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Review article

Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect

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ABSTRACT

For over half a century extensive research has been undertaken for the control of cancer. However, success has been limited to certain malignancies, and surgical intervention is potentially curative for early stage patients. For the majority of patients with advanced stage of cancer, the treatment is limited to chemotherapy or radiation. Chemotherapy in particular has limitations due to the lack of selectivity with severe toxicity. Under these circumstances tumor-targeted delivery of anticancer drugs is perhaps one of the most important steps for cancer chemotherapy. We reported such a drug for the first time, styrene-maleic acid copolymer-conjugated neocarzinostatin (SMANCS) in 1979, and it eventually led to formulate the concept of the enhanced permeability and retention (EPR) effect of solid tumors in 1986. Monoclonal antibody conjugates are another direction, of which interest is increasing recently though with limited success. The EPR-effect appears as a universal phenomenon in solid tumors which warrants the development of other polymeric drugs or nanomedicine.

EPR-effect is applicable for any biocompatible macromolecular compounds above 40 kDa, even larger than 800 kDa, or of the size of bacteria; thus complexed molecules like micelles and liposomes containing anticancer drugs are hallmark examples. The drug concentration in tumor compared to that of the blood (T/B ratio) can be usually as high as 10–30 times. In case of SMANCS/Lipiodol given via tumor feeding artery, the T/B ratio can be as high as 2000, a real pin-point targeting. EPR-effect is not just passive targeting for momentary tumor delivery, but it means prolonged drug retention for more than several weeks or longer.

This review describes the pathophysiological mechanisms of the EPR-effect, architectural difference of tumor blood vessel, various factors involved and artificial augmentation of EPR-effect with respect to tumor-selective delivery, and then advantages and problems of macromolecular drugs.

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1. Introduction

The field of drug delivery systems (DDS) utilizing synthetic polymers either by covalent conjugation or by composite of micellar drugs has become a new domain for new drug development for numerous diseases. Synthetic polymers become an indispensable component for micellar or stealth liposome drugs and protein–polymer conjugates [1–3]. These polymer-based new drug entities are called "polymer therapeutics" [2,3] or macromolecular drugs, and they overlap with nanomedicine that becomes popular in recent years [4]. The polymer therapeutics or nanomedicines are designed to improve drug performance by utilizing pathophysiological uniqueness of solid tumor, of which conventional low molecular weight drugs are incapable. Macromolecular drugs or nanomedicines show improved tumor-selective targeting; the improved therapeutic efficacy and fewer side effects are their primary benefits, in which prolonged circulation time plays a crucial role [4–9].

Most conventional low molecular weight anticancer drugs have inherent character to traverse in and out of blood vessels freely, unless the drug is linked with a tumor-specific molecular ligand having high binding constant. For instance, low molecular weight

Abbreviations: AT-II, angiotensin-II; AUC, area under the concentration curve (vs time); CML, chronic myeloid leukemia; EPR, enhanced permeability and retention effect (of macromolecular drugs in solid tumor); HPMA, poly(hydroxypropyl methacrylic acid); HCC, hepatocellular carcinoma (hepatoma); i.v., intravenously; i.a., intra-arterially; MDR, multidrug resistance; NCS, neocarzinostatin; NO, nitric oxide; NOS, nitric oxide synthase; $ONOO^-$, peroxynitrite; PEG, polyethylene glycol (also called polyoxyethylene); PGs, prostaglandins; PEG-poly(Asp), block copolymer (polyethylene glycol) linked to poly (aspartic acid-benzyl ester); SMA, copolymer of styrene-maleic acid; SMANCS, copoly (styrene-maleic acid) conjugated neocarzinostatin; SOD, superoxide dismutase; T/B, tumor to blood ratio of drug (delivered concentration); VPF, vascular permeability factor; VEGF, vascular endothelial growth factor.

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drugs injected intramuscularly reach a distant site of the body in 10 min. Consequently, their undesirable indiscriminatory distribution in normal tissues causes severe systemic side effects in case of anticancer agents. Namely, free diffusion of toxic drugs in a non-selective manner in the body, and their inability to accumulate selectively in tumor tissues make them disastrous to patients.

Experiences in antibiotic research tell us that selective toxicity is possible in prokaryotic kingdom, where different types of biochemical machinery are used from the animal kingdom. For instance, machinery for protein-synthesis (ribosomes) in prokaryotes is different from eukaryotic cells. Similarly, the cell-wall synthesis of peptidoglycan in bacteria (a target for penicillin) does not exist in eukaryotes. On the contrary, it is difficult to get tumor-selective toxicity because the biological events taking place in cancer cells are essentially the same as that of the host cells. Namely, there is little difference in biochemical or molecular machinery between cancer and normal cells at a cellular or molecular level. Therefore, to target tumor cells more selectively, active targeting based on antibodies or the receptor-mediated targeting with cancer-specific ligands are developed. However, recent clinical results of molecular target-based drugs were somewhat disappointing, if not completely.

Tumor cells have inherent heterogeneity and epitopic diversification as a result of great magnitude of mutation frequency even amongst the same cancer patient [10,12]. The recent results of cancer genomics showed that most human solid tumors were not only single gene-based events, but also multiple genomic alterations. Namely, there were no specific alterations or gene mutations common among individual patients if not name p53, a cancer suppressor gene. Extensive genomic studies of 11 colon cancer and 11 breast cancer patients revealed numerous genetic variants arising from a single solid malignancy. On average about 90 or so such variants were found in a patient. This makes the task of specific antibodies for each of these diverse epitopic targets inefficient if not unrealistic from antibody therapy [10,11].

Furthermore, according to recent reports in the annual meeting of *American Society of Clinical Oncology* (*ASCO),* efficacy of molecular target drugs exhibited only 4–5% of response rate despite very high expectation and very high cost of manufacturing. It is generally thought this much efficacy is only useful as adjuvant or supplementary. Although it is beyond this review, the costs of antibody drugs are so expensive that public and national insurance systems may be at risk aside from the low response rate achieved with these therapies. In this regard, for example, Avastin was not recommended in the UK for reimbursement of national insurance [13]. Thus, 'the cost-benefit' will be considered more than ever for drug approval. Recently, the editorial in the Lancet criticized these issues one step further for drug appraisal [14].

Under these circumstances, a more universal and efficient strategy for anticancer drug design having high selectivity to tumor tissues must be developed. To solve this problem, the phenomenon of "enhanced permeability and retention (EPR)-effect" discovered by Maeda and Matsumura is now becoming the gold-standard in cancer-targeting drug designing that is based on macromolecular, micellar and lipidic particles [5–9], all utilizing EPR-effect as a guiding principle, and the EPR-effect is applicable for almost all rapidly growing solid tumors [7–9,15–18].

