Host Stromal Bradykinin B2 Receptor Signaling Facilitates Tumor-Associated Angiogenesis and Tumor Growth

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ABSTRACT

We evaluated the significance of the host kallikrein-kinin system in tumor angiogenesis and tumor growth using two rodent models genetically deficient in a kallikrein-kinin system. Inoculation of Walker 256 carcinoma cells into the s.c. tissues of the back of normal Brown Norway Kitasato rats (BN-Ki rats) resulted in the rapid development of solid tumors with marked angiogenesis. By contrast, in kininogen-deficient Brown Norway Katholiek rats (BN-Ka rats), which cannot generate intrinsic bradykinin (BK), the weights of the tumors and the extent of angiogenesis were significantly less than those in BN-Ki rats. Daily administration of B2 receptor antagonists significantly reduced angiogenesis and tumor weights in BN-Ki rats to levels similar to those in BN-Ka rats but did not do so in BN-Ka rats. Angiogenesis and tumor growth were significantly suppressed in B2 receptor knockout mice bearing sarcoma 180 compared with their wild-type counterparts. Immunoreactive vascular endothelial growth factor (VEGF) was localized in Walker tumor stroma more extensively in BN-Ki rats than in BN-Ka rats, although immunoreactive B2 receptor also was detected in the stroma to the same extent in both types of rats. Cultured stromal fibroblasts isolated from BN-Ki rats and BN-Ka rats produced VEGF in response to BK (10^-8-10^-6 M), and this stimulatory effect of BK was abolished with a B2 receptor antagonist, Hoe140 (10^-5 M). These results suggest that BK generated from kininogens supplied from the host may facilitate tumor-associated angiogenesis and tumor growth by stimulating stromal B2 signaling to up-regulate VEGF production mainly in fibroblasts.

INTRODUCTION

Angiogenesis is a process involved in many pathologic conditions such as diabetic retinopathy and rheumatoid arthritis, in which angiogenesis is responsible for the progression of such diseases (1–4). Angiogenesis also is a prerequisite for solid tumor growth and metastasis, and vigorous angiogenesis has been associated with a poor prognosis for cancers originating at several primary sites (5–8). It seems highly plausible that the excess supply of oxygen and various plasma components, including growth factors from newly formed microvessels, may facilitate tumor growth. Angiogenesis is a complex and multistage process, in which a variety of cells are involved in the construction of new blood vessels. Various defined or putative proangiogenic factors have been shown to up-regulate neovascularization. However, the critical factors responsible for the tumor angiogenesis still await further elucidation. Although some of these factors are produced by the tumor itself or are supplied from the circulation, the tumor stromal cells surrounding the tumor also may play an important role in the production of proangiogenic factors (9). Many investigators (10–14) have suggested the significance of interactions between tumor tissue and its stroma to facilitate tumor growth, tumor metastasis, or tumor angiogenesis. Stroma-derived factors can play a role in tumor cell migration and proliferation. However, the molecular basis for these interactions remains unclear. It recently has been indicated that cyclooxygenase-2 expressed in stromal fibroblasts is implicated in colorectal carcinoma growth and angiogenesis (12), which led us to evaluate the importance of the stroma for tumor growth and angiogenesis.

Bradykinin (BK) is a biologically active peptide that can induce vasodilatation, increase vascular permeability, cause pain, and accelerate glomerular filtration and excretion of sodium from the kidney mainly via B1 receptor signaling (15–17). BK is generated through the action of kallikreins from the precursor proteins, kininogens (KGNs). It has been demonstrated that BK stimulates angiogenesis in a sponge model (18, 19). We previously have shown that BK contributed to tumor-induced angiogenesis and tumor growth via the B2 receptor, not the inducible B1 receptor, in sarcoma 180-bearing mice (20). BK also is reported to be involved in the enhancement of tumor growth via increased permeability of the tumor vasculature (21, 22). The expressions of (a) a kinin-generating enzyme, kallikrein; (b) KGNs, which are substrates for kallikrein; and (c) BK receptors (B1 or B2) in various types of tumor cells were reported (23–27). This accumulating evidence suggests that BK may be one of the primary mediators responsible for tumor angiogenesis, and thereby for tumor growth, but the precise process of BK production associated with tumor growth and the mechanisms underlying BK-induced angiogenesis have not been fully demonstrated.

