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Kinin-generating Cascade in Advanced Cancer Patients and *in vitro* Study

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The role of the bradykinin-generating system in the pathogenesis of cancer was explored by simultaneously measuring plasma prekallikrein (PK), the precursor of kallikrein, which is the major enzyme responsible for kinin generation, and plasma kininogens (KNG), which are precursors of kinin, in patients with various cancers. The mean value of plasma PK in healthy volunteers was 2.5 ± 0.5 (mean ± SD) units/mg plasma protein and that in cancer patients (all stage IV) was 1.7 ± 0.7 units/mg plasma protein. The mean value of plasma KNG in healthy volunteers was 12.5 ± 2.0 ng kinin equivalents/mg plasma protein and that in cancer patients was 10.9 ± 2.8 ng. These data showed that plasma PK and plasma KNG values were significantly lower in cancer patients compared with healthy volunteers ($P \leqq 0.0005$ for PK; $0.0005 < P \leqq 0.005$ for KNG; n = 28 for healthy subjects; n = 29 for cancer patients). These data appear to indicate that conversion of PK to kallikrein would probably occur with concomitant consumption of KNG by newly generated kallikrein for kinin generation in cancer patients. Early stage cancer patients showed little difference from healthy volunteers. For the *in vitro* study, activation of purified Hageman factor (HF) and PK was examined by using cancer cell lines and virus-transformed cells that produced plasminogen activator (PA) at a high rate. Both HF and PK were activated in the presence of plasminogen. Diploid cell lines and primary fibroblasts, which did not produce PA, activated neither HF nor PK. Taking all these data together, we conclude that kinin generation does occur in the plasma of patients with advanced cancer, and that one of the initiation mechanisms of the kinin-generating cascade appears to be mediated by plasmin and to depend on cancer cell-derived PA activity.

Key words: Bradykinin — Cancer — Vascular permeability — Kallikrein — Plasminogen activater

It is important to understand the microvascular structure and physiology of tumor tissue for cancer chemotherapy. We have advocated principles and methodology for targeting chemotherapy with macromolecular anticancer agents that are based on the vascular function of tumors, particularly tumor vascular permeability.[1–4] The tumoritropic accumulation of macromolecules and lipids is related to the following characters; (i) the hypervasculature; (ii) enhanced vascular permeability, which depends on some effector substances that are generated by tumor tissues; (iii) incomplete vascular architecture; and (iv) little drainage of macromolecules and lipids because of a poor lymphatic system in solid tumor tissue.[3–8]

During our study of tumor vascular permeability, we found that bradykinin (henceforth referred to as kinin) and the kinin-generating cascade were present in the tumor compartment.[9,10] Kinin is generated from high-molecular-weight kininogen (KNG)[6] by limited proteolysis with a serine protease (*kallikrein*), which is present in plasma as a precursor, *prekallikrein* (PK)[11,12] (see Fig. 1). Kallikrein is most effectively inhibited by Kunitz-type soybean trypsin inhibitor (SBTI).[13] It is also well known that kinin is degraded rapidly in plasma or tumor effusion by kininases such as angiotensin-converting enzyme (kininase II) and carboxypeptidase N (kininase I).[14–16]

Using a mouse ascitic tumor model, we tested the effect of SBTI or kininase inhibitors on the accumulation of tumor ascitic fluid.[9] SBTI effectively inhibited the accumulation of tumor ascites due to suppressed vascular permeability (Fig. 1). On the other hand, kininase inhibitors enhanced the vascular permeability to radiolabeled albumin which was injected intravenously (iv) and thereby the accumulation of this protein into tumor ascites was facilitated. In this situation, the breakdown of kinin was assumed to be prevented by the kininase inhibitors, and thus the enhanced vascular permeability facilitated fluid accumulation in the ascitic compartment. We also found that human ascitic tumor fluid contained

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[6] Abbreviations: KNG, kininogen; HF, Hageman factor; PK, prekallikrein; PA, plasminogen activator; LBTI, lima bean trypsin inhibitor; SBTI, soybean trypsin inhibitor (Kunitz type); PBS, 0.01 *M* phosphate-buffered 0.15 *M* saline (pH 7.0); E-MEM, Eagle's minimum essential medium; D-MEM, Dulbecco's minimum essential medium; NBCS, newborn calf serum; FITC, fluorescein isothiocyanate; ZFR-MCA, carbobenzoxyphenylalanylarginine-4-methylcoumaryl-7-amide; PFR-MCA, prolylphenylalanylarginine-4-methylcoumaryl-7-amide; AMC, 7-amino-4-methylcoumarin.

