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Suppression of basic fibroblast growth factor-induced angiogenesis by a specific chymase inhibitor, BCEAB, through the chymase-angiotensin-dependent pathway in hamster sponge granulomas

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1 We investigated the profound involvement of mast cell chymase, an alternative angiotensin II-generating enzyme, in angiogenesis using a specific chymase inhibitor. We also studied the functional profiles of this novel inhibitor in basic fibroblast growth factor (bFGF)-induced angiogenesis.

2 In this study, angiogenesis was induced by daily injections of bFGF (0.3 μg site^{-1} day^{-1}), angiotensin I (2 nmol site^{-1} day^{-1}) or angiotensin II (2 nmol site^{-1} day^{-1}) into sponges implanted to male hamsters subcutaneously for 7 days. Angiogenesis in the granulation tissue surrounding sponges was evaluated by measuring the haemoglobin (Hb) content and local blood flow as the parameters for angiogenesis.

3 A chymase inhibitor, BCEAB (4-[1-{[bis-(4-methyl-phenyl)-methyl]-carbamoyl}-3-(2-ethoxy-benzyl)-4-oxo-azetidine-2-yloxy]-benzoic acid), was simultaneously administered into the implanted sponges (2 or 5 nmol site^{-1} day^{-1}, for 7 days) treated with bFGF and strongly suppressed the haemoglobin contents in sponge granulomas. In the studies using a laser doppler perfusion imager, BCEAB (5 nmol site^{-1} day^{-1}) also attenuated the bFGF-induced increase of local blood flow around the implanted sponge granuloma.

4 In bFGF-induced angiogenesis, chymase activity in sponge granulomas was substantially increased. It was also confirmed that the chymase activity increased by bFGF was significantly and dose-dependently inhibited by BCEAB (2, 5 nmol site^{-1} day^{-1}).

5 BCEAB inhibited the Hb contents and the expression of vascular endothelial growth factor (VEGF) mRNA induced by angiotensin I but not by angiotensin II.

6 These results suggest that the significance of chymase in bFGF-induced angiogenesis was confirmed, and a novel inhibitor, BCEAB, strongly suppresses the bFGF-induced angiogenesis through the chymase-angiotensin II-VEGF dependent pathway.

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Abbreviations: ACE, angiotensin converting enzyme; bFGF, basic fibroblast growth factor; Hb, haemoglobin; VEGF, vascular endothelial growth factor

Introduction

A disabled regulation of angiogenesis is strongly implicated in the pathogenesis of numerous diseases including chronic inflammatory diseases, diabetic retinopathy and tumour growth (Folkman, 1971; Michaelson, 1948; Wise, 1956; Peacock *et al*., 1992; Colville-Nash & Scott, 1992). Angiogenesis is a multistep event and is regulated by various factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), or epidermal growth factor (EGF) positively or negatively (Fan & Brem, 1992; Folkman & Shing, 1992).

Angiotensin II also enhances neovascularization in several animal models and increases blood flow in ischaemia-induced angiogenesis (Fernandez *et al*., 1985; Le Noble *et al*., 1991; Emanueli *et al*., 2002; Sasaki *et al*., 2002; Tamarat *et al*., 2002). From the results of experiments in a mouse sponge model and in a laser-induced macular degeneration model using angiotensin II receptor antagonists, it has been suggested that the effects of angiotensin II in angiogenesis are mediated by the angiotensin type I (AT1) receptor (Walsh *et al*., 1997; Hikichi *et al*., 2001).

Angiotensin converting enzyme (ACE) has been believed to predominate in the production of angiotensin II. An alternative pathway for generating angiotensin II, i.e. the chymase-dependent angiotensin II-generating system, has been identified (Okunishi *et al*., 1984). Chymase is a chymotrypsin-like serine protease originally found as a mast cell protease (Langunoff & Benditt, 1963). The enzymatic character of chymase has been identified in some species, it cleaves the C-terminal of peptides just after aromatic amino acids such as Phe, Tyr and Trp. There are species differences in the cleavage of angiotensin I (Okunishi *et al*., 1993). In humans, monkeys, dogs and hamsters, chymase generates angiotensin II from angiotensin I by cleaving the Phe^{8}-His^{9} bond, but, in rats, mice and rabbits, chymase cleaves Tyr^{4}-Ile^{5}