Most importantly, EPR-effect can be observed in almost all human cancers with the exception of hypovascular tumors such as prostate cancer or pancreatic cancer. As clinical examples for this, we have experienced that SMANCS/Lipiodol given via the hepatic artery accumulated selectively in hepatocellular carcinoma distinctively [7,17,19–22]. A similar result in clinical setting was also reported for Doxil, a liposomal type of doxorubicin. Namely, Doxil mimic was prepared for radio scintigraphy, and clear tumor accumulation was seen in the whole body scintigram [23]. Another clinical example of EPR-effect can be demonstrated in the traditional tumor imaging in the clinic that utilizes (γ)-emitting gallium scintigraphy based on the selective accumulation of radioactive gallium (used as citrate) in the tumor. ^{65}Gallium ion as injected i.v. will bind to plasma protein transferrin (90 kDa) in the blood, thus radioactive transferrin will accumulate in the tumor by EPR-effect, which will take more than 24 h. Usually radio-scintigram is obtained 2–3 days after intravenous injection of ^{65}Ga when signal/noise ratio is improved; while its clearance from the normal tissues will take place in a day or so via the lymphatic system. The tumor, however, retains this ^{65}Ga-transferrin for several days at high levels by EPR-effect.

Another case of EPR-effect observed in human tumor is the selective accumulation of Lipiodol in the tumor after intra-arterial infusion of Lipiodol which is visualized by X-ray CT-scan [19–21]. This tumor detection method by use of Lipiodol staining after our report [19–22] is now becoming a routine examination before hepatic tumor resection.

Conventional angiography for tumor detection uses water-soluble low molecular weight radio contrast agent, and its high electron density yields staining of tumor as it is infused intra-arterially. This means increased uptake (staining) of this contrast agent by the tumor mass, which is a part of EPR-effect though it is washed out rapidly by diffusion due to the small molecular size (i.e. no retention). Thus, the tumor staining in this angiography is only transitory, less than a few minutes or so. This is a passive delivery of drug, but not EPR-effect which requires long time retention.

Based on EPR-effect, many polymeric drugs are being developed as a new class of antitumor agents [1,24–28], including nanoparticles [16], polymer micelles [27,28] and liposomes [28–31]. Further, EPR-effect is not only limited to these nanoparticles, but it is also valid for tumor-imaging contrast agent Lipiodol as described above, where Lipiodol shows virtually pin-point targeted delivery to the tumor, i.e. tumor/blood (T/B) ratio of more than 2000 can be obtained [19–22,32]. Further, bacterial cells as well as quantum dots (QDs) as ultra-sensitive imaging probe showed more selective accumulation into tumor tissue, which can be explained by EPR-effect [33–35].

In this review, we will describe current problems in cancer chemotherapy, the mechanism of EPR-effect and factors involved, artificial augmentation of EPR-effect for polymeric or macromolecular drugs under the angiotensin-II (AT-II) induced hypertension, and advantages of macromolecular drugs are also discussed.

2. How good is cancer chemotherapy; status quo

In the past 40–50 years, low molecular weight anticancer drugs have been the main treatment modality for many cancers of advanced stage, but have offered no improvement in the cure rate [36,37]. The biggest limitation of these therapeutic agents is overwhelming toxicity due to lack of selectivity. Scientists realized this fact finally towards the end of 20th century and thus cancer-selective targeting became one of the most important goals.

Theoretically, in drug development, molecular target drug is considered more ideal not only in cancer but also in other diseases such as inflammation, primarily to avoid side effects. In this context, *Meares*, editor in chief of *Bioconjugate Chemistry,* a journal of *American Chemical Society*, commented that drug targeting was the most popular subject recently in this journal [38]. In any event, cancer still remains a major cause of death in most developed countries, and the lack of effective control of many cancers is becoming increasingly burdensome on the health care system [36].

As discussed in above references [36,37], the death rate of major cancers such as lung, breast, colon, prostate, and pancreas at advanced stage, has not changed much in the last half century. Further, the clinical benefit of chemotherapy in the most common cancers, for instance the breast cancer and the prostate cancer, is

only marginal when the stage of cancer is advanced. Among the breast cancer patients who underwent surgery only, or who had surgery plus chemotherapy, the benefit of chemotherapy is only 2–3%. Namely, survival extension was 2 months in 60 months, while extensive medical cares including blood transfusion, platelets and leukocytes transfusion, injection of hematogenic factors (EPO, GCSF, etc.), antibiotics, ambulatory care or hospitalization are required for chemotherapy patients, which requires a huge expense. In addition, severe loss of appetite, hair loss, and poor quality of life (QOL) are more frequent in chemotherapy [39,40].

There have substantial increases in survival rate in some malignancies such as early stage gastric cancer by surgical intervention. Clinical results in lymphocytic leukemia and Hodgkin's disease have also been improved more effectively with chemotherapy than before. Early stage cervical cancer has responded well to surgery, radiation, and chemotherapy or a combination approach. Another most recent successful agent is *Gleevec*® (Imanitib) against chronic myeloid leukemia (CML) which has demonstrated 90% regression at least for 6 months. CML is one of the few cancers caused by the activation of *ABL/BCR* oncogene. However, at 6 months when leukemic cells began to propagate (called blastic crisis), drug-resistant mutants concurrently emerge in many or most, if not all patients, making a cure for CML far from reality. Life expectancy of CML patients is about five years or longer if patients remain stable and if it does not turn to blastic crisis.

Prostate cancer is one of the most common cancers amongst males in developed countries. Among the prostate cancer patients, 10–20% have metastatic diseases, most commonly to the bone. These metastatic tumors are less frequently treated by radiotherapy or by surgery, and chemotherapy is used as the next best alternative. In one recent study with more than one thousand prostate cancer patients with hormone refractory metastasis, clinical efficacy of docetaxel plus prednisolone, or mitoxantrone plus prednisolone, was evaluated [41]. The median survival was 17.4 months and 18.9 months, respectively. Although PSA levels decreased by more than 50% in 30–50% of the patients and the QOL was improved, adverse side effects remain a severe problem compared with the slight survival benefit. Similar studies without steroid resulted in 10–21 months of median survival span. An earlier study with mitoxantrone plus prednisone or prednisone alone showed no difference in overall survival benefit [42]. In any event about 65–70% of all patients died within three years, which means a not so remarkable cure rate, and also PSA is not a so accurate marker.

A few kinases, growth factors, DNA polymerases, transporters, etc. are also believed to be unique molecular targets, but the problems are that therapeutic windows are too narrow due to dose limiting toxicity, followed by emerging resistance. One such protein kinase inhibitor trastuzumab (Herceptin®) is an antibody against epidermal growth factor receptor (HER-2) and is used for breast cancer. Herceptin® targets to oncogene product "HER-2", it is thus only suitable for HER-2 positive patients, which comprises only 20% ~ 30% of the total breast cancer patients. Furthermore, among this group, only 20–30% will respond to Herceptin. Therefore, the value will end up at about 4–6% as an overall response rate.

Whatever the chemotherapeutic agents currently available, their excessive and prolonged exposure will ultimately damage normal cells. More serious of all is emergence of drug resistance, even to Gleevec®. Therefore, importance of more universal tumor-targeted drug delivery becomes obvious under these circumstances.

