A Potential Role of Bradykinin in Angiogenesis and Growth of S-180 Mouse Tumors

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ABSTRACT—Angiogenesis is an important event in tumor growth. We evaluated the contribution of endogenous bradykinin to tumor-associated angiogenesis and tumor growth using pharmacological approaches in mice bearing sarcoma 180 cells. The weight of implanted tumors increased in parallel with increased hemoglobin contents (a parameter to evaluate angiogenesis) over a 20-day experimental period. Daily administration of bradykinin B₂-receptor antagonists, Hoe140 (0.1 and 1 mg/kg per day, local injection) or FR173657 (30 mg/kg per day, p.o.), significantly suppressed the increment in angiogenesis and tumor weight, but a B₁-receptor antagonist, desArg¹⁰-Hoe140 (1 mg/kg per day), did not. Administration of a plasma kallikrein inhibitor, soybean trypsin inhibitor (3 mg/site per day), significantly suppressed angiogenesis and tumor growth. In contrast, bradykinin-degrading enzyme inhibitors, captopril and phosphoramidon (500 μg/site per day), enhanced angiogenesis and increased tumor weight. Our results suggest that bradykinin, produced by plasma kallikrein or plasma kallikrein-like enzymes, promote tumor-associated angiogenesis and tumor growth in vivo.

Keywords: Angiogenesis, Kallikrein-kinin system, Bradykinin B₂ receptor, Kallikrein, Sarcoma 180

Several groups have established the enhancing role of angiogenesis on tumor growth. For example, it has been demonstrated experimentally that tumors do not grow beyond a size of 2 – 3 mm³ unless they are able to attract the growth of new vessels from the existing vascular network (1 – 3). Apart from their importance for tumor growth, the new blood vessels provide an essential entry route for metastatic tumor cells. It is likely that the excess supply of oxygen and various plasma components including growth factors and fibrinogen facilitate tumor growth and angiogenesis (4, 5). The significance of angiogenesis has been demonstrated also by several clinical studies; enhanced neovascularization is a poor prognostic factor in breast cancer (6), cervical cancer (7) and bladder cancer (8). Therefore, one of the primary targets for tumor chemotherapy is perhaps the inhibition of angiogenesis.

Bradykinin (BK) is a potent, biologically active peptide with several functions such as vasodilation, increased vascular permeability, pain sensation, regulation of glomerular filtration rate and excretion of sodium from the kidney (9, 10). Thus, BK is considered to be involved in various pathological states, including inflammation (11, 12), shock (13, 14), hypertension (15, 16) and airway diseases (17). Recent studies have demonstrated that BK stimulates angiogenesis in vivo (18). Although BK is also thought to enhance vascular permeability in tumors and promote tumor growth (19, 20), its involvement in enhancement of angiogenesis and consequently tumor growth has not been fully elucidated.

The aim of the present study was to investigate the role of endogenous BK in tumor-associated angiogenesis and tumor growth in tumor-bearing mice. By using selective bradykinin receptor antagonists, kallikrein inhibitors and kininase inhibitors, we suggested the significant role of BK in angiogenesis and tumor growth in mice, which were subcutaneously implanted with sarcoma 180 cells.

MATERIALS AND METHODS

Animals

Male 5-week-old ICR mice (SLC Japan, Hamamatsu)
were used in the present experiment. All mice were housed at a controlled humidity of 60 ± 5% and temperature if 25 ± 1°C, with a 12-h light/dark cycle. Experiments were performed in accordance with the Guideline Principles for the Care and Use of Laboratory Animals of the Animal Care Committee of Kitasato University.

Antagonists and protease inhibitors

The bradykinin B₁-receptor antagonist d-Arg[Hyp³, Thi⁴, D-Tic⁵, Oic⁶] bradykinin (Hoe140) was purchased from Peptide Institute (Osaka) (21), and FR173657 ((E)-3-(6-acetamido-3-pyridyl)-N-[2,4-dichloro-3-[(2-methyl-8-quinolinyl) oxymethyl] phenyl]-N-methylaminocarbonyl-methyl]acrylamide) was kindly provided by Fujisawa Pharmaceutical Co. (Osaka) (22). The B₁-receptor antagonist desArg²-d-Arg[Hyp³, Thi⁴, D-Tic⁵, Oic⁶] bradykinin (desArg¹⁰-[Hoe140]) (23) was obtained from Peninsula Laboratories (San Carlos, CA, USA). Captopril, an inhibitor of angiotensin converting enzyme (ACE) that is also known as a kinin-degradating enzyme, kininase II, was a gift from Sankyo Pharmaceutical Co. (Tokyo). Phosphoramidon was purchased from Pure Chemical Industries, Osaka) and MGPA (500 μg/site), captopril (500 μg/site), phosphoramidon (500 μg/site), SBTI (500 μg/site) and Hoe140 (1 mg/kg, suspended with sterile physiological saline at 200 μg/ml) were dissolved in sterilised physiological saline.

