



Targeting tumor stroma and exploiting mature tumor vasculature to improve anti-cancer drug delivery

Caroline Bouzin, Olivier Feron*

UCL Medical School, Unit of Pharmacology and Therapeutics (FATH5349), Angiogenesis and Cancer Research Group, 52 Avenue E. Mounier, B-1200 Brussels, Belgium

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Abstract

The identification of a critical role of tumour stroma in the regulation of tumour **interstitial fluid pressure** and the simultaneous discovery of the impact of anti-angiogenic drugs on **tumour hemodynamics** have provided new potential for improving tumour delivery of anti-cancer drugs. Here, we review the most recent studies investigating **how tumour-associated fibroblasts and macrophages as well as the extracellular matrix itself may be targeted to facilitate delivery of both low-molecular weight drugs and macromolecules**. In addition, we summarize the current understanding of the use of vasoactive compounds, radiotherapy and vascular-disrupting agents as potential adjuvants to maximize tumour delivery of anti-cancer drugs. The impact of these strategies on the diffusive and convective modes of drug transport is discussed in the light of Fick's and Starling's laws. Finally, we discuss how transcytosis through caveolae may also be exploited to optimize the selective delivery of conventional chemotherapy to the subendothelial tumour cell compartment.

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1. Introduction

Anti-angiogenic strategies target tumour blood vessels in an effort to deprive tumours of vital oxygen and nutrients (Jain et al., 2006). Recently, different reports have documented that anti-angiogenic therapy could also improve drug delivery through the *normalization* of the tumor vasculature (Jain, 2005). This effect is presented as paradoxical since destroying the tumor vasculature of solid tumours would be expected to compromise delivery of chemotherapy (and of oxygen which should also render radiation less effective). The paradox is however limited if one considers that the targets of anti-angiogenic drugs are the endothelial sprouts and not the mature tumour blood vessels. The term *normalization* describes the expected result of the pruning of immature

blood vessels, leading to a vascular network made of fewer, larger and less permeable vessels. Moreover, this normalization of the tumour vasculature is limited to a short window, as recently emphasized in patients with recurrent glioblastoma treated with a VEGF receptor tyrosine kinase inhibitor (Batchelor et al., 2007). In addition, the tumour vascular network, remodelled or not, should not be considered as normal in regard of the specificity of another class of drugs aimed at damaging the tumour vasculature, the “vascular-disrupting agents” (VDA) (Tozer et al., 2005). Indeed, VDA exert their cytotoxic activity against the existing non-angiogenic component of tumour blood vessels and leave largely unaltered the rest of the vasculature in the body (Neri and Bicknell, 2005; Tozer et al., 2005), underlying the existence of proper characteristics of the apparently normal mature tumor vessels *versus* the healthy vasculature.

In this review, we will juxtapose these recent data on improved drug delivery after anti-angiogenic treatments with older paradigms on drug transport in tumors. We will then extend the restrictive concept of tumour vasculature

* Corresponding author at: Unit of Pharmacology and Therapeutics, UCL-FATH5349, 52 Avenue E. Mounier, B-1200 Brussels, Belgium.
Tel.: +32 2 764 5264; fax: +32 2 764 5269.

E-mail address: feron@mint.ucl.ac.be (O. Feron).

normalization as an adjuvant approach to chemotherapy to the larger concept of dynamic modulation of tumour microenvironment to improve drug delivery.

2. The tumour vasculature maturation: the true rationale to combine therapies

Despite the restrictions to the concept of tumour vasculature normalization mentioned above, one must recognize that it has focused more interest on the mature component of the tumor vasculature. With the major interest of researchers and pharmaceutical companies focused on inhibition of tumor neovascularisation during the last decade, the role of the mature tumor vessels in tumor therapy was largely ignored or at least underestimated in the literature. There are indeed very few studies examining and comparing the extent of angiogenic versus mature vasculature components in human tumors. In one of these rare studies, Eberhard and colleagues examined the functional status of tumor vascular bed by quantifying endothelial cell proliferation and recruitment of pericytes (Eberhard et al., 2000). Proliferating capillary index (PCI) values of 2–10% led to the conclusion that angiogenesis was present in all tumours, but of considerable less intensity than physiological angiogenesis in such context as the growing ovarian corpus rubrum (PCI = 40%). The microvessel pericyte coverage index was found to be variable according to the tumor origin with lower values (10–20%) for glioblastomas and renal cell carcinomas, and the highest index (>60%) for colon and mammary carcinomas. This study implicitly indicated that important differences may exist in the functional status of tumour vasculature.

This observation has only very recently been recognized as one of the major reasons for the disparity between the results of human clinical trials and the preclinical evaluations of anti-angiogenic agents. Animal studies have revealed that the pericyte coverage of tumour endothelium is a source of resistance towards anti-angiogenic drugs (Feron, 2004; Bender et al., 2004). A deficient coverage in pericytes is indeed associated with a defect in the structural and biochemical protection of endothelial cells, the latter through the release of a variety of pro-survival factors. The tumor vasculature of most preclinical tumor models (notably transplanted mouse tumors) is consequently more susceptible to anti-angiogenic drugs, leading to tumor regression and/or eradication. In human tumours, or in some slow-growing models of mouse tumours where the maturation of tumor vasculature is more complete, anti-angiogenic drugs are essentially cytostatic. Combination with other anticancer therapies which directly target tumour cells, such as chemotherapy and radiotherapy, is therefore a rational strategy to favour tumor eradication. Different studies (mostly in animal models) have recently led to the identification of key insights on the advantage of altering the tumor microenvironment to increase the efficacy of conventional anticancer treatments including chemotherapy.