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of angiotensin I to inactive fragments. Recently, many pathophysiological possibilities for chymase through the formation of angiotensin II have been reported such as vascular proliferation in dog grafted vein (Takai *et al*., 2000) and remodelling after myocardial infarction (Jin *et al*., 2001; 2002). We have demonstrated that chymase acts as a pro-angiogenic factor because the exogenous injections of chymase gene or purified chymase into implanted sponges strongly facilitate angiogenesis in a hamster sponge implant model (Muramatsu *et al*., 2000a). The source of this chymase is mainly mast cells and it is a major mediator in mast cell-associated angiogenesis (Muramatsu *et al*., 2000b).

In the previous report (Muramatsu *et al*., 2000a), we have also demonstrated the involvement of endogenous chymase in bFGF-induced angiogenesis using chymostatin, limabean trypsin inhibitor (LBTI) and soybean trypsin inhibitor (SBTI). The inhibitions of chymase activities are common among these inhibitors. These inhibitors are able to inhibit chymase activity, while it was a problem that each inhibitor has wide effects against other serine proteases such as elastase, tryptase and trypsin.

In this study, we investigated the precise significance of endogenous chymase in bFGF-induced angiogenesis using a specific chymase inhibitor, BCEAB and also studied the functional profiles of it on inhibition of angiogenesis.

Methods

Hamster sponge model

Angiogenesis model was produced by the method described previously (Muramatsu *et al*., 2000a). Briefly, polyurethane sponge discs (5-mm thick, 1.3 cm in diameter) were implanted in the subcutaneous air pouch in the dorsum of individual male Syrian hamsters (6 weeks old) purchased from Japan SLC (Shizuoka, Japan), under light anaesthesia with pentobarbital (50 mg kg^{-1}, i.p.). Angiogenesis was induced by the injection of bFGF (0.3 μg $site^{-1}$ day^{-1}), angiotensin I (2 nmol $site^{-1}$ day^{-1}) or angiotensin II (2 nmol $site^{-1}$ day^{-1}) into the implanted sponges for 7 days. One hundred microlitres of saline were injected into the sponges as a control. A chymase inhibitor, BCEAB (2 or 5 nmol $site^{-1}$ day^{-1}) was administered into the sponge simultaneously with the inducer. At the end of the experimental period, the animal was sacrificed, and the granuloma tissues were excised immediately, together with the enclosed sponge implants. The obtained tissues were divided into halves, one half for measurement of haemoglobin contents and the other half for measurement of chymase activity, detection or analysis of VEGF mRNA expression.

Measurement of haemoglobin content

We measured the haemoglobin contents of the implant samples according to the method of Majima *et al*. (1997) as a parameter for angiogenesis. Briefly, the sample granuloma tissues were weighed and homogenized with a Polytrone homogenizer in distilled water (4 ml g^{-1} wet weight). After centrifugation at $5000 \times g$ for 20 min, the haemoglobin concentration in the supernatant was determined by means of haemoglobin assay kit (Hemoglobin B-test WAKO).

Measurement of local blood flow in sponge granuloma

The local blood flow in sponge granuloma tissues was measured by a laser doppler perfusion imager (PIM II, Lisca Developments Co., Linköping, Sweden). After the experimental period, the hamsters were anaesthetized with pentobarbital sodium (50 mg kg^{-1}, i.p.) and were put into the prone position, keeping a precise distance to the laser. Defined areas on the whole back or the site around the sponge granuloma tissues were scanned at 2176 or 2500 pixels, respectively, and local blood flow was detected. The obtained data were visualized with colour-coded images and were quantified by the mean value for blood flow in defined areas.

Measurement of chymase activity

To measure chymase activity, each sample was homogenized in 20 mM phosphate buffer (pH 7.4) containing 2 M KCl and 0.1% Nonidet P-40. After centrifugation at 15,000 r.p.m. for 30 min, the obtained supernatant was used for the measurement of chymase activity. Aliquots of the tissue extracts were incubated for 1 h at 37°C with 770 μM angiotensin I in 48.5 mM borax-borate buffer (pH 8.5) containing 8 mM dipyridyl, 770 μM diisopropyl phosphorofluoridate, and 5 mM ethylenediaminetetraacetic acid as inhibitors of ACE and angiotensinases. The reactions were terminated by addition of 15% cold trichloroacetic acid and the mixtures were centrifuged at 15,000 r.p.m. for 5 min. The concentration of His-Leu, an enzymatic cleavage product of angiotensin I, in the supernatant was determined using 10% *o*-phthaldialdehyde. One unit of chymase activity was defined as the amount of enzyme that cleaved 1 μmol His-Leu min^{-1}.

