EXHIBIT B

PART 2 OF 5

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Table 1 *(Continued)*

The following agents have been shown to lower tumor interstitial fluid pressure					
Agent name	Injected dose	Injection route	Tumor type	Species/strain	Result summary/Conclusions
(4) Dexamethasone	0.3–30 mg/kg	i.p.	*LS174T	SCID	*(Kristjansen et al., 1993)* 0.3 mg/kg = no significant effect on IFP observed 1.0 mg/kg = marginal ↓ IFP between *day 1 versus day 4* 3–10 mg/kg = significant ↓ IFP between *day 1 versus day 4*
(5) Pentoxifyllin	25 and 100 mg/kg	i.p.	*FSaII (fourth generation)	female C3Hf/Sed	*(Lee et al., 1994)* *2 hrs post injection:* 25 mg/kg = no effect on PO2 100 mg/kg = ↑PO2; ↑RBC flux; ↓IFP by 40% 100 mg/kg = no effect on MABP was observed
(6) Thalidomide	200 mg/kg	i.p.	*FSA II	male C3H/HeOuJIco	*(Ansiaux et al., 2005)* 2 days post injection = ↑ in tumor reoxygenation ↓ IFP; effect due to tumor vascular remodeling
(7) Cereport (Bradykinin agonist)	0.1, 1.0, and 0.15 μg/kg/min	I.V. infusion	*MATB-III	male Fisher/rats	*(Emerich et al., 2001)* *I.V. infusion 5–10 min:* 0.1 mg/kg/min = ↓ IFP significantly ($P < 0.01$) 1.0 mg/kg/min = IFP ($P > 0.1$); 66% ↓ in perfusion ($P < 0.001$) 0.15 mg/kg/min = ↑ vascular pore size; ↑ vol. surface area

Table 1 *(Continued)*

The following agents have been shown to lower tumor interstitial fluid pressure

Agent name	Injected dose	Injection route	Tumor type	Species/ strain	Result summary/Conclusions
					(Peters et al., 1997)
(8) Nicotinamide	100, 500, 1000 mg/kg	i.p.	*NT(CaNT)	mice CBA	*20 minutes post-injection:* 100 mg/kg = no effect on IFP ($P = 0.05$) 500–1000 mg/kg = ↓ IFP ($P = 0.0001$) 1000 mg/kg = ↓ IFP ($P < 0.0001$) Radiosensitizing effect after 80 min but not after 10 min.
					(Pietras et al., 2002)
(9) ST1571 (PDGF-β inhibitor)	12 mg/kg/day	i.p.	Kat-4 (in mice)/ PROb (in rats)	SCID mice/ BDIX rats	*40 minutes post-injection:* ST1571 blocked PDGF signaling; ↓ IFP ; ↑ 51Cr-EDTA uptake " ↑ antitumor effect of taxol against Kat-4 " ↑ antitumor effect of 5FU against PROb
					(Rubin et al., 2000)
(10) PGE(1)	15 μg	s.c. (area around tumor)	PROb *C-IMC	Rats	*8 and 24 hrs post-injection:* PEG(1) ↓ IFP by 30% ↑ transcapillary transport by 39.6%-(by microdialysis)($P < 0.05$) ↑ uptake of 51Cr-EDTA by 86.9% ($P < 0.05$) Well developed collagen and hyaluronan stromal content

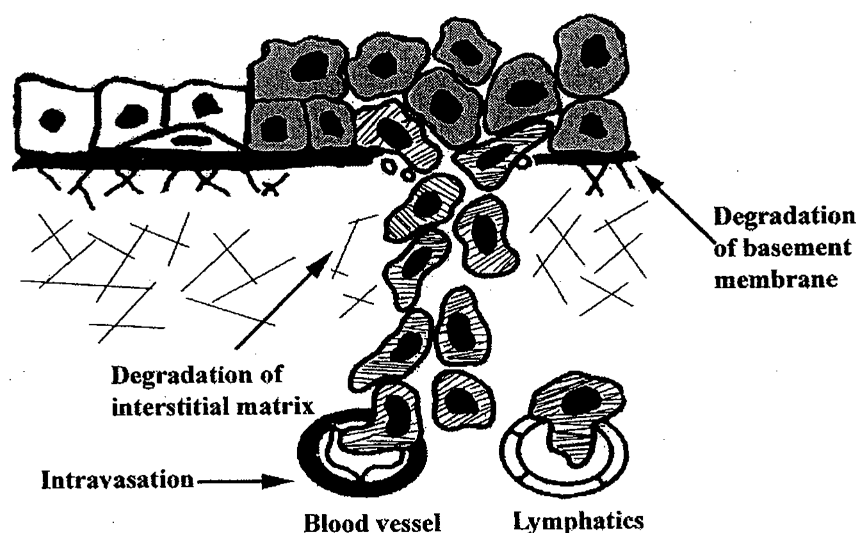

Fig. 1 This figure models the process of invasion. The continuous basement membrane is penetrated by neoplastic cells. These cells gain access to components of the interstitial matrix. After degradation of the interstitial matrix tumor cells invade blood vessels and lymphatics. In order for the cells to enter the blood stream the cells must penetrate the continuous endothelial basement membrane. Blood vessels are surrounded by a continuous endothelial cell membrane but lymphatics lack this form of structural support.

administration, investigated tumor model(s), species and strain of tumor bearing rodents, and conclusions as they relate to IFP and other interesting related findings. The tumor IFP lowering agents (among other therapeutic functions) were also evaluated under different experimental conditions and should be applied with the understanding that what works under one set of conditions, may not work in another situation. The effects of a few tumor IFP lowering agents will be discussed here; for more information see published findings in respective journals.[93,94,96–103]