a high level of kinin (about 1–40 ng/ml ascitic fluid).[9] In addition we found there were two types of kinins in the tumor ascites from human gastric and other cancers, one being bradykinin and the other being a novel one, hydroxyprolyl^3-bradykinin.[9, 10, 16]

In the present report, we describe the kinin-generating system in the tumor by using both clinical materials and *in vitro* study. The mechanism of initiation of kinin generation in tumor tissue was studied in an *in vitro* system. The objective of the present study was to prove enhanced kinin generation in human cancer patients, as observed in the mouse model. The results should improve our understanding of the pathophysiological role of kinins in cancer.

MATERIALS AND METHODS

Reagents Guinea pig Hageman factor (HF) was prepared according to the method previously described by Fujikawa and Davie.[17] Guinea pig prekallikrein was purified by the method of Yamamoto *et al.* [18] Guinea pig plasminogen was prepared by using affinity chromatography with Lysine-Sepharose obtained from Pharmacia A.B., Uppsala, Sweden.[19] Fibrinogen was purchased from Green Cross Co., Ltd., Osaka. Fluorescein isothiocyanate (FITC) was obtained from Dojin Chemical Laboratories, Kumamoto. Bovine urokinase, bovine thrombin, bovine trypsin, and lima bean trypsin inhibitor (LBTI) were obtained from Sigma Chemical Co., St. Louis, Mo., USA. Kunitz-type SBTI was a gift of Fuji Seiyu Co., Ltd., Osaka. Acid-treated kaolin powder was obtained from Wako Pure Chemical Co., Ltd., Osaka. Carbobenzoxyphenylalanylarginine-4-methylcoumaryl-7-amide (ZFR-MCA) and L-prolylphenylalanylarginine-4-methylcoumaryl-7-amide (PFR-MCA) were purchased from Peptide Institute, Inc., Minoh, Osaka. Markit A, a bradykinin assay kit, was from Dainippon Pharmaceutical Co., Ltd., Osaka. All other chemicals were purchased from local commercial sources.

Collection of plasma Blood samples were collected from 28 healthy volunteers (40–60 years old) and 29 stage IV cancer patients with various cancers (see Table I) by using 21-gauge needles. Each siliconized glass tube contained a blood sample plus 3.8% sodium citrate solution at 0.1 volume with respect to the blood sample. Tubes were centrifuged at 1000*g* for 5 min. These samples were stored at -70°C until use.

Cells used The cells used were Balb/c mouse embryonic fibroblasts, Balb/3T3; SV40 virus-transformed Balb/3T3, SV-T2; primary cultured mouse embryonic fibroblasts; methylcholanthrene A-induced mouse fibrosarcoma, Meth-A; human embryonic lung fibroblasts, HEL; normal peripheral lymphocytes; human cervical cancer cell line, HeLa; human hepatoma, Hep G2; human epidermoid carcinoma cell line, KB; a human neuroblastoma cell line, BE; and Epstein-Barr virus-transformed B cell line, EB-B. Cells were maintained in *in vitro* culture in either Eagle's minimum essential medium (E-MEM) or Dulbecco's MEM (D-MEM), containing 10% newborn calf serum (NBCS).