3. Fick's and Starling's laws

Dissection of the adjuvant effects of anti-angiogenic drugs when combined with chemotherapy has provided new insights on the determinants of drug transport from capillaries to tumor interstitial tissue. The original question was whether the efficacy of co-administered chemotherapy could be improved by rectifying abnormal endothelial barrier function (increased permeability) and pruning the branches of the tumor vascular tree. We know today that the driving force for the extravasation of chemotherapeutic drugs is increased by angiogenesis inhibitors through both *reduced luminal resistance* and *decreased* interstitial fluid pressure (IFP).

The ability of pharmacological treatments to promote drug delivery through the tumor microvasculature is a process dependent on both the size of the chemotherapeutic molecule administered but also on tumor hemodynamics. Fick's and Starling's laws, although originally established not for rationalizing the diffusion and convection of drugs towards the tumor compartment, enable one to understand the key determinants of the drug delivery process (Box 1). Fick's law is applicable to the transport of low-molecular-weight drugs across the capillary walls. The equation states that the diffusion rate depends on the drug concentration gradient and is proportional to the surface of exchange. Convection fluxes, and not diffusion, are generally proposed to account for the extravasation towards the interstitial space of entities above a cut-off size of 10 kDa, such as antibodies or liposomes. The determinants of the capillary filtration pressure for these molecular entities are described by Starling's law (Box 1). Like for diffusion, there is a direct correlation with the surface area of exchange. The resulting flux is however mostly dependent on the net pressure difference between hydrostatic and colloid osmotic pressures. Actually, since the oncotic gradients in tumors are almost zero, convection mostly relies on the difference in the hydrostatic pressure between the capillary (microvascular pressure, MVP) and the interstitium (interstitial fluid pressure, IFP).

We will focus below on some parameters from Fick's and Starling's equations, in particular those for which the understanding of their biological roles and pharmacological regulation in tumors have evolved during the last decade, namely IFP and the area of exchange (A).

4. Targeting the tumor stroma to improve drug delivery

In Starling's law, the mathematical weight of IFP is obvious since it represents a direct obstacle for MVP to determine the extent of drug delivery to the tumor. IFP reduces the transvascular convection and generates an outward interstitial flux toward the periphery of the tumor.

Interstitial pressure is normally regulated through interactions between the extracellular matrix (ECM) and stromal cells (Fig. 1). At the onset of tumor development, the extrava-

Box 1: Fick's and Starling's laws

Extravasation of low-molecular-weight drugs is dominated by diffusion, whereas convection represents the principal mechanism of macromolecular extravasation.

- Fick's equation (diffusion):

$$J_s = PA(C_p - C_i)$$

J_s is the diffusive molecule flux; P vascular permeability coefficient; C_p and C_i are the drug concentrations in plasma and in the interstitium; A vascular surface area of exchange.

Modified equation:

$$J_s = \frac{PA(C_p - C_i)}{MW^{1/2}d}$$

The diffusion of a small molecule is inversely proportional to the root of its molecular weight (MW) and the distance (d) from the vessel.

- Starling's equation (convection):

$$J_v = L_p A [(MVP - IFP) - \sigma(\pi_p - \pi_i)]$$

J_v is the convective fluid flux; L_p hydraulic conductivity; A vascular surface area of exchange; $MVP - IFP$, difference in hydrostatic pressures in the vessel and the interstitium; MVP , microvascular pressure; IFP , interstitial fluid pressure; $\pi_p - \pi_i$ = difference in osmotic pressure in the plasma and the interstitium; σ osmotic reflection coefficient.

sation of plasma macromolecules such as fibrinogen through the angiogenic, permeable vasculature and a high deposition of collagen lead to the formation of a very dense network of matrix molecules in the tumor. Fibroblasts can easily proliferate in this specific microenvironment and eventually gain contractile function through the acquisition of smooth muscle cell properties (Desmouliere et al., 2004). This mode of differentiation arises from the release of various cytokines by tumor cells but also by other cells recruited in the tumor stroma such as macrophages and other types of immune cells. The binding of these "activated" fibroblasts to the interstitial fibres via a variety of integrins results in a constant increase in the tension within the tumor connective network (Desmouliere et al., 2004). Together with the obvious consequences of tumor cell proliferation on the blood and lymphatic vessel integrity, the stroma of solid tumors actively participates in the increase in IFP in tumors.

Different approaches have been recently identified to decrease the IFP, focusing on altering the extracellular matrix or targeting stromal cells.

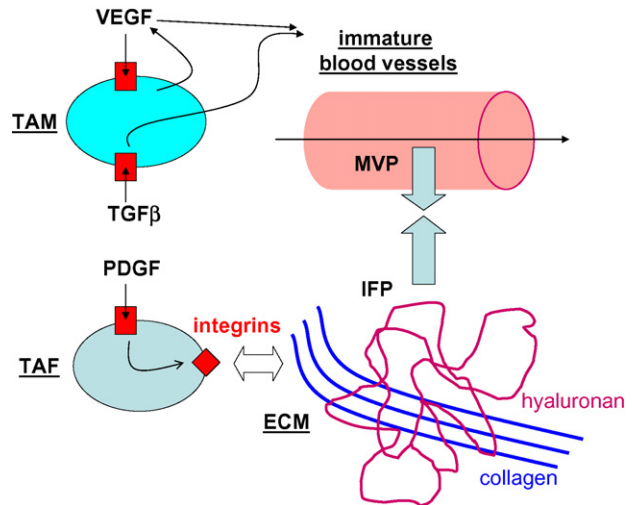


Fig. 1. Scheme of the different targets within the tumor stroma for adjuvant therapies to improve anti-cancer drug delivery. Tumor-associated macrophages (TAM) through the production of VEGF or their phenotype conversion by TGF β stimulation inhibit the maturation process of the tumor vasculature. In tumor-associated fibroblasts (TAF), PDGF stimulation promotes the interaction of integrins with the extracellular matrix (ECM), thereby contracting the tumor stroma and increasing the interstitial fluid pressure (IFP). The dense network of collagen and hyaluronan per se also participates in the tumor interstitial hypertension. The resulting equilibrium between the elevated IFP and the moderate microvascular pressure (MVP) opposes the homogenous distribution of anti-cancer drugs and in particular, prevents the extravasation of macromolecules.

