Involvement of the Kinin-generating Cascade in Enhanced Vascular Permeability in Tumor Tissue

Yasuhiro MATSUMURA,^{*1} Masami KIMURA,^{*1} Tetsuro YAMAMOTO^{*2} and Hiroshi MAEDA^{*1, *3}

^{*1}Department of Microbiology and ^{*2}Department of Allergy, Institute for Medical Immunology, Kumamoto University Medical School, 2-2-1 Honjo, Kumamoto 860

Enhanced vascular permeability in tumor tissue has profound pathological consequences in tumor biology. However, details of the mechanism involved are not clear. The present work on tumor vascular permeability has led to the following three findings. (i) Ascitic tumor fluid contained kinin (about 1-40 ng/ml), which is known to enhance vascular permeability and induce pain *in vivo*. (ii) Kinin is generated via the kallikrein-dependent cascade in the ascitic tumor fluid. By blocking this kinin-generating cascade with Kunitz-type soybean trypsin inhibitor the formation of ascites was suppressed. (iii) Blocking of kinin-degrading enzymes (kininases I and II) by an appropriate kininase inhibitor (e.g., captopril) may result in increased permeability, leading to accumulation of the ascitic fluid. This phenomenon was verified by an about 1.2-1.5 fold increase in leakage of ⁵¹Cr-labeled bovine serum albumin into the ascites when kininase inhibitors had been administered orally 30 min before intravenous administration of the bovine serum albumin.

Key words: Enhanced extravascular permeability in tumor — Bradykinin in tumor ascites — Kinin-generating cascade — Manipulation of kinin level — Macromolecular drug delivery

At least two pathologically different lesions exhibit common aspects of vascular permeability. One lesion is observed during inflammation (such as that noted with a bacterial infection) and is caused by bacterial proteases, which activate the kinin-generating cascade in normal tissue.¹⁻³⁾ The other is caused by a permeability-enhancing factor secreted by various solid tumors.^{4,5)} The active principle in tumor, reported to have an approximate molecular weight of 40,000, is not related to any known vascular dilators or constrictors. Both lesions, tumorous and inflammatory, however, seem to share a common cascade of kinin generation, as described in this report.

^{*3} To whom correspondence should be sent.

^{*4} Abbreviations used: SBTI, soybean trypsin inhibitor (Kunitz type unless otherwise specified); CPNI, carboxypeptidase N inhibitor (see page 1328); ELISA, enzyme-linked immunosorbent assay; EPR effect, enhanced permeability and retention effect in tumor tissue; DTPA, diethylenetriaminepentaacetic acid; smancs, a generic name of an antitumor agent which is a derivative of neocarzinostatin conjugated with styrene-maleic acid copolymer (refs. 6, 7).

We have also been working on a macromolecular anticancer agent called smancs (Mr = 16,000),^{6,7)} and we found that an experimental solid tumor seems to possess the unique property of accumulating plasma proteins and macromolecular drugs more effectively than normal tissue.^{8,9)} A tumor-to-blood drug concentration ratio of more than 5 is readily accomplished. This enhanced permeability and retention effect (EPR effect)^{*4} in the solid tumor has also been noted with lipids injected into the tumor-feeding artery.¹⁰⁻¹³⁾ In this case the tumor-to-blood ratio exceeded 2,500.¹⁰⁾ We have thus proposed a new concept for macromolecular therapeutics in cancer chemotherapy.⁸⁾

The basic reasons for this unique tumor-tropic accumulation of macromolecules (EPR effect) are related to: (i) the hypervascularity; (ii) enhanced vascular permeability, which depends on some effector substances generated by tumor tissues; and (iii) little recovery of macromolecules and lipid from lymphatics and blood capillaries in the solid tumor.⁸⁻¹³⁾ There seems to be no lymphatic system in solid tumors.⁸⁻¹³⁾ Furthermore, a recent study by Suzuki *et al.* on hepatoma

demonstrated architectural defects of tumor vasculature such as extreme hypoplastic appearance of medial smooth muscle and much thinner arterial wall than the control.¹⁴⁾ This would obviously facilitate the EPR effect.

