Significance of Vascular Endothelial Cell Growth Factor Up-Regulation Mediated via a Chymase-Angiotensin-Dependent Pathway during Angiogenesis in Hamster Sponge Granulomas

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ABSTRACT
Chymase is a serine protease responsible for local production of angiotensin (Ang) II from its precursor Ang I in several species, including humans, dogs, and hamsters. We have previously reported that chymase facilitates angiogenesis in sponge granulation tissues via local production of Ang II. Herein, we report the significance of vascular endothelial growth factor (VEGF) up-regulation mediated by Ang II during angiogenesis in hamster sponge granulomas. Treatment of granulation tissues with an anti-VEGF neutralizing antibody or antisense oligomers against VEGF mRNA significantly reduced Ang II-induced angiogenesis, supporting a significant role for VEGF during angiogenesis. In cultured fibroblasts prepared from granulation tissues, VEGF mRNA was up-regulated in response to Ang II within 2 h and this enhanced expression was abolished in the presence of an Ang II type 1 receptor-selective antagonist, an inhibitor of nuclear factor-κB activation, or an activator protein-1 inhibitor. To study the significance of local production of Ang II by chymase, we examined the effects of chymostatin on in vivo angiogenesis. We found that chymostatin markedly inhibited both up-regulation of VEGF mRNA and angiogenesis in granulation tissues treated by compound 48/80 or basic fibroblast growth factor. Our results suggest that Ang II directly acts on fibroblasts in granulation tissue to up-regulate VEGF mRNA and thereby induce angiogenesis. Furthermore, a chymase-Ang II-VEGF pathway may operate in granulation tissue as the primary mediator of angiogenesis.

Angiogenesis is a process involved in many physiological events, including embryonic development, placental implantation, and wound healing. It also occurs under certain pathological conditions such as cancer, diabetic retinopathy, and rheumatoid arthritis, where angiogenesis is responsible for the progression of such diseases (Folkman, 1995; Koch, 1998; Brenchley, 2000; Campochiaro, 1999; and Carmeliet and Jain, 2000; Griffioen and Molema, 2000). Angiogenesis is a complex and multistep process in which a variety of cells are involved in the construction of new blood vessels. Numerous factors, including growth factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), and epidermal growth factor, are involved in the regulation of neovascularization process, both positively and negatively.

Angiotensin II (Ang II) is a multifunctional bioactive peptide. One important role of this peptide is regulation of blood pressure and blood flow by modification of vascular tone. Recently, many reports have suggested the significance of Ang II as a growth factor. Several in vitro studies have shown that Ang II promotes proliferation, migration, and growth factor synthesis in several types of vascular cells, including smooth muscle cells (Lyall et al., 1988; Bell and Madri, 1990; Paquet et al., 1990) and pericytes (Nadal et al., 1999; Otani et al., 2000), suggesting a particular role in vascular remodeling. Other studies have also investigated the angiogenic effects of exogenously administered Ang II in several in vivo angiogenesis models (Fernandez et al., 1985; Le Noble et al., 1991; Andrade et al., 1996; Hu et al., 1995). The role of locally generated Ang II in angiogenesis, however, remains to be elucidated.

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generation of Ang II in vascular tissues in several species, including humans, dogs, monkeys, and hamsters (Okunishi et al., 1993; Balcells et al., 1997). In our previous study (Muramatsu et al., 2000a), we showed that angiogenesis in hamster sponge granulomas was mediated by chymase-dependent Ang II formation. These results suggest that the chymase-mediated local Ang II-generating system plays a significant role in angiogenesis under some physiological and/or pathophysiological conditions. Many questions, however, remain to be answered regarding the mechanism and importance of this chymase-Ang II-dependent angiogenesis. One of our specific interests is the mechanism of induction of new vessel formation by locally produced Ang II. The aim of the present study was to elucidate the mechanisms underlying chymase-dependent angiogenesis.