4.1. Targeting the extracellular matrix

The proof of principle that degradation of the ECM leads to a favorable change in the transvascular pressure gradient came from studies where hyaluronidase or collagenase were injected intra-tumourally (Brekken et al., 2000; Eikenes et al., 2004; Netti et al., 2000). The interstitium consists of a protein network (e.g., fibrillar collagen) embedded in a hydrophilic gel of glycosaminoglycans (e.g., hyaluronan) (Fig. 1). Intra-tumoural injection of collagenase or hyaluronidase was shown to reduce IFP and to increase the uptake of macromolecules, in particular of antibodies. Interestingly, collagenase reduced both IFP and MVP, restricting *a priori* the gain in pressure difference. The transcapillary pressure gradient is, however, favorable to antibody delivery because MVP usually recovers by the time IFP reaches its minimum level (Eikenes et al., 2004). In these experiments, mean arterial blood pressure (MABP) was measured and was found not to be affected; the reduction in MVP was attributed to reduced vascular resistance. The beneficial effects of the disintegration of the ECM also impacts on other parameters of Starling's law. For instance, an increase in the surface of exchange is directly associated to the reduced resistance and the vascular hydraulic conductivity is increased up to a factor of 10 (Weinberg et al., 1997).

Comparison of collagenase and hyaluronidase effects indicates that the degradation of the structural protein network is more efficient than the degradation of the hyaluronan

gel, with respect to antibody uptake (Brekken et al., 2000; Choi et al., 2006; Netti et al., 2000). More recently, collagenase also revealed to be an efficient adjuvant strategy for oncolytic herpes simplex virus (HSV) (McKee et al., 2006), confirming that fibrillar collagen is an important barrier to the distribution of macromolecules within the tumor. The use of collagenase may however not be clinically relevant because the enzyme is not tumor selective and may facilitate the formation of metastases.

In a recent study, McDonald and colleagues (Nakahara et al., 2006) reported that inhibition of VEGF signaling decreases the overall distribution of extravasated antibodies, in large part due to reduced tumour vascularity. However, when expressed per surviving vessel the distribution was found to increase. Antibodies were actually found to accumulate in sleeves of basement membrane left behind by regressing tumor vessels. The same group showed that this route of distribution for macromolecules within tumours also provides a scaffold for rapid revascularization of tumors after removal of anti-VEGF therapy (Mancuso et al., 2006). This observation opens a new framework for the development of strategies delivering drugs capable of altering the **extracellular matrix** and/or preventing blood vessel re-growth.

4.2. Targeting tumor-associated fibroblasts (TAF)

As mentioned above, activated fibroblasts which proliferate during tumor progression actively participate in transforming the loose connective network of normal tissue into a denser and more rigid network (Desmouliere et al., 2004). Fibroblasts bind collagen fibres in an integrin-dependent manner, thereby increasing the tension within the **extracellular matrix**. Among the growth factors and cytokines that activate the TAF, platelet-derived growth factor (PDGF) was recently identified as a key promoter of the interaction between integrins and **ECM** molecules (Fig. 1). PDGF injection was already known to correct the lowered IFP during anaphylactic reactions (Rodt et al., 1996). The PDGF receptor expression in the stroma cells of a broad range of solid tumours therefore logically led several investigators to examine whether conversely, PDGF inhibitors could be used to block the contraction of myofibroblasts in the tumors and thereby reduce tumor IFP. Imatinib (Glivec) was among the first molecules with inhibitory effects on the PDGF receptor tyrosine kinase to be found to decrease the interstitial hypertension in tumours (Pietras et al., 2001, 2002, 2003). Interestingly, low molecular weight drugs including taxol, 5-fluorouracil and epothilone B, normally the lesser sensitive to elevated tumor IFP, were the first to be shown to benefit from the imatinib-driven increase in capillary-to-interstitium transport in subcutaneously (s.c.) growing thyroid carcinomas (Pietras et al., 2002, 2003). Although it cannot be excluded that inhibition of other molecular targets of imatinib (c-kit, abl) contributes to the effects of imatinib, similar results were obtained with PDGF aptamers, suggesting that inhibition of the beta-subunit of the platelet-derived growth factor recep-

tor (PDGFR- β) is the prime molecular mechanism for the observed effects of imatinib. Significantly, in most of these studies, PDGF receptors were found to be expressed in blood vessels and stromal cells but not in tumor cells, and PDGF antagonists had no effect on tumor growth (Pietras et al., 2001). However, it is not known whether the anti-angiogenic effects of PDGF inhibitors, through the targeting of PDGF receptors on pericytes and the consecutive destabilization of more mature tumor blood vessels, contribute to improved drug uptake.

More recently, the combination of imatinib and radiolabeled antibody against mucin-like, tumor-associated glycoprotein was reported to result in a dose-dependent growth inhibition of s.c. xenografts of colorectal and pancreatic adenocarcinoma in athymic mice (Baranowska-Kortylewicz et al., 2005, 2007). These authors documented that the improved antibody uptake and the concomitant reduction in tumor hypoxia were both direct consequences of the attenuation of tumor IFP and contributed to the increased efficacy of these radioimmunotherapy approaches. Whether these changes in IFP also account for the effects on low molecular weight drug uptake is a matter of debate.

Whether PDGFR- β inhibition may also reduce tumor IFP in humans was recently addressed using CDP860 in patients with advanced ovarian or colorectal cancer (Jayson et al., 2005; Board and Jayson, 2005). CDP860 is an engineered Fab' fragment-polyethylene glycol conjugate, which binds to PDGFR- β and blocks its activity. In this small clinical trial, serial dynamic contrast-enhanced magnetic resonance imaging studies revealed that the ratio of vascular volume to total tumor volume increased significantly within 24 h following CDP860 administration in some patients. This observation is in good agreement with the expected recruitment of previously non-functioning vessels due to the decrease in intra-tumor hypertension. Importantly however, most of the patients showed evidence of fluid retention and about half of them developed significant ascites. This observation warrants further studies to determine the optimal drug regimen of PDGF inhibitors used as adjuvant strategy to increase drug delivery. Another obvious caveat in the use of PDGF inhibitors is their negative impact on the maturation of the tumor vasculature and the risk to render the already poorly efficient tumor vasculature yet more chaotic and less susceptible to deliver drugs in an efficient manner.