Cell culture

The murine sarcoma 180 (CCL-8, S-180) tumor cells were purchased from Dainippon Pharmaceutical Co. (Osaka). S-180 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The cells were harvested then washed three times with phosphate buffered saline (PBS; NaCl at 8 g/l, KCl at 0.2 g/l, NaHPO₄·12H₂O at 2.9 g/l, KH₂PO₄ at 0.2 g/l). The cells were pelleted by brief centrifugation at 300 × g at 25°C. The supernatant was aspirated, and the cells were resuspended in PBS and counted using a hemocytometer.

Tumor-bearing mice

The dorsal hair of mice anesthetized lightly with ether was shaved and the skin was disinfected with 70% ethanol. S-180 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The cells were harvested then washed three times with phosphate buffered saline (PBS; NaCl at 8 g/l, KCl at 0.2 g/l, NaHPO₄·12H₂O at 2.9 g/l, KH₂PO₄ at 0.2 g/l). The cells were pelleted by brief centrifugation at 300 × g at 25°C. The supernatant was aspirated, and the cells were resuspended in PBS and counted using a hemocytometer.

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Quantification of angiogenesis and tumor growth

Mice were sacrificed with excess dose of ether and tumor tissues were excised from the back for evaluation of tumor growth. The tumor was weighed immediately after harvesting and hemoglobin contents were determined using the following method. Excised tumor tissues were homogenized in distilled water using four times the weight of the tumor tissue, in a Polytron homogenizer (Kinematica, Luzern, Switzerland). After centrifugation at 5,000 × g for 30 min at 4°C, the hemoglobin concentration in the supernatant was measured using a commercial hemoglobin assay kit (Hemoglobin B Test Wako, Wako Pure Chemical Industries). The hemoglobin contents of tumor tissues were expressed as mg/g wet tumor tissue.

Histology

Tumor tissues together with the stroma were excised from the back of mice and immediately fixed with 10% formalin at 4°C. Thin paraffin sections were prepared for von Willebrand factor (vWF) immunostaining and for routine staining with hematoxylin and eosin. For immunohistochemical staining, deparaffinized sections were treated with proteinase K (Dako Japan, Kyoto) for 6 min at room temperature and then incubated with anti-vWF polyclonal antibody (Dako Japan) overnight at 4°C, followed by the treatment with anti-rabbit IgG polyclonal antibody. For quantitative studies, four randomly selected sections were photographed on 35-mm film using a ×10 objective lens and the number of microvessels per field (1.8 mm²) was counted. The relative lumen area (%) of microvessels in the same photographs was also quantified using NIH image software.

Statistical analyses

Data are shown as the mean ± S.E.M. The statistical difference between two groups was examined using unpaired Student’s t-test. Multiple comparisons were performed by using one-way ANOVA with Bonferroni’s
correction. A $P$ value of less than 0.05 was considered statistically significant.

RESULTS

Hemoglobin contents in tumor tissue

Figure 1A shows a significant correlation ($r = 0.837$, $P < 0.0001$) between hemoglobin contents and number of microvessels in the tumor tissues, which were isolated from mice at days 6, 7 and 12. As shown in Fig. 1B, there was also a linear relationship between hemoglobin contents and the relative lumen area of microvessels ($r = 0.887$, $P < 0.0001$). Thus, in the present study, we evaluated tumor-associated angiogenesis using hemoglobin contents.

Time-course of tumor-associated angiogenesis and tumor growth in tumor-bearing mice

S-180 tumor cells were inoculated into the dorsal subcutaneous space of each mouse, and tumor growth and tumor-associated angiogenesis were quantified at days 5, 10, 15 and 20 after inoculation. As shown in Fig. 2, tumor weight increased gradually in parallel with hemoglobin contents over 20 days of the experimental period.

