilast was preincubated for 10 min prior to stimulation of PDGF-BB. DNA synthesis was determined by measuring the incorporation of [^{3}H]-thymidine as described in Methods. Data are expressed as mean$\pm$s.e.mean ($n=4$). Basal: PDGF-BB was not added to the medium. Control: PDGF-BB alone was added to the medium. **$P<0.01$, ***$P<0.001$ as compared with the control.

immunoblotting using an anti-phosphotyrosine antibody. Since a molecular mass of 180 kDa is close to the previously reported one for the human PDGF β-receptors (Grotendorst *et al*., 1991), we estimated that the tyrosine-phosphorylated 180 kDa substrates might be PDGF β-receptor-related proteins. By reprobing of the stripped membranes with a specific antibody for PDGF β-receptor, we confirmed definitely that the observed single band was coincident with a 180-kDa tyrosine-phosphorylated protein (data not shown). In all experiments, basal phosphorylation of the receptor was undetectable (Figure 4A, lane 1). Similarly to MPA kinase, the preincubation with tranilast (100 or 300 μM) also resulted in a significant reduction of PDGF-stimulated TP of 180 kDa protein, which identified with PDGF receptor, as shown by immunoblots of total cellular proteins (Figure 4B). The IC_{50} value for the inhibitory effect of tranilast was 149.6 μM. In addition, some lower molecular weight protein bands of 125, 118, 75, and 70 kDa exhibiting tyrosine phosphorylation immunoreactivity could also be observed in cell extracts. The intensity of the 118-kDa band appeared to decrease by preincubation of tranilast in a concentration-dependent manner, whereas that of the 125-kDa band appeared to increase. In contrast, the intensity of the 75- and 70-kDa bands remained almost unchanged. In the present study, however, we did not investigate the origin or identity of these bands.

Effect of tranilast on receptor binding

Having documented the inhibitory effect of tranilast on MAP kinase activation and receptor TP, we next tested whether tranilast could affect the binding of PDGF-BB to cell surface receptors with a radioligand receptor binding

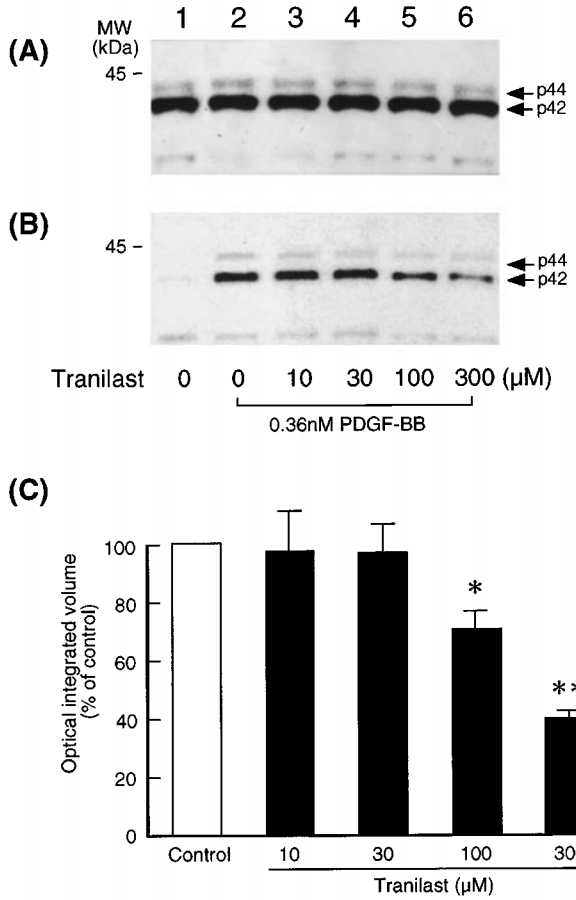

Figure 3 Effect of tranilast on MAPK activation. Confluent monolayers of serum-starved cells were stimulated for 5 min with 0.36 nM PDGF-BB in the absence (control, lane 2) or presence (lane 3–6) of the indicated concentrations of tranilast. Western blots showing the effects of tranilast on the total MAP kinase protein levels (A) and on PDGF-BB-induced phosphorylated (activated) MAP kinase protein levels (B). The figure shows one representative experiment out of four with similar results. The molecular sizes are shown in kilodalton (kDa) and the positions of the p44 (ERK1) and p42 (ERK2) proteins are indicated with arrows. (C) A summary of the densitometric analysis to compare relative levels of phosphorylated MAP kinase protein. The sum of p44 and p42 activities is referred to as MAP kinase activity. $n=4$. $^*P<0.05$, $^{**}P<0.01$ as compared with the control.

