Inhibition of chymase reduces vascular proliferation in dog grafted veins

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Abstract We investigated the effect of a chymase inhibitor Suc-Val-Pro-Phe^P(OPh)$_2$ on the proliferation of the grafted vein in dog. By 28 days after the operation, the mean intimal area of the grafted vein in the placebo group was 3.24 ± 0.32 mm^2. The intimal area of the grafted vein in the chymase inhibitor-treated group was reduced to 63.9%. In the placebo group, the activities of chymase and angiotensin-converting enzyme in grafted vein were significantly increased 15- and 2-fold, respectively. In the chymase inhibitor-treated group, chymase activity in the grafted veins was decreased significantly. These findings suggest that inhibition of chymase appears useful for preventing vascular proliferation.

Key words: Chymase; Inhibitor; Angiotensin II; Angiotensin-converting enzyme

1. Introduction

Vascular proliferation after vascular surgery and coronary angioplasty has been thought to be dependent on an increase of growth factors such as platelet-derived growth factor [1]. Angiotensin (ANG) II activates these growth factors, and directly acts on extracellular signal-regulated kinase, which leads to mitogenic or hypertrophic responses, resulting in inducing the proliferation of vascular tissues [2,3]. In general, it is known that ANG II is generated from ANG I by angiotensin-converting enzyme (ACE). However, in vascular tissues in human, monkey, dog and hamster, chymase also converts ANG I to ANG II [4–8]. On the other hand, vascular tissues in rat and rabbit do not contain ANG II-forming chymases [6]. Chymase hydrolyzes the C-terminal side of proteins after aromatic amino acids such as Phe, Tyr and Trp. However, using the substrate ANG I, human-type chymase cleaves the Phe^8-His^9 bond of ANG I to yield the active substance ANG II, while rat-type chymase cleaves the Tyr^4-Ile^5 bond of ANG I to form inactive fragments [9,10].

ANG II produced from ANG I in vascular tissues is also known to induce vascular contraction, but the conversion of ANG I to ANG II is very different in various species. Vascular contractions after injection of ANG I in isolated arteries of rat were completely inhibited by an ACE inhibitor only, suggesting that vascular tissues in rat contain ACE only as the ANG II-forming enzyme. On the other hand, ANG I-induced vascular contractions in human, monkey and dog were not suppressed completely by an ACE inhibitor [6]. The remaining contraction was suppressed by chymostatin, which inhibits chymotrypsin-like enzymes including chymase, thus suggesting that this remainder is due to chymase-dependent ANG II formation. Thus, the chymase-dependent ANG II-forming system has been shown by in vitro experiments, while that of in vivo experiments has not as yet been clarified because there are no chymase inhibitors that are useful in in vivo experiments.

In vascular proliferative responses of ANG II, an ACE inhibitor was effective in preventing the vascular proliferation of post-balloon injury of vessels in rat [11]. Rat vascular tissues contain ACE only as an ANG II-forming enzyme, and this result suggests that vascular ANG II formation plays a crucial role in tissue proliferation. Based on this report, it was studied whether an ACE inhibitor suppresses human vascular restenosis after percutaneous transluminal coronary angioplasty (PTCA), but the result was negative [12]. In grafted vessels, an ACE inhibitor also prevented the vascular proliferation in rat, but not in a baboon model [13,14]. Such species differences in the effects of ACE inhibitors on the proliferation of vascular tissues may depend on whether or not a given species possesses ANG II-forming chymase in vascular tissue. Therefore, the chymase-dependent ANG II formation in vascular tissue may be closely related to promoting growth in vivo.

In this report, we investigated whether Suc-Val-Pro-Phe^P(OPh)$_2$, which inhibits strongly chymase activity [15], could prevent vascular proliferation in grafted veins.

2. Materials and methods

2.1. Animal treatments

Ten dogs weighing 8 to 10 kg were obtained from Japan SLC (Shizuoka, Japan), and were divided into two groups, a group treated with chymase inhibitor, Suc-Val-Pro-Phe^P(OPh)$_2$, and a vehicle group. Each dog underwent right common carotid artery bypass grafting with the ipsilateral external jugular vein. The animals were anesthetized with sodium pentobarbital (35 mg/kg). In the placebo group, the right external jugular vein was removed and was infiltrated for 20 min in saline containing isosorbid dinitrate (50 mg/kg) and dipyridamole (100 mg/kg), which were used as agents for vascular dilation and antithrombosis, respectively. In the group treated with the chymase inhibitor, the vein was infiltrated for 20 min in the solution used for the placebo group, but with the addition of Suc-Val-Pro-Phe^P(OPh)$_2$ (10 μM). Then, the vein was grafted to the ipsilateral artery. 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).

