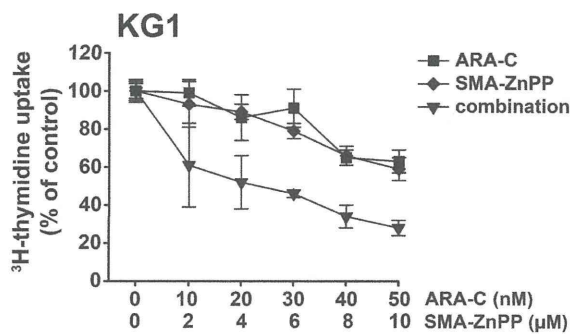
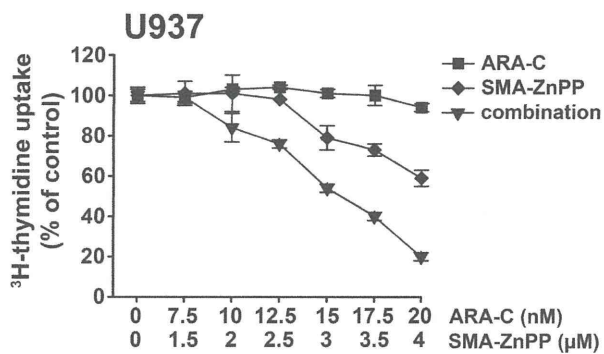


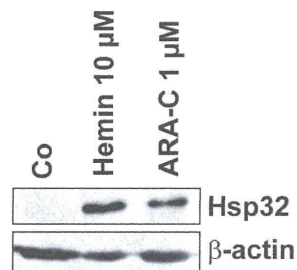
Herrmann et al, Figure 5A



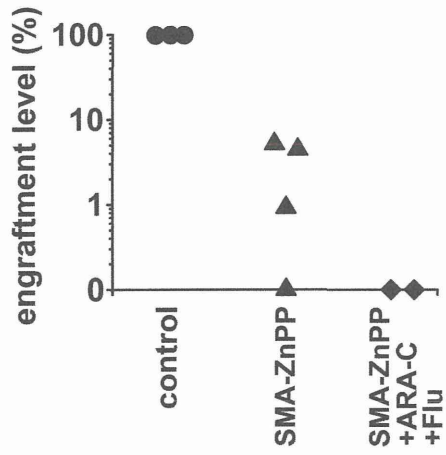
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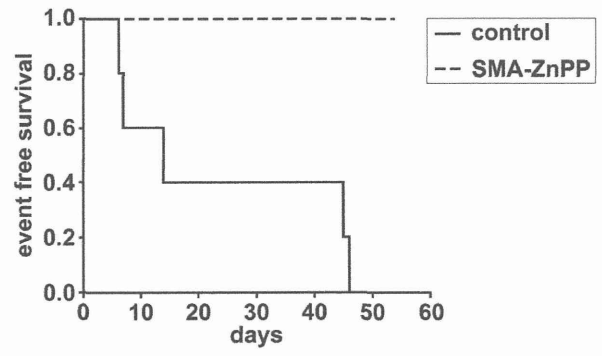
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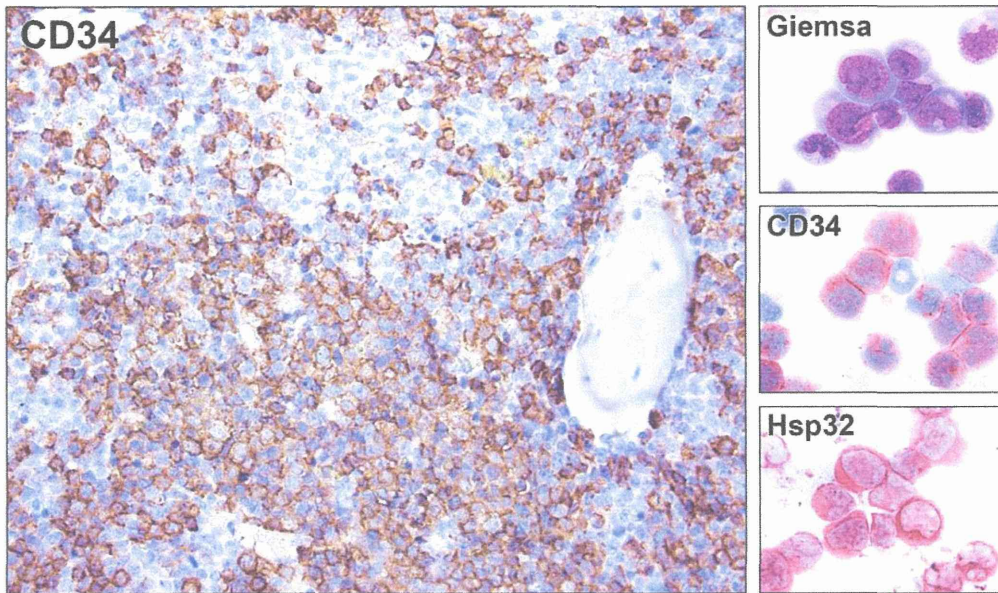
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Herrmann et al, Figure 6A



Herrmann et al, Figure 6B



Herrmann et al, Figure 6C

## Review

**Vascular permeability in cancer and infection as related to macromolecular drug delivery, with emphasis on the EPR effect for tumor-selective drug targeting**By Hiroshi MAEDA<sup>\*1,†</sup>

(Communicated by Takashi SUGIMURA, M.J.A.)

**Abstract:** Tumor and inflammation have many common features. One hallmark of both is enhanced vascular permeability, which is mediated by various factors including bradykinin, nitric oxide (NO), peroxyinitrite, prostaglandins etc. A unique characteristic of tumors, however, is defective vascular anatomy. The enhanced vascular permeability in tumors is also distinctive in that extravasated macromolecules are not readily cleared. We utilized the enhanced permeability and retention (EPR) effect of tumors for tumor selective delivery of macromolecular drugs. Consequently, such drugs, nanoparticles or lipid particles, when injected intravenously, selectively accumulate in tumor tissues and remain there for long periods. The EPR effect of tumor tissue is frequently inhomogeneous and the heterogeneity of the EPR effect may reduce the tumor delivery of macromolecular drugs. Therefore, we developed methods to augment the EPR effect without inducing adverse effects for instance raising the systemic blood pressure by infusing angiotensin II during arterial injection of SMANCS/Lipiodol. This method was validated in clinical setting. Further, benefits of utilization of NO-releasing agent such as nitroglycerin or angiotensin-converting enzyme (ACE) inhibitors were demonstrated. The EPR effect is thus now widely accepted as the most basic mechanism for tumor-selective targeting of macromolecular drugs, or so-called nanomedicine.

**Keywords:** enhanced permeability and retention (EPR) effect, infection and cancer, nanomedicine, vascular permeability, tumor targeted drug delivery, nitric oxide/super oxide

**1. Introduction**

In the 1980s–1990s, we demonstrated a mechanism that mediates extravasation upon bacterial infection via the activation of protease cascades involving kallikrein system that generates bradykinin (kinin).<sup>1)–6)</sup> Subsequently we found a similar cascade of kinin generation did involve in vascular permeability in the solid tumor.<sup>7)–10)</sup> Concomitant generation of nitric oxide (NO) was also discovered. Then prostacyclins were also found involved in vascular permeability in solid tumors as was also the case in inflamed tissues.<sup>11)–16)</sup>

This finding was important because of our interest of drug delivery to solid tumor using macromolecular drugs, more specifically polymer [SMA] conjugated neocarzinostatin [NCS] designated as SMANCS.<sup>17),18)</sup> SMA is a synthetic polymer of styrene-co-maleic acid of about 1.2 kDa with high lipophilicity, which was covalently conjugated to NCS via amide bond. SMA also conferred albumin binding capacity to SMANCS, as well as quite different pharmacological properties *in vivo*. Using this polymer conjugate macromolecular drug SMANCS, we found its remarkable accumulation in the tumor tissue. Then we clarified that it was extravasated more selectively in solid tumor as was a case observed by bacterial infected site where blue albumin was extravasated due to kinin generation.<sup>8)–10)</sup>

Because of the high lipophilicity of SMANCS we also found that it could be solubilized in lipid contrast agent [Lipiodol<sup>®</sup>] (SMANCS/Lipiodol) (see

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discussion later). We further found that arterial injection of Lipiodol, or SMANCS/Lipiodol truly targeted tumor tissue selectively.<sup>19),20)</sup> There was no other method that can target an anticancer agent with so much selectivity.

In this review article, I will therefore discuss the unique vascular pathophysiology, microanatomy of tumors and the biochemical mechanisms involved in the enhanced permeability and retention (EPR) effect. More important, I also describe how we can augment the tumor selective drug delivery and hence improve the therapeutic efficacy using macromolecular anticancer drugs—the issue of greatest importance that addressed in this article.

