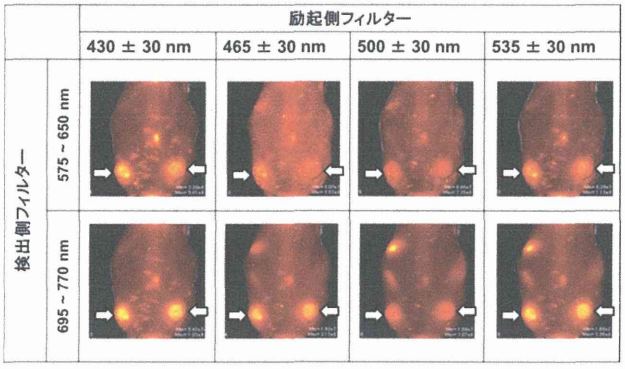
C. 結果



矢印は腫瘍を示す

HPMA-ZnPP(7.0)を静脈注射し、5時間後の腫瘍集積と蛍光イメージング。異った波長帯の励起フィルタと蛍光フィルタを用いた際の蛍光像の違いの検討。

D. 考察

ZnPP はソーレーバンドに 420 nm の強い吸収を持ち、Q バンドに 530nm, 560nm の比較的小さな吸収を持つ。また、蛍光として 590nm および 630nm 付近になだらかな蛍光を発する。しかし、生体内を考慮すると、波長による光の透過率に大きな違いが見られるため、in vitro における励起光および蛍光波長フィルタが最適とはいえない。そこで、S-180 担癌マウスを用い蛍光イメージングを行った。ここでは HPMA-ZnPP (7.0) を用い、投与 5 時間後の蛍光イメージング結果を示す(③-C)。ZnPP の分光特性のみを考慮にいれた場合は、励起 (430±30 nm) と蛍光 (575 ~ 650 nm) の組み合わせが最も適している。腫瘍の検出は可能であり、蛍光強度も最も強いが、非特異的な蛍光も強く見られる。一方、蛍光フィルタを 695 ~ 770nm にしたものでは、腫瘍がより均一かつ選択的に検出されていることが分かる。

④P-THP による腫瘍の蛍光イメージング

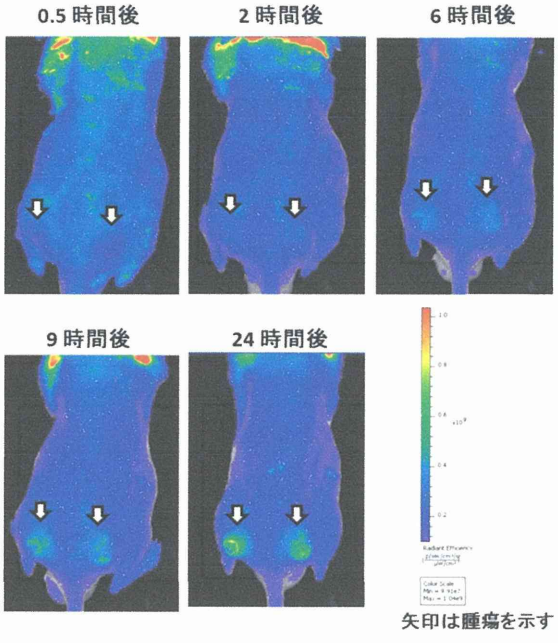
A. 研究目的

In vivo における腫瘍の蛍光イメージングが P-THP により可能かを検討する。

B. 方法

マウス肉腫 (S-180 細胞) を ddY マウス背部皮下に移植し、その直径が 3~5mm になったときに、20 mg/kg の P-THP を尾静脈より投与し、経時的に IVIX Lumina-XR を用い、in vivo 蛍光イメージングを行った。

C. 結果



矢印は腫瘍を示す

P-THP の投与後、時間の経過と共に、腫瘍部に強い蛍光が認められた。

D. 考察

P-THP の投与直後 (0.5 時間後) では全身に蛍光が認められるが、時間経過とともに徐々に腫瘍部のみに蛍光が見られるようになっていることが分かる。P-THP はおよそ 26nm の大きさの分子として挙動し、EPR 効果により、徐々に腫瘍組織に集積していることを示している。

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III. 知的財産権の出願・登録状況

特許出願

発明の名称：高分子型蛍光分子プローブ
国際出願番号：PCT/JP2012/072640
国際出願日：2012 年 9 月 5 日
発明者：前田 浩、方 軍、中村 秀明 他
状況：公開

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出願番号：特願 2013-239222
国際出願日：2013 年 11 月 19 日
発明者：前田 浩、方 軍、中村 秀明
状況：出願

別添５

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
H. Nakamura, H. Maeda	Nanomedicine and cancer drug delivery based on the EPR effect and EPR augmentation	I. Uchegbu	Fundamentals in Pharmaceutical Nanosciences	Springer	New York	2013	401-427

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研究成果の刊行物・別刷

Running Title: Nanomedicine for EPR effect based tumor targeting

Nanomedicine and cancer drug delivery based on the EPR effect and EPR augmentation

