

## Tranilast Suppresses Intimal Hyperplasia in the Balloon Injury Model and Cuff Treatment Model in Rabbits

Juichi Fukuyama<sup>1</sup>, Kiyoshi Ichikawa<sup>1,\*</sup>, Keiji Miyazawa<sup>1</sup>, Shuichiro Hamano<sup>1</sup>, Nobuo Shibata<sup>2</sup> and Arai Ujiie<sup>1</sup>

<sup>1</sup>Pharmacological Laboratories and <sup>2</sup>Second Laboratories, Kissei Pharmaceutical Co., Ltd., Hotaka, Nagano 399–83, Japan

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**ABSTRACT**—Intimal hyperplasia is a serious problem after percutaneous transluminal coronary angioplasty (PTCA). In this study, we investigated the effects of tranilast on intimal hyperplasia in both in vivo and in vitro experiments. For the in vivo experiments, we used the balloon injury model and the cuff treatment model of rabbits fed regular chow. In the balloon injury model, tranilast decreased intimal area, intima/media ratio, stenosis ratio and vascular DNA content after endothelial injury. Also in the cuff treatment model, tranilast suppressed the intimal hyperplasia. In the in vitro experiments, we assessed the effects of tranilast on platelet-derived growth factor-induced rabbit vascular smooth muscle cell (VSMC) migration and proliferation and on collagen synthesis by VSMCs. Tranilast inhibited VSMC migration, proliferation and collagen synthesis. These results suggest that tranilast has a suppressive effect on intimal hyperplasia after a vascular injury such as PTCA.

**Keywords:** Tranilast, Intimal hyperplasia, Balloon injury, Cuff treatment, Vascular smooth muscle cell

Percutaneous transluminal coronary angioplasty (PTCA) is one of the widely used therapies for patients with ischemic coronary artery disease. Although PTCA is successful, 30–50% of patients suffer from vascular restenosis within 6 months after the operation (1). The restenosis results from vascular smooth muscle cell (VSMC) migration from the media to the intima, where cells proliferate and cause on excessive accumulation of extracellular matrix produced by themselves (2–4). The precise mechanism of the restenosis is unknown; however, it is reported that some cytokines, such as platelet-derived growth factor (PDGF), are implicated in the development of the restenosis (4–6).

Tranilast is used for not only the patients with bronchial asthma and allergic rhinitis but also those with proliferative diseases such as keloid and hypertrophic scars. Tranilast is reported to inhibit the release of chemical mediators and cytokines from various cells (7–9) and the accumulation of collagen (10–12). On the other hand, it is reported that an angiotensin II (Ang II) takes part in intimal hyperplasia (13–15) and that angiotensin-converting enzyme inhibitor or Ang II-receptor antagonist prevents the intimal hyperplasia (16–19).

In this study, we investigated the effects of tranilast and

losartan, an Ang II-receptor antagonist, on the intimal hyperplasia in the balloon injury model and the cuff treatment model in rabbits fed regular chow. Moreover, to investigate the mechanisms involved, we assessed the effect of tranilast on PDGF-induced VSMC migration and proliferation and collagen synthesis.

### MATERIALS AND METHODS

#### Materials

Tranilast, *N*-(3,4-dimethoxycinnamoyl) anthranilic acid and losartan (Dup 753) were synthesized in our laboratories. Fetal bovine serum (FBS) was purchased from Gibco Laboratories (Grand Island, NY, USA). Human recombinant PDGF-BB was purchased from R&D System, Inc. (Minneapolis, MN, USA). <sup>3</sup>H-Thymidine, <sup>3</sup>H-proline and <sup>3</sup>H-leucine were obtained from Du Pont-NEN (Boston, MA, USA). DNA dye (Hoechst 33258) was purchased from Eastman Kodak Company (Rochester, NY, USA).

#### Animal

Japanese White male rabbits (body weight: approx. 3 kg) obtained from Kearsy Co., Ltd. (Osaka) were used. The rabbits were housed individually from 1 week before the experiment in a temperature (23 ± 2°C)–humidity

\* To whom correspondence should be addressed.

(55±5%) controlled room and were fed regular chow throughout the experimental periods.