In the present report we describe the results of our search for the presence of both bradykinin and its generating cascade, in which kallikrein cleaves off bradykinin from high-molecular-weight kininogen. Bradykinin is the most potent permeability factor and at the same time a pain inducer. Kinins may be continuously generated by the cascade but would also be degraded immediately by kininases (see Fig. 1). Therefore, inhibition of kininases should result in a higher kinin concentration in the tissue. We tested inhibitors of the kinin cascade for their effect on kinin generation and degradation: upstream with Kunitz-type soybean trypsin inhibitor (SBTI) which inhibits plasma kallikrein, and downstream with captopril, enalapril and CPNI, known kininase inhibitors (see Fig. 1).¹⁵⁻¹⁷⁾ The model systems employed here were ascitic tumors of rat AH-130 and mouse S-180.

Microvascular permeability was evaluated by determining the amount of leakage of radiolabeled albumin from the circulation and its accumulation in the ascitic fluid. Ascites formation was also quantified in mice with ascitic tumor, in which the effect of SBTI was also tested. The Hageman factor-prekallikrein-kinin system can be activated by experimental artifacts, so that stringent precautions to prevent this were taken in the present experiments. The homogenate of the solid tumor was not used because the homogenization process would activate the Hageman factor and this cascade.

The present results clarified a role of the kinin system in the solid tumor. Namely, mechanisms of microvascular permeability and pain induction in the tumor can now be attributed at least partly to the kinin-generating cascade.

MATERIALS AND METHODS

Reagents *p*-Amidinophenylmethylsulfonyl fluoride (APMSF) was obtained from Wako Pure Chemical Co., Ltd., Osaka. Captopril, D-2-methyl-3-mercaptopropionyl-L-proline (kininase II inhibitor), was a gift from Sankyo Co., Ltd., Tokyo. Enalapril maleate, (–)-{N-(S)-1-ethoxycarbonyl-3-phenyl-

propyl]-L-alanyl}-L-proline maleate (kininase II inhibitor) was a gift from Banyu Co., Ltd., Tokyo. Carboxypeptidase N inhibitor, DL-2-mercapto-methyl-3-guanidino-ethyl-thiopropionic acid (CPNI; kininase I inhibitor), was obtained from Calbiochem-Behring, San Diego, CA. SBTI was a gift from Fuji Seiyu Co., Ltd., Osaka. Polybrene, hexadimethrine bromide, was from Aldrich Chemical Co., Inc., Milwaukee, WI. Diethylenetriamine-pentaacetic acid anhydride (DTPA) was obtained from Dojin Chemical Laboratories, Kumamoto. ⁵¹CrCl₃ was obtained from New England Nuclear, Boston, MA. Bovine serum albumin (BSA) and all other chemicals were obtained from local commercial sources. A bradykinin assay kit, Markit-A, was from Dainippon Pharmaceutical Co., Ltd., Osaka.

Tumor and Ascites Rat ascitic hepatoma AH-130 cells were maintained in ascitic form in Donryu rats. An inoculation of 5×10^6 AH-130 cells was made into the peritoneal cavity of rats 8–10 weeks old. Rats were fed with regular feed *ad libitum* (F-2 Funabashi Farm K.K.). Then, in most instances, after 10–14 days, ascitic fluid was collected for assay of the permeability factor (bradykinin). About $2-8 \times 10^7$ tumor cells/ml were usually found. The mouse S-180 ascitic tumor (about 5×10^6 tumor cells/mouse) was injected intraperitoneally (ip) and was maintained in ddY mice 8–10 weeks old. Human ascitic fluid from patients with gastric, pancreatic, hepatic and ovarian cancers, and pleural effusion from a patient with lung cancer, were obtained from our university hospital.