Quantification of plasma PK The concentration of plasma PK was measured after converting PK to kallikrein by means of the potential amidolytic assay as described previously.[20] Briefly, citrated plasma (50 μl) was mixed with 850 μl of acetone-containing buffer (700 μl of 0.02 *M* Tris-HCl/0.15 *M* NaCl, pH 8.0, and 150 μl of acetone) and was allowed to stand for 10 min at room temperature for inactivation of endogenous kallikrein inhibitor. Then 100 μl of kaolin suspension (at 10 mg/ml in the same acetone-containing buffer) was added to activate latent forms of HF and PK completely by mixing vigorously with a magnetic stirrer for 15 s (tube A). The amount of kaolin was more than a saturating quantity as described previously.[21] At timed intervals after the addition of kaolin, 20 μl of this mixture was removed from tube A and incubated for 10 min at 37°C with 960 μl of assay buffer (0.05 *M* tris-HCl, 0.1 *M* NaCl, 0.01 *M* $CaCl_2$, pH 8.0), 10 μl of 1 m*M* ZFR-MCA, and 10 μl of 4 mg/ml LBTI, which inhibits plasmin. This makes up tube B. Further, a third tube (C) was prepared with the same mixture as in tube A plus 10 μl of 4 mg/ml SBTI to inhibit kallikrein as well as plasmin. Tube C served as a background control. Plasmin needs to be inhibited because it could hydrolyze ZFR-MCA (a substrate for kallikrein), although slowly. The reaction was terminated by the addition of 1 ml of 17% acetic acid, and kallikrein activity was measured by using a fluorescence spectrophotometer with excitation at 380 nm and emission at 411 nm. The difference in the values between tube B and tube C (value B–C) was considered to be kallikrein activity, thus giving the amount of PK indirectly. The results are given in arbitrary units, with one unit being defined as the amount of enzyme that releases 1×10^{-7} *M* aminomethylcoumarin (AMC)/10 min as described. As shown in Fig. 2, the time course of kallikrein generation was initially examined in 5 of 28 healthy volunteers and in four cancer patients, one patient each with esophageal, stomach and pancreatic cancer, and hepatoma. Because the maximal activation of PK was obtained at 30 min after the start of incubation with kaolin and remained unchanged for 1 h, a 1 h activation period with kaolin was used in the following studies.

Quantification of plasma KNG Quantification of KNG was carried out by a standard method which was reported previously.[20, 22] Briefly, 0.03 *M* HCl (1.65 ml) and citrated plasma (200 μl) were mixed in plastic tubes,

and the mixture was incubated at 37°C for 15 min to inactivate all plasma protease inhibitors. To this solution were added 50 μl of 1.0 *M* NaOH, 500 μl of 0.2 *M* Tris-HCl, pH 7.8, and 100 μl of 0.2% bovine trypsin (130 U/ml, Type II). This mixture was incubated for 30 min at 37°C to release kinin from KNG. Then, 20% trichloroacetic acid (500 μl) was added for deproteinization, and the supernatant containing kinin was obtained by centrifugation (1000*g*, 10 min). The supernatant (500 μl) and 500 μl of 0.5 *M* Tris-HCl, pH 7.0, containing 0.2% gelatin and 0.9% NaCl, were mixed, and kinin was quantified by using an enzyme immunoassay, Markit A. In this assay the antibody is directed at the COOH-terminal sequence of kinin, and the value was assumed to represent native kinin.[22, 23]

Preparation of culture supernatants for experiments Adherent-type cells, including 1×10^5 cells of BE, Balb/3T3, HEL, SV-T2, HeLa, Hep G2, KB, and mouse primary fibroblasts, were seeded on a 16-mm × 24-well multiplate (Falcon 3047) and grown to the confluent stage in their respective growth media. Confluent cells were washed three times with PBS (0.01 *M* phosphate-buffered 0.15 *M* saline, pH 7.0) and were incubated for 48 h in serum-free E-MEM without phenol red. The conditioned media thus obtained were stored at −20°C until use.

Floating-type cells, EB-B, normal peripheral lymphocytes, and Meth-A were maintained in D-MEM with 10% NBCS and were washed 3 times with PBS and resuspended at a density of 3×10^5 cells/ml in serum-free D-MEM without phenol red for 48 h. The cultured media were collected, centrifuged at 1000*g* for 5 min to remove cells, and stored at −20°C until use.

PA assay. (1) Labeling of fibrinogen with FITC The labeling with FITC was conducted at room temperature with a 10-fold molar excess of FITC over fibrinogen in 0.5 *M* $NaHCO_3/Na_2CO_3$, pH 9.8, for 3 h as described previously.[24] The FITC-labeled fibrinogen contained an average of 0.9 mol of fluorescein per mole of protein, as judged by fluorospectroscopy using free fluorescein as a standard.

