reach normal tissues and organs, which would lead to adverse side effects in these tissues and organs. Macromolecular drug size therefore plays a critical role in EPR effect-dependent drug accumulation in tumors.

2.2 Biocompatibility and stability of macromolecular integrity

Biocompatibility is another requirement for longer drug retention in the circulation. For example, intrinsic native proteins including albumin, IgG, and macroglobulin are the most biocompatible and in systemic circulation have long half-lives (several days). However, excessive chemical modifications of native proteins have resulted in loss of biocompatibility, with the result being a much shorter half-life in plasma [33]. α_2-Macroglobulin (α_2M) is an inhibitor of various proteases, and after the native form binds to plasmin and forms the E/I complex plasmin/α_2M, the plasma half-life (>90 h) becomes only a few minutes, although the ratio of plasmin to α_2M is only 1:1 [20, 33]. This means that conformational integrity may have been destroyed by the formation of the complex, which is cleared from plasma. The reticuloendothelial system (RES) is the intrinsic system that clears non-natural or foreign particulates. The liver and spleen have a rich RES, so non-biocompatible materials may rapidly accumulate in these tissues, and the plasma concentration rapidly decreases. Earlier failure of liposomal drugs in the 1960s and early 1970s may be attributed to this problem [34].

To avoid elimination by the RES, macromolecular drugs or nanoparticles must have good biocompatibility. For this purpose, the surface of macromolecular drugs may be conjugated with numbers of biocompatible polymers, such as polyethylene glycol (PEG) or poly-*N*-(2-hydroxypropyl)methacrylamide (PHPMA), which confer both hydrophilicity and biocompatibility [35-39]. PEG modification of liposomes and proteins greatly improves their plasma half-lives, and many PEG-modified drugs such as Doxil (doxorubicin HCl liposome), Pegasys (peginterferon alfa-2a), Cimzia (certolizumab pegol, an anti-TNF-α antibody), and Krystexxa (pegloticase, a recombinant modified mammalian uricase) are available [34, 40-43].

2.3 Surface charge

The luminal side of the blood vasculature is rich in the negative charges of carboxyl, phosphate, and sulfate. Therefore, positively charged drug molecules are easily absorbed on the surface of vascular endothelial cells and then rapidly disappear from the systemic circulation [31, 32]. He et al. reported that the plasma concentration of chitosan nanoparticles with a positive charge (zeta potential = +10 to +35 mV) decreased to 2–3% of the injected dose in 1 h after i.v. injection [44]. In contrast,

negatively charged chitosan nanoparticles (zeta potential = −10 to −40 mV) circulated for a long time in the blood circulation; 15–20% remained in the blood at 1 h after i.v. injection. More important, the tumor area under the curve of negatively charged chitosan was higher than that of positively charged chitosan [44]. One should therefore consider the effect of the particle charge during development of drug preparations with a longer circulation time, and thereby one can accomplish favorable accumulation of drugs in tumor by virtue of the EPR effect.

3. Augmenting the EPR effect, and overcoming the problem of the heterogeneity of the EPR effect in tumor tissues

Many tumors such as metastatic liver cancer have both necrotic and hypoxic features, particularly in the tumor mass interior, which may be attributed to thrombus formation or hypercoagulation resulting in obstructed blood flow [45]. These features may lead to a heterogeneous EPR effect, with poor accumulation of macromolecular drugs in tumor tissues, and thus an insufficient therapeutic response. Furthermore, metastatic liver cancer and prostate cancer are rich in stroma or fibrous tissue, which will suppress drug diffusion, with the result being poor access and uptake of drugs, so a poor therapeutic response would result. Diffusion of macromolecular drugs will be poor in stromal tissues and will decrease parallel to the increase in molecular size. Diffusion of macromolecular drugs in such tissues is thus limited. Under these circumstances, artificial augmentation of the EPR effect should achieve better delivery of drugs to tumors and, more important, greater therapeutic success.