Effect of BK receptor antagonists on tumor-associated angiogenesis and tumor growth

To examine the involvement of BK in angiogenesis and tumor growth in our model, a $B_1$-receptor antagonist, desArg$^{10}$-[Hoe140], and $B_2$-receptor antagonist (Hoe140 and FR173657) were administered to S-180-bearing mice from the day of tumor inoculation. As shown in Fig. 3: A and B, both angiogenesis and tumor growth were significantly suppressed by administration of Hoe140 at doses of 0.1 mg/kg per day or higher in S-180-bearing mice. Furthermore, as shown in Table 1, FR173657 (10 and 30 mg/kg per day, p.o., twice daily) also attenuated angiogenesis and tumor growth in a dose-dependent manner, giving a % inhibition of about 50% at 30 mg/kg per day as compared with the control group. On the contrary, desArg$^{10}$-[Hoe140] did not have any effects on angiogenesis and tumor growth at 1 mg/kg per day (once daily, local injection) (Figs. 3: C and D).

Effect of kallikrein inhibitors on tumor-associated angiogenesis and tumor growth

BK is produced from kininogens by tissue and/or plasma...
kallikrein. To examine which enzyme is involved in the generation of BK in our model, we examined the effects of two kallikrein inhibitors. As shown in Fig. 4, both angiogenesis and tumor growth were significantly suppressed by administration of SBTI, which inhibits plasma kallikrein, but not tissue kallikrein (24, 25), at doses exceeding 1 mg/site per day. Additional administration of aprotinin (an inhibitor of both tissue and plasma kallikreins (24, 25), at 1,000 U/site per day) did not induce further suppression of angiogenesis and tumor growth. No additive inhibition on the hemoglobin contents and tumor weight was observed following coadministration of SBTI (3 mg/site per day) and aprotinin compared with SBTI alone.

### Table 1. Reduction in angiogenesis and tumor weight by B2-receptor antagonist in tumor-bearing mice

| Dose (mg/kg, p.o.) | Inhibition (%) |  |
|--------------------|----------------|-----------------|
|                    | day 6          | day 12          |
| Hb contents        |                |                |
| 10                 | 19.0 ± 8.7%    | n.d.            |
| 30                 | 58.4 ± 7.7%*   | 42.4 ± 11.5%*  |
| Tumor weight       |                |                |
| 10                 | 27.9 ± 8.5%*   | n.d.            |
| 30                 | 50.0 ± 9.0%*   | 40.7 ± 9.3%*   |

FR173657, a bradykinin B2-receptor antagonist, was orally administered twice a day for 6 or 12 days. Values are the mean ± S.E.M. of 5 mice. n.d. = not determined. *P<0.05 vs vehicle control.
Histological evaluation of effects of a B<sub>2</sub>-receptor antagonist and kallikrein inhibitors

The formation of new blood vessels in developed tumor tissues and encapsulation of tumor stroma were confirmed by microscopic examination. As shown in Fig. 5A, the tumor was surrounded by a well-developed stroma tissue mainly composed of fibroblast-like cells, together with matrix formation. Most microvessels were localized within the stroma tissue. These microvessels were not uniformly distributed in the stroma and a cluster of vessels was often observed. In contrast, the number of newly formed vessels was markedly reduced in tumor tissues treated with FR173657 (30 mg/kg per day) (Fig. 5B) or SBTI (3 mg/site per day) (Fig. 5C).

Effect of kininase inhibitors on tumor-associated angiogenesis and tumor growth

As shown in Fig. 6: A and B, daily local injection of captopril (a kininase II inhibitor, 500 μg/site per day) enhanced angiogenesis by 120% and increased tumor weight by 90%, respectively, compared with those of the vehicle control group. On the other hand, additional administration of Hoe140 (bradykinin B<sub>2</sub>-receptor antagonist, at 1 mg/kg per day) significantly suppressed the angiogenesis and tumor growth (Table 2).

Phosphoramidon (a neutral endopeptidase inhibitor (26), 500 μg/site per day) but not MGPA (a kininase I inhibitor (27)), significantly promoted angiogenesis (Fig. 6A).

DISCUSSION

In the present study, we suggested that BK plays an important role in tumor-associated angiogenesis and tumor growth. Although the involvement of BK in ascitic fluid accumulation in ascitic tumor has been reported (19, 20, 28), the present study is the first report demonstrating the roles of BK in tumor-related angiogenesis. As shown in Fig. 3 and Table 1, Hoe140 and FR173657, selective bradykinin B<sub>2</sub>-receptor antagonists, dose-dependently suppressed tumor growth together with angiogenesis. By contrast, blockade of B<sub>1</sub> receptors with a selective antagonist, desArg<sup>10</sup>-[Hoe140], did not result in significant inhibition. These results suggest that BK may be involved in angiogenesis and tumor growth via the B<sub>2</sub> receptor. The significance of endogenous BK was also suggested from the present results showing that inhibition of BK degradation by captopril or phosphoramidon enhanced both angiogenesis and tumor growth (Fig. 6). Because kininase II and neutral endopeptidase are major enzymes responsible for BK degradation in the connective tissue of normal mouse.
It is plausible that inhibition of BK degradation by these inhibitors may elevate the tissue levels of BK, and thus facilitate angiogenesis in our model.

