Tranilast Suppresses Vascular Chymase Expression and Neointima Formation in Balloon-Injured Dog Carotid Artery

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Background—Activation of vascular chymase plays a major role in myointimal hypertrophy after vascular injury by augmenting the production of angiotensin (ANG) II. Because chymase is synthesized mainly in mast cells, we assumed that the chymase-dependent ANG II formation could be downregulated by tranilast, a mast cell–stabilizing antiallergic agent. We have assessed inhibitory effects of tranilast on neointima formation after balloon injury in the carotid artery of dogs, which share a similar ANG II–forming chymase with humans, and further explored the pathophysiological significance of vascular chymase.

Methods and Results—Either tranilast (50 mg/kg BID) or vehicle was orally administered to beagles for 2 weeks before and 4 weeks after balloon injury. Four weeks after the injury, remarkable neointima was formed in the carotid arteries of vehicle-treated dogs. Chymase mRNA levels and chymaselike activity of vehicle-treated injured arteries were increased 10.2- and 4.8-fold, respectively, those of uninjured arteries. Angiotensin-converting enzyme (ACE) activity was slightly increased in the injured arteries, whereas ACE mRNA levels were not. Tranilast treatment completely prevented the increase in chymaselike activity, reduced the chymase mRNA levels by 43%, and decreased the carotid intima/media ratio by 63%. In vehicle-treated injured arteries, mast cell count in the adventitia showed a great increase, which was completely prevented by the tranilast treatment. Vascular ACE activity and mRNA levels were unaffected by tranilast.

Conclusions—Tranilast suppressed chymase gene expression, which was specifically activated in the injured arteries, and prevented neointima formation. Suppression of the chymase-dependent ANG II–forming pathway may contribute to the beneficial effects of tranilast. (Circulation. 1999;99:1084-1090.)

Key Words: angiotensin ■ leukocytes ■ restenosis ■ angiogenesis ■ polymerase chain reaction

Percutaneous transluminal coronary angioplasty (PTCA) is an established therapy for obstructive coronary artery diseases. However, its long-term efficacy is limited by frequent occurrence of restenosis. Migration and proliferation of smooth muscle cells (SMCs) with subsequent formation of neointima are considered important in late stenosis after PTCA. In this process, angiotensin (ANG) II supposedly plays a major role. Involvement of ANG II in the pathogenesis of restenosis was further confirmed by the facts that ACE inhibitors as well as ANG II receptor antagonists effectively prevented neointima formation in balloon-injured rats. On the basis of these experimental findings, ACE inhibitors were applied in clinical trials for preventing restenosis after PTCA. However, ACE inhibitors proved to be ineffective on human restenosis.

Such species differences in the ACE inhibitor effects on neointima suggested the presence of alternative ANG II–forming pathways in humans. Previously we demonstrated that chymase contributed to ANG II formation in human, monkey, and dog vascular tissues. Chymases of these species cleave the Phe-His bond of ANG I and produce ANG II efficiently. In contrast, rat chymase (rat mast cell protease I [RMCP-1]) hydrolyzes the Tyr-Ile bond to yield inactive fragments. Therefore the vascular ANG II–forming system in primates and dogs is quite different from that in rats. Such species differences in the vascular ANG II–forming systems may explain the differential effects of ACE inhibitors on myointimal hypertrophy. To confirm our hypothesis, we established balloon-injured models of dogs that have the dual ANG II–forming systems like that in humans and compared the effects of an ACE inhibitor and an ANG II receptor antagonist. Balloon injury remarkably activated canine vascular chymase, and an ANG II receptor antagonist substantially prevented neointima formation, whereas an ACE inhibitor did so only modestly. These results indicate that chymase contributes to the pathogenesis of intimal hyperplasia by augmenting the local ANG II production to a greater extent than does ACE.

Chymase is synthesized mainly in connective tissue–type mast cells and secreted into the interstitium. Some antiallergic
drugs are capable of stabilizing mast cell functions. Therefore the chymase-dependent ANG II formation may be attenuated by such agents that suppress mast cell activation. Tranilast, N-(3,4-dimethoxycinnamoyl) anthranilic acid, is an antiallergic drug that has clinical indications such as bronchial asthma, allergic rhinitis, atopic dermatitis, and keloid and hypertrophic scar. Thus we investigated in dogs whether tranilast inhibits neointima formation and whether vascular chymase is involved in the pathogenesis of neointimal hyperplasia after balloon injury.