TNF-α was shown to reduce IFP and MABP by approximately 50–70% ($P < 0.05$) and 30% ($P < 0.01$), respectively. In this study, human melanoma tumors were implanted in nude mice and a reversible effect of TNF-α on IFP was observed 5 hours post-injection ($P < 0.05$), with no observable influence on IFP after 24 hours.[94]

TGF-β lowered IFP in a KAT-4 anaplastic thyroid carcinoma model. Affymetrix microarray studies and other protein expression assays confirmed that the chimeric protein (FC:TβRII) exerted its effect in part through modulating macrophage activity. When KAT-4 was pretreated with Fc:TβRII, a significant effect of doxorubicin on tumor growth was observed.[93]

Concentration dependent effects of dexamethasone on IFP in LS174T models were also investigated. Injected doses ranged from 0.3 to 30 mg/kg with i.p. injections administered over 4 consecutive days. Relatively high doses resulted in lower IFP values, reduced permeability and vascular hydraulic conductivity.[96]

As observed with the effects of other tumor IFP lowering agents, PTX was found to lower tumor IFP without altering MABP.[97] Based on concentrations per/kg of body weight, dexamethasone exerted a more potent effect on IFP than PTX. PTX lowered IFP at concentrations around 100 mg/kg but failed to effectively lower IFP at the 25 mg/kg dose. Prior to a decrease in tumor IFP, a dose-specific effect of PTX on RBC flux near the tumor center was observed.[97] Inducing RBC velocity in tumor areas near zone 3 could potentially create new opportunities to deliver peptide and protein therapeutics to the deeper regions of the tumor. Studies of this type could eventually contribute to resolving issues related to radioresistant hypoxic tumor cells.

Taxol (aka ~ paclitaxel), a popular chemotherapeutic agent used in the treatment of a variety of solid malignancies, has also been shown to lower tumor IFP, decompress blood vessels and improve oxygenation (PO_2) in patients.[95,99] Clinical data now supports the role of taxol as a tumor IFP lowering agent over other chemotherapeutic agents. In a study involving breast cancer patients, taxol significantly lowered IFP by 36% and increased the PO_2 by approximately 100%, whereas doxorubicin did not exert a similar or significant effect on IFP. The ability of taxol to lower IFP is a bonus effect of this chemotherapeutic agent.[95,99] It is possible that other drugs belonging to its class may exert similar or additional anti-barrier functions. It is also entirely possible that some of the conventional and experimental therapeutics that have made their way into the clinic may lower tumor IFP in addition to exerting other widely accepted antitumor effects. Although taxol was never initially used in a clinical setting to limit a major physiological barrier such as interstitial hypertension, it has the potential to improve penetration of small therapeutic peptides and proteins into the interior tumor mass when used in combination with other agents proven to lower tumor IFP.[93,94,96–103] Subsequent clinical investigations are warranted.

4. Concluding Remarks

Given the urgency for development of novel therapeutics against cancer, an understanding of the major barriers hindering optimal delivery and transport is necessary. In view of the time and energy invested in the discovery, clinical testing, and marketing of a new agent, the physiology of tumors should be taken into account as soon as possible to ensure the greatest level of clinical success in a time-sensitive manner.

Although a number of researchers have managed to achieve some success with interstitial tumor targeting methods, tumor vascular targeting still represents a viable alternative. Improving methods to selectively deliver therapeutic peptides and proteins to tumor vessels is certainly time well invested, but other methods of circumventing barriers are also needed. Another approach is to reduce the burdens of tumor physiology on drug delivery and transport. Experiments and technologies developed to optimize the use of agents already shown to limit tumor IFP and/or modify the tumor interstitial matrix, will likely assist the next generation of treatments against cancer and progression of disease.

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3

Enhanced Permeability and Retention (EPR) Effect and Tumor-Selective Delivery of Anticancer Drugs

Khaled Greish, Arun K. Iyer, Jun Fang, Michio Kawasuji and Hiroshi Maeda

1. Introduction

Research aimed at achieving an effective cure for cancer — a major killer of humans in many nations — is a great challenge. This effort is reflected in the immense number of scientific research articles, which numbered more than 1 million in 2002. In that same year, cancer took the lives of more than 6.7 million patients throughout the world.[1] A major focus in cancer research concerns the unique characteristics of tumor cells or tumor tissues. Understanding of these characteristics will aid development of strategies for selective destruction of abnormal cancer cells without any harm to patients. Tumor vasculature is an ideal and attractive target for such strategies because it demonstrates more extensive abnormalities than vessels in normal tissues or organs. For example, it exhibits uniquely different fluid and molecular transport dynamics to meet an ever-increasing demand for nutrients and oxygen of the cancer cells.

Among these hallmark characteristics is the enhanced permeability and retention (EPR) effect of macromolecular agents in solid tumors, or the EPR effect, which was described about 20 years ago.[2–4] By means of the EPR effects, accumulation of macromolecules at the interstitium of tumor tissues is facilitated. One can take advantage of this macromolecular accumulation, via the EPR effect, for the delivery of polymeric or macromolecular drugs. EPR effect-based drug design is thus becoming more important for tumor-selective drug delivery, and the development of anticancer agents of polymeric or nanosize particles, which are believed to be more effective and safer than conventional chemotherapeutic agents, is of great interest. In this chapter, we briefly discuss the principles and factors involved in this mechanism, as well as the contribution of various fluid dynamic forces working across blood vessels in and near tumor tissue.

