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Enhanced Vascular Permeability in Solid Tumor Involving Peroxynitrite and Matrix Metalloproteinases

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Peroxynitrite ($ONOO^-$), which is generated from nitric oxide (NO) and superoxide anion ($O_2^{\cdot-}$) under pathological conditions, plays an important role in pathophysiological processes. Activation of matrix metalloproteinases (MMPs) contributes to tumor angiogenesis and metastasis. NO mediates the enhanced vascular permeability and retention (EPR) effect in solid tumors, and $ONOO^-$ activates proMMP to MMP *in vitro*. In this study, we examined the role of $ONOO^-$ in the EPR effect in solid tumors and normal tissues as related to MMP activation. Authentic $ONOO^-$, at 50 nmol or higher concentrations, induced the enhanced vascular permeability in normal dorsal skin of mice. $ONOO^-$ scavengers ebselen and uric acid significantly suppressed the EPR effect in mouse sarcoma 180 (S-180) tumors. Indirect evidence for formation of $ONOO^-$ in S-180 and mouse colon adenocarcinoma (C-38) tumors included strong immunostaining for nitrotyrosine in the tumor tissue, predominantly surrounding the tumor vessels. MMP inhibitor BE16627B (66.6 mg/kg i.v., given 2 times) or SI-27 (10 mg/kg i.p., given 2 times) significantly suppressed the $ONOO^-$-induced EPR effect in S-180 tumors and in normal skin. Soybean trypsin inhibitor (Kunitz type), broad-spectrum proteinase inhibitor ovomacroglobulin, and bradykinin receptor antagonist HOE 140 also significantly suppressed the $ONOO^-$-induced EPR effect in normal skin tissues. These data suggest that $ONOO^-$ may be involved in and promote the EPR effect in tumors, which could be mediated partly through activation of MMPs and a subsequent proteinase cascade to generate potent vasoactive mediators such as bradykinin.

Key words: Peroxynitrite — Matrix metalloproteinases — Nitric oxide — Bradykinin — Enhanced permeability and retention (EPR) effect

Solid tumors exhibit unique pathophysiological characteristics such as angiogenesis, rapid growth, and metastasis. Enhanced permeability of tumor vasculature allows tumor tissue to meet the great demand for nutrients and oxygen. Such enhanced permeability is thus a prerequisite for rapid tumor growth. We previously demonstrated that solid tumors have a unique characteristic of enhanced vascular permeability and retention of macromolecular and lipidic agents, which we termed the *enhanced permeability and retention effect* (EPR effect).[1, 2] This concept has now been accepted as valid in anticancer drug design for targeted delivery of macromolecular therapeutics.[1–5] However, better understanding of tumor vascular permeability and various vascular mediators is of fundamental importance for gaining insight into the molecular mechanisms of tumor growth and for successful cancer-targeted drug delivery in anticancer chemotherapy.

We and others previously clarified that the EPR effect is mediated by bradykinin (BK),[6–8] nitric oxide (NO),[8–10] and prostaglandins[8] as well as vascular permeability factor/vascular endothelial growth factor (VPF/VEGF).[11] NO, an inorganic free radical generated from L-arginine by a family of nitric oxide synthases (NOSs), has diverse effects on various biological systems, such as neuronal signal transduction, host defense, angiogenesis, and vascular modulation (vasodilatation) in normal and disease states.[12–14] It has been well documented that excess NO is generated in rodent tumors and in human carcinomas.[8–10, 15–18] It appears that macrophages that infiltrate tumor tissue may generate VEGF, and NO upregulates VEGF activity in angiogenesis and permeability enhancement.[19] In addition, superoxide anion ($O_2^{\cdot-}$) may be generated in tumor tissue, primarily by the recruited or infiltrated macrophages and neutrophils.[20] Xanthine oxidase in pathological tissue including solid tumors also generates $O_2^{\cdot-}$.[14, 21] Furthermore, at a low concentration of L-arginine, or in its absence, NOS can generate $O_2^{\cdot-}$.[22, 23] NO and $O_2^{\cdot-}$ can react with each other extremely rapidly (the rate constant of 6.7×10^9 M^{-1} s^{-1} is more than 3 times faster than that for superoxide dismutase and $O_2^{\cdot-}$) to form another highly reactive compound, peroxynitrite ($ONOO^-$).[24, 25]

Matrix metalloproteinases (MMPs) are also known to play important roles in tumor invasion, metastasis, and angiogenesis.[26–29] We previously demonstrated that $ONOO^-$

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produced remarkable activation of human neutrophil-derived procollagenase (i.e., changed proMMP to MMP) in an *in vitro* system,[30] and hence we speculated that $ONOO^-$ might induce vascular permeability via activation of proMMP and/or other proteinase cascades. The current study is therefore aimed at clarifying whether $ONOO^-$ is involved in vascular permeability in tumor tissue as well as in normal tissue, and at exploring the molecular mechanism of $ONOO^-$-dependent extravasation, with a focus on activation of MMPs and BK.

MATERIALS AND METHODS

Animals and tumors The subcutaneous dorsa of five- to six-week-old male ddY mice (SLC, Shizuoka) were inoculated with mouse sarcoma 180 (S-180) (inoculum size 2×10^6 cells per site). Similarly, mouse colon adenocarcinoma 38 (C-38) was injected subcutaneously (3×3×3 mm piece) with a trocar into the dorsal skin of male C57BL/6n mice (Seac Yoshitomi, Ltd., Fukuoka). The ddY mice and male Hartley guinea pigs (male; weight, 400–450 g; Seac Yoshitomi, Ltd.) were also used for assessment of enhanced vascular permeability caused by $ONOO^-$ and proteinases. All experiments were carried out according to the guidelines of the Laboratory Manual of Animal Handling, Kumamoto University School of Medicine.

Substances $ONOO^-$ was synthesized in our laboratory according to the method described previously.[30, 31] Ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one) was a gift from Daiichi Pharmaceutical Co., Tokyo. Uric acid and 4-aminophenylmercuric acetate (APMA) were from Wako Pure Chemicals, Ltd., Osaka. HOE 140 (D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-bradykinin) was a gift from Professor B. A. Schölkens, Hoechst Marion Roussel, Frankfurt am Main, Germany. MMP inhibitors BE16627B (L-*N*-(*N*-hydroxy-2-isobutylsuccinynamoyl)-seryl-L-valine; molecular weight, 375.2)[32] and SI-27 (L-*N*-(*N*-hydroxy-2-isobutylsuccinamoyl)-leucyl-isobutylamide; molecular weight, 357.5),[33] from Tsukuba Research Institute, Banyu Pharmaceutical Co., Tsukuba, were dissolved in dimethylsulfoxide (final concentration of 1%) and were then diluted with physiological saline. *o*-Phenanthroline (molecular weight, 180.2) was obtained from Dojindo Laboratories (Kumamoto). proMMP-9 from human leukocytes and *Serratia marcescens* 56-kDa metalloproteinase were purified as reported previously.[34, 35] Ovomacroglobulin (ovoM), a proteinase inhibitor from chicken egg white (720 kDa), was from JIMRO (Takasaki), and soybean trypsin inhibitor (SBTI, Kunitz type, 20 kDa) was from Fujimoto Pharmaceutical Co., Osaka. α-Gelatin monomer was a product of Serva Feinbiochemica, GmbH, Heidelberg, Germany. 2,7-Dichlorodihydrofluorescein (DCDHF) was purchased from Molecular Probes, Inc. (Eugene, OR). All other reagents were from commercial sources.