In addition to the above pharmacological approach, an immunotherapeutic approach aimed at specifically killing TAF was recently proposed (Loeffler et al., 2006). In contrast to genetically unstable tumour cells, tumour stroma fibroblasts represent targets for cancer immunotherapy. An oral DNA vaccine targeting fibroblast activation protein (FAP) was reported to successfully suppress primary tumor cell growth and metastasis of multidrug-resistant murine colon and breast carcinoma. FAP is a type II transmembrane protein that functions as a serine protease implicated in **ECM** remodeling and is selectively overexpressed in over 90% of stromal fibroblasts associated with colon, breast, and lung carcino-

mas (Kelly, 2005). Vaccinated mice revealed a markedly decreased collagen type I expression and up to 70% greater uptake of chemotherapeutic drugs, associated with a longer lifespan (Loeffler et al., 2006).

4.3. Targeting tumor-associated macrophages (TAM)

Macrophages are, together with fibroblasts, another major type of cells present in the tumor stroma. Recently, bevacizumab, a humanized anti-VEGF antibody, was reported to reduce the density of macrophages in xenograft human anaplastic thyroid carcinoma (Salnikov et al., 2006). Interestingly, the plasma protein leakage from tumor vessels, the density of vascular structures and the tumor IFP were found to be concomitantly decreased whereas the tumor plasma volume remained unchanged. The authors concluded that carcinoma cell-derived VEGF either directly or indirectly participates in maintaining an inflammatory tumor microenvironment.

The same group had previously documented that blocking TGF- β signaling could also reduce the macrophages-driven IFP elevation in experimental carcinoma (Salnikov et al., 2005) (Fig. 1). TGF- β is a multifunctional cytokine which potently inhibits the proliferation of epithelial cells and thereby participates in the resistance to cancer formation. However, after tumours have escaped TGF- β surveillance, TGF- β can promote metastases by increasing tumor cell viability and migratory potential (Muraoka et al., 2002). TGF- β secretion by cancer and/or stromal cells can also promote the remodeling of extracellular matrix and may therefore potentially impact on the IFP. Lammerts et al. (2002) reported that the use of a soluble TGF- β receptor type II-murine Fc:IgG_{2A} chimeric protein (Fc:T β RII) could lower IFP in experimental thyroid carcinoma in a dose- and time-dependent manner. As mentioned above, TAM were finally identified by the same group as the ultimate targets of the Fc:T β RII treatment (Salnikov et al., 2005). In this study, the *in vivo* antitumor efficacy of doxorubicin was shown to be increased by the concomitant administration of the TGF β -targeting treatment. Significantly, the growth inhibitory effect of doxorubicin on cultured tumor cells was not influenced by the presence of either Fc:T β RII, thereby confirming the tumor stroma as the target of this treatment.

TGF β can induce the expression of different key angiogenic factors including VEGF and MMP9 expression in macrophages (Hiratsuka et al., 2002). Although some of the effects of TGF- β inhibition could therefore be related to a modulation of tumor angiogenesis, microvessel density was found not to be dramatically affected in the above studies using the Fc:T β RII treatment. The observed lowering of tumour IFP is therefore likely to reflect a maturation of the carcinoma vasculature, as supported by the reduced plasma protein leakage. Also, the decrease in NG2 expression (a marker of activated pericytes) and the relative increase in α -SMA-positive vessels indicate a change towards more differentiated smooth muscle cells as reported in normal and

mature microvessels (Salnikov et al., 2005) (Fig. 1). One may therefore postulate that here again the maturation of the tumor vasculature, in particular after targeting macrophages in the tumor stroma, may have beneficial roles for the transcapillary diffusion of drugs.

In conclusion, TAM appears to be a particularly well-suited target considering the impact of suppressing a major source of cytokines and growth factors favoring angiogenesis and/or increasing the tumor IFP. Interestingly, strategies aimed at killing TAM directly (*versus* targeting macrophage-driven signaling pathways) have recently emerged. A DNA vaccine against legumain, an endopeptidase overexpressed by TAMs, was recently reported to induce a robust CD8⁺ T cell response against TAM (Luo et al., 2006). Also, treatment with clodronate encapsulated in liposomes (clodrolip) efficiently depleted macrophages in mouse tumour models (Zeisberger et al., 2006). Synergistic effects were also observed by combining clodrolip and a VEGF-neutralising antibody. In both approaches, significant inhibition of tumour growth, reduction in blood vessel density and marked decrease in proangiogenic factors released by TAMs (such as VEGF, TGF- β , TNF- α and MMP-9) were observed. These results validate TAM-targeting therapies as a promising novel strategy for an indirect cancer therapy.

5. Targeting the tumor vasculature to improve drug delivery

5.1. Increasing the area surface of exchange and the pressure perfusion

The changes in tumor blood flow caused by non-tumor-selective vasoactive treatments are fundamentally influenced by the structural relationship between the tumor vascular bed and the vascular bed of the surrounding normal tissue. Should the tumor and the surrounding beds be *in parallel*, an increase in normal tissue blood flow due to vasodilation would shunt away the blood from the tumor to the normal tissue, resulting in a decrease in tumor blood flow. Conversely, vascular beds *in series* would coincide with similar changes of blood flow in the normal and tumor tissues because the blood that leaves the normal tissue vascular bed could directly flow into the tumor. The situation in most existing tumours is obviously complicated because both vascular organizations (parallel and series) tend to be intimately mixed.

