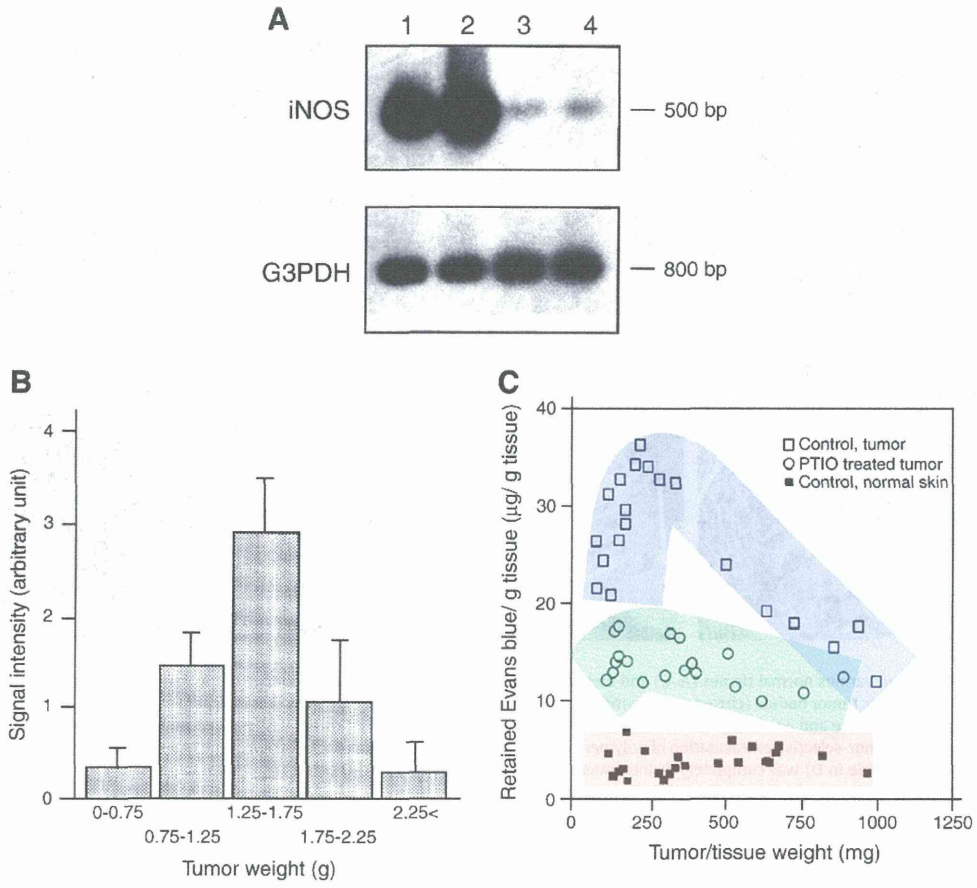

Fig. 5. NO production in different tumors. (A) RT-PCR for iNOS mRNA expression in rat AH136B solid tumor is shown: lanes 1 and 2 are results for AH136B tumor obtained from two different rats, and lanes 3 and 4 are results for two normal livers. (B) Correlation of the concentration of NO, as determined by the signals of NO-*N*-dithiocarboxysarcosine (DTCS)-Fe^{2+} being formed in solid tumor (AH136B) with tumor weight. Rats received i.v. injections of DTCS-Fe^{2+} complex, followed by electron paramagnetic resonance measurement at 110 K. Data are expressed as means ± SE. (C) Association between S-180 tumor weight in mice and the extent of extravasation of Evans blue/albumin in tumor (the EPR effect), and the concentration of NO in tumors of different size. PTIO is an NO scavenger [130]. Tumors weighing up to 1.75 g in rats (B) and 250 mg in mice (C) showed size-dependent NO production and extravasation of Evans blue/albumin.
Modified from Refs. [77] and [78].

In addition, the high reactivity of $ONOO^-$ leads to rapid production of nitrate or nitrosated aromatic residues including proteins and nucleic acids, and thus generation of nitrotyrosine and nitroguanosine [79,97–99,101,108]. These nitro compounds are likely to release nitrite (NO_2^-) and may serve as a source of NO. As discussed later, nitroglycerin (NG) would be converted to NO to facilitate the EPR

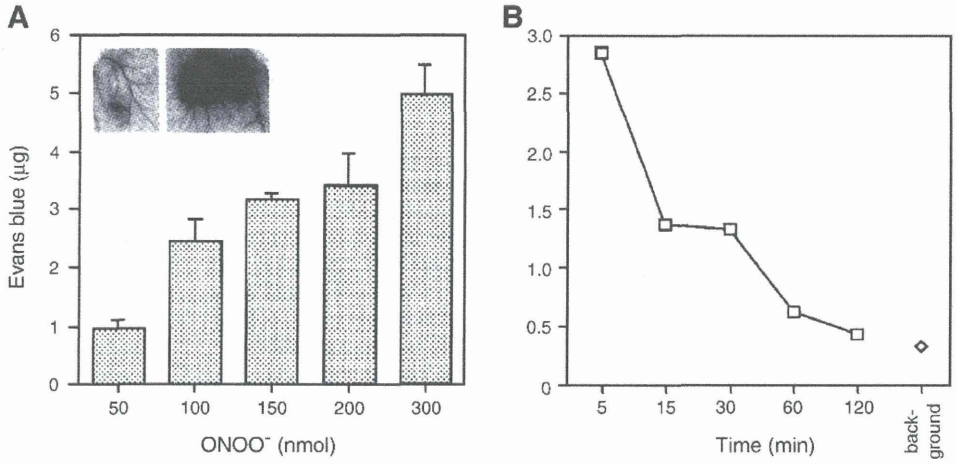

Fig. 6. Dose-dependent effect of $ONOO^-$ on vascular permeability of dorsal skin in normal mice (A) and duration of the enhancement of vascular permeability (B). (A) $ONOO^-$ was injected intradermally at the indicated concentrations. The inset shows authentic $ONOO^-$-induced vascular permeability in mouse skin (left: decomposed $ONOO^-$, right: $ONOO^-$ 100 nmol). (B) Evans blue (10 mg/kg) was given by i.v. injection at 10, 15, 30, 60, or 120 min after intradermal injection of 100 nmol $ONOO^-$ into dorsal skin; dye extravasated for 1 h.
From Ref. [79] with permission.

effect. Multiple mechanisms of enhancement of vascular permeability by $ONOO^-$ therefore exist and are a consequence of cross-talk in the vascular mediator network.