2. Theory Underlying the EPR Effect

When a tumor reaches a diameter of about 2–3 mm,[5–6] or under hypoxic state, it starts to induce formation of its own vasculature, or neovasculature. In response to an increasing need for a supply of nutrients and oxygen, cancer cells influence the host tissue to produce various factors such as vascular endothelial growth factor (VEGF) [which was initially named as vascular permeability factor (VPF)], bradykinin, nitric oxide (NO) and hypoxia-responsive element, and others.[7–17] Also, this induced vasculature in tumor tissues differs greatly from its counterpart in normal tissues in terms of microscopic morphology.[18–19] The defective anatomy, alone or together with functional abnormalities, results in considerable extravasation of blood plasma contents. These findings prompted us to investigate the possibility of delivering macromolecular anticancer drugs to tumor tissues in a selective fashion. For example, 20 years ago, we found that Evans blue dye, which bound with plasma albumin, concentrated selectively in tumor tissues[2] (Fig. 1). Other plasma proteins, including transferrin (90 kDa) and IgG (160 kDa), that were labeled with radioisotopes behaved similarly, whereas smaller proteins such as neocarzinostatin (12 kDa) and ovomucoid (29 kDa) did not.[2] We named the phenomenon of macromolecule or microparticle accumulation in solid tumor tissue the EPR effect. Since then, we have focused our efforts on analyzing the vascular mediators that facilitate the EPR effect, as well as on influencing the extravasation (a consequence of the EPR effect), so as to enhance this phenomenon for

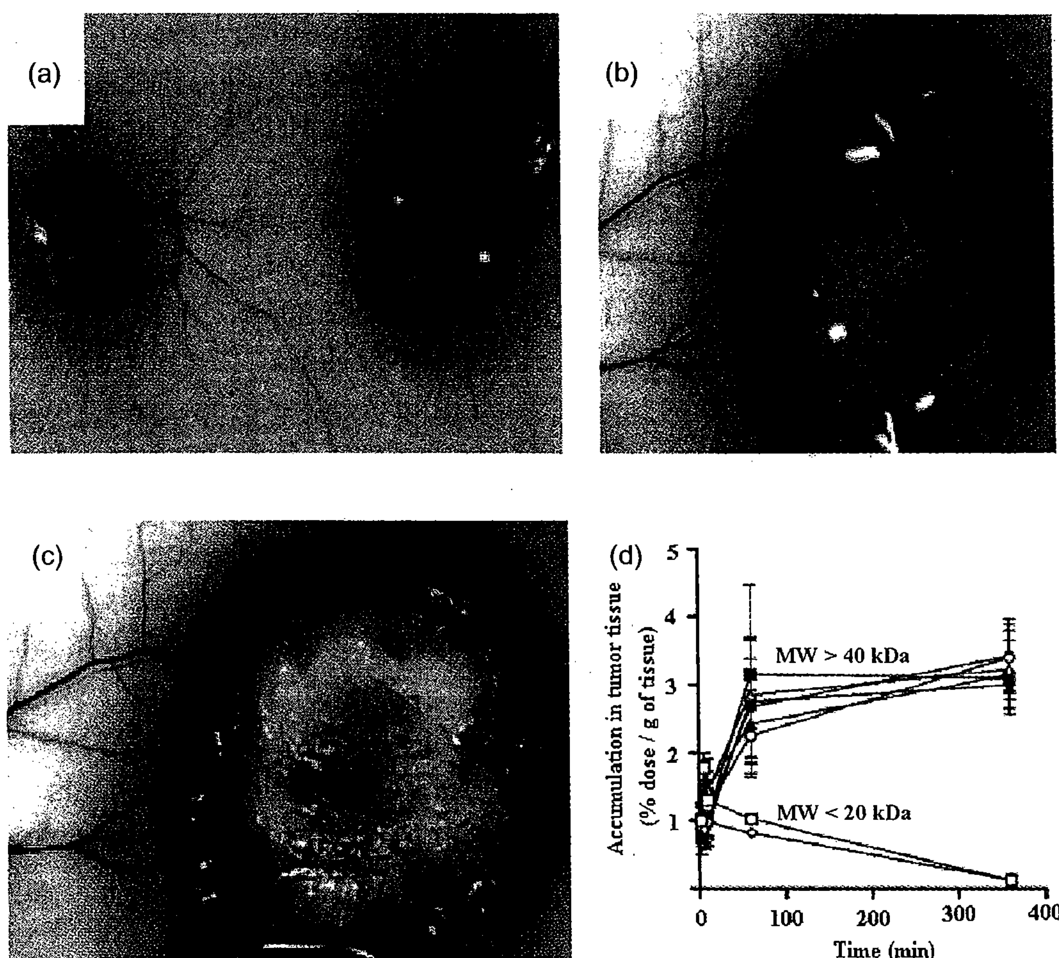


Fig. 1 Selective accumulation of Evans blue dye bound to albumin (70 kDa) in a small tumor (a), large tumor (b), and cross-section of the tumor in (b) (c). The depth of penetration of the dye was showed. The central region of the tumor is necrotic and avascular, and hence does not facilitate the uptake of macromolecules, because it is not a growing area (from Ref. 7) (d) shows the accumulation of ^{125}I-labeled N-(2-hydroxypropyl)methacryalmide (HPMA) copolymer in the solid tumor tissues (from Ref. 3). Note that large macromolecules, but not smaller ones, manifest progressive accumulation.