By contrast to anti-angiogenic strategies, we have proposed the term “provascular” for any attempt to temporarily increase tumor perfusion/oxygenation through pharmacological/physical interventions (Feron, 2004). As emphasized above, a non-negligible fraction of blood vessels in human tumours are covered by pericytes and therefore are capable of autoregulation. Although the non-uniform coverage does not allow the same sophisticated mode of regulation as that of coronary arteries, tumour blood vessels have been shown to respond to a variety of substances (Feron, 2004).

Vasoactive drugs able to selectively increase tumour blood flow have the potential to act as radio- and/or chemosensitizers. Transient effects are however needed to avoid a long-lasting increase in tumor pO_2 that could promote tumor growth. Although many drugs tested so far do not meet this last requirement, they were used in early studies to test whether modulation of the functional vascular reactivity within tumours is at least a feasible objective. These studies essentially proved that tumour vessels were not maximally dilated at a given time or in a given area of the tumour.

5.1.1. Exploiting the differential vasoreactivity of tumour versus healthy organ

The potential for tumour vessel dilation to impact drug delivery in response to pharmacological modulators mostly relates to the surface area of exchange parameter (Fig. 2). If considering circular vessels, any increase in the vessel diameter should lead to a π -fold larger increase in vessel surface. The size of the molecule will then determine whether an increase in the surface of exchange is positive or not for its delivery to the tumor compartment. Indeed, while for larger drug molecules (such as antibodies) the hydrostatic force will determine whether the *convection* current is favorable, it is mainly the difference in concentration between plasma and tumor compartments that will govern the *diffusion* of small molecules. Furthermore, the level of diffusion will be inversely related to the distance from the blood vessels.

Bradykinin and angiotensin II are well-known physiological peptides exerting opposite vasodilatory and vasoconstricting effects, respectively. Amazingly, they have both been documented to improve drug delivery in tumors. Labradimil, a bradykinin agonist, was shown to increase the delivery of carboplatin through a decrease in IFP (Emerich et

al., 2001a,b). However, considering the associated increase in tumor vessel permeability (in part through the increase in vascular pore size), the major reason for this synergy has to be searched in other variables and most likely in the concomitant increase in the tumour vascular surface area (absent or less important in healthy tissues) (Fig. 2). Whether the tumour vasculature contained a higher density of B_2 receptors in these studies is not known; however for maximal efficacy, the bradykinin agonist had to be administered at low dosage and after the chemotherapeutic drug infusion (Emerich et al., 2001a,b).

Angiotensin II was reported to induce an increase in the tumour vascular resistance in tumor-bearing animals, but to a much lesser extent than in the normal vasculature (Jirtle et al., 1978) (Fig. 2). The resulting effects are a proportionally higher increase in tumour perfusion pressure (especially in large tumours (Thews et al., 2000)), preventing the intermittent collapse of tumor microvessels or temporary flow stasis phenomena. In another study, periodic alterations of MABP induced by systemic injection of angiotensin II were shown to lead to an enhancement of fluid exchange between the vascular and interstitial space in tumours (Netti et al., 1999). Using a non-specific antibody, however, this protocol of modulations of MABP failed to promote macromolecule accumulation in the interstitium, probably because extravasated antibodies were reabsorbed within a blood pressure cycle.

Calcium antagonists were historically among the first agents evaluated for their effects on the tumour vasculature. Contradictory reports exist in the literature making difficult any consensus on the use of these drugs as adjuvant strategy. In the context of isolated limb perfusion however, nifedipine application was recently shown to enhance tumour microcir-

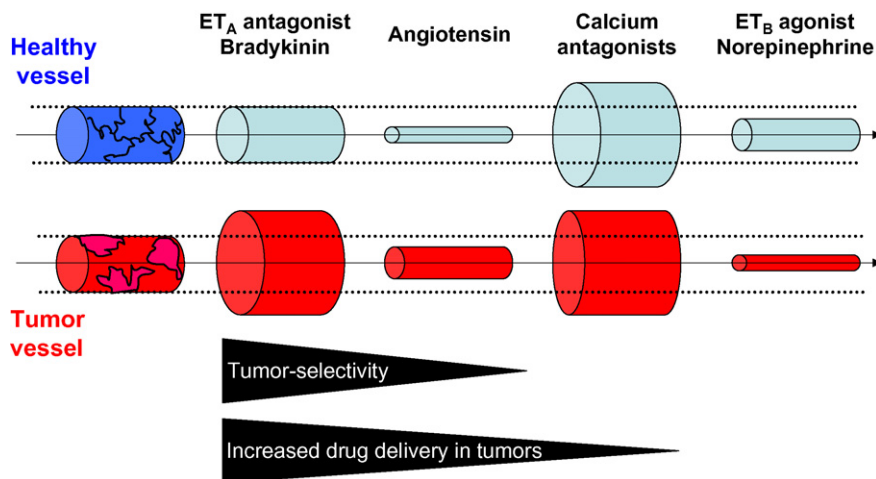


Fig. 2. Comparison of the effects of various vasomodulatory compounds on the tumor vs. healthy organ vascular tone. The differential effects between healthy and tumor blood vessels explain why both vasoconstrictor (e.g., ET_A antagonists and bradykinin) and vasodilating agents (Angiotensin II) have been reported to increase anti-cancer drug delivery in tumors. Note that these vasomodulatory effects are possible because of the largely underestimated pericyte coverage of tumor blood vessels (although incomplete when compared with healthy vessels). Note that drugs like calcium antagonists may improve drug delivery in tumors but not selectively (except if some protocols such as isolated limb perfusion are used) and that others can exert opposite effects by selectively reducing blood perfusion in tumors and not that of major healthy organs (note that such effects could still theoretically be exploited in monotherapy to deprive tumors of oxygen and nutrients).

ulation (Thews et al., 2002). Since this technique allows the direct application of therapeutic agents to a tumour-bearing extremity, the combination of chemotherapy with dihydropyridines could theoretically be exploited. Interestingly, “steal phenomena” (i.e., blood re-oriented towards some healthy tissues) and oedema formation were not observed.