(2) FITC-labeled fibrin coating of wells FITC-labeled fibrinogen (100 μl) at 0.5 mg/ml in 0.1 *M* phosphate buffer, pH 7.4, containing 20 m*M* Ca^{2+}, was poured into the 24-well multiplate (Falcon 3047) and the fibrinogen was transformed into FITC-labeled fibrin by addition of bovine thrombin (1 NIH unit/ml) as reported previously.[25]

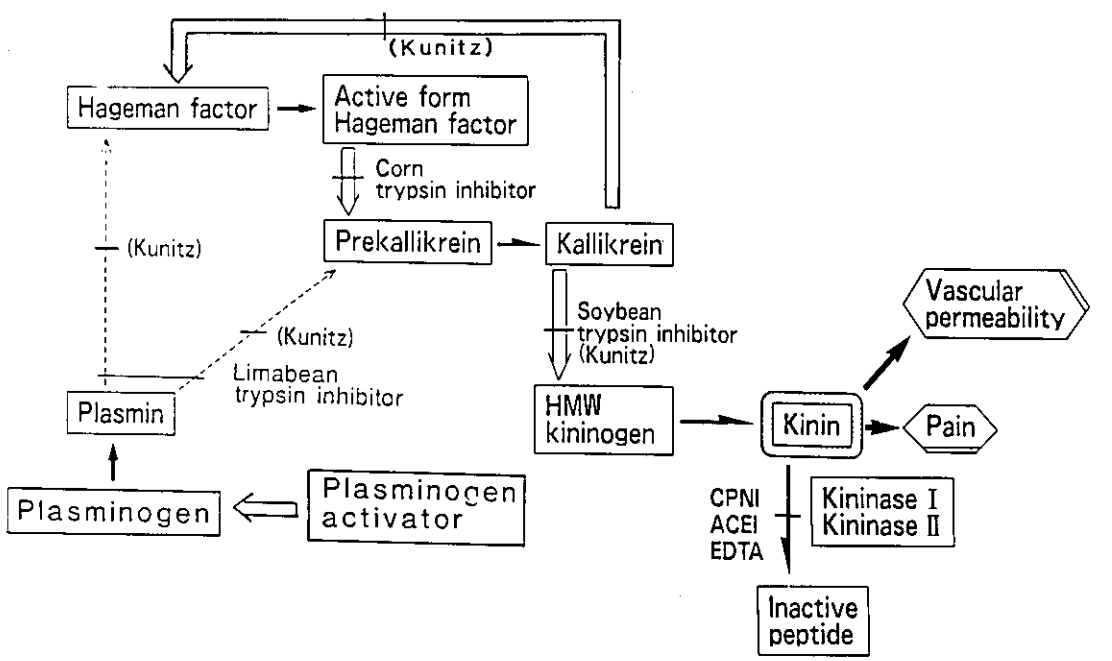

Fig. 1. Kinin generation and degradation cascade and points of inhibition by various inhibitors. →, Limited proteolysis resulting in activation or inactivation; ⇒, activation of precursor zymogen; ---→, proposed activation. Cross bars on the arrows show inhibition. CPNI, carboxypeptidase N inhibitor; ACEI, angiotensin-converting enzyme inhibitor; HMW, high molecular weight.

(3) Assay of plasminogen activator (PA) activity PA assay was conducted by using the solid-phase fibrin plate method using the FITC-labeled fibrin-coated well described above, with one plate for each culture supernatant (1.0 ml).[26] Then 10 μl of guinea pig plasminogen (purified by using a Lysine-Sepharose column) with A_{280} nm 0.90 was added to the FITC-labeled fibrin-coated well and incubated at 37°C for 1 h. Aliquots (500 μl) were removed and the fluorescence intensity was read by using a fluorescence spectrophotometer with excitation at 490 nm and emission at 520 nm. The fluorescence intensity increased in the presence of PA activity derived from the cells which was correlated with that of the human urokinase standard. The increase in fluorescence intensity exhibited a linear correlation with the increase in the urokinase concentration (or PA activity), at least from 0.03 to 3 mU/ml.

To standardize the amount of cells, they were lysed in 1 ml of 20 m*M* Tris-HCl buffer, pH 8.5, containing 0.2% sodium dodecyl sulfate. Aliquots of the lysed cell solution were used for quantification of the protein content by the method of Lowry *et al.*,[27] using bovine serum albumin as the standard.