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Abbreviations

ACE	Angiotensin converting enzyme
AT-Ⅱ	Angiotensin Ⅱ
BSA	Bovine serum albumin
CO	Carbon monoxide
COX	Cyclooxygenase
CT	Computer topography
EPR	Enhanced permeability and retention
HIF-1α	Hypoxia inducible factor 1 alpha
HO-1	Heme oxygenase-1
HPMA	Hydroxypropyl methacrylamide
ISDN	Isosorbide dinitrate
IgG	Immunoglobulin G
NADPH	nicotinamide adenine dinucleotide phosphate
NCS	neocarzinostatin
NG	Nitroglycerin
NO	Nitric oxide
NOS	Nitric oxide synthase
PEG	Polyethylene glycol
SCID	Severe combined immune deficiency
SEM	Scanning electron microscopy
SERCA	Sarcoplasmic/endoplasmic reticulum ATPase
SMA	Styrene maleic acid
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

Abstract

The enhanced permeability and retention (EPR) effect is the first essential step for selective delivery of macromolecular drugs to tumor tissues. The EPR effect is based on the aberrant architecture of tumor blood vessels and the impaired lymphatic drainage system in tumor tissue. This effect is facilitated by overproduction of multiple vascular mediators such as bradykinin, nitric oxide, prostaglandins, VEGF, and other cytokines in tumor tissue, which may also affect surrounding normal tissues. The biocompatibility, molecular size, and surface charge of macromolecular drugs i.e. nanomedicines are critical determinants of tumor-targeted drug delivery based on the EPR effect. However, ineffective treatment can result from the heterogeneity of the EPR effect in tumor tissues, which impedes drug delivery to some tumors. In this chapter, we also discuss how to overcome this problem by using specific therapeutic methods, such as angiotensin (AT) II-induced high blood pressure, angiotensin-converting enzyme inhibitors, nitric oxide-releasing agents, tumor necrosis factor-α, transforming growth factor-β, and heme oxygenase-1 inducer, some of which were demonstrated to be effective in clinical settings.

1. Introduction

One primary goal of cancer drug development is to produce safe and effective drugs. At the end of the 19th century, Paul Ehrlich proposed the concept of a "*magic bullet,*" that is, a drug should selectively target pathogens and not harm normal cells of a host. The discovery of antibiotics successfully advanced this concept, because most antibiotics target specific molecules that are unique to bacteria and do not occur in mammalian cells or tissues. Most antibiotics are quite safe and have a large therapeutic safety widow. Extension of Ehrlich's concept led to the development of molecular-targeted anticancer drugs. Since the 1990s, many attempts were made to develop such drugs, to affect only tumor-specific kinases or receptors. As part of this strategy, focusing on monoclonal antibodies that target such epitope molecules became a mainstream trend. However, recent clinical data related to these molecular drugs revealed that this approach, despite a few significant prolongation of survival of treated patients in clinical settings using such as imatinib, has limitations and is far from a panacea for cancer treatment [1-5]. In a similar vein, cancer treatment by means of vaccines has been extensively investigated by using multiple cancer antigen-specific peptidyl epitopes based on certain multiple motifs. However, recent multi-institutional phase II/III studies of 153 patients with pancreatic cancer that were carried out in Japan yielded no significant benefits of cancer vaccine treatment [6].

Most solid tumors diagnosed in patients are essentially polyclonal in origin. In the last 10–20 years, these tumors have evolved extensive genetic diversification, as demonstrated by recent results for technological advances in cancer genomics. For example, Maeda and Akaike, et al showed excessive generation of reactive oxygen species and reactive nitrogen species during infection and inflammation [7-12], and this excessive generation resulted in formation of mutants or drug resistant bacteria accompanying damage to the RNA or DNA, or DNA repair systems. Consequently, Sjöblom et al, Wood et al, and others reported highly diversified genetic polymorphism in the human cancer patients [13,14].

These data are in clear contrast to those for the experimental severe combined immunodeficiency (SCID) mouse tumor model, which utilizes a human tumor xenograft that does not elicit a host reaction (immunological or inflammatory reaction) and produces no reactive oxygen species, and thus no mutation such as genetically altered epitopes or genetic polyclonality is expected. In this content, strategies using molecular-targeted drugs such as monoclonal antibodies, however, would not be as effective as hoped because human cancer do indeed produce such genetically altered epitopes or genetic polyclonality. In fact, the therapeutic benefit for patients treated with these molecular-targeted drugs is usually extension of a survival time at most 1-2 months for 3–5 years of expected overall survival of untreated controls or those given conventional anticancer agents [3-5], in addition to the high cost of such drugs [2,3].

In view of these data, anticancer drugs with greatly improved efficacy and affecting more broad cancer types are clearly needed. Development of macromolecular or nanoparticle-based drugs, which utilize the enhanced permeability and retention (EPR) effect for selective drug delivery to tumors, will meet such requirements.