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Abbreviations: DFP, diisopropyl phosphorofluoridate; EDTA, ethylenediaminetetraacetic acid

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2.2. Preparation of vascular tissue

At 28 days after the operation, the animals were anesthetized with sodium pentobarbital (35 mg/kg), and then the grafted and the symmetrical veins were removed. We cut the tissue in half. One segment was used for the measurement of the cross-sectional area, and the other segment was used for assay of the ANG II-forming enzymes, ACE and chymase. Immediately after excision, the tissues used for histological analysis were formalin-fixed, and the tissues used for the determination of enzyme activity were frozen in liquid nitrogen. The symmetrical carotid artery in the placebo-treated group was removed and was used for the study of ANG I-induced vascular contraction.

2.3. Vascular contraction

The dog carotid arteries were cut into helical strips, 15 mm in length and 2.0 mm in width. The strips from dog were placed on an organ bath under a resting tension of 1.5 g in the bathing medium [16]. The medium was continuously aerated with O_2/CO_2 (95:5), which was maintained at 37°C. The strip was equilibrated for 2 h before the experiments of ANG I-induced contractile response. The contractile response to 50 mM KCl was obtained first, and then the bathing medium was washed out. The medium was washed out three times for 20 min, each time with fresh Tyrode's solution, and equilibrated for 40 min. ANG I (final concentration of 100 nM) was added to the bathing medium. The steps for the ANG I-induced response were repeated once again, and the second ANG I-induced response was regarded as the control ANG I-induced response. After the response, the medium was washed out three times for 20 min, each time with fresh Tyrode's solution. An ACE inhibitor, lisinopril (final concentration of 1 μM), was added and preincubation was conducted for 20 min and then the ANG I-induced response was observed. In the same manner, the ANG I-induced responses were observed after preincubation for 20 min with a combination of 1 μM lisinopril, and chymostatin or Suc-Val-Pro-Phe^P(OPh)$_2$.

2.4. Histological analysis of vascular tissue

The vessel segments were fixed in 10% neutral buffered formalin, embedded in paraffin and cut into 5-μm 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, Japan).

2.5. Measurement of ACE and chymase activities

The vessels were homogenized in 10 volumes (w/v) of 20 mM Na-phosphate buffer, pH 7.4. The homogenate was centrifuged at 20 000 × *g* for 30 min. The pellets were re-suspended and homogenized in 5 volumes (w/v) of 10 mM Na-phosphate buffer, pH 7.4, containing 2 M KCl and 0.1% (v/v) Nonidet P-40. The homogenate was stored overnight at 4°C, and centrifuged at 20 000 × *g* for 30 min at 4°C. The supernatant was used for the measurements of ACE and chymase activities. The ACE activity was measured using a synthetic substrate, hippuryl-His-Leu (HHL), specifically designed for ACE [17]. One unit of ACE activity was defined as the amount of enzyme that formed 1 μmol hippuric acid/min. The chymase activity was measured by incubating tissue extracts for 30 min at 37°C with 770 μM ANG I in 150 mM borax-borate buffer, pH 8.5, containing 8 mM dipyridyl, 770 μM DFP and 5 mM EDTA, as described previously [18]. The enzyme reaction was terminated by addition of 15% (w/v) trichloroacetic acid. One unit of chymase activity was defined as the amount of enzyme that formed 1 μmol ANG II/min.

2.6. Statistical analysis

All experiments were done with five dogs per group. Statistical analysis was done using ANOVA followed by a Student's *t*-test. Values of $P < 0.05$ were considered significant. Data are expressed as mean ± standard error of the mean (S.E.M.).

3. Results

Fig. 1 shows the effects of chymostatin and Suc-Val-Pro-Phe^P(OPh)$_2$ on the vascular contractions induced by 100 nM ANG I in the presence of an ACE inhibitor in the isolated dog vascular tissues. Suc-Val-Pro-Phe^P(OPh)$_2$ suppressed the ANG I-induced vascular contraction in isolated dog arteries in the presence of an ACE inhibitor. The IC_{50} values of chymostatin and Suc-Val-Pro-Phe^P(OPh)$_2$ in dog artery were 97 nM and 2.8 nM, respectively.