Analysis of VEGF mRNA by reverse-transcription polymerase chain reaction (RT–PCR)

Total RNA was extracted from the excised sponge granuloma tissues, which were immediately frozen with liquid nitrogen according to the acid guanidium-phenol-chloroform (AGPC) method. First-strand cDNA was synthesized by the reverse transcription reaction using oligo $(dT)_{12\text{-}18}$ primer and reverse transcriptase (SuperScript II). As PCR primers, 5′-ggaccctggctttactgctg-3′ (sense) and 5′-gtgattttctggctttgttc-3′ (antisense) for hamster VEGF and 5′-ccaaggccaaccgcgagaa-gatgac-3′ (sense) and 5′-agggtacatggtggtgccgccagac-3′ (antisense) for β-actin cDNA were used for the amplifications. The amplifications for VEGF and β-actin were performed at 25 cycles of 94°C for 30 s 50°C for 30 s, and 68°C for 30 s. PCR products were separated on a 2% agarose gel.

Drugs

All chemicals were reagent grade. Recombinant human bFGF was purchased from Genzyme/Techne (Minneapolis, USA). Angiotensin I and angiotensin II were purchased from Peptide Institue Inc. (Osaka, Japan). Hemoglobin B-test®, diisopropyl phosphorofluoridate and *o*-phthaldialdehyde were purchased from Wako Pure Chemical Industries (Osaka, Japan). *Taq* DNA polymerase and reverse transcriptase (SuperScript II) were purchased from Life Technologies (Rockville, USA). Oligo $(dT)_{12\text{-}18}$ primer was purchased from Invitrogen (Groningen, Netherlands). Primers for VEGF and

β-actin were ordered from Funakoshi (Tokyo, Japan). BCEAB (4-[1-{[bis-(4-methyl-phenyl)-methyl]-carbamoyl}-3-(2-ethoxy-benzyl)-4-oxo-azetidine-2-yloxy]-benzoic acid) was a kind gift from Shionogi Co. (Osaka, Japan).

Statistics

Results are given as mean ± standard error of the mean (s.e.mean). Differences among groups were examined for statistical significance using one-way ANOVA. P values of lower than 0.05 indicated significant differences.

Results

Inhibition of bFGF-induced angiogenesis by a chymase inhibitor

In the specimens treated with bFGF alone, granuloma tissues rich with newly formed vessels were observed (Figure 1a). On the other hand, BCEAB strongly suppressed the microvessel formation and granulation. As shown in Figure 1b, significant decreases of haemoglobin contents due to BCEAB of 2 and 5 nmol site^{-1} were observed and this inhibition was dose-dependent.

To confirm that the haemoglobin contents in sponges are not stagnated blood, the local blood flow in sponge granuloma tissues was measured by the laser doppler perfusion imager. Figure 2a,b show the intensity of local blood flows in the whole back of the hamster. The area circled with the dotted line indicating the site of the sponge implant exhibited a strong signal with bFGF treatment. This signal was clearly reduced by simultaneous administration of BCEAB (Figure 2c, d). In quantitative analysis, BCEAB (5 nmol site^{-1} day^{-1}) also inhibited the local blood flow induced by bFGF (Figure 3).

Chymase activity in sponge implants

On day 7 after the continuous administration of bFGF with or without BCEAB, the granuloma tissues were excised and the chymase activities in the extracts obtained from granulomas were assessed. The administration of bFGF strongly promotes chymase activity in sponge granulomas in comparison to saline-treated implants. The increase of chymase activity with bFGF treatment was completely suppressed by BCEAB of 5 nmol site^{-1} (Figure 4).

VEGF mRNA expression induced by angiotensin II

We examined whether the inhibitory effect of angiogenesis by BCEAB was mediated *via* the angiotensin II/VEGF dependent pathway. Daily administrations of angiotensin II (2 nmol site^{-1} day^{-1}) into implanted sponges for 7 days induced angiogenesis and 2 nmol site^{-1} of angiotensin I, which is the inactive precursor of angiotensin II, also induced angiogenesis to an almost equal extent (Figure 5a). As shown in Figure 5a, BCEAB significantly blocked the angiogenesis induced by angiotensin I but not by angiotensin II.