**Materials and Methods**

**Agents.** Ang II, chymostatin (chymase inhibitor), H7 (protein kinase C inhibitor), pyrrolidine dithiocarbamate (PDTC, an inhibitor of NF-κB activation), and curcumin (an AP-1 inhibitor) were purchased from Sigma-Aldrich (St. Louis, MO). Bisindolylmaleimide (GFx, a selective inhibitor of protein kinase C) was obtained from Calbiochem-Novabiochem (San Diego, CA). Hemoglobin B-test WAKO was purchased from Wako Pure Chemicals (Osaka, Japan). LipofectAMINE was purchased from Invitrogen (Carlsbad, CA). A messenger RNA Capture kit was purchased from Roche Applied Science (Mannheim, Germany). VEGF-neutralizing antibody was purchased from Pepro (Rocky Hill, NJ). This antibody was originally developed against human VEGF. We confirmed that it neutralized the biological activity of hamster VEGF in a series of preliminary experiments in which mitogenic activity in conditioned medium of HPD-NR cells (VEGF-producing hamster pancreatic cancer cells; Egawa et al., 1995) was completely inhibited by addition of the antibody. A neutralizing antibody against interleukin-1β (Serotec, Washington, DC) was also used in the present study. It is reported that this antibody neutralizes the biological effects of hamster interleukin-1β (Takikita et al., 2001). We also used anti-bovine bFGF polyclonal antibody (R & D Systems, Minneapolis, MN). We confirmed that this antibody detected a single 22-kDa protein band when the tissue extract of hamster uterus was analyzed by Western blotting and that it inhibited the fibroblast proliferating activity of heparin-binding fraction obtained from hamster uterus extract in an in vitro study. An anti-TGF-β antibody was also purchased from R & D Systems. The cross-reactivity of this antibody was examined in preliminary experiments, where it was found to recognize 12- and 25-kDa protein bands in hamster sponge granuloma extract sample under reducing and nonreducing conditions, respectively. Antibodies against vimentin, von Willebrand factor (vWF), or muscle actin (HHF35) were obtained from Dako Japan (Kyoto, Japan).

**Hamster Sponge Model.** Circular sponge discs (5 mm in thickness, 13 mm in diameter, weighing 14.2 ± 0.1 mg) were prepared from a sheet of polyurethane foam using a wad pouch. The discs were soaked in 70% ethanol overnight then sterilized by autoclaving. The sponge discs were implanted aseptically in a subcutaneous air pouch surgically prepared in the dorsum of male Syrian hamsters (6 weeks old) purchased from SLC (Shizuoka, Japan), under light ether anesthesia. At the end of the experimental period, the animals were sacrificed and the granuloma tissues were excised immediately, together with the enclosed sponge implants. All experimental procedures were approved by the Animal Care Committee of Kitasato University of Medicine and conformed with international guidelines.

**Measurement of Hemoglobin Content.** In advance of the present study, we investigated the correlation between hemoglobin content and the degree of angiogenesis in sponge granulomas, using histological sections or quantification of CD31 expression, an endothelial cell marker (Newman, 1994; Giromano et al., 1997). For histological evaluation, thin sections were prepared from each granuloma at different time points (days 2, 4, and 7), and the vascular lumen area was quantified by using NIH Image software in five randomly selected sections. The mean lumen area of five sections was plotted against hemoglobin content in the corresponding granuloma. For evaluation using CD31 expression, sponge granulomas were divided into two halves, one for measuring the hemoglobin content and the other for determination of CD31 mRNA expression by RT-PCR. As shown in Fig. 1, hemoglobin content in sponge granulation tissues correlates well with the degree of angiogenesis, in agreement with our previous results (Muramatsu et al., 2000b). Therefore, we measured hemoglobin content of sponge granulation tissues as a surrogate parameter for angiogenesis, according to the method of Majima et al. (1997). Briefly, sample granulation tissues were weighed and homogenized with a Polytron homogenizer (Kinematica, Cincinnati, OH) in distilled water (4 ml/g of wet sample weight). After centrifugation at 5000g, the hemoglobin concentration in the supernatant was determined using a commercially available hemoglobin assay kit (Hemoglobin B-test WAKO; Wako Pure Chemicals).

**Semi-quantitative RT-PCR.** RT-PCR to detect VEGF mRNA was conducted using poly(A) mRNA extracted from sponge granulation tissue as templates. Briefly, sponge granulation tissues were frozen in liquid nitrogen immediately after excision to isolate total RNA. Poly (A) mRNA was purified from total RNA using an mRNA Capture kit. Primer sequences for detecting VEGF mRNA were 5′-ggacgctgcatgcc-3′ and 5′-ggtattctggctgttgc-3′, designed according to the sequence of rat VEGF mRNA. Each cycle of the reaction consisted of 30 s at 94°C (denaturation), 30 s at 55°C (annealing), and 60 s at 72°C (extension), followed by a final 10-min