## **2. Tumor selective macromolecular drug, SMANCS, and lipid formulation for arterial infusion; The method for pin-pointed tumor delivery**

Following the structural study of proteinaceous antitumor agent neocarzinostatin, or NCS, we synthesized a polymer (SMA) conjugated derivative of NCS, designated SMANCS in 1978.<sup>17),18)</sup> The main objective was to develop a drug that would accumulate in the lymphatic system utilizing SMA-component that would confer lipophilic as well as and macromolecular nature. In lymphology macromolecules or lipidic particle are known to be recovered via the lymphatic system.<sup>21)</sup> Therefore, a lymphotropic drug would be preferred character to deliver the drug to the lymphatics system, thus ideal drug against lymphatic metastasis.<sup>22),21)</sup> That is one of the major problems in cancer treatment.

When SMANCS dissolved in water was injected intravenously, it was accumulated predominantly in the lymph nodule.<sup>23)</sup> Furthermore, because of its lipophilic nature of SMANCS, I found it could be dissolved in the lipid contrast agent (Lipiodol<sup>®</sup>, iodinated poppy seed oil ethyl ester). In collaboration with Toshimitsu Konno, Surgery Department, Kumamoto University, we pursued arterial infusion of SMANCS/Lipiodol (SX/LP) into the tumor feeding artery of VX-2 tumor implanted in the rabbit liver to facilitate more tumor targeted delivery. When SMANCS/Lipiodol was infused into the hepatic artery, extraordinary high intratumor concentration was found. Namely the drug concentration in the tumor compared to that of blood was more than 2000 fold in the tumor.<sup>19)</sup> This pharmacological data of tumor uptake using SMANCS/Lipiodol was so remarkable, we pursued for its clinical development.

In the clinical front then, we had many advanced hepatoma patients in our hospital, and Professor Ikuzo Yokoyama of Department Surgery decided to undertake the evaluation of SX/LP for treatment of primary hepatoma, which exhibition unprecedented targeting and therapeutic response in VX-2 tumor. The method not only offers truly pin-point targeting drug delivery, but it also offers diagnostic information, such as exact tumor size, location, intrahepatic spread, and amount of drug delivered to tumor because the remaining of Lipiodol could be clearly detected by X-ray CT-scan.<sup>24)–26)</sup> This was the first example of theranostic modality in early 1980s, which is now becoming popular. This therapeutic method using SMANCS was later approved for hepatoma treatment in 1993 by the Japanese Government.

## **3. Discovery of the EPR effect of macromolecules in solid tumors and its elaboration**

We first observed prolonged tumor-selective accumulation of blue albumin (67 kDa) (Fig. 1A,B), which can be readily visualized by injecting Evans blue intravenously as a blue tumor.<sup>27)</sup> The amount of Evans blue-albumin in the tumor and other organs were quantified, and time dependent progressive accumulation was only seen in the tumor tissues (Fig. 2A). The tumor concentration of blue albumin was about 10 fold higher than that of blood at 145 hrs (Fig. 2A). This phenomenon was coined the enhanced permeability and retention (EPR) effect. We also confirmed the EPR effect by using radiolabeled serum proteins; IgG (170 kDa), transferrin (90 kDa), albumin (67 kDa), and ovalbumin (48 kDa). However, we did not observe the EPR effect with low molecular weight proteins such as ovomucoid (29 kDa) and neocarzinostatin (12 kDa).<sup>27)</sup>

To confirm the molecular weight dependency of the EPR effect, we used biocompatible synthetic copolymers of hydroxypropyl methacrylate (HPMA), which ranged in size from 4.5 to 800 kDa,<sup>28)–31)</sup> that was kindly provided by Professors Karel Ulbrich and Ruth Duncan. We reconfirmed our earlier findings: most biocompatible plasma proteins and HPMA polymers between 4.5 and 800 kDa exhibited the EPR effect (Fig. 3A,B).<sup>28)–33)</sup> A more detailed time course study showed that the tumor uptake of HPMA copolymers after intravenous injection was relatively rapid: some showed a marked tumor uptake within a few hours, while smaller HPMA copolymers, about 30 kDa or smaller, showed no EPR effect. Figure 3B illustrates the relationship among tumor uptake, plasma concentration, and renal



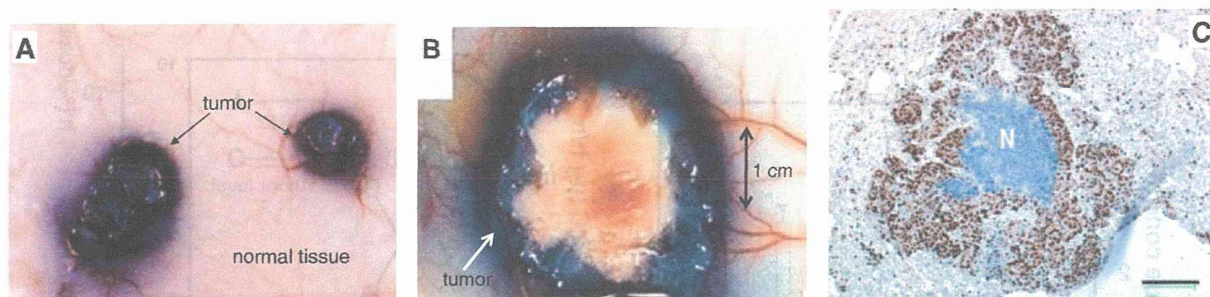


Fig. 1. (A and B): Extravasation of Evans blue-bound albumin in S-180 tumor in mice. Blue staining (extravasation) is homogeneous in A, but heterogeneous in B. Note the lack of leakage in the normal skin tissue in the background in A and B. (C) Heterogeneity of a micro tumor nodule at microscopic level (Ki-67 immunohistochemistry/DAB staining for viable cells: brown,  $\times 100$ ).<sup>44</sup> Note central portion of the nodule is necrotic (N) and the periphery is viable. Nodule size is about 0.5 mm in diameter. The tumor used was methylhydrazine-induced colon cancer in inbred CBA mice that was implanted in the spleen and allowed to metastasize to the liver (See refs. 35), 44), 84)).

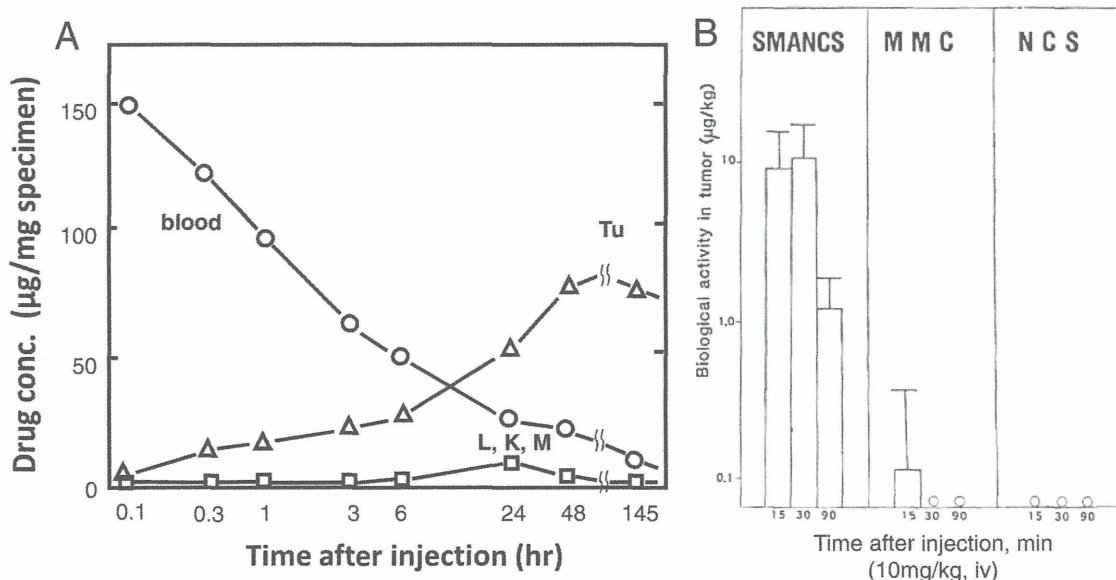


Fig. 2. (A) Quantitation of time dependent accumulation of Evans blue/albumin; in the tumor, blood and normal tissues (L, liver; K, kidney and M, muscle) (from ref. 27)). Evans blue (10 mg/kg) was injected i.v. and it will bind to plasma albumin. Levels of Evans blue in the blood and in the tumor (S-180 in mice) were inversely related, as the plasma level decreased, the tumor concentration increased. After 100 h or more, the T/B was about 10; ratios for normal tissues such as the liver (L), kidney (K), and muscle (M) to tumor were less than 1/40. (B) Tumoritropic accumulation of macromolecular anticancer agent SMANCS, and low molecular weight drugs mitomycin [MMC] and the parental drug neocarzinostatin [NCS] at different time point; at 15, 30, and 90 min in S-180 tumor in mice.

clearance in terms of drug size (MW). A relatively long circulation time after intravenous injection was needed for the EPR effect to occur. For progressive drug accumulation to tumor, high drug concentrations in circulation is needed. For albumin and transferrin (not shown), the T/B becomes much higher after 6 h or more (Fig. 2A).<sup>27)–30)</sup> Once these macromolecules accumulate in solid tumor tissues, they are not cleared within a few days or weeks, in

contrast to the situation in normal tissue. Biocompatible proteins and lipid particles are known to be removed from normal tissues via the lymphatic system in just days, however.<sup>19)–22),27)</sup> The prolonged retention time of macromolecular drugs in tumor tissue is thus another important aspect of the EPR effect.<sup>27),34)–36)</sup>

The upper size limit of macromolecular drugs exhibiting the EPR effect is actually quite large.