Activation of HF and PK Fibrin-coated wells were prepared as described above except that fibrinogen was used instead of FITC-labeled fibrinogen. To 500 μl of culture medium in the fibrin-coated wells were added 10 μl of plasminogen (A_{280} nm, 0.90) and 10 μl of purified HF (A_{280} nm, 0.23) or 10 μl of purified PK (A_{280} nm, 0.12); the mixture was incubated at 37°C for 1 h. The presence of fibrin augmented the activity of PA. Then, 10 μl of LBTI (4 mg/ml), a potent inhibitor of plasmin, and 10 μl of HF substrate, PFR-MCA (5 m*M*), or PK substrate, ZFR-MCA (5 m*M*), were added, the mixture was incubated at 37°C for 10 min, and the fluorescence intensity was read with excitation at 380 nm and emission at 441 nm. LBTI did not inhibit the activated HF or kallikrein activity at this concentration.

Statistical evaluations Student's *t* test was used for this purpose.

RESULTS

KNG contents As shown in Tables I and II, plasma KNG contents from 28 healthy volunteers ranged from 9.3 to 17.7 ng kinin equivalents/mg plasma protein (mean$\pm$SD, 12.5$\pm$2.0 ng), whereas that in 29 cancer patients was lower, with the range from 5.9 to 15.7 ng kinin equivalents/mg plasma protein (mean$\pm$SD, 10.9$\pm$2.8). The difference between these values was statistically significant ($0.0005 < P \leq 0.005$). Hepatoma patients (n=9) showed a tendency to have slightly lower KNG contents (9.6$\pm$2.8 ng) than the other cancer patients (10.9$\pm$2.4 ng), but there was no statistically significant difference between different types of cancer ($0.05 < P \leq 0.1$).

Time course of kallikrein generation from plasma of cancer patients and healthy volunteers The enzyme involved in direct generation of kinin from KNG is kallikrein. By activating HF, PK can be converted to kallikrein (Fig. 1). The time course of this process measured at 10, 30 and 60 min is shown in Fig. 2. The results showed that plasma of cancer patients exhibited a significantly lower rate of conversion of PK to kallikrein than that of healthy subjects. The kallikrein-convertible PK content was thus lower in cancer patients than healthy subjects whose PK remained unconverted due to inactive PA (Fig. 1).

PK contents As shown in Tables I and II, plasma PK contents from 28 healthy volunteers ranged from 1.8 to 4.2 U/mg plasma protein (mean$\pm$SD, 2.5$\pm$0.5), whereas the plasma PK contents from 29 cancer patients ranged from 0.5 to 2.8 U/mg plasma protein (mean$\pm$SD, 1.7$\pm$0.7). Plasma PK of cancer patients was significantly lower than that of healthy volunteers ($P < 0.0005$). The PK contents measured were 1.2$\pm$0.3 U/mg in hepatoma patients and 2.0$\pm$0.7 U/mg in other cancer patients, and both were significantly lower than those of healthy volunteers (2.5$\pm$0.5 U/mg) ($P < 0.0005$ for both). In contrast to the case with KNG, there was a statistically significant difference between PK values from hepatoma patients and those of the other cancer patients ($0.0005 < P \leq 0.005$).

Involvement of PA in activation of HF and PK in various cells in culture As shown in Table III, high PA production was found in the culture media of cancer cell lines such as BE (1.7 mU/mg cell protein), KB (0.7 mU/mg cell protein), and Meth-A (4.6 mU/mg cell protein), and the virus-transformed cell lines SV-T2 (2.0 mU/mg cell protein) and EB-B (1.2 mU/mg cell protein). On the other hand, PA production was not found in the media of the diploid cells Balb/3T3, mouse primary fibroblasts, etc. PA production in HeLa and Hep G2 cells was also low and seemed to be balanced by production of a significant amount of PA inhibitor (see Table III, legend).

An increasing production of PA was correlated positively with the increasing activation of HF or PK in these tumors or tumorigenic cells *in vitro* (Table III).

DISCUSSION

It is well known that enhanced vascular permeability occurs in the tumor compartment.[3-6, 28-33] We showed that severe vascular permeability (caused by generated kinin) occurs at sites of bacterial infection.[21, 34, 35] Based on these studies, we recently found that a similar kinin-generating system occurs in tumor tissue, although it is

Table I. Contents of Total Kininogen and Prekallikrein in Plasma from Patients with Cancer