**Fig. 1.** Hemoglobin content in sponge granulomas as a parameter for angiogenesis. Ang II (2 μg/site/day) was injected into the implanted sponge once daily for 7 days. The sponge granulomas were excised at day 7 and divided into halves, one for measuring hemoglobin content and the other for histological evaluation or quantification of CD31 by RT-PCR. A, histological section of 7-day-old sponge granuloma treated with Ang II. Microvessels, which appear as tubular structures often filled with erythrocytes, are indicated by arrows. B, relationship between hemoglobin content and lumen area of microvessels. Thin sections were prepared from each granuloma at different time points (days 2, 4, and 7) and the vascular lumen area was quantified by using NIH Image software on five randomly selected sections. The mean lumen area of five sections was plotted against hemoglobin content in the corresponding granuloma. Each symbol represents the value from one granuloma (mean ± S.E.M.). C, relationship between hemoglobin content and CD31 mRNA expression in granulation tissue. Excised sponge granulomas were divided into two halves, one for measuring the hemoglobin content and the other for determination of CD31 mRNA expression by RT-PCR. Expression level of CD31 mRNA was presented as the ratio of CD31 mRNA to β-actin, used as an internal control, in each sponge granuloma. Each symbol represents the value from one granuloma. The regression line was drawn by the least-squares method.
extension at 72°C. We confirmed the amplified products to be hamster VEGF by DNA sequencing. The amount of poly(A) RNA template (30 ng) and the number of amplification cycles (20 cycles) were selected to be within quantitative ranges. The reaction proceeded linearly, as determined by plotting the signal intensity as a function of the amount of template and number of cycles. Signal intensities were quantified by densitometric analysis.

Semi-quantitative RT-PCR for CD31 (platelet-endothelial cell adhesion molecule-1) was performed according to the method described previously by Redick and Bautch (1999). Polymerase chain reaction primers were 5'-caggggtctctgacatccag-3' and 5'-caggggtctctgacatccag-3'. Each cycle of the reaction consisted of 45 s at 94°C (denaturation), 60 s at 55°C (annealing), and 120 s at 72°C (extension), followed by a final 15-min extension at 72°C. The amount of poly(A) RNA template (30 ng) and the number of amplification cycles (35 cycles) were selected to be within quantitative ranges. Expression of β-actin mRNA was used as an internal control and VEGF mRNA or CD31 mRNA expression level was presented as the ratio of VEGF mRNA or CD31 mRNA to β-actin mRNA expressed in the sponge granulomas.

Preparation of Fibroblasts from Granulation Tissue and Normal Skin Tissue. Preparation of fibroblasts from granulation tissue and skin was carried out according to the methods described previously (Williams et al., 2000). Briefly, sponge granulation tissues were cut into small pieces and placed into 60-mm culture dishes, which were filled with Dulbecco’s modified Eagle’s medium (DMEM) containing 20% fetal calf serum. After incubation for 1 h before 10 nM Ang II treatment and then treated with Ang II inhibitors, the cultured cell was preincubated with each inhibitor for 1 h before 10 nM Ang II treatment and then treated with Ang II concomitant with each inhibitor for 4 h. Total RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). For semi-quantitative RT-PCR, poly (A) RNA was purified from total RNA using an mRNA Capture kit.

Treatment with Antisense Oligodeoxynucleotide against VEGF mRNA. A 20-mer antisense oligodeoxynucleotide complementary to VEGF mRNA and its scrambled oligodeoxynucleotide were synthesized as phosphorothioate DNA. The sequences of the antisense and scrambled oligomer were 5'-agaggcaggaatgctctgta-3' and 5'-agaggcaggaatgctctgta-3', respectively. These sequences were designed according to the partially determined sequence of cloned hamster VEGF cDNA. Administration of antisense and scrambled oligomers was performed by the lipofection method. Briefly, 5 nmol of antisense or scrambled oligomer was mixed with 25 μL of LipofectAMINE. The mixture was incubated for 40 min at room temperature to form a liposome complex. Liposome-DNA complexes were injected into subcutaneous tissue near the sponges implanted into the back of the hamster once daily for 4 days (5 μmol/site/day). Ang II (2 μg/site/day) was administered into the sponges once daily during the experiments. On day 7, the granulation tissues were excised and the hemoglobin content determined. Expression of VEGF protein after treatment with the antisense oligonucleotide was examined by Western blot. Protein (5 μg), obtained from each granuloma tissue, was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed for VEGF protein content. The result was then used as a band of which molecular mass was estimated to be 20 to 22 kDa.