Other investigators, including ourselves, also focused on another vasomodulatory molecule, endothelin-1 (ET-1). ET-1 is known as powerful vasoconstrictor molecule even though, when delivered intravenously, the normal vasculature first responds by a transient vasodilation. These effects are largely dependent on the distribution of ET_A (but also ET_B) receptors on smooth muscle cells and involved in contraction *versus* ET_B receptors located on the endothelium (i.e., the first to be reached after i.v. injection) and mediating vasorelaxation through stimulation of NO release (Marasciulo et al., 2006). Although several reports have described how altering the ET-1 axis may impact on blood flow and how to theoretically take advantage of the differential response of the tumour vascular bed *versus* that of other tissues (Cemazar et al., 2005), the effects of ET-1 antagonists on drug and oxygen delivery to the tumour have only recently been reported (Martinive et al., 2006; Sonveaux et al., 2004). In a first study, we documented that blocking the tumour-selective increase in the vascular endothelin-1/ET_A pathway could unravel an important reserve of vasorelaxation (Fig. 2). Endothelin-1 binding to ET_A receptor was actually shown to support the development of a myogenic tone within the mature tumour blood vessels contrary to size-matched healthy arterioles. Administration of ET_A antagonists to tumour-bearing mice was reported to lead to a significant increase in tumor blood flow and oxygenation (Sonveaux et al., 2004). In another study, we have also reported that these effects could contribute to a better delivery of cyclophosphamide into the tumour and a subsequent significant tumour growth delay (Martinive et al., 2006). The net effect of the ET_A antagonist on the microvascular pressure (MVP) translated in an increase in IFP which probably excludes the use of ET_A antagonist to improve the delivery of macromolecules (*versus* small drugs).

5.1.2. Exploiting local radiation effects

In non-tumoural vessels, the eNOS pathway leading to vasodilation is activated by receptor-dependent agonists that increase intracellular Ca²⁺ levels and by shear forces exerted by the blood stream. Similar eNOS-dependent vasodilatory effects were reported in tumour blood vessels, as elicited by the use of pharmacological NOS inhibitors. Limitations however are the systemic effects of NO-mediated vasodilation and the associated risk of the stealing effects. Recently, however, we showed that nitric oxide (NO)-mediated vasorelaxation (found to be defective in large tumour vessels) could be increased in response to local tumour irradiation (Sonveaux et al., 2002, 2003). We further showed that the stimulation of the NO-dependent pathway induced a marked increase in tumor blood flow. Two major consequences of these effects were identified: (i) an elevation in tumour

pO₂ increased tumour sensitivity to subsequent radiation exposure and (ii) the delivery of large cationic lipid complexes was dramatically increased (Sonveaux et al., 2002). Importantly, we recently reported that the combination of this priming irradiation protocol and the administration of a plasmid encoding for a dominant-negative form of Akt complexed into the cationic lipids resulted in significant anti-angiogenic effects due to the endothelial expression of the transgene (Sonveaux et al., 2007). Thus, low-dose irradiation of endothelial cells within a tumor appears to selectively modify NO-regulated tumor blood flow, thereby offering a unique strategy to improve radiotherapy and to deliver therapeutic macromolecules including lipid-containing entities.

5.2. Reducing the local and temporal heterogeneities of tumour blood flow

Our data on the beneficial effects of ET_A antagonists on tumor oxygenation and drug delivery (Martinive et al., 2006; Sonveaux et al., 2004) led us to focus on another key parameter of tumour hemodynamics. Our results revealed that the tumour vasculature could benefit from the ET_A antagonist treatment, mostly through the correction of local, temporal ischemia within the tumour at the time of chemotherapy administration (Martinive et al., 2006) (Fig. 3). These data emphasize that besides the well-characterized structural defects in tumor vasculature, functional alterations in tumor blood vessels also constitute a source of heterogeneities in tumor blood flow (TBF) which importantly, appear to be reversible. This may be related to the so-called acute or intermittent hypoxia (Dewhirst, 2007; Dewhirst et al., 2007),

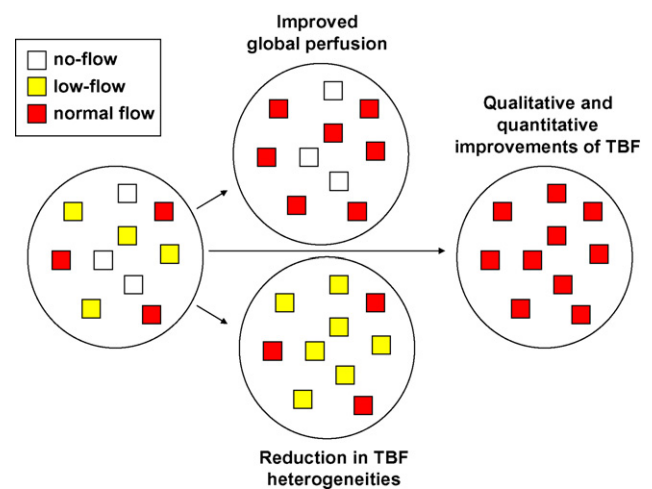


Fig. 3. Representation of the potential effects of drugs targeting the tumor vasculature on tumor blood flow. Vasomodulatory substances may increase the blood flow in the (already) perfused areas of tumors and lead to a global increase in tumor blood flow (TBF). Reduction in TBF heterogeneities can also be achieved by blocking intermittent ischemia/hypoxia, thereby promoting a qualitative increase in the tumor perfusion. Combination of these two quantitative and qualitative modes of TBF improvements by drugs such as ETA antagonists may considerably increase the extent and the distribution of co-administered anti-cancer drugs.

characterized by cyclic alterations in tumour pO_2 due to rapid fluctuations in haematocrit and slower local vascular remodeling due to angiogenesis. Our data support the hypothesis that alterations in tumor vascular tone may also contribute to intermittent ischemia and hypoxia. Therefore, pharmacological reductions in the vascular tone could dampen TBF heterogeneities and consequently increase drug delivery into the tumour. Any treatment improving the perfusion in those areas of low- or no-flow at a given time should also increase the absolute amounts of drug delivered into the tumor.