Patient No.	Age	Sex	Cancer[a]	Total kininogen[b]	Prekallikrein[c]	Plasma albumin[d]	Liver cirrhosis
1	62	M	Esophagus	14.8	2.8	—	−
2	80	F	〃	13.4	2.7	4.1	−
3	66	M	Stomach	14.1	2.8	—	−
4	56	M	〃	11.3	1.1	3.5	−
5	66	M	〃	11.6	2.2	4.0	−
6	36	M	〃	9.3	2.4	4.2	−
7	54	M	〃	8.1	1.3	3.4	−
8	45	M	〃	11.0	2.0	3.9	−
9	41	M	Colon	13.6	2.6	4.0	−
10	48	M	Rectum	10.8	2.7	4.2	−
11	59	M	Gall bladder	9.2	2.4	—	−
12	42	F	Choledochus	15.2	2.8	4.1	−
13	75	F	〃	12.0	1.2	—	−
14	55	F	〃	8.3	0.8	—	−
15	55	M	〃	7.6	1.4	2.7	−
16	69	M	〃	10.3	2.3	3.1	+
17	70	M	Hepatoma	8.4	1.4	2.7	+
18	48	M	〃	10.3	1.0	—	−
19	68	M	〃	9.4	1.4	3.8	−
20	53	M	〃	14.0	1.4	3.3	+
21	68	M	〃	7.3	1.1	3.7	−
22	55	F	〃	5.9	1.5	2.7	+
23	55	M	〃	12.6	1.4	3.6	+
24	60	M	〃	6.9	0.8	2.2	+
25	42	M	〃	11.6	0.5	4.0	+
26	56	M	Cholangiocarcinoma	15.7	1.1	—	−
27	76	F	Pancreas	10.0	1.2	2.7	−
28	63	M	〃	15.2	2.7	2.2	−
29	64	M	Lung	8.0	1.8	3.7	−
Mean±SD				10.9±2.8[e]	1.7±0.7[e]	3.4±0.6	

a) All patients were stage IV, and blood samples were obtained prior to or after 2 weeks of chemotherapy.
b) ng kinin equivalent/mg plasma protein.
c) Unit/mg plasma protein. One unit is defined as that amount of enzyme which releases 1×10^{-7} *M* AMC/10 min/mg plasma protein at 37°C.
d) g/dl.
e) Age-adjusted kininogen and prekallikrein values (to the control value in Table II) for patients in their 40s and 50s were 11.0±2.8 and 1.6±0.7 (n=15).

weaker than that in the case of bacterial infection,[9,10] in which we showed that by manipulating the kinin level, either lowering it with SBTI, an inhibitor upstream, or elevating it with kininase inhibitors downstream (see Fig. 1), we could regulate putative kinin concentration in the tumor compartment and thus, the vascular permeability, as discussed above.[9]

Dvorak *et al.* found a protein-type, tumor-specific, permeability-enhancing factor, with an approximate molecular weight of 40,000.[29,30,32] In earlier studies, Greenbaum *et al.* had proposed that a product of an acid protease (such as cathepsin D) was responsible for the enhanced vascular permeability; this product was designated leukokinin.[33] Both of these substances appear to be different from bradykinins in chemical structure and biochemical mechanism of formation.

In this study, we found that the contents of both KNG and PK in plasma from various cancer patients were lower than those of healthy volunteers (Tables I and II). These plasma levels were measured as relative values to the total plasma protein concentration. The plasma levels of albumin in cancer patients taken into the present study were within the normal range (3.4 g/dl), although those of hepatoma patients were slightly lower (3.1 g/dl).

Table II. Contents of Total Kininogen and Prekallikrein in Plasma from Healthy Volunteers

Healthy volunteer No.[a]	Sex	Total kininogen[b]	Prekallikrein[c]
1	M	17.7	2.8
2	M	11.0	2.1
3	M	13.1	2.5
4	M	12.6	2.3
5	M	13.5	2.7
6	M	14.7	2.7
7	M	15.2	3.1
8	M	11.9	1.9
9	M	12.2	2.1
10	M	12.2	2.1
11	M	13.2	3.0
12	M	12.5	2.9
13	M	14.7	4.2
14	M	11.6	2.1
15	M	12.3	2.7
16	F	16.5	2.6
17	F	12.1	3.0
18	F	8.1	1.8
19	F	9.8	1.9
20	F	13.3	2.4
21	F	11.7	2.7
22	F	11.2	2.8
23	F	11.2	2.5
24	F	11.7	2.7
25	F	10.8	2.8
26	F	9.3	2.1
27	F	13.3	1.8
28	F	11.4	2.7
Mean±SD		12.5±2.0	2.5±0.5

a) Volunteers were aged 50±9 years old.
b) ng kinin equivalent/mg plasma protein.
c) Unit/mg plasma protein. One unit is defined as that amount of enzyme which releases 1×10^{-7} *M* AMC/10 min/mg plasma protein at 37°C.