3. Phenomenon and mechanism of the EPR-effect

3.1. Historical background

During the study of in vivo behavior for antitumor protein antibiotic, neocarzinostatin, and its polymer conjugate SMANCS, Maeda et al. discovered a great difference in tumor uptake between low molecular weight drugs and biocompatible macromolecular drug SMANCS in solid tumors [17,24,43–46]. In a series of studies, the EPR-effect was discovered using the most biocompatible macromolecule drug candidates such as albumin (65 kDa), transferrin (90 kDa), IgG (immunoglobulin, 150 kDa), α_2-macroglobulin (240 kDa), ovomucoid of chicken eggwhite (29 kDa, highly glycosylated protein), SMANCS (16 kDa, with albumin binding character becomes 80 kDa) and neocarzinostatin (12 kDa) [6,7]. Macromolecules such as albumin, transferrin, IgG, SMANCS and lipid particles (Lipiodol) accumulated in the solid tumor so remarkably, and they were retained for a very extended period up to 3 months or longer [5–9,17,22].

In general, they found the areas under the concentration curve vs time (AUC) of a drug and its tumor uptake are positively related, whereas the rate of urinary clearance is inversely related to tumor uptake [5,7,46,47]. In collaboration with Prof. K. Ulbrich and Prof. R. Duncan, a series of biocompatible synthetic polymers, HPMA (4-hydroxypropylmethacrylate copolymer) ranging from 20 to 800 kDa in various tumor models (S-180, meth A, colon 38, VX-2 of rabbit, Walkar-256 carcinomas and AH66 of rats) were investigated, and the findings are consistent with previous studies [5–7,46,47]. These polymers displayed remarkable tumor accumulation in mouse and human xenograft tumors up to ~20% dose/g in some case, and 1–5% dose/g usually in small tumors. However, in larger tumors, i.e. more than 0.5 g, the tumor uptake of Evans-blue albumin became lower [46,47,64]. From these results we concluded that, in order to exhibit features of the EPR-effect, a drug must have a higher molecular weight than the renal excretion threshold (typically > 40 kDa) [5–9,17,25–27,46,47].

3.2. Factors involved in EPR-effect

Maeda et al. had previously demonstrated that bacterial proteinases initiated bradykinin generation via factor XII (Hageman factor) activation followed by activation of prekallikrein to kallikrein, which releases bradykinin from kininogen. Bradykinin is known to induce vascular permeability and plays an important role in the EPR-effect. Thus the site of infection/inflammation where excess of bradykinin is generated also exhibits EPR-effect [50–53]. This study was extended to identify the cause of ascitic and pleural effusions of carcinomatosis in peritoneal and pleural compartments. In these studies Maeda et al. were able to identify that bradykinin formation was responsible for the extravasation of ascetic fluid [54–59]. The only difference between infection-induced EPR-effect and that of cancer is duration of retention period; the retention in normal tissue, where inflammation occurs, is shorter than cancer because the lymphatic drainage system is still operative, thus swelling may dissipate in a matter of a few days. In contrast, macromolecular or lipidic drug retention in cancer tissue can be as long as weeks, a great contrast to that of infection-induced inflammation [7,17,19–22]. Under these circumstances, it was realized that vascular phenomenon was important not only because of its critical role in tumor growth and metastasis, but also for tumor-selective delivery of anticancer agents, on which Maeda's group had been working [19–22]. Then, they demonstrated the EPR-effect [5]. Namely, EPR-effect is a phenomenon of enhanced extravasations of macromolecules from tumor blood vessels, and their retention in tumor tissues, which is not observed in normal vasculature.

We then investigated vascular mediators involved in EPR-effect and found that nitric oxide (NO) also facilitates this effect [58,59,64,65] in addition to bradykinin. Oxidized product of NO (peroxynitrite) also shows this effect with or without activation of collagenase [66,67]. A similar phenomenon was observed with other proinflammatory mediators such as prostaglandins [58,66,68] and vascular endothelial growth factor (VEGF) [54,58,

69–71] which was formerly identified by Dvorak et al. as vascular permeability factor (VPF) [71]. In these connections, dual function of VEGF, namely, angiogenesis and enhancement of vascular permeability, both facilitate and sustain rapid growth of tumor. Furthermore, NO is also involved in tumor growth [72], VEGF and bradykinin are also known to be potent activators of NOS which generates NO from L-arginine. Following is the summary of factors facilitating EPR-effect:

(1) Vascular endothelial growth factor (VEGF/VPF).
(2) Bradykinin/3-hydroxyprolyl bradykinin (BK).
(3) Nitric oxide (NO).
(4) Peroxynitrite ($ONOO^-$) (reaction product of superoxide anion radical and NO).
(5) Prostaglandins (PGs).
(6) Matrix metalloproteinases (MMPs); proMMP is activated to MMP by $ONOO^-$.
(7) Other proteinases (e.g. kinin/kallikrein system) involving various protease cascade.
(8) Other cytokines (e.g. tumor necrosis factor, IL-2, and TNF-α.) also facilitating EPR-effect.

3.3. Anatomical and pathophysiological difference of tumor vasculature

As to the anatomical or architectural difference between normal and tumor vasculature, O'Brien's group has demonstrated remarkably unique difference using scanning electron microscope (SEM) [60,61]. Namely, a water-soluble acrylic monomer that forms polymeric resin in the blood vessels was injected into the blood vessel of tumor-bearing mice, thus the cast of the blood vessels of tumor was obtained, which is made of plastic resin. As shown in Fig. 1(C and D), plastic resin is extravasculated extensively outside of blood vessels in the metastatic tumor nodule; more importantly these features are not seen in normal capillary (Fig. 1A and B).

These photographs (Fig. 1C and D) clearly show completely different morphology of tumor blood vessels in microtumor nodules (Fig. 1C) compared with that in normal blood capillary (Fig. 1A and B). More recently Hashizume et al. also observed this phenomenon in mouse mammary carcinoma using scanning electron microscopy [62] and identified structural abnormalities in the endothelium of tumor blood vessels, whose intercellular openings were found to be as large as 4.7 μm (mean size 1.7 μm) in diameter. In other studies Yuan et al. have measured the pore size of tumor vessel in LS-147 human colon adenocarcinoma implanted in SCID mouse and found that it could be as large as 0.4 μm in diameter [63]. All these studies demonstrate that the abnormal vascular architecture plays a major role in excessive vascular permeability of tumor, i.e. EPR-effect in tumor for selective macromolecular drug targeting at tissue level, that can be summarized as follows:

(1) Extensive angiogenesis and high vascular density (hypervasculature).
(2) Lack of smooth-muscle layer; pericytes; no AT-II-induced vasoconstriction; more leakage at tumor vessels under hypertensive state.
(3) Defective vascular architecture; irregular vasculature networks, large openings, fenestration.
(4) Whimsical blood flow; no constant blood flow and direction.
(5) Meager lymphatic clearance that leads to enhanced retention of macromolecular drugs and lipidic particles in the interstitium of tumors.
(6) Slow venous return that leads to accumulation of macromolecular drugs and lipidic particles from the interstitium of tumor.