#### *Balloon injury model*

Rabbits were anesthetized with 30 mg/kg, i.v. sodium pentobarbital. Bilateral common carotid arteries were exposed and dissected from the surrounding tissues. An arterial embolectomy catheter (model 2F; Baxter Healthcare Corporation, Santa Ana, CA, USA) was inserted about 5 cm into the right common carotid artery through an incision made on the wall of the vessel. The balloon was inflated with the pressure of 1200 mmHg, and the intraluminal surface of the artery was rubbed by pulling back the balloon. After rubbing, the embolectomy catheter was pulled out from the vessel, and the incision on the wall of vessel was sutured. The left common carotid artery was used for a sham-operation. Four weeks after injury, the rabbits were sacrificed by exsanguination under anesthesia. Right and left common carotid arteries were removed, and each were cut into two segments. One segment was fixed in a 10% neutral solution of formaldehyde for morphometric analysis, and the other was frozen at -40°C for later measurement of DNA content.

#### *Cuff treatment model*

Rabbits were anesthetized and bilateral common carotid arteries were exposed as described above. The right common carotid artery was sheathed with a cuff (length: 2 cm, inner diameter: 2.38 mm, outer diameter: 3.96 mm) made of a polyethylene tube (TYGON<sup>®</sup>, R3603; Norton KK, Tokyo). The cuff was ligated with a silk thread according to the method described by Hirosumi et al. (20). The left common carotid artery was used for a sham-operation. Three weeks later, the rabbits were sacrificed by exsanguination under anesthesia, and then the bilateral common carotid arteries were removed. These arteries were fixed in a 10% neutral solution of formaldehyde for morphometric analysis.

#### *Morphometric analysis*

Transverse-cut specimens of fixed vessels were embedded in paraffin, and cross sections were cut from these blocks. The sections were stained by the Elastica-Van Gieson method. The intimal, medial and luminal area were measured with a morphometric analyzer (Lussex III; Nikon, Tokyo). Results were given as the intimal area, intima/media (I/M) ratio and stenosis ratio. The stenosis ratio was calculated as follows:

$$\begin{aligned} \text{Stenosis ratio (\%)} \\ = (\text{intimal area} \times 100) / (\text{intimal} + \text{luminal area}) \end{aligned}$$

#### *DNA content*

DNA content was measured by a fluorometric assay as described by Labarca and Paigen (21). In brief, a 5-mm length of vessel was homogenized in phosphate-buffered saline (50 mM Na<sub>2</sub>PO<sub>4</sub>, 0.2 M NaCl, pH=7.4) and centrifuged at 3,000 rpm for 5 min. DNA dye (Hoechst 33258) was added to the supernatant at the final concentration of 1 µg/ml. The resulting fluorescence was measured at 458 nm. The change in DNA content was expressed as follows:

$$\begin{aligned} \Delta \text{DNA } (\mu\text{g}/5 \text{ mm}) = & \text{DNA in injured side } (\mu\text{g}/5 \text{ mm}) \\ & - \text{DNA in sham-operated side } (\mu\text{g}/5 \text{ mm}) \end{aligned}$$

#### *Drugs*

For the in vivo experiments, we chose the doses of tranilast according to the serum concentration that showed efficacy in the granuloma model of rats and in clinical use. The serum concentration of tranilast administered at the dose of 200–300 mg/kg to rabbits was nearly equal to those in rats given 100 mg/kg and humans given 600 mg/body/day. The doses of losartan were selected according to the report of Azuma et al. (17). Tranilast and losartan were suspended or dissolved in 0.5% carboxy methylcellulose solution and were administered perorally once a day from the day after the operation.

The serum concentration of tranilast after peroral administration at the dose of 200–300 mg/kg in rabbits is about 100 µM, so we used a tranilast concentration of 30 µM to 300 µM, which did not cause cell death, in this in vitro experiments.