Drug Application. Ang II and neutralizing antibodies were dissolved with sterile phosphate-buffered saline (PBS) and administered daily into the sponge for 7 days. Chymostatin was solubilized with dimethyl sulfoxide and then diluted with PBS (the final concentration of dimethyl sulfoxide was 0.05%) just before administration. Administration of each drug was conducted under aseptic conditions.

Statistical Analysis. All data are expressed as mean ± S.E.M. Differences between two groups were examined using the unpaired Student’s t test. Multiple comparisons were performed by using one-way analysis of variance with Bonferroni’s correction. A P value less than 0.05 was considered statistically significant.

Results

Ang II-Induced Angiogenesis in Hamster Sponge Granulomas. As previously described by our group (Muramatsu et al., 2000a), administration of exogenous Ang II into the implanted sponges promoted angiogenesis and development of granulation tissue encapsulating the sponges. As shown in Fig. 1A, many microvessels were formed in the granulation tissue and in the stroma, which infiltrated the sponge matrix. The vascular lumen area, estimated in randomly selected histological sections prepared from Ang II-treated sponge granulomas, correlated well with hemoglobin content in the sponge granulomas (Fig. 1B). There was also a linear relationship between hemoglobin content in granulation tissue and CD31 mRNA content determined by RT-PCR (Fig. 1C). Therefore, hemoglobin content in the granulation tissue was measured as a surrogate parameter to quantify the extent of angiogenesis.

Involvement of VEGF in Ang II-Induced Angiogenesis. We have previously demonstrated that chymase, an alternative enzyme responsible for Ang II generation, promotes angiogenesis through the local generation of Ang II (Muramatsu et al., 2000a). To investigate the mechanism underlying Ang II-induced angiogenesis, we used several neutralizing antibodies against cytokines or growth factors such as interleukin-1α, TGF-β, bFGF, and VEGF. Of these, the neutralizing antibody against VEGF significantly suppressed Ang II-induced angiogenesis (Table 1). At concentrations of 1 and 10 μg/sponge/day, this antibody inhibited the increment of hemoglobin content by 54 and 95%, respectively. Injections of nonimmune IgG had no effect on angiogenesis (data not
TABLE 1
Effects of neutralizing antibodies on angiogenesis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Hemoglobin Content</th>
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<tr>
<td>Vehicle</td>
<td>4.25 ± 0.42</td>
<td></td>
</tr>
<tr>
<td>Anti-bFGF</td>
<td>3.86 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>Anti-IL-1β</td>
<td>4.62 ± 0.34</td>
<td></td>
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<tr>
<td>Anti-TGFβ</td>
<td>4.75 ± 0.25</td>
<td></td>
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<tr>
<td>Anti-VEGF</td>
<td>1.96 ± 0.08</td>
<td></td>
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<tr>
<td>Anti-TGFβ</td>
<td>0.23 ± 0.03</td>
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*P < 0.01, **P < 0.005 versus vehicle control.

Effects of neutralizing antibodies on angiogenesis. Implanted sponges were treated with Ang II (2 μg/site/day) and each neutralizing antibody (1 μg/site/day, 100 μl/site/day) were simultaneously injected once daily into the sponges for 7 days. At day 7, the sponge granulomas were excised and their hemoglobin content determined. Data are mean ± S.E.M. of samples.

Promotion of VEGF Expression by Ang II. To further examine the role of VEGF in Ang II-induced angiogenesis, we investigated VEGF mRNA expression in Ang II-treated sponge granulomas. As shown in Fig. 2A, hemoglobin content in sponge granulomas increased from day 2 to day 11 in Ang II (2 ng/site/day)-treated sponges, compared with saline-injected control sponges. VEGF mRNA expression was examined by semiquantitative RT-PCR using the same sponge granuloma samples. A 395-bp cDNA fragment, corresponding to hamster VEGF mRNA, was detected from day 2. This signal increased gradually and reached a peak at day 7, followed by a gradual decrease until day 11 (Fig. 2, B and C). In contrast, VEGF mRNA expression was obscure in saline-treated sponges until day 11. As shown in Fig. 2D, up-regulation of VEGF mRNA expression was dose-dependent at day 7.