Effect of $ONOO^-$ and MMPs on vascular permeability in normal skin tissues Mice were anesthetized with sodium pentobarbital (83 mg/kg) administered intraperitoneally (i.p.). $ONOO^-$ at pH 12 was stored at −80°C and was used after appropriate dilution in 20 m*M* phosphate-buffered physiological 0.15 *M* saline (PBS, pH 7.0). After addition of an aliquot of stock solution of $ONOO^-$ into PBS, the pH changes to about 9.5 to 10. At this pH, $ONOO^-$ is relatively stable, at least during its dilution and administration to the animals. Its concentration was quantified spectrophotometrically at 302 nm just before intradermal (i.d.) injection (50 to 300 nmol in 100 μl/injection). Diluted $ONOO^-$ solution was immediately injected i.d. into the dorsal skin of normal mice. Vascular permeability was determined by assessing extravasation of Evans blue dye, which was injected intravenously (i.v.) at 10 mg/kg just before the $ONOO^-$ injection. The animals were killed 1 h after Evans blue injection, the skin tissue was removed, and the amount of extravasated dye in the skin was quantified after extraction with formamide as described previously.[1] The control vehicle (0.1 *M* NaOH diluted with PBS to pH 10) induced very little vascular permeability. The effect of $ONOO^-$ on vascular permeability was also examined with the guinea pig skins in a similar manner to the mouse skin model.

For assay of the duration of $ONOO^-$-induced enhanced vascular permeability, Evans blue solution was administered i.v. at different time points after i.d. injection of 100 nmol $ONOO^-$ into the dorsal skin. The dye extravasation was quantified as just described.

Dye extravasation in the skin tissue was also examined after injection of various amounts of purified MMP-9 from human leukocytes.[34] Specifically, the purified proMMP-9 was first activated fully by incubating with 1 m*M* APMA in 100 m*M* Tris-HCl buffer (pH 7.6) containing 0.4 *M* NaCl and 10 m*M* $CaCl_2$ at 35°C for 2 h, and APMA was completely removed from the proMMP-9-containing solution with an ultrafiltration tube (Ultrafree-MC, Millipore Corp., Bedford, MA; cutoff size 30 000 Da). Various amounts of activated MMP-9 diluted with PBS (pH 7.0) were injected i.d. into the dorsal skin of mice or guinea pigs (100 μl/injection), followed by Evans blue administration (10 mg/kg). The extravasation reaction during 1 h after MMP-9 injection was measured by quantifying the amount of the dye extravasated in the skin tissue as just described. In addition, the vascular permeability-enhancing potential of MMP-9 was compared with that of *Serratia* 56-kDa metalloproteinase, that we identified previously as a potent activator of the BK-generating cascade, leading to strongly enhanced vascular permeability.[36, 37]

Immunostaining for nitrotyrosine in solid tumor tissues Tumor tissues were excised, after which they were immediately embedded in tissue-embedding medium (Miles, Elkhart, IN) and were then frozen in liquid nitrogen. Sec-

tions of S-180 and C-38 tumors (6 μm thick) were prepared with a cryostat at −14°C and were then mounted on gelatin-coated slides. The sections were fixed with cold acetone for 10 min and dried for 2 h, followed by use of the indirect immunoperoxidase method described previously.[38] Briefly, after inhibition of endogenous peroxidase activity with 0.3% H_2O_2/methanol, sections were incubated at room temperature for 60 min (the first step) with a rabbit polyclonal anti-nitrotyrosine antibody (1:1000) (Upstate Biotechnology Inc., Lake Placid, NY). Sections were washed three times with PBS and then were incubated for 60 min with a donkey anti-rabbit immunoglobulin $F(ab')_2$ conjugated with peroxidase (Amersham, Little Chalfont, UK) diluted 1:100. For analysis and visualization of this reaction, 0.02% of 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) was used as a substrate in 50 m*M* Tris-HCl buffer (pH 7.6) containing 0.01% H_2O_2. Nuclei were counterstained with hematoxylin. In some experiments, tissue sections were stained with anti-nitrotyrosine antibody in the presence of authentic nitrotyrosine (1 m*M*).

Effect of $ONOO^-$ scavengers ebselen and uric acid on vascular permeability in tumor-bearing animals Ebselen reacts rapidly (~10^6 $M^{-1}s^{-1}$) with $ONOO^-$ and thus serves as an $ONOO^-$ scavenger.[39] Uric acid, another natural $ONOO^-$ scavenger,[40] was also used. Ebselen, suspended in 3% arabic gum/saline solution, was administered orally (p.o.; 200 μl) to S-180 solid tumor-bearing ddY mice at 0.1 or 0.3 g/kg. Uric acid was administered similarly, at 0.5 or 1.0 g/kg p.o., to tumor-bearing mice. Evans blue was injected i.v. 30 min after administration of ebselen or uric acid. Six hours after administration of Evans blue, tumor tissues were excised and vascular permeability was quantified.

Effect of various proteinase inhibitors or antagonists on $ONOO^-$-induced vascular permeability in normal and tumor-bearing animals MMP inhibitor SI-27, administered i.v. (100 μl) at 3.3 or 10 mg/kg, was given twice to normal mice, 5 min before and 10 min after $ONOO^-$ (100 nmol/site) and Evans blue administrations. Similarly, SI-27 was administered twice to S-180 tumor-bearing mice at an interval of 15 min. BE16627B (100 μl) was given i.p. at 66.6 mg/kg once or twice, similar to the SI-27 treatment. Evans blue was injected 5 min after the first administration of MMP inhibitors to normal mice, followed by dye extravasation for 1 h; an interval of 3 h was used for Evans blue extravasation after the second administration of MMP inhibitors to S-180 tumor-bearing mice. Proteinase inhibitor ovoM or SBTI was administered i.v. at 33.3 mg/kg/100 μl to normal mice. HOE 140, a potent BK (B_2) receptor antagonist,[41, 42] was injected i.d. at 30 or 60 nmol/kg (100 μl per injection) into normal mice at a site distant from that used for the $ONOO^-$ injection. Treatment with SBTI, ovoM, or HOE 140 was given 5 min before the i.d. $ONOO^-$ injection at 50 and/or 100 nmol/site, and Evans blue was injected i.v. before $ONOO^-$ administration and extravasated for 1 h.