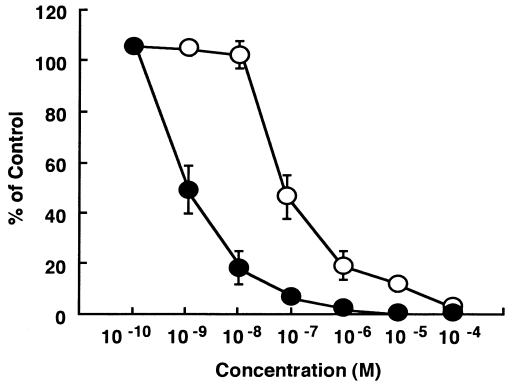

Fig. 1. Effects of chymostatin (○) and Suc-Val-Pro-Phe^P(OPh)$_2$ (●) on the contraction induced by 100 nM ANG I in the presence of 1 μM lisinopril in the isolated dog carotid arteries ($N = 5$).

The intimal formation of the grafted veins in the placebo group was developed from 14 days and was completely formed at 28 days, and we decided on investigating the vessels 28 days after the operation (data not shown). As shown in Fig. 2 as typical sections, the chymase inhibitor suppressed the area of intima. The areas of intima and media from the grafted and symmetrical veins treated with placebo or the chymase inhibitor are shown in Table 1. In the placebo group, the intimal area of the grafted vein was 3.24 ± 0.32 mm^2, but that of the symmetrical vein was hardly measured. The mean intimal area of the grafted veins in the placebo group was regarded as 100%, while that in the group treated with chymase inhibitor was reduced to 63.9%.

In the placebo group, the chymase activity in the grafted veins was increased significantly when compared with that in the symmetrical veins (the grafted veins vs. the symmetrical veins: 12.4 ± 1.10 vs. 0.82 ± 0.15 mU/mg protein) (Fig. 3). The ACE activities in the grafted and symmetrical veins were 4.46 ± 0.58 and 2.16 ± 0.26 mU/mg protein, respectively, and this difference was also significant (Fig. 3). In the chymase inhibitor-treated group, the chymase activity was 5.73 ± 1.17 mU/mg protein and was decreased significantly compared to the levels of the grafted veins in the placebo group (Fig. 4). The ACE activities in the grafted veins were not affected by treatment with the chymase inhibitor (Fig. 4).

4. Discussion

In the present study, we demonstrated for the first time that a chymase inhibitor suppressed vascular proliferation in grafted veins in dog, suggesting that the chymase-dependent ANG II-forming system has an important role in vascular

Table 1
The areas of intima and media in the placebo- and chymase inhibitor-treated groups

Group	Area (mm^2)	
	Intima	Media
Placebo	3.24 ± 0.32	3.43 ± 0.39
Chymase inhibitor	1.17 ± 0.21*	3.26 ± 0.36

Values represent the mean ± S.E.M. ($N = 5$).
*$P < 0.001$ vs. the placebo-treated group.