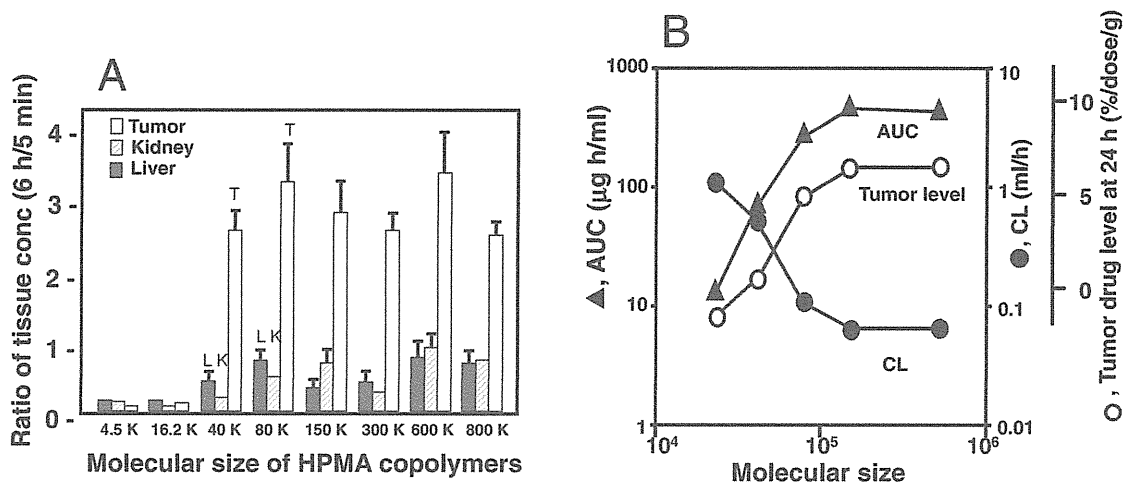


Fig. 3. Tumortropic uptake of biocompatible macromolecule HPMA (see text for detail). (A) The EPR effect is seen with HPMA copolymers (>40 kDa): tumor (□) compared to the liver (■) and the kidney (▨). Amount of tumor uptake is compared with 6h to that of 5 min. (B) Relationship between molecular sizes of putative polymeric drugs (HPMA) and their plasma levels (AUC, area under the concentration-time curve, ▲), tumor uptake, ○, and renal clearance rate (CL, ●) (Adapted from Noguchi *et al.*<sup>28</sup>).

Kimura *et al.*<sup>37</sup>) showed earlier that *Lactobacillus* sp., 1–2 μm in size, accumulated more in tumor tissue than in normal. Recently, Zao *et al.* and Hoffman<sup>38,39</sup>) showed that *Salmonella typhimurium* also preferentially accumulated in tumor compared with normal tissue. Konerding *et al.*<sup>40</sup>) and Hashizume *et al.*<sup>41</sup>) examined tumor vasculature and found that endothelial openings in the vessels could be as large as 4.7 μm (a mean of 1.7 μm) (Fig. 4D vs H) (see Section 4) which are not found in the normal vasculature. These results indeed agree well with the observation noted above that bacteria preferentially accumulated in tumor tissues after intravenous injection. Therefore the upper limit of molecular size to exhibit the EPR effect can be far greater than 10<sup>6</sup> Da, although the rate of intracellular uptake of such large molecules may not be as fast as that for 100-kDa.

With regard to the detection and imaging the tumor based on the uptake of low molecular weight radio contrast agents, which was infused into a tumor-feeding artery, we observed tumor staining lasted only short time (<1 min) in the angiogram. Tumors such as hepatoma and renal cell carcinoma can take up the contrast agent more efficiently than do normal tissues, resulting more tumor-selective staining, and hence detection. However, such X-ray images using a low MW contrast agent will disappear within 10s or so because of rapid diffusion of the contrast agent. This type of tumor selective

imaging may be called *passive targeting*, but such passive targeting has little value for therapeutic purpose, because conventional low MW anticancer agents also disappear within a few minutes or so.

What I emphasize here is that prolonged retention of drugs in tumor is critically important for therapeutic purpose, and that such retention is possible primarily with macromolecular or lipidic drugs. Arterial infusion of SMANCS/Lipiodol can aid tumor-selective imaging as well as drug delivery.<sup>19)–22),36)</sup>

#### 4. Microanatomy of tumor blood vessels and extravasation of macromolecular drugs: the cause of EPR effect

Morphology of blood vasculature in tumors and normal tissues is uniquely different. Microarchitecture of blood vessels can be visualized clearly by scanning electron microscopy (SEM) using vascular casts of plastic resin (Figs. 4A–H). Vascular image of metastatic liver cancer and colon cancer were elegantly described by Professor Paul E. O'Brien and his associates of the University of Melbourne<sup>42)–44</sup>) (Fig. 4 A/B, normal, and E/F, tumor), and by Professor Moritz Konerding of the University of Mainz (Fig. 4 D/E, normal, and G/H, tumor)<sup>40</sup>) respectively. One can notice a great contrast to the normal blood vasculature in Fig. 4 A/B (liver), C/D (colon) on the top vs tumor vasculature on the bottom (E–H). The metastatic tumor micronodule

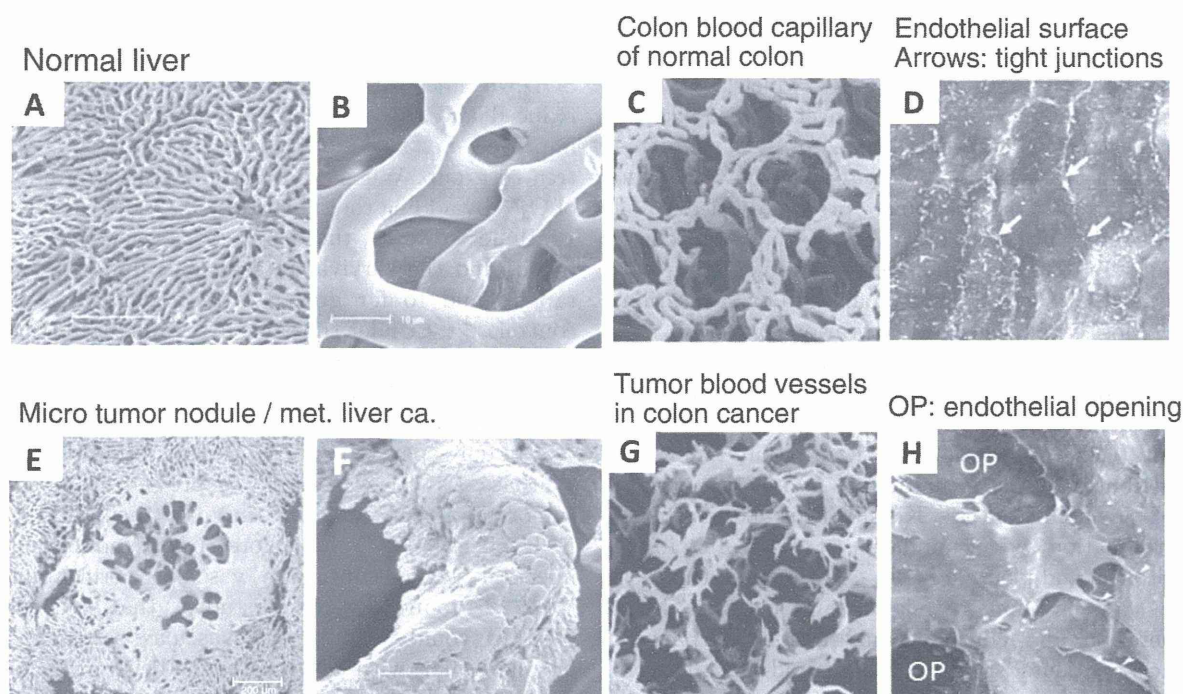


Fig. 4. Comparison of SEM images of blood vessels in normal healthy tissue (A–D on the top) and tumor tissues (E–H, lower figures) of metastatic tumor nodules in the liver from colon cancer. Blood vessels in healthy tissue in (A)–(C) show a clear, smooth, regular feature and no leakage of polymeric resin. In contrast the tumor vessels show polymer leakage (E) at the capillary level or in the early phase of leakage in (F). (See refs. 34, 35)). In (D) and (H) shown are SEM images of the luminal surface of normal blood vessel (D) which shows tight cell-cell junctions of endothelium, whereas blood vessels in the tumor (H) have large openings (OP in H) in the endothelium. (C) shows the vascular cast of normal colon with regularly shaped capillary, whereas an irregular vascular network can be seen in vascular cast of colon tumor (G) from ref. 36). (C), (D), (G), (H) courtesy of Prof. Konerding of Mainz.