Zymography of S-180 tumor tissue homogenates S-180 tumor tissues, normal skin, and muscle tissues were separately homogenized with a Polytron homogenizer in a 5-fold volume of PBS containing phenylmethylsulfonyl fluoride (1 m*M*) and leupeptin (10 μg/ml) and were then stored at −80°C. The supernatant of the homogenate of each sample obtained by centrifugation (10 000*g* for 20 min at 4°C) was diluted with PBS, and each aliquot containing 2 μg of tissue homogenate protein was subjected to gelatin zymography.[43] proMMP-9 used for the positive control was purified from human leukocytes as described above.[34] Briefly, samples underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide gel) containing 1 mg/ml gelatin under nonreducing conditions. The gel was washed three times with 2.5% Triton X-100 in an incubation buffer (50 m*M* Tris, pH 7.5, 0.02% Brij35, 10 m*M* $CaCl_2$, 2 μM $ZnCl_2$) for 15 min, followed by incubation at 37°C for 18 h in a sealed airtight box. After the gel was fixed with 50% methanol and 10% acetic acid, it was stained with Coomassie brilliant blue R250. To confirm the specificity of gelatinolysis, after electrophoresis the gelatin-containing gel was incubated in the presence of the MMP inhibitor SI-27 (0.3 mg/ml in the incubation buffer), followed by gel fixation and staining.

***In situ* zymography** S-180 tumor and skin tissue sections were embedded in tissue-embedding medium (Miles) and then frozen in liquid nitrogen. Sections 4 μm thick were prepared with a cryostat at −14°C and were then immediately mounted on gelatin-coated film slides (gifts from Mr. R. Nemori at Ashigara Research Laboratories, Fuji Film Co., Ltd., Kanagawa). The film slides were incubated in a wet box at 37°C for 12 h, after which they were dried at room temperature and then stained with 0.8% Ponceau-3R in 70% ethanol plus 1.8% trichloroacetic acid to reveal the gelatin remaining on the slides.[44] The gelatinase activity in the tissue sections was readily identified *in situ* on the basis of the gelatinolysis. MMP activity in each tissue section was blocked by incubating the tissue sections with 50 μl of MMP inhibitor (10 mg/ml BE16627B or 3 mg/ml SI-27 in PBS). In some experiments, gelatin-coated films containing MMP inhibitors were used to further confirm the specificity of the MMP activity. Specifically, the MMP inhibitors such as SI-27 and *o*-phenanthroline are incorporated into the gelatin film by overlaying 100 μl of 50% ethanol solution containing either SI-27 (8.4 m*M*) or *o*-phenanthroline (100 m*M*) onto the film, followed by drying at room temperature. Then, *in situ* zymography was performed with the solid tumor tissues by using the gelatin-coated film treated with MMP inhibitors or solvent control (50% ethanol).

Effect of various inhibitors for $ONOO^-$, proteinases, and BK on MMP activity To confirm the specificity of the pharmacological inhibitions for $ONOO^-$, proteinases, and BK employed in this study, the effects of various inhibitors on the gelatinolytic activity of MMP-9 were examined *in vitro* by using human MMP-9 activated as described above. Specifically, MMP-9 (1.3 μM) was incubated with 1.0 mg/ml α-gelatin monomer (95 kDa) in the presence or absence of $ONOO^-$ scavengers (14.6 μM ebselen; 14.9 μM uric acid), proteinase inhibitors (2.8 μM ovoM; 12.5 μM SBTI), or a BK antagonist HOE 140 (15.3 μM) in 100 m*M* Tris-HCl buffer (pH 7.6) containing 0.4 *M* NaCl and 10 m*M* $CaCl_2$ at 35°C for 5 h. The gelatinolysis was then measured by 7.5% SDS-PAGE.

Effect of various compounds on $ONOO^-$ stability To further verify that various proteinase inhibitors and BK antagonist used in this study specifically affect the MMPs or other endogenous proteinases without nonspecific inhibitory effects on $ONOO^-$ *per se*, we employed DCDHF oxidation assay for assessment of chemical reactivity of authentic $ONOO^-$.[45] $ONOO^-$ was added as a bolus (5 μM) to react with 0.1 m*M* DCDHF in PBS (pH 7.0) containing various proteinase inhibitors such as BE16627B (6.5 μM), SI-27 (6.5 μM), ovoM (5.2 μM), SBTI (6.5 μM), and HOE 140 (6.5 μM), at room temperature for 30 min. Ebselen was used as a positive control for the $ONOO^-$ scavenging reaction. The oxidation of DCDHF by $ONOO^-$ with or without various substances tested was determined by measuring the increase in absorbance at 500 nm, so that the scavenging potential of each substance was assessed in terms of its inhibitory activity on DCDHF oxidation.

Statistical analysis Data are shown as means±SEM. Statistical analysis was performed by a two-tailed unpaired *t* test comparing the drug-treated groups and the control vehicle groups. A P value of <0.05 was considered statistically significant.

RESULTS

$ONOO^-$-induced enhanced vascular permeability in normal mouse skin Dose-dependent $ONOO^-$-induced vascular permeability was clearly observed in normal dorsal mouse skin (Fig. 1A). Vascular permeability was similarly enhanced in guinea pig skin treated with $ONOO^-$ (data not shown). $ONOO^-$-induced vascular permeability continued for an appreciable time, even 30 min after $ONOO^-$ administration (Fig. 1B), despite the very short half-life of $ONOO^-$, which is presumed to be only a few seconds in normal physiological states. This result suggests that $ONOO^-$ induced enhanced permeability not only by direct action on the vasculature, but also by an indirect mechanism through formation of some other endogenous vascular mediators.

Nitrotyrosine formation in solid tumor tissues Generation of $ONOO^-$ in tumor tissue was analyzed by immunohistochemistry for nitrotyrosine formation. Immunostaining was used with S-180 and C-38 tumor sections to determine the presence of nitrotyrosine produced by efficient nitration by $ONOO^-$. The results showed clear staining for