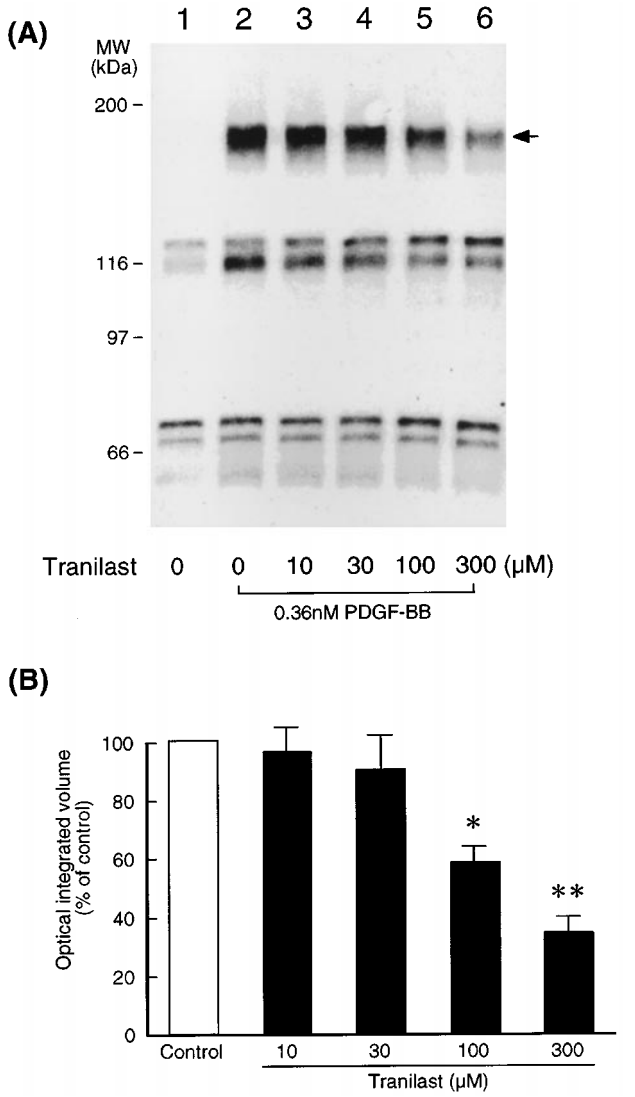

Figure 4 Effect of tranilast on receptor tyrosine phosphorylation. Confluent monolayers of serum-starved cells were stimulated for 1 min with 0.36 nM PDGF-BB in the absence (control, lane 2) or presence (lanes 3–6) of indicated concentration of tranilast. Tranilast was preincubated for 10 min prior to stimulation of PDGF-BB. (A) Western blots showing the effects of tranilast on tyrosine phosphorylated protein levels stimulated by PDGF-BB. The figure shows one representative experiment out of four with similar results. The putative PDGF β-receptor autophosphorylation product is visualized at 180 kDa (arrow) as well as several other phosphotyrosine-containing proteins with size of 125, 118, 75, and 70 kDa. Molecular sizes are shown in kilodalton. (B) A summary of the densitometric analysis of the four separate experiments is shown. Densitometric scanning of fluorograms was performed to compare relative levels of the proteins. $^*P<0.05$, $^{**}P<0.01$ as compared with the control.

assay using [^{125}I]-PDGF-BB. The ability of tranilast to inhibit the binding of [^{125}I]-PDGF-BB to monolayer cultures of human CASMCs was determined by using a binding assay. As shown in Figure 5, exposure of the cells to tranilast (30–1000 μM) inhibited the binding of [^{125}I]-PDGF-BB to cell surface receptor in a concentration-dependent manner, with an IC_{50} value of 268.0 μM. [^{125}I]-PDGF-BB binding to CASMCs in the presence of 1000 μM tranilast was $\approx$10% of that in the absence of tranilast. The addition of tranilast in highest concentration (1000 μM) for the present incubation time (2 h) produced by visible effects on CASMCs and did not affect viability as assessed by trypan blue exclusion (data not shown).

To establish the characteristics of the antagonism exerted by tranilast, we next determined its effect on the saturation curve of PDGF-BB binding by carrying out competition studies with [^{125}I]-PDGF-BB in the presence of 300 μM (a concentration close to the IC_{50} value for PDGF receptor binding) tranilast. The results for tranilast are shown in Figure 6 in the form of a Scatchard analysis. Tranilast (300 μM) induced a reduction of the B_{max} value for PDGF-BB from 27.6 to 18.0 fmol 10^6 cells^{-1}, whereas hardly any influence could be observed in the K_d value for that (from 0.159 to 0.147 nM). These results indicate that tranilast inhibits PDGF-BB binding to its receptors in a non-competitive manner.