2. Background and characteristics of the EPR effect

In 1986, Matsumura and Maeda [15] originally discovered the EPR effect, in which plasma proteins

with large molecular weight, such as albumin (65 kDa), transferrin (75 kDa), and IgG (160 kDa) exhibited tumor-tropic accumulation (Fig. 1), whereas small proteins such as ovomucoid (29 kDa) and neocarzinostatin (NCS, 12 kDa) did not exhibit this phenomenon. Later, Maeda et al clearly demonstrated the EPR effect by using a synthetic biocompatible polymer of hydroxypropyl methacrylate (HPMA) larger than 40–800 kDa [16-19] (Fig. 2a). This unique feature of the EPR effect in tumor tissue is based on the defective nature of tumor blood vessels, which have an aberrant architecture and are highly permeable, and on the impaired lymphatic drainage system of tumor tissue (see discussion below). To manifest the EPR effect, anticancer drugs must satisfy specific criteria such as biocompatibility, molecular size range, and surface charge, as described below.

<Figure 1>

That the EPR effect does not occur in a few minutes, as does passive targeting, should be emphasized; macromolecules must remain in circulation for several hours, and drug retention by tumor tissue occurs in days to weeks. Passive targeting, however, can last a few minutes, as seen by means of tumor angiography with low-molecular-weight (MW) contrast reagents.

This unique, universal phenomenon—the EPR effect—which occurs in most solid tumors, is believed to be the first requirement before the uptake of drugs by cancer cells, in the development of tumor-targeted nanomedicines. Table 1 presents the molecular criteria required for the EPR effect to occur in solid tumors.

<Table 1>

2-1. Factors involved in enhanced vascular permeability and the reason for retention of macromolecules in tumor tissue

Enhanced vascular permeability in tumor tissue is a key factor contributing to the accumulation of macromolecules in the tumor. Elevated vascular permeability was originally discovered in inflamed tissue: extravasation of plasma protein in inflamed tissue is now known as edema. We also know that tumor tissue and inflamed tissue share many common parameters, which affecting the elevated vascular permeability [18-24]. Enhancement of vascular permeability in both tumor and normal tissue types is mediated by various factors including bradykinin, nitric oxide (NO), prostaglandins, vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF-α), heme oxygenase-1 (HO-1), which generates carbon monoxide (CO), and others [19-25]. These factors open the intercellular gap in blood vessels and facilitate extravasation of plasma components and other macromolecules into the tissue interstitium. Leaky blood vessels in tumors can be readily seen with the use of a scanning electron microscope, which can show vascular casts of extravasated polymer resin in microtumor nodules less than 0.2 mm in diameter (Fig. 1b) [23,24,26]. Leaky tumor vessels are observed even in tumors larger than 10 mm in diameter (Fig. 1a) [24]. The EPR effect is thus seen in most solid tumors, and it ensures a sufficient supply of nutrients for rapidly growing tumors.

<Figure 2>

Aberrant vascular architecture including unusually large pores in the vessel walls of many tumor blood vessels, which range from 200 nm to 2 μm or larger [26], contributes to extravasation of large

nanoparticles. Hashizume et al reported similar findings [27]. Bacteria such as *Lactobacillus* and *Salmonella* spp. also reportedly accumulated preferentially in tumor nodules in mice [28-30].

Another key factor contributing to the EPR effect is impairment of the lymphatic drainage system in tumor tissue, which is the primary reason for prolonged retention of macromolecules in tumor tissue. Lymphatic drainage is the main route of clearance of macromolecules or lipid particles from the tissue matrix or the interstitial space of normal tissues [31]. For instance, Lipiodol® (iodinated poppy seed oil) is used as a contrast agent for lymphangiography (imaging of lymphatic ducts and networks), because Lipiodol is largely recovered via the lymphatic system in normal tissues, and its presence is evidence of lymphatic vessels, which can be visualized with X-ray imaging [31-35]. Once Lipiodol is delivered to tumor tissues, it remains there for a prolonged time [33-35].

2-2. Biocompatibility, molecular size, and surface charge of macromolecules and the EPR effect

2-2-1. Biocompatibility

(1) Plasma proteins and plasma residence time. One of the most important factors that govern the EPR effect is the biocompatibility of macromolecules. We demonstrated earlier that even the most biocompatible plasma proteins, which have very long plasma circulation times (more than weeks), would lose this characteristic after chemical modification. Also, in vivo examination of the circulation time of proteins that had originated in different species (xenogenous proteins) showed that macrophages or scavenger receptors identify these proteins as nonself proteins, capture them, and clear them rapidly. Interaction of a protease and an inhibitor, such as plasmin and α_2-macroglobulin, causes a great reduction in the plasma residence time ($t_{1/2}$), from about 140 h to a few minutes (Tables 2 and 3). These findings mean that not only the size of the macromolecules but also their affinity to the host, or biocompatibility, controls the residence time in plasma during circulation.