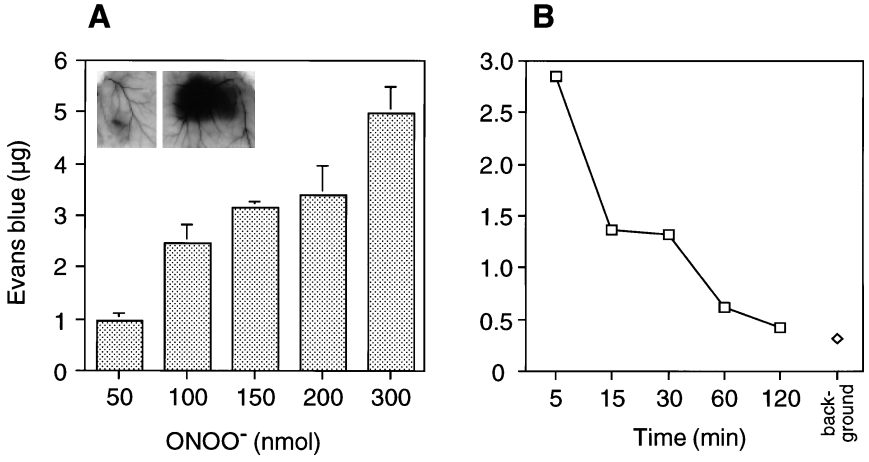

Fig. 1. Dose-dependent effect of $ONOO^-$ on dorsal skin vascular permeability in normal mice (A), and duration of the vascular permeability enhancement (B). A, $ONOO^-$ was administered i.d. at 50, 100, 150, 200, or 300 nmol/100 μl as described in "Materials and Methods." Evans blue was injected i.v. at 10 mg/kg just before the $ONOO^-$ injection, and the dye was extravasated for 1 h. Data are means±SEM (n=3). The inset shows authentic $ONOO^-$-induced vascular permeability (left: decomposed $ONOO^-$; right: $ONOO^-$ 100 nmol). B, Evans blue (10 mg/kg) was injected i.v. at 10, 15, 30, 60, or 120 min after i.d. injection of 100 nmol $ONOO^-$ into dorsal skin; dye extravasated for 1 h. Data are means of duplicate determinations.

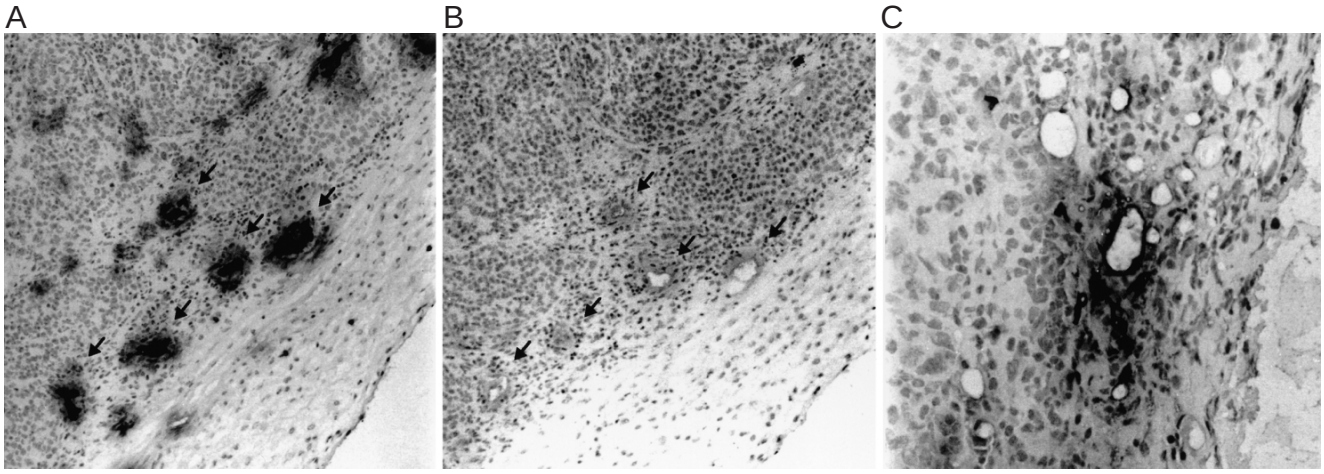

Fig. 2. Immunostaining for nitrotyrosine in C-38 and S-180 tumor tissue sections. A, A section of C-38 solid tumor immunostained without free nitrotyrosine absorption; B, a serial section of A stained with free nitrotyrosine absorption; C, S-180 tumor section without free nitrotyrosine absorption. The area of strong immunostaining for nitrotyrosine in the section (A) is not observed in the corresponding area of its serial section (B) as indicated with arrows. Magnification, ×59 (A, B); ×117 (C). See the text for details.

nitrotyrosine, predominantly in the peripheral tumor vasculature in both C-38 (Fig. 2, A and B) and S-180 (Fig. 2C) tumors.

Involvement of $ONOO^-$ in enhanced vascular permeability in S-180 solid tumor As demonstrated in Fig. 3, the $ONOO^-$ scavenger ebselen at 0.3 g/kg significantly suppressed extravasation of Evans blue into tumor tissue. Uric acid produced similar suppression. These data suggest that $ONOO^-$ may be involved in enhanced tumor vascular permeability, i.e., the EPR effect.

Production of MMPs in solid tumor tissues Gelatin zymography of homogenates of the tumor tissues and other tissues identified MMP expression. Strong proMMP-9 activity and a low level of proMMP-2 activity were found in S-180 tumor tissues (Fig. 4A, lane 2). Both proMMP-9 and proMMP-2 (MMP-2) activities were weakly expressed in normal skin tissue (Fig. 4A, lane 3). No appreciable MMP activity was observed in muscle tis-

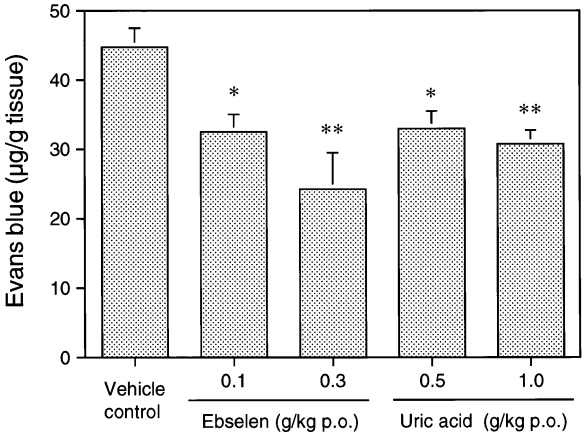

Fig. 3. The effect of ebselen and uric acid on tumor vascular permeability in mice bearing solid S-180 tumor. Ebselen was suspended in 3% arabic gum/saline solution and was administered at 0.1 or 0.3 g/kg p.o. Uric acid was administered similarly, at 0.5 or 1.0 g/kg p.o. Evans blue (at 10 mg/kg) was injected i.v. 30 min later; dye extravasated for 6 h. n=5. * P<0.05; ** P<0.01. See the text for details.

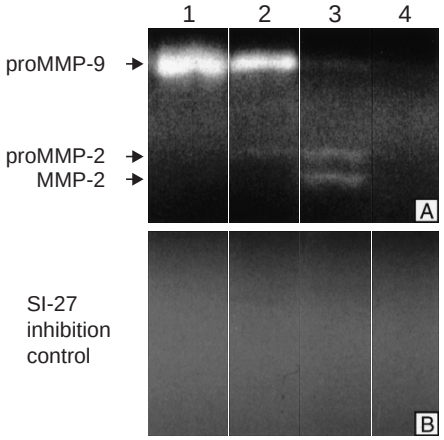

Fig. 4. Zymography of tissue homogenates. A, Lane 1, purified proMMP-9 from human leukocytes; lane 2, S-180 tumor; lane 3, normal mouse skin; lane 4, normal mouse muscle. B, The same polyacrylamide gels as those used in (A) were incubated with SI-27 (0.3 mg/ml) after electrophoresis. See the text for details.

