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Tranilast inhibits the growth of rat mesangial cells

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Abstract

We investigated the effects of tranilast on the growth of cultured rat mesangial cells. The number of mesangial cells increased fivefold during a 5-day incubation in RPMI 1640 with 20% fetal bovine serum. The number of cells was significantly lower in the presence of tranilast than in its abscence. Tranilast (0 ~ 500 μM) inhibited platelet-derived growth factor (PDGF)-induced DNA synthesis of rat mesangial cells cultured in RPMI 1640 medium containing 0.5% fetal bovine serum in a dose-dependent manner. The inhibition of DNA synthesis by tranilast was not affected by the presence of indomethacin (1 μg/ml) or N^G-monomethyl-L-arginine (0.5 mM). Tranilast did not stimulate nitrite oxide synthesis in PDGF-stimulated cells. Mitogen-activated protein kinase activity in mesangial cells was significantly increased by exposure to PDGF, while the effect was significantly suppressed in the presence of tranilast. The present study revealed that tranilast inhibits the growth of rat mesangial cells, independently of nitric oxide or prostacycline synthesis.

Keywords: Nitric oxide (NO); Prostacyclin; Glomerulonephritis

1. Introduction

Tranilast (*N*-(3,4-dimethoxycinnamoyl) anthranilic acid) has long been used clinically to treat allergic diseases such as bronchial asthma, atopic dermatitis and allergic rhinitis (Mori et al., 1995). The efficacy of tranilast in the treatment of these allergic diseases is based on the inhibition of antigen-induced chemical mediator release from mast cells and basophilis (Koda et al., 1985). Recently, it has also been shown that tranilast inhibits collagen accumulation in rat and human fibroblasts (Isaji et al., 1987, Yamada et al., 1994), and therapeutic application of tranilast has been approved for treatment of hypertrophic scarring and keloid (Nanba et al., 1987), formation of which is thought to be associated with excessive proliferation of, and collagen synthesis by, fibroblasts. Tranilast has also been shown to suppress vascular smooth muscle proliferation induced by platelet-derived growth factor (PDGF) or angiotensin II (Miyazawa et al., 1995, 1996; Tanaka et al., 1994).

The glomerular mesangium plays a central role in the evolution of immune-mediated glomerular disease. Immune deposits localize in the glomeruli and activate the circulating inflammatory cells (Fries et al., 1988). Infiltrating leukocytes and mesangial cells produce cytokines and polypeptide growth factors which regulate the growth of mesangial cells (Horii et al., 1989; Ikeda et al., 1991, 1992; Lovett et al., 1983) and increase matrix synthesis (Border and Noble, 1993). Mesangial cell proliferation and extracellular matrix formation are hallmarks of chronic glomerulonephritis. Thus, inhibition of mesangial cell growth is thought to prevent the advance of glomerulonephritis.

Recently, it was reported that tranilast significantly prevents restenosis after precutanous transluminal coronary angioplasty (TREAT Study Investigators, 1994; Ueda et al., 1995) as well as after directional coronary atherectomy (Kosuge et al., 1995). To evaluate the possibly use of tranilast for prevention of the advance of glomerulonephritis, we examined whether tranilast affects the growth of mesangial cells.

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2. Materials and methods

2.1. Mesangial cell culture

Mesangial cells from male Sprague-Dawley rats (100–150 g) were isolated and cultured as previously described (Ikeda et al., 1992). Briefly, rat renal cortices were minced and gently pressed though 200, 150, 75-μm stainless steel sieves (V.S. Tyler, Mentor, OH, USA). The collected glomeruli were resuspended in RPMI 1640 culture medium (Gibco, New York, NY, USA), buffered with 10 mM HEPES at pH 7.4, supplemented with 20% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Culture dishes were incubated under a humidified 95% air/5% CO_2 atmosphere incubator at 37°C.

Mesangial cells were identified by positive staining for α-actin, myosin and desmin, and by negative staining for the endothelial cell maker, factor VIII (Ikeda et al., 1992). Angiotensin II-induced contraction of mesangial cells was also detected by phase contrast microscopy. Cells were used at passages 6–15 for the following experiments.