in Fig. 4E is less than 0.5 mm in diameter, and it also demonstrates extravasation of the polymeric resin. This indicates clear architectural difference of neovasculature, compare with normal vessels. It should be noted that this neovasculature formation of tumor nodule is seen even as small as 200  $\mu\text{m}$ , much smaller than the size reported by Folkman: size of 2–3 mm.<sup>44)–47)</sup> Konerding *et al.*<sup>40)</sup> demonstrated large endothelial openings at the luminal surface of tumor vessels (Fig. 4H), and a lack of discrete arteriole-capillary-venule systems (not shown), while revealing the tight cell-cell junctions of vascular endothelial cells in normal vessels (Fig. 4D). Tumor blood vessels also lack smooth muscle cell layer. These structural abnormalities and vascular permeability factors make tumor vessels more leaky than vessels in normal tissue. Polymer resin is being extravasated (Fig. 4E) into the tumor nodule, or in the beginning of leakage process to the outside of blood vessels (Fig. 4F). These extravasations did not occur in normal tissues. Further SEM images of vascular casts

indeed demonstrate the facts of leakage of polymeric resin into tumor interstitium selectively, and a clear evidence of the defective functionality of tumor vessels. These defects make tumor vessels being highly permeable to albumin, IgG, and other macromolecules including the lipid contrast agent Lipiodol, and so called nanoparticles.<sup>24)–36),42)–44)</sup>

## 5. Factors involved in vascular permeability and the EPR effect in infection and solid tumors

**5-1. Bradykinin.** Kinin (bradykinin) is one of the most potent endogenous pain inducing nonapeptide, and it is continuously generated at sites of infection and cancer tissues by kallikrein and other proteases, from kininogen. However, it is degraded rapidly with a half-life ( $t_{1/2}$ ) of a few seconds in plasma by host proteases such as kininase and angiotensin converting enzyme (ACE) *in vivo*.

We studied the vascular permeability of bacterial proteases with regard to the pathogenic mechanism in 1980s, and found that all bacterial proteases



activate one or more steps in the kallikrein-kinin cascade.<sup>1)-4)</sup> Thereby extravasation of plasma components occurs and edema ensues, which are events similar to those occurring in cancer. The kallikrein-kinin cascade is stimulated as a result of activated kallikrein (or Hageman factor/Factor XII and prekallikrein), or microbial proteases cleave off bradykinin directly from either high-molecular-weight or low-molecular-weight kininogen in the plasma<sup>3)-11),13)</sup> which causes pain and extravasation.

Most cancer cells produce various proteases, *e.g.*, serine-type proteases, cathepsins, and collagenases. These proteases are also involved in kinin production and thus result in vascular permeability enhancement (the EPR effect). We found that kinin also contributes to the formation of ascitic and pleural fluids in carcinomatosis. Kinin is thus a key factor in extravasation and accumulation of ascetic fluid in the peritoneal and pleural cavity.<sup>7)-10)</sup> Based on this fact, one can envision a relationship between kinin generation and clinical manifestations of pain in cancer patients similar event found in infection. However, very few studies on this issue have been undertaken, and investigation of kinin antagonists in cancer patients appears interesting.

Dvorak *et al.* reported vascular permeability factor (VPF) of peptide nature that was generated by cancer cells.<sup>48),49)</sup> Later it was found identical to the vascular endothelial growth factor (VEGF).<sup>47),50)-53)</sup> This means that neoangiogenesis of tumor tissue is reflecting other side of the same coin, vascular permeability, to supply nutrients for tumor growth.<sup>52),53)</sup>

**5-2. Biological free radicals in vascular permeability in infection, cancer and related issues: ROS and RNS.** ROS (reactive oxygen species), such as superoxide anion radical ( $O_2^{\bullet-}$ ), and RNS (reactive nitrogen species) such as NO and peroxynitrite ( $ONOO^-$ ) are induced in viral and bacterial infections. In influenza virus infection, we found that a critical cause of viral pneumonia was attributable to the excessive generation of  $O_2^{\bullet-}$ .<sup>54)-56)</sup>  $O_2^{\bullet-}$  production was increased more than 300-fold in the lung or alveolar lavage fluid of the virus-infected mice compared with healthy control mice. The cause was attributable to the activation of xanthine oxidase (XO).<sup>54)-56)</sup> This finding indicates that ROS may be an important direct etiological agent in influenza infection. To confirm this possibility, we removed  $O_2^{\bullet-}$  from the site of infection *in vivo* by using a pyran polymer-conjugated derivative of Cu, Zn-superoxide dismutase (SOD); this derivative showed

a prolonged *in vivo*  $t_{1/2}$  of SOD, and sustained enzyme activity. When the polymer conjugated SOD was injected, the disease severity was markedly reduced and the survival rate of mice was greatly improved, *i.e.*, 97% survived vs. 3% of the SOD non-treated group. When we augmented the production of  $O_2^{\bullet-}$  by adding the substrate of XO, *i.e.*, inosine or xanthine, however, the disease was severely exacerbated, and the survival rate of mice dropped more rapidly and to far greater extent compared with controls.<sup>54),55)</sup> Under the same experimental settings, we found that NO was generated in parallel with the production of  $O_2^{\bullet-}$  in infection with influenza, herpes, and Sendai viruses and *Salmonella* sp. in mice.<sup>57)-62)</sup>

In our separate research, we had found that NO synthase (NOS) was highly expressed in a solid tumor, and demonstrated that NO facilitated the EPR effect.<sup>11)-13),63)</sup> Therefore, iNOS induction in infection would contribute vascular permeability. We<sup>11)-13)</sup> found in other studies that NO and  $ONOO^-$  could facilitate the vascular permeability of solid tumor, with  $ONOO^-$ , which being a reaction product of NO and  $O_2^{\bullet-}$  (*i.e.*,  $NO + O_2^{\bullet-} \rightarrow ONOO^-$ ). Then  $ONOO^-$  can activate pro-matrix metalloproteinases to generate active matrix metalloproteinases (proMMPs  $\rightarrow$  MMPs or collagenase).<sup>12)-14)</sup> This collagenase also facilitated vascular permeability.<sup>11)-13)</sup> These three components (NO,  $ONOO^-$ , collagenase) can thus enhance the vascular permeability of normal blood vessels as well as tumor blood vessels, as described below.

Investigations in the same line of research revealed that  $ONOO^-$ , in addition to affecting vascular permeability by itself and via activation of procollagenase ( $\rightarrow$  metastasis), it facilitates genomic mutation via oxidative cleavage of DNA/RNA or via nitration, oxidation and hydroxylation of guanosine at 8th position, with formation of 8-nitroguanosine and 8-oxoguanosine.<sup>60),62),64)-67)</sup> Furthermore, we found 8-nitroguanosine can be a preferred substrate of cytochrome  $b_5$  reductase (as well as the reductase domain of NOS),<sup>68)</sup> and thus, in the presence of NADPH it can generate superoxide, which reacts with NO generated by NOS, with concomitant formation of  $ONOO^-$ .  $ONOO^-$  then reacts with guanosine, which results in nitration of guanosine and formation of 8-oxoguanosine.<sup>65)-67)</sup> This type of propagation reaction involves a stoichiometry greater than 1:1 (Fig. 5), in that 8-nitroguanosine facilitates formation of both  $O_2^{\bullet-}$  and thus  $ONOO^-$ . The consequence of this process in solid tumor and inflamed tissue is an accelerated production of



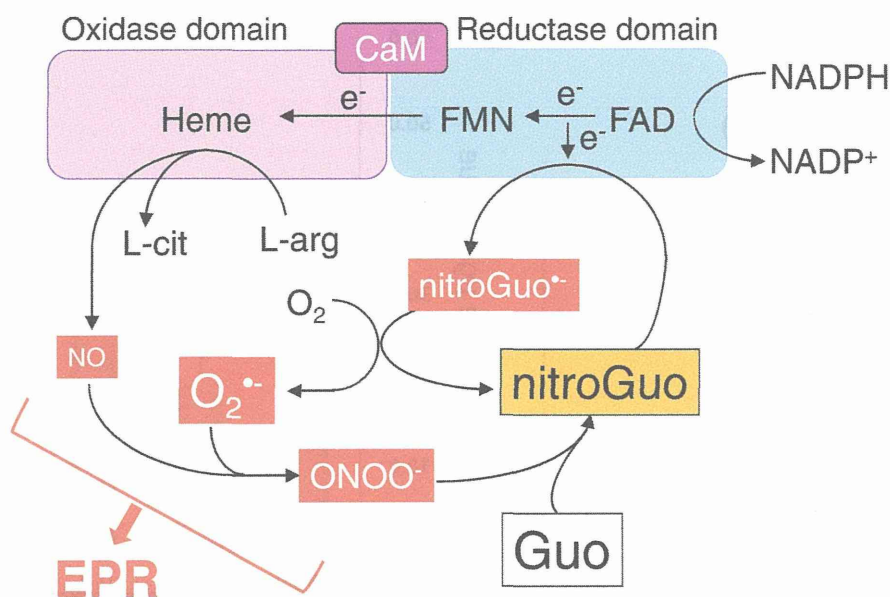


Fig. 5. Scheme of the reaction generating  $O_2^{\bullet-}$  via 8-nitroguanosine (nitroGuo) involving nitric oxide synthase (NOS) or cytochrome  $b_5$  reductase in the presence of oxygen and L-arginine.<sup>68)</sup> Simultaneous generation of NO and  $O_2^{\bullet-}$  results formation of  $ONOO^-$ , which nitrates guanosine to form 8-nitroGuo; it then becomes substrate of NOS (reductase domain) generating either NO or  $O_2^{\bullet-}$ , and this reaction continues on. NOS consists of an oxidase domain and a reductase domain. This reductase is similar to cytochrome  $b_5$  reductase that is responsible for  $O_2^{\bullet-}$  generation. CaM, calmodulin; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; Guo, guanosine; L-cit, L-citrulline.