Methods

Balloon Catheterization of Carotid Arteries

Twelve male beagles weighing 9 to 12 kg (Nihon Nosan, Yokohama, Japan) underwent balloon catheterization as described previously with modification. Under pentobarbital anesthesia, the anterior thyroid arteries of both right and left sides were exposed. Through the right side artery, a balloon catheter (3F, VERMED) was inserted into the right common carotid artery with caution to minimize unintended surgical damages to the outer wall of the common carotid artery and pushed further into the proximal direction. The balloon was inflated with water to distend the common carotid artery and pulled back. After this procedure was repeated thrice, the catheter was removed and the insertion port was ligated. The left common carotid artery was kept intact and used as the control. All other procedures including heparin and antibiotic dosing were performed as previously noted.

Drug Administration

The beagles were placed randomly into 2 groups. One group (n=5) was orally given tranilast (50 mg/kg BID; Kissei Pharmaceutical) for 2 weeks before balloon injury and for 4 weeks thereafter throughout the experimental period. The other group (n=7) received the vehicle during the same period. Experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (Animal Research Laboratory, Osaka Medical College).

Histological Analysis

Four weeks after ballooning, the animals were given an overdose of pentobarbital and exsanguinated by heart excision. The common carotid arteries of both sides were excised and divided into 5 parts, respectively. From each part, a small segment (3 mm long) was obtained and fixed with neutral buffered formalin, paraffin-embedded, and cut into 3-μm-thick sections. These sections were stained with Carazzi’s hematoxylin. Cell density was determined by counting vasa vasorum in the total adventitial area and expressed as density (vessels/mm²). The number and localization of mast cells were estimated with toluidine blue-stained sections. Because almost all mast cells are located in the adventitia, its density was expressed as total mast cell count in adventitia/total adventitial area.

Correlation analyses were performed between (1) intimal and adventitial areas, (2) intimal area and mast cell density, and (3) adventitial area and mast cell density for the injured carotid arteries of vehicle-treated and tranilast-treated dogs.

Measurement of ACE and Chymaselike Activities

ACE activity was measured with hippuryl-His-Leu substrate (Peptide Institute). Chymaselike activity was measured with ANG I substrate as described previously.

Measurement of Plasma Renin Activity and Plasma ANG II Content

Blood samples for measurement of plasma renin activity (PRA) and plasma ANG II content were taken into chilled tubes containing 5 mmol/L EDTA and 1 mmol/L PMSF (final concentrations) 2 hours after the final dosing. PRA was measured by radioimmunoassay with a commercial kit (SRL). Plasma ANG II content was measured by radioimmunoassay after fractionation by reverse-phase high-performance liquid chromatography with the use of anti-ANG II antiserum (a gift from Dr. S. Kim, Osaka City University).

Quantitative Reverse-Transcriptase Polymerase Chain Reaction Analysis

Chymase and ACE mRNA levels of carotid arteries were determined with quantitative reverse-transcriptase polymerase chain reaction (PCR) analysis described previously with modifications. The PCR primers for dog chymase were selected according to the dog chymase cDNA sequence (sense primer: 5'-ATCCTACCTCTCCCCAGGATT-CCTCCT-3', position 130 to 153; antisense primer: 5'-CACAAAGTTTGATCCCAAGGCGG-3', position 741 to 718). The competitor DNA for dog chymase was obtained by inserting a 360-bp external DNA fragment to the BstXI site. For determination of the dog ACE cDNA sequence, PCR primers were designed at first based on the human ACE cDNA sequence (sense primer: 5'-AACAGGCTCTGAACACATG-3', position 483 to 463; antisense primer: 5'-CTGAGGTTGCTCGTCTGAGT-3', position 924). The PCR product was subcloned, and a 504-bp dog ACE cDNA sequence was determined, then the specific PCR primers for quantification of dog ACE transcripts were designed according to the already-determined dog ACE cDNA sequence (sense primer: 5'-AACAGGATCTATTCCACAGGACC-3', position 464 to 484; antisense primer: 5'-AGGCAGACCCTCTGAGT-3', position 919 to 899). The competitor DNA for dog ACE was prepared by inserting a 360-bp external DNA fragment to the BstXI site. The amplification conditions for dog chymase were 94°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute over 40 cycles. The conditions for dog ACE were 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute over 40 cycles. The integrated density of PCR products was measured as described previously.