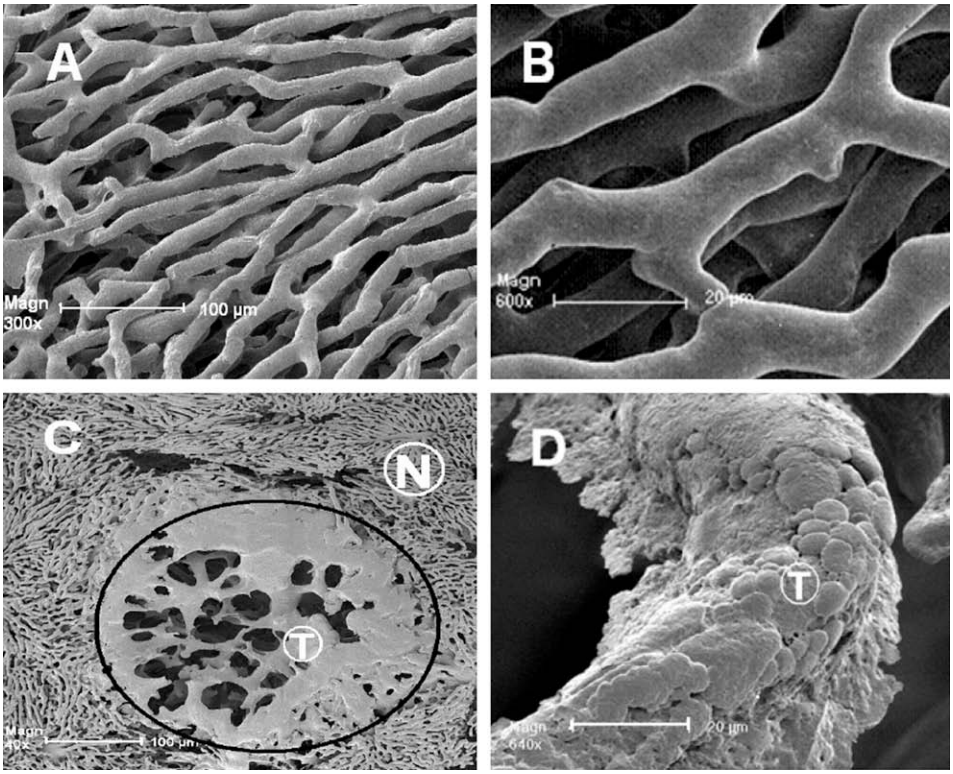

Fig. 1. Scanning electron micrograms of blood vessels at capillary level in normal tissue [liver] (A and B) and blood vessels of tumor nodules (C and D) in the liver. Enlarged image of capillary network of (A) is shown in (B). No leakage of the polymer is seen in the normal liver as indicated by Ⓝ whereas tumor-selective extravasation of polymer (by EPR-effect) in tumor nodule (Ⓣ) is seen, but it is not observed in normal vascular area Ⓝ, or in (A) or (B). (D) Tumor vessels in which extravasation of acrylic polymer is seen. This will lead to selective delivery of polymeric drug in tumor as seen Ⓣ in (C) and (D) [58,61].

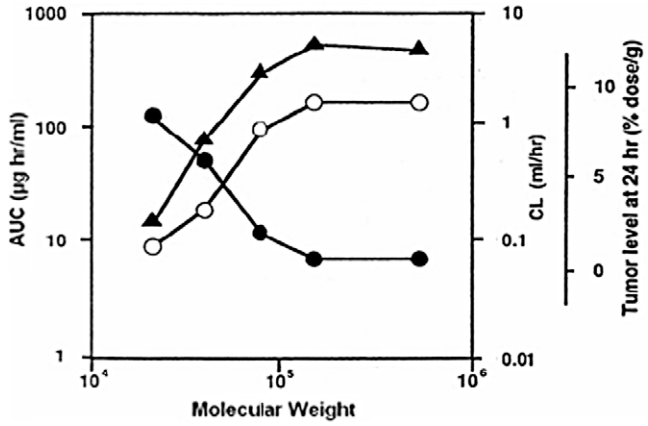

Fig. 2. Relationships between molecular weights and tumor uptake and clearance of ^{125}I-tyr-HPMA-polymer of varied molecular weight drugs. Mice bearing S-180 solid tumor received about 1.8×10^6 cpm/mouse by i.v. injection. ●, CL – renal clearance rate; ▲, AUC – area under the concentration curve for different Mw polymers in plasma. Both values are based on 72 h after injection; ○, tumor uptake values are based on 24 h data [18].

Demonstration of the EPR-effect in metastatic micronodules of tumor in the liver is seen in (Fig. 1C) by using SEM, and blood capillary of normal liver is shown in (Fig. 1A and B). Tumor blood vessels illustrated here show that monomer of plastic resin penetrated the vascular wall during polymerization (Fig. 1C and D), and the vascular network of tumor vessels in the nodule looks abnormal and shows appearance of leaked resin; these are clearly different from the blood capillary of normal liver tissue. Namely, the tumor nodule is selectively almost filled with plastic resin (Fig. 1C).

In the case of SMANCS/Lipiodol, it is administered via the tumor feeding artery. This arterial infusion will result in direct drug capturing, that is known as the first path effect, in which EPR-effect works additively [20–22]. Remarkable success of the SMANCS/Lipiodol therapy can be attributed to the hypervascular nature as well as EPR-effect due to excessive vascular mediators in the hepatoma. This trend of efficient uptake of SMANCS/Lipiodol is also seen for renal cancer. Most water-soluble polymers can accomplish EPR-effect by i.v. infusion as seen by T/B ratio ranging 5–30 [5,7,15–18,64] and EPR-effect becomes obvious even 10 min after i.v. injection [46]. The tumor/normal tissue ratio is as high as 30- to 300-folds at 6 h or later, of which the values depend largely on time of quantification. Most of the macromolecular drugs exhibit prolonged plasma $t_{½}$ (>several hours) and very low renal clearance as seen in Fig. 2.

4. Recovery of macromolecules from the tissues interstitium: the lymphotropism and lymphatic metastasis

When lipids and lipidic particles or macromolecules are injected into the interstitial space of normal tissue, they are known to be recovered via the lymphatic systems. Now it should be noted that this is the route of clearance of lipidic or macromolecular drugs from the normal tissue, but it is also the route of lymphatic metastasis of cancer cells. Initially, Maeda et al. found that when NCS (12 kDa) or SMANCS (16 kDa, by albumin binding it becomes 80 kDa) was injected into normal tissue subcutaneously, they accumulated most effectively in the regional lymph nodes, and very slowly traversed into the general circulation [73–76]. The lymphotropic property is more clearly seen for lipids in general. Namely, the lymphographic contrast agent Lipiodol® (Laboratoire Guerbet, France), which is iodinated poppy seed oil, has been used for lymphography because it moves into the lymphatics and its exclusive presence in the lymphatic duct can be detected under the X-ray system. It should be worthwhile to count these lymphotropic characters in the drug designing aiming at antimetastatic property. The control of lymphatic metastasis is so important because lymphatic metastasis of tumor is the more frequent cause of therapeutic failure. Therefore, suppression of lymphatic metastasis by lymphatic drug delivery with the use of liposomes or nanoparticles may be of critical importance [24,73–75].