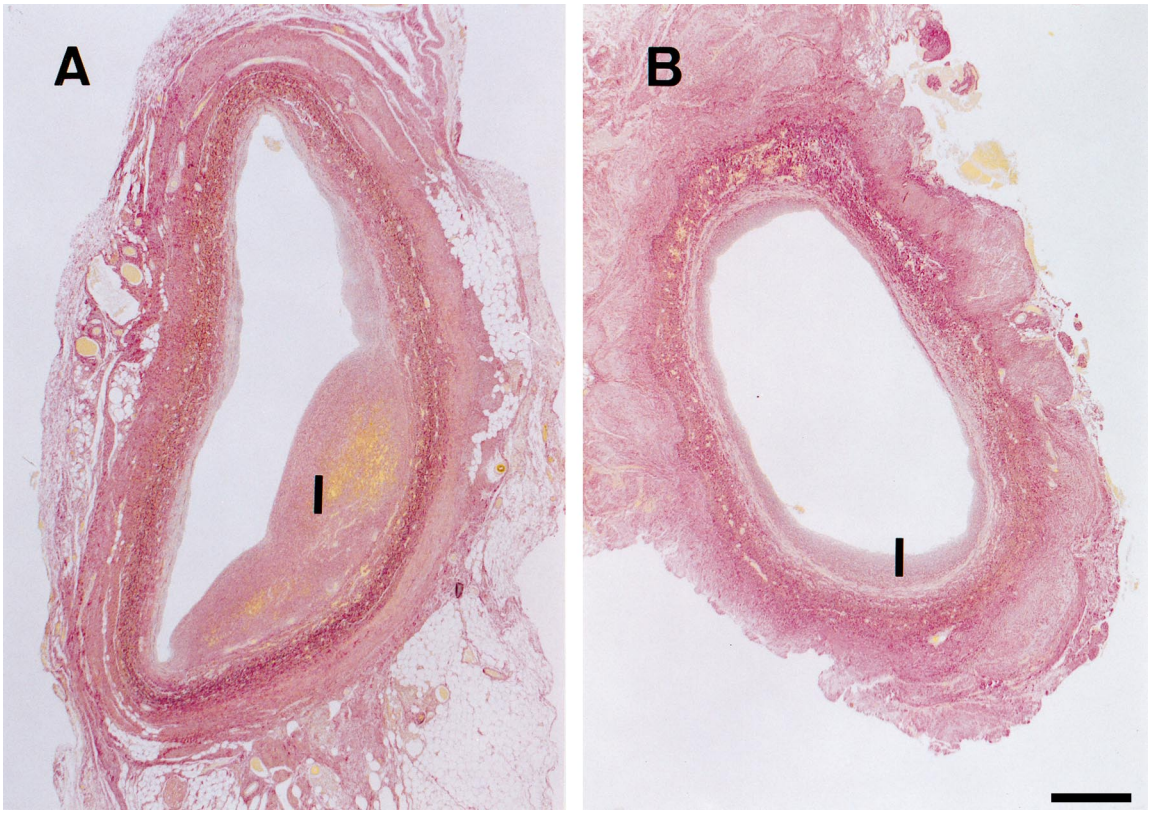

Fig. 2. Histological cross-sections stained with elastica-van Gieson of the grafted vein (A) from the dog treated with placebo, and of the grafted vein (B) from the group treated with Suc-Val-Pro-Phe^{P}(OPh)$_2$. I, intima. Bar, 1 mm. These sections are representative of the findings summarized in Table 1.

proliferation in vivo. Previously, Oleksyszyn and Powers [15] reported that Suc-Val-Pro-Phe^{P}(OPh)$_2$ is a specific chymase inhibitor and the half-degradative time is about 20 h in human plasma. Suc-Val-Pro-Phe^{P}(OPh)$_2$ suppressed the ANG I-induced vascular contraction in isolated dog arteries in the presence of an ACE inhibitor, thus indicating a chymase-dependent ANG II formation (Fig. 1). The IC_{50} value of Suc-Val-Pro-Phe^{P}(OPh)$_2$ in dog artery was 2.8 nM. The IC_{50} value of Suc-Val-Pro-Phe^{P}(OPh)$_2$ was about 35 times larger than that of chymostatin, which is typical of a compound that inhibits chymase [4–8]. These findings suggest that Suc-Val-Pro-Phe^{P}(OPh)$_2$ may be a stable and strong chymase inhibitor in vivo; however, because the inhibitor cannot be orally administered, it has been thought that the compound could not

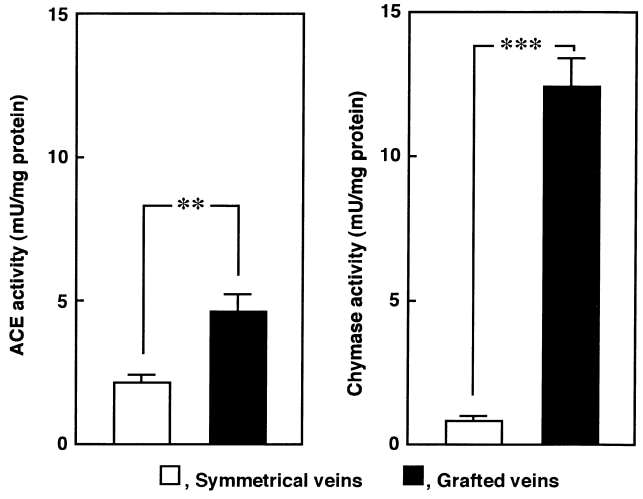

Fig. 3. ACE and chymase activities of the symmetrical veins and grafted veins from dogs treated with placebo. Each bar represents the mean ± S.E.M. ($N = 5$). **$P < 0.01$ and ***$P < 0.001$ vs. the symmetrical veins.