mutations and thus genetic diversification. Under such circumstances, which generates great genetic diversification, however, it poses a problem against the development of molecular target drug. Recent advances in cancer genomics have provided ample evidence that many human solid cancers undergo disease progression for 10–30 years, and individual malignancies such as breast cancer or colon cancer often have numerous genetic alterations in the same patients.<sup>69)–71)</sup>

Molecular target drug development has aroused great expectations in the cancer treatment community. However, clinical responses to many of these drugs such as antibody drugs are no better than those of conventional anticancer agents.<sup>72),73)</sup> In addition to the very low response rate to these drugs, they are so expensive that the National Institute of Clinical Excellence (NICE) and other agencies in the UK expressed concern about their use,<sup>73)–75)</sup> notwithstanding one rather exceptional success of molecular target drug, imatinib used for chronic myelogenous leukemia.

In this context, tumor targeting via the EPR effect appears to have a more universal application to solid tumors in general, with improved drug delivery,

and thus better clinical outcomes (see, *e.g.*, the AT-II-based arterial infusion of SMANCS/Lipiodol section 6.2.1.).

**5-3. Prostaglandins, VEGF, carbon monoxide (CO), and cytokines.** Prostaglandins and VEGF will result in activation of NOS, and thus generating consequently NO, facilitate the EPR effect, as we described elsewhere.<sup>13),15),16),32)</sup> Other cytokines, including TNF- $\alpha$ , transforming growth factor- $\beta$  (TGF- $\beta$ ) inhibitor, IL-8, and other inflammatory factors,<sup>76)–78)</sup> may be also involved in the EPR effect involving various mechanisms. Typical examples of cross talk in such factors include bradykinin by inducing cyclooxygenase (COX) II, and eNOS activation<sup>30)–32),34),35)</sup> These cytokines cross talk each other and affect vascular tone.<sup>32),77)</sup>

Very recently, we found that HO-1 (heme oxygenase-1)<sup>79)</sup> is upregulated in many cancers. Consequently the product of HO-1, CO appears to be actively produced during catabolic degradation of heme. This process will facilitate vascular permeability of tumor vessels (Fig. 6A).<sup>79),80)</sup> We also found earlier that suppression of HO-1 leads to tumor regression.<sup>81)–83)</sup> Direct proof of CO as a factor of EPR effect is shown in Fig. 6B.<sup>80)</sup>

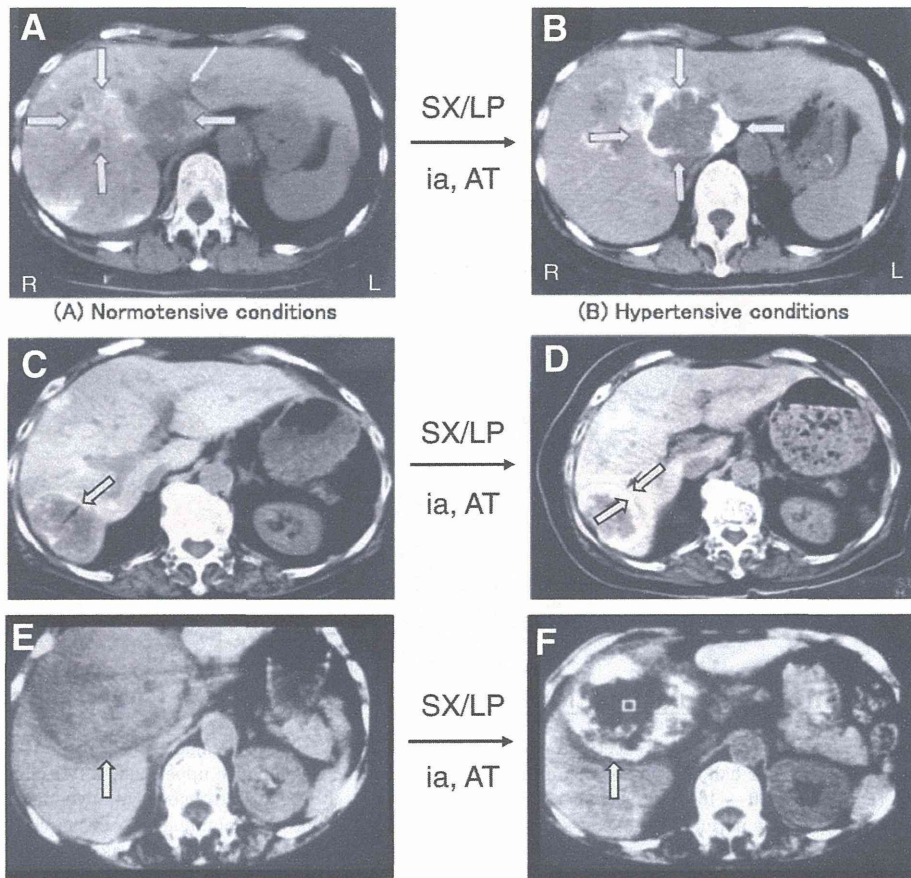


Fig. 7. Retention of SMANCS/Lipiodol in the metastatic liver cancer (A/C/E). (A) and (C) CT scans of images of colon cancers metastasized to the liver. SX/LP (SMANCS/Lipiodol) was infused *via* the hepatic artery under normotension. Retention of drug SX/LP given *ia* (intraartery) under normotensive state reveal very little drug retention. Three and five days later to (A) and (C), respectively, SX/LP was infused similarly but under AT(angiotensin)-II-induced hypertension; clearly more deposition of the drug near the tumor periphery is clearly seen in (B) and (D). (E) is a massive cholangio cell carcinoma in the liver which did not take-up SX/LP administered *ia* under normotensive state. Then SX/LP was infused *ia* under AT-II induced hypertension, and the CT scan reveal intense peripheral staining (F) (*cf.* Fig. 8). All (B), (D) and (F) are classified as type B staining.<sup>26)</sup>

concentrations in such tumors are usually significantly lower than the drug concentrations in plasma. Thus chemotherapy of these tumors is often, if not always, very poor. The hypovascular characteristic and poor blood flow of these tumors with poor drug delivery, can be improved, however, by modulating vascular effectors or by elevating systemic blood pressure via the slow infusion of angiotensin II (AT-II), as well as other means as described below.

*6-2-1. Augmentation of the EPR effect by modulating tumor blood flow by raising blood pressure.* As demonstrated by Suzuki *et al.*<sup>85)</sup> and Hori *et al.*<sup>86)</sup> of Tohoku University, blood flow in tumor tissues can be increased by raising the systemic blood pressure; for instance, from 100 mmHg (normal) to 150 mmHg

by slow intravenous infusion of AT-II. The blood flow volume (rate) in normal tissue, in contrast, remains constant regardless of artificially elevated blood pressure. Under this AT-II-induced hypertension, a relatively hypovascular tumor image can be changed to well vascularated image with rich blood flow<sup>86)</sup> as seen in angiogram being highly stained (Fig. 9A vs. 9B). The same angiographic enhancement will be seen in the clinical setting. For example, a metastatic abdominal tumor, originating from liver cancer was visualized in abdominal cavity clearly (Fig. 9C vs. 9D, see circled area at right), only by using AT-II induced hypertension. This hypertension facilitates tumor selective drug delivery and contrast agent (*cf.* Figs. 8–11), while reducing leakage of drug from