4.3. Prostaglandins

PGs are lipid compounds that are derived enzymatically from arachidonic acid by means of cyclooxygenases (COXs) [76,80]. Similar to bradykinin, PGs are important mediators in inflammation and can be upregulated by inflammatory cytokines (e.g., interleukin-1 and tumor necrosis factor-α) as well as kinin [109,110]. Among the various PGs, PGE_1 and PGI_2 exhibit effects similar to those of NO, i.e., preventing platelet aggregation, leukocyte adhesion, and thrombosis formation and facilitating extravasation and the EPR effect. As we anticipated, we found significantly decreased accumulation of the Evans blue/albumin complex in tumors after administration of a COX inhibitor to tumor-bearing mice [76]. Injection of a stable analogue of PGI_2, beraprost sodium (Dorner), which has a long plasma half-life in humans (1.1 h compared with several seconds for native PGI_2), resulted in significantly increased extravasation. Beraprost enhanced the accumulation of the Evans blue/albumin complex 2- to 3-fold [111]. An important and interesting finding was that systemic blood pressure did not change significantly, nor was blood flow of normal tissues and organs affected, whereas blood flow in tumors was dramatically suppressed (70–90%). Beraprost not only enhanced the EPR effect but also inhibited tumor growth [111]. The therapeutic potential and the possible diagnostic applications of beraprost thus warrant more detailed studies.

4.4. Angiotensin-converting enzyme (ACE) inhibitors

ACE inhibitors are a landmark class of drugs for hypertensive patients. They inhibit conversion of angiotensin I to AT-II by carboxypeptidase. The amino acid sequence of angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) is similar to that of bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) at the C-terminal end, so inhibition of ACE blocks degradation of bradykinin as well. ACE inhibitors therefore potentiate the pharmacological actions of kinin, which is a major vascular permeability factor as discussed above, the result being augmented extravasation of the Evans blue/albumin