Discussion

In this study, we demonstrated that tranilast is a potent inhibitor of PDGF-BB-stimulated mitogenesis in human CASMC. Growth factor-stimulated CASMC proliferation was inhibited by tranilast in a concentration-dependent manner. We found that tranilast exhibited weaker activity in cell proliferation (IC_{50} 118.3 μM) than that in DNA synthesis (IC_{50} 81.2 μM). This may be explained by contribution of other growth factors or cytokines contained in FBS to cell growth. On the other hand, tranilast did not show any cytotoxicity even at 300 μM, which is 3 fold higher than the maximum plasma concentration achieved with a standard clinical dosage (300 mg day^{-1}), for 4 days incubation. In all experiments in this study, tranilast exerted inhibitory effects at a concentration of 100 μM, which is in the range of therapeutic plasma concentration levels of the compound (Tanaka *et al*., 1994).

As the first step of current study of the molecular mechanism(s) of tranilast's inhibitory action against PDGF-driven cell proliferation, we assessed the effect of tranilast on MAP kinase activation enhanced by PDGF-BB *in vitro*. Pyles *et al*. (1997) have described that MAP kinase was activated in response to balloon overstretch injury in porcine carotid arteries. Moreover, in a recent study using rat carotid arteries following balloon injury, it has been demonstrated that MAP kinase signalling, in particular ERK activity, was increased and medial cell replication following injury was reduced by PD 098059, a MAP kinase kinase (MEK) inhibitor (Koyama *et al*., 1998). The above results imply that MAP kinase pathway may be crucial in the neo-intimal thickening progression in injured vessels. In the present study, tranilast significantly inhibited MAP kinase activation in response to PDGF-BB in cultured CASMCs, suggesting that the clinical prevention of restenosis by tranilast after PTCA may be due to the inhibition of MAP kinase pathway. In addition, our results have shown that tranilast suppressed the activation of MAP kinase stimulated by PDGF-BB without affecting the protein expression of MAP kinase in CASMCs. These results indicate that tranilast could inhibit the PDGF-BB-induced activation (phosphorylation) of MAP kinase by any mechanism in these cells.

The next question to be answered was how tranilast interferes with ERK activity. Therefore, we have investigated whether the interruption of receptor tyrosine kinase, an upstream activator of MAP kinase, may account for the antimitogenic actions of tranilast in CASMCs. Tranilast markedly inhibited PDGF β-receptor tyrosine autophosphorylation. Thus, inhibition of receptor tyrosine autophosphorylation could explain the interruption of ERK pathway by tranilast. This study confirmed that changes in tyrosine phosphorylation of PDGF receptor substantially correlated with the changes in MAP kinase activity. Both receptor

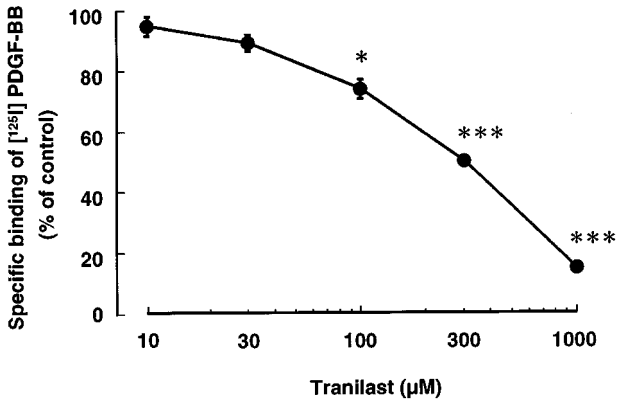

Figure 5 Effect of tranilast on PDGF-BB binding to its receptors in human CASMCs. Confluent monolayers of serum-starved cells were incubated with 0.05 nM [^{125}I]-PDGF-BB in the presence of indicated concentration of tranilast. In each experiment, each value was determined in triplicate and results given are means for four separate experiments. Specific binding was determined by subtracting the nonspecific binding obtained in the presence of 36.3 nM unlabelled PDGF-BB from total binding. The specific binding in the absence of tranilast was set to 100%. *$P<0.05$, ***$P<0.001$ as compared with the control.