<Tables 1-3>

(2) Conjugation with polyethylene glycol (PEG) and other polymers. Conjugation of proteins with PEG, or pegylation of proteins, is one way to overcome the disadvantage of rapid clearance of macromolecules or foreign proteins. PEG is nonadhesive, nonimmunogenic, and usually nonantigenic, and it confers high solubility in water. Thus, pegylation, although attachment of the PEG chain increases the size of target molecules, results in a longer plasma circulation time (Tables 2 and 3) [36-41]. PEG-modified proteins also have increased resistance to proteolytic degradation [36,38,40]. Maeda et al reported that conjugation of poly(styrene-co-maleic acid) (SMA) to NCS (MW 12 kDa), to produce an agent named SMANCS [42], had an effect similar to that of pegylation. We also showed that conjugation with divinyl ether-maleic acid (pyran) copolymer (DIVEMA), or polyvinylpyrrolidone, as well as gelatin, yielded the same advantage [9,43-46].

2-2-2. Molecular size

We stated earlier that the EPR effect is characteristic of macromolecules (>40 kDa). Low-MW drugs (<40 kDa) diffuse more freely through blood vessels, so they distribute in tumor as well as normal tissues

throughout the body. Low-MW drugs given by infusion via a tumor-feeding artery first reach the tumor tissue because of the "first-path" effect and will distribute throughout the tumor tissue in only a few minutes, but they then quickly diffuse out from the interstitial space into the bloodstream and are excreted via the kidney into the urine. Therefore, in most cases, low-MW drugs are not retained in tumor tissue for long periods [16,23,24,47,48]. However, because of the large endothelial gap openings (0.2–2 μm) in the tumor vasculature, macromolecules extravasate specifically into tumor tissue and do not enter normal tissue because of tight endothelial intercellular gaps (<5 nm) [24,26]. This finding indicates that macromolecules cannot leak out of vasculature in normal tissue. Furthermore, extravasated macromolecules are not cleared from the tumor tissue as described above. These two advantages—tumor-selective leakage and retention of macromolecules—result in tumor-selective drug delivery [22-24,46,47]. It should be also emphasized that molecular size (> 40 KDa) is not the main criteria for the EPR effect, but biocompatibility governs the prolonged circulation that result in sustained extravasation for the EPR effect as discussed above, and shown in Table 2 and 3.

2-2-3. Surface charge

With regard to the surface charge of macromolecules, polycations such as a cationic liposome-DNA complex, poly(L-lysine)/DNA, at 50–100 nm in diameter, were rapidly cleared from systemic circulation with a half-life in blood of less than 3 min [48-50]. This rapid clearance is similar to that of small proteins such as superoxide dismutase [9,10,44] and NCS [42,43,45], but these are excreted via urine. Rutter and Wade reported that macromolecular proteins with different isoelectric points, and thus different surface charges in physiological solution, clearly affected the blood half-life of proteins in circulation [39] (Fig. 1). Chunbai et al also reported that chitosan nanoparticles with both negative and positive charges: ζpotential = −10 to −40 mV have a longer blood half-life and accumulate more in tumors than with a positive charge (ζpotential = +10 ~ +35 mV [51]. These results suggest that a slightly negative to a neutral charge is preferable for prolonged systemic circulation and facilitation of the EPR effect (Tables 2 and 3).

3. Heterogeneity of the EPR effect

Although the EPR effect is a common phenomenon in most solid tumors, the effect varies from one tumor type to another. Various tumors have different histopathological characteristics, vascularity, genetic diversity, implanted or spontaneous origins of primary or metastatic, hypoxic or normoxic features in the microenvironment, and so on. For instance, a tumor exhibiting the EPR effect could be as small as <1 mm in diameter [23,24,52]. However, clinical and experimental tumors can be as large as 50–100 mm in diameter , which means that some part of a large tumor may be necrotic or rich in fibrotic tissue or clotted vessels. When we studied a large S-180 tumor (>3 cm), accumulation of blue albumin (or a putative macromolecular drug) was extensive primarily in the peripheral tumor tissue; the inner core of the tumor held much less dye [23,24,34,52,53]. However, after intravenous (i.v.) injection of the dye-albumin complex into a small S-180 tumor (4–6 mm), the tumor tissue had more uniform uptake throughout the whole tumor.

Such heterogeneity occurs in human cases as well. For example, when SMANCS/Lipiodol was injected into patients with metastatic liver cancer, the staining, visualized by computed tomography (CT), was seen predominantly at the tumor periphery [23,24,52,53]. The reasons for this may be sparse, compressed, or occluded blood vessels, or an extremely limited blood supply in the inner core of the tumor. These tumors were identified as hypoxic or avascular, or they frequently became necrotic, and thus drug delivery to the inner core of the tumor would be limited. However, tumor periphery showed the most active angiogenesis and rich blood flow. These features are accompanied by the generation of vascular mediators, as discussed in 2-1, which would result in active tumor growth and extravasation of plasma components and other macromolecules. This heterogeneous vascular environment would lead to heterogeneous drug delivery to tumor tissues. Pancreatic and prostatic cancers, both of which are hypovascular tumors, exhibit a poor EPR effect [24,35,54]. Such a poor EPR effect would result in ineffective treatment with conventional as well as macromolecular anticancer drugs. Therefore, overcoming the problem of heterogeneity of the EPR effect and augmenting drug delivery are of great importance. Accordingly, we developed methods to augment the EPR effect, as described below.