2.2. Analysis of DNA synthesis

DNA synthesis of mesangial cells was assayed by measuring the incorporation of the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU) into the cells (Muir et al., 1990), using a cell proliferation kit (BrdU labeling detection kit III, Boehringer-Mannheim, Mannheim, Germany). Subconfluent mesangial cells cultured in 96-well dishes were preincubated in RPMI 1640 medium with 0.5% fetal bovine serum for 24 h. PDGF and/or tranilast were then added, and 10 μM BrdU was added to each culture during the last 2 h. Cells were then fixed with 70% ethanol/HCl for 30 min. After addition of nucleases, mesangial cells were incubated with peroxidase-labeled anti-BrdU antibody for 30 min at 37°C. Incorporation of BrdU into mesangial cells was determined by absorbance at 405 nm on an ELISA reader.

2.3. Measurement of nitrite

The production of nitric oxide (NO) by the cultured cells was determined by measuring nitrite contents of the culture medium. Mesangial cells plated in 24-well dishes were incubated in RPMI 1640 containing 0.5% fetal bovine serum at 37°C. The nitrite contents of the culture medium were determined by mixing 500 μl of the medium with an equal volume of Griess reagent (1 part 0.1% naphthylethylenediamine dihydrochloride to 1 part 1% sulfanilamide in 5% phosphoric acid) (Green et al., 1982). Absorbance at 550 nm was measured and nitrite concentration was determined from a curve calibrated with sodium nitrite standards. Nitrite levels were corrected by protein measurement (Bio-Rad assay kit, Herucles, CA, USA) and data are shown as nmol per mg protein.

2.4. Assay of MAP kinase activity

Mitogen-activated protein (MAP) kinase activity in the cells was assayed as described previously (Ishikawa et al., 1994). The measurement of MAP kinase was performed using quiescent cells incubated with serum-free RPMI1640 for 24 h. After washing twice with physiological saline solution (PSS), the cells were exposed to PDGF and/or tranilast for a specified times at 37°C. The reaction was stopped by addition of 1 ml of ice-cold extraction solution (20 mM Tris/HCl, 6 mM EGTA, 6 mM $MgCl_2$, 0.1 mM sodium fluoride, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 20 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) on dry ice. The cell extracts were centrifuged at $1500 \times g$ for 5 min. The supernatant was then incubated with 6 mM of a synthetic peptide (sequence APRTPGGER, amino acids 95–98 of bnoxine myelin basic protein (MBP) with the Thr-97 phosphorylation site for MAP kinase) and reaction mixture containing 75 mM β-glycerophosphoric acid, 2 mM dithiotheritol, 6 mM $MgCl_2$, and 50 μM [^{32}P]-γATP (specific activity; 10 Ci/mmol, New England Nuclear, Wilmington, DE, USA) for 10 min at 25°C. This synthetic peptide is more selective as a substrate for MAP kinase than MBP (Clark-Lewis et al., 1991). The reaction products were placed on P-81 phosphocellulose papers (Whatman International, Maidstone, UK) and washed in 20 ml of ice-cold 10 mM phosphoric acid three times. The radioactivity was counted with a liquid scintillation counter (Aloka LSC-671, Tokyo, Japan). Specific radioactivity was determined by subtracting the radioactivity of the synthetic peptide-free reaction from the synthetic peptide-directed radioactivity. MAP kinase activity is represented as pmol ATP incorporated/mg cell extract per min.

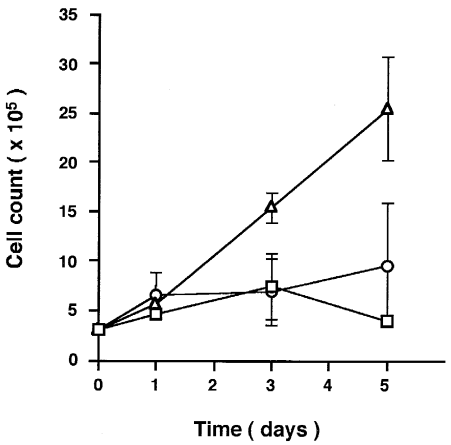

Fig. 1. Effects of tranilast on the number of mesangial cells during a 5-day period of incubation. Mesangial cells (2×10^5/well) in 24-well dishes were incubated in the presence (○) or absence (△) of tranilast (300 nM) in 20% fetal bovine serum containing RPMI 1640 for 5 days. Mesangial cells were almost quiescent in 0.5% fetal bovine serum containing RPMI 1640 (□). The cell number in each well was counted with a hemocytometer. Data are means ± S.E.M of four samples.