#### *Cell culture*

Rabbit VSMCs were prepared by the explant method described by Ross (22). First, thoracic aortas were obtained from three rabbits. The adventitias were carefully removed, and the inner surfaces were scraped to remove endothelial cells. Then the medial explants were minced into 1-mm pieces and then cultured in DMEM supplemented with FBS (20%), penicillin (50 units/ml), streptomycin (50 units/ml), kanamycin (50 units/ml) and amphotericin B (1 mg/ml). After 2–3 weeks, the cells that had migrated from the explants were removed by trypsinization and seeded into 25-cm<sup>2</sup> flasks. Confluent VSMCs at the second passage were subcultured in DMEM supplemented with 10% FBS at a 1:5 split ratio. The identification of VSMCs was confirmed by typical morphological characteristics (spindle shape, hill and valley patterns) and indirect immunofluorescent staining with a monoclonal anti-α-smooth muscle actin antibody (clone: 1A4; Dako Japan Co., Ltd., Kyoto) (23). VSMCs were used within 10 passages in this study.

### Migration assay

The migration of cells was assayed by a modified Boyden's chamber method using a 96-well Boyden chamber apparatus (Neuroprobe, Inc., Cabin John, MD, USA) (24). PDGF-BB (50 ng/ml) was first diluted in DMEM with or without tranilast and then loaded into the lower wells of the Boyden chamber. The wells were subsequently covered with a standard 8-mm pore filter (Nucleopore Corp., Pleasanton, CA, USA) coated with type I collagen. Cell suspensions ( $1 \times 10^4$  cells) in DMEM containing 0.1% bovine serum albumin (BSA) with or without tranilast were then loaded into the upper wells of the chamber, after which the chamber was incubated for 4 hr at 37°C in an atmosphere of 95% air and 5% CO<sub>2</sub>. Non-migrated cells on the upper surface were scraped off. The filters were then fixed in methanol and stained with Diff-Quick staining solution (International Reagent Corp., Kobe). The number of VSMCs per 400× high-power field (HPF) that had migrated onto the lower surface of the filters was then determined microscopically. Four HPFs were counted per well, and the values were averaged.