4. Augmentation of the accumulation of macromolecular drugs in tumors

4-1. Arterial infusion of Lipiodol and augmentation of its accumulation under angiotensin II (AT-II)-induced high blood pressure

We developed several techniques to overcome the EPR-related heterogeneous accumulation of macromolecular drugs in tumors. The most direct delivery of anticancer agents to tumors involves the infusion of a drug into the tumor-feeding artery, which utilizes the first-path effect. However, only marginal therapeutic benefit occurs with conventional low-MW anticancer agents because these drugs diffuse rapidly and return to the circulation, an effect that would be similar to that after i.v. infusion [53]. When a lipid-formulated drug such as SMANCS/Lipiodol is given via this arterial route, however, its delivery to the tumor is more efficient. The high affinity of SMANCS to Lipiodol, because of its highly lipophilic nature, means that it mixes well in Lipiodol. This type of drug and the method of administration offer many advantages, as follows. First, SMANCS/Lipiodol can most effectively target tumors. The tumor/blood ratio of the drug concentration favored the tumor more than 2000-fold. Second, SMANCS/Lipiodol stays in the tumor interstitium for several weeks because of the sticky nature of the lipid and the macromolecular characteristics of the agent [24,32-35,54], and it thus has a markedly prolonged antitumor effect. This arterial infusion was applied to primary hepatoma and renal cancer, which are hypervascular. Other tumors such as metastatic liver cancer and cancers of the gallbladder and pancreas do not demonstrate such a remarkable effect, so forced intraarterial infusion is preferred. The third advantage is the clear CT imaging of the target tumor; for example, the image of CT scan reveals the tumor size and the spread in the liver as a bright area, which indicates Lipiodol deposition. The last advantage is semiquantitation of the drug delivered to the tumor, which informs clinicians about any needed additional administration.

In contrast to primary hepatoma and renal cancer, certain tumors such as prostatic, pancreatic, and metastatic liver cancers are hypovascular, as revealed by angiography, and thus demonstrate a poor EPR effect,

as mentioned earlier. To address the poor drug delivery to these hypovascular cancers, or difficult-to-treat tumors, the method of arterial infusion under AT-II-induced hypertension was developed [54]. Tumor blood vessels lack a vascular smooth muscle layer or pericytes surrounding the vessels that mediate vasoconstriction. Tumor blood vessels therefore exhibit an extremely small constrictive response to AT-II, whereas normal blood vessels constrict in response to i.v. AT-II, which leads to systemic hypertension. Under such hypertensive conditions, as Figure 3 shows, tumor vessels open by means of hydrodynamic pressure, and thereby the blood flow volume in tumor vessels increases [55], accompanied by polymeric drugs being forced out, into the tumor tissue. This tumor-specific event leads to more effective drug delivery to tumor tissue than to normal tissue. Under AT-II-induced hypertension (e.g., 100 mmHg → 160 mmHg), therefore, nanomedicines (including albumin, SMANCS, IgG, HPMA-drug conjugates, and SMA micelles, as well as liposomes, and lipid particles) leak out more into the interstitial space of tumor tissue than into the interstitial space of normal tissue, which results in an augmented therapeutic effect [47,53,54] (Fig. 3). A 2- to 3-fold increase in drug accumulation in tumor tissue occurred, and drugs remained there for more than 6 h. This situation is a great contrast to the one in normal blood vessels.

Also important is a significantly reduced adverse effect of these drugs on bone marrow and intestines [53,54]. Further, under AT-II-induced hypertension, delivery of low-MW drugs to tumors had increased only transiently (20–25%) at 15 min after injection, so no significant therapeutic advantage occurred, in contrast to the situation with high-MW agents [53]. AT-II-induced hypertension during normal clinical procedures usually maintained for 10–20 min, when AT-II is given by arterial infusion by an experienced angiographer [54]; it will return to normotensive state within 3-5 min as AT-II infusion is ended.

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Although delivery of macromolecules to solid tumors was reportedly difficult because of an elevated interstitial fluid pressure in tumors [58], we achieved progressively increased drug delivery by using various plasma proteins, Lipiodol, and a monoclonal antibody against a tumor [32-35,54,57]. Therefore, AT-II-induced hypertension, which led to significantly increased blood flow in tumor tissue, is an effective way to overcome obstacles such as interstitial fluid pressure in tumors [54,56,57,59]. Also important is a significantly reduced adverse effect of these drugs on bone marrow and intestines [53,54].

In the future, after many polymeric drugs and nanomedicines become available, AT-II-induced hypertension may be recommended as a therapeutic modality that not only enhances bioavailability of the drug to tumor but also reduces drug delivery to normal tissues and hence produces fewer adverse effects.