Quantification of Ascitic Kinin Nine volumes of ascites were obtained by ip puncture at 10 and 14 days for mice and rats, respectively, by using 3- or 10-ml polyethylene syringes with 26 gauge needles containing 1 volume of an inhibitor cocktail of 1 mM APMSF or 0.1 mM SBTI, 500 µg/ml of polybrene, 1.5% EDTA, 1 mM captopril (kininase II inhibitor), and 1 mM CPNI (kininase I inhibitor). The inhibitors in the cocktail were used to prevent proteolytic degradation of a putative permeability factor. Polybrene was used to inhibit the activation of Hageman factor. APMSF or SBTI was used to stop serine-type protease, EDTA to stop metallo-enzyme/peptidase, and kininase I and II inhibitors (enalapril or captopril) to prevent the breakdown of kinin(s). The ascites was then centrifuged at 1600g and 1 ml of supernatant was used for quantification of kinin by enzyme-linked immunosorbent assay (ELISA) (Markit-A) and by bioassay using rat uterus contraction.¹⁸⁾

Effect of Soybean Trypsin Inhibitor on Ascitic Fluid Accumulation The increase in the volume of ascitic fluid was estimated by comparison of the total body weight of mice bearing S-180 tumor and

non-tumor-bearing mice. Increase of body weight exhibited a positive correlation with increase of ascitic fluid within two weeks under the present experimental conditions (data not shown). In an additional experiment, at the indicated time, each mouse was sacrificed and after laparotomy, the ascites was absorbed with cotton pads for collection and the ascitic amount determined by weighing. SBTI was injected at a dose of 3 mg/mouse per day for 10 or 14 days, starting on the day of tumor inoculation (5×10^5 cells).

Effect of Kininase Inhibitor on the Degradation of Bradykinin in Ascitic Fluid Ten μl of the supernatant of mouse S-180 tumor ascites which might contain kininase(s), 10 μl of 1mM synthetic bradykinin solution, and 10 μl of 1mM captopril (or 1mM CPNI) were added to 90 μl of the reaction buffer, 0.02M Tris-HCl buffer in 0.15M NaCl (pH 7.4). For the control run the volume of the reaction buffer was 100 μl and no aliquot of captopril (or CPNI) of 10 μl was added. The mixture was incubated for 1 hr at 37° and 100 μl of incubation mixture was applied to the HPLC system.

High-performance Liquid Chromatography HPLC analyses were performed with a column (4.6 mm $\times$ 25 cm) of ODS-120A from Toyo Soda Manufacturing Co., Ltd., with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid for the analysis of breakdown peptides using a pump (model CCPM) and a UV detector (model UV-8000, Toyo Soda, Tokyo). A flow rate of 1.0 ml/min was maintained at ambient temperature in all systems. The products detected by measuring the absorption at 214 nm were identified by coelution with standard peptides as reported previously.¹⁹ The inhibitory effect of captopril on the degradation of bradykinin was calculated by comparing the integrated peak area with the peak area of a known amount of original bradykinin.

Enhancement of Accumulation of ^{51}Cr -albumin in Ascites by Kininase Inhibitors The accumulation of intravenously (iv) injected radioactive albumin into the ascitic fluid was examined. The radioactive albumin was prepared essentially as described by Hnatowich *et al.*²⁰ and others,^{8, 21, 22} by utilizing the bifunctional chelating agent DTPA. The chelate-tagged protein is chelated with radioactive chromium using $^{51}\text{CrCl}_3$. Approximately one amino group per mol of BSA was labeled with DTPA. It should be noted that when radioiodinated proteins were used in this type of experiment not only did the *in vivo* fate of the modified proteins differ from the actual *in vivo* behavior of native proteins, but also rapid transiodination reaction made the results very difficult to interpret.²¹ On the contrary, use of DTPA and radioactive metal nuclei can make proteins more stable, and is less problematic as com-