The aim of the present study was to investigate the kinin-generating systems and the roles of kinin receptor signaling in tumor-associated angiogenesis and tumor growth in tumor-bearing models. To demonstrate the significance of the host stromal kallikrein-kinin system, we used two experimental types of rodents whose kallikrein-kinin systems were intrinsically impaired.

MATERIALS AND METHODS

Animals. Genetically KGN-deficient Brown Norway Katholiek rats (BN-Ka rats) were obtained from the Katholiek Universiteit of Leuven, Belgium. Normal rats of the same strain were transferred from the Microbiological Association (Frederick, MD) and kept at Kitasato University [designated as Brown Norway Kitasato rats (BN-Ki rats)]. Male rats of both strains (8–10-weeks-old) were used. In BN-Ka rats, only trace levels of KGNs are observed (21, 22). The expressions of (a) a kinin-generating enzyme, kallikrein; (b) KGNs, which are substrates for kallikrein; and (c) BK receptors (B1 or B2) in various types of tumor cells were reported (23–27). This accumulating evidence suggests that BK may be one of the primary mediators responsible for tumor angiogenesis, and thereby for tumor growth, but the precise process of BK production associated with tumor growth and the mechanisms underlying BK-induced angiogenesis have not been fully demonstrated.

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backcrossed over >10 generations. These procedures minimize problems related to the genetic background and are accepted as a standard method of excluding any bias from the genetic background.

Although genetic factors could control individual angiogenic potential, angiogenic activities do not differ greatly between species whose genetic backgrounds are closely related, as shown by Rohan et al. (32). Therefore, it is reasonable to conclude that most of the difference between angiogenic activities in the B2 knockout mice from those in age- and sex-matched wild-type mice resulted from the difference in availability of B2 receptors. Knockout mice and their wild-type counterparts were purchased from The Jackson Laboratory.

All of the rats and mice were housed at a controlled humidity of 60 ± 5% and a temperature of 25 ± 1°C with a 12-h light/dark cycle. All of the animal experiments were performed in accordance with the guidelines for animal experiments of Kitasato University School of Medicine and Kansai Medical University.

**Determination of KGN Levels in Plasma.** Citrated plasma was prepared from the rats and mice, and the levels of high molecular weight KGN (HMW-KGN) and low molecular weight KGN (LMW-KGN) were determined as described previously (33). We determined the circulating KGN levels in B2 knockout mice and wild-type mice. The plasma levels of HMW-KGN and LMW-KGN in B2 knockout mice were 94 ± 6.3 ng BK equivalent/ml plasma protein (n = 5) and 52 ± 3.9 ng BK equivalent/ml plasma protein (n = 5), respectively, whereas those in wild-type mice were 79 ± 3.4 ng BK equivalent/ml plasma protein (n = 5) and 55 ± 3.7 ng BK equivalent/ml plasma protein (n = 5). These results indicate that there are no significant differences in plasma KGN levels between the two strains.

The plasma HMW-KGN and LMW-KGN levels in BN-Ki rats were 15.4 ± 0.5 ng BK equivalent/mg protein plasma (n = 6) and 9.5 ± 0.3 ng BK equivalent/mg plasma protein (n = 6), respectively. In BN-Ka rats, the levels were <0.5 ng BK equivalent/ml plasma protein (n = 6).

**Cell Culture.** Rat Walker 256 (CCL-38) carcinoma cells and murine sarcoma 180 (CCL-8; S-180) tumor cells were provided by Chugai Pharmaceutical (Tokyo, Japan) and Dainippon Pharmaceutical (Osaka, Japan), respectively. Walker 256 cells and S-180 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). We confirmed that in vitro growth of Walker 256 and S-180 cells was not affected by the addition of BK (10−4-10−8 M at final concentration) or the BK antagonist Hoe140 (10−3 M at final concentration).