2.5. Statistical analysis

Values are shown as means ± S.E.M, of at least three separate experiments. In experiments involving comparisons between multiple groups, the significance of differences between the means of the groups was determined by analysis of variance using the least significant difference for multiple comparisons. P values of less than 0.05 were considered to indicate a statistically significant difference.

2.6. Miscellaneous

Tranilast was a gift from Kissei Pharmaceutical (Matsumoto, Japan). Human recombinant PDGF-BB was obtained from Genzyme (Boston, MA, USA). Human recombinant interleukin-1β (IL-1β) was a gift from Otsuka Pharmacy (Tokushima, Japan). N^G-Monomethyl-L-arginine (L-NMMA) and indomethacin were obtained from Sigma (St. Louis, MO, USA). Other chemicals were of the highest grade commercially available.

3. Results

We first investigated the effects of tranilast on the number of mesangial cells. As shown in Fig. 1, the number of viable mesangial cells increased fivefold during a 5-day period of incubation in RPMI 1640 with 20% fetal bovine serum. The number of cells was significantly lower in the presence of tranilast (300 μM) than in its absence.

We then determined the effect of tranilast on DNA synthesis of mesangial cells. After pre-incubation in 0.5% fetal bovine serum containing RPMI 1640 for 24 h cells were further cultured with PDGF (100 ng/ml) and varying concentrations of tranilast (0–500 μM) for 24 h, and the incorporation of BrdU into the cells was determined. Incorporation of BrdU into mesangial cells was taken as a measurement of the extent of DNA synthesis. As shown in Fig. 2, tranilast significantly inhibited PDGF-induced incorporation of BrdU in a dose-dependent manner.

Mesangial cells release prostacyclin which exhibits a growth-inhibiting effect on mesangial cells (Pfeilschifter et

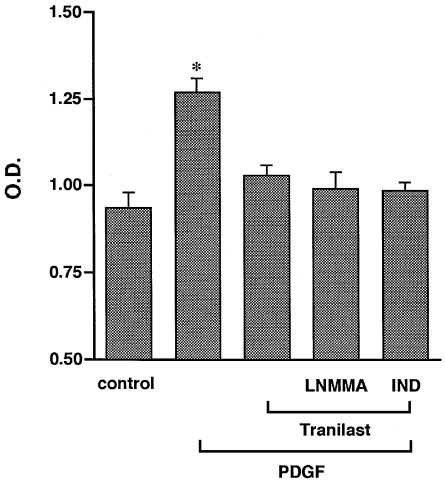

Fig. 3. Effects of N^G-monomethyl-L-arginine and indomethacin on BrdU incorporation into mesangial cells. Mesangial cells in 96-well plates were preincubated in 0.5% fetal bovine serum containing RPMI 1640 for 24 h, and then cultured with PDGF (100 ng/ml) and tranilast (300 μM) in the presence of 0.5 mM N^G-monomethyl-L-arginine (L-NMMA) or 1 μg/ml indomethacin (INDO) for 24 h. BrdU incorporation was measured during the last 2 h. Optical density (O.D.) was determined at 405 nm. Data are means ± S.E.M of eight samples. $^*P < 0.05$ compared with control samples.