The expression of VEGF mRNA was detectable in the sponge granuloma stimulated with angiotensin I or angiotensin II. As shown in Figure 5b, BCEAB suppressed the expression of VEGF mRNA stimulated with angiotensin I but not with angiotensin II.

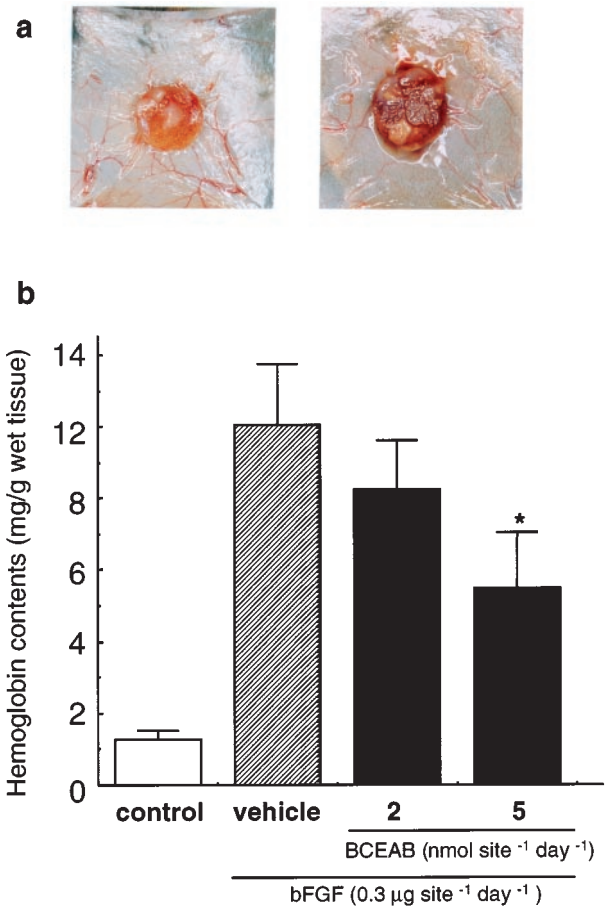

Figure 1 Inhibition of bFGF-induced angiogenesis by BCEAB in hamster sponge model. Angiogenesis was induced by the administration of bFGF (0.3 μg site^{-1} day^{-1}) for 7 days and a chymase inhibitor, BCEAB, was also injected into implanted sponges (2 or 5 nmol site^{-1} day^{-1}). (a) Typical photographs of a sponge treated with bFGF alone (right) and a sponge treated with bFGF and BCEAB in the concentration of 5 nmol site^{-1} (left). (b) Haemoglobin contents in sponge granuloma tissues were significantly suppressed by BCEAB in a dose-dependent manner. Data are the means ± s.e.mean of five hamsters. *$P<0.05$ versus the bFGF-treated group.

Discussion

Chymase is a serine protease contained in granules of mast cells and several of its pathophysiological roles based on the production of angiotensin II have been reported (Takai *et al*., 2000; Jin *et al*., 2001; 2002). Previously, we demonstrated that hamster chymase is angiogenic when injected exogenously as genes or purified proteins into implanted sponges (Muramatsu *et al*., 2000a) and we also confirmed that the origin of chymase was the mast cells that accumulated in the sponge granulomas (Muramatsu *et al*., 2000b). We have also investigated the significance of endogenous chymase in angiogenesis induced by bFGF using several protease inhibitors such as chymostatin, LBTI and SBTI (Muramatsu *et al*., 2000a). Because of the absence of a specific chymase inhibitor, we previously demonstrated our hypothesis by using a multiple number of inhibitors. These inhibitors are able to inhibit chymase, but also have inhibitory effects against other serine proteases. It was problematic that chymostatin also inhibits elastase and cathepsin G, and that