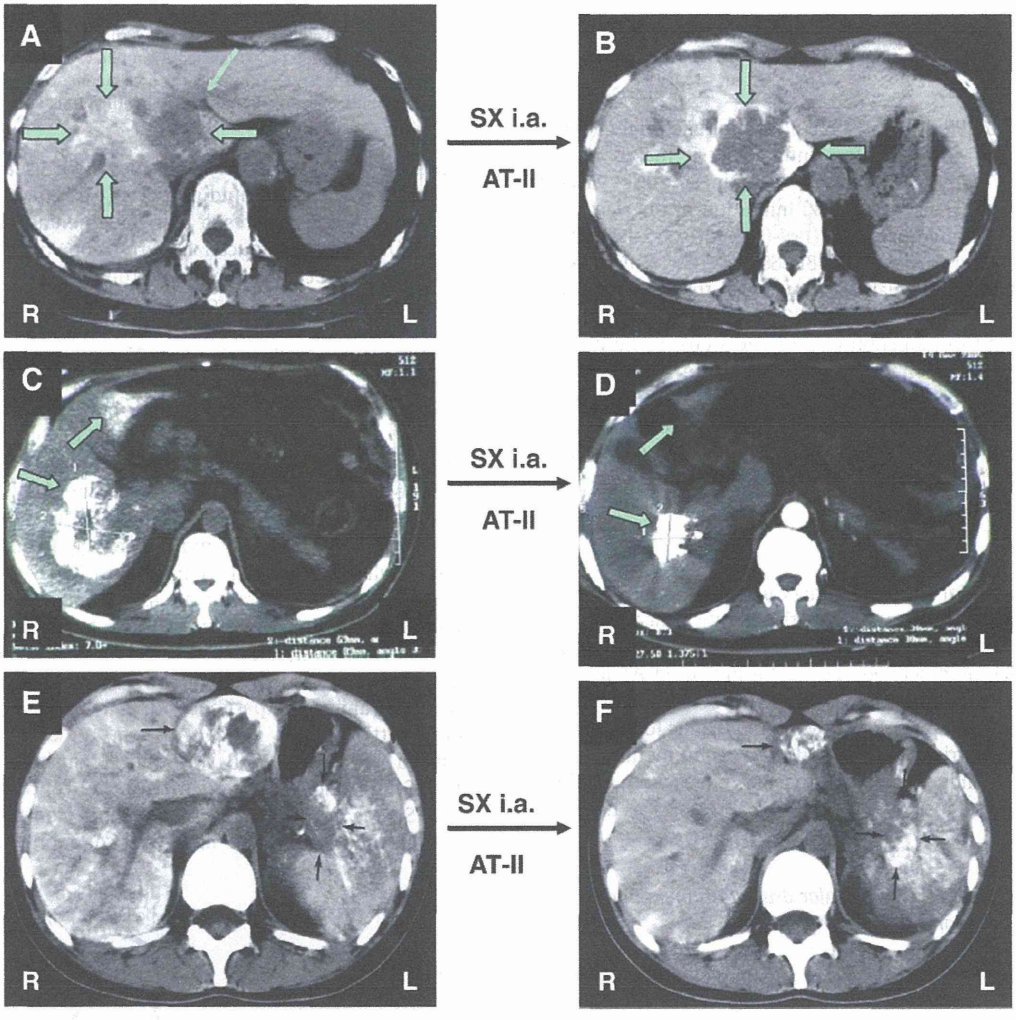

Fig. 7. Responses of human metastatic liver cancers to SMANCS/Lipiodol (SX/LP) under AT-II-induced hypertension. (A and B) CT scans of a patient with metastatic liver cancer after an i.a. injection of SX/LP under normotensive (A) and hypertensive (B) conditions. A clear difference can be seen from normotensive to hypertensive conditions in tumor uptake of SX/LP, as indicated by arrows pointing to high-density areas. B-type staining (i.e., a peripheral ring shape), which is usually seen in metastatic tumors on CT scans (B), indicates greater drug uptake under AT-II-induced hypertension. A massive metastatic liver cancer that originated from stomach cancer is shown in (C). At 50 days after SX/LP treatment, this liver cancer had regressed considerably (D). (E and F) A massive metastatic liver cancer originating from pancreatic cancer. A CT scan shows the metastatic mass in the liver (center front) and the primary pancreatic cancer (left middle) at the time of the first SX/LP infusion (E). The large metastatic mass at center front did regress markedly at 5 months after four SX/LP injections given under AT-II-induced hypertension (F). The primary pancreatic cancer did also take up SX/LP (F). R and L indicate the right and left sides of the patient, respectively. Modified from Ref. [19] with permission.

complex into tumor tissues, an effect similar to that of beraprost [66,111].

4.5. Vascular endothelial growth factor

VEGF is the angiogenesis factor that is highly upregulated in most tumors, and it has a crucial function in angiogenesis and solid tumor growth [60,81,83,84,112]. Folkman [56–58] and Ferrara and Henzel [82] played a most important role in the discovery and clinical development of VEGF. However, Dvorak's group first reported VEGF as VPF in 1983 [81,113]. Since those discoveries, researchers have conceived of an inhibitor of VEGF as being a useful therapeutic tool against solid tumors. After decades of research, inhibitors of VEGF have been developed as therapeutics, e.g., bevacizumab (Avastin) and ranibizumab (Lucentis). Other researchers and we previously reported that the amount of VEGF was higher (2- to 30-fold) in many implanted murine tumors than in most normal tissues and organs [60]. The degree of potency of VEGF in inducing extravasation (the EPR effect) was comparable to that of kinin in guinea pig skin seen after intradermal injection [60]. Additional studies by different groups indicated that VEGF action involves upregulating NO production [114,115].

4.6. Summary of EPR-related factors

Thus, briefly, the EPR effect is a result of multifactorial events *in vivo*, and interaction among factors influencing it makes it quite complicated. Like many inflammatory cytokines, these vascular factors affect each other via cross-talk. Inhibition of one factor, for example by the MMP inhibitors SI-27 (L-*N*-(*N*-hydroxy-2-isobutylsuccinamoyl)-leucyl-isobutylamide) and BE16627B (L-*N*-(*N*-hydroxy-2-isobutylsuccinamoyl)-seryl-L-valine), may block the EPR effect to a significant extent but not completely [79]. Activation of one factor, however, would lead to induction of multiple steps of the cascade, thereby involving activation of many factors. A greater than 1:1 correlation between the initial activation and the end result would occur, so that a significant EPR effect would rapidly ensue. Studies of these factors may therefore help development of new strategies to modulate the EPR effect, angiogenesis and thereby tumor growth.

5. Augmentation of the EPR effect

Many macromolecular anticancer drugs are being developed on the basis of the EPR effect. To improve the therapeutic efficacy of these drugs, we focused on the unique pathophysiological features associated with the EPR effect, as described above. We first used AT-II, which produces systemic hypertension. Under these hypertensive conditions, a macromolecular drug is pushed out by hydrodynamic forces into the interstitial space or matrix of tumor tissues. A second method utilizes NO-releasing compounds to enhance the EPR effect. Both methods exhibited favorable therapeutic effects in clinical settings, as described here.

5.1. Increased delivery of macromolecular drugs to tumors under AT-II-induced hypertension

Tumor blood vessels usually lack a smooth muscle layer or pericytes needed for vasoconstriction, so tumor blood vessels show very little response to infusion of AT-II, whereas blood vessels of normal tissues show constriction (hence hypertension). One would expect, therefore, that during induction of the hypertensive state by AT-II, normal blood vessels would constrict but tumor blood vessels would be open, which would facilitate the vascular leakage. The outcome would be increased blood flow volume in tumor tissues and hence increased drug delivery.

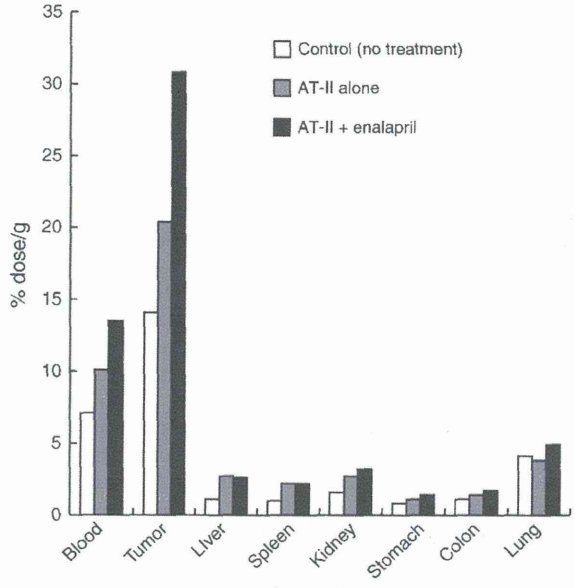

Fig. 8. Effects of AT-II alone or AT-II plus enalapril on accumulation of macromolecular drugs. Tissue distribution of ^{125}I-labeled A7 antibody 3 days after i.v. injection. Nude mice ($n=4$) bearing SW1116 human colon carcinoma cells were treated with AT-II alone or AT-II plus enalapril. From Ref. [131] with permission.