**Tumor Implantation Models.** The dorsal hair of rats anesthetized lightly with ether was shaved, and the skin was sterilized with 70% ethanol. Walker 256 cells were harvested and washed three times with PBS [NaCl at 8 g/l, KCl at 0.2 g/l, Na2HPO4·12H2O at 2.9 g/l, and KH2PO4 at 0.2 g/l (pH 7.2)]. The cells were pelleted by brief centrifugation at 3000 × g at 25°C. The supernatant was aspirated, and the cells were resuspended in PBS at a density of 2 × 107 cells/ml. The cell suspension (0.2 ml) was injected into the dorsal s.c. tissue of each rat using a 25-gauge needle. The day of inoculation was defined as day 0. On designated experimental days, the rats were sacrificed, and the hemoglobin contents of the tumor tissues and the tumor weights were determined as described below. Some BN-Ki rats received topical injections of the BK B1 receptor antagonist des-Arg10-Hoe140 (0.01–0.1 mg/site, twice a day) or of the BK B2 receptor antagonist Hoe140 (0.1 mg/site, twice a day). Control mice were administered vehicle solution (physiologic saline) topically. All of the injections were made in the vicinity of the tumor with the needle pointed in the direction of the tumor. FR173657 (suspended with 5% gum Arabic at 6 mg/ml in distilled water) was administered orally (10–30 mg/kg, twice a day) to the tumor-bearing BN-Ki rats. Control rats received 5% gum Arabic solution orally.

Similarly, tumor-bearing mice were prepared by s.c. injections of 0.1 ml/site of S-180 cell suspension (2 × 107 cells/ml). Seven days after the injection of S-180 cells, the mice were sacrificed, and the hemoglobin contents of the tumor tissues, the tumor weight, and tumor volume were determined as described below.

**Determination of Angiogenesis and Tumor Growth.** Rats and mice were killed with an excess dose of ether, and the tumor tissues with encapsulating stromal tissues were excised from the back. Tumor volume was determined following our previous methods (13). The total amount of excised tumor tissues was weighed immediately after harvesting, and the hemoglobin contents were determined using the following method (13). Briefly, tumor tissues were homogenized in distilled water (four times the weight of the tumor tissue) in a Polytron homogenizer (Kinematica, Lucerne, Switzerland). After centrifugation at 5000 × 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, Osaka, Japan; Ref. 13). The hemoglobin contents of the tumor tissues were expressed as mg/g wet tissue of tumor (13).

**Histologic and Immunohistochemical Studies.** Tumor tissues together with the stroma were excised from the backs of rats and mice and were immediately fixed with ice-cold 4% paraformaldehyde in 0.1 M PBS (pH 7.4). After fixation, the tissues were dehydrated with a graded series of ethanol and embedded in paraffin. Thin sections (4 μm) prepared from the paraffin-embedded tissues were mounted on glass slides, deparaffinized with xylene, and then placed in cold (4°C) acetone for immunostaining. Some sections were prepared and stained with H&E and then processed for light microscopic examination. The manufacturer confirmed the cross-reactivity of the anti-B2 receptor antibody with rat B2 receptor.

For quantitative studies, the excised tumor tissues were divided into two halves, one for measuring the hemoglobin content and the other for quantification of the lumen area. Thin sections were prepared and then immunostained with anti-von Willebrand’s factor’s serum. 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.

**Determination of VEGF mRNA Levels in Tumor and Stroma with Taqman Reverse Transcription-PCR.** Total RNA was isolated from the excised tumor tissues including stroma by the standard acid-guanidinium-phenol-chloroform alcohol extraction method. Vascular endothelial growth factor (VEGF) mRNA expression levels in the tumor tissues were determined with TaqMan reverse transcription-PCR. The following primers and TaqMan probes were used: mouse VEGF (M05200) upstream primer, 5′-GACACAAGTCCCATGGAAGTGAT-3′; downstream primer, 5′-AGATGGTCCAC-GAGGGTCTCAAT-3′; and TaqMan probe, 5′-(FAM)-AGGTCTACGCCAGGACTCTGCTC-(TAMRA)-3′. Rat VEGF upstream primer, 5′-CTACTCACCACCGCATTTTGGC-3′; downstream primer, 5′-CACACAGGAGGCTTGGAAGAT-3′; and TaqMan probe, 5′-(FAM)-TGGACATCTTCCAGGAGTACCGG-(TAMRA)-3′. The β-actin mRNA level in each tumor tissue was used as an internal standard. The thermal cycler parameters were 95°C for 10 min (for heat activation of Taq-polymerase), followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. We isolated tumor tissues with stroma at day 7 and estimated the expressed levels of VEGF in terms of the relative VEGF mRNA to that of β-actin as an internal standard. The mean ratio of mRNA of VEGF to that of β-actin in the tumor and normal tissues from BN-Ki rats was set as 100%. The relative VEGF mRNA to that of β-actin of wild-type mice was compared with that of B2 receptor knockout mice. The relative VEGF mRNA also was compared between BN-Ki rats and BN-Ka rats.