Because a rapidly proliferating tumor cell mass leads to mechanical compression of either blood or lymphatic vessels in tumors, blood or lymphatic flow may be suppressed, which would lead to poor lymphatic clearance. Also, poor development of the lymphatic system may also suppress clearance of interstitial fluid by tumor tissues. Avascular tumor tissues, which we frequently see via angiography in prostate, pancreatic, and metastatic liver cancers in clinical settings, result from hypoxia-induced apoptosis of blood vessels, which reduces vessel leakiness and accumulation of drugs in tumor tissues.

In contrast to normal vasculature, tumor vasculature is disorganized. That is, in tumor blood vasculature, differentiating between venules and arterioles is difficult, and the vasculature often manifests connections by bidirectional shunts and a disordered direction of blood flow [46, 47]. Therefore, blood flow in the tumor vasculature is irregular and discontinuous, which often leads to insufficient blood flow in tumor tissues, so drug distribution or the EPR effect becomes inhomogeneous or poor.

3.1 Hypertension induced by infusing angiotensin II

Physiological induction of hypertension by infusing angiotensin II i.v. may constrict normal blood vessels and tighten endothelial gaps. In contrast, such hypertension may lead to more selective passive opening of vessels and endothelial cell-cell gaps in tumor tissues, because the neovasculature of tumor tissue frequently has defective vascular functions, with missing smooth muscle cell layers and pericytes surrounding blood vessels (for constriction), or missing of angiotensin II receptors, which are responsible for vasoconstriction of vascular smooth muscle. Passive opening of endothelial cell gaps by the high physical fluid pressure induced by the hypertension would occur. This result would counteract the high osmotic fluid pressure, which would impede penetration of tumor tissues by fluid. This induced hypertension method was initially described by Suzuki and colleagues [48, 49]. However, clinical applications have used primarily low-MW anticancer agents such as mitomycin C. In these cases, low-MW drugs with high diffusion constants dissipated rapidly from tumor tissue, and the therapeutic gain was marginal [50]. In contrast, macromolecules and lipid formulations (with Lipiodol), for example, SMANCS/Lipiodol administered via a tumor-feeding artery, have demonstrated excellent clinical results [26-29]. These patients had advanced, stage IV, difficult-to-cure cancers, such as gallbladder, pancreas, and kidney tumors and metastatic liver cancers originating from gastric and colon cancers [29]. Li et al. described significantly decreased adverse effects such as damage to bone marrow and intestines with angiotensin II-induced hypertension [50]. Thus, this method has greater advantages with macromolecular drugs than with conventional drugs.

3.2 Angiotensin II-converting enzyme (ACE) inhibitors

Bradykinin is a peptide generated in inflamed and tumor tissues via proteolytic cleavage of kininogen by kallikrein. Bradykinin, thus generated in tumor tissue, dilates blood vessels and increases vascular permeability, which may also involve prostacyclins and NO (endothelial NO synthase) generation [19]. Maeda and colleagues previously reported that bradykinin and hydroxypropyl bradykinin were found in the systemic circulation and the peritoneal and pleural fluids of advanced cancer patients.

Furthermore, Wu et al. previously reported that tumor accumulation (via the EPR effect) of Evans blue-bound albumin was suppressed about 46% by administration of a bradykinin receptor antagonist (HOE-140) [51]. This report clearly indicated that

bradykinin is a major mediator of the EPR effect. Therefore, increasing the bradykinin concentration by preventing its degradation by kininase or ACE inhibitors, i.e. ACE inhibitors such as enalapril, would enhance the EPR effect. The *in vivo* half-life of bradykinin is about 15 s, so bradykinin in tumor tissues is rapidly degraded in normal settings [52]. Because of this rapid degradation, bradykinin may be useless as an injected agent or may cause pain. Therefore, administration of ACE inhibitors such as enalapril would result in a more selective increase in bradykinin concentration in tumor tissues, because this tissue is the only one that produces bradykinin, except for inflamed tissues in different settings.