In such situations, macromolecules such as albumin, SMANCS, micelles, liposomes and lipid particles (e.g., Lipiodol) leak out more from tumor vessels, the result being augmentation of the EPR effect [19,29]. Under AT-II-induced hypertension, blood flow volume in normal tissues, in contrast to that in tumor vessels, remains constant by means of an autoregulatory mechanism, even though the blood vessels are constricted. This important tumor-selective phenomenon was first reported by Suzuki et al. [62] in 1981. They first applied this method of hypertensive chemotherapy with conventional, low-molecular-weight anticancer agents, but with little success. Even though AT-II-induced hypertension should increase drug delivery, the tumor accumulation was maintained for no more than 15 min for low-molecular-weight agents, as we found for [^{14}C]methylglucose delivered to tumors in a rat model [116]. The therapeutic benefit is thus quite limited for low-molecular-weight anticancer drugs. In contrast, when we utilized AT-II-induced hypertension with macromolecular drugs, we observed an approximate 2-fold increase in the EPR effect, as well as less adverse effect to normal tissues including bone marrow, liver, kidney and colon [19,29,116]. Not only has augmentation of the EPR effect by means of AT-II proved effective in preclinical (animal) experiments, but it has also been validated in clinical settings with difficult-to-treat tumors, even those of an advanced stage [19]. AT-II was utilized during an i.a. infusion of SMANCS for patients with such advanced cancers. For these cases, SMANCS was formulated with the lipid contrast agent Lipiodol (SMANCS/Lipiodol), which has been commonly used in Japan to

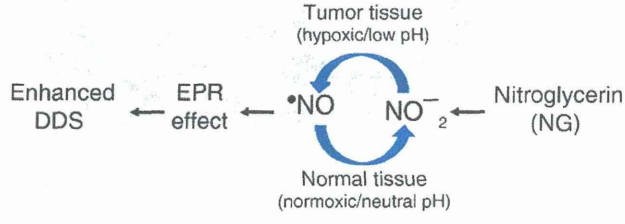

Fig. 9. Mechanism of NO generation from nitroglycerin in tumor. NO was generated from NO_2^- predominantly in hypoxic tumor tissues compared with normal tissues. DDS, drug delivery system.
From Ref. [121] with permission.

treat hepatocellular carcinoma (HCC) since 1993, although under normotensive conditions.

To augment drug delivery, AT-II was first given by slow i.v. infusion to induce a hypertensive state (i.e., to increase mean arterial blood pressure by 20–25%, for example, from a systolic blood pressure of 110 mm Hg to 150 mm Hg). The drug was then administered by i.a. infusion (via Seldinger's method). With this method, we obtained a marked therapeutic effect, not only for HCC but also for metastatic liver cancers [19]. A few typical examples are described here. As Fig. 7A shows, with SMANCS/Lipiodol infusion first performed under normotensive conditions for colon cancer metastatic to the liver, only a very limited area of the tumor mass evidenced staining, and most of the other area (especially the central part of the tumor) was dark (i.e., was of low density on the CT scan), which suggests no uptake of SMANCS/Lipiodol under normotension. However, 1 week later, after SMANCS/Lipiodol was administered under AT-II-induced hypertension, marked staining with SMANCS/Lipiodol was evident, especially at the tumor periphery (Fig. 7B). This typical pattern of staining of metastatic tumors is classified as B-type staining [18]. This result demonstrates the central hypovascularity of the tumor. After 3 months, another infusion of SMANCS/Lipiodol was performed under AT-II-induced hypertension, which produced a marked accumulation of the drug, a reduction in levels of tumor markers (carcinoembryonic antigen and immunosuppressive acidic protein), and an improved performance status of the patient; in about 8 months, the tumor volume was one-fifth of the original [19]. The second example shown here confirmed a marked therapeutic effect of similar treatment (under hypertension) used for a patient with massive metastatic liver cancer originating from stomach cancer (Fig. 7C,D). Still another patient with metastatic liver cancer originating from pancreatic cancer (Fig. 7E,F) also evidenced marked regression of the tumor mass (to about 15% of the original) after four SMANCS/Lipiodol injections in 5 months. In this last patient (Fig. 7E,F), the original pancreatic cancer also accumulated SMANCS/Lipiodol. AT-II-induced hypertensive conditions produced complete filling of SMANCS/Lipiodol even in a hypovascular pancreatic tumor (Fig. 7F). These and many other examples confirmed that i.a. SMANCS/Lipiodol administered under AT-II-induced hypertension could augment the EPR effect and drug delivery and thereby induce a remarkable therapeutic effect. Similar results were observed with gallbladder, kidney and other intractable cancers. AT-II-induced hypertension can therefore be a powerful tool for augmenting the EPR effect, and we anticipate great improvements in results with macromolecular anticancer therapeutics under AT-II-induced hypertension.