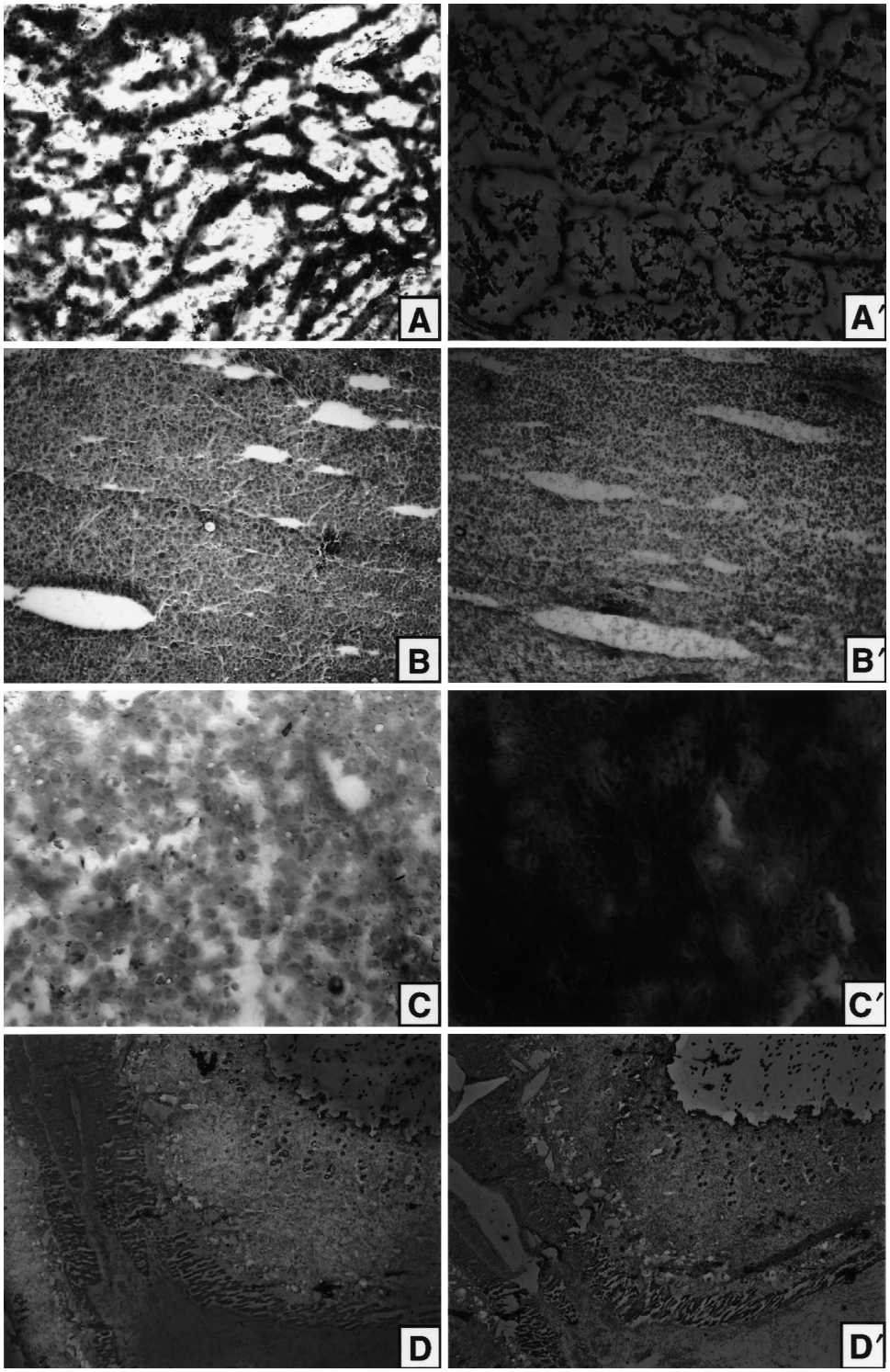

Fig. 5. *In situ* zymography of S-180 tumor and normal skin tissue sections. A, B, and C, S-180 tumor without MMP inhibitor; D, normal mouse skin without MMP inhibitor; A′, S-180 tumor treated with MMP inhibitor SI-27; B′, S-180 tumor treated with MMP inhibitor BE16627B; C′, S-180 tumor section mounted on gelatin-coated film containing SI-27; D′, normal skin treated with BE16627B. The transparent area (A, B, C, D) indicates the presence of proteinase (gelatinase), which is blocked by the MMP inhibitors, as seen in the corresponding serial sections (A′, B′, C′, D′). Similarly, gelatinolysis caused by the tissue sections was completely inhibited on the gelatin-coated film incorporated with *o*-phenanthroline (data not shown). Magnification, ×35 (A, B, D, A′, B′, D′); ×138 (C, C′). See the text for details.

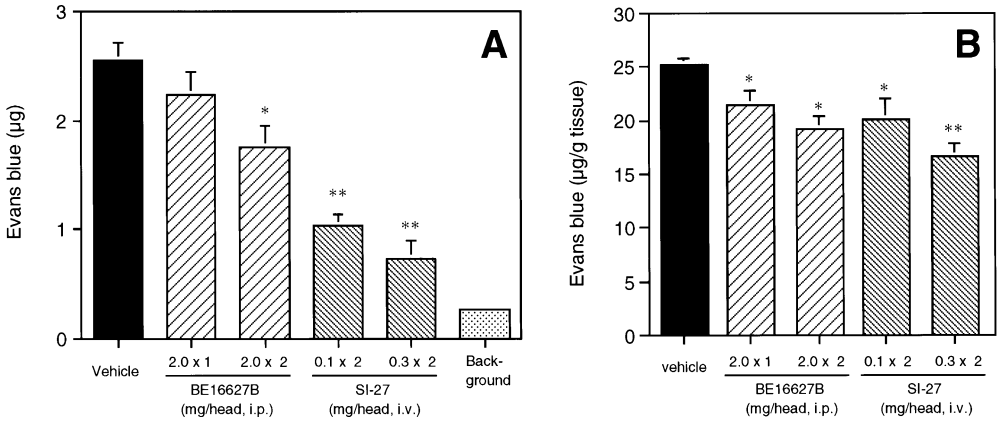

Fig. 6. Effect of the MMP inhibitors SI-27 (3.3 or 10 mg/kg (0.1 or 0.3 mg/head) i.v., given two times) and BE16627B (66.6 mg/kg (2.0 mg/head) i.p., given once or twice) on $ONOO^-$-induced vascular permeability in normal mouse skin (A) and on S-180 tumor vascular permeability (B). $ONOO^-$ was given at 100 nmol/injection i.d. Evans blue (10 mg/kg) was injected i.v., and the dye was extravasated for 1 h (normal mice) and 3 h (tumor-bearing mice). $*$ $P<0.05$; $**$ $P<0.01$. See the text for details.

sue (Fig. 4A, lane 4). All gelatinolytic bands identified in tumor and normal skin tissues completely disappeared after use of the specific MMP inhibitor SI-27, confirming that these gelatinolytic activities are attributable solely to MMPs expressed in the tissues (Fig. 4B). In contrast, the level of proMMP-9 and proMMP-2 expression in the normal skin tissue did not change significantly after i.d. injection of $ONOO^-$ (data not shown), suggesting that $ONOO^-$ may directly activate the proMMPs rather than upregulate proMMP expression.