Measurement of Plasma and Vascular Tissue Concentration of Tranilast

Plasma samples were obtained 2 and 12 hours after drug administration the day before the euthanasia. Plasma aliquots (100 μL) were incubated for 10 minutes at room temperature with 200 μL of internal standard solution (N-cinnamoylthranilic acid, 100 μg/mL) and 2 mL of ethanol and centrifuged at 3000 rpm for 10 minutes. Supernatants were evaporated to dryness, and the residues were resuspended with 150 μL of 50% acetoniwater and 10 μL of acetic acid and then fractionated by reverse-phase high-performance liquid chromatography. Common carotid arteries of both sides were dis-
sected 2 hours after the final dosing on the day of euthanasia. The excised arteries (200 mg) were minced and homogenized in 1 mL of 0.02 mol/L NaOH and 200 µL of internal standard solution. The homogenate was incubated for 30 minutes at room temperature with 3 mL of acetonitrile and centrifuged at 3000 rpm for 10 minutes. The supernatant was processed in the same manner as for plasma. The final recovery of tranilast from tissues or plasma after overall procedures was >90%.

Direct Effect of Tranilast on Chymase Activity In Vitro
The direct inhibitory effect of tranilast on the catalytic activity of chymase was analyzed with purified human chymase (from gastro-epiploic arteries24) and dog carotid arterial preparations. One milli-unit of human chymase was preincubated with 10⁻⁶, 10⁻⁵, 10⁻⁴, and 10⁻³ mol/L tranilast in 20 mmol/L Tris-HCl buffer (pH 8.0) containing 0.5 mol/L KCl and 0.1% (vol/vol) Triton X-100 for 1 hour at 37°C and then incubated with 100 µmol/L ANG I for 10 minutes. ANG II was determined as mentioned earlier. After tissue extract from dog carotid artery was preincubated with the same series of concentrations of tranilast for 1 hour at 37°C, its chymase-like activity was measured as described earlier.

Statistical Analyses
All numerical data are expressed as mean±SEM. Significant differences between the means of different groups were evaluated by Student’s t test for unpaired data and by the modified t test for multiple comparison (Tukey’s method) after 2-way ANOVA. Simple linear regression was used to determine correlation between several morphological indexes mentioned in the “Histological Analysis” section.

Results
Effect of Tranilast on Vascular Remodeling
The balloon injury elicited remarkable neointima formation (Figure 1A). Tranilast markedly prevented this neointima formation and decreased the intima/media ratio of injured arteries by 63% (P<0.05, Figures 1B and 2). The lumen area of vehicle-treated injured arteries (1.00±0.09 mm², n=7) was smaller than that of vehicle-treated uninjured arteries (1.56±0.17 mm², n=7, P<0.05), indicating luminal stenosis. After treatment with tranilast, luminal stenosis as well as neointima formation were prevented: The lumen area of injured arteries (1.66±0.21 mm², n=5) was not significantly different from that of uninjured arteries (1.87±0.20 mm², n=5). The cross-sectional area of the media was not altered by the injury, nor differed between the injured arteries of the vehicle-treated and the tranilast-treated dogs. After the injury, fibrosis (Figure 1G), angiogenesis (Figure 1I), and an increase in mast cell count (Figure 1D) were observed in the adventitia. The adventitial area/medial area ratio of injured arteries of vehicle-treated dog (I) and tranilast-treated dog (J). Scale bar, 100 µm for A through C; 50 µm for D through J.
uninjured arteries ($P<0.05$, Figure 2). In the tranilast-treated
group, adventitia/media ratio did not differ between injured
and uninjured arteries (Figure 2). Adventitial fibrosis was
analyzed with Azan-stained sections. In vehicle-treated dogs,
the blue-stained area in the adventitia of injured arteries
(3.82±0.50 mm$^2$, n=7) was 2.2 times that of uninjured
arteries (1.72±0.20 mm$^2$, n=7, $P<0.05$). In tranilast-treated
dogs, the blue-stained area in the adventitia of injured arteries
(2.15±0.21 mm$^2$, n=5) did not significantly differ from that
of uninjured arteries (1.76±0.28 mm$^2$, n=5). In vehicle-
treated dogs, the vasa vasorum density in injured arteries
(60.4±8.5 vessels/mm$^2$, n=7) was 1.5 times that of uninjured
arteries (40.3±4.6 vessels/mm$^2$, n=7, $P<0.05$). In tranilast-
treated dogs, vasa vasorum density in injured arteries
(46.9±2.8 vessels/mm$^2$, n=5) was not significantly different
from that of uninjured arteries (35.9±7.3 vessels/mm$^2$, n=5).
PCNA-positive cells were found mainly in the neointima
(Figure 1E), whereas few were found in the media and
adventitia. The PCNA index in the neointima of tranilast-
treated injured arteries (0.018%±0.011%, n=5) was significantly
lower than that of vehicle-treated injured arteries (0.35%±0.13%, n=7, $P<0.05$).