improved delivery of anticancer drugs, especially macromolecular agents, as discussed later. During this period, investigations of the EPR effect continued by use of various biocompatible polymers.[3,20,21]

3. Anatomical and Pathophysiological Abnormalities Related to EPR Effect

The density of the vasculatures in many tumors is often higher than that in normal tissues. The newly developed tumor vessels have abnormally wide pores between endothelial cells lining the lumina. Davies *et al.*[22]

measured the rate of diffusion of IgG (160 kDa) and was estimated to be 100 μm/hr. A macromolecular anticancer drug, copoly(styrene-maleic acid)-conjugated neocarzinostatin, or SMANCS (16 kDa, also bound to albumin) diffuses at about 1 mm/hr. In view of the fact that functional cells can be located as far as 20–30 μm from tumor blood vessels, one can understand the easy access of macromolecular drugs to tumor tissue.

Furthermore, tumor vessels were found to lack a smooth muscle layer, which usually surrounds capillary endothelial cells.[18–19] In normal blood vessels, the smooth muscle layer is essential for the proper response to vascular mediators such as acetylcholine, NO, bradykinin, and calcium, and hence for maintaining a constant blood flow volume. Vascular mediators cause relaxation of the smooth muscle layer or vasoconstriction, as a function of smooth muscle constriction. Normal blood vessels have an amazingly sophisticated system for monitoring the blood supply to healthy organs such as the heart, brain, liver, and kidney. The vascular smooth muscle tone is controlled by the autonomic nervous system, as well as by various vascular mediators, via receptors on smooth muscle cells. The tonus of the muscles is involved in maintaining a constant blood flow volume by means of blood pressure and blood flow rate in normal tissues. Thus, angiotensin II (AT-II)-induced hypertension will cause high blood pressure (generated by constriction of smooth muscle that leads to a narrowing of vascular diameter) and faster blood flow, but blood flow volume in the capillaries in normal tissues remains constant.[23] This blood flow control mechanism does not operate in tumor vessels because of the lack of smooth muscles. Therefore, in the presence of high blood pressure, capillaries of tumor tissue show much higher blood flow volume than do those in normal tissue[24] (Fig. 2).

In addition to the enhanced permeability, other important relevant abnormalities include a lack of receptors for vascular mediators, and a lack of functional lymphatic drainage which is needed for clearing lipidic or macromolecular particles.[2–4,23,25,26] Also, as tumors grow, hypoxia may result, causing activation of genes responsive to this state, and thus generation of factors such as hypoxia-induced factor,[16,17] VEGF, NO and others.[7–15] Table 1 summarizes the factors that contribute to the EPR effect in solid tumors.

Tumor cells and tissues, as well as surrounding normal cells and especially leukocytes that have infiltrated the tumor, produce large amounts of

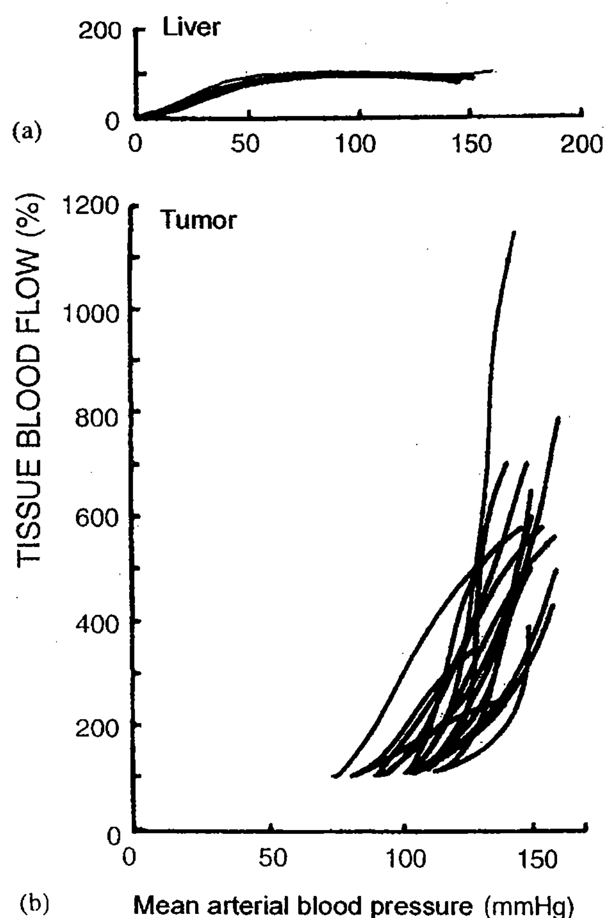


Fig. 2 Effect of AT-II-induced hypertension on blood flow volume in (a) normal organs as represented by the liver and (b) tumor. Other normal organs, including the brain, bone marrow, and kidney, were also valuated; the tumor used was AH109B hepatoma in Donryu rats (from Ref. [24]).

angiogenic factors that lead to neoangiogenesis. Most of these angiogenic factors function as both permeability factors and vasodilators. For instance, VEGF is linked to the generation of NO. We have demonstrated excessive production of bradykinin, NO, and prostaglandin in tumors.[7,8,10,27] We found that matrix metallo proteinase (MMP) (collagenase), which is known to facilitate metastasis and angiogenesis to support tumor growth, also enhances the vascular permeability of solid tumor in mice.[7,8,14,25] This effect is inhibited by many MMP inhibitors.[14] NO and superoxide are simultaneously generated at sites of inflammation and cancer, and they react very rapidly with each other to form peroxynitrite, which can activate proMMP and affect the EPR effect and tumor metastasis.[28]

Macrophages play an important role in host defense in infection and cancer. However, they secrete VEGF and NO, which potentiate

Table 1 Factors contributing to the EPR effect: anatomical aspects and overproduction of vascular permeability mediators.