pared with iodination.²² Nine days after tumor inoculation (S-180; 5×10^6 cells), enalapril, captopril or CPNI was given orally to each group of mice at various doses; after 30 min, the radio-labeled BSA at 3.1×10^5 cpm/mouse (3.8×10^5 cpm/mg) was injected iv. A control group without kininase inhibitors was also given ^{51}Cr -labeled BSA similarly. Three hours were allowed for accumulation of ^{51}Cr -labeled BSA in the ascites, and then the ascitic fluid and blood were collected. Radioactivity of the ascites and blood was counted by an auto- γ -counter (Packard).

Statistical Analysis Data were compared by using Student's *t* test. Data are reported as the mean $\pm$ SEM ($n=5$).

RESULTS

Kinin Concentration in Ascitic and Pleural Fluid and Effect of Soybean Trypsin Inhibitor

As shown in Table I, considerable amounts of kinin ranging from 0.5 to 20 ng/ml were detected by ELISA. A high kinin content (40 ng/ml, by the bioassay method using rat uterus smooth muscle) was also confirmed in the ascites with peritoneal carcinomatosis of a gastric cancer patient and in the pleural effusion of a lung cancer patient.

To test for the presence of the kinin-generating cascade shown in Fig. 1, SBTI was administered ip and the amount of ascitic fluid accumulated was measured. The results (Fig. 2A) indicate less accumulation of the fluid with SBTI. Also, as shown in Fig. 2B, on day 7 after tumor inoculation, the mean weight of ascitic fluid accumulated was 1.02 g in the SBTI group, while it was 2.95 g without inhibitor ($P < 0.025$). Also, in day 11 after tumor inoculation, the mean weight of ascites was 8.3 g in the SBTI group, but 12.7 g without inhibitor. In terms of survival, mice administered SBTI had a significantly prolonged life span (data not shown). Extensive leakage of plasma proteins into the ascites might have resulted in a feeble state and the early death of tumor-bearing mice.

Inhibition of Bradykinin Degradation by Captopril and Carboxypeptidase N Inhibitor (CPNI) Bradykinin was effectively degraded into smaller peptides (including des Arg⁹-bradykinin) even with 12-fold-diluted tumor ascitic fluid and intact bradykinin disappeared completely within one hour (Fig. 3B vs. Fig. 3A). Addition of captopril (Fig. 3C) and CPNI (Fig. 3D) prevented kinin degra-

Table I. Kinin Content in Various Tumor Effusions

Ascites	Kinin content (ng/ml) ^{a)}	
	Bioassay	Enzyme immunoassay
1. S-180 (mice) ^{b)}	1-4	0.625-2.5
2. AH-130 (rats) ^{b)}	1-8	0.625-2.0
3. Pancreas cancer (human) ^{c)}	1	0.625
4. Stomach cancer (human) ^{c)}	30	8.0
5. Stomach cancer (human) ^{c)}	40	10.0
6. Hepatoma with liver cirrhosis (human) ^{c)}	2.5	— ^{d)}
7. Ovarian cancer (human) ^{c)}	2.5	— ^{d)}
8. Lung cancer (human) ^{c)}	40	20

a) Synthetic bradykinin was used as a standard.

b) Values are from several rodent ascites. Different ascites samples were used for bioassay and for enzyme immunoassay.

c) Individual cases.

d) Not done.