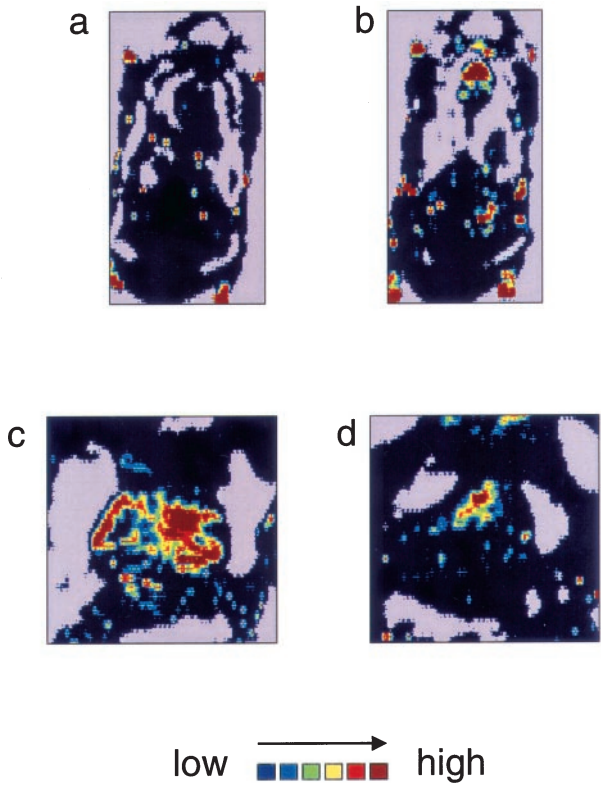

Figure 2 Increase in blood flow in sponge granulomas induced by bFGF treatment and inhibitory effect of BCEAB on this blood-flow increase. On day 7 after the sponge implantation, the local blood flow around the sponge granulomas was measured by the laser doppler perfusion imager. The scanned data are shown by the colour-coded image, which indicates blood flow in the whole back of hamsters treated with saline (a) or bFGF (0.3 μg site^{-1} day^{-1}) (b). The areas circled with dotted lines indicate the sites of sponge implantation. Panels (c) and (d) show the local blood flow around the sponge implantations, which were treated with bFGF alone (c) or bFGF plus BCEAB (5 nmol site^{-1} day^{-1}) (d).

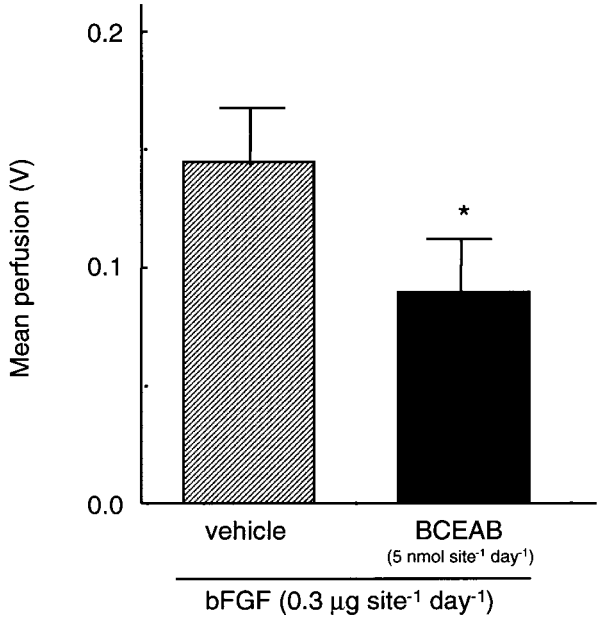

Figure 3 Quantitative analysis of local blood flows in sponge granuloma tissues. On day 7 after the sponge implantation, the local blood flow around the sponge granuloma was quantified by the laser doppler perfusion imager and is shown as the mean value for the defined area. Data are presented as means±s.e.mean of four hamsters. *$P<0.05$ versus the bFGF-treated group.