In our study exploiting the ET_A antagonist (Martinive et al., 2006), both parameters, i.e., drug concentration and distance are very likely to be positively influenced. Indeed, local vasodilation increases the amount of drug present at a given time in the tumor and, more importantly, by decreasing the proportion of vessels with lower perfusion leads to a more homogeneous tumour perfusion, thereby increasing the chance for chemotherapy to diffuse towards a larger population of tumour cells (Fig. 3).

Nicotinamide is another example of a drug thought to influence tumor vessel function through an effect on heterogeneities in tumour perfusion. Its proposed action on tumour oxygenation is clinically exploited to radiosensitize tumors (Kaanders et al., 2002) and a few experimental studies have also documented its use as adjuvant for chemotherapy (Gupta et al., 2006). Whether those effects derive from inhibition of tumor vasospasms, sparing of O_2 by reduction in O_2 consumption or decrease in IFP is not completely resolved.

5.3. Decreasing the microvascular pressure (MVP) by VDA

VDA are another example of drugs capable to reduce IFP by acting on tumour blood flow. However, the mechanism here is more complex than mere modulation of tumour vascular tone. VDA attack the existing vasculature, contrary to anti-angiogenic agents that target the developing neovasculature (Tozer et al., 2005; Pasquier et al., 2006). Disruption of the tumour vasculature results in increased microvascular permeability and probably also occlusion of the precapillary vessels that feed the tumour, leading to a cascade of reduced blood flow, vascular congestion, thrombus formation, metabolic starvation, and tumor cell necrosis. The impact of vascular disrupting drugs on IFP is *a priori* difficult to anticipate. Indeed, from a theoretical point of view, the increase in capillary permeability and the intravascular coagulation should favour an increase in IFP whereas the development of central necrosis should lower it by increasing the interstitial hydraulic conductivity. Recent data however indicate that the effects of VDA on capillary blood flow are those that determine the decrease in IFP with these agents (Hori and Saito, 2003; Skliarenko et al., 2006).

In tumours, the extent of macromolecular extravasation is often close to zero, because microvascular pressure (MVP) is very close to the IFP. Moreover, both parameters are intimately linked. The high vascular permeability of tumor blood

vessels and the absence of a functional lymphatic circulation suggest that the hydrostatic microvascular pressure (MVP) is the main force governing IFP in tumors. Arteriolar constriction by VDA leads to an increase in blood flow resistance and a reduction in downstream tumor blood flow and IFP (Skliarenko et al., 2006). Although the later development of capillary thrombosis may considerably increase capillary blood flow resistance and restore IFP toward its initial value, the development of central tumor necrosis accounts for the decline in IFP generally observed 24 h after VDA administration. It is also thought that VDA are most effective against vessels deep inside the tumor, possibly because the high interstitial pressure in these regions contributes to vascular collapse. But a compromise is probably necessary because of the need for the VDA to have access to the microvascular network in the interior of the tumor. The persisting rim of malignant cells after VDA treatments is perfused by more normal host vessels and is characterized by a low blood flow resistance and IFP.

Enhancement of anti-tumour efficacy of conventional anti-cancer drugs by VDA has started to be investigated and although the role of the IFP reduction was not directly addressed, the first successful combinatorial approaches have been reported (Curnis et al., 2002; Siemann and Rojiani, 2002). More work is needed to examine whether the window for alterations in IFP is wide enough or can be used repeatedly to maximize drug delivery. Other possibilities, such as the encapsulation of drugs in nanoparticles containing VDA, seem also to be exploitable for the specific trapping of large amounts of drugs inside the hemorrhagic/necrotic tumour center (Sengupta et al., 2005).

6. Directing the drug to the tumor vasculature

Interference with the IFP or TBF heterogeneities represents only one aspect of affecting drug delivery to the tumour. Indeed, the drug itself may be modified to be transported more efficiently or simply, its affinity for a specific receptor present on tumour cells may modify the mode of accumulation into the tumour. This is also where lies the possibility to introduce determinants for directing a drug selectively to tumours, thereby limiting its side effects. Recent extensive reviews have addressed how specific markers can be targeted by antibodies, peptides, aptamers, small molecules, viruses or cationic liposomes (Dass and Choong, 2006; Hajitou et al., 2006; Liu and Deisseroth, 2006; Lu et al., 2006). These specific markers enable the selective delivery of effector molecules to the tumor EC, including radiolabeled compounds, toxins, photosensitizers, genes, immunomodulators or procoagulant substances.

Recently, specific organelles called caveolae have been proposed to fulfill the double role of offering specific antigens (to be targeted) characteristic of the endothelial bed of a given tissue including tumors as well as the structure which favors the drug delivery to the subendothelial space (Fig. 4). Cave-