Anatomical Factors

(1) Extensive angiogenesis: High vascular density, irregular formations
(2) Lack of smooth muscle layer: In a hypertensive state, passive opening of gaps induced by vasoconstrictors, e.g. AT-II
(3) Microanatomical defect: Wide endothelial gap junctions
(4) Lack of functional lymphatic system: Prolonged retention of macromolecules after extravasation
(5) Slow venous return

Permeability Mediators

(1) VEGF/(VPF)
(2) Low-molecular-weight mediators: NO, bradykinin, prostaglandins and others
(3) Matrix metalloproteinase (MMP)/collagenase: Facilitates metastasis; peroxynitrite and proteinases also activated (proMMP)
(4) Lack of response to vasoconstrictors (lack of smooth muscle layer, AT-II receptor)

angiogenesis and promote tumor growth. Moreover, the thrombin clotting system is also involved in angiogenesis and tumor progression via multiple mechanisms, including interaction with and enhancement of VEGF.[29]

4. Augmentation of the EPR Effect in Solid Tumor by Influencing Vascular Mediators

As discussed earlier, tumor vasculature has irregularities that make it highly leaky, even for macromolecular plasma components that are not found in the vasculature of normal tissues or organs. The absence of a smooth muscle layer means that tumor vessels cannot respond to vasoconstrictors. Therefore, administration of a vasoconstrictor such as AT-II that affects normal vessels and increases blood pressure would be expected to have no effect on tumor vessels. However, hypertension would have mechanical effects and cause dilation of tumor vasculature in a passive manner. Enhanced extravasation would result as discussed below. Indeed, Hori *et al.* showed clearly in a window model of solid tumor that some tumor vessels cannot be seen under normotensive conditions but can when an AT-II-induced hypertensive state is generated.[30] This finding means that

apparently avascular tumor tissue actually does have vessels but that they are visible or functional sporadically, e.g. once every 15 min or 25 min, and that the blood flows in unpredictable directions.[30] However, the absence of smooth muscle in tumor vessels accounted for a three- to five-fold increase in blood flow volume under conditions of induced hypertension, when systolic pressure increased from 100 to 160 mmHg by infusion of AT-II.[23,30,31]

With regard to macromolecular agents, deposition of radiolabeled albumin and SMANCS in the tumor tissues increased two- to three-fold compared with that in normal tissues.[30] This greater delivery of macromolecules to tumor tissue was also observed with many solid cancers, including hepatoma, cholangiocarcinoma, metastatic liver cancer, pancreatic cancer, and others, after arterial injection of SMANCS in Lipiodol (SMANCS/Lipiodol) under hypertensive conditions induced by AT-II.[23,31,32] In a converse approach, we also utilized vasodilators, such as the NO-releasing agent isosorbide dinitrate (ISDN; Nitrol), to enhance the EPR effect via widening the tumor-feeding artery. This result was accomplished by infusing ISDN by catheter (31, 32 and unpublished results, Maeda H, Greish K, *et al.*).

It is well known that bradykinin induces intense pain and also increases vascular permeability and that NO promotes angiogenesis.[33] Interplay between bradykinin and other mediators including NO, prostaglandins (PGs), and VEGF will also lead to angiogenesis. Biosynthesis of PGs particularly prostaglandin E_2 via cyclooxygenase isozymes (COX-1 and -2) is markedly elevated in inflammation and cancer. These increased levels of PGs can also enhance vascular permeability in solid tumor, as evidenced by significant suppression of vascular permeability in sarcoma 180 and other solid tumor models by the COX inhibitor indomethacin and salicylic acid.[7,8,25] We showed that a prostaglandin I_2 analogue (beraprost sodium) with a much longer *in vivo* half-life (about 30 min vs 3 sec for prostaglandin I_2) was useful for the delivery of macromolecules,[34] although a therapeutic advantage of beraprost sodium needs to be demonstrated.

We had also reported earlier a significant activation of the bradykinin-generating cascade in the tumor compartment, and bradykinin was shown to be involved in the accumulation of malignant ascitic and pleural fluid.[9,10,27] So-called angiotensin-converting enzyme (ACE) inhibitors such as enalapril and other similar agents can inhibit degradation of bradykinin *in vivo* and lead to higher bradykinin concentrations at sites of tumor

and infection, because of an amino acid sequence homology near the C-termini. Consequently, ACE inhibitors did enhance the EPR effect[9,27,35] mediated by either bradykinin or NO. It should be noted that the ACE inhibitors or prostaglandin I_2 analogue beraprost, in addition to enhancing vascular permeability, significantly suppressed blood flow only in tumor tissue.[27,34,35] It should be remembered that bradykinin is actively generated only at sites of infection, inflammation and cancer, and not in normal tissues[8,25]; ACE inhibitors and beraprost have only a slight, if not negligible effect on systemic blood pressure in the normotensive population. Therefore, increasing the local concentration of bradykinin by means of an ACE inhibitor, and thereby improving tumor-selective delivery of macromolecular drugs should be possible. It should also be mentioned that when [^{14}C]methylglucose, a representative low-molecular-weight drug mimic, was studied, accumulation of this agent in tumor was much less than that of polymeric drugs and lasted no longer than 10 min.[36] Such low-molecular-weight drugs seem to be washed out rapidly into the general circulation and are excreted via the urine.