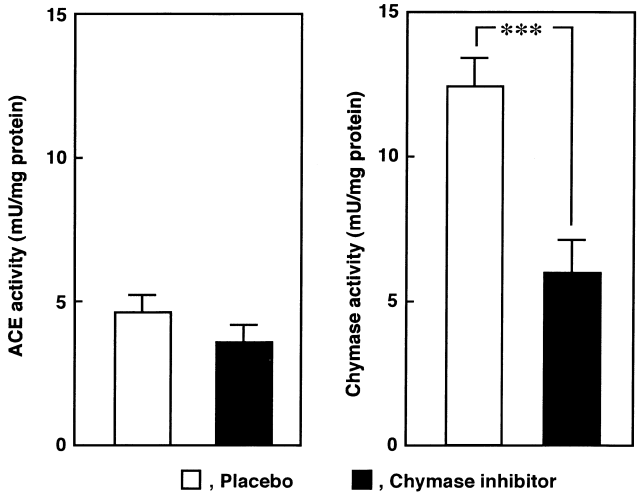

Fig. 4. Effects of Suc-Val-Pro-Phe^{P}(OPh)$_2$ on ACE activity and chymase activity of the grafted veins from dogs treated with placebo or Suc-Val-Pro-Phe^{P}(OPh)$_2$. Each bar represents the mean ± S.E.M. ($N = 5$). ***$P < 0.001$ vs. the placebo group.

be used in vivo. In the present study, we demonstrated that local infiltration, and not oral administration, of Suc-Val-Pro-Phe^{P}(OPh)$_2$ (10 μM) could prevent vascular proliferation in grafted veins.

Clinically, stenosis of the coronary arteries is primarily treated by intracoronary therapies such as PTCA or by surgical therapies such as grafts of arteries or veins. Previously, in dog, which has the chymase-dependent ANG II-forming pathway, the activities of both ACE and chymase in arteries injured by a balloon catheter were increased compared with those in uninjured arteries; an ANG II type 1 receptor antagonist was effective in preventing intimal formation of vessels after injury, but an ACE inhibitor was ineffective [19]. These findings suggest that the chymase-dependent ANG II-forming system may be closely related to promoting the growth of vascular tissues, while the role in the chymase-dependent ANG II-forming system could not be investigated, because there are no chymase inhibitors that are useful in vivo. In the present model, the chymase and ACE activities in the grafted veins were increased 15- and 2-fold, respectively, whereas treatment with the chymase inhibitor significantly decreased the chymase activity, but not the ACE activity. From the relation between the pathogenesis and the activities of these ANG II-forming enzymes, the chymase-dependent ANG II formation may be more important for stenosis than the ACE-dependent ANG II formation. In fact, treatment with the chymase inhibitor only suppressed the development of the intimal formation in this model.

The chymase inhibitor was used only during the operation and not continuously, but it suppressed the intimal formation of the grafted vein even 28 days after the operation. Clinically, the veins for grafting are infiltrated with the agents for vascular dilation and antithrombosis. In the present study, we only added the chymase inhibitor into the solution, and this method may be useful easily in clinical study. However, it is interesting that the chymase inhibitor, applied to the grafted vein only once after the operation, inhibited the chymase activity even 28 days after the operation. It is reported that chymase, an enzyme that is present in mast cell granules, is released from the granules upon strong stimulation, binds to extracellular matrix, and continues to function for several weeks [20–22]. In this study, mast cells derived from the grafted vessels were activated 1 day after the operation (unpublished data), indicating that chymase was bound to the extracellular matrix immediately after the operation, and thereby continued to function for several weeks. The chymase inhibitor used in this study functions irreversibly, which means that the inhibitor, once bound to the enzyme, continues to inhibit it for a long time. In fact, up to 7 days after the operation, the chymase activity was completely inhibited by treatment with the chymase inhibitor (unpublished data). It is thought that the inhibitor was irreversibly bound to the chymase that was released upon degranulation immediately after the operation, which then bound to the extracellular matrix, resulting in the inhibition of chymase for several weeks. This may be the reason why chymase activity remained inhibited even 28 days after the operation. Moreover, chymase is known to activate stem cell factor, a typical cytokine that has the ability to induce accumulation of mast cells [23]. In this study, the chymase inhibitor may also suppress the accumulation of mast cells, resulting in the inhibition of chymase 28 days after the operation.

In conclusion, chymase plays an important role in vascular proliferation in vivo, and thus inhibition of chymase may be useful for preventing vascular diseases such as vascular proliferation in grafted vessels.

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