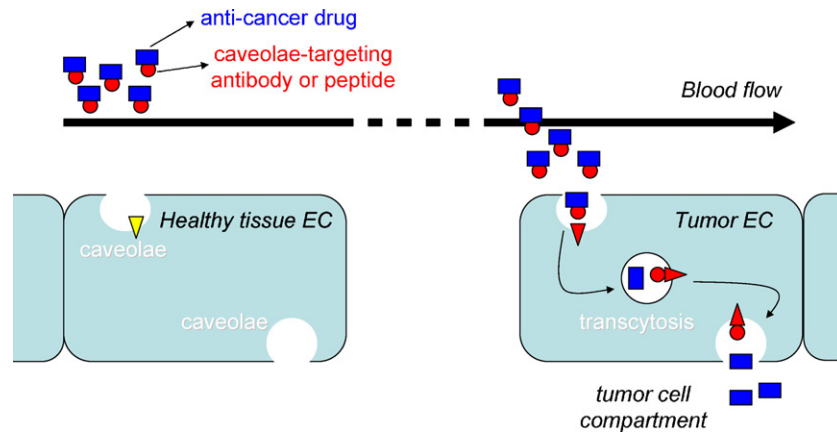


Fig. 4. Double roles of caveolae in the delivery of anti-cancer drugs to the subendothelial space. Caveolae are plasmalemmal invaginations actively participating in transcytosis. Expression of specific antigens in the caveolae of endothelial cells (EC) lining tumor blood vessels (but not of EC from healthy tissues) accounts for the tumor-selective recruitment of therapeutic complexes made of an antibody directed against the caveolar antigen and a therapeutic moiety such as radioisotopes or toxins. The capacity of caveolae to pinch off and to shuttle from the luminal to the abluminal pole of EC then favors the anti-cancer drug delivery to the subendothelial tumor cell compartment.

olae are dynamic, detergent-resistant microdomains that are enriched in cholesterol and glycosphingolipids. They contain signaling molecules, such as select heterotrimeric G proteins, non-receptor tyrosine kinases and endothelial nitric oxide synthase (eNOS), and seem to act as organized transducing centres that concentrate key signaling molecules (Feron and Balligand, 2006). Importantly, they can also mediate transcytosis—the transcellular movement of macromolecular entities. The best physiological example is albumin which is transported through caveolar vesicles from the luminal to the basal poles of the membrane after binding to its receptor gp60 (Carver and Schnitzer, 2003). Recently, Millan and colleagues showed that this route was also used by T lymphocytes to migrate through endothelial cells (Millan et al., 2006). In contrast to the classic clathrin-dependent endocytosis, the caveolar internalization pathway seems to avoid the fusion with lysosomes. Caveolae constitute therefore with their tissue-specific molecules a trafficking pathway that might be worth targeting—not only for site-directed drug and gene delivery but also to overcome the normally restrictive endothelial-cell barrier to reach underlying tumour cells.

The administration of tumour-specific antibodies or peptides which recognize antigens expressed only on the surface of cancer cells is limited for solid tumours partly because of physical barriers formed by endothelial cells (and increased interstitial pressure). Dvorak and colleagues documented for instance that less than 1% of the injected dose of tumour-specific antibodies accumulates in the tumor after intravascular injection in animal models (Dvorak et al., 1991). Recently, Schnitzer and colleagues showed that the ability to target caveolae in the vascular endothelium to mediate tissue-specific delivery and transcytosis of therapeutics was an achievable goal in normal tissue (McIntosh et al., 2002), but also in tumors (Oh et al., 2004). According to these authors, within minutes after entering the bloodstream, caveolae-targeted antibodies enter the caveolae (which then

pinch off), cross the endothelium of the vessels lining the targeted organ and are released to underlying tissue cells.

The tumour vascular caveolae-targeting strategy depends on the existence of unique targets at the endothelial cell surface in tumours. To identify these tissue- and tumour-specific proteins, sub-fractionating methods which allow the isolation of pure luminal endothelial cell plasma membranes (including caveolae) were combined to 2D-gel-based proteomic analyses (Oh et al., 2004) or screening of phage display libraries (Valadon et al., 2006). Oh and colleagues identified differentially expressed proteins already known to be implicated in tumour angiogenesis, including VEGF receptor and Tie2 as well as other proteins such as the Annexins A1 and A8, of which the selective association with the tumour vasculature was not yet known (Oh et al., 2004). Using ^{125}I -labelled monoclonal antibodies directed against Annexin A1, these authors observed in tumour-bearing rats, distinct focus of radioactivity in the lung and little signal elsewhere. One-third of the injected dose was found to accumulate in the tumour after 2 h, leading later to a significant remission among the treated rats. For other proteins identified like endoglin, the tumoral specificity of the expression is not complete but recent work confirmed their caveolar location (Toporsian et al., 2005) and their potential use as carrier to the underlying parenchyma had been previously established (Bredow et al., 2000).

Finally, it should be mentioned that other approaches exploit the capacity of albumin to be transported through endothelial cell to more efficiently address chemotherapy to tumor. Recently, paclitaxel, a hydrophobic chemotherapeutic agent disrupting microtubule disassembly has been combined to albumin covering nanoparticles (Altundag et al., 2006). This galenic form, named nab-paclitaxel, was shown to deliver paclitaxel to the tumor tissue after transcytosis. Clinical studies have confirmed increased antitumor activity and tolerability, and higher intratumoral paclitaxel levels for nab-paclitaxel compared to paclitaxel (Gradishar, 2006). Another

study also describes the rapid, specific and persistent targeting of an antibody directed against a tumor-associated antigen when it is combined with albumin which favors an altered route of clearance and metabolism (Dennis et al., 2007).

7. Concluding remarks

The emergence of anti-angiogenic and vascular-disrupting strategies has critically expanded the therapeutic arsenal for cancer treatment. They have also shed new light on the complexity of the tumor vascular compartment. The tumour vasculature can no longer be considered as a non-functional network of endothelial tubes. Differences are now acknowledged in the degree of maturation of blood vessels between aggressive mouse tumours with high metabolic demand and slow-growing human tumors. Other apparently more subtle differences in tumor hemodynamics also exist within a given tumor between the periphery and the center, or according to the moment where blood flow parameters are evaluated. These differences arise in part from differential pericyte coverage of the tumour blood vessels but also from intrinsic characteristics of the tumour such as interstitial fluid pressure. TAF and TAM dynamically influence the status of both the tumor vasculature and the tumour stroma. The in-depth analysis of these features has begun to reveal new targets to improve qualitatively and quantitatively drug delivery inside the tumour, together with a reduction of the access of toxic chemotherapy to healthy organs.

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