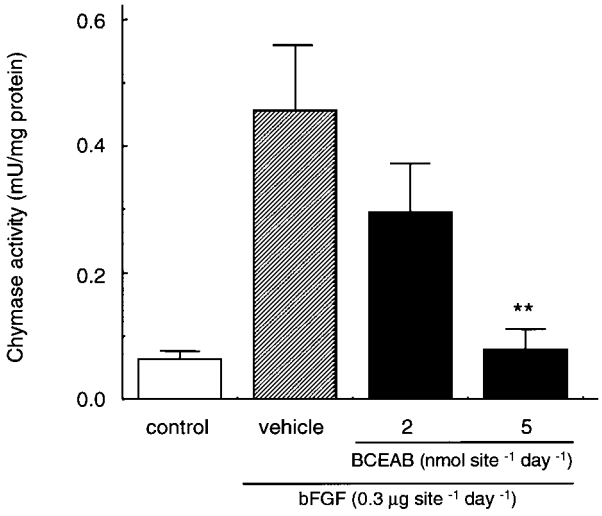

Figure 4 Effects of BCEAB on chymase activity in sponge granuloma tissues. The sponges with or without BCEAB treatment (2, 5 nmol site^{-1} day^{-1}) were excised and an extract buffer was obtained from each sponge. The chymase activity in the buffered extract was determined as the His-Leu releasing activity from angiotensin I. Data are presented as means±s.e.mean of five hamsters. **$P<0.01$ versus the bFGF-treated group.

LBTI inhibits tryptase and trypsin. To clarify the precise role of endogenous chymase in angiogenesis, a specific chymase inhibitor was sought.

BCEAB inhibited purified hamster chymase activity with an IC_{50} value of 5.4 nM but it did not suppress ACE, elastase and tryptase activities (Takai *et al*., 2001), and it weakly inhibits trypsin activity ($IC_{50}=6400$ nM). Tryptase is reported as an angiogenic factor (Blair *et al*., 1997). Elastase, which is mainly present in neutrophils, generates an anti-angiogenic factor, angiostatin (O'Reilly *et al*., 1994). As BCEAB did not inhibit the activities of these other proteases involved in angiogenesis, by using it, we could observe an unambiguous effect of endogenous chymase in bFGF-induced angiogenesis. We also investigated the anti-angiogenic profile of a novel chymase inhibitor, BCEAB, in bFGF-induced angiogenesis.

To investigate the significance of endogenous chymase in pathophysiological angiogenesis, the typical and potent angiogenic factor, bFGF, was used as an inducer in this model. It was reported that bFGF is released from various cancer cells (Relf *et al*., 1997) and it is a chemoattractant of mast cells (Gruber *et al*., 1995). By treatment with bFGF, implanted sponges grew into granulation tissues rich in fibrous matrix and CD31-positive microvessels (data not shown). BCEAB strongly reduced the Hb content induced by bFGF in a dose-dependent manner and its maximum inhibition was about 60% (Figure 1b). Furthermore, we also detected the blood flow in the sponge granuloma. The continuous administration of bFGF caused an apparent increase of blood flow in the sponge granuloma, but BCEAB (5 nmol site^{-1} day^{-1}) suppressed it (Figures 2 and 3). Since BCEAB is an inhibitor highly selective for chymase rather

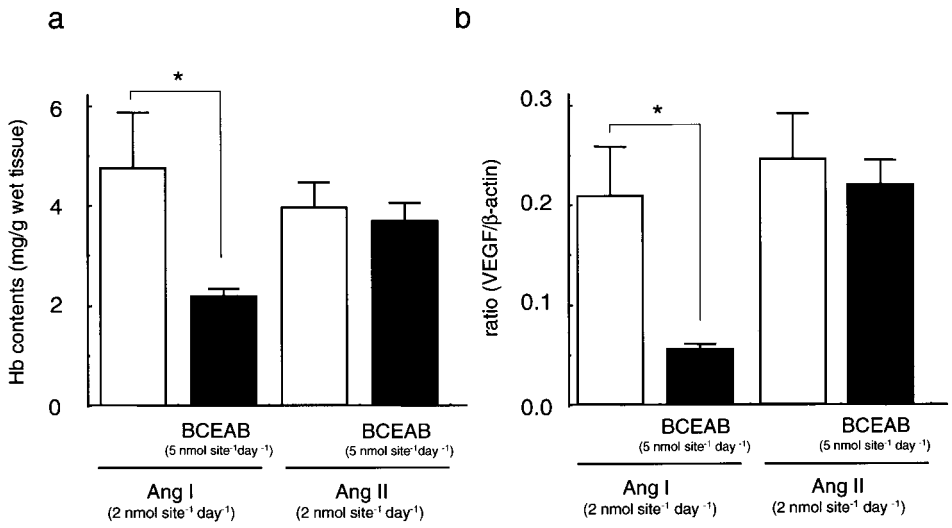

Figure 5 Angiogenesis and VEGF mRNA expression in angiogenesis induced by angiotensin I or angiotensin II. After the administration of angiotensin I (2 nmol site^{-1} day^{-1}) or angiotensin II (2 nmol site^{-1} day^{-1}) for 7 days with or without BCEAB (5 nmol site^{-1} day^{-1}), haemoglobin contents and VEGF mRNA expression were determined in the sponge granulomas. Haemoglobin contents in sponge granuloma tissue (a). The ratio of VEGF mRNA expression against β-actin (b). Results are shown as means$\pm$s.e.mean of five sponge granulomas. $^{**}P<0.01$ versus each vehicle-treated group.