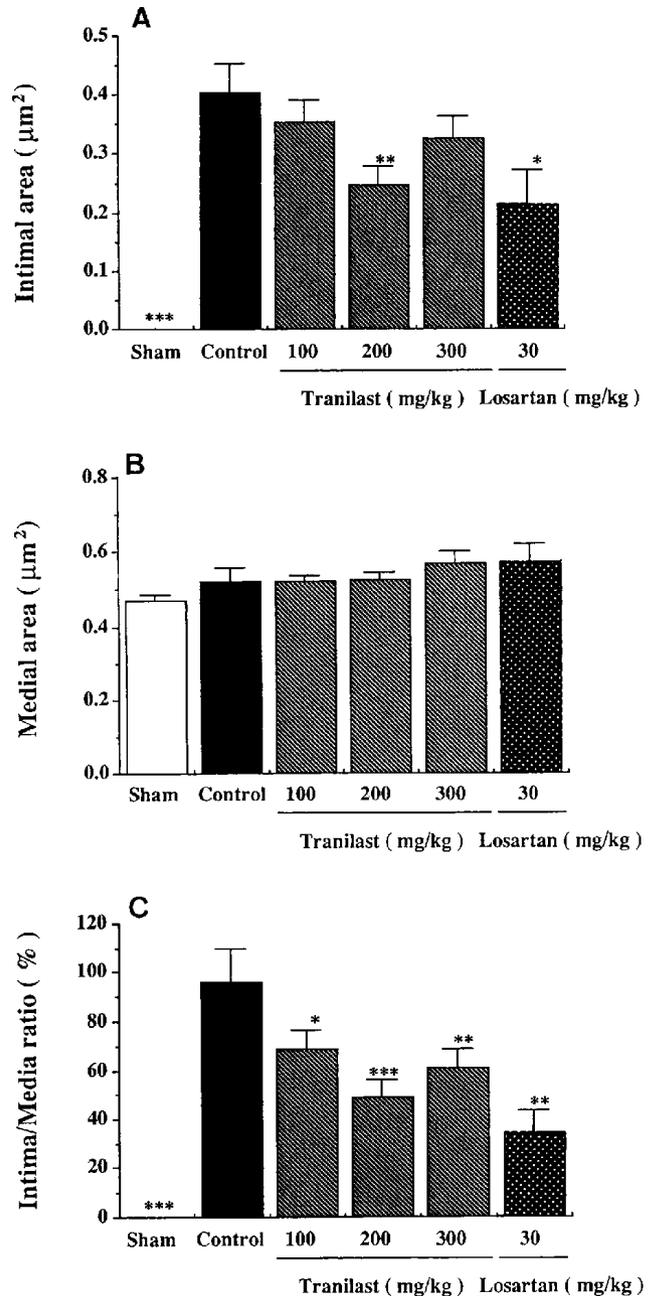
### Measurement of DNA synthesis

Cells were grown to confluence in 96-well tissue culture dishes and the growth was arrested for 48 hr in a serum-free medium consisting of DMEM supplemented with 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml selenium (ITS). The DMEM/ITS medium was employed to maintain the VSMC in a quiescent, but not catabolic, state, a condition resembling that of healthy cells in the normal arterial wall in vivo (25). The DMEM/ITS medium was then removed, and fresh DMEM containing PDGF-BB (50 ng/ml) was added to the quiescent cells. The cells were subsequently incubated for 22 hr in the absence or presence of tranilast. The cells were then incubated with <sup>3</sup>H-thymidine (46 kBq/ml) for 2 hr 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 then harvested onto Unifilter plates (GF/B 96; Packard Instrument Company, Meriden, CT, USA) with a cell harvester. The extent of <sup>3</sup>H-thymidine incorporation was determined by scintillation counting.

### Measurement of collagen synthesis

Confluent cells were incubated for 6 hr at 37°C in DMEM containing ascorbic acid (100 µg/ml), β-aminopropionitrile (100 µg/ml), and <sup>3</sup>H-proline (74 kBq/ml) with or without tranilast. Subsequently, the medium and cell layer were collected and then precipitated with 10% trichloroacetic acid and 0.5% tannic acid. After centrifugation, the pellet was washed with acetone

and dissolved in 0.1 N NaOH. Collagen synthesis was then quantified by a bacterial collagenase digestion method (*Clostridium histolyticum* type III; Wako, Tokyo) modified from procedure of Peterkofsky and Diegelmann (26). Collagen synthesis was determined by measuring the dpm of <sup>3</sup>H-proline per mg of DNA.



**Fig. 1.** Effects of tranilast and losartan on intimal area (A), medial area (B) and intima/media ratio (C) after balloon-induced endothelial denudation of carotid artery in rabbits. The arteries were examined 4 weeks after the injury. Data are each the mean ± S.E. of 4–10 animals. \*, \*\* and \*\*\*: Significantly different from the control at  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ , respectively.

### Measurement of total protein synthesis

Confluent cells were incubated for 6 hr at 37°C in DMEM containing  $^3\text{H}$ -leucine (74 kBq/ml) with or without tranilast. Subsequently, the medium and cell layer were collected and then precipitated with 10% trichloroacetic acid and 0.5% tannic acid. After centrifugation, the pellet was washed with 5% trichloroacetic acid and 0.25% tannic acid, and dissolved in PBS. Total protein synthesis was determined by measuring the dpm of  $^3\text{H}$ -leucine per  $\mu\text{g}$  of DNA.

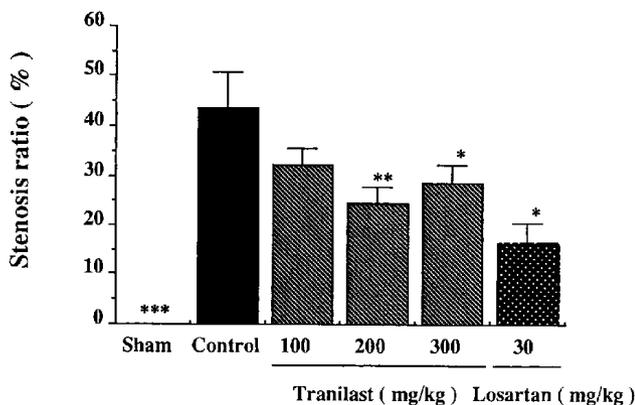
### Data analyses

Statistical analyses were performed by ANOVA and Fisher's PLSD test on a Stat View 4.0 software program (Abacus Concepts, Inc., Berkeley, CA, USA). Data are represented as means  $\pm$  S.E.