The mechanism underlying the BK-induced angiogenesis is still unclear. Whether BK could directly induce proliferation or migration of vascular cells such as endothelial cells, smooth muscle cells or fibroblasts (pericytes) is still controversial. BK promotes the growth of endothelial cells from post-capillary venules in vitro (34). However, this proliferation of venular endothelial cells is mediated by the B1 receptor, not by the B2 receptor. Because tumor angiogenesis is mainly mediated by the B2 receptor as shown in Fig. 3 in the present study, it is unlikely that the B1-dependent mitogenic activity of BK is involved in tumor angiogenesis in our model. The mitogenic activity of BK on other cell types is not clear, as it depends to a large extent on the culture condition (32).

In our preliminary experiment, the growth of S-180 cell in skin (29), it is plausible that inhibition of BK degradation by these inhibitors may elevate the tissue levels of BK, and thus facilitate angiogenesis in our model.

Fig. 5. Histological sections of sarcoma 180 tumors implanted into mice treated with a B2-receptor antagonist and a kallikrein inhibitor. Thin sections were immunostained with anti-von Willebrand factor (vWF) antibody, followed by the counterstaining with hematoxylin. A) Tumor tissue from a control mouse. B) Tumor tissue isolated from a representative mouse treated with FR173657 (30 mg/kg per day). C) Tumor tissue from a representative mouse treated with local injections of SBTI (3 mg/site per day). Newly formed microvessels are indicated by arrows. Bar = 100 μm.

Fig. 6. Effects of kininase inhibitors on angiogenesis and tumor growth in tumor-bearing mice. Sarcoma 180 cells were inoculated into the dorsal subcutaneous tissue of each mouse. At day 7, tumors were excised and the hemoglobin contents (A) and tumor weight (B) were determined. Captopril (kininase II inhibitor), MGPA (kininase I inhibitor) and phosphoramidon (NEP inhibitor) were injected locally (500 μg/site per day) once a day for 7 days. Open columns represent control mice. Values are the mean ± S.E.M. of five mice. *P<0.05, **P<0.01. Hb: Hemoglobin.
A selective antagonist for the B2 receptor is involved in angiogenesis and tumor growth, because different pathways, angiogenesis-dependent and -independent ones. It is possible that BK may stimulate the proliferation of tumor cells directly or indirectly via up-regulation of growth factors or cytokines. BK-induced vascular hyperpermeability might be also implicated in tumor growth because enhanced vascular permeability induces extravasation of blood-derived factors including nutrients or plasma proteins. We also evaluated BK generating systems involved in our experiments. As shown in Fig. 5, SBTI, a plasma kallikrein inhibitor, inhibited angiogenesis and tumor growth in a dose-dependent manner, and maximum inhibition was obtained at a dose of 3 mg/site per day. Co-administration of aprotinin, which inhibits both plasma and tissue kallikreins, with the highest dose of SBTI (3 mg/site per day) did not exhibit further inhibition. These results suggest that plasma kallikrein or plasma kallikrein-like enzymes, which are sensitive to SBTI, are mainly implicated in the generation of BK in our experiment.