**Preparation of Tumor Stromal Fibroblasts and Determination of VEGF Released.** The tumor stromal tissues encapsulating the tumors from BN-Ki rats and BN-Ka rats were chopped into small pieces, placed into 60-mm culture dishes, and then covered with DMEM/10% FBS containing 0.01% collagenase, 100 units/ml penicillin, and 100 mg/ml streptomycin. After 24 h of culture, the tissues were washed and transferred into 75-cm2 dishes containing DMEM/10% FBS. Seven days later, the cells extending over the surface of the dishes were collected and seeded at a 1:3 dilution. These cells homogenously exhibited a typical fibroblast-like configuration. They were identified as fibroblasts through immunostaining and by their morphologic features. Briefly, the cells were positively stained with vimentin antibody and not with antibodies against von Willebrand’s factor or HHF-35, which are specific markers for endothelial cells and smooth muscle cells, respectively. No contamination of macrophages was confirmed by microscopic observation of hematoyxin-stained cells using the criterion that fibroblasts have elongated cytoplasmic extensions and large pale nuclei, whereas unstimulated macrophages have an oval cytoplasmic border and a smaller and more darkly staining nucleus. It is known that some of the original characteristics of stromal fibroblasts may be lost long-term adaptation to in vitro culture conditions. In the present study, therefore, we used only first-or second-passage fibroblasts. We confirmed that the signaling pathway for VEGF induction was not...
lost through the use of cobalt chloride (CoCl₂), which is known to mimic hypoxic conditions.

Walker 256 cells were cultured in DMEM supplemented with 10% FBS. When these cells became 80% confluent, they were collected and counted.

VEGF concentrations in the culture media were determined as follows. Fifty thousand fibroblasts from rats and Walker 256 cells were seeded into 35-mm² culture dishes and incubated for 24 h in DMEM/10% FBS. The medium was discarded, and the cells were washed with PBS; serum-free DMEM then was added, and they were incubated for an additional 6, 12, and 24 h in the presence of BK (10⁻⁸ and 10⁻⁷ M), a B₂ receptor antagonist (Hoe140 at 10⁻⁵ M), or CoCl₂ (10⁻⁵ M). CoCl₂ is widely accepted as a standard agent to enhance VEGF expressions (34). Therefore, we used 10⁻⁸ M CoCl₂ as a reference agent and compared the B₂ receptor-mediated induction of VEGF in isolated fibroblasts with that in CoCl₂-treated fibroblasts. The culture media were collected, and the concentrations of VEGF were determined using a VEGF immunoassay kit (MMV00; R&D Systems, Minneapolis, MN). Because the absolute concentrations of rat VEGF were not determined in the present study, the data were expressed as values relative to the VEGF concentration of the culture medium treated with 10⁻⁸ M CoCl₂, which was taken as 100%.

Agents. A BK B₂ receptor antagonist, FR173657 [(E)-3-(6-acetamido-3-pyridyl)-N-[N-[2,3-dichloro-4-[2-methyl-8-quinolinyl]oxymethyl][phenyl]-Nmethylnctinobmethyl]acrylamide], was provided by Fujisawa Pharmaceutical (Osaka, Japan; Ref. 35), and d-Arg[Hyp3, Thr5, d-Tic7, Oic8] BK (Hoe140; Ref. 36) was purchased from the Peptide Institute (Osaka, Japan). A B₁ receptor antagonist, des Arg⁹-d-Arg[Hyp³, Thr⁵, d-Tic⁷, Oic⁸] BK (des Arg⁹-Hoe140; Ref. 37) was obtained from Peninsula Laboratories (San Carlos, CA).

Statistical Analysis. Data are shown as mean ± SE. The statistical difference between the two groups was examined using Student’s unpaired t test after confirming that the variance of data was not heterogeneous. Multiple comparisons were performed using one-way ANOVA with Bonferroni’s correction. P < 0.05 was considered statistically significant.