Blockade of VEGF mRNA with an Antisense Oligodeoxynucleotide. We also investigated the significance of VEGF in Ang II-induced angiogenesis using an antisense oligodeoxynucleotide complementary to hamster VEGF mRNA. The antisense or a scrambled oligomer was injected once daily (5 nmol/site/day) for 4 days into the subcutaneous tissue near sponges treated with Ang II (2 μg/site/day). As shown in Fig. 3, hemoglobin content in the sponge granulomas decreased after treatment with the antisense oligomer. At day 7, inhibition was about 65%. Treatment with the scrambled oligomer did not inhibit angiogenesis. As shown in Fig. 3B, expression of VEGF protein in the granuloma tissue was reduced after treatment with the antisense oligonucleotide.

Up-Regulation of VEGF by Ang II in Fibroblasts Prepared from Granulomas. To examine whether Ang II directly induces up-regulation of VEGF, we isolated fibroblasts from normal skin or Ang II-treated granulation tissues and evaluated their ability to produce VEGF in response to Ang II. Fibroblasts isolated from normal skin and granulation tissue did not express significant VEGF mRNA in basal culture conditions. Treatment with 10 nM Ang II markedly stimulated VEGF mRNA expression in fibroblasts isolated from granulation tissue, whereas up-regulation of VEGF was much less remarkable in fibroblasts of normal skin after the same treatment (Fig. 4, A and B). This up-regulation of VEGF mRNA in granuloma fibroblasts in response to Ang II was abolished by 1 μM losartan, an AT1-selective antagonist. VEGF mRNA up-regulation in stromal fibroblasts was dose-dependent (Fig. 4C). Significant up-regulation was ob-

Fig. 2. VEGF mRNA expression in Ang II-treated granulation tissue. Ang II (2 μg/site/day) was injected into the implanted sponge once daily for 7 days. Sponge granulomas were excised at day 7, and the hemoglobin content and VEGF mRNA expression were determined. A, time course of the increment in hemoglobin (Hb) content in sponge granulomas. Closed circles, Hb content of Ang II-treated granulomas; open circles, vehicle control. Data are mean ± S.E.M. values of eight hamsters. *P < 0.05; **P < 0.01 versus vehicle control. B, time-dependent changes in VEGF mRNA expression in Ang II-treated granulomas. Poly(A)+ RNA was prepared from Ang II-treated (2 μg/site/day) granulation tissue at days 2, 4, 7, and 11 and VEGF mRNA expression was determined by semiquantitative RT-PCR. The 395-bp band corresponds to the amplified product of hamster VEGF mRNA. Expression of β-actin (586-bp) was used as an internal control, and VEGF mRNA expression level is presented as the ratio of VEGF mRNA to β-actin mRNA expressed in sponge granulomas. C, densitometric analyses of the VEGF mRNA/β-actin mRNA ratio in sponge granulomas treated with Ang II. Closed circles and open circles represent VEGF mRNA levels in Ang II-treated and control granulomas, respectively. Data are mean ± S.E.M. values of four sponges. *P < 0.05; **P < 0.01 versus vehicle control. D, dose dependence of Ang II-induced up-regulation of VEGF mRNA. Implanted sponges were treated with Ang II solutions at various concentrations for 7 days. Results of the RT-PCR studies were quantified by densitometry. Data are mean ± S.E.M. values of four sponges. *P < 0.05 versus vehicle control.

Fig. 3. Effect of an antisense oligodeoxynucleotide complementary to VEGF mRNA on Ang II-induced angiogenesis. Antisense or scrambled oligomers (5 nmol/site/day) were injected from day 0 to day 3. Ang II (2 μg/site/day) was injected 1 h after the injection of the oligomers for 7 days. On day 7, the sponge granulomas were excised and their hemoglobin content determined (A). Data are mean ± S.E.M. values of eight hamsters. *P < 0.01 versus vehicle control. ns, not significant. Expression of VEGF protein in the sponge granulomas on day 7 was determined by Western blot analysis (B). Lane 1, vehicle-treated granuloma; lane 2, antisense oligomer-treated granulomas; and lane 3, scrambled oligomer-treated granulomas.
served after treatment with Ang II at more than 10 nM for 4 h. The time course of Ang II-induced up-regulation of VEGF is shown in Fig. 4D. The mRNA level rapidly increased upon addition of Ang II and reached a peak level at 4 h, followed by a plateau over 8 h.