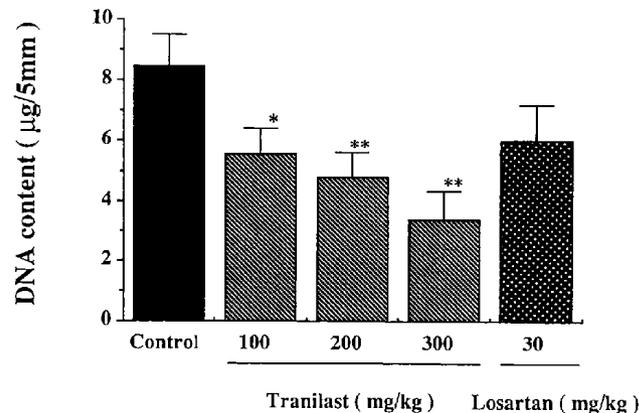
## RESULTS

### Effect of tranilast on intimal hyperplasia in balloon injury model

After vascular injury, although the medial area showed no significantly thickening, intimal hyperplasia occurred (Fig. 1: A and B). The I/M ratio in the control group increased to  $95.9 \pm 13.8\%$ . Tranilast (100, 200 and 300 mg/kg/day) significantly suppressed it to  $68.9 \pm 7.8\%$ ,  $49.0 \pm 7.1\%$  and  $60.8 \pm 8.1\%$ , respectively. Losartan (30 mg/kg/day) also suppressed the I/M ratio to  $33.8 \pm 9.5\%$  (Fig. 1C). The stenosis ratio in the control group also increased to  $43.6 \pm 7.0\%$ . Tranilast (200 and 300 mg/kg/day) and losartan (30 mg/kg/day) significantly inhibited this ratio (Fig. 2). Furthermore, vascular DNA content in the control group increased after vascular in-



**Fig. 2.** Effects of tranilast and losartan on stenosis ratio after balloon-induced endothelial denudation of carotid artery in rabbits. The arteries were examined 4 weeks after the injury. Data are each the mean  $\pm$  S.E. of 4–10 animals. \*\* and \*\*\*: Significantly different from the control at  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ , respectively.

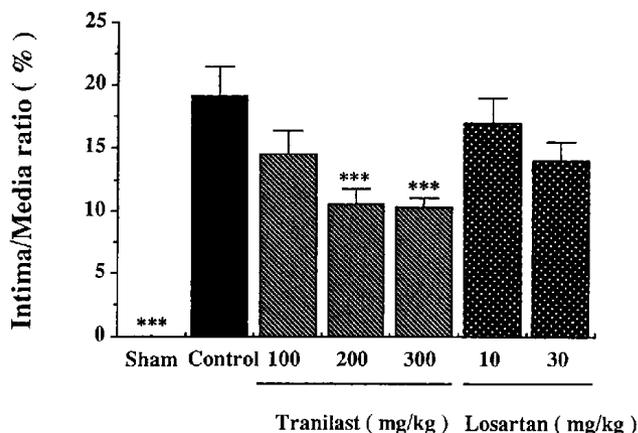


**Fig. 3.** Effects of tranilast and losartan on the vascular DNA contents after balloon-induced endothelial denudation of carotid artery in rabbits. The arteries were removed 4 weeks after the injury and analyzed for DNA content. Data are each the mean  $\pm$  S.E. of 4–8 animals. \* and \*\*: Significantly different from the control at  $P < 0.05$  and  $P < 0.01$ , respectively.