Antitumor efficacy is usually judged by the effect on the major or primary cancer, which is the target of current chemotherapy or surgery, and concomitant effect on micrometastasis in the lymph nodes or elsewhere will become ultimately very important, which can be more effectively achieved by lymphotropic drugs. Further, definition of the regression (response) is traditionally defined as size reduction to less than half of the original size. For instance, metastatic micronodules as shown in Fig. 1C are invisible under conventional methods of cancer detection. Thus, evaluation of response in these microtumor by tumor size is not possible. More recently, prolongation of survival time span is being considered as an important parameter. However, it is often difficult to control the lymphatic metastasis. If one could control lymphatic metastasis by the lymphotropic drug delivery, one would contribute survival span. In the SMANCS therapy, we experienced that there are many patients who become tumor stabilized (but not regression) after treatment without further disease progression, nor emergence of new tumor. As stated above, lymphotropic nature of macromolecular drugs will contribute on this point and it will be a highly preferred character. In this connection, a recent report of PEG-interferon 2b treatment resulted in prolonged tumor-free survival of patients with malignant melanoma, which is interesting [77].

5. Augmentation of EPR-effect and drug delivery by angiotensin-II induced hypertension

The vascular density of many tumors, if not all, is higher than that of normal tissues. Furthermore, tumor blood vessels frequently lack the smooth-muscle layer, which plays a vital role in regulating blood pressure and flow volume. In normal blood vessels, the smooth-muscle layer responds to vascular mediators such as bradykinins, acetylcholine and NO via receptor binding on vascular smooth-muscle cells, which modulate intracellular calcium levels, helping to maintain constant blood-flow volume. In normal tissues, when hypertension is induced by infusing angiotensin-II (AT-II) intravenously, vasoconstriction of the smooth-muscle layer will result in higher blood pressure and higher flow velocity. Interestingly, however, the blood-flow volume remains constant [78]. In contrast, in tumor tissues under the AT-II-induced hypertensive state, the blood-flow volume does not remain constant. Tumor blood-flow volume actually increases in response to elevated blood pressure. In tumor-bearing rats, raising the systolic blood pressure from 90 to 160 mm Hg resulted in nearly 2–6 times increase in tumor blood-flow volume depending on the blood pressure attained, whilst blood-flow volume in the normal tissues such as the kidney and the bone marrow remained constant [78]. Therefore, we anticipated that the induction of the hypertensive state would augment the EPR-effect, and hence, the delivery of macromolecular drugs would increase. In fact, when ^{51}Cr-labeled SMANCS or ^{51}Cr-labeled albumin was injected i.v. in rats bearing Walkar-256 carcinoma and when the blood pressure was raised from 90 to 150 mm Hg only for 15 min, there was a 1.3- to 3.0-fold increase in accumulation of these macromolecular drugs in the tumor tissue [79]. At the same time, a significant reduction of the drug delivered to healthy organs (e.g. intestine, kidney, liver and bone marrow), and thereby lesser toxicity, was observed because of vasoconstriction and tightening of endothelial gap junctions, which suppressed the extravasation of polymeric drugs.

Similar results were obtained with SMANCS/Lipiodol administered under the AT-II-induced hypertensive state, in many patients with solid tumors including hepatocellular carcinoma (HCC), metastatic liver cancer, cholangiocarcinoma, pancreatic cancer, and renal cell carcinoma [7,9,18,80 – and unpublished data]. It is known that in common cancer chemotherapy with low molecular weight anticancer agents, the anticancer agents should be administered no more than the recommended dose, because of severe dose limiting toxicity. However, under the hypertensive state, using macromolecular drugs in animal models as well as in the clinical setting, it was possible to achieve more than 1.3- to 5-fold higher tumor concentration in the tumor with the same dosage. More importantly, sufficient augmentation of tumor-targeting effect could be achieved even when hypertension was maintained for only 20 min, meanwhile no apparent toxicity was seen [9,80]. Having these obvious advantages and evidences, we warrant AT-II-induced hypertension chemotherapy should be applied for macromolecular anticancer chemotherapy. This method is approved in Japan for conventional drugs but not yet for nanomedicines.

6. Consideration in designing of macromolecular anticancer drugs: for utilization of tumor targeting mechanism by EPR-effect

6.1. Release rate and pharmacokinetics

In recent years, many efforts were directed towards designing of macromolecular drugs utilizing EPR-effect for tumor-seeking anticancer agents. The method involves conjugates or encapsulation of low molecular weight anticancer drugs, or proteins by chemical conjugation or micellar or liposomal encasement, respectively, to water-soluble, biocompatible, biodegradable polymers, or nanoparticles such as encapsulated liposomes or polymeric micelles [1–4,25–28,81]. The crucial point of all is the optimally sustained rate of drug release from these nanoparticles to attain the required drug level.

In this context, when liposomes are too stable and have slower release rate than the optimum rate for drug release, the drug concentration cannot reach the required concentration within the adequate time span. Doxil, adriamycin encapsulated liposome, belongs to this category, and it is used for first line of defence against Kaposi's sarcoma and second line drug for breast cancer and others, but not universal for solid tumor in general. On the other hand, such liposomes also need to be stable enough for storage period, and on the shelf, or refrigerator to the bed side. As an example, adriamycin-encapsulated block copolymer micelles (PEG-polyAsp), designated NK-911, on the contrary showed initial rate of drug release too fast, it thus could not release adriamycin in a sustained manner for adequate time span of few days. Therefore, it cannot attain adequate pharmacological drug concentration inside tumors in human. As 50% of the drug was released less than an hour after i.v. injection, this micellar formulation has no advantage (Fig. 3A-(b)) [25,26,28,81]; also the results in phase I discouraged for further development.

In Fig. 3B, plasma concentration of another synthetic polymer micelle SMA-doxorubicin is shown, which exhibits longer residence time than NK-911. Further, when one compares between SMA-micelles of doxorubicin and pirarubicin, the latter micelle showed higher AUC and very higher tumor uptake than SMA-doxorubicin. Namely, SMA-pirarubicin micelles showed 100 × higher plasma AUC and 27 × higher tumor concentration than parental-free pirarubicin [25,26]. This means a combination of a drug and chemical constituent of the micelles is an important factor to have preferred characteristics.