In our previous unpublished studies, when AT-II was combined with the ACE inhibitor enalapril, which blocks kinin degradation, the accumulation of macromolecular drugs in tumors was significantly increased, which indicated a synergistic and/or additive effect (Fig. 8); enalapril had no influence on blood flow in normal tissues. The clinical importance of AT-II-induced hypertensive chemotherapy may thus be

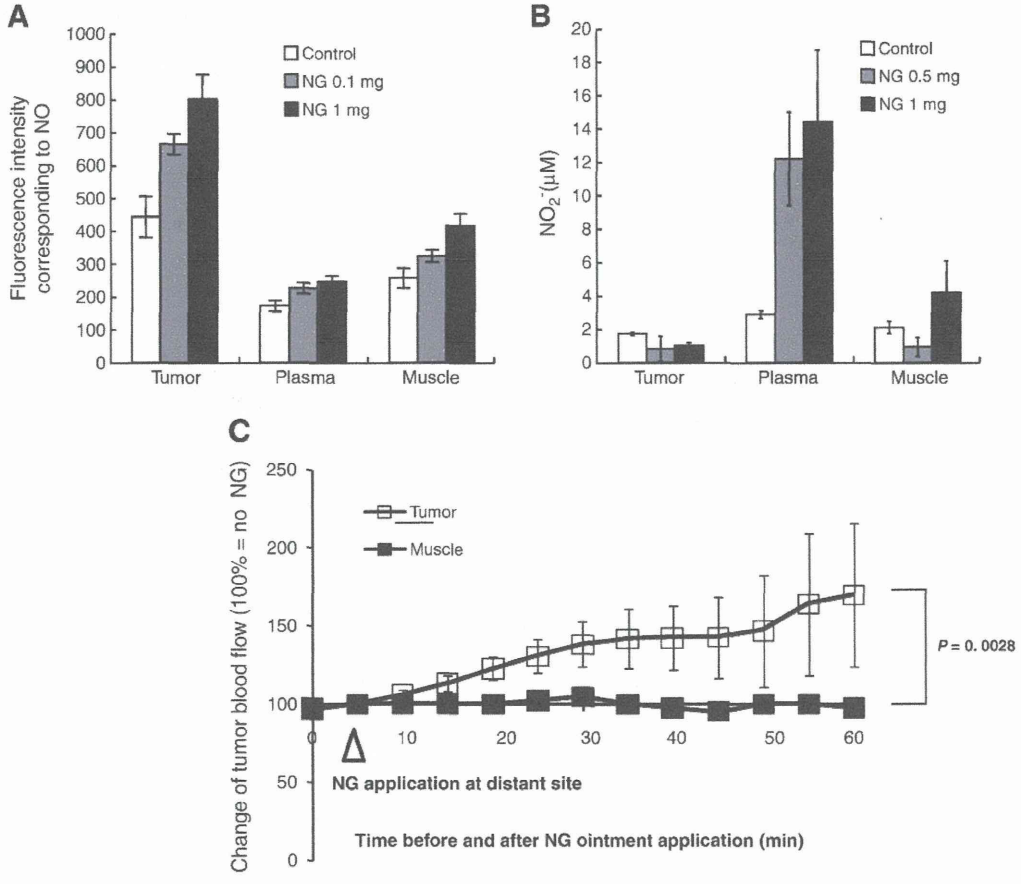

Fig. 10. NG-induced intratumor production of NO (A) and NO_2^- (B) and increased tumor blood flow (C). NG ointment was applied to S-180 tumor-bearing mice on the skin distal to the tumor at the indicated concentrations. After specified intervals, mice were killed and tumors were collected and homogenized. NO was detected in the supernatant of tumor tissue homogenate by using the fluorescent probe DAF (A) or by measuring NO_2^- with HPLC (B). (C) Blood flow in normal tissues and in tumors with diameters of 6–7 mm was measured with a laser Doppler flowmeter, after mice had been anesthetized and placed on a warm pad (30–35 °C). Blood flow was monitored for the first 5 min to confirm that it was stable, and then NG at dose of 1.0 mg/tumor was applied on the tumor. Blood flow in the thigh muscle was measured after application of the same dose of NG on the skin. Modified from Ref. [121] with permission.

further improved by including an ACE inhibitor, which is known to be a very safe, routinely used drug. Additional investigations of this method are warranted.

5.2. Enhanced delivery of drugs to tumors by means of NG

Because NO is a major factor that facilitates the EPR effect, we expected that the EPR effect or tumor-targeted delivery of macromolecular anticancer drugs might be enhanced by use of NO or NO-releasing compounds.

With reference to a separate but related topic, even though Jordan et al. [117] did not discuss drug delivery or the EPR effect, they reported that isosorbide dinitrate (ISDN) increased the partial oxygen pressure (pO_2) in tumor tissues as related to increased blood flow, and Mitchell et al. [118] showed that NO_2^- increased tumor cell radiosensitivity because of increased tissue pO_2.

In our laboratory, we investigated NG and ISDN to determine whether they would enhance the EPR effect. NG is a well-known NO-generating agent and has been used as medication for angina pectoris for more than a century. In cardiac infarct tissue, NO_2^- is first liberated from NG and is then converted to NO under hypoxic conditions (Fig. 9) [119,120]. Vasodilatation and increased blood flow can thereby be attained in infarcted tissues. The pO_2 in cardiac infarct tissue is known to be low, and the pH is slightly acidic [117,118]. These conditions are similar to those in cancer tissues, which are hypoxic and slight acidic. We thus hypothesized that NG may induce the same processes in tumor tissues as in cardiac infarct tissues (Fig. 9). If the same mechanism does operate in solid tumors, macromolecular drug delivery (the EPR effect) and hence therapeutic efficacy of these drugs may be enhanced by applying NG.