### Cell Density of Intimal, Medial, and Adventitial Layers

<table>
<thead>
<tr>
<th></th>
<th>Intimal Layer</th>
<th>Medial Layer</th>
<th>Adventitial Layer</th>
</tr>
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<tbody>
<tr>
<td>Vehicle-treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injured (n=7)</td>
<td>21.4±1.74</td>
<td>12.7±0.45</td>
<td>7.68±0.37</td>
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<td>Uninjured (n=7)</td>
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<td>11.8±0.67</td>
<td>7.48±0.59</td>
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<tr>
<td>Tranilast-treated</td>
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<td>Injured (n=5)</td>
<td>25.2±0.69</td>
<td>12.5±0.49</td>
<td>7.83±0.66</td>
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<tr>
<td>Uninjured (n=5)</td>
<td>ND</td>
<td>11.4±0.28</td>
<td>7.43±0.35</td>
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</tbody>
</table>

ND indicates not determined.

Cell density expressed as total number of cells per area (mm$^2$) of intimal,
medial, and adventitial layers.

few mast cells in uninjured vascular walls, whereas numerous
cells were found in the injured vascular wall (Figures 1D and
3A). Mast cells were located primarily in the adventitia and
occasionally seen in the outer media (Figure 1D). In the dogs
treated with tranilast, mast cell density in the injured arteries
was decreased to the level of the uninjured arteries ($P<0.05$,
Figure 3A). Some correlation existed between adventitial and
intimal areas ($r=0.388$, $P=0.0026$, Figure 3B), between
the change in mast cell density and the magnitude of neointima
formation ($r=0.639$, $P<0.0001$, Figure 3C), and between
the change in mast cell density and the magnitude of adventitial
thickening ($r=0.756$, $P<0.0001$, Figure 3D).

### Chymaselike and ACE Activities of Carotid Arteries and Plasma ACE Activity

The chymaselike activity of vehicle-treated injured arteries
exhibited an increase 10.2-fold that of uninjured arteries
($P<0.05$). The long-term tranilast treatment completely
suppressed the chymaselike activity of injured arteries to the
normal level (Figure 4A). The ACE activity of vehicle-treated
injured arteries was increased 1.3 times that of uninjured
arteries ($P<0.05$), whereas that of tranilast-treated injured
arteries was 1.2 times that of uninjured arteries ($P<0.05$).
Thus tranilast did not affect vascular ACE activity (Figure
4B). The plasma ACE activity of the tranilast-treated dogs
(11.2±1.8 mU/mL, n=5) did not differ from that of the
vehicle-treated controls (10.5±0.8 mU/mL, n=7).

### PRA and Plasma ANG II Content

The PRA (0.95±0.20 ng/mL per hour, n=5) and the plasma
ANG II content (14.6±6.3 pg/mL, n=5) of tranilast-treated
dogs did not differ from the PRA (0.84±0.38 ng/mL per hour, n=7) and the plasma ANG II content (15.2±3.8 pg/mL, n=7) of vehicle-treated controls.

ACE and Chymase mRNA Levels of Carotid Arteries

The chymase mRNA level of vehicle-treated injured arteries exhibited an increase 4.8-fold that of uninjured arteries (P<0.05). Tranilast treatment blunted the increase of chymase mRNA level in the injured arteries to 57% that for the vehicle-treated group (P<0.05, Figure 5A), although the level still remained significantly higher than that of the uninjured arteries after tranilast treatment (Figure 5A). In contrast, there was no difference in the ACE mRNA levels between the injured and the uninjured arteries in vehicle-treated dogs (Figure 5B). Tranilast did not affect the ACE mRNA levels of injured or uninjured arteries (Figure 5B).

Plasma and Tissue Tranilast Concentrations

Plasma tranilast concentrations 2 and 12 hours after its dosing on the penultimate day of the experiment were 297±33 μmol/L and 55.0±9.2 μmol/L, respectively. Tissue tranilast concentration 2 hours after the final dosing was 32±7 nmol/g tissue in injured carotid arteries and 29±3 nmol/g tissue in uninjured arteries.