RESULTS

Tumor-Associated Angiogenesis and Tumor Growth in KGN-Deficient BN-Ka Rats. To elucidate the significance of the supply of KGN from the host, Walker 256 carcinoma cells were inoculated into the dorsal s.c. space of BN-Ka and BN-Ki rats, and tumor-associated angiogenesis and tumor growth were determined at days 7 and 14 after inoculation. We confirmed that the hemoglobin contents of the tumor tissues were well correlated with the histologically examined neovascularization. Tumor-associated angiogenesis, tumor weights, and tumor volume were increased over the experimental period, suggesting vigorous tumor growth and angiogenesis without spontaneous regression (Fig. 1, A–C). In BN-Ka rats, increments in the hemoglobin contents, tumor weights, and tumor volume were significantly suppressed at day 7 (Fig. 1, A–C). At day 14, tumor weights showed a tendency to be reduced in BN-Ka rats compared with those in BN-Ki rats (the difference was not significant; P = 0.09). The hemoglobin contents and tumor volume in BN-Ka rats were significantly lower even at day 14. These results indicate that BK generated from KGN supplied from the host circulation plays significant roles in tumor angiogenesis and tumor growth in the initial phase of tumor development.

Fig. 2, A and B, depicts the typical appearance of tumors at day 7 implanted in BN-Ki rats and BN-Ka rats, respectively. Tumor-associated angiogenesis may be enhanced in BN-Ki rats, judging from the redness of the tumor (Fig. 2A). As Fig. 2C shows, in the immunohistochemical examination of BN-Ki rats at day 7, the tumor was surrounded by a well-developed stromal tissue mainly composed of fibroblast-like cells, together with a matrix formation. Tumor cells often invaded the stromal tissue, and the boundary between the tumor and the stromal tissue was not clear. Many microvessels were observed (arrows in Fig. 2C), most being localized within the stromal tissue. By contrast, in BN-Ka rats at day 7 (Fig. 2D), the stromal tissue encapsulating the tumor was poorly developed compared with that in BN-Ki rats, invasion of the stroma by the tumor cells was less frequent, and the extent of angiogenesis, as estimated by the immunohistochemical examination, was markedly reduced in BN-Ka rats.

Effect of BK Receptor Antagonists on Tumor-Associated Angiogenesis and Tumor Growth. To elucidate the BK receptor subtypes responsible for the tumor growth and angiogenesis, we examined the effects of BK receptor antagonists on the increments in tumor weight, tumor volume, and the hemoglobin contents in BN-Ki rats bearing Walker 256 cells. The tumor weight and the tumor volume of tumors at day 7 were markedly reduced with B₂ antagonist FR173657 (30 mg/kg, p.o., twice a day) by 30% and 35%, respectively (Fig. 3, A and C). The hemoglobin contents at day 7 also were reduced with FR173657 (Fig. 3A). By contrast, B₁ antagonist des Arg¹₀-[Hoe¹⁴⁰] (0.01–0.1 μg/site, local injections; data not shown) did not suppress these parameters. The reduced levels in FR173657-treated BN-Ki rats were levels similar to those in BN-Ka rats that were not administered FR173657 (Fig. 3, right open columns in A–C). However, administration of FR173657 to BN-Ka rats did not result in a further reduction in hemoglobin content, tumor weight, or tumor volume (Fig. 3, right closed columns in A–C).
Tumor-Associated Angiogenesis and Tumor Growth in B₂ Receptor Knockout Mice. The significance of the host B₂ receptor signaling also was investigated using B₂ receptor knockout mice, which were inoculated with S-180 cells. As shown in Fig. 4, A–C, angiogenesis and tumor growth at day 7 were significantly suppressed in B₂ receptor knockout mice compared with their wild-type counterparts. The percentage of inhibition of angiogenesis, tumor weight, and tumor volume in B₂ receptor knockout mice at day 7 was 48%, 60%, and 61%, respectively. These low figures suggest that the site action of kinins was located in the host stroma.

Immunostaining of B₂ Receptors and VEGF. To ascertain the cellular distribution of the B₂ receptor within the tumor tissues, we conducted an immunohistochemical study using an anti-B₂ receptor monoclonal antibody in BN-Ki rats and BN-Ka rats. As Fig. 5A indicates, B₂ receptor expression was obvious in the fibroblast-like cells within the stromal tissues. The stroma and vascular endothelial cells also were strongly stained. By contrast, practically no staining was detected in the tumor cells themselves. When Fig. 5A was compared with Fig. 5B, the results of B₂ receptor staining show no significant difference between BN-Ka and BN-Ki rats.

