Full Paper

A Single Treatment With a Specific Chymase Inhibitor, TY-51184, Prevents Vascular Proliferation in Canine Grafted Veins

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Received December 19, 2003; Accepted February 17, 2004

Abstract. In this study, we evaluated whether a specific chymase inhibitor, TY-51184 (2-[4-(5-fluoro-3-methylbenzo[*b*]thiophen-2-yl)sulfonamido-3-methanesulfonylphenyl]oxazole-4-carboxylic acid), prevents the vascular proliferation in canine grafted veins. In the placebo- and chymase inhibitor-treated groups, the external jugular vein was infiltrated with saline and 10 μ M TY-51184, respectively, and then it was grafted to the ipsilateral carotid artery. The non-surgical dogs were used as the control group. By 28 days after grafting, the chymase and ACE activities were significantly increased in the injured arteries. TY-51184 significantly reduced the chymase activity in the grafted veins, while it did not affect the ACE activity. The intimal areas in the placebo- and TY-51184-treated groups were 3.32 ± 0.16 and 1.96 ± 0.52 mm², respectively, and this difference was significant. The ratios of intimal area to medial area in the placebo- and TY-51184-treated groups were $66.8 \pm 3.5\%$ and $34.9 \pm 9.2\%$, respectively, and this difference was also significant. There was a significant relationship between vascular proliferation and chymase activity, but not ACE activity. In this study, we demonstrated that a single treatment with a specific chymase inhibitor, TY-51184, could prevent the vascular proliferation in canine grafted veins.

Keywords: chymase, inhibitor, graft, vascular proliferation

Introduction

Vascular proliferation after percutaneous transluminal coronary angioplasty (PTCA) or bypass graft is mainly dependent on an increase of extracellular matrix components. Angiotensin (Ang) II is known to activate extracellular matrix components, resulting in inducing the proliferation of vascular tissues (1–3). In general, Ang II is known to be generated from Ang I by angiotensin-converting enzyme (ACE). However, human, monkey, dog, and hamster vascular tissues contain chymase as an Ang II-forming enzyme, while rat vascular tissue does not (4–9). Previous reports demonstrated that an ACE inhibitor was effective in preventing vascular proliferation in grafted arteries or injured vessels by a balloon catheter in rat (10, 11). Rat vascular tissue contains ACE only as an Ang II-forming enzyme, and these reports suggest that Ang II plays a crucial role in vascular proliferation. In fact, an Ang II type 1

(AT₁)-receptor antagonist could also prevent vascular proliferation after balloon injury (12). However, in clinic, an AT₁-receptor antagonist, but not an ACE inhibitor, could prevent restenosis after PTCA (13, 14). Dog vascular tissues, like humans, contain ACE and chymase as Ang II-forming enzymes, and an AT₁-receptor antagonist was effective in preventing vascular proliferation after balloon injury and bypass graft in the dog, but an ACE inhibitor was ineffective (15, 16). Such species differences in the effect of ACE inhibitor on the proliferation of vascular tissues may depend on whether or not a given species possesses Ang II-forming chymase in vascular tissue. In fact, we reported that treatment with Suc-Val-Pro-Phe^p(Oph)₂, which inhibits chymase activity, to the vein only once before grafting could prevent the intimal hyperplasia in canine grafted veins 28 days after the operation (17). However, this peptide inhibitor inhibits chymotrypsin-like enzymes such as cathepsin G which can produce Ang II from Ang I, and it is not a specific inhibitor against chymase (18, 19).

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Fig. 1. The chemical structure of TY-51184.

In this study, we evaluated a specific chymase inhibitor, 2-[4-(5-fluoro-3-methylbenzo[*b*]thiophen-2-yl)sulfonamido-3-methanesulfonylphenyl]oxazole-4-carboxylic acid (TY-51184), on the vascular proliferation in canine grafted vein (20, 21). TY-51184 used in this study inhibits human, dog, and hamster chymases by IC_{50} at concentrations of 37, 58, and 128 nM, respectively. However, this inhibitor does not suppress cathepsin G even at 100 μ M. In this study, we investigated whether a single treatment of a specific chymase inhibitor, TY-51184, could prevent the vascular proliferation in canine grafted veins.