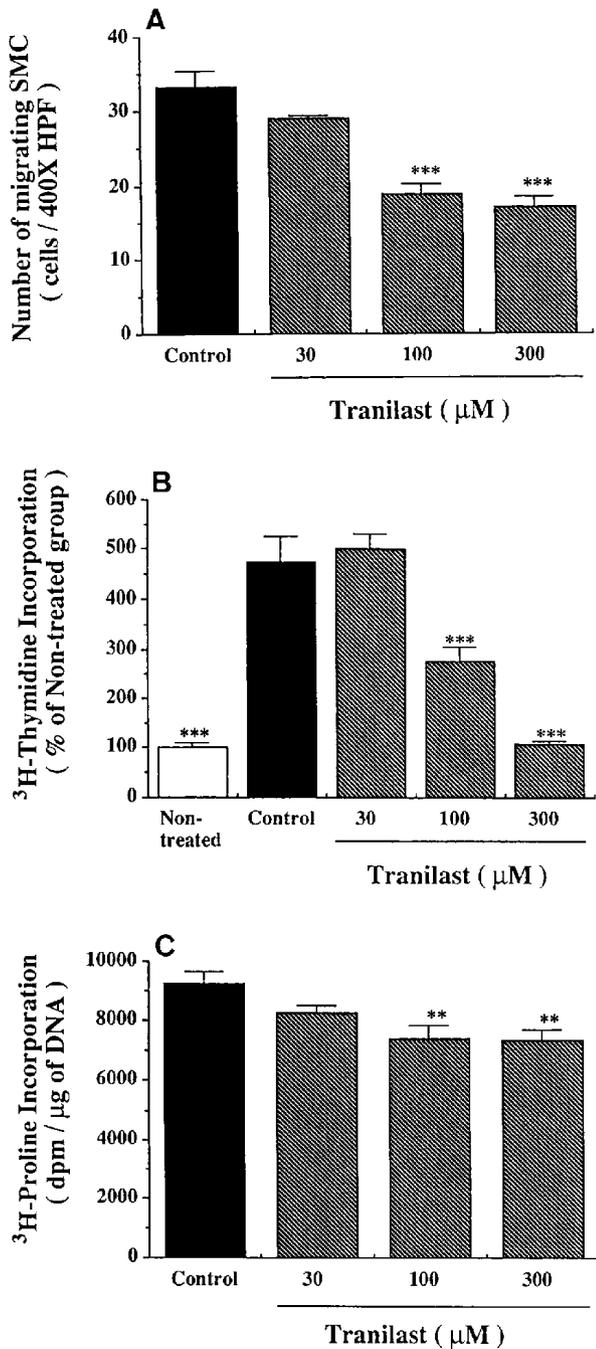
jury. Tranilast (100–300 mg/kg/day) inhibited dose-dependently and significantly the increase in DNA content. On the other hand, losartan showed no significant effect on the DNA content after vascular injury (Fig. 3).

### Effect of tranilast on intimal hyperplasia in cuff treatment model

Intimal hyperplasia also occurred by cuff treatment. That is, the I/M ratio in the control group increased to  $19.1 \pm 2.3\%$ . Tranilast (200 and 300 mg/kg/day) significantly inhibited the intimal hyperplasia to  $10.6 \pm 1.2\%$  and  $10.3 \pm 0.7\%$ , respectively. Losartan showed no effect on intimal hyperplasia (Fig. 4).



**Fig. 4.** Effects of tranilast and losartan on intimal/medial ratio after cuff treatment around the common carotid artery in rabbits. The arteries were examined 3 weeks after treatment. Data are each the mean  $\pm$  S.E. of 6–11 animals. \*\*\*: Significantly different from the control at  $P < 0.001$ .



**Fig. 5.** Effect of tranilast on PDGF-induced VSMC migration of (A), PDGF-induced VSMC proliferation by (B) and collagen synthesis in (C) rabbit VSMCs. Concentration of PDGF was 50 ng/ml. Data are each the mean  $\pm$  S.E. of 5 experiments. \*\* and \*\*\*: Significantly different from the control at  $P < 0.01$  and  $P < 0.001$ , respectively.

#### Effect of tranilast on VSMC migration

PDGF-BB induced VSMC migration ( $33.2 \pm 2.2$  cells/400  $\times$  HPF) in the control group. Tranilast (100 and 300  $\mu$ M) concentration-dependently and significantly decreased the number of migrating cells (Fig. 5A).

#### Effect of tranilast on DNA synthesis

<sup>3</sup>H-Thymidine incorporation was measured as a marker of DNA synthesis. <sup>3</sup>H-Thymidine incorporation increased to  $474.1 \pm 50.1\%$  after stimulation by PDGF-BB in the control group. Tranilast (100 and 300  $\mu$ M) significantly decreased <sup>3</sup>H-thymidine incorporation (Fig. 5B).