Fig. 1. Cascade of kinin generation and degradation and points of inhibition by various inhibitors. ACEI, angiotensin converting enzyme inhibitor. Captopril and enalapril inhibit kininase II (ACE). CPNI, carboxypeptidase N inhibitor. Dotted lines show possible activation.

dation to a significant extent; remaining kinin amounted to 24% and 19% compared with controls. Namely, 0.08mM inhibitor in the incubation mixture with ascites prevented

kinin degradation 24 and 19% in a 1-hr incubation period (Fig. 3). Therefore, captopril and CPNI are effective inhibitors of kinin degradation, though they are not complete

Fig. 2. Effect of SBTI on ascitic fluid formation. (A) SBTI was administered ip for 14 days beginning from the day of tumor inoculation. Each group consisted of seven mice. Control mice (●) weighed about 34 g on day 16. Ascitic volume can be estimated from the difference between the weight of controls with and without tumor. Tumor-bearing and nontumor-bearing mice (controls) are shown by ○ and ●, respectively. SBTI (□) was given at 3 mg/mouse per day. (B) SBTI was administered ip for 10 days beginning from the day of tumor inoculation. Each group consisted of seven mice. Each mouse was sacrificed at day 7 or day 14 and each ascites was weighed directly (see "Materials and Methods"). White and dark columns show the control group and SBTI-treated group, respectively. A and B were separate experimental groups.

inhibitors. It was found that 3mM EDTA inhibited the degradation completely (data not shown). Thus, to enhance the vascular permeability by elevating bradykinin level for

Fig. 3. Elucidation by HPLC of kinin degradation by mouse S-180 tumor ascites and inhibition by kininase inhibitors. A. Synthetic bradykinin on HPLC. B. Bradykinin incubated in tumor ascites for 1 hr at 37°. BK had completely disappeared. C. Similar to B with 0.08mM captopril. D. Similar to B with 0.08mM CPNI. The dark peak indicates the peak of bradykinin. The retention times, in minutes, are shown in parenthesis. Peaks at 29.9 min and 39.4 min are due to des-Arg⁹-bradykinin and des-Phe⁸-Arg⁹-bradykinin, respectively. The small peak marked with a star is due to an unidentified component. See the text for details.

macromolecular drug delivery, it became apparent that a kininase inhibitor would be useful, and the following experiment was designed.

Intraascitic Accumulation of Radiolabeled BSA: a Prototype Macromolecular Drug Per-

Table II. Extravascular Leakage into and Accumulation of ^{51}Cr -labeled BSA in the Peritoneal Cavity of Mice with Ascitic S-180 Tumor

Inhibitor used		Radioactivity of ^{51}Cr -labeled BSA in ascites (cpm/ml)	P-value versus control
Control (saline) ^{a)}		7809 $\pm$ 428	—
Captopril (mg/kg, po)	0.1	8211 $\pm$ 541	0.1 < P < 0.375
	1	9340 $\pm$ 359	0.01 < P < 0.025
	10	9496 $\pm$ 643	0.025 < P < 0.05
Enalapril (mg/kg, po)	0.1	8347 $\pm$ 537	0.1 < P < 0.375
	1	10749 $\pm$ 1489	0.025 < P < 0.05
	10	12239 $\pm$ 666	0.0005 < P < 0.005
CPNI (mg/kg, po)	1	9501 $\pm$ 441	0.01 < P < 0.025

a) Each group consisted of 5 mice; data are expressed as mean $\pm$ SEM. See the text for details.

itoneal vascular permeability was quantified by determining the accumulation of ^{51}Cr -labeled BSA in the ascites. Accumulation was enhanced after oral administration of enalapril and other kininase inhibitors. The effect was most prominent with enalapril at 10 mg/kg. Radioactivity in the ascites in this group increased 1.5 fold more than that of the control group (Table II). The administrations of 1 and 10 mg/kg of captopril, 1 and 10 mg/kg of enalapril, and 1 mg/kg of CPNI resulted in significantly increased vascular leakage compared with the control (Table II).