As we hypothesized, we obtained astonishing results in various rodent tumor models [121]. We first quantified the amount of NO in tumor and normal tissues after NG treatment, by using an NO-specific fluorescent agent, diaminofluorescein (DAF) [122]. Administration of NG induced a significant (e.g., 2 folds) increase in NO dose-dependently in tumor tissue, whereas normal tissues showed no significant increase (Fig. 10A). Similarly, NO_2^- production in tumor tissue after NG administration increased in a dose-dependent manner, as quantified by using Griess reagent (Fig. 10B), and the high level of NO_2^- lasted at least for 3 h (data not shown). Consistent with the increased NO level, blood flow in tumor tissue was significantly elevated (Fig. 10C) [121]. This finding is similar to that for ISDN in the report mentioned previously [117]. These results in tumor-bearing animals are therefore analogous to the effects of NG seen in cardiac patients.

Subsequently, when tumor-bearing mice received topical applications of NG ointment, over the tumor or on skin opposite or distal to the tumor site, at doses of 1.0 μg/mouse to 1.0 mg/mouse, the EPR effect was greatly increased in a dose-dependent manner. Namely, NG treatment significantly augmented accumulation of both the Evans blue/albumin complex and the macromolecular anticancer drug PEG-conjugated zinc protoporphyrin (PZP) in all tumors including S-180, Meth-A and colon-38 in mice, and breast cancer in rats induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) [121]. The NG-enhanced EPR effect lasted more than 24 h after a single application [121], which was consistent with the sustained efficacy of NG given to patients for angina pectoris. Consequently, the therapeutic effect of PZP was markedly enhanced when PZP was combined with NG in all the mouse tumor models (Fig. 11) [121]. The NG/NO-enhanced drug delivery to tumor and hence improved antitumor effect were found as well with the anthracycline antitumor agent aclarubicin (Fig. 11B), which is a conventional low-molecular-weight drug [121]. It is

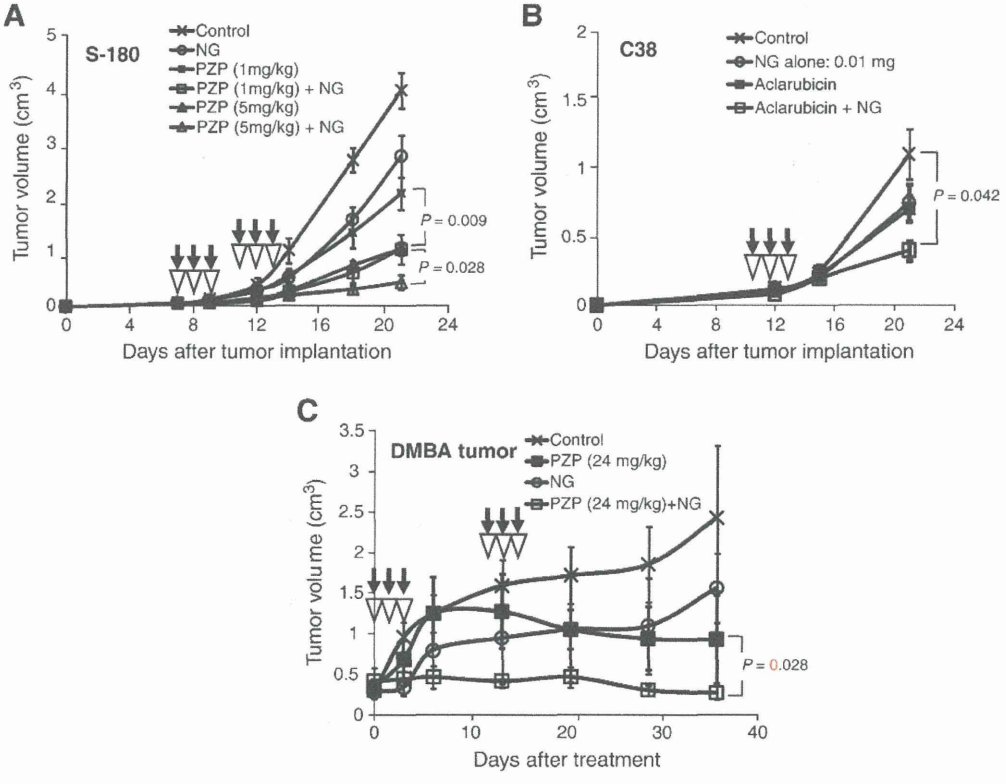

Fig. 11. Enhancement of the therapeutic effect of antitumor drugs by NG treatment. (A) The effect of the combination of NG (0.1 mg/tumor) and the macromolecular drug PZP against S-180 solid tumors. (B) The effect of NG (0.01 mg/tumor), alone and in combination with the low-molecular-weight drug aclarubicin (5 mg/kg), against colon-38 solid tumors. (C) Therapeutic effect of PZP plus NG (0.1 mg/tumor) in DMBA-induced breast tumors in rats. Black arrowheads indicate the times of drug administration; open inverted triangles indicate the times of NG treatment.
Modified from Ref. [121] with permission.

intriguing that NG alone also had significant suppressive effects on tumor growth (Fig. 11B) [121]. All these findings therefore suggest that use of NG may become a promising strategy for cancer treatment in patients, to enhance the effect of not only macromolecular anticancer drugs but also conventional chemotherapeutics.