than other proteases such as elastase, tryptase and trypsin, this result indicated that endogenous chymase is obviously involved in angiogenesis induced by bFGF. As the administration of BCEAB *in vivo* apparently inhibited chymase activity in the sponge granuloma, the suppression of angiogenesis by BCEAB may be mediated through the inhibition of chymase activity (Figure 4). BCEAB strongly suppressed bFGF-induced angiogenesis, but did not completely inhibit it. Basic FGF itself powerfully facilitates angiogenesis by acting on endothelial cells, and it also synergistically accelerates angiogenesis together with various other factors. It was reported that bFGF increased the expression of the KDR receptor for VEGF (Hata *et al*., 1999) and a VEGF receptor antagonist suppressed bFGF-induced angiogenesis (Tille *et al*., 2001). In addition, bFGF also promotes the expression of matrix metalloproteinase-1 (MMP-1), which is important when endothelial cells invade the extracellular matrix (Okamura *et al*., 1991). Thus, the reason why BCEAB did not inhibit angiogenesis completely might be that the effects of bFGF itself or downstream factors partly contributed to the bFGF-induced angiogenesis.

It has been reported that angiotensin II facilitates angiogenesis *via* the augmentation of VEGF mRNA expression *via* the angiotensin II type I receptor in several types of cells *in vitro* (Chua *et al*., 1998; Otani *et al*., 2000; Pupilli *et al*., 1999). To confirm the inhibitory characteristics of BCEAB in this model, the expression of VEGF mRNA in angiotensin-induced angiogenesis was examined. BCEAB blocked the angiogenesis and VEGF mRNA expression in sponge granulomas induced by angiotensin I but not by angiotensin II (Figure 5a). These results show that the suppression of angiogenesis by BCEAB is mediated *via* the inhibition of chymase activity that converts angiotensin I to angiotensin II, and the reduction of VEGF mRNA expression as a result of the attenuation of angiotensin II stimuli.

In this model, we hypothesize that chymase derived from mast cells, which were accumulated as a result of bFGF stimuli, generates angiotensin II. Angiotensin II facilitates the up-regulation of VEGF but also the synthesis and expression of bFGF (Itoh *et al*., 1993; Peifley & Winkles, 1998). Basic FGF induced by angiotensin II could further accelerate the accumulation of mast cells and angiotensin II generation by chymase. Thus the significance of angiotensin II in angiogenesis is strongly suggested.

We primarily examined the angiotensin-VEGF dependent pathway for the angiogenic effect of chymase in this study. Interestingly, chymase cleaves not only angiotensin I but also various substrates associated with angiogenesis such as interleukin (IL)-β (Mizutani *et al*., 1991), latent transforming growth factor (TGF)-β (Taipale *et al*., 1995; Lindstedt *et al*., 2001) and pro-gelatinase B (Fang *et al*., 1997). Thus, the possibility that chymase promotes angiogenesis synergistically through other pathways by the activation of these substrates, is also suggested. This hypothesis is supported by the result that TCV-116, an angiotensin II type 1 receptor antagonist, did not completely inhibit the angiogenesis induced by purified chymase (Muramatsu *et al*., 2000a).

In the present study, we demonstrated the significant role of endogenous chymase in angiogenesis induced by bFGF by using a specific chymase inhibitor and this effect was mediated *via* the chymase-angiotensin-VEGF-dependent pathway. Moreover, several possibilities for treatment with chymase inhibitors in angiogenesis-associated diseases such as cancer, diabetic retinopathy, rheumatoid arthritis and so on, are suggested. Further studies are required to clarify the actual roles of chymase in angiogenesis other than through the angiotensin II-VEGF-dependent pathway.

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