Materials and Methods

Agents and animals

TY-51184 was a kind gift from Toa Eiyo Ltd. (Oomiya). Figure 1 shows the chemical structure of TY-51184, and the molecular weight of TY-51184 is 510.5. Eighteen dogs weighing 10 to 12 kg were obtained from Japan SLC (Shizuoka). The animals were anesthetized with sodium pentobarbital (35 mg/kg, i.v.). In the groups treated with the placebo ($n = 6$) and the chymase inhibitor ($n = 6$), the external jugular vein was infiltrated with saline and 10 μ M TY-51184, respectively, and then it was grafted to the ipsilateral carotid artery. The non-surgical dogs were used as the control group ($n = 6$). The experimental procedures for animals were in accordance with the Guide for the Care and Use of Laboratory Animals (Animal Research Laboratory, Osaka Medical College).

Preparation of vascular tissue

Twenty-eight days after the operation, the animals were anesthetized with sodium pentobarbital (35 mg/kg, i.v.). After anesthetizing, the grafted veins were removed. Each vein was cut in half, and one segment was used for the measurement of cross-sectional area, and the other segment was used for the assay of the Ang II-forming enzymes.

Measurement of chymase and ACE activities

Chymase activities were measured by incubating the tissue extracts for 1 h at 37°C with 4 mM Ang I

in 150 mM borax-borate buffer, pH 8.5, containing 5 mM ethylenediaminetetraacetic acid, 8 mM dipyridyl, and 0.77 mM diisopropylfluorophosphate, as described previously (17). The enzyme reaction was terminated by addition of 15% trichloroacetic acid (w/v) and the reaction mixture was placed in ice water for 10 min. After centrifugation of the reaction mixture at 15,000 rpm for 5 min, we applied 50 μ l of the supernatant to an octadecyl silica reversed-phase column (4.6 mm $\times$ 25 cm; Tohso, Tokyo), which had been equilibrated with 30% methanol in 10 mM phosphoric acid and eluted it with a linear gradient of 30–90% methanol in 10 mM phosphoric acid at a rate of 1 ml/min. Ang II was detected by ultraviolet absorbance at 226 nm. One unit of chymase activity was defined as the amount of enzyme that cleaved 1 μ mol Ang II/min.

ACE activities were measured by incubating the tissue extracts for 1 h at 37°C with 5 mM hippuril-His-Leu as a substrate in 100 mM phosphate buffer, pH 8.3, containing 800 mM NaCl (17). The enzyme reaction was terminated by addition of 3% metaphosphoric acid (w/v) and the reaction mixture was placed in ice water for 10 min. After centrifugation of the mixture at 15,000 rpm for 5 min, we applied 50 μ l of the supernatant to an octadecyl silica reversed-phase column (4.6 mm $\times$ 25 cm, Tohso), which had been equilibrated with 10 mM KH_2PO_4 and CH_3OH (1:1, pH 3.0), and eluted it with the same solution at a rate of 0.3 ml/min. Hippuric acid was detected by ultraviolet absorbance at 228 nm. One unit of ACE activity was defined as the amount of enzyme that cleaved 1 μ mol hippuric acid/min.

Histochemical analysis of vascular tissue

The vessel segments were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 5- μ m-thick sections. These sections were stained with elastica-van Gieson, and the cross-sectional areas of intima and media were quantified with an image analysis system (VM-30; Olympus Optical Co., Tokyo).

Statistical analyses

All data shown in the text are expressed as means $\pm$ S.E.M. Significant differences between the means of the two groups were evaluated by Student's *t*-test. Significant differences among the mean values of multiple groups were evaluated by 1-way ANOVA followed by a post-hoc analysis (Fisher's test).

Results

Chymase and ACE activities in vascular tissues

Figure 2 shows the activities of vascular Ang II-

Fig. 2. Effects of TY-51184 on ACE and chymase activities 28 days after the operation. ** $P < 0.01$ vs grafted veins in the placebo-treated group.

forming enzymes, chymase, and ACE, 28 days after the operation. The chymase activity in the grafted veins was increased about 11.5-fold compared with that in the control veins (control veins, 1.03 ± 0.07 mU/mg protein; grafted veins, 11.8 ± 1.05 mU/mg protein) and the ACE activity was also increased about 1.9-fold (control veins, 0.92 ± 0.06 mU/mg protein; grafted veins, 1.78 ± 0.17 mU/mg protein). In the group treated with TY-51184, the increased chymase activity in the grafted veins was reduced significantly. The percent inhibition of chymase by treatment with TY-51184 was

64.2%. However, TY-51184 did not affect the ACE activity in the grafted veins.

Effects of TY-51184 on vascular proliferation in grafted vein

Typical photographs of grafted veins treated with placebo and TY-51184, stained with elastica-van Gieson, are shown in Fig. 3. The intimal areas in the placebo- and TY-51184-treated groups were 3.32 ± 0.16 and 1.96 ± 0.52 mm², respectively, and this difference was significant (Fig. 4). The ratios of intimal area to

Fig. 3. Typical photographs of the injured arteries in the placebo- and TY-51184-treated groups 28 days after the operation. I, intima; M, media.