The combination therapy of NO with anticancer drug was also reported by Passut et al. by using a polymer–drug conjugates carrying epirubicin (EPI) and NO (EPI-PEG-NO) that co-release the anticancer drug EPI and NO [123,124]. Even though no enhancement of EPR effect was discussed, they showed that NO released from EPI-PEG-NO inhibited cellular respiration followed by mitochondrial membrane depolarization and cell death in cancer cells but not in normal cells, which significantly increased the cytotoxic (antitumor) effect of EPI whereas remarkably reduced the cardiac toxicity of EPI [123–125]. Moreover, in clinical settings, Yasuda et al. [126–128] and Siemens et al. [129] independently reported direct therapeutic benefits of NO donors. They indicated that the possible anticancer mechanism of NO is probably due to down-regulation of hypoxia-inducible factor-α, VEGF and P-glycoprotein. Yasuda et al. [126,127] demonstrated clinical benefits of NG in a randomized phase II study with patients with non-small cell lung cancer, in which NG significantly increased chemosensitivity to conventional anticancer drugs such as docetaxel, carboplatin, vinorelbine and cisplatin. Siemens et al. [129] analyzed the effect of an NO donor on prostate cancer patients after primary therapy (either surgery or radiotherapy) and showed that the doubling time of the prostate-specific antigen value was significantly extended with NG treatment. These reports suggest not only that NG/NO may show synergistic and/or additive effects with other anticancer drugs, probably by augmenting tumor drug delivery, but also that NO itself appears to have a tumor-suppressive effect.

These findings together indicate that AT-II-induced hypertension and NG-derived NO can augment the EPR effect and hence the chemotherapeutic effect of macromolecular drugs. Further investigations of this new anticancer strategy are consequently warranted.

6. Other issues related to the EPR effect

The discussion just presented indicates that the EPR effect is clearly understood to improve targeting of drugs to tumors. However, the EPR effect has other consequences: for example, it facilitates the transport to tumors of nutrients and oxygen that sustain rapid tumor growth. Tumor growth can thus be suppressed by inhibiting or blocking the EPR effect in tumor tissues, i.e., by reducing angiogenesis and extravasation. Certain drugs in clinical use may suppress the EPR effect, for example, VEGF inhibitors such as bevacizumab and ranibizumab, as described above.

Also, with regard to the kallikrein–kinin cascade, Maeda's group reported suppression of ascitic and solid tumors by use of a kallikrein inhibitor (SBTI, Kunitz type) and the kinin antagonist HOE-140 [73–76]. In a mouse S-180 ascitic tumor model, intraperitoneal (i.p.) administration of SBTI significantly reduced the ascitic volume (Fig. 12A) [74]. Similarly, HOE-140 treatment (i.p. injection) significantly inhibited accumulation of ascites in tumor-bearing mice (Fig. 12B), as well as significantly increasing the survival of these mice (Fig. 12C). Furthermore, a therapeutic effect was found for HOE-140 in S-180 solid tumor model: HOE-140 treatment caused an approximate 50% reduction in tumor volume (Fig. 12D) [76]. These findings suggest that inhibition of

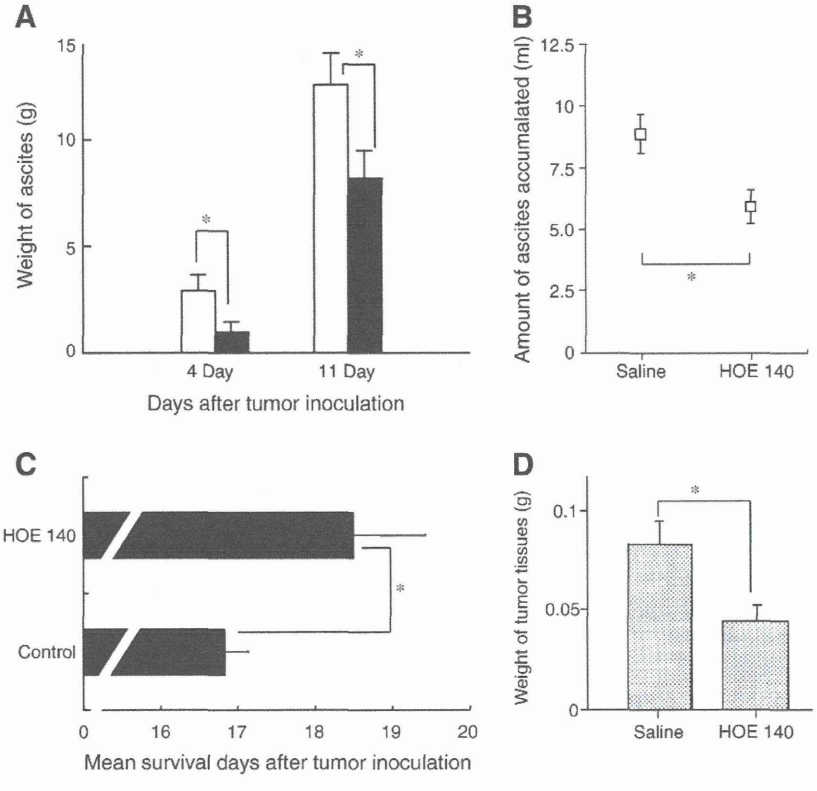

Fig. 12. Effects of SBTI and HOE-140 on tumor growth and survival of mice. (A) SBTI (3 mg/mouse per day i.p.) was administered for 10 days beginning from the day of S-180 ascitic tumor injection. White and black columns indicate the control group and the SBTI-treated group, respectively. (B) HOE-140 was given at a dose of 13 μg/kg i.p. starting immediately after tumor injection, with ascitic fluid collected 10 days after tumor injection. The effects of this HOE-140 treatment on survival of ascitic tumor-bearing mice (C) and S-180 solid tumor growth in mice (D) are shown. *$P<0.05$.
Modified from Refs. [74] and [76] with permission.

the EPR effect, by using agents such as HOE-140 and SBTI, may produce an antitumor effect via an alternative mechanism. In patients with carcinomatosis, ascitic or pleural effusion is one of the most dangerous conditions; however, it is often overlooked in drug development. The findings that we just discussed may suggest a clue for the solution to this problem.