To further examine MMP expression in solid tumor tissue, *in situ* gelatin zymography was performed with or without MMP inhibitor treatment. As shown in Fig. 5, MMP activity is expressed mainly in the interstitial tissue of S-180 tumor (Fig. 5, A, B and C); it was less clearly expressed in skin tissue (Fig. 5D). MMP inhibitors such as SI-27, BE16627B, and *o*-phenanthroline, nullified gelatinolytic activities in the tumor tissue sections (Fig. 5, A′, B′ and C′), indicating that the gelatinolytic area was produced by the MMPs expressed in the solid tumor. BE16627B also suppressed the gelatinolysis in normal skin tissue (Fig. 5D′).

Involvement of MMP in $ONOO^-$-induced vascular permeability in normal skin and in the EPR effect in S-180 solid tumors MMP inhibitor SI-27 strongly suppressed $ONOO^-$-induced vascular permeability in normal mouse skin (Fig. 6A). SI-27 also significantly suppressed the EPR effect in S-180 tumor (Fig. 6B). Another MMP inhibitor, BE16627B, moderately attenuated the vascular permeability not only in normal mouse dorsal skin treated with $ONOO^-$ (100 nmol/injection), but also in S-180 tumor tissues (Fig. 6, A and B). These results may suggest a possible involvement of MMPs in both $ONOO^-$-induced enhanced vascular permeability in normal skin and the EPR effect in S-180 solid tumors.

To further confirm the extravasation-inducing potential of MMPs, the purified MMP-9, that was a fully activated form as judged by its strong gelatinolytic activity (Fig. 7A), was injected into the normal skin tissue (Figs. 7B and

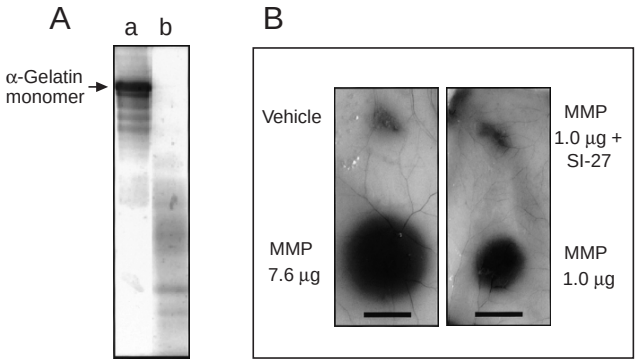

Fig. 7. Enhanced vascular permeability induced by exogenous MMP-9 in normal mouse dorsal skin. A, The gelatinolytic activity of MMP-9 purified from human leukocytes. proMMP-9 (1.65 μM) with (b) or without (a) APMA activation was incubated with 1.0 mg/ml α-gelatin monomer (95 kDa) in 100 m*M* Tris-HCl buffer (pH 7.6) containing 0.4 *M* NaCl and 10 m*M* $CaCl_2$ at 35°C for 2 h. The cleavage of α-gelatin monomer (5.0 μg/lane) was assessed by 7.5% SDS-PAGE. B, MMP-9 thus activated was administered i.d. to mice after incubation with or without SI-27 (30 μg/ml) for 30 min at 37°C, followed by Evans blue i.v. injection. The extensive dye extravasation is evidenced by the strong blue spots produced at the site of MMP-9 i.d. injection. Bar, 1 cm. See the text for details.

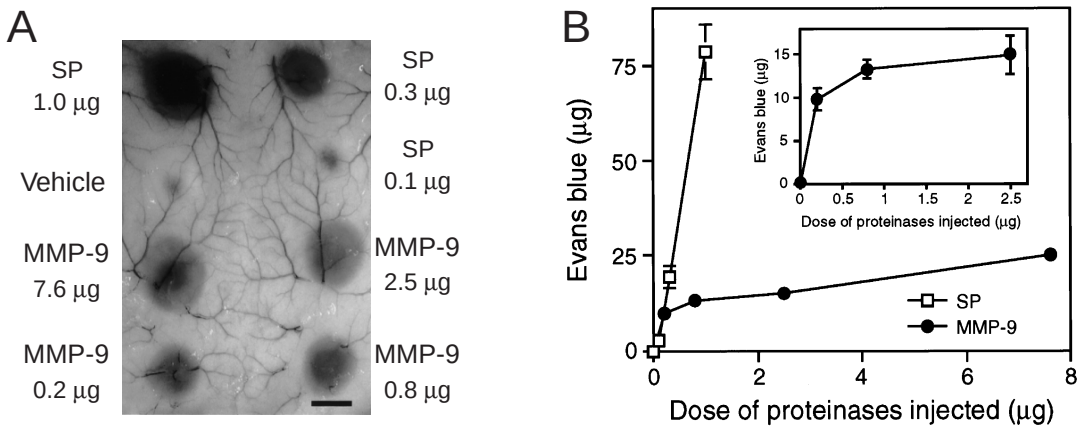

Fig. 8. Enhanced vascular permeability induced by exogenous MMP-9 and *Serratia* proteinase (SP) in normal guinea pig dorsal skin. A, Various amounts of MMP-9 and SP were injected i.d. into the normal skin of guinea pigs (100 μl/injection). The blue spot of the dye extravasation caused by each dose of proteinases are demonstrated. Bar, 1 cm. B, The amount of Evans blue extravasated in the skin tissues shown in (A) was extracted and quantified. The dye extravasation induced by low doses of MMP-9 is illustrated in the inset. n=4. See the text for details.