Noguchi et al. previously reported that accumulation of ^{125}I-labeled A7 antibody in SW1116 tumors in mice increased twofold after administration of angiotensin II and ACE inhibitors [53]. Dr. Felix Kratz at the University of Freiburg confirmed the effect of ACE inhibitors in enhancing the EPR effect in different tumor types, by using albumin attached to doxorubicin (personal communication, unpublished data). Therefore, using ordinary non-toxic drugs such as ACE inhibitors may be of benefit for improving tumor-selective delivery of nanomedicines.

3.3 NO

NO is a gaseous radical molecule that plays an important role in vasodilation by activating soluble guanylyl cyclase (200 times) in endothelial cells, followed by activation of the sarcoplasmic or endoplasmic reticulum Ca-ATPase, which causes smooth muscle cell relaxation (vasodilation) and thus the EPR effect in tumor tissues. In fact, accumulation of Evans blue bound-albumin (via the EPR effect) was reduced 39% after administration of an NO scavenger (2-phenyl-4,4,5,5,-tetramethylimidazoline-3-oxide-1-oxyl) [51]. In clinical settings, NO-releasing agents such as nitroglycerin, isosorbide dinitrate (ISDN), and amyl nitrite are used for angina pectoris. These agents liberate nitrite, which is converted to NO in hypoxic tissues as in angina pectoris. Administration of such NO-releasing agents should thus release NO and enhance the EPR effect. Indeed, Seki et al., in our laboratory, reported that transdermal administration of nitroglycerin ointment (0.01–2.0 mg/mouse) anywhere on the skin selectively increased blood flow in tumor tissues, and macromolecular drug accumulation increased 1.5–3.0 times [54]. The enhanced accumulation of macromolecular drugs led to marked improvement in the antitumor effect in clinical settings, even with conventional low-MW drugs and radiotherapy [55-57]. These beneficial effects may be attributed to the elevation of pO_2, suppression of hypoxia-inducible factor-1α in tumor tissue, and suppression of the multidrug efflux

pump [55].

3.4 TNF-α

TNF-α is a pleiotropic proinflammatory cytokine secreted mainly by immune cells. In vascular endothelial cells, TNF-α induces NO production [58], and thus alteration of cytoskeletal actin [59] and destabilization of platelet endothelial cell adhesion molecule-1 [60]. These alterations of vascular endothelial cells lead to vascular relaxation and then increased permeability. TNF-α has been used to enhance perfusion of isolated limbs with the intra-arterial administration of an antitumor agent, melphalan, against sarcoma and melanoma in clinical settings [61]. Recently, Seki et al. reported that i.v. administration of TNF-α at 1 μg/mouse enhanced the accumulation of Evans blue-bound albumin and delivery of adenovirus in EL4 ascites lymphoma in mice [62].

3.5 CO and HO-1

CO is a well-known toxic gaseous molecule when inhaled at high doses. Recently, the physiological importance of CO in vascular functions was reported [63, 64]. CO mediates vascular relaxation, as NO does, via the cGMP-dependent pathway [65, 66]. CO is naturally produced in the body, and HO is responsible for 80% of CO generation *in vivo* [65]. Among HOs, HO-1 is the inducible form, which is upregulated in many pathological conditions such as inflammation reactive oxygen species-related diseases and disorders after various cellular stresses [65, 67]. More important, many solid tumors have high expression of HO-1 to support their rapid growth, so elevated CO production in tumor tissues leads to relaxation of vascular tonus and increased blood flow as well as increased permeability (via the EPR effect) [68, 69]. We recently reported that administration of the CO-releasing agent CORM2 significantly increased vascular permeability; also, induction of CO production in tumors by using pegylated hemin (an inducer of HO-1) markedly increased tumor blood flow, which resulted in significantly enhanced accumulation of a putative macromolecular drug, Evans blue-bound albumin, in S-180 solid tumors in mice [70].