Localization of VEGF proteins in the tumor and stromal tissue of Walker 256-bearing BN-Ki rats at day 7 also was examined by immunohistochemistry (Fig. 5C). Intense staining of immunoreactive VEGF was detected in the tumor stromal tissues. Not only fibroblasts but also the fibrous matrix was positively stained. Such positive staining was not observable with the use of nonimmune control IgG (data not shown). A faint staining of VEGF was observed in the tumor cells. In contrast, in Walker 256-bearing BN-Ka rats, in which the stroma and microvessels were poorly developed (Fig. 5D), staining of immunoreactive VEGF in the tumor stromal tissues was much weaker than in BN-Ki rats.

VEGF mRNA Levels in Tumor and Stroma. VEGF mRNA levels in tumor tissues including stroma were determined by quantitative PCR (TaqMan). The relative VEGF mRNA expression level in BN-Ki rats was 100 ± 27.2 (n = 6), which was significantly (P < 0.05) higher than that in BN-Ka rats (34 ± 9.1; n = 6). As shown in Fig. 4D, VEGF mRNA levels in the tumor and stromal tissues isolated from B₂ receptor knockout mice also were significantly lower than those from their wild-type counterparts.

VEGF Production in Tumor Stromal Fibroblasts and Tumor Cells Treated with BK. Our results presented previously suggest that one of the target cells of BK for inducing angiogenesis may be the host stromal fibroblasts rather than the tumor cells. Therefore, we examined the responses of stromal fibroblasts and tumor cells to BK in vitro. Stromal fibroblasts were prepared from tumor stromal tissues from BN-Ki rats and BN-Ka rats as described in “Materials and Methods.” These cells exhibited a typical fibroblast-like configuration and did not show morphologies peculiar to endothelial cells or macrophages. Treatment of the stromal fibroblasts from BN-Ki rats with BK for 12 h in the absence of FBS resulted in a marked increase in the release of VEGF into the culture medium (Fig. 6A). The maximal effect was obtained at 10⁻⁷ M. The effect of BK was nearly completely abolished by the presence of the B₂ selective antagonist Hoe140 (10⁻⁶ M). This also occurred with another B₂ antagonist, FR173657, at 10⁻⁵ M (data not shown). The number of fibroblasts during the present experiment did not change significantly in any experimental group, suggesting that BK did not induce proliferation of the stromal fibroblasts. The same was true in fibroblasts from BN-Ka
FR173657 (B₂ receptor antagonist, 30 mg/kg) was orally administered twice a day to rats (normal). From day 1, kininogen-deficient) and Brown Norway Kitasato (BN-Ka); mean open columns administered to BN rats (Fig. 6B). However, as shown in Fig. 6C, under stimulation with BK for 12 h in the absence of FBS, no significant VEGF release from Walker 256 carcinoma cells was observed in any experimental group.

**DISCUSSION**

In the present study, we first demonstrated that tumor growth and the development of tumor-associated angiogenesis was distinctly suppressed in KGN-deficient BN-Ka rats compared with normal BN-Ki rats (Fig. 1). These results suggest that the tumor growth together with tumor-associated angiogenesis may significantly depend on the supply of KGNs from the host circulation. The KGNs in the circulation were plasma proteins belonging to a globulin fraction and were secreted from the hepatocytes. BN-Ka rats, whose hepatocytes cannot secrete KGN because of one-point mutation of the KGN moiety, lack the circulating KGN (19, 14). Because kinins were generated from KGN through the action of kinin-releasing enzymes, kallikreins, supplementation of deficient KGN molecules resulted in the generation of kinin in the body, such as in the kidneys, in BN-Ka rats (38). There have been several reports showing that KGNs or KGN-like proteins are produced by tumor cells (39, 40), but the present results indicate the possibility that tumor-derived KGNs, if present, are not involved in tumor-associated angiogenesis in our model because the prolonged administration of B₂ antagonist did not reduce angiogenesis in KGN-deficient BN-Ka rats (Fig. 4). The contribution of kinin to facilitation of tumor-associated angiogenesis is obvious in the early phase of the experimental period (Fig. 1). This may be explained by the fact that the enhanced extravasation of plasma, which will become a source of kinin generation, was rather limited in the early phase of the experimental period, as we reported previously (41). Further, because the plasma exudation in the early stage was B₂ dependent, it is highly plausible that the kinin generation, which leads to facilitation of angiogenesis, may be enhanced by a B₂-mediated increase in plasma exudation (41). Because of this positive feedback mechanism, the kinins generated may effectively facilitate tumor-associated angiogenesis.