8). As demonstrated in Fig. 7B, MMP-9 produced remarkable extravasation in the mouse skin, which was almost completely inhibited by a specific MMP inhibitor SI-27. Also, strong and dose-dependent extravasation was observed with MMP-9 in the guinea pig skins (Fig. 8). Although the extravasation caused by 1.0 μg of *Serratia* proteinase was more remarkable than that by MMP-9, MMP-9 at less than 1.0 μg induced appreciable extravasation, which was almost comparable to that by *Serratia* proteinase. The extravasation by MMP-9, however, reached a plateau at doses more than 1.0 μg. The time profile of the enhanced vascular permeability induced by MMP-9 was consistent with that by *Serratia* proteinase: maximal extravasation was observed between 10–20 min after intradermal injections and rapidly declined within 1 h after administration. This suggests that a similar mechanism for vascular permeability enhancement may be operative with these different proteinases. It is also important that the vascular permeability-inducing potential of MMP-9 was found to be more than 10 times stronger than that of authentic BK when compared on the molar basis of each compound introduced to skin tissue (data not shown).[36, 37]

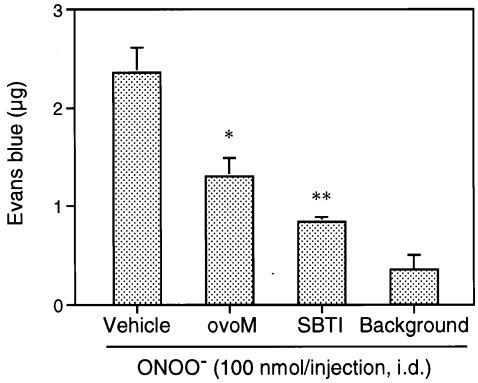

Fig. 9. Effects of the proteinase inhibitors ovomacroglobulin (ovoM) (33.3 mg/kg i.v.) and soybean trypsin inhibitor (SBTI, Kunitz type) (33.3 mg/kg i.v.) on the vascular permeability-inducing property of $ONOO^-$ (100 nmol/injection i.d.) in dorsal skin of normal mice. Evans blue was administered i.v. at 10 mg/kg just before $ONOO^-$ injection, and extravasated for 1 h. n=5. * P<0.05; ** P<0.01. See the text for details.

Effect of various proteinase inhibitors on $ONOO^-$-induced vascular permeability in normal skin ovoM, an inhibitor of various proteinases from chicken egg white, and the serine proteinase inhibitor SBTI, the most potent plasma kallikrein inhibitor,[7, 46] were administered i.v. at 33.3 mg/kg to mice 5 min before the i.d. injection of $ONOO^-$. These two substances significantly inhibited vascular permeability induced by $ONOO^-$ in normal skin (Fig. 9), suggesting that not only MMP, but also other proteinases, e.g., kallikrein, may be involved in $ONOO^-$-induced vascular permeability.

Effect of BK in $ONOO^-$-induced vascular permeability The activation of serine proteinases, particularly of Hageman factor (factor XII), kallikrein and plasmin, will trigger the activation of the kallikrein-kinin system and subsequently result in the generation of BK,[7, 46–48] which is a potent vascular permeability-inducing factor. As illustrated in Fig. 10, the BK antagonist HOE 140 at 30 or 60 nmol/kg i.d. had a dose-dependent suppressive effect on $ONOO^-$-induced vascular permeability in normal skin tis-

sues. HOE 140 at 60 nmol/kg i.d. significantly suppressed the vascular permeability induced by 50 nmol $ONOO^-$, and about 50% inhibition was obtained for 100 nmol $ONOO^-$. These results indicate involvement of BK in $ONOO^-$-induced vascular permeability. HOE 140 produced similar suppression of the EPR effect in S-180 solid tumor.[8] Therefore, a new BK-generating pathway may be operating for enhanced vascular permeability in solid tumor via $ONOO^-$-induced proMMP activation.

Direct effect of $ONOO^-$ scavengers, proteinase inhibitors, and BK antagonist on MMP activity To examine whether $ONOO^-$ scavengers, proteinase inhibitors and a BK antagonist directly affect the MMP activity, the effects of various inhibitors on the gelatinolytic activity of the purified MMP-9 were examined *in vitro*. The results showed that the compounds tested including ebselen, uric acid, SBTI, and HOE 140, have no inhibitory activities against MMP-9, except that ovoM significantly suppressed the gelatinolytic activity of MMP-9 (data not shown). It is thus suggested that the suppressive effect of ovoM on $ONOO^-$-induced extravasation may be brought about by its multiple inhibitory actions against MMPs and other endogenous proteinases which are involved in the BK-generating cascade as well.

Substantiation of pharmacological effects of various proteinase inhibitors and HOE 140 in $ONOO^-$-induced extravasation To test the specificity of the proteinase inhibitors and to confirm their lack of $ONOO^-$-scavenging actions, DCDHF oxidation by $ONOO^-$ was measured in the presence or absence of various proteinase inhibitors. However, $ONOO^-$ did not react with any of the substances tested, such as MMP inhibitors (BE16627B and SI-27), other proteinase inhibitors (ovoM and SBTI), and HOE 140, whereas ebselen efficiently scavenged $ONOO^-$, at least under the present assay conditions (data not shown). It is therefore suggested that the inhibitory effects of these proteinase inhibitors and HOE 140 on the $ONOO^-$-induced enhanced vascular permeability (Figs. 6, 9, and 10) were brought about through the specific inhibitory actions of these inhibitors, and were not due to nonspecific effects on $ONOO^-$.

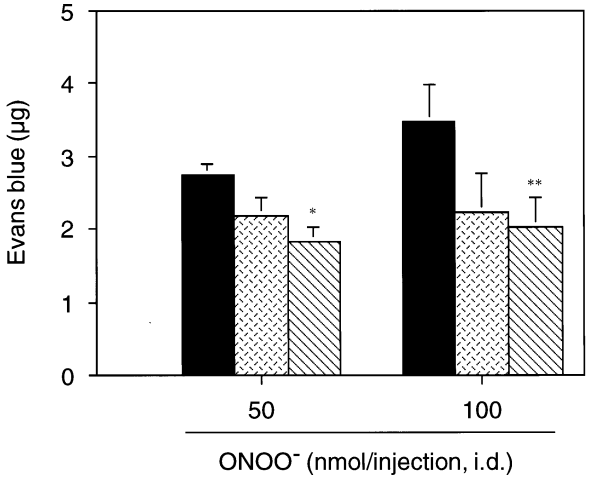

Fig. 10. Effect of the bradykinin antagonist HOE 140 at 30 or 60 nmol/kg i.d. on $ONOO^-$-induced vascular permeability. Dye extravasation was assessed in the same manner as in Fig. 9. $n=5$. $*$ $P<0.05$; $**$ $P<0.01$. See the text for details. ■ without HOE 140, ▩ HOE 140 (30 nmol/kg i.d.), ▨ HOE 140 (60 nmol/kg i.d.).