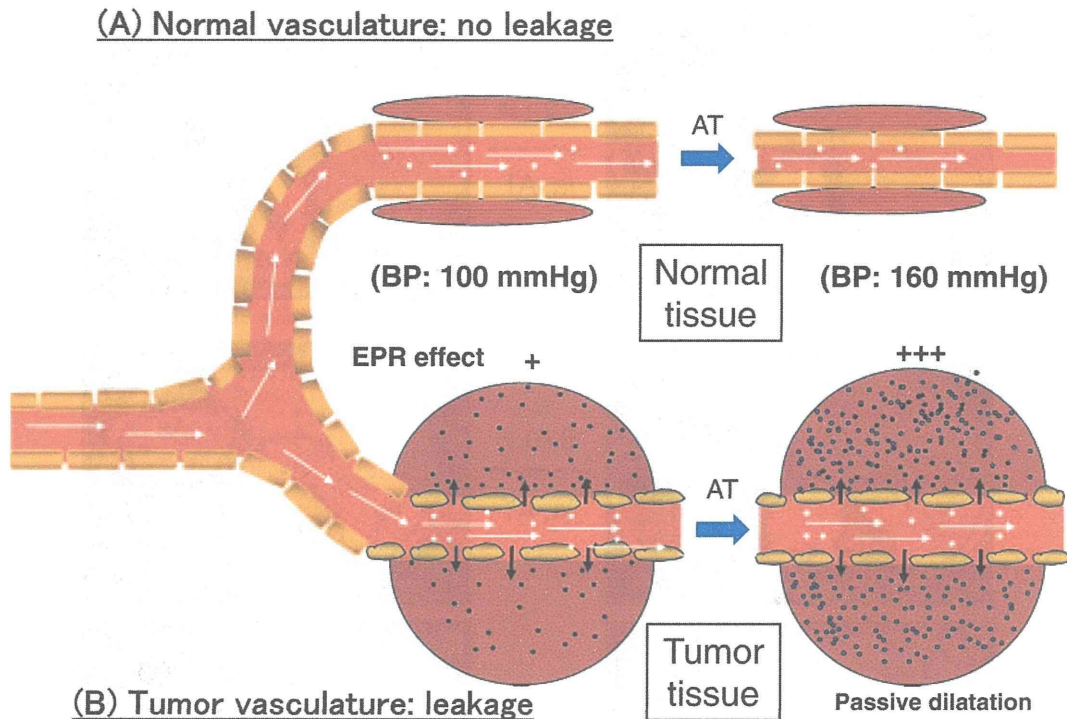


Fig. 8. Diagrammatic representation of EPR effect and effect of angiotensin(AT)-II-induced enhancement of the EPR effect and drug delivery in tumor tissue (lower half). Please compare the situation in normal tissue in the upper half. AT-II infusion induced high blood pressure (BP) (e.g., 100 → 160 mmHg) that caused endothelial cell-cell junctions to open hydrodynamically only in tumor vasculature (lower right). The normal blood vessels (upper right), in contrast, have surrounding smooth muscle-cell layer that will contract and tighten the cell-cell junctions, and a narrowing of the vascular diameter will cause less leakage of drugs. AT-II induced hypertension thus result in more delivery of macromolecular drugs to tumor (See lower right) (Adapted from Maeda<sup>36</sup>) with modification).

blood vasculature in normal tissue, thus preventing adverse side effects.<sup>87),88)</sup> The arterial infusion of SMANCS/Lipiodol under AT-II-induced hypertension markedly enhanced the delivery (Fig. 7 A,C,E → B,D,F/ Fig. 10 C,E → D,F/ Fig. 11 A,C → B,D) and therapeutic effect was much improved (Figs. 10,11).<sup>30)–32),88)</sup>

The most remarkable therapeutic consequences of AT-II-induced hypertension were observed in patients with difficult-to-treat advanced cancers—metastatic liver cancer, cancers of the gallbladder, bile duct, pancreas, and kidney, and primary liver cancers<sup>88)</sup> (Figs. 10,11). Beneficial effects included a shorter time to achieve tumor regression. In the normotensive state, massive HCCs (diameters >5 cm) needed three arterial infusions in 6 months to reduce the tumor volume to 10% of the original size. With AT-II-induced hypertension, however, only one infusion produced a remarkable response in one month.<sup>88)</sup> Many metastatic liver cancers regressed to less than 10% of the original tumor

volume in 1–2 months without appreciable toxicity such as bone marrow suppression, or damage to the kidney or the liver.<sup>88)</sup>

*6-2-2. Augmentation of the EPR effect by utilizing NO-releasing agents.* As described above, we found that NO plays an important role in the vascular permeability and growth of solid tumors.<sup>11)–13),35)</sup> During my research, I conceived an analogy between hypoxic solid tumor (hence showing less staining) and infarcted cardiac tissue (as in angina pectoris), because both are similarly hypoxic.<sup>89),90)</sup> In the latter case use of nitroglycerin (NG) has been recognized for more than a century. NG liberates nitrite by action of denitrase, and nitrite is then converted by nitrite reductase to NO in the infarcted tissue.<sup>91)–93)</sup> This pharmacological benefit of NG was also validated in mouse tumor models *in vivo* that showed an improved therapeutic effect when anticancer agents were combined with NG.<sup>35),89)</sup> Figure 12A illustrates the mechanism of NO generation in solid tumor. An application of NG ointment to the skin of tumor-

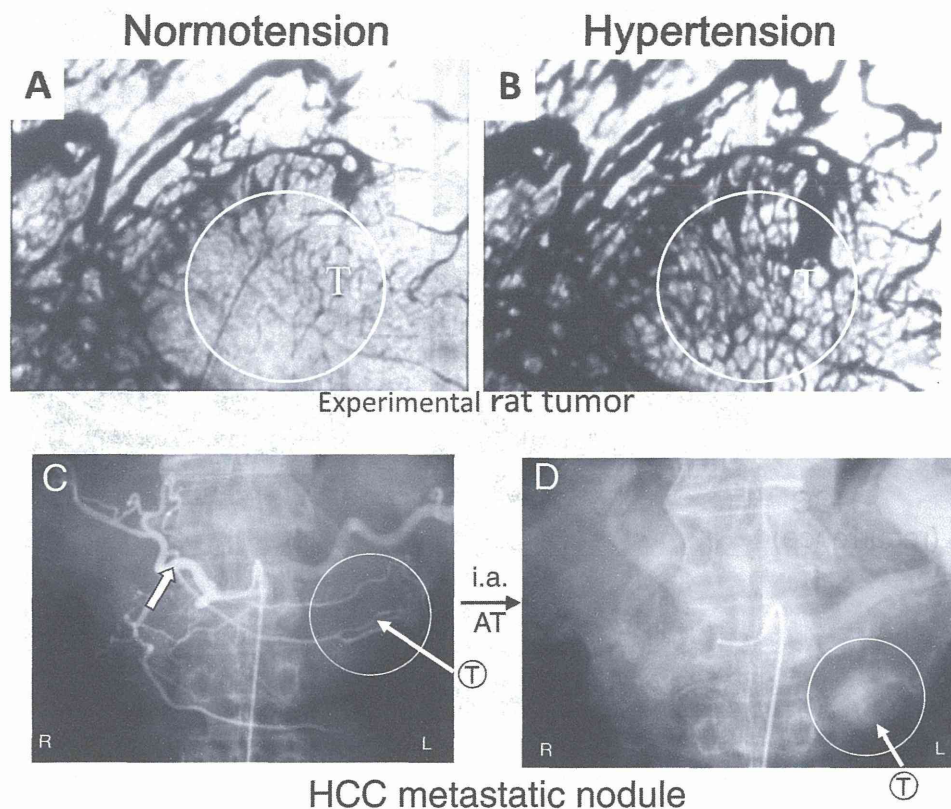


Fig. 9. Visualization of hypovascular tumor by AT-II-induced hypertension. (A) Under normal blood pressure, this tumor, encircle mark T, in which rat Yoshida LY80 tumor was clamped with Lucite<sup>®</sup> window, shows only weak blood flow. (B) Blood flow became highly visible under AT-II-induced hypertension (adapted from Hori *et al.*<sup>86)</sup>). This type of enhanced angiographic staining can be observed with human case. As seen in the angiogram (C), (D), which show an abdominal metastatic tumor (encircled T) from the primarily liver cancer; it is only visible after AT-II-induced hypertension (D). Under the normotensive state of arterial phase angiography, no tumor was revealed in the circle area at the lower left (C). But AT-II-induced angiography at the venous phase demonstrated easily detectable tumor in (D) (arrows in circled area at the lower right) (C and D adapted from ref. 36)).

bearing mice produced increased blood flow in only tumor tissue, but not in normal tissue, and hence increased macromolecular drug delivery to tumor was achieved (Fig. 12 B,C).