6.2. General advantages of polymer conjugates or nanomedicine

6.2.1. Prolongation of half-life, stealth character and reduced antigenicity

One of the important advantages of polymer conjugation is the stabilization and prolongation of the plasma half-life of the low molecular weight drugs or proteins. SMANCS, a conjugate of SMA and neocarzinostatin (NCS), showed this character as well as polyethylene glycol (PEG)-conjugated bilirubin oxidase and others [24,76,82]. PEG-conjugated interferon, PEG-INFα2a (Pegasys®), showed increase in plasma half-life to about 10-fold of native INFα2a (8 h vs 80 h) when injected s.c. in human [83]. Second advantage is solubilization of insoluble low MW compounds as discussed below. Further, polymer conjugates or micellar formulation under specific condition also confers 'stealth' characteristics and diminishes uptake by the reticuloendothelial system (RES) or macrophages, and hence the half-life in blood circulation can be extended [29–31,82,83]. These merits cannot only improve the chemotherapeutic efficacy, but also reduce the time and cost of frequent injections to a great extent. This had been observed in SMANCS/Lipiodol, of which arterial injection is required at most once a month, usually once in 2–3 months or less [7].

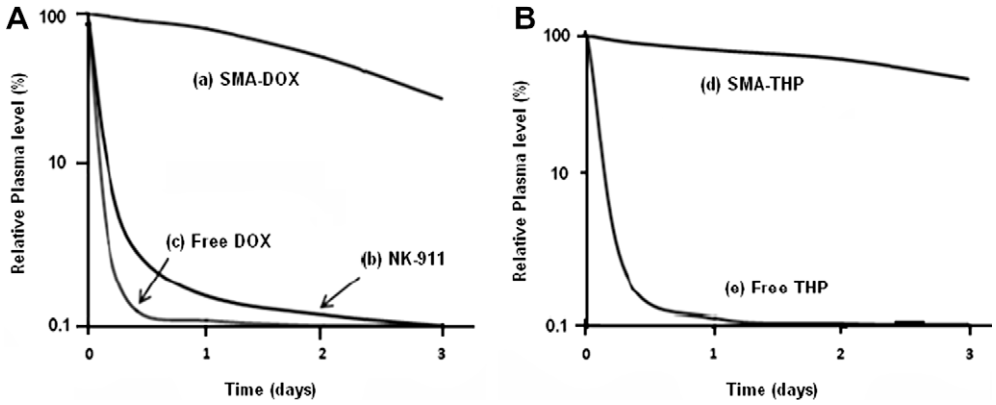

Fig. 3. Improved plasma half-life of anthracyclines by micellar encapsulation, and different micelle materials and different drugs are shown. (A) Plasma half-life of free doxorubicin (DOX) and that of SMA or PEG-Asp block copolymer micelles. SMA-micelles of doxorubicin shows much prolonged plasma residence time [25], while PEG-Asp block copolymer (NK-911) bursted very rapidly within short time after injection. The data values were simplified from published literature [28]. Higher stability of SMA-DOX micelle is apparent. (B) SMA-THP (4-O-tetrahydropyranyladriamycin) micelles are compared with that of free THP in human cancer patients. Advantage of SMA-THP micellar formulation in the plasma residence time over free THP is apparent as well as higher tumor targeting by EPR-effect [25,26]. Free THP is more rapidly taken up by cancer cells than free doxorubicin. THP has higher affinity to SMA than DOX so that SMA-THP micelles in plasma stayed longer and more stable than SMA-DOX (A vs B).

6.2.2. Biological response modification or immunopotentiation by polymers

Some polymer conjugates, such as SMANCS, for instance, displayed various immunomodulating properties, including macrophage (Mϕ) activation, induction of interferon-γ production [84–87], and augmentation of splenic and peritoneal natural killer (NK) cells [86,87]; thus it stimulates non-specific cytotoxicity to tumors in mice bearing syngeneic tumors. Similar findings were also reported for pyran copolymer and other anionic polymers, which have the capacity to induce interferons and other cytokines [86].

6.2.3. Resistance against free radicals, proteolytic degradation, addition of metal chelating capacity and surface lubrication

The pathological and inflammatory lesions including cancer are constantly under the exposure of endogenously formed free radicals such as superoxide anion radical $O_2^{\cdot-}$, hydrogen peroxide (H_2O_2), NO, $ONOO^-$ and hypochlorite (HClO) [88–91]. Many proteins, enzymes, nucleic acids or active drugs, when exposed to these radicals, may be readily inactivated or modified. However, polymer conjugation or micellar shield will confer better stability against these radicals. To clarify this point, we studied the effect of oxygen radicals (ROS) to functional proteins in model systems [92,93]. One example is superoxide dismutase (SOD), which catalyzes the dismutation of superoxide anion radical $O_2^{\cdot-}$ to H_2O_2. Subsequently, if catalase is not available in the milieu, H_2O_2 will accumulate in the lesion. We previously showed that Cu, Zn-SOD was disintegrated by exposure to H_2O_2 (the product of SOD), and then free Cu^{2+} ion was liberated, which will serve to catalyze ·OH generation from H_2O_2 effectively by Fenton type reaction [92,93]. However, polymer conjugates of SOD with pyran copolymer {DiVEMA, poly(divinylether-maleic acid)} remarkably reduced this ·OH generation, suggesting the protective and stabilizing effect of DiVEMA modification to SOD against free radicals. Further, we extended the study to an acute phase plasma protein, α_1-protease inhibitor (α_1-PI) as the target using various polymers *per se* to suppress inactivation by ·OH. Among the polymers tested, pyran copolymer showed the most potent inhibitory activity against ·OH generation and greatest suppressive effect against α_1-PI inactivation, whereas BuSMA (butylated-SMA) or PEG alone suppressed α_1-PI inactivation only weakly (Table 1 and Fig. 4). The effect of pyran copolymer may be attributed to the chelating activity of Cu^{2+} by dicarboxylate ion in pyran copolymer, which was found to exhibit high activity comparable to the potent chelating agent, DTPA, whereas not all polymers have bicarboxylic groups thus showing no chelating effect, but shielding effect.

Along this line, we further examined the time course of H_2O_2-induced inactivation of native SOD and various polymer-conjugated SOD, and those of α_1-PI by ·OH radical similarly. Namely, assuming the metal (Cu^{2+}) would catalyze Fenton reaction, through which H_2O_2 will become hydroxyl radical in the presence of various SODs and α_1-PI. We found that polymers having paired carboxyl groups and OH groups such as pyran copolymer and PVA effectively suppressed the inactivation of SOD as well as α_1-PI. It is probably attributed either by direct scavenging of ·OH (by PVA) or by chelating transition metal ion Cu^{2+} [88,92,94].