<Figure 3>

4-2. Bradykinin (kinin) and ACE inhibitors

Kinin (a nonapeptide) is a major mediator of inflammation that is also induced in infection and cancer. Kinin dilates blood vessels (vasodilation) and enhances vascular permeability and thus causes extravasation of body fluids in inflamed (edema) and cancerous tissues [20,21,24,60-64]. Maeda et al reported that kinin and [hydroxyprolyl$_3$] bradykinin were found in the plasma and peritoneal fluid in advanced cancer patients [20,62-64]. During activation of the protease cascade of kinin generation, called the kallikrein-kinin cascade, serine prostease kallikrein and cancer cell-derived plasminogen activator are responsible [20,62,63]. Administration of nanomolar amounts of kinin into guinea pig skin significantly increased vascular permeability, and extravasation of Evans blue-bound albumin at the injection site was observed [20,21,45,60,63,64]. In the peritoneal fluid of patients with ascites tumor and pleural effusion of lung cancer patients, the kinin level was significantly elevated (1-40 ng/ml) and was sufficient to result in an enhanced permeability effect [62].

Administration of a kinin receptor antagonist (HOE 140) suppressed vascular permeability and inhibited extravasation of body fluid in a subcutaneous tumor and ascites in ascites tumor-bearing mice [20,21]. Administration of soybean trypsin inhibitor, a known inhibitor of plasma kallikrein, also suppressed kinin generation and thus reduced ascites formation [64].

With regard to the effect of kinin, NO and prostaglandins interacted during mediation of the EPR effect in a tumor model. Extravasation of the Evans blue-albumin complex was suppressed about 46% after administration of HOE 140, 39% after administration of the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), and 49% after administration of a cyclooxygenase inhibitor (indomethacin) [20,21,63]. These studies thus showed that kinin is one of the most potent mediators of vascular permeability in tumors, and an increased kinin level in a tumor would lead to augmented vascular permeability in the tumor. One method of increasing the kinin level in the tumor is direct local infusion. However, the half-life of kinin in plasma is only a few seconds, and the infusion of intact kinin does little to enhance the EPR effect while possibly inducing pain.

Another way to increase the kinin level in the tumor is to inhibit the degradation pathway of kinin. Kinin is known to be degraded by kininase 2, which is also called angiotensin-converting enzyme (ACE). ACE inhibitors such as enalapril and captopril are widely used as standard antihypertensive drugs that can inhibit both conversions of angiotensin I (AT-I) to AT-II as well as degradation of kinin. Because of the similar amino acid sequences of AT-I and kinin at the C-terminal end, ACE inhibitors can inhibit the degradation of kinin and the conversion of AT-I to AT-II, so administering ACE inhibitors will increase the kinin level at the site of its generation (tumor). This working hypothesis was confirmed by using ^{51}Cr-labeled bovine serum albumin and ^{125}I-labeled monoclonal antibody in tumor-bearing mice [57,63,64] (Fig. 4). Dr. Felix Kratz, University of Freiburg, also confirmed this effect of ACE inhibitors in different tumor models; in tumor targeting of albumin doxorubicin conjugate (personal communication; unpublished data).

<Figure 4>

4-3. NO and other NO-releasing agents

NO is an endogenous free radical molecule that plays important roles as a signaling messenger in cells. NO is naturally synthesized from L-arginine by nitric oxide synthase (NOS) in the presence of O_2, NADPH, and calmodulin. With respect to blood vessels, NO enhances the activity of soluble guanyl cyclase (200-fold), which leads to dephosphorylation of myosin light chain followed by smooth muscle relaxation and vasodilation [65,66]. NO also induces calcium uptake by the sarcoplasmic reticulum by activating calcium ATPase in the sarcoplasmic/endoplasmic reticulum, which results in a reduced intracellular calcium level and smooth muscle cell relaxation [65]. NO also serves as a growth promoter in solid tumor and can enhance vascular permeability and increase blood flow [21,67].

In clinical settings, nitroglycerin, isosorbide dinitrate (ISDN), amyl nitrite, and other NO releasing agents are frequently used to treat angina pectoris and myocardial infarction. These NO-releasing drugs are administered orally or applied transdermaly and are converted to nitrite NO_2^- in hypoxic conditions such as those found in infarcted or tumor tissues. In such tissues, nitrite is further reduced to NO by nitrite reductase [68,69]. The tissue environment in many tumors is hypoxic and acidic, similar to the environment in infarcted heart tissue. We would therefore expect NO production to occur preferentially in tumor tissue after application of an NO-releasing agent such as nitroglycerin. In fact, Seki et al in our laboratory reported that transdermal application of nitroglycerin (0.01–2.0 mg/site over the skin) increased blood flow in the tumor but not in the normal tissue (muscle), which led to enhanced accumulation of macromolecular drugs in the tumor [69] (Fig. 5). The application site of nitroglycerin ointment can be anywhere, even at a site distal from the tumor, because nitroglycerin reaches the blood circulation readily.