5. Relation of Fluid Dynamics in Cancer Tissue to the EPR Effect

Physiologists have studied fluid dynamics in normal tissue for centuries.[23] E.H. Starling referred to the constant blood volume between the arterial end and the venous end of a capillary under normal conditions. In both normal and tumor vessels, the difference between the hydrostatic and colloid osmotic pressures is known to affect the movement of fluid and solutes through the capillary vessel wall.[23,37] In normal human tissue blood vessels, the arterial end of a capillary has an average hydrostatic positive pressure of about 25 mmHg.[23,37] This value drops to about 10 mmHg in the venous end of the capillary. The interstitial colloid osmotic and hydrostatic pressures remain constant at both arterial and venous ends of the capillary. This pressure difference facilitates leakage of fluid and nutrients into the interstitial space at the arterial end of the capillary, and then reabsorption at the venous end. This continuous translocation of fluid from the arterial end to the venous end of capillary through the interstitial space, ensures a continuous supply of oxygen and nutrients for cells, as well as an efficient removal of metabolic waste products.

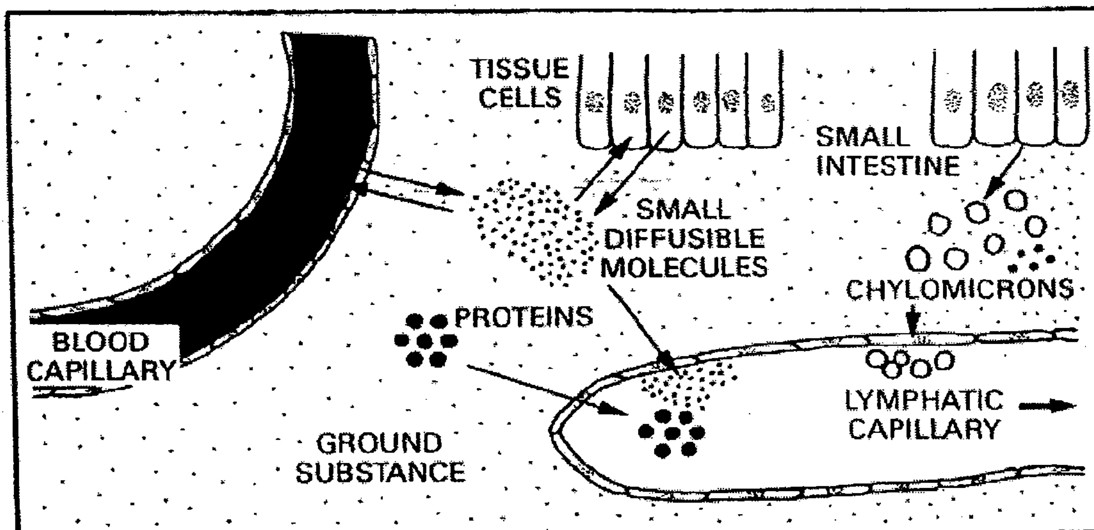
Tumor vessels, however, demonstrate two main differences compared with normal vessels. Firstly, significantly enhanced or almost unrestricted leakage of plasma proteins occurs because of the wide endothelial gap openings with large pore sizes estimated to be 0.2~0.5 μm.[22,38] Secondly, the lack of functional lymphatics in tumor tissue,[2,4,25,39] as described in an earlier section, would lead to higher interstitial accumulation of macromolecules or nanoparticles than in normal tissue because these components cannot be cleared. As can readily be perceived, the raised interstitial colloid osmotic pressure would facilitate transfer of low-molecular-weight components as well as macromolecules from tumor vesseles into the interstitial space of the tumor tissues, due to the higher solute level there. Also, the increased interstitial hydrostatic pressure at the arterial end of the capillaries will drive low-molecular-weight components in fluid from the interstitial space into the venous end of the capillaries and back to the luminal circulation (Fig. 3).

Only the lymphatic system can clear macromolecules, nanoparticles, and lipids in normal tissues, whereas small molecules can move into and out of blood vessels in both normal and tumor tissues more freely.[2,3,25,26,38,40–42] However, transfer of macromolecules to the luminal side of blood capillaries does not occur effectively.[30,40] Actively dividing tumor cells with accelerated metabolic activity can eliminate low-molecular-weight waste products via venous return, but the venous return in tumor tissues was found to be an order of magnitude lower than that in normal tissue.[26,34] Thus, the impaired lymphatic clearance of macromolecules and lipidic components can also account, in part, for the EPR effect in solid tumors.

6. Implications for Delivery of Drugs to Tumors

In cancer treatment, the concentration of a drug in tumor tissue is of utmost importance. However, it is not usually possible to achieve a drug concentration in tumor tissue that is higher than that in plasma by using conventional anticancer drugs, which are often low-molecular-weight agents without carriers. However, the EPR effect allowed achievement of anticancer drug concentrations that were several to 10 times higher in tumor tissue than in normal tissues or organs. For example, Lipiodol is a lipid contrast medium and becomes minute particles after arterial injection. In our experience,