Mechanisms Underlying Ang II-Induced VEGF mRNA Up-Regulation. Having demonstrated that primary cultured fibroblasts prepared from granulation tissues express VEGF mRNA in response to Ang II, we next examined the molecular basis for Ang II-induced VEGF mRNA up-regulation. It has been suggested that protein kinase C may be implicated in Ang II-induced VEGF up-regulation in rat heart endothelial cells (Chua et al., 1998). We examined whether protein kinase C inhibitors, H7 and GFX, attenuate VEGF expression in primary fibroblasts. As shown in Fig. 5A, VEGF up-regulation elicited by Ang II (10 nM for 4 h) was significantly reduced in the presence of H7 (10 μM). A more selective protein kinase C inhibitor, GFX, also suppressed the induction at 10 μM. To elucidate which transcription factors are responsible for the induction of VEGF mRNA by Ang II in fibroblasts, we examined the effects of PDTC, an inhibitor of NF-κB activation, and curcumin, an AP-1 inhibitor. PDTC (100 μM) inhibited VEGF mRNA induction, but its effect was moderate. Treatment of fibroblasts with curcumin (10 μM) considerably attenuated the response (Fig. 5B).

Fig. 5. Intracellular signaling of Ang II-induced VEGF mRNA induction. Granuloma fibroblasts were treated with each inhibitor for 1 h and then treated with 10 nM Ang II concomitant with each inhibitor. After 4 h, mRNA was extracted from the fibroblasts. Results of the RT-PCR studies were quantified by densitometry and expressed as relative VEGF mRNA level (VEGF mRNA/β-actin mRNA ratio). A, effects of protein kinase inhibitors H7 and GFX. Both inhibitors were used at 10 μM. Data are mean ± S.E.M. values of four independent experiments. B, effects of PDTC and curcumin on Ang II-induced VEGF mRNA induction in the granuloma fibroblasts. PDTC (100 μM), an inhibitor of NF-κB activation, and 10 μM curcumin, an AP-1 inhibitor, were used. Data are mean ± S.E.M. values of four independent experiments.

Functional Linkage between Chymase and VEGF Up-Regulation. The next study was performed to determine whether endogenous Ang II, produced by chymase, is functionally related to VEGF up-regulation in the sponge granuloma tissues. In a recent study, we reported that local injection of compound 48/80, a mast cell activator, promotes angiogenesis in sponge granulomas through a chymase-dependent mechanism (Muramatsu et al., 2000a). As shown in Fig. 6A, VEGF mRNA expression was markedly enhanced by the treatment with compound 48/80 (100 μg/site/day, for 7 days). Combination of compound 48/80 with chymostatin (daily injections of 100 μl of 1 or 10 μM solution/site for 7 days) significantly attenuated VEGF mRNA up-regulation compared with treatment with compound 48/80 alone. Compound 48/80-induced VEGF mRNA expression was also reduced with losartan (daily injection of 100 μl of 1 or 10 μM solution/site for 7 days) but not with an AT2 antagonist (daily injection of 100 μl of 10 μM solution/site for 7 days) (Fig. 6B). Captopril did not affect up-regulation of VEGF mRNA after daily injections of 100 μl of 1 mM solution/site for 7 days (data not shown). These results suggest that locally produced Ang II by mast cell chymase would be functionally linked to VEGF mRNA expression in the sponge granulomas.

Endogenous Chymase-Ang II-VEGF Pathway in bFGF-Induced Angiogenesis. As shown previously (Muramatsu et al., 2000a), exogenously administered bFGF induces angiogenesis in part through a chymase-dependent pathway. To estimate the contribution of the endogenous chymase-Ang II-VEGF pathway to bFGF-induced angiogenesis, VEGF mRNA expression was determined in bFGF-treated sponges. As shown in Fig. 7, daily administration of
bFGF (0.3 μg/site/day) resulted in a significant increase in the hemoglobin content in granulation tissues, together with marked VEGF mRNA expression at day 7. Treatment with chymostatin (100 μl of a 1 μM solution/site/day for 7 day) significantly reduced both the increment in hemoglobin content and VEGF mRNA expression (percentage of inhibition, 66 and 64%, respectively). VEGF neutralizing antibody (1 μg/site/day for 7 days) also inhibited the elevation of hemoglobin but did not affect VEGF mRNA expression. Inhibition of angiogenesis by the neutralizing antibody was significantly more prominent than that observed after chymostatin treatment (P < 0.05). Treatment with TCV-116 (5 mg/kg/day p.o.), a selective antagonist of the AT1 receptor, also inhibited the increment in hemoglobin content and enhanced VEGF mRNA expression.