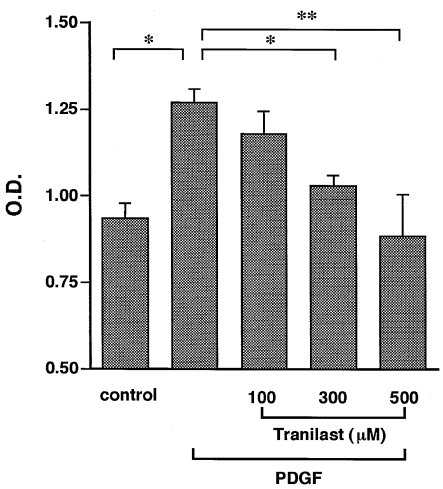

Fig. 2. Effects of tranilast on incorporation of BrdU into mesangial cells. Cultures of mesangial cells in 96 well plates were preincubated in 0.5% fetal bovine serum containing RPMI 1640 for 24 h, and then cultured with tranilast (0 ~ 500 μM) for 24 h. BrdU was added for the last 2 h of the incubation. The BrdU incorporation into mesangial cells was determined by measuring the optical density (O.D.) at 405 nm. Data are means ± S.E.M of eight samples. $^*P < 0.05$; $^{**}P < 0.01$.

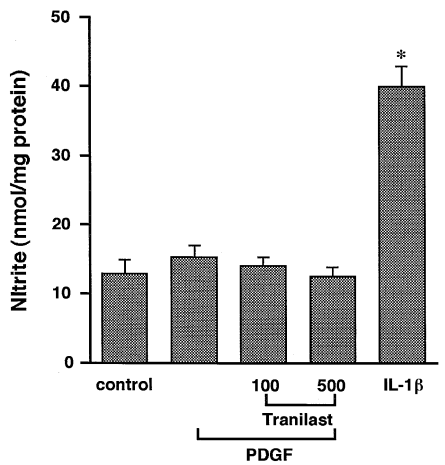

Fig. 4. Effects of tranilast on nitrite accumulation in PDGF-stimulated mesangial cells. Cells were incubated with PDGF (100 ng/ml) for 24 h in the presence or absence of tranilast (100, 500 μM). As a positive control, mesangial cells were stimulated with IL-1β (10 ng/ml) for 24 h. Nitrite accumulation in the culture medium was measured, and the values were normalized to the protein content per dish. Data are means ± S.E.M. of four experiments. $^*P < 0.01$ compared with control samples.

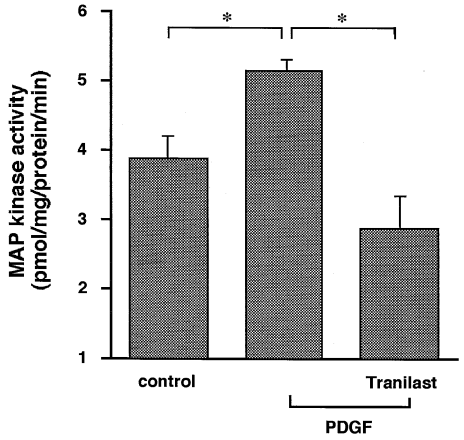

Fig. 5. Effects of tranilast on MAP kinase activity in mesangial cells. Mesangial cells incubated in serum-free RPMI 1640 with or without tranilast (300 μM) for 1 h were exposed to PDGF (100 ng/ml) for 5 min. MAP kinase activity was measured as described in Section 2. Data are means ± S.E.M of four separate dishes. $^{*}P < 0.05$.

al., 1989). We thus investigated whether prostacyclin synthesis was related to the effect of tranilast. However, the inhibitory action of tranilast on BrdU incorporation was not affected by treating mesangial cells with indomethacin (1 μg/ml) (Fig. 3), suggesting that the effect of tranilast is independent of prostacyclin synthesis.

It has been reported that NO also inhibits the proliferation of mesangial cells (Garg and Hassid, 1989a). Previously, we showed that cultured rat mesangial cells express inducible NO synthesis in response to cytokines (Ikeda et al., 1994). To determine whether NO synthesis was involved in the inhibitory effect of tranilast on mesangial cell proliferation, we used L-NMMA, an analogue of L-arginine which inhibits NO synthesis. Addition of L-NMMA (0.5 mM) to the cultures showed no effect on the tranilast-induced inhibition of BrdU incorporation into PDGF-stimulated mesangial cells (Fig. 3), suggesting that the effect of tranilast is not dependent on NO synthesis.