(a) Normal tissue



(b) Tumor tissue

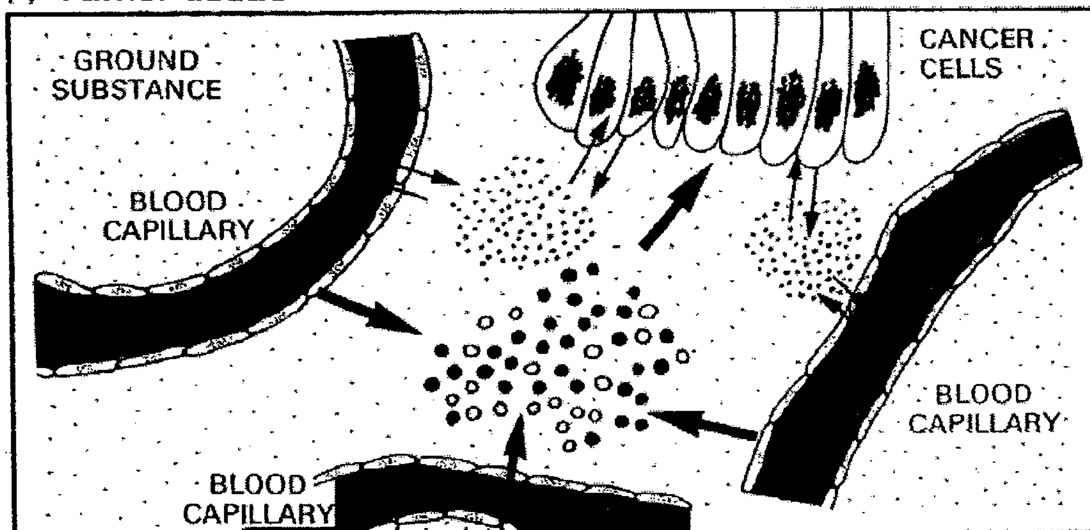


Fig. 3 Diagrammatic representation of vessel structures and transport of various substances into and out of blood capillaries in normal (a) and tumor (b) tissue. Leakage of macro molecules and nanoparticles from blood capillaries to tumor tissues is enhanced but no lymphatic recovery occurs. Furthermore, in tumor tissues there is no flow of macromolecules back into the blood capillaries, and lymphatic ducts are absent (from Ref. 40).

injection of SMANCS/Lipiodol into tumor tissue via the tumor-feeding artery resulted in drug retention for more than several weeks, and the drug level was 2000-fold higher in tumor tissue than in blood plasma.[4,43,44] In this case, the EPR effect and the first-pass effect work additively. Eventually, the drug disappeared from the tumor together with degraded tumor tissues.

Thus, the advent of SMANCS therapy and the discovery of the EPR effect, which applies to macromolecular anticancer drugs in general, permitted EPR effect-based anticancer drug targeting to solid tumor in becoming a reality.[2,39,40,43–50] For this type of therapy, the plasma level of macromolecules, nanoparticles, or lipidic particles (drugs), as expressed by the area under the concentration curve (AUC), must remain high, preferably for more than 6 hours. Extravasation of these agents into the tumor tissue increases progressively with time (in several hours to days), whereas clearance from tumor does not proceed because of a lack of lymphatic function. It should be noted that one can maintain a high plasma concentration or AUC for many hours, even with low-molecular-weight agents, by slow continuous intravenous infusion. However, low-molecular-weight agents do not exhibit EPR-effect because they can move freely into and out of blood vessels (capillaries), and the plasma level quickly becomes quite low because of excretion. Thus, targeting of drug to tumor does not take place.

Macromolecular conjugates or particles that contain active drugs of low molecular weight are usually required, the active principle being released to react with the molecular target in tumor cells. Therefore, the release rate, enhanced not only tumor delivery, but become a key issue for the development of truly effective anticancer agents. For example, we found that when SMANCS/Lipiodol is infused via the tumor-feeding artery, it is delivered to and deposited throughout the tumor but is not cleared quickly (clearance takes several weeks to months). The concentration of SMANCS administered via the artery was 20–30 μg/g of tumor tissues even 2–3 months after injection of 1 mg/ml (SMANCS/Lipiodol), and this remaining activity was more than 1000 times higher than the minimal inhibitory concentration against tumor cells (i.e. SMANCS had a minimal effective concentration below 0.05 μg/ml).[43] Also, SMANCS is itself biologically active without release from the polymeric matrix. For these reasons, SMANCS therapy is highly effective.

It should be mentioned that not all tumors are highly vascular; some appear to be hypovascular or avascular.[30] Tumors of the pancreas and the prostate, for example, are hypovascular. Many avascular tumor tissues such as the central portion of metastatic tumors demonstrate central necrosis and circulatory insufficiency. Avascular areas are not growing and need little, if any, attention.

The EPR effect functions not only in subcutaneous implanted tumors in mouse tumor models, but also in many other tumors such as VX-II carcinoma implanted in the liver (rabbit); Walker 256 carcinoma which metastasizes to the omentum in rats; and other metastatic liver and colon cancers chemically induced in a mouse model, as well as patients given SMANCS.[31,32,43,44] In the clinical setting, visualization of tumor is possible by use of radioscintigraphy with ^{67}Ga citrate, a radioactive isotope compound, which binds to transferrin (90 kDa) in blood plasma and selectively accumulates in the tumor by means of the EPR mechanism.[2]

The advantage of polymeric drugs with regard to multidrug resistance has also been discussed.[51–53] The lymphotropic nature of polymeric drugs discussed previously, is of prime importance for controlling lymphatic metastasis, which is the cause of many therapeutic failures.[50,54–57]

7. Conclusion

The EPR effect can be considered as a hallmark concept that exploits the anatomical and pathophysiological defects in the tumor vasculature. It plays a critical role in more selective delivery of macromolecular anticancer agents to cancer tissues. Understanding and manipulating the factors contributing to the EPR effect can further improve the selective targeting of high-molecular-weight biocompatible or nanoparticle anticancer drugs to tumor. The EPR effect can be enhanced in many ways, such as by using vascular mediators and by arterial injection to attain concentrations in tumor tissues that can never be possible with conventional intravenous chemotherapeutics. With achievement of the targeting of macromolecular drugs to tumors, the rate of release of free drugs from the composite becomes the key for successful anticancer therapeutics. In summary, EPR-based strategies portend a bright future for cancer chemotherapy.