Fig. 6. Implication of a chymase-Ang II pathway in compound 48/80-induced VEGF mRNA up-regulation in the sponge granulomas. The sponge granulomas, induced by daily injections of compound 48/80 (100 μg/site/day) for 7 days, were excised and VEGF mRNA expression was determined by RT-PCR. One hundred microliters of chymostatin (1 or 10 μM solution), losartan (1 or 10 μM solution), and PD123319 (10 μM solution) were injected into the sponges 30 min before the injections of compound 48/80 for 7 days. Data are mean ± S.E.M. values of four sponges.

The major finding of the present study was that Ang II-induced angiogenesis was mediated via up-regulation of VEGF in the hamster sponge angiogenesis model. Several studies have reported Ang II-induced angiogenesis when exogenously administered in models using rat sponge granulomas (Andrade et al., 1996; Hu et al., 1996), rabbit cornea (Fernandez et al., 1985) or chick chorioallantoic membrane (Le Noble et al., 1991). However, the mechanism underlying Ang II-induced angiogenesis has not yet been fully elucidated. Because Ang II itself does not exert proliferation-stimulating activity on vascular endothelial cells, other factors are implicated in angiogenesis as a secondary mediator(s). However, few in vivo studies have reported Ang II-induced regulation of growth factors, especially VEGF. In the present study, both the neutralizing antibody against VEGF (Table 1) and an antisense oligomer complementary to VEGF mRNA (Fig. 3) markedly inhibited Ang II-induced angiogenesis, suggesting the involvement of VEGF in angiogenesis in hamster granulomas. The maximum inhibition was 95 and 65% by treatment with the antibody and antisense oligomer, respectively, indicating that VEGF could be a crucial factor in this process.

VEGF mRNA expression in granulation tissues was verified by RT-PCR experiments (Fig. 2), which demonstrated up-regulation of VEGF mRNA by Ang II in a time- and dose-dependent manner. This up-regulation was followed by an increase in hemoglobin content in the sponge granulomas, supporting a significant contribution of VEGF to angiogenesis.

Up-regulation of VEGF mRNA in response to Ang II was also demonstrated in primary fibroblasts prepared from granulation tissue (Fig. 4). The results of our in vitro experiments indicate that Ang II directly acts on AT1 receptors on fibroblasts to induce VEGF mRNA expression. Interestingly, the response of fibroblasts prepared from normal back skin of the same hamster to the same stimuli was much less than that of granulation tissue fibroblasts. The most plausible explanation for these seemingly contradictory results would be that fibroblasts in the granulation tissue might be in an activated state in response to various factors during granuloma development. In our preliminary experiments, fibroblasts from these two different locations responded to cobalt chloride, which is often used for mimicking hypoxic conditions, in somewhat different manners (J. Katada, unpublished data). As reported previously (Chua et al., 1998; Otani et al., 2000), induction of VEGF mRNA involves a protein kinase C-dependent pathway in rat heart endothelial cells and bovine retinal microcapillary endothelial cells. As shown in Fig. 5A, protein kinase C would be also involved in Ang II-induced up-regulation of VEGF mRNA in granuloma fibroblasts because both H7 and GFX significantly attenuated the induction. Our results also showed that AP-1 and NF-κB may act downstream of protein kinase C. Implication of these transcription factors in Ang II-induced VEGF mRNA induction has been indicated in rat heart endothelial cells (Chua et al., 1998). Although the present results shown in Fig. 5B suggest that AP-1 may be functionally more important than NF-κB because the effect of PDCT on induction of VEGF mRNA was only moderate even at a higher concentration, more detailed studies should be conducted to determine the
relative contribution of AP-1 and NF-κB. The promoter of human VEGF gene was shown to contain potential binding sites for signal protein-1 (SP-1), AP-1, and AP-2 (Tischer et al., 1991). The mouse VEGF promoter contains not only binding sites for signal protein-1 (SP-1), AP-1, and AP-2 but also NF-κB (Shima et al., 1996). Although hamster VEGF gene has not been investigated, our present results suggest that NF-κB and AP-1 would be important regulators of VEGF expression in hamsters. To conclude the functional significance of these transcription factors, further investigation using specific inhibitors of these factors, such as antisense oligonucleotides, would be necessary.