<Figure 5>

Jordan et al also reported that ISDN treatment of patients increased the blood flow in prostate tumors, which led to increased tissue pO_2 in this hypoxic tumor [70]. Use of ISDN therefore improved the therapeutic response [70]. They suggested that the increased blood flow and accompanying higher pO_2 would result in decreased VEGF, with tumor growth suppression and reduced generation of P-glycoprotein in the tumor, via rapid decreases in the expression of hypoxia-inducible factor-1α [71]. Similarly, NO donors such as *S*-nitroso-*N*-acetyl-D-penicillamine increased the intracellular accumulation of doxorubicin in a doxorubicin-resistant cancer cell line via reduction of doxorubicin efflux by tyrosine nitration in multidrug resistance-associated protein 3 [72]. In a different context, radiosensitization by inducing iNOS using γ-interferon was also reported in hypoxic EMT-6 mammary adenocarcinoma, in which NO is the key player [73].

In addition, Yasuda et al observed an improved clinical response with vinorelbine and cisplatin in non-small cell lung carcinoma [74], and with docetaxel and carboplatin in lung adenocarcinoma [71], both studies demonstrating a significant benefit of using nitroglycerin. Our interpretation of the beneficial effect of nitroglycerin is that the benefit is primarily due to enhanced drug delivery (via the EPR effect). In addition, findings of both Yasuda et al [71] and Seki et al [69] showed that nitroglycerin alone significantly suppressed tumor growth.

4-4. TNF-α and Rho kinase

TNF-α is a pleiotropic proinflammatory cytokine that has permeability-enhancing and direct toxic effects on tumor-associated vasculature in vivo [75]. TNF-α substantially alters the integrity of endothelial monolayer cells and remodels the cytoskeleton [76] as well as redistributes cell-cell adhesion molecules such as PECAM-1 [77] and vascular endothelial cadherin [78]. All these events lead to enhanced vascular permeability in vivo. In subcutaneous tumor models, systemic administration of TNF-α increased the vascular permeability of only the tumor, which resulted in higher tumor uptake of monoclonal antibodies [79] and viral particles as well as the Evans blue-albumin complex [80], without any change in the tumor blood flow volume. The TNF-α-induced increase in vascular permeability can be reversed by treatment with Rho kinase inhibitor (Y-27632), which suggests the important role of Rho signaling in TNF-α-induced enhancement of vascular permeability. Van Nieuw Amerongen et al reported that a Rho signaling inhibitor (simvastatin) suppressed vascular leakage of the Evans blue-albumin complex into the thoracic and abdominal aorta in the atherosclerosis-induced Watanabe heritable hyperlipidemic rabbit model [81]. In a rat sarcoma model, isolated limb perfusion with TNF-α increased the concentrations of melphalan and doxorubicin in the tumor, whereas no increased uptake occurred in the normal muscle and skin [82,83].

Notwithstanding above effects of TNF-α, it was used as anticancer biologic agent n cancer patients. The major effect was severe fever and therapeutic effect was marginal or not beneficial.

4-5. Transforming growth factor-β (TGF-β) type 1 receptor inhibitor

TGF-β is a multifunctional cytokine, similar to TNF-β, that plays a pivotal role in the suppression of growth and the differentiation of tumor cells and the tumor environment: TGF-β signaling suppresses the growth of both epithelial and endothelial cells and induces the production of extracellular matrix [84]. Hypovascularity and extensive fibrosis found in pancreatic adenocarcinoma and diffuse-type gastric carcinoma are thought to be major obstacles to achieving efficient delivery of drugs to tumors in conventional chemotherapy as well as EPR effect-based therapy [85,86]. Inhibition of the TGF-β receptor in tumor tissue should produce an effect similar to inhibition of VEGF: growth progression of endothelial cells and decreased production of extracellular matrix and fibrosis, so that delivery of anticancer drugs to tumors should be facilitated.

Treatment with low doses of TGF-β type 1 receptor inhibitor (LY364947; 1 mg/kg) inhibited TGF-α signaling in the vascular endothelium but not in cancer cells and most interstitial cells [87]. Abnormal blood vessels with aberrant and irregular dilation were found in typical untreated tumors in mice; whereas the vasculature of mice treated mice with TGF-β type 1 receptor inhibitor was narrower and rounder than untreated controls, which suggests vascular normalization [87]. It is interesting that TGF-β type 1 receptor inhibitor treatment normalized blood flow in the tumor, i.e., unidirectional constant flow (as in normal blood flow) was

observed [88]. Minowa et al [88] reported that inhibiting TGF-β receptor (by using A-83-01) increased the number of pericytes surrounding the tumor vasculature. Kano et al [87] however, reported a reduced number of pericytes surrounding the tumor vasculature. This controversial issue may be due to the different tumor types and/or different types of TGF-β receptor inhibitor used.