3.6 Hyperthermia

Hyperthermia, which was described in the mid-20th century, is one modality used in cancer therapy [71]. Blood flow in tumor tissues is frequently lower than that in normal tissues, as discussed above. Tumor cells *in vitro* are more susceptible to high temperature than are normal cells. During hyperthermia therapy, the temperature of

the tumor tissue is increased to about 42°C. Thus, tumor growth *in vivo* may be suppressed by exposing the tumor to high temperature. Kong et al. reported that hyperthermia enhanced extravasation of liposomal drugs into the interstitial space of tumor tissues without any adverse effects on normal tissues in mice [72, 73]. Buckway et al. also reported that selective hyperthermia of tumors, which was achieved by tumor accumulation of gold nanorods irradiated with near-infrared light, increased the accumulation of the ^{111}In-labeled HPMA polymer-DOTA conjugate by tumors [74].

However, in practical terms, increasing the local temperature of tumor tissues by applying heated probes or immersion of the tissues or organs in hot baths is rather difficult, because excessively high temperature may cause tissue to burn. Furthermore, tissue temperature cannot be readily increased because of vascular blood flow in the tissue, which would cool the tissue, similar to a motor engine being cooled by radiator fluid going to the inside of the engine. For this reason, hyperthermia did not become as popular as expected more than 60 years after discovery. The utilization of nanomedicines, like that of gold nanoparticles as described elsewhere [74], may lead to a more beneficial outcome than will use of conventional low-MW drugs or hyperthermia alone.

4. Conclusion

We discussed here the mechanisms of tumor-selective drug delivery based on the EPR effect and the required characteristics of macromolecular drugs. We also described methods of enhancing the EPR effect and the tumor accumulation of such antitumor nanomedicines by modulating the EPR effect. These methods can significantly improve the delivery of drugs to tumors, as well as the therapeutic effect, while significantly lowering toxicity.

This strategy can also be applied to tumor imaging by using fluorescent nanoprobes and photodynamic therapy (PDT). Previously in PDT, little tumor-selective accumulation of photosensitizers was explored, because only low-MW probes were traditionally used. The combination of macromolecules carrying either tumor-suppressing agents or photosensitizers is expected to be more beneficial for tumor imaging and therapy, as demonstrated by PHPMA-conjugated Zn-protoporphyrin (Zn-PP) (see the Expert Opinion below) compared with conventional cytotoxic antitumor drugs or photosensitizers used alone. In both methods, the EPR phenomenon is a prerequisite first step.

5. Expert Opinion

5.1 Importance of the EPR effect for delivery of drugs to tumors

A major problem with conventional antitumor therapeutics is non-selective delivery of cytotoxic drugs to normal vital organs and tissues, but little delivery to tumor tissues. Recent advances in tumor drug delivery, for instance, utilize encapsulation or conjugation of low-MW antitumor drugs such as liposomes, macromolecular micelles, conjugates to synthetic polymers, and plasma proteins. Such nanomedicine-based drug development appears to hold promise for tumor-selective delivery of antitumor drugs by virtue of the EPR effect, which utilizes the unique leaky characteristics of the blood vasculature of solid tumors and various vascular permeability factors to facilitate this leakiness. The EPR effect and its tumor selectivity are thought to apply universally, and this important finding is comparable to the selective toxicity proposed by Paul Ehrlich as discussed by Lammers et al. [18, 75]. That is, the importance of the EPR effect is that the EPR effect is valid in most, if not all, solid tumor types regardless of pathology, tumor size, extensive genetic diversification by mutations, or different tumor markers.

Recent studies revealed that 100–200 mutants per tumor exist in lung cancer and 50–100 mutations per tumor in esophageal and colon cancers [14]. Thus, chances are high that a molecular drug with a specific molecular target will not reach the target in the tumor cells. Genetic polymorphism may therefore pose a serious problem for molecular target drugs and antibody drugs. In addition, the cost of these drugs is now becoming intolerable, and many are voicing criticisms with regard to cost and efficacy [8, 57].