Seemingly contradictory effects of ACE inhibitors on angiogenesis have been reported. Captopril is shown to inhibit angiogenesis in experimental tumors in rats and in human Kaposi’s sarcoma (40, 41). On the contrary, Cameron et al. (42) and Maxfield et al. (43) suggested that there was an increase in capillary density in sciatic nerve and in extensor digitorum longus muscle with an ACE inhibitor, lisinopril. Furthermore, long term treatment with spirapril, another ACE inhibitor, in adult spontaneously hypertensive rats resulted in a 28% increment in the number of coronary capillaries per unit area in left ventricle (44). Thus, effects of ACE inhibitors (that is, kininase II inhibitors) on angiogenesis are different and the precise reason for the discrepancy of the effects of ACE inhibitors is uncertain. It is plausible that the effects of ACE inhibitors differ, depending on the animal models used or experimental conditions. Gohlke et al. have shown that BK is implicated in the ACE inhibitor-induced increase in cardiac angiogenesis in stroke-prone spontaneously hypertensive rats because blockade of BK B2 receptors prevents the increase in cardiac capillary density induced by ramipril (45). The present results have also shown that angiogenesis associated with S-180 tumor growth was enhanced by the treatment with captopril and was attenuated by inhibition of the kallikrein-kinin system. We also confirmed that increased angiogenesis by captopril was cancelled by the simultaneous treatment with a B2-receptor antagonist (Table 2). Taken together, it seems likely that in circumstances where BK production is enhanced, ACE inhibitors may promote angiogenesis through inhibition of BK degradation. In contrast, ACE inhibitors would prevent neovascularization by inhibiting generation of angiotensin II, known as an angiogenic factor, probably when the tissue renin-angiotensin system is up-regulated. Recently, Volpert et al. suggested that inhibition of angiogenesis by captopril would be mediated through the direct inhibition of zinc-dependent endothelial cell-derived 72- and 92-kD metalloproteinases (MMPs) (40). It is not clear that inhibition of these MMPs would be involved in the effects of captopril in our model. Further studies are necessary to elucidate the effects of captopril on angiogenesis and the underlying mechanisms.

In the present study, angiogenesis was evaluated by the hemoglobin contents contained in the tumor tissues. Several reports have suggested a discrepancy between the hemoglobin contents and the extent of angiogenesis in solid tumor tissues and pointed out the possibility of overestima-

| Table 2. Effect of kininase inhibitor and B2-receptor antagonist on tumor-associated angiogenesis and tumor growth |
|---------------------------------------------------------------|
|                | Hb contents (mg/g wet tissue) | Tumor weight (g)         |
| Vehicle        | 3.63 ± 0.28                   | 0.66 ± 0.09              |
| Captopril      | 5.82 ± 0.61*                  | 0.97 ± 0.09*             |
| Captopril + Hoe140 | 2.58 ± 0.27*                | 0.34 ± 0.02*             |

Captopril (500 μg/site per day) and Hoe140 (1 mg/kg per day), a bradykinin B2-receptor antagonist, were administered locally once a day for 7 consecutive days. Values are the mean ± S.E.M. of 5 mice. *P<0.05 vs vehicle control. #P<0.01 vs captopril alone. Hb: Hemoglobin.
tion of angiogenesis because of the erythrocyte-rich plug formation in tumor vasculature due to enhanced production of tissue factor by tumor cells (46–48). Taking account of these reports, we have performed some experiments to obtain a rationale for using the hemoglobin contents as a quantitative marker for tumor angiogenesis. As shown in Fig. 1, we examined the relationship between the hemoglobin contents and the number of newly formed microvessels, and confirmed that the hemoglobin content is a good marker for angiogenesis in our S-180 tumor tissue model. Increment in the number of microvessels was also confirmed histologically by using thin sections stained with anti-vWF antibody. Furthermore, no plug formation was observed on these histological sections as far as we examined. Hemoglobin has been used as a quantitative marker to evaluate angiogenesis in various solid tumors (49–51). Therefore, whether or not hemoglobin could be used for evaluation of tumor angiogenesis would be largely dependent on the tumor cell types, especially on the ability to produce procoagulant factors.

We did not directly demonstrate the enhanced generation of BK in the tumor stroma because the BK generated is degraded at an early time, and the estimated amounts of BK to be generated are too small to be quantitatively determined. Generation of kinins by tumor tissues was demonstrated in the ascitic fluid of experimental ascitic tumors (19). The molecular basis for the generation of BK in the tumor tissues has not been fully proved. As shown in Fig. 4, BK is suggested to be generated by plasma kallikrein. Matsumura et al. (28) demonstrated that conditioned media of various tumor cell lines contain the activities to produce active kallikrein from prekallikrein. They suggested that plasmin, generated from plasminogen by plasminogen activator derived from tumor cells, might be implicated in the activation of plasma prekallikrein into kallikrein. Therefore, it is likely that plasmin may be involved in the generation of kinins in the vicinity of tumor tissue in our tumor-bearing models.

In conclusion, we demonstrated that BK, produced by plasma kallikrein or plasma kallikrein-like enzymes, promote angiogenesis and tumor growth through B2 receptor in vivo. These results suggest that BK could be a possible target for anti-angiogenic therapy and that B2 antagonists and/or kallikrein inhibitors are promising therapeutic agents against solid tumors.

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