DISCUSSION

As shown in Table I, all tumorous exudates exhibited relatively high kinin content. It is well known that bradykinin is produced from high-molecular-weight kininogen via limited proteolysis by plasma kallikrein (Fig. 1). Thus, we suspected that this kinin-producing cascade would exist in tumor ascites. Because there are numerous proteolytic enzymes, including many serine types and other peptidases, the kinins present in the ascites must be constantly produced and degraded. Thus, we employed inhibitors to prevent kinin generation and breakdown during harvesting of ascites, purification, and assay. The ascites formation was suppressed in the presence of SBTI (Fig. 2). In preliminary experiments, when HeLa cells were exposed to SBTI at 1.0 mg/ml, no cytotoxicity was observed. Fur-

ther, in *in vivo* studies in mice, there was no lethality at 30 mg/mouse ($n=5$). These facts show that SBTI is neither toxic nor has anti-tumor activity. Thus, the present findings indicate that the kinin-generating cascade was blocked upstream, namely, at kallikrein, and thus bradykinin formation decreased (Fig. 1). It is, however, also possible that plasmin, which is known to be inhibited by SBTI, might play some role by activating Hageman factor and/or prekallikrein, so that inhibition of plasmin might also result in suppressed ascites formation (Fig. 2). Under these circumstances, the result in Fig. 2 is concordant with the known fact that kinin is a potent permeability-enhancing factor *in vivo*.

By suppressing the degradation of bradykinin by using kininase I or kininase II inhibitor (see Fig. 1), a higher kinin level had been expected to result in the tumorous compartment. Indeed, the experiment on the vascular permeability showed it was enhanced (Table II). The dose range of kininase II inhibitors used in the present experiment, which is known to be effective in hypertensive patients based on the inhibition of angiotensin converting enzyme, did not induce hypotension in normotensive subjects according to the manufacturer's information. In the case of inflammatory tissue a dose of 10 mg/kg ip resulted in enhanced permeability.²³⁾ At a much higher dose range, namely 50–100 mg/kg of captopril, it was reported that the drug did

suppress vascular permeability.²⁴⁾ Furthermore, we speculate that this effect was brought about by decreased blood pressure.

The above results demonstrate that kinin is produced in the tumor tissue via the kallikrein-dependent pathway (Fig. 1), and that one can manipulate the vascular permeability with various inhibitors. We enhanced the EPR effect particularly with 10 mg/kg of enalapril in mice (Table II). This suggests that we can direct the delivery of macromolecular anticancer agents more in tumor tissues than in their normal counterparts. SBTI or other appropriate plasma kallikrein inhibitors would be beneficial for the control of ascites, as shown in Fig. 2.

The principle of the EPR effect may thus be applied to radioemitting macromolecules for radiosciintigraphic purposes, or even to monoclonal antibody-toxin conjugates for diagnosis or treatment. Treatment experiments using the macromolecular anticancer agent smancs in combination with enalapril are under way, with the aim of enhancing drug delivery and improving the therapeutic outcome.

It is unclear at this stage whether the permeability-enhancing factor produced by various tumor cells reported by Dvorak *et al.*^{4,5)} may act at the same point in the cascade described here, or whether it manipulates a completely different cascade reaction involving kinin or other molecules such as leukotrienes or prostaglandins. These questions need to be clarified.

Further characterization of the kinins described here is now being performed in our laboratory. We have recently found that there are two types of kinins in the tumor ascites from human gastric cancer; one is bradykinin and the other is a novel type [hydroxyprolyl³-bradykinin] (hydroxyproline in place of proline³).¹⁹⁾ However, the possibility of a pathway independent of kallikrein/high-molecular-weight kininogen (Fig. 1) for the generation of this new kinin needs to be explored.

In earlier studies Greenbaum *et al.* examined ascites formation and the inhibitory effect of the protease inhibitor, pepstatin.^{18,25)} They have proposed that a product of acid protease (cathepsin D) is responsible for the enhanced vascular permeability and designated it as leukokinin; this was, however,

generated *in vitro* by the treatment of ascites at acidic pH. Therefore, the chemical entity and biochemical mechanism of its formation appear to be different from those of the present kinins. It is possible that both bradykinin and leukokinin are operating additively in the tumor tissue.

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