Fig. 4. Effects of TY-51184 on the intimal area, medial area, and the ratio of the intimal area to the medial area in the grafted veins 28 days after the operation. Open and filled columns show the placebo- and TY-51184-treated groups, respectively. * $P < 0.05$ vs grafted veins in the placebo-treated group.

Fig. 5. The correlations between Ang II-forming enzymes and vascular proliferation.

medial area in the placebo- and TY-51184-treated groups were $66.8 \pm 3.5\%$ and $34.9 \pm 9.2\%$, respectively, and this difference was also significant (Fig. 4).

Correlation of Ang II forming enzymes and vascular proliferation

There was a significant relationship between vascular proliferation and chymase activity, but not ACE activity (Fig. 5).

Discussion

In this study, a single treatment with a specific chymase inhibitor, TY-51184, to the vein before grafting was able to prevent the intimal hyperplasia in grafted veins 28 days after the operation. Previously, we reported that a single treatment with a chymase inhibitor, Suc-Val-Pro-Phe^P(OPh)₂, to the vein also prevented the vascular proliferation of the grafted vein 28 days after the operation (17). However, Suc-Val-Pro-Phe^P(OPh)₂ is not a specific inhibitor against chymase, and this

inhibitor inhibits not only chymase but also cathepsin G which can produce Ang II from Ang I. On the other hand, TY-51184 is a highly specific chymase inhibitor; the IC₅₀ values of TY-51184 for chymotrypsin, elastase, and cathepsin G were higher than 100 μ M. The dose of TY-51184 used in this study completely inhibits dog chymase, but it does not suppress cathepsin G at all. Therefore, we demonstrated that a single treatment with a specific chymase inhibitor, TY-51184, could prevent the vascular proliferation in canine grafted vein.

In the present study, both the ACE and chymase activities in the grafted veins 28 days after the operation were increased 1.9- and 11.5-fold compared with that in the control veins, respectively. However, in a previous study, we reported that the ACE activity in the grafted veins was significantly decreased up to 7 days after the operation; and especially after 1 and 3 days, it was suppressed in the grafted veins to less than 10% of the control veins (22). The reason why the ACE activity was decreased at acute periods after the operation is thought to be dependent on the loss of the endothelium. Because the endothelium in grafted veins is put under arterial pressure, it results in the loss of the endothelium including ACE (23). On the other hand, 7 days after the operation, the chymase activity was significantly increased in the grafted veins (20). Considering these findings, up to 7 days after the operation, the Ang II formation in the grafted veins is thought to depend mainly on the chymase-dependent Ang II-forming pathway. In fact, the Ang II concentration and the mRNA levels of fibronectin, collagen I, and collagen III, all of which are induced by an increase of Ang II action, were significantly increased in the grafted veins 7 days after the operation, while they were completely suppressed by a single treatment with Suc-Val-Pro-Phe^P(OPh)₂ (22). These findings suggest that chymase-dependent Ang II

formation may play an important role in the development of vascular proliferation via increases of extracellular matrix such as fibronectin, collagen I, and collagen III. Therefore, chymase inhibition at the acute phase after the operation may play a crucial role in the preventive mechanism of vascular proliferation in the grafted veins.

Coronary artery bypass grafting is offered to patients with ischemic heart disease (24, 25). The internal thoracic artery and saphenous vein have been frequently used as coronary artery bypass conduits. However, the poor performance of the saphenous vein compared with the internal thoracic artery is well known (26, 27). In isolated human saphenous vein, the contractile response of Ang II is greater than that in the internal thoracic artery, suggesting that the saphenous vein exhibits greater Ang II-mediated action than the internal thoracic artery (28). Moreover, the chymase activity and total Ang II-forming activity, but not the ACE activity, is significantly higher in human saphenous vein than in the internal thoracic artery (29). This high level of total Ang II-forming activity in the saphenous vein is thought to be dependent on the upregulated chymase activity. In the present study, there was a significant relationship between vascular proliferation and chymase activity, but not ACE activity. These findings suggest that the chymase-dependent Ang II-action may be more important rather than ACE-dependent Ang II-action in the development of vascular proliferation in the grafted veins. However, to clarify the effect of ACE-dependent Ang II action on vascular proliferation in a dog graft model, further studies, such as the effect of ACE inhibitors, may be needed.

In this study, we demonstrated that a single treatment with a specific chymase inhibitor, TY-51184, could prevent the vascular proliferation in canine grafted veins.

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