Both reports did show, however, the positive effect of TGF-β receptor inhibitor for enhanced drug delivery to tumors in a molecular size-dependent manner, as seen with the EPR effect. At 24 h after injection of macromolecular drugs, i.e., 2×10^6 Da dextran with a 50-nm hydrodynamic diameter, Doxil® with a 108-nm diameter, and adriamycin (ADR) micelles with a 65-nm diameter, and treatment with TGF-β type 1 receptor inhibitor, all drugs effectively accumulated in the tumors [87] (Fig. 6). However, treatment with TGF-β type 1 receptor inhibitor did not significantly enhance accumulation of the low-MW free ADR (MW 543) and bromodeoxyuridine (MW 307) in the tumors (Fig. 6). This augmented accumulation of macromolecular therapeutic agents (Doxil and ADR micelles) resulted in a marked suppression of tumor growth, even in the hypovascular solid tumor, which is difficult to treat. Thus, augmenting the EPR effect by using TGF-β type 1 receptor inhibitor warrants further exploitation.

<Figure 6>

4-6. HO-1 induction and the CO-based enhanced EPR effect

Similar to NO, CO, a gaseous molecule that has a critical role in vascular physiology, has recently been receiving considerable attention. An excessive dose of CO may be hazardous, but CO given as a microdose is now considered to be an important vascular modulator, particularly in transplantation surgery [89,90]. CO causes the vascular tonus to dilate and facilitates erythrocyte trafficking, which transports molecular oxygen to peripheral tissues. CO is naturally generated in the body by HO-1 together with biliverdin during heme catabolism. Heme oxygenase-1 (HO-1) is responsible for 80% of CO production in the body [91] and it is highly upregulated in most cancers in vivo [92]. Recently, Fang et al in our laboratory reported that a subcutaneous injection of recombinant HO-1 facilitated extravasation of Evans blue-albumin complex as a result of the EPR effect [25]. More direct evidence of involvement of CO in the EPR effect was demonstrated by subcutaneous administration of a CO-releasing agent, tricarbonyldichlororuthenium (II) dimer (CORM-2) [25] (Fig. 7). Furthermore, the EPR effect-based macromolecular drug delivery was enhanced by inducing HO-1 in cancer tissue, which was accomplished by administering pegylated hemin as a polymeric drug, which, because of the EPR effect, is selectively delivered to tumor tissue rather than to normal tissue. Hemin is a typical inducer of HO-1, and we developed a water-soluble micellar form of hemin for tumor targeting by means of the EPR effect [25] (Fig. 7).

<Figure 7>

5. Concluding remarks

Nanomedicine, i.e., macromolecular drug, development for cancer chemotherapy, is thought to

represent a new paradigm in cancer treatment and has been the focus of considerable attention. The major advantage of nanomedicine in cancer chemotherapy is the ability of drugs to target tumor tissue on the basis of the EPR effect, to improve therapeutic efficacy and reduce the adverse effects. Tumor tissue usually has aberrant blood vasculature: irregular networks of vessels, lack of surrounding smooth muscle layer cells, irregular blood flow, and impaired lymphatic clearance. Vascular permeability factors such as kinin, prostaglandins, NO, and inflammatory cytokines are overexpressed in both cancer and inflamed tissues. Clearly, these factors also affect normal vessels near the tumor tissue. These upregulated vascular factors and the architectural defects of tumor blood vessels are the major contributors to the EPR effect. Once macromolecules extravasate into the interstitial space of tumor tissue, they are retained in the tumor for long periods, more than a week; they are not cleared because of the impaired lymphatic recovery system in the tumor, and thus they have a sustained pharmacological effect.

Tumor-specific accumulation and retention of drugs that function based on the EPR effect pertain to biocompatible macromolecules with MW of >40 kDa. Nevertheless, the EPR effect is frequently heterogeneous, especially in large tumors. Also, tumors such as pancreatic, metastatic liver, and prostatic cancer have hypovascular features and thus demonstrate less of an EPR effect. These obstacles to drug delivery must be overcome to achieve more efficient and more uniform delivery of drugs to tumors.

In this chapter, we discussed different methods of augmenting the EPR effect and thereby providing more homogeneous drug delivery to tumors. AT-II-induced hypertension increases leakage of drugs, such as SMANCS/Lipiodol given by intraarterial infusion, into the tumor interstitium. Similarly, ACE inhibitors, NO-releasing agents such as nitroglycerin, TGF-β receptor inhibitors, and HO-1 inducers may be useful for an augmentation of EPR effect. Early clinical evaluation of drug administration based on the EPR effect did improve drug delivery, as demonstrated with SMANCS/Lipiodol administered by the intraarterial route under induced hypertension, the result with this specific formulation being improved therapeutic efficacy without serious adverse effects [54]. Also, use of nitroglycerin or other NO-releasing agents in combination with conventional cancer chemotherapeutic agents was reportedly beneficial in clinical settings [70,71,74,93]. In conclusion, under these circumstances as described herein, EPR-based drug design is becoming an important issue for development of macromolecular drugs used to treat solid tumors.