DISCUSSION

Of great importance are issues involved in the EPR effect and targeting of macromolecular drugs to tumors, and anticancer drug development based on this mechanism. The EPR effect allows macromolecules (>40 kDa)[1, 49] and lipids[50, 51] to traverse the tumor vascular endothelial barrier to enter the interstitium of the tumor and surrounding tissues. Because of the lack of effective lymphatic clearance, the EPR effect results in time-dependent accumulation of these molecules in the tumor.[1, 8, 50] Not only can this principle be applied to satisfy the great demand for nutrients and oxygen for rapid growth of solid tumors, it also offers an important opportunity to achieve selective and passive drug delivery of macromolecular or lipidic anticancer agents to solid tumor. The EPR effect occurring in solid tumors is a universal phenomenon, with selective accumulation of polymeric or macromolecular drugs in all such tumors.[1–5, 49, 50, 52, 53] This effect has now become a "gold standard" in the field of drug design for polymer-conjugated macromolecular anticancer drugs.[1–5]

Ridger *et al*. showed that $ONOO^-$ induced plasma extravasation in normal rat dorsal skin, although the mechanism was not clarified.[54] Our present results indicate that $ONOO^-$ is involved in the EPR effect in tumor tissues, and further that at least some of the $ONOO^-$-induced vascular permeability is most likely mediated by activation of MMP and other proteinases. The enhanced vascular permeability induced by $ONOO^-$ was retained for a relatively long time (an approximate half-time of 15 min) after $ONOO^-$ administration (Fig. 1B), despite the half-life of $ONOO^-$ under neutral physiological pH being only a few seconds.[30] This suggests that secondary or indirect mechanisms upregulating vascular permeability are involved in $ONOO^-$-induced vascular permeability enhancement. Related to this, activation of MMP (gelatinase B, MMP-9) was suggested to contribute to increased permeability of the blood-brain barrier in ischemia-reperfusion injury of the brain,[55] which is consistent with our direct evidence showing potent enhancement by MMP-9 of vascular permeability (Figs. 7 and 8).

The purified MMP-9 from human leukocytes at less than 1.0 μg showed a strong vascular permeability-induc-

ing potential, which is almost comparable to that by *Serratia* proteinase, a well-established BK generator and thus a potent inducer of extravasation, as we reported previously.[36, 37] However, the level of extravasation induced by MMP-9 reached a plateau at more than 1.0 μg, whereas *Serratia* proteinase showed an almost linear increase in the vascular permeability. This may be explained by some putative endogenous inhibitors for MMP such as tissue inhibitor of metalloproteinases (TIMPs) that may abrogate the excessive MMP activity added exogenously. In contrast, endogenous proteases could not function biologically against the *Serratia* proteinase, as we revealed previously, so that such a linear dose-dependent extravasation may be produced with *Serratia* proteinase.[37] Intriguingly, TIMPs are reported to be readily inactivated by $ONOO^-$.[56] It is thus suggested that the MMP activities could be potentiated indirectly by $ONOO^-$ through its inhibitory action for TIMPs. In any event, the present results unequivocally show that MMP-9 is a potent enhancer of vascular permeability *in vivo*.

Also, our present data indicate that not only $ONOO^-$ scavengers but also two different MMP inhibitors produced an appreciable suppression of tumor vascular permeability in the S-180 solid tumor model (Fig. 6B). The inhibitory effect of MMP inhibitors on the extravasation in solid tumors is moderate compared with their effects in $ONOO^-$-induced extravasation. This may be due to the low molecular weight of BE16627B and SI-27, which diffuse rapidly in and out of tumor tissues, and thus are not retained at effective concentrations for long periods. Targeted delivery of these MMP inhibitors to tumor tissue should improve this situation. Previous studies demonstrated that there are multiple factors involved in the enhanced vascular permeability in solid tumors.[6–11] It is thus conceivable that not only MMP, but also several other mediators including BK, NO, and VEGF (VPF) mutually interact to synergistically affect the tumor vasculatures. For example, we reported earlier that the inhibitory effect of the individual antidote for each vascular permeability factor is not strong *per se*, but remarkable attenuation of the vascular permeability was achieved by the combined administration of various inhibitors for these mediators.[8] We therefore believe that MMP could be partly contributing to solid tumor extravasation.

It appears that the MMP activity is expressed in the interstitial tissue of the tumor rather than the tumor cells, as identified by *in situ* gelatin zymography (Fig. 5). The activated macrophages are known to be the major contributor to MMP (MMP-2 and MMP-9) expression.[57] In addition, our earlier study revealed that high NO production in experimental solid tumor is derived mainly from tumor-infiltrating macrophages expressing inducible NOS.[8–10, 58] We interpret these results to suggest that induction of $ONOO^-$ formation and MMP activation, both simultaneously produced by macrophages in tumor tissues, may contribute in critical ways to the EPR effect in solid tumor.

To date, there is substantial evidence that $ONOO^-$ exhibits many biologically relevant functions, even though it is chemically reactive and thus unstable due to its short half-life in neutral solution.[14, 25, 45] In fact, although we have no direct evidence for MMP activation by $ONOO^-$ generated either endogenously or given exogenously *in vivo*, our previous study indicated that $ONOO^-$ remarkably activated proMMPs through chemical modification of the sulfhydryl moiety of their autoinhibitory domains[30, 59] (partly unpublished work). Because no apparent change in the molecular size of proMMPs was observed during the $ONOO^-$-induced activation on SDS-PAGE gels, it seems to be difficult to clearly identify such activation by $ONOO^-$ occurring *in vivo*. Alternatively, the pharmacological intervention using MMP inhibitors provided indirect evidence for the involvement of MMPs in $ONOO^-$-induced vascular permeability and the EPR effect in the solid tumor, as demonstrated in Fig. 6.

SBTI suppressed extravasation induced by $ONOO^-$ in normal skin, probably by inhibiting formation of BK, a potent permeability-enhancing factor. The contribution of BK to the EPR effect in solid tumor has been verified by our previous studies.[8, 47, 48] In addition, we found in the present study that the BK antagonist HOE 140 showed significant inhibition of vascular permeability induced by $ONOO^-$, suggesting involvement of BK generation in $ONOO^-$- and MMP-induced enhanced vascular permeability in solid tumor. It was recently reported that stromelysin-1 (MMP-3) can activate plasminogen to generate so-called miniplasminogen.[60] We also demonstrated that plasmin activation as well as urokinase-type plasminogen activator induced the kallikrein-kinin system.[6, 7, 46] All these results may indicate a possible new pathway of BK generation via activation of kallikrein triggered by MMPs activated by $ONOO^-$: $ONOO^- \rightarrow$ proMMP $\rightarrow$ MMP $\rightarrow$ plasminogen $\rightarrow$ plasmin $\rightarrow$ kallikrein $\rightarrow$ BK. More detailed experiments may be needed to clarify whether activation of Hageman factor is involved in $ONOO^-$-induced BK generation.

On the basis of these findings, it is suggested that $ONOO^-$ not only may induce vascular permeability involving MMPs, but also may activate kallikrein (or BK), and hence the EPR effect in tumor tissues. We reported previously that BK facilitates dissemination of bacteria from local sites of infection via the systemic circulation to distant organs.[61, 62] The activated MMPs and other proteinases may potentiate tumor invasion and metastasis by accelerating inflammatory responses through formation of BK. Therefore, use of noncytotoxic MMP inhibitors, antagonists of BK, $ONOO^-$ scavengers such as ebselen, or other proteinase inhibitors in cancer chemotherapy may help in suppressing the EPR effect, and hence ascitic or pleural

fluid accumulation, tumor invasion and metastasis, and tumor growth.

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