We next identified the site of action of kinin generated from circulating KGNs. Tumor-associated angiogenesis and tumor growth were markedly attenuated in B₂ receptor knockout mice (Fig. 4) despite the implantation of the same number of tumor cells. These results indicated that B₂ receptor signaling in the host stroma would be one of the pathways via which tumor angiogenesis and tumor growth are promoted, although other mediators such as prostat glandins and angiotensin also may facilitate angiogenesis, as reported by us previously (13, 14, 42–44). Because a B₁ receptor antagonist did not significantly reduce tumor growth or angiogenesis, the B₁ receptor signaling did not contribute markedly to kinin-mediated facilitation of tumor growth and angiogenesis, although in another experimental inflammatory angiogenesis model, BK has been shown to induce angiogenesis via B₁ receptor partly (19).

The present immunohistochemical examination of rats bearing Walker tumor cells further revealed that the localization of immunoreactive B₂ receptor was mainly observed in the tumor stromal tissues, suggesting that the target cells of kinins may be the host stromal cells (Fig. 5, A and B), although the presence of B₁ and B₂ receptors in some tumor cells was reported previously (23, 26). Of all of the constituents of the stroma, the fibroblasts may be the cells that respond to B₂ signaling because, as mentioned below, we found that the isolated cultured fibroblasts prepared from tumor stroma increased the production of VEGF in response to a B₂ receptor agonist. These findings together strongly suggested that the host stromal B₂ receptor present on the fibroblasts is relevant to the facilitation of tumor-associated angiogenesis.

The mechanism of stromal B₂-mediated facilitation of angiogenesis was further elucidated in the present study. Although BK-induced angiogenesis has been reported in several in vivo angiogenesis models, the molecular basis for the kinin-mediated tumor angiogenesis has been incompletely understood (45, 46). We evaluated the contribution of VEGF in this tumor-associated angiogenesis model. Because we identified the significant role of VEGF in sponge-induced angiogenesis using VEGF-neutralizing antibody and antisense oligonucleotide against VEGF (42, 47) and because tumor growth implanted in mice was strongly suppressed with VEGF antibody (13, 43), we focused on this growth factor in the present experiment. As mentioned previously, immunohistochemical study revealed that VEGF was up-reg-
ulated, not in the tumor cell itself, but in the tumor stroma (Fig. 5), which corresponds to the region where tumor angiogenesis was markedly induced. The intensity of the immunostaining was well correlated with the extent of angiogenesis in the stromal tissue. Furthermore, stromal fibroblasts, which expressed a considerable amount of B2 receptor, also stained with an anti-VEGF antibody. These results strongly suggest that VEGF may be involved in BK-induced angiogenesis in our models. At day 14, there is no major difference in the hemoglobin content or the weight of the tumors in BN-Ki versus BN-Ka rats (Fig. 1). Accordingly, when tumors are more hypoxic after extensive growth, the tumor growth is probably mainly dependent on the VEGF secreted from the tumor rather than from the tumor stroma. The mediator-dependent inhibition of angiogenesis may be limited. The angiogenic response in the larger tumors may be highly dependent on the hypoxia-dependent factors, such as hypoxia-inducible factor 1α, because the promoter regions of the genes of these factors were sensitive to hypoxia. Thus, the VEGF that was responsible for tumor growth and tumor angiogenesis after extensive growth may be derived from tumor cells, not from stromal cells.

To investigate this possibility, we conducted two experiments. The VEGF mRNA levels in the tumor and stroma from BN-Ki rats and from normal mice were significantly higher when compared with those from KGN-deficient BN-Ka rats and B2 receptor knockout mice (Fig. 4D), respectively. Further, in an in vitro experiment using fibroblasts prepared from tumor stroma tissues, we found that VEGF production was enhanced in response to BK at physiologic concentrations. If these facts are taken together, it is reasonable to conclude that VEGF, produced by the stromal fibroblasts in response to BK, promoted new vessel formation in the stromal tissues in the present experiment. We previously reported that angiogenesis in a sponge model was dependent on the activation of activator protein-dependent pathways (44). The signal transduction pathway of B2 receptor has not

Fig. 4. Tumor-associated angiogenesis, tumor growth, and vascular endothelial growth factor (VEGF) mRNA levels in tumor and stroma tissues in B2 receptor knockout mice and wild-type counterparts. Sarcoma-180 cells (2 × 10^6 cells/0.1 ml in PBS) were inoculated into the dorsal s.c. tissue of B2 receptor knockout mice and wild-type mice. At day 7, tumor tissues were excised with the encapsulating stromal tissues and dissected free of fat and connective tissues. The tumor weight (B), tumor volume (C), and the hemoglobin content of the tumor tissue (A) were determined. VEGF mRNA levels in tumor and stroma tissues (D) were determined as described in “Materials and Methods;” mean ± SE (A–C, n = 6; D, n = 8); Hb, hemoglobin.