We further investigated the effects of tranilast on NO synthesis in PDGF-stimulated mesangial cells. Accumulation of nitrite in the medium represents the summation of NO synthase activity during the time period studied, since NO secreted by cells is decomposed to the more stable products nitrite and nitrate. As shown in Fig. 4, addition of tranilast did not affect nitrite accumulation in PDGF-stimulated cells.

We then studied the effect of tranilast on PDGF-induced MAP kinase activity in mesangial cells. Addition of PDGF to the cultures caused a significant increase in MAP kinase activity, while the effect was significantly suppressed in the presence of tranilast (Fig. 5).

4. Discussion

This study demonstrates that tranilast inhibits proliferation of mesangial cells, although the mechanism of this effect is still unclear. It has been reported that NO stimulates soluble guanylate cyclase, the enzyme which catalyzes the synthesis of cGMP, and suppresses the proliferative activity of a variety of cells including vascular smooth muscle cells (Garg and Hassid, 1989b; Radomski et al., 1990) and mesangial cells (Garg and Hassid, 1989a). Previously, Hishikawa et al. (1995) reported that tranilast abolished PDGF-induced inhibition of NO synthesis in mesangial cells, suggesting that the inhibitory effect of tranilast was dependent on NO production. However, in the present study, we observed no alteration in the potency of tranilast in the presence of L-NMMA, and tranilast did not affect NO production by mesangial cells. From the present results, the antiproliferative effect of tranilast on mesangial cells observed here was not likely to be the result of synthesis of NO.

The prostacyclin released by mesangial cells has growth-inhibiting effects on these cells via a cAMP-dependent pathway (Pfeilschifter et al., 1989). Furthermore, it has been reported that tranilast increases the level of cAMP in platelets (Iwasa et al., 1988). We found that adding indomethacin, an inhibitor of prostacyclin biosynthesis, to the culture medium had no significant effect on tranilast-mediated inhibition of DNA synthesis, indicating that the cyclooxygenase-cAMP pathway is not involved in the ability of tranilast to inhibit the growth of mesangial cells.

Recently, we observed that tranilast inhibits the increase in fura-2-estimated cytosolic Ca^{2+} levels of the rat aortic vascular smooth muscle induced by PDGF (unpublished observation). This action of tranilast might be related to its inhibitory effect on cell proliferation, since elevation of cytosolic Ca^{2+} levels and the action of protein kinase C via formation of inositol-1,4,5-triphosophate and diacylglycerol induce the expression of protooncogenes and cell proliferation. The protein kinase C pathway is commonly activated by a variety of growth factors such as PDGF, epidermal growth factor and angiotensin II, followed by activation MAP kinase via phosphorylation of both tyrosine and serine/threonine residues. MAP kinase then reactivates the phosphatase-inactivated S6 kinase II (Sturgill et al., 1988). S6 kinase II has been shown to phosphorylate nuclear laminin C in addition to ribosomal protein S6 (Ward and Kirschner, 1990). Moreover, it has recently been demonstrated that MAP kinases phosphorylate the products of the protooncogenes c-*jun*, c-*fos* and c-*myc* (Pulverer et al., 1991). Thus, MAP kinase is considered to play an important role in mediating signals from the growth factor receptors to the ribosomes and the nucleus, and to play a critical role in the control of cell growth and differentiation including mesangial cells (Xiaomei Li et al., 1995). In the present study, MAP kinase activity in mesangial cells was increased by exposure to PDGF, while the effect was significantly suppressed in the presence of tranilast, compatible with the inhibitory effect of tranilast on DNA synthesis of mesangial cells.

In conclusion, the present experiments revealed that tranilast inhibits the growth of rat mesangial cells, independently of NO or prostacyclin synthesis. This study does not lead to any conclusions concerning the role of tranilast in vivo, but we are tempted to speculate that tranilast prevents the development of glomerulonephritis by inhibiting the proliferation of mesangial cells. Further studies are necessary to determine its mechanism of action and clinical usefulness for prevention of glomerulonephritis.

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