Clinical evaluations of NG used in combination with conventional low MW anticancer agents were recently undertaken by Yasuda *et al.*<sup>94-96)</sup> and Siemens *et al.*<sup>97)</sup> and both studies showed significant clinical improvement in therapeutic response. In earlier study NG was found to enhance the therapeutic effect of radio therapy of cancer.<sup>98)</sup> By the administration of NG, enhanced blood flow resulted higher  $pO_2$  of tumor tissue, consequently better therapeutic outcome.<sup>93),98)</sup> Higher  $pO_2$  of cancer tissue also facilitated the modulation of cellular signaling such as HIF-1 $\alpha$  (hypoxia inducible factor) that down-regulation of growth signals (VEGF) or their further signaling pathway.<sup>94),96)</sup> These data

therefore indicate that NO clearly benefits patients undergoing chemotherapy. Also, nitro agents per se showed tumor suppression in animal models and in humans.<sup>89),95)</sup>

6-2-3. Augmentation of EPR effect by using ACE inhibitors and a prostaglandin  $I_2$  agonist (beraprost sodium). Another line of research on enhancing tumor drug delivery concerns the use of angiotensin converting enzyme (ACE) inhibitors. ACE can degrade angiotensin-I (AT-I) to generate AT-II, which results in hypertension. On the other hand, ACE also inhibits degradation of (brady)kinin, whose C-terminal amino acid sequence is similar to that of AT-I. Consequently, it results in an increased local concentration of kinin, and hence vascular permeability of tumor will be increased, as will delivery of drugs, including the antibody IgG.<sup>31),35),99),100)</sup> ACE inhibitors are nontoxic, usually producing no adverse



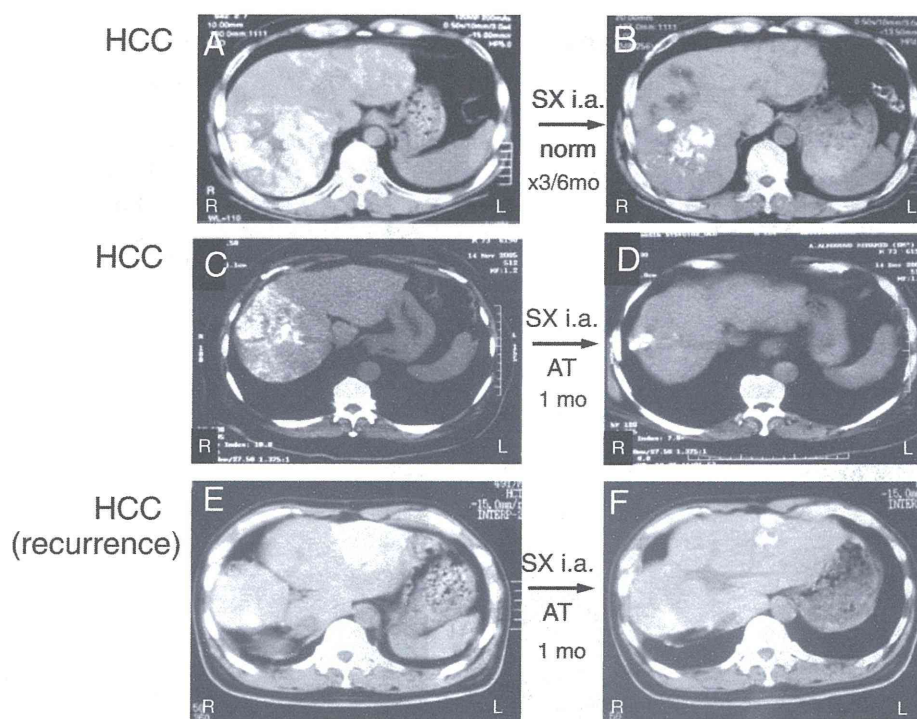


Fig. 10. CT scan images of primary and recurrent hepatocellular carcinoma (HCC). Patient on the top with massive primary liver cancer received about 5–6 mg SMANCS(SX)/5–6 mL Lipiodol(LP) (A). Subsequently, the patient received three injections of the drug i.a. under normotensive state in 6 mon, then marked regression was obtained ( $\rightarrow$  B). CT scans in (C) and (E) patients received i.a. infusion of SX/LP under hypertension induced by intravenous infusion of AT-II. In about 1 mon, the size of tumors in both cases was reduced to less than 10% of the original size (Fig. 10 C  $\rightarrow$  D; E  $\rightarrow$  F) (adapted from ref. 88).

effects in healthy people and are active only in hypertensive patients and more selectively at tumor sites in this case. Data indicate that ACE inhibitors, such as enalapril increased drug delivery about 2- to 3-fold (Fig. 13).<sup>99),100)</sup> Professor Felix Kratz also confirmed this effect in different tumor models with different polymeric drugs (personal communication).

We likewise found that beraprost sodium, a stable analogue (prolonged plasma  $t_{1/2}$ , 30 times) of prostaglandin  $I_2$ , which administered orally, enhanced tumor-targeted drug delivery in mouse tumors.<sup>78)</sup>

### 7. Concluding remarks

Tumor vasculature is structurally unique and different from normal vasculature. Tumor tissue also shows highly up-regulated production of vascular effectors. As a consequence extravasation of macromolecules into the interstitial space would occur. The effectors affecting vascular permeability factors includes (brady)kinin, NO, prostaglandins, VEGF (or vascular permeability factor), and CO (by HO-1) in

or near the most solid tumors. This enhanced vascular permeability also commonly occurs in inflamed tissue at the sites of infection that is affected by many similar vascular mediators.

Once macromolecules extravasate from the circulation or blood vessels into the tumor interstitium, they remain in the tumor for a long time without being cleared. This situation is in great contrast to normal tissue, in which macromolecules are cleared via the lymphatic system. Thus, prolonged retention of macromolecules—for more than days to weeks—is a unique characteristic of the EPR effect in tumor tissue. These features led to this phenomenon being named the *enhanced permeability and retention (EPR) effect* of macromolecules in solid tumor.<sup>26)–32),35),36)</sup> The EPR effect is applicable to biocompatible macromolecules with MW > 40 KDa.

However, the EPR effect occurs frequently heterogeneously, which means that tumor selective macromolecular drug delivery based on the EPR effect may not proceed homogeneously. Consequently, drug delivery may be less efficient to

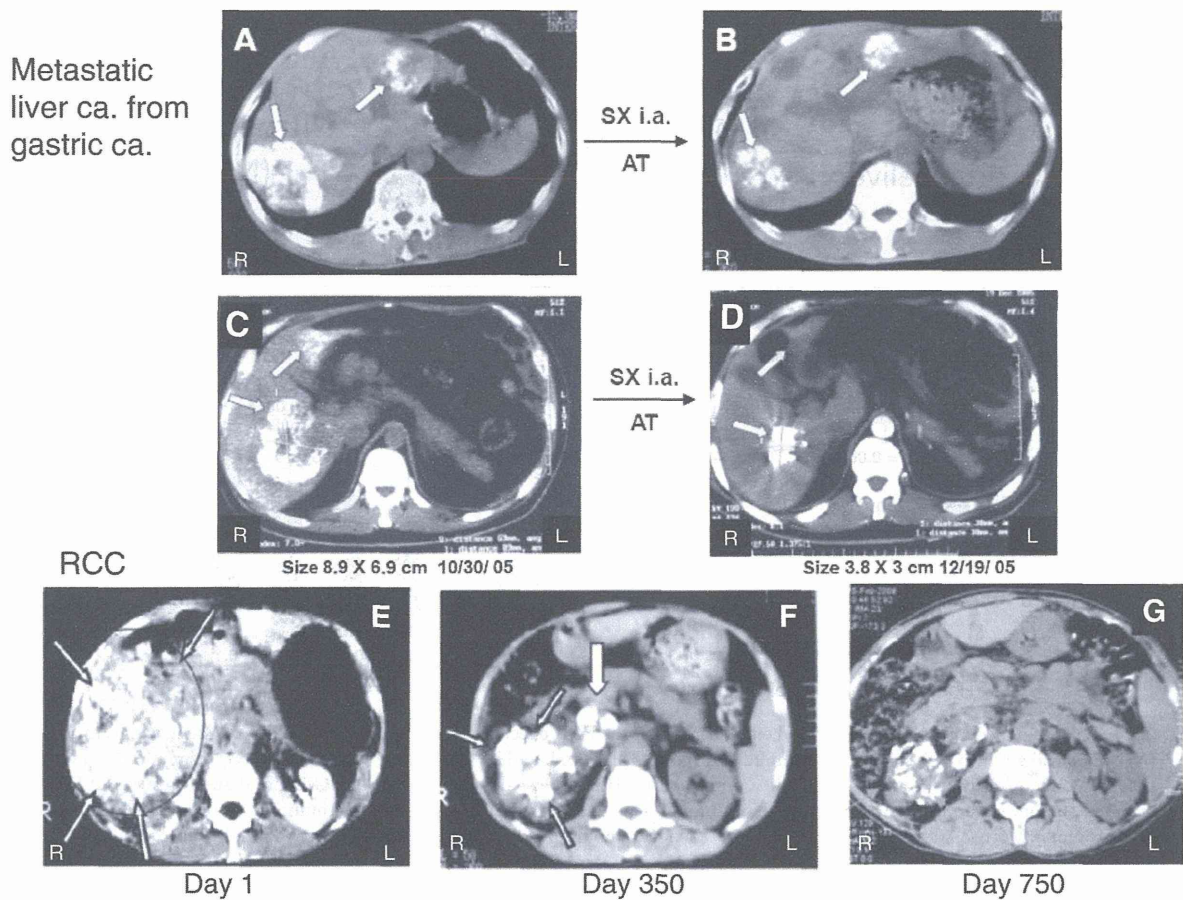


Fig. 11. CT scan images of (A) and (C) show stomach cancer metastasized to the liver. They received SX/LP(SMANCS/Lipiodol) i.a. under AT-II-induced hypertension, at about 150–160 mmHg. The notable reduction to about 10% of the original volume of tumor was observed in 1–2 months (see B,D) (Adapted from ref. 88)). In the bottom three CT images showing a case of massive renal carcinoma. SX/LP was administered via the renal artery under AT-II-induced hypertension. CT scans at left was taken at day 1, middle at day 350, and on lower right at day 750 showing remarkable regression. Also please note a metastatic tumor nodule at the inferior vena cava (middle; large white arrow). This metastatic tumor (nodule) regressed to a small spot in the scan at day 750 (right). Dose of SX/LP administration was 3–4 mg in about 2 ml at a time, and the patient received total of 14 times i.a. infusion in three years. This patient is still alive after 9 years with good QOL.