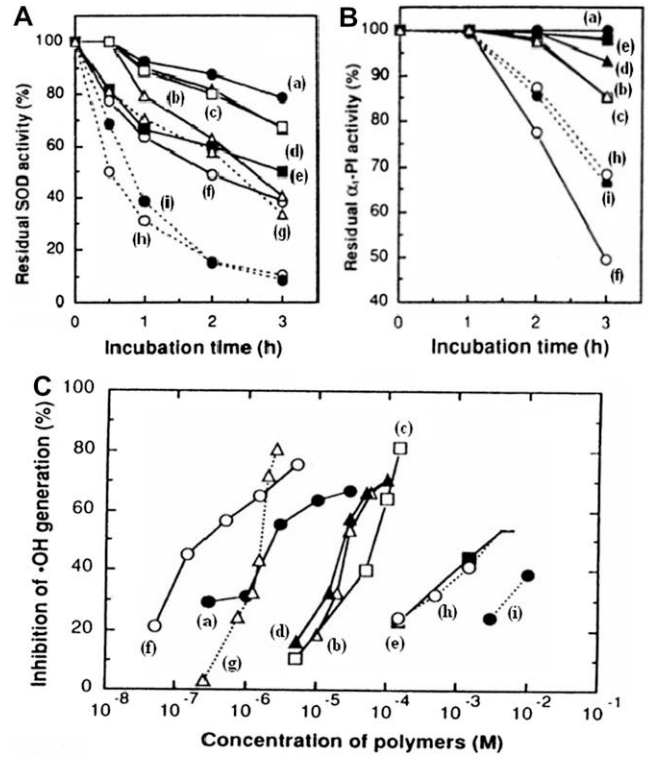

Fig. 4. Protective effect of enzyme activity of SOD by various polymer conjugation (A), and (B) inactivation of α_1-protease inhibitor (α_1-PI) both by exogenously adding H_2O_2 [94]. (C) The extent of ·OH generation by SOD or various polymer-conjugated SODs upon addition of H_2O_2. Concentration of SOD and each polymer-SOD conjugates (3.7 μM) were incubated with 0.1 mM H_2O_2 in 25 mM sodium phosphate buffer (pH 7.4) for the indicated time at 37 °C. In (A), (B) and (C), (a) -●-, high molecular weight pyran(H)-SOD; (b) -△-, low molecular weight pyran (L)-SOD; (c) -□-, PVA-(L)-SOD; (d) -▲-, high molecular weight PVA(H)-SOD; (e) -■-, succinylated-gelatin SOD; (f) -○-, native Cu, Zn-SOD; (g) --△--, PEG-SOD; (h) --○--, carboxyl HSMA-SOD; (i) --●--, BuSMA-SOD. The [·OH] radical was quantified by electron spin resonance spectroscopy by using of spin trapping agent [See text for details and Ref. [92,94]]. (For abbreviation, PVA, etc. see Table 1).

Another advantage of polymer conjugations is that they also confer resistance against proteolytic degradation without much affect on biological activity. We previously showed PEG-conjugated bilirubin oxidase becomes more resistant against proteolytic degradation than native enzyme [73,95].

Viscosity of high molecular weight polymers for injection-drug at high concentration may impede blood flow. We investigated previously the effect of PVA, PEG and gelatin on the lubricating

Table 1
Inhibitory potentials of various free polymers and DTPA against ·OH generation, and their protective against inactivation of α_1-protease inhibitor (α_1-PI) by ·OH[a].

Polymers (abbrev.)	Approx. Mw.	Concentration of 50% inactivation [μM]
Pyran copolymer; divinylether-maleic acid (DIVEMA)	30,000	0.48
N-ethylsuccinimidyl-S-poly(vinylalcohol) (PVA)	11,000	37.0
Succinylated-gelatin fragment (gelatin)	23,000	16.0
Poly(styrene-co-maleic acid) half-butyl ester (SMA)	1600	>1000
Monomethoxypoly(ethylene glycol) (PEG)	5000	> 100
Diethylenetriaminopenta acetic acid (DTPA, chelating agent)[b]	393	1.6

[a] Hydroxyl radical (·OH) was generated in the presence of 7.4 μM Cu^{+2} and 3 mM H_2O_2. Concentration of α_1-PI, 20 μg/ml [data are from Ref. [92]].
[b] DTPA is one of the best chelating agent; it is not a polymer. Potent suppression of ·OH generation by DTPA is due to removal of Cu^{+2} from the ·OH generating system, whereas all the polymers will scavenge ·OH, or chelating Cu^{+2} (e.g. DIVEMA) and suppress ·OH generation.

effect of the erythrocyte passage through micropore up to 5 μm in pore size [96,97,93]. Gelatin and PEG showed preventive effect of red blood cell from bursting (hemolysis) and facilitated the passage through the micropore. Modification of SOD with succinyl gelatin also conferred this beneficial effect in addition to the enhancement of pharmaceutical effect [93].

6.2.4. Enhancement of solubility, cellular uptake, intestinal absorption, and physical stability

Besides the increase in $t_{½}$ and EPR-effect, we can confer different characteristics and thus drug formulations from the parent drugs by polymer conjugation or micellar encapsulation. It is well known that many promising drug candidates fail to become therapeutic agents because of their poor water-solubility. The conjugation or encapsulation of such drugs to water-soluble nanoparticles confers remarkable solubility. For instance, Taxol®, caplitaxel, KRN 5500, SN38, Zn-protoporphyrin are converted to highly water-soluble drugs [25–28,98–100]. Moreover, many proteins and drugs which have poor intestinal absorption may be rendered orally active by conjugation with appropriate polymers in combination with oily formulation [101]. One such formulation is SMANCS in medium chain triglyceride (MCT) solution (oily SMANCS); when it was orally administered to mice, it was more efficiently absorbed than aqueous solution (PBS) from the intestine. Plasma concentration or AUC of oily SMANCS given orally was about 11 times greater than that of aqueous SMANCS. In comparison to parent NCS, total AUC could be improved by more than 100-folds. Further the stability (and half-life) of SMANCS was remarkably improved in oily formulation with Lipiodol®, not to mention of its 20 times increased stability at 37 °C in vitro [102], which could be given orally, and enhancement in peak plasma level was 9-fold higher than aqueous formulation of SMANCS.

Further, enhanced intracellular uptake to tumor cells without any known specific receptor was observed for SMANCS than for NCS. Interestingly, it was more efficient in acidic pH (5.5–6.5) than neutral pH [103,104]. However, receptor-mediated internalization with tagged ligand following the EPR-effect-based vascular crossing delivery is considered efficient and important steps for this type of drugs. One such example is galactose-containing HPMA copolymer-doxorubicin targeted to the liver [48,49,105]. Another probably more promising receptor is transferrin receptor which is known to be over expressed in tumor cells. Consequently, the transferrin–methotrexate conjugate was developed, as well as liposomes [106,107]. More recently, a similar result was seen by the use of folate receptor, where folate-mitomycin C is cross-linked on polymer conjugates [108]. There are several other examples of receptor-mediated drug delivery reported elsewhere [16,109–112].

A naive question is how transferrin conjugates be so effective despite excessive presence of free transferrin in the circulation that would compete with the binding of the transferrin-conjugated drugs. The same is true, while antibody–drug conjugates are reaching the tumor-associated antigen on tumor cells, free tumor-associated antigen being produced by the tumor cells is in circulation. Then, how can it effectively reach the tumor cells despite large amount of tumor antigens in circulation, which will also bind to the target; like α-feto protein of hepatoma or CEA of colon cancer present in circulation. The answer may be that these conjugates utilize EPR-effect more than free antigens. Consequently, even though antibody function of antigen binding may be nullified by free antigens in circulation, the antigen-antibody complexes in circulation may exert EPR-effect and reach the target because of their large size, and thus they are capable of easy access to tumor cells followed by endocytosis. In any event, dividing tumor cells are known to have higher endocytotic uptake capacity than non-dividing normal cells [112], which makes another unique advantage of macromolecular drugs.