7. Conclusions

Macromolecular anticancer drugs are receiving more attention than ever in cancer chemotherapy, because the most important mechanism for targeting of drugs to tumors—the EPR effect—would improve therapeutic efficacy and reduce adverse effects, compared with conventional chemotherapy with low-molecular-weight drugs. The EPR effect is the unique and most crucial phenomenon occurring in tumor tissues, in that it accounts for the anatomical and pathophysiological characteristics of tumor blood vessels. The EPR effect is mediated by various upregulated vascular factors such as kinin, NO, VEGF and PGs. A strategy to augment the EPR effect and hence anticancer drug effects by modulating these factors seems reasonable. Among methods to augment the EPR effect, AT-II-induced hypertension has been validated as effective in both experimental and clinical studies. Also, the NO-releasing compound NG is promising for enhancing both the EPR effect and consequent therapeutic effects of anticancer drugs, especially macromolecular drugs.

In summary, the EPR effect is the gold standard for macromolecular anticancer drug design. However, its limitations—such as the PEG dilemma and the heterogeneous consequences of the effect—must be addressed, perhaps by making use of different strategies such as SMA micelles. For example, we found better cellular uptake (about 5-fold better) for SMA micelles than for PEG micelles. We thus anticipate great progress in the development of macromolecular therapeutics because of the advantages of the EPR effect.

Acknowledgements

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Intracellular uptake and behavior of two types zinc protoporphyrin (ZnPP) micelles, SMA-ZnPP and PEG-ZnPP as anticancer agents; unique intracellular disintegration of SMA micelles

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ABSTRACT

SMA-ZnPP and PEG-ZnPP are micellar drugs, encapsulating zinc protoporphyrin IX (ZnPP) with styrene maleic acid copolymer (SMA) and covalent conjugate of ZnPP with polyethylene glycol (PEG) respectively. Their intracellular uptake rate and subcellular localization were investigated. We found SMA-ZnPP showed higher and more efficient (about 2.5 times) intracellular uptake rate than PEG-ZnPP, although both SMA-ZnPP and PEG-ZnPP micelles were localized at endoplasmic reticulum (ER) and inhibited the target enzyme heme oxygenase 1 (HO-1) similarly. Both micellar ZnPP were taken up into the tumor cells by endocytosis. Furthermore SMA-ZnPP and PEG-ZnPP were examined for their drug releasing mechanisms. Liberation of ZnPP from the SMA micelle appears to depend on cellular amphiphilic components such as lecithin, while that for PEG-ZnPP depends on hydrolytic cleavage. These results indicate that these micelle formulations make water insoluble ZnPP to water soluble practical anticancer agents.

1. Introduction

Heme oxygenase-1 (HO-1), also known as heat shock protein 32 (HSP 32), is a stress related protein, that is involved in cellular defense against oxystress. HO-1 is induced by various stimuli such as ultraviolet radiation, oxidative stimuli, metalloporphyrins, heavy metals, nitric oxide and the others [1–4]. This phenomenon is ubiquitously seen in mammalian cells including normal cells and cancer cells. It is intriguing that most cancer cells lack cellular defense systems against oxidative stress such as catalase, superoxide dismutase, glutathione peroxidase and the others. Alternatively cancer cells rely on either HO-1 alone or with glutathione S-transferase against oxystress for survival by defending cells [5,6]. Under these circumstances, it was reported that inhibition or suppression of HO-1 activity by a specific inhibitor or siRNA leads to cancer selective cell death [7–9]. It was also reported that enzymatic activity of HO-1 seemed to be located at the endoplasmic reticulum (ER) in cells and anchored via the single transmembrane peptide segment located at the C-terminus of HO-1 protein [10].

In zinc protoporphyrin (ZnPP), zinc atom is coordinated in the center of protoporphyrin IX, and we found that ZnPP becomes one of the specific inhibitors against HO-1 and exhibits a cytotoxic effect against various cancer cells [8,9,11]. However water insoluble property of ZnPP hampers its therapeutic application. To solve this problem we have developed water soluble ZnPP derivatives, polyethylene glycol (PEG) conjugated ZnPP (PEG-ZnPP) and ZnPP encapsulated with styrene maleic acid copolymer (SMA) micelle (SMA-ZnPP) (Fig. 1A and B) [9,11]. PEG-ZnPP and SMA-ZnPP micelles were exhibited to have mean particle size of around 180 nm and 50 nm, respectively. These biocompatible macromolecular drugs in general, for example SMANCS (SMA-neocarzinostatin), SMA-THP (SMA-pirarubicin) micelle, PEG-DAO (PEG-conjugated D-amino acid oxidase), SMA-AHPP (4-amino −6-hydroxypyrazolo[3,4-d]pyrimidine SMA conjugate) and the other tend to accumulate preferentially at tumor site by the mechanism called enhanced permeability and retention (EPR) effect [12–18].

Most macromolecular drugs are transported into cells by active transport mechanisms, namely, endocytosis [19–22], where clathrin dependent, caveolae mediated and fluid phase endocytosis pathways are well known in the endocytosis pathway of eukaryotic cells, as a common cellular internalization pathway of macromolecular drugs. Cellular internalization is the critical step to exert therapeutic effect, where both receptor dependent and independent endocytosis are well investigated for macromolecular drugs. So far, to facilitate the cellular uptake of macromolecular drugs, or to increase selectivity to target cancer cells, the specific tissues, or specific receptors for such as transferin, asialoglycoprotein, folate, epidermal growth factor and chemokine, are frequently utilized [23–25].

Cellular internalization of SMA-micells or the PEG-ZnPP conjugates, and further delivery of free ZnPP to specific intracellular target

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