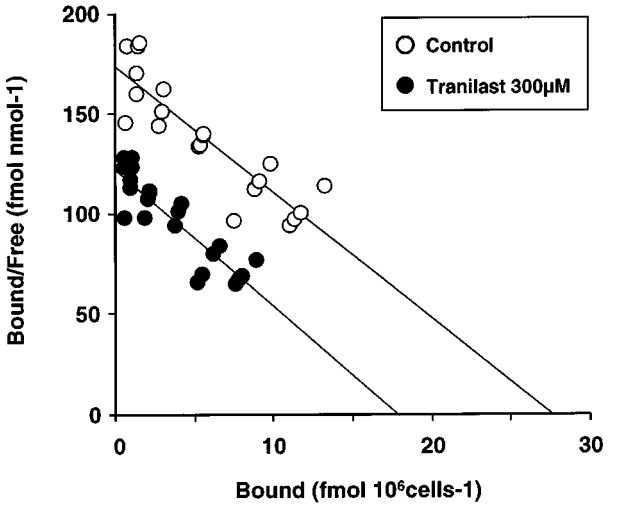

Figure 6 Scatchard analysis of binding of PDGF-BB to its receptors with or without tranilast in human CASMCs. Confluent monolayers of serum-starved cells were incubated with increased amounts (0.01–0.3 nM) of [^{125}I]-PDGF-BB in the presence or absence of 300 μM tranilast. Each point represents the mean value from triplicate experiments. Binding was expressed as fmol PDGF-BB bound per 10^6 cells, with cell number determined sister cultures. Specific binding was determined as described in the legend of Figure 5. The specific binding of [^{125}I]-PDGF-BB in the absence of tranilast (K_d = 0.159 nM, B_{max} = 27.6 fmol 10^6 cells^{-1}) differed from that in the presence of 300 μM tranilast (K_d = 0.147 nM, B_{max} = 18.0 fmol 10^6 cells^{-1}).

tyrosine autophosphorylation and MAP kinase activation induced by PDGF-BB were inhibited by tranilast to almost the same magnitude.

Next, to clarify the inhibitory mechanism of receptor tyrosine autophosphorylation by tranilast, radioligand-binding assays were carried out in monolayer cultures of CASMCs. As a result, tranilast exhibited a non-competitive inhibition of PDGF-BB binding to its specific receptors with an IC_{50} of 268.0 μM. This result suggests that the suppression of PDGF-BB-mediated receptor tyrosine phosphorylation and MAP kinase activation by tranilast may be attributable to inhibition of the PDGF-BB-receptor binding. To the best of our knowledge, there is so far no study with the effect of tranilast on PDGF binding to its cell-surface receptors. Therefore, we demonstrate for the first time that tranilast could interfere with ligand (PDGF-BB)-receptor interactions at the human CASMC surface *in vitro*. The remaining problem for the above explanation is the discrepancy in inhibitory potency of tranilast; the IC_{50} value for the receptor binding inhibition is approximatly twice as high (268.0 μM) than that needed to inhibit the receptor autophosphorylation (149.6 μM) or cell growth (118.3 μM). This difference in the IC_{50} values suggest that, in the PDGF-BB-stimulated cells, other mechanisms whereby tranilast disturbs the receptor tyrosine may exist. Recently, it has been reported that H_2O_2 plays a key role in PDGF signal transduction, and the responses of VSMCs to PDGF, which includes tyrosine phosphorylation, MAP kinase activation, and DNA synthesis, are inhibited when PDGF-BB-stimulated rise in H_2O_2 concentration was blocked by anti-oxidant agents (Sundaresan *et al*., 1995). Moreover, it has been demonstrated previously that tranilast decresed H_2O_2 generation in the presence of polymorphonuclear leukocytes system and exhibited an antioxidant action (Miyachi *et al*., 1987). From these findings, it is likely that antioxidant activity of tranilast may also be involved in the inhibition of receptor tyrosine phosphorylation.

Nie *et al*. suggested that tranilast may inhibit cell proliferation through the inhibition of PDGF-BB-induced Ca^{2+} entry, without affecting PDGF-BB-mediated receptor autophosphorylation or MAP kinase activation in cultured rat VSMCs (Nie *et al*., 1996). However, in the present study, we have shown evidence for the inhibition of PDGF receptor tyrosine autophosphorylation and of MAP kinase activation in human CASMCs. The discrepancies between our findings and those of Nie *et al*. may reflect differences concerning the experimental conditions including cell-lines used. They performed their experiments in rat aortic SMCs in culture, whereas we used human-derived coronary SMCs. Beside that, tranilast has been reported to antagonize angiotensin II, which may stimulate PDGF synthesis (Jin *et al*., 1998) and to induce the cyclin-dependent kinase inhibitor $p21^{waf1}$ (Kusama *et al*., 1999; Takahashi *et al*., 1999). There remains some possibility that these factors may also contribute to inhibitory effect of tranilast.

In conclusion, the present study demonstrated that tranilast exhibited antagonistic action towards PDGF-BB-mediated cell signal transduction in human CASMC *in vitro*. From the current results, it is suggested the possibility that such antagonistic actions of tranilast against PDGF in cell growth might be exerted at least partly by the inhibition of PDGF binding to cell surface receptors. However, the elucidation of the more detailed mechanism, which governs this inhibitory action of receptor binding, requires further investigation and may lead to a better understanding of the unknown action of tranilast at the molecular level.

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