Our results concord with those of previous in vitro studies using cultured cells, where up-regulation of growth factors (including VEGF) by Ang II via the AT₁ receptor has been reported in vascular smooth muscle cells (Williams et al., 1995), heart endothelial cells (Chua et al., 1998), and mesangial cells (Pupillì et al., 1999). Because the vasculature developed in the granulation tissue at day 7 would be immature, it is unlikely that vascular smooth muscle cells contribute significantly to the promotion of VEGF expression. The significance of endothelial cells in VEGF up-regulation is not clear from this in vivo angiogenesis model. Our present results strongly suggest that fibroblast-like cells in the granulomas may be the primary cells responsible for generation of VEGF in response to Ang II, resulting in angiogenesis.

We have demonstrated previously that local injections of compound 48/80, a potent mast cell activator, promote angiogenesis in granuloma tissues via chymase-dependent Ang II production (Muramatsu et al., 2000a). Our present results (Fig. 6) show that treatment with compound 48/80 induced up-regulation of VEGF mRNA in the sponge granulomas, which was markedly attenuated by inhibition of chymase with chymostatin and by AT₁ receptor blockade. These results suggest that local production of Ang II mediated by mast cell chymase would be functionally linked to VEGF mRNA up-regulation.

Furthermore, our results (Fig. 7) also implicate the chymase-Ang II-VEGF pathway in angiogenesis induced by bFGF, one of the most important endogenous angiogenic factors. These results indicate the presence of active chymase and a functional chymase-Ang II-VEGF pathway in granulation tissues, suggesting a significant role for this pathway in physiological and/or pathological angiogenesis. Chymase is a major protein contained in mast cell granules (Schechter et al., 1983; Wintroub et al., 1986; Sayama et al., 1987). Mast cells are normal residents of vascular tissues, and a number of these cells have also observed in the vicinity of microvessels under pathological conditions. Therefore, it is most likely that activation of mast cells results in increased local formation of Ang II through a chymase-dependent pathway, which up-regulates the local production of VEGF and enhances angiogenesis. Human chymase has substrate specificity similar to that of hamster chymase (Okunishi et al., 1993; Balcells et al., 1997) and can generate Ang II locally. Considering that mast cell distribution in normal and pathological tissues is almost similar between humans and hamsters, it is possible that this chymase-Ang II-VEGF pathway may be implicated in the pathogenesis of angiogenesis-dependent diseases in humans. However, further studies, in particular studies using animal models of angiogenesis-dependent diseases, are necessary to elucidate the significance of this pathway in human diseases.

Figure 8 shows the proposed chymase-mediated pathway in bFGF-induced angiogenesis. It is reported that bFGF could act as a chemoattractant for mast cells (Gruber et al., 1995), suggesting the significant role for mast cell recruitment into extravascular tissues. It is also probable that bFGF could stimulate proliferation and activation of perivascular fibroblasts, which may facilitate Ang II-induced VEGF production by fibroblasts. In contrast, the involvement of ACE in this pathway is not clear. We previously showed that captopril moderately inhibits bFGF-induced angiogenesis (Muramatsu et al., 2000a), but it is not known whether ACE-generated Ang II can also stimulate VEGF expression in fibroblasts. Effects of ACE inhibitors on angiogenesis are conflicting (Vogt and Frey, 1997; Fabre et al., 1999; Takeshita et al., 2001; Yoshiji et al., 2001). Therefore, further studies should be conducted to elucidate the role of ACE in angiogenesis and define the relationship between chymase-dependent pathway and ACE-dependent pathway.

In conclusion, we have demonstrated in the present study that a neutralizing antibody and an antisense oligodeoxynucleotide against VEGF suppressed and Ang II-induced angiogenesis in a hamster sponge granuloma model. Up-regulation of VEGF mRNA was confirmed in the granulation tissue by RT-PCR, and this up-regulation was attenuated by treatment with chymostatin. VEGF mRNA expression significantly increased in response to Ang II in primary cultures of fibroblasts prepared from granulation tissue. These results suggest that Ang II directly acts on fibroblasts in granulation tissue to induce up-regulation of VEGF mRNA, consequently resulting in angiogenesis. Furthermore, our results invoke a functional chymase-Ang II-VEGF pathway in granulation tissue, as a primary mediator of angiogenesis.

References
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