Fig. 5. Immunohistochemical staining of bradykinin B2 receptor and vascular endothelial growth factor (VEGF) in the tumor tissues in Brown-Norway rats. Walker 256 cells (4 × 10^6 cells/0.2 ml in PBS) were inoculated into the dorsal s.c. tissue of Brown Norway Katholiek (BN-Ka) rats (genetically kininogen-deficient) and Brown Norway Kitasato (BN-Ki) rats (normal). Thin histologic sections were prepared from the tumor tissues excised with the encapsulating stromal tissues in BN-Ki rats (A and C) and BN-Ka rats (B and D). The sections were stained with antihuman B2 receptor antibody (A and B) or antirat VEGF antibody (C and D); T, tumor; St, stromal tissue. Arrow indicates newly formed microvessels; bar, 100 μm.
been fully investigated in fibroblasts, but it has been reported that stimulation of B2 receptor results in activator protein translocation to the nuclear fraction through the mitogen-activated protein kinase pathway or by activation of the protein kinase C pathway in several cell lines (48–50). Because the promoter region of VEGF gene contains an activator protein binding site, these mitogen-activated protein kinase- or protein kinase C-dependent pathways, or both, may be implicated in BK-induced VEGF up-regulation in stromal fibroblasts (51). One of the possible mechanisms of VEGF induction by B2 receptor signaling may be activator protein dependent.

The angiogenic switch was induced in the early stage of tumor development even in human cancer cases. The examination of the early phase of angiogenesis is useful for the study of human cancer. It is widely known that the inhibition of angiogenic switches is critical for management of cancer if a tumor is found in the early phase. The chemopreventive approaches using agents that block the angiogenic switches may be effective. As the present study indicates, B2 receptor-dependent inhibition of tumor development in the early stage may be an effective strategy of chemoprevention even in human disease. In conclusion, one of the most important findings in the present study is the critical role of tumor stromal B2 receptor signaling in tumor angiogenesis and tumor growth. Many investigators (10, 11) have alluded to the significance of the interaction between the tumor and the tumor stroma in tumor growth. We found in the present study that kinin is one of the key factors acting on the host stroma cells, promoting tumor angiogenesis and tumor growth, and that local kinin generation was dependent on the supply of KGN from the host. Our results also suggested that B2 receptor signaling facilitates tumor angiogenesis by acting on stromal fibroblasts to up-regulate the production of VEGF, a potent proangiogenic factor. Consequently, the host kallikrein-kinin system is probably one of the targets for chemoprevention of tumors, and B2 antagonists appear to have promise as therapeutic agents against solid tumors.

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Fig. 6. Vascular endothelial growth factor (VEGF) production in tumor stromal fibroblasts and tumor cells treated with bradykinin (BK). Walker 256 cells (4 × 10⁶ cells/0.2 ml in PBS) were inoculated into the dorsal s.c. tissue of Brown Norway Katholiek (BN-Ka) rats (genetically kininogen-deficient) and Brown Norway Kitasato (BN-Ki) rats (normal). At day 7 after inoculation, tumor stromal tissues were carefully dissected of tumor, fat, and connective tissue under sterile conditions. Primary cultures of the stromal fibroblasts (A, BN-Ki rats; B, BN-Ka rats) and tumor cells (C) were obtained as described in “Materials and Methods.” The stromal fibroblasts (A and B) and tumor cells (C) were treated with BK (10⁻⁵ M and 10⁻⁷ M) in the absence of fetal bovine serum for 12 h, and the VEGF concentrations in the culture media were determined by enzyme immunoassay. The effects of B2 receptor antagonist (Hoe140, 10⁻⁵ M) were evaluated with cells treated with 10⁻⁷ M BK. The VEGF concentration in the culture medium of 10⁻⁵ M cobalt chloride (CoCl₂)-treated fibroblasts was defined as 100%, and the data were expressed as relative values, mean ± SE (n = 6); Hb, hemoglobin. **P < 0.01; #P < 0.05 (compared with vehicle); n.s., not significant.
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