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4

Basic Strategies for PEGylation of Peptide and Protein Drugs

Gianfranco Pasut, Margherita Morpurgo and Francesco M. Veronese

Abstract

The term PEGylation defines the modification of a protein, peptide or non-peptide molecule by the chemical linking of one or more poly(ethylene glycol) (PEG) chains. PEG is the polymer of choice for drug conjugation, being non-toxic, non-immunogenic, non-antigenic and highly soluble in water; all properties that are conferred on the conjugated drugs. The PEG derivatives have the following advantages: (1) a prolonged residence in the body due to a reduced kidney clearance, as a consequence of the increased molecular weight; (2) a decreased degradation by proteolytic enzymes, thanks to the shielding effect of the polymer chains; (3) reduction or elimination of protein immunogenicity.

Thanks to these favorable properties, PEGylation now plays an important role in enhancing the use and potential of peptides and proteins as therapeutic agents that, in the native form, often encounter severe limitations in their use. So far, several chemical strategies have been developed to perform the linking of the polymer to a biologically active molecule, and a wide range of methods of which a selection is reported here. A clear demonstration of the potential of PEGylation is the numbers of products

that have already reached the market or that will soon be available. In addition to a review of PEGylation methods, this chapter also describes recent achievements in PEGylation of protein and peptide drugs.

1. Introduction

Research in polymer-protein conjugation had its beginnings in the sixties through the seventies when dextran was studied as a coupling polymer. Since then, many studies have been carried out using both natural and synthetic polymers and suitable chemical strategies for conjugation have been developed along with the identification of conditions for selective modification and easy purification as well as tailor-made analytical procedures. Among all of the polymers studied so far, PEG emerged as the best candidate for modification, thanks to its uncommon properties that are conferred on the final conjugate. The most interesting characteristics of PEG are: (a) the lack of immunogenicity, antigenicity and toxicity; (b) high solubility in water and in many organic solvents; (c) high flexibility of the chain and; (d) FDA approval for human use. Many pharmaceutical companies are now looking to this technology to improve their products, most of them being proteins or peptides (an increasing portion among all drugs after the human genome sequence release) since PEGylation appears to be powerful solution to many of their shortcomings. Common limits for therapeutic application of peptides and proteins are their tendency to promote an immunological response (in particular when their sequence is not identical to the human protein); their chemical instability (both *in vivo* and in the formulation); and their short body residence time, the last mainly evident for the low molecular weight peptides. Most of these problems are overcomed by PEGylation, thanks to the combination of increased molecular weight, coverage or blockage of critical protein's sites (epitopes or sequences degraded by enzymes) and the enhanced solubility of the conjugates in water. As reported in several articles and reviews (Refs. 1–4), PEGylation increases the hydrodynamic volume of the molecule so that the first common positive effect of conjugation is an extended blood residence time due to reduced kidney clearance. Consequently, the derivatives need a reduced frequency of administration with respect to the unmodified drug. Moreover, PEGylated molecules possess increased solubility. The shielding effect of a PEG chain on specific sites on the protein surface

Table 1 PEG conjugates that are already on the market.

Conjugates	Year to market	Disease
PEG-adenosine deaminase (*Adagen*®)[20]	1990	Severe combined immunodeficiency disease (SCID)
PEG-asparaginase (*Oncaspar*®)[21]	1994	Acute lymphoblastic leukemia
Linear PEG-interferon α2b (*PEG-Intron*®)[33,77]	2000	Hepatitis C and clinical trials for cancer, multiple sclerosis, HIV/AIDS.
Branched PEG-interferon α2a (*Pegasys*®)[34,35]	2002	Hepatitis C
PEG-growth hormone receptor antagonist (*Pegvisomant, Somavert*®)[36]	2002	Acromegaly
PEG-G-CSF (*pegfilgrastim, Neulasta*®)[37]	2002	Treating of neutropenia during chemotherapy
Branched PEG-anti-VEGF aptamer (*Pegaptanib, Macugen*™)[38]	2004	Macular degeneration (age-related)

prevents or reduces the expression of immunogenicity and antigenicity, and enhances stability towards both chemical and enzymatic degradation.[5–7] For small drugs, it may yield more convenient biodistribution, selected cellular uptake[8–10] and, thanks to a specific linkers or molecules, a controlled drug release or drug targeting into specific organs or cells.[11]

The first application of PEG as a bioconjugation polymer has been proposed in the late 1970s by Professor Frank Davis at Rutgers University.[12] Following this pioneering study, large number of drugs with different structure (proteins, peptides, small molecular weight drugs, polynucleotides) have been PEGylated, thus creating a new class of drugs,[13] some of which have become blockbuster products. In Table 1, PEGylated products that have already reached the market are reported.

2. Features of PEG as Bioconjugation Polymer

Raw poly(ethylene glycol) is synthesized by ring opening polymerization of ethylene oxide. The reaction is initiated by methanol or water,