#### Effect of tranilast on collagen synthesis and total protein synthesis

<sup>3</sup>H-Proline incorporation was measured as a marker of collagen synthesis. Tranilast (100 and 300  $\mu$ M) concentration-dependently and significantly inhibited the <sup>3</sup>H-proline incorporation by VSMCs (Fig. 5C). On the other hand, tranilast had no effect on total protein synthesis by VSMCs (data not shown).

## DISCUSSION

Animal models of vascular intimal hyperplasia are separable into two categories: the first is intraluminal manipulation such as in vascular endothelial denudation using a balloon catheter, and the second is perivascular manipulation such as in positioning a cuff around an artery (27). In the intraluminal manipulation model, platelets aggregate and leukocytes accumulate on the endothelial denudated area, where they release cytokines (4, 28). These cytokines induce VSMC migration from the media to the intima and VSMC proliferation there. The VSMCs that have migrated and proliferated not only release cytokines, which make other VSMCs migrate and proliferate, but also produce an extracellular matrix (2-4). The precise mechanism of intimal hyperplasia in the perivascular manipulation model is yet unknown; However, it is reported that leukocytes accumulate in the intima of the perivascularly manipulated area, where their products cause endothelial denudation (20, 27). After endothelial loss, intimal hyperplasia then occurs by the same mechanism as in the intraluminal manipulation model.

In this study, we investigated the effect of tranilast on intimal hyperplasia in both intraluminal manipulation and perivascular manipulation models, i.e., in the balloon injury model and the cuff treatment model, respectively. In both animal models, intimal hyperplasia occurred and tranilast suppressed it. Moreover, in the balloon injury model, tranilast inhibited the increase in vascular DNA content, whose increase may indicate VSMC migration and/or proliferation. These results suggested that tranilast suppresses the intimal hyperplasia after vascular injury.

On the other hand, losartan suppressed the intimal hyperplasia in the balloon injury model, like tranilast. However, losartan had no effect on the intimal hyper-

plasia in the cuff treatment model, although tranilast suppressed it. In the cuff treatment model, the leukocytes accumulated in the intima play an important role in endothelial denudation via the inflammatory response, including the production of oxygen radicals (20). Tranilast is reported to prevent the inflammatory response and production of oxygen radicals (11). On the other hand, losartan is an Ang II antagonist, and it may be ineffective to prevent inflammation. Because of these reasons, we speculated that the difference of the effects of tranilast and losartan in the cuff treatment model is due to the effect on the inflammatory responses. In addition, it is reported that tranilast has an Ang II-receptor antagonistic effect; however, the  $IC_{50}$  of tranilast is about 1/1000 that of losartan (29). From these things, we thought that tranilast shows the suppressive effect on the intimal hyperplasia via the different mechanisms from losartan.

Tranilast had no effect on platelet aggregation (data not shown). So, the effect of tranilast on intimal hyperplasia depends on the inhibition of the pathway subsequent to platelet aggregation. Previous studies suggested that some cytokines, such as PDGF, released from vascular endothelial cells, aggregated platelets and VSMCs, play an important role in the development of intimal hyperplasia (4–6). Because of these findings, we investigated the effects of tranilast on the PDGF-induced VSMC migration and proliferation. Also, we observed the VSMC synthesis of collagen, a protein component of the extracellular matrix, because it is reported that the accumulation of collagen produced by VSMCs is one of the causes of the intimal hyperplasia (2, 28). In this study, tranilast inhibited both PDGF-induced VSMC migration and proliferation. Moreover, tranilast inhibited collagen synthesis by VSMCs but not total protein synthesis, suggesting a specific effect on collagen synthesis. Thus tranilast prevents VSMC migration and proliferation, and accumulation of the extracellular matrix.

From the findings in this study, we conclude that tranilast manifests its suppressive effect on intimal hyperplasia after vascular injury by preventing VSMC migration from the media to the intima, VSMC proliferation in the intima, and accumulation of the extracellular matrix produced by the VSMCs. Recently, it was reported that tranilast suppressed intimal hyperplasia in photochemically-induced intimal hyperplasia in spontaneously hypertensive rats (30). These results suggest that tranilast is useful for preventing restenosis after PTCA.

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