metastatic liver cancers and to less vascularized cancers, *e.g.*, cancers of the pancreas and prostate, rendering poor EPR effect. Therefore better therapeutic outcomes for such cancers depends on further augmentation of drug delivery to such tumors. We thus developed measures to enhance the EPR effect. One method involves raising the systemic blood pressure, *e.g.*, from 100 to 160 mmHg by using angiotensin II during arterial infusion of a macromolecular drug, *e.g.*, SMANCS/Lipiodol. This method produced excellent clinical results even in advanced and difficult-to-treat tumors such as metastatic liver cancer, cholangiocarcinoma, and cancers of the pancreas, and others.<sup>88)</sup>

Another method is utilization of NO-releasing agents such as NG for advanced and poor-EPR tumors. Hypoxic tumor tissues and infarcted cardiac tissue (as in angina pectoris) seem to possess similar NO-related mechanisms. Topical application of NG results the site-selective increase of NO concentration, which did facilitate an improved EPR effect<sup>89),90)</sup> and clinical benefit.<sup>94)–98)</sup> Similarly, ACE inhibitors can increase the local kinin concentration, and thus enhanced EPR effect without any adverse effects.<sup>99),100)</sup> These methods of enhancing the EPR effect will likely achieve better clinical outcomes for cancer patients without any adverse effects and warrant continuing development.



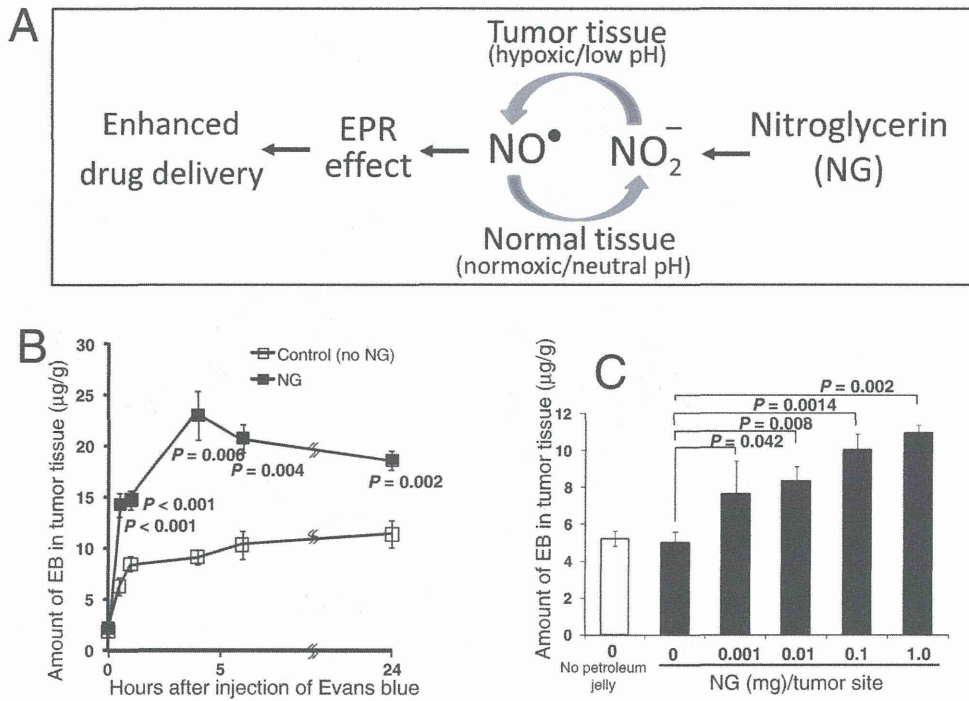


Fig. 12. (A) Mechanism of NO generation and enhancement of the EPR effect by topical application of NO-releasing agent, nitroglycerin (NG). NG will get into circulation in a few min, and NO<sub>2</sub><sup>-</sup> is liberated, and in the hypoxic tumor tissue NO<sub>2</sub><sup>-</sup> is reduced to nitric oxide (NO) which induces the vascular permeability. Time course (B) and dose dependence (C) of NG-induced augmentation of EPR effect in S-180 tumors were probed with Evans blue/albumin accumulation in tumor. Chemically induced rat tumor with (DMBA) and other tumors (Meth A fibrosarcoma and colon 38 adenocarcinoma) also showed similar results (Adapted from ref. 89)).

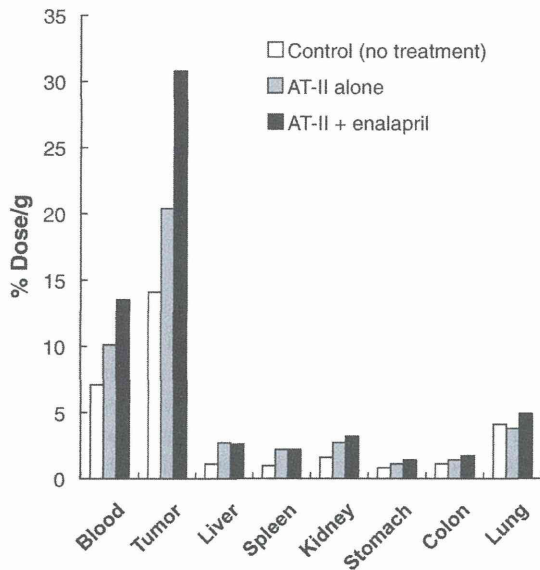


Fig. 13. Augmentation of EPR effect and macromolecular drug delivery to tumor by using AT-II and ACE inhibitor enalapril. The drug used here was monoclonal antibody A7 against human colon cancer in athymic mice bearing SW-1116 tumor cells. IgG was labeled with <sup>125</sup>I (Adapted from ref. 99)).

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## Profile

Hiroshi Maeda, born in 1938 and received BS from Tohoku University, started his research career since 1964 at Department of Bacteriology, Tohoku University Medical School, Sendai, Japan, after completing MS degree from University of California, Davis, CA as Fulbright student. He studied biochemical characterization of an antitumor protein neocarzinostatin [NCS] isolated from *Streptomyces*, and then completed the amino acid sequence at Children's Hospital/Harvard Medical School, Boston. He was conferred Ph.D., and M.D. degree from Tohoku University. After moving to Kumamoto University Medical School 1971, he studied molecular mechanism of pathogenesis of infection, and discovered two unique pathological events; (i) bradykinin yielding protease cascade (kalikrein-kinin system) and (ii) induction of superoxide generation and nitric oxide synthase, both upon microbial infections.

Another line of research he undertook was on the tumor drug delivery. He pioneered synthesis of the first polymer conjugated antitumor drug (NCS) in 1978, designated SMANCS. SMANCS, being highly lipophilic, it could be solubilized in lipid contrast agent Lipiodol, and developed arterial injection therapy of SMANCS/Lipiodol with Toshimitsu Konno. By this way drug will target to and remain in tumor selectively for several weeks. This therapeutic modality was approved by Japanese Government in 1993. Meantime, he discovered tumor accumulation mechanism of macromolecules. Namely, the drug size larger than 40 KDa exhibited tumor selective accumulation. As a result, ratio of drug in tumor to normal tissues could be more than 20 to 100 in favor of tumor, and they remained in the tumor for weeks. This phenomenon of enhanced permeability and retention effect in solid tumor was coined EPR-effect of macromolecules in 1986. This discovery of EPR-effect paved the way to the tumor drug delivery using nanomedicine. He was professor of Microbiology of Kumamoto University Medical School (1981–2004), then upon retirement moved to Sojo University Faculty of Pharmaceutical Sciences till now. He received many awards such as Award from Princess Takamatsu Cancer Research Fund, Asakawa Award of Japanese Bacteriological Society, Tomizo Yoshida Award from Japan Cancer Association, Gold Medal Award from Frey-Werle Foundation, Germany, Nishinippon Culture Award from Nishinippon Shimbun, Fukuoka, Japan, Nagai Outstanding Innovation Award from Controlled Release Society, U.S.A., among others.