Direct Effect of Tranilast on Chymase Activity

In Vitro

Tranilast at concentrations of 10⁻⁶, 10⁻⁵, 10⁻⁴, and 10⁻³ mol/L inhibited the ANG II-forming activity of purified human chymase by 2%, 4%, 7%, and 25%, respectively. Tranilast in concentrations up to 10⁻³ mol/L did not inhibit the chymaselike activity in the tissue extract of dog carotid artery.

Discussion

In the injured arteries, chymaselike activity and chymase mRNA level were remarkably increased, whereas a slight increase in ACE activity and no increase in ACE mRNA expression were detected. The current study demonstrated for the first time that tranilast prevented vascular chymase expression and effectively inhibited neointima formation and luminal stenosis. Our previous study showed that the vascular ANG II content doubled in the injured arteries compared with that in the uninjured arteries and that an ANG II receptor antagonist but not an ACE inhibitor prevented the neointima formation. In the current study, tranilast treatment did not affect plasma ANG II concentration, plasma ACE activity, or PRA. Vascular ANG II–forming activity of chymase increased 4.8-fold in vehicle-treated dogs, whereas tranilast treatment completely prevented the increase in chymase activity. Therefore, the ANG II–forming rate in local vascular tissues supposedly increased after vascular injury but returned to the normal level by tranilast treatment. Tranilast decreased significantly the intima/media ratio and the PCNA index of the neointima. Tranilast may exhibit an antiproliferative effect on neointimal cells. These results suggest that the activated vascular chymase promotes tissue remodeling by augmenting the local ANG II production in injured vascular tissues.

Long-term treatment with tranilast completely suppressed the increase of vascular chymaselike activity in vivo. However, tranilast up to a concentration of 10⁻³ mol/L did not directly affect the chymaselike activity in vitro. Chymase derives mainly from connective tissue–type mast cells. In the current study, mast cell density was markedly increased in the balloon-injured arteries, but such an increase was almost completely prevented by the long-term tranilast treatment. A clinical finding also showed that the mast cell counts were reduced in lesions of urticaria pigmentosa after long-term tranilast treatment. Mast cell count was greatly increased in the adventitia after balloon injury, but few PCNA-positive cells were there, indicating that the mast cell number might be increased primarily because of migration into the adventitia. Therefore tranilast might inhibit the mast cell migration, thereby indirectly suppressing vascular chymaselike activity. Cell-to-cell interaction between fibroblasts and mast cells through regulation of stem cell factor (SCF)/c-kit expression is essential for the development of mast cells. Tranilast is also known to downregulate the activity of fibroblasts. Although the precise mechanism is yet unknown, tranilast may inhibit the development as well as degranulation of mast cells.

In addition to neointima formation, adventitial thickening occurred in the injured arteries. PCNA-positive cells were
found mainly in the neointima, whereas few were found in the adventitia. A recent study with pig balloon-injured models indicated that the PCNA index of each vascular wall compartment increased maximally 3 to 7 days after injury and returned to baseline after 28 days.26 Our study analyzed the proliferative activity of arteries 4 weeks after balloon injury, when the adventitia might become largely quiescent. Furthermore, adventitial cell density of injured arteries did not differ from that of uninjured arteries, whereas Azan-stained areas of collagen fibers increased in the adventitia of injured arteries. These results suggest that adventitial thickening can be ascribed mainly but not simply to collagen accumulation. Tranilast prevented adventitial fibrosis as well as neointimal hyperplasia. Recent observations indicated the close interaction between adventitial remodeling and neointima formation. After deep medial coronary injury, adventitial myofibroblasts migrated across the EEL toward the intima and contributed to intimal thickening by diverse actions described above, a dose of >50 mg/kg (BID) is required to exhibit those actions sufficiently in vivo. Instead, tranilast at the dose of 50 mg/kg completely inhibited the increases in mast cell numbers and ANG II–forming chymase activity in the injured vascular walls. Accordingly, inhibition of chymase-dependent ANG II–forming pathway by suppressing both activation and development of cellular matrix and is associated with fibrotic conditions.36

The pathophysiological role of tryptase, another major secretory product of mast cells, remains unclear, but recent studies proved tryptase as a potent mitogen for fibroblasts.37 Tranilast completely inhibited the injury-induced increase of mast cells. Therefore it is possible that tranilast treatment may suppress these potent fibroproliferative factors together with chymase. The current study explored the beneficial effect of tranilast from the aspect of inhibition of chymase-dependent ANG II–forming pathway. However, in view of the overall functions of vascular mast cells, tranilast is potentially more beneficial than a simple ANG II receptor antagonist.

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References

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