6.2.5. Advantage of polymeric drugs over p-glycoprotein-dependent multidrug resistance

To overcome the multidrug resistance (MDR), we worked with SMANCS, and found that p-glycoprotein-resistant MDR cells are sensitive to SMANCS, probably by preventing the binding of the polymeric drug to p-glycoprotein that leads to suppression of the efflux of the drug, because the macromolecular size of the drug does not fit to the efflux transporter system [43,44]. Minko et al. reported that HPMA copolymer-linked doxorubicin affected the signal transduction system of MDR cells and induced apoptosis which was not seen in the parent drug [113–115].

6.2.6. Patient compliance and quality of life (QOL)

The polymer-conjugated drugs or nanomedicines require less frequent administrations due to the increased tissue and blood half-life ($t_{½}$) which is usually 10 times or more than the free drugs, that is of great benefit to patients [1,8,97,93]. For instance, for the treatment of lymphocytic leukemia the use of native enzyme L-asparagine having a short $t_{½}$ (8–30 h) necessitates daily administration for 4 weeks, whereas the PEG-L-asparaginase conjugate (Oncaspar®, $t_{½}$ approx 14 days) requires only one infusion every 2 weeks [116–118]. The same is true for native interferon-α vs pegylated interferon-α (Pegasys®, MW 50 kDa), the former requires more than 2–3 injections per week, while the latter requires only once a week intramuscular injection [83].

6.3. Disadvantages and limitations of polymer conjugates or unsolved issues

In view of clearance from the body, a size or unit of polymer being used should be preferably smaller than the size of renal excretion threshold (40 kDa), or biodegradable, or evidence of clearance via fecal route; in this case molecular weight may be of secondary importance. Further, the biocompatibility of polymers to be conjugated has to be well established. Hydrophobic polymers, if resistant to degradation in vivo, may tend to localize eventually in the skin or lipid-rich tissues, although mostly they may be eliminated via the bile. Therefore, the total amount of polymers to be administered may become important if the dose becomes large (e.g. 10 g). Some polymers may elicit immune response or allergic reactions, more likely in the subsequent injections, although it may be controlled by corticosteroid and antihistamine. There were reports in earlier days on the so called "macromolecular syndrome", or more lately on the infusion-related reaction that is observed at first usage even with antibody drugs or liposomal drugs, that is not observed in the second administration [119].

7. Species difference and optimization of release rate from the drug complex

The rate of initial burst of free drugs in vivo is frequently a problem in many polymeric drug conjugates, micellar, or liposomal drugs. However, the release rate from the given polymer micelles or from liposomes is purely physical matter exhibiting zero order rate kinetics. In contrast, species difference in the renal or the hepatic functions or metabolic rate and their time scale in different animal species may need to be considered in terms of time course of drug release. For instance mice may tolerate toxicity well because of the more rapid metabolic rate or urinary clearance compared to human time scale. The metabolic rate particularly in the liver or urinary clearance rate in mouse may be far faster than that in human. We may not see effective therapeutic effect if the conjugates were not designed for human metabolic rate. Therefore, ideal experimental data in mice or rats do not guarantee good therapeutic response in human. We also encountered that SMA copolymer has little binding to rabbit albumin, in contrast to mouse or human

albumin (unpublished data). This means that some animal models may not reflect the actual drug behavior for optimization in the human situation. Therefore optimization of the parameters has to be targeted for human setting, but not for mice.

Particular notion in this regard should be addressed to the nature of chemical bonding between the drug and polymers. For instance, chemical bond in the conjugates either linked by ester- or amide-bond makes a great difference. Namely, cleavage of ester bond could be 10- to 30-folds faster than that of amide bond in mice than in humans [119,120].

Another discrepancy in animal model vs real human cancer treatment is the tumor model using ascetic form (i.p.) tumor and the drug given i.p. or i.v. route. Frequently one injects the drug i.p. within one day after tumor inoculation into the peritoneal cavity, and hence the drug will be accessible to the tumor cells directly at a very early stage and it will be so effective. However, this protocol is an unrealistic model to develop a drug for solid tumor. In this system of the i.p. tumor/i.p. drug, there is no solid tumor being developed yet, thus no EPR-effect. Also, there are no human patients with 10^6 tumor cells (very small inoculums size) to be diagnosed to have cancer at that early stage. If this is a case, cancer cells are easy to eradicate by helping immune surveillance. When both tumor cells and drug were administered i.p., easy access of drug to the tumor is guaranteed. Thus it is not a good model because the peritoneal compartment becomes a type of test tube, and the drug concentration in the compartment is very high and effective, resulting in more chance to eradicate tumor cells.

8. Conclusion

For the tumor-targeted drug delivery system, EPR-effect is now widely accepted as a guiding principle (see recent review) [121]. The EPR-effect is observed in most macromolecular drugs that include polymer conjugates, micellar drugs, and liposomal drugs; all exhibiting tumor-targeting characteristics. Once these macromolecular drugs are delivered to the tumor, they remain in the tumor tissues for extended period (days to weeks), which is different from just passive targeting and is different from the behavior in the normal tissue. Even cancer-targeting drugs that are conjugated with monoclonal antibodies will rely on this EPR-effect at first crossing of vascular barriers to be accessible to tumor tissue. In this connection, it was shown previously that normal IgG exhibited tumor-selective accumulation by EPR-effect [5]. Various factors that mediate EPR-effect such as bradykinin, NO, VEGF, and prostaglandins are reviewed and discussed.

EPR-effect can be further augmented by elevating the systemic blood pressure artificially by infusing AT-II [79], and also by prostaglandin I_2 agonist [67,68]. Consequently, the delivery of macromolecular drugs is facilitated significantly even to metastatic tumor which has low vascular density [122]. Thereby therapeutic effect of macromolecular drugs will be enhanced while side effect will be lesser. Whilst most micellar nanoparticle drugs and liposome formulations require proper rate of drug release in order to exert optimal therapeutic effect, these formulations need special attention involving stability and modulation of release kinetics in vivo. This involves meticulous adjustments in the formulation, or selection of materials, or chemistry during development of drugs for human use, not for mice.

We discussed here the advantages of polymer therapeutics or nanomedicine including tumoritropic and lymphotropic nature; enhanced residence time in circulation and in tumor; lesser side effect than parent drugs; and ways to augment the delivery of polymeric or macromolecular drugs, e.g. by elevating blood pressure. Further, benefits of macromolecular drugs are also found against multidrug resistance. All these improved characteristics warrant the promising future of macromolecular therapeutics for many diseases, especially for cancer.

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