Sample No. 18, No. 19 and No. 21 (Table I) were from hepatoma patients without liver cirrhosis. The average values of plasma KNG and plasma PK were 9.0 ng kinin equivalent/mg plasma protein and 1.2 U/mg plasma protein, respectively for these three patients. The seven other hepatoma patients were positive for liver cirrhosis. The average values of KNG and PK were 10.0 ng kinin equivalent/mg plasma protein and 1.2 U/mg plasma protein, respectively. Thus the PK value in hepatoma patients with or without cirrhosis appeared to be only slightly lower than in other cancer patients. This may partially be explained by a decreased synthesis of PK in the damaged liver. However, the KNG and PK values of

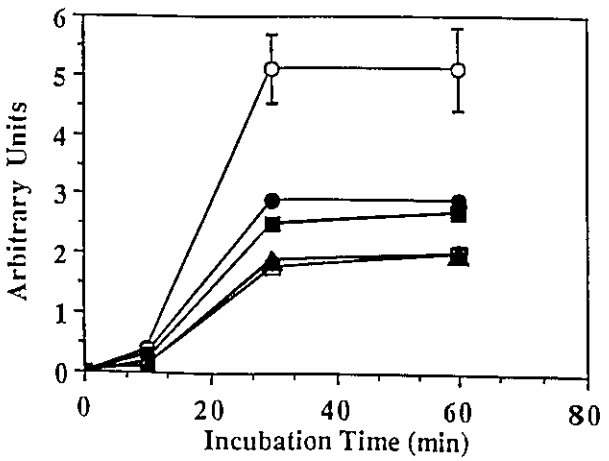

Fig. 2. Amount of activatable PK in plasma from healthy volunteers and cancer patients. ○, Healthy volunteers (n=5); ●, esophageal cancer (Patient No. 1); □, stomach cancer (Patient No. 4); ■, hepatoma (Patient No. 20); ▲, pancreatic cancer (Patient No. 27). The arbitrary unit is fluorescence intensity of the amidolytic product, 7-amino-4-methylcoumarin (AMC), based on fluorescence at 441 nm under excitation at 380 nm (see the text for details). Patients No. is that in Table I. See text for details.

the other cancer patients (without hepatoma) were lower than those of healthy volunteers. This implies that the decrease in KNG and PK values was not caused by impairment of synthesis in the damaged liver. This assumption is also supported by the similar values of albumin level. Namely, hepatoma patients showed 3.1 g/dl while all others showed 3.4 g/dl. Therefore, the lower concentrations of KNG and PK in all the cancer patients can be attributed to catabolism. These values in patients in their 40s and 50s (n=15) were compared with those of age-matched healthy volunteers in this range, and they were clearly lower (Table I) than in the healthy group (see footnote of Table I). Thus, the HF-kallikrein-kinin cascade (Fig. 1) ought to be activated in the tumor tissue, which would then facilitate the processing of both PK and KNG to kallikrein and kinin, respectively, in the plasma of cancer patients. These notions are consistent with the low values observed in the present experiments (see Fig. 2, Tables I and II). When plasma KNG and PK from patients with early stage cancer were compared with those from healthy volunteers, no difference was found. At this stage, the tumor burden is too small to affect their systemic level. Therefore, even if the kinin-generating cascade is occurring in localized tumor tissue, the associated reduction of KNG and PK values in plasma may not be detectable.

Table III. Generation of Hageman Factor Activity or Kallikrein Activity in a Mixture of Hageman Factor or Prekallikrein and the Culture Supernatants from Various Cell Lines

Cell	PA activity[a] (mU/mg cell prot)	HF activation[b] (U/mg cell prot)	PK activation[b] (U/mg cell prot)
Normal origin			
HEL	0	0	0
BALB/3T3	0.4	0	0
Mouse fibroblasts	0.2	0	0
Normal lymphocytes	0	0	0
Tumorous origin			
SV-T2	2.0	2.7	4.3
BE	1.7	1.4	3.8
HeLa[c]	0[c]	0[c]	0
Hep G2[d]	0[d]	0[d]	0
KB	0.7	0.5	1.5
EB-B	1.2	0.9	2.4
Meth-A	4.6	3.1	4.6

See text for details of cell lines.
a) Their activities were expressed in Sigma urokinase equivalent units.
b) 10^{-10} *M* AMC release/10 min = 1 unit.
c) In this culture supernatant, the inhibitory activities against tissue-type PA and urokinase-type PA were found to be 0.41 U and 0.17 U equivalent, respectively.
d) Similar to *c*. The inhibitory activities against tissue-type PA and urokinase-type PA were 0.35 U and 0.21 U, respectively.