5.2 The EPR effect in various tumors: rodent versus human tumors, primary versus metastatic tumors, implanted versus autochthonous tumors

Macromolecular drugs selectively accumulate in tumor tissues, and this finding can be easily visualized in tumor-bearing mice (Figure 1a–c). The EPR effect has thus far been studied primarily in implanted mouse tumors, and the effect in metastatic and autochthonous tumors was not rigorously explored. As Figure 2 shows, we demonstrated the EPR effect in metastatic lung cancer in mice, in which metastatic tumor nodules in the lung were selectively stained blue. The tumor (colon 26) was initially implanted in the dorsal skin, and after 30–40 days, metastatic foci were visually apparent, and the tumor size was about 1–5 mm. Figure 2c shows a different metastatic tumor model, a nodule in the liver (originating from an implanted tumor in the spleen), in which enhanced tumor vascular permeability (the EPR effect) was

demonstrated by the accumulation of plastic resin that leaked out in this vascular cast. This finding indicates that a metastatic tumor nodule size of about 250 μm (less than 1 mm) manifests the EPR effect [76]. Both of these metastatic cases, lung and liver, had clear evidence that the EPR effect operated in metastatic tumor as in primary tumor.

These data therefore indicate that macromolecular drugs or nanomedicines have advantages for control of metastatic tumors. Indeed, we found that i.v. polymeric SMA-pirarubicin at 15 mg/kg completely eradicated lung metastasis of colon 26 tumors (unpublished data), as well as liver metastasis in a different model (MoCR tumor in the CBA mouse) [76].

Another issue concerns whether the EPR effect also operates in spontaneous and autochthonous tumors, as in implanted tumors. As Figure 1dillustrates, tumor in the colon induced by azoxymethane and dextran sodium sulfate also manifested tumor-selective staining of rhodamine-conjugated albumin (67 kDa). However, the low-MW drug Laserphyrin (Figure 1e) and free Zn-PP (Figure 1c) did not show selective tumor staining. We also observed that 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced breast cancer in rats had selective accumulation of polymer-drug conjugates, i.e. HPMA polymer-conjugated ZnPP (HPMA-ZnPP) (unpublished data). On the basis of these two cases and others, we determined that the EPR effect operates in autochthonous tumors and that it is also important for tumor imaging with fluorescent nanoprobes, which selectively accumulate in tumor tissues.

In addition to EPR-related findings in tumor-bearing mice, similar data for humans have been reported. In Maeda's group, Konno et al. and Maki et al. showed that the lipid contrast agent Lipiodol was selectively retained in primary liver cancer even 3 weeks after administration, when Lipiodol was infused via the tumor-feeding hepatic artery [26-29]. We also found that Lipiodol accumulation in liver tumors was about 2,000-fold greater than that in blood, when tested in rabbits with VX-2 cancer implanted in the liver [25]. Similar to the observations mentioned above for humans, the tumor concentration of pegylated liposomal doxorubicin (Doxil) was 4- to 16-fold higher than that of parental low-MW doxorubicin at days 3–7 after i.v. drug administration [77].

5.3 Augmentation of drug delivery to tumors based on the EPR effect

Here, we discussed factors affecting the EPR effect such as NO, bradykinin, and leakiness of tumor blood vessels under angiotensin II- induced hypertension (e.g. 110 → 150 mmHg). NO-releasing agents such as nitroglycerin ointment can open occluded blood vessels, as seen with angina pectoris during heart attacks, and nitroglycerin

enhanced blood flow and vascular permeability in tumors [16, 54]. Macromolecular drug delivery can thus increase 2- to 3-fold because of an enhanced EPR effect. Also, application of ISDN just before the drug infusion in lung cancer patients, in combination with hypertention during intra-arterial (bronchial) infusion of SMANCS/Lipiodol, produced remarkable clinical results [78]. Likewise, a bradykinin potentiator, i.e. an ACE inhibitor such as enalapril, had the same effect.