In the case of disseminated intravascular coagulation,[36, 37] the levels of plasma HF and PK are known to decrease similarly because of conversion by the activation of the HF-dependent kinin-generating cascade, which is similar to the present observations. If this is the case, what triggers the kinin-generating cascade to result in higher kinin contents in the tumor compartment?

There have been many reports showing that several different tumor cells produce a urokinase-type PA. Normal cells upon transformation also produce PA (for instance, refs. 38–42). PA is a serine protease, which catalyzes the cleavage of plasminogen to generate the active protease, plasmin (see Fig. 1). In addition to the involvement of the PA/plasmin system in fibrinolysis, proteases have been implicated in several aspects of expression of the malignant phenotype, including tumorigenicity,[41, 43] angiogenesis,[43, 44] and increased invasive and metastatic potential.[45, 46] In the present study, involvement of PA in activation of HF and hence PK, resulting in facilitation of the kinin-generating cascade, became apparent using the *in vitro* model.

HF is a glycoprotein circulating in plasma in zymogen form and it participates in the early or contact phase of blood coagulation, fibrinolysis, and kinin formation when these reactions are initiated by surface activation.[47–49] A latent form of HF is known to be converted to the active form HF (HFa) by plasmin, although this activation is weaker than that by plasma kallikrein.[50] As shown in Table III, the high PA activity of cancer cells and virus-transformed cell lines with the exception of HeLa and Hep G2 cells correlates well with their high capability to activate HF or PK. The possibility that PA inhibitor is produced by HeLa and Hep G2 cells was verified in the present study. We have not determined which type of PA, either urokinase or tissue type, was produced in each cell line. A recent report indicated that urokinase-type PA was also elevated in bladder cancer tissue.[51] In the experiments on activation of HF or PK, we used fibrin-coated wells because not only tissue-type PA but also urokinase-type PA can convert plasminogen into active form plasmin more effectively in the presence of fibrin than in its absence,[52] although the affinity for fibrin of urokinase-type PA is significantly lower than that of tissue-type PA.[52] With all these data, it is reasonable to assume that kinin generation occurs in the tumor tissues and perhaps systemically as well, and that it is caused by activation of upstream proteases of the kinin generation system, i.e., HF and PK, and perhaps by plasmin that is produced from activation of plasminogen by PA derived from tumor cells (see Fig. 1).

What then would the presence of kinin mean in the clinical setting and in the pathogenesis of cancer? It is natural to assign kinin, together with permeability-enhancing factors,[29, 30] as the cause of accumulation of

ascitic and pleural effusion related to leakage of plasma proteins.[9, 10] Local and systemic kinin generation, if any, which enhances vascular permeability, might result in the hypoalbuminemia that is observed in many cancer patients.[31] It may also be that enhanced vascular permeability in the tumor tissue with carcinomatosis would facilitate a greater supply of nutrients and oxygen to tumor cells. These events could thus contribute to acceleration and/or maintenance of cachexia in cancer patients.

We have proposed that this uniquely enhanced tumor vascular permeability should be exploited for cancer chemotherapy using macromolecular therapeutics and a lipid carrier (Lipiodol),[3-8] and we should be able to achieve more effective and selective drug delivery to tumor tissue, resulting in very much reduced side effects. We also have shown that manipulation of local kinin contents is possible, and thus control of formation of ascites or delivery of macromolecules to the site of tumor may become possible.[4, 9]

Takada *et al.* reported that plasminogen is more prone to activation by both tissue-type PA and urokinase-type PA in the presence of fibrin because the conformational change of plasminogen takes place by the binding of plasminogen to fibrin.[52] There are extensive studies showing that fibrin deposition occurs in tumor tissue,[29, 53-55] which would facilitate the kinin cascade. Thus, the local microenvironmental concentration of PA might be greater than in plasma or ascites, in addition to counteracting inhibitors.

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