5.4 A new future for PDT with EPR-based delivery of fluorescent nanophotosensitizers to tumors

The principle of PDT has been known for more than 100 years. PDT became more popular with the advent of laser beam-based photoirradiation, in which the He/Ne laser, with an emitting light at 633 nm, was used to circumvent the absorption of heme compounds at around 430 nm. Photosensitizers developed thus far are derivatives of tetrapyrroles, i.e. analogs of heme compounds, such as chlorin, Photofrin, and Laserphyrin, but they are not macromolecular photosensitizers. Current photosensitizers include free Zn-PP, but no benefit of the EPR effect can be seen in tumor-selective delivery (Figure 1c, e). As Figure 1b demonstrates, PHPMA-conjugated nanophotosensitizer was localized in the tumor. After tumor irradiation (430-nm), complete regression was observed in the breast cancer model induced by DMBA (Figure 3).

The second drawback of current PDT is that the light irradiation being used comes from the He/Ne laser (633 nm) or the Nd:YAG laser (532 nm). These wavelengths are not on the Soret band (about 430 nm) and thus would not give the highest quantum yield as well as singlet oxygen generation, with the result being little therapeutic effect from $^1O_2$. Any therapeutic effect probably results from heat generated, as is the case for the laser knife. Most tumors are not like spleen or liver, which contain many heme proteins, and thus light at about 430 nm does not penetrate. Instead, most surface tumors such as breast, esophageal, and colon cancers are readily accessible to PDT or endoscopic light, and a marked therapeutic effect is actually observed (Figure 3 and unpublished data). Therefore, by improving photosensitizers via polymer conjugation and using the proper wavelength, one can now achieve much better therapeutic results with PDT, as well as sensitive tumor detection. One major adverse effect of current PDT is skin damage from mild ambient in-house light. However, we did not observe this damage with polymeric nanophotosensitizers in rats.

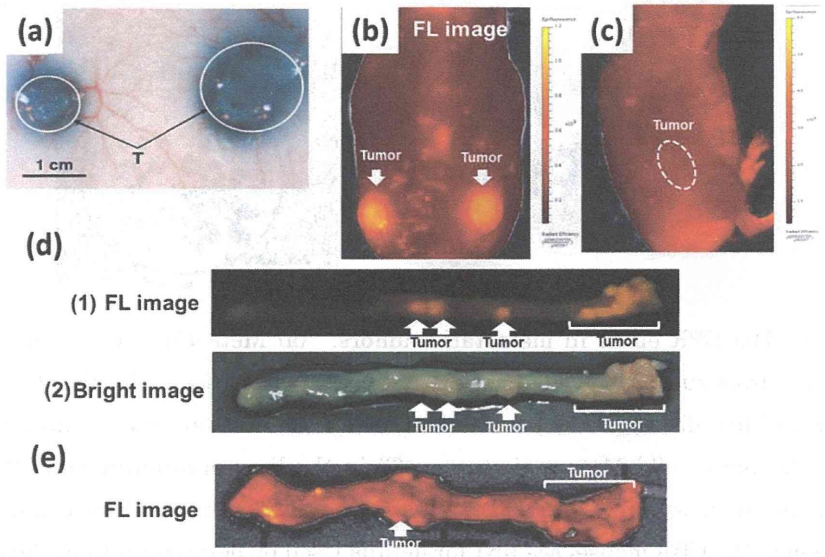

Figure 1. Visualization of the EPR effect. (a) Selective accumulation of the putative macromolecular drug complex Evans blue-albumin in a mouse S180 tumor (T) implanted bilaterally in the dorsal skin. (b) Fluorescent image of PHPMA-ZnPP. (c) Free ZnPP in the S180 implanted tumor model. The fluorescent image obtained 5 h after i.v. administration via IVIS Lumina XR shows no tumor-selective accumulation. (d) (1) Fluorescent (FL) image of autochthonous colon cancer from a mouse after using bovine serum albumin-conjugated rhodamine. The tumor was induced by azoxymethane and dextran sodium sulfate. The image was obtained 24 h after i.v. drug administration via a standard digital camera equipped with bandpass filter. (2) The normal light image, of the same colon sample as that seen in (1), shows no distinct tumor. (e) Fluorescent image of colon cancer (same as above) 5 h after i.v. injection of Lasephyrin. The image, obtained via IVIS Lumina XR, shows fluorescence that is not tumor-selective.