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Delivery of Lipoplexes for Genotherapy of Solid Tumours: Role of Vascular Endothelial Cells

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Abstract

The cells constituting a solid tumour may vary considerably due to biological disparities, but for a solid tumour to pose as a threat to its host, an adequate blood supply has to be established. Although neovascularisation may have dire consequences for the host, it provides a common route by which tumours in general may be reached and eradicated by drugs. The fact that a tumour's vasculature is relatively more permeable than healthy host tissue means that selective delivery of drugs may be achieved. A closer examination of the role played by the cells making up the tumour vascular bed, vascular endothelial cells (VECs), is required to facilitate design of ways for enhancing drug delivery to solid tumours via the vascular route.

VECs have two major roles in the body, barrier and transport, both of which are highly pertinent to drug delivery. This review discusses the factors regulating VEC function, and how these cells may be manipulated in-vivo to improve the selective delivery of lipoplexes, carriers for gene therapy constructs, to solid tumours. It also discusses how genotherapeutic drugs may be targeted against tumour VECs on the premise that by killing these cells, the tumour itself will perish.

Biological and Physiological Factors in the Treatment of Solid Tumours

Current therapies fail to destroy solid tumours for three primary reasons. Firstly, when initially diagnosed, most tumours are well advanced. A 1-g, 1-cm^3 growth (the smallest size clinically detectable) contains approximately a billion cells (Fidler 1991). Eradication of even 99·9% of these cells would leave a million viable cells. For complete cure, all cells need to be destroyed. Secondly, in nearly 50% of patients, surgical excision of the primary tumour is not curative due to metastasis, the process whereby mutated cells spread from the primary site via the bloodstream to establish neoplasms in secondary sites such as the liver or lung (Fidler & Ellis 1994). Metastases are often undetectable due to their small size (<5 mm diameter) and may persist in a dormant state for years following removal of the primary tumour (Meltzer 1990). The third reason and greatest obstacle to success of therapy is the heterogeneous composition of tumours. Individual cells within a tumour vary in terms of genetic (Grover et al 1999), biochemical (Rockwell 1992), immunological (Nanda & Sercarz 1995) and biological characteristics (Stewart 1994). These differences may involve cell-surface receptors, enzymes, karyotypes, cell morphologies, cell cycling times, sensitivity to various therapeutic agents and metastatic potential. Such heterogeneity reduces the ability of both surgery and therapeutic agents to kill all neoplastic cells.

Additionally, particularly in solid tumours of the colon, kidney and adrenal glands, over-expression of the p-glycoprotein gene (multidrug resistance 1, *mdr-1*) causes tumour cells to acquire drug-resistance to therapeutic agents such as cisplatin (Tsuruo & Tomida 1995). Drug resistance is due to the *mdr-1* glycoprotein that excretes cytotoxic drugs from the cell via its ATPase action (Hamada & Tsuruo 1988). Dense packing of tumour cells is believed to be a further major hindrance to movement of anticancer agents from the

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bloodstream into tumour interstitium (Burrows & Thorpe 1993; Thorpe & Burrows 1995). Furthermore, all solid tumours lack an efficient lymphatic drainage system resulting in an elevated interstitial pressure in the tumour (Boucher et al 1995). Increased interstitial pressure, together with the rapid and aberrant nature of tumour cell growth (Rockwell 1992), is believed to be largely responsible for the compression and occlusion of blood vessels characteristic of solid tumours (Murray & Carmichael 1995).

Neovascularisation

At the early avascular stage, supply of nutrients and oxygen to, and removal of wastes from, a solid tumour is performed entirely by diffusion through the surrounding host tissue. This limits the neoplasm to a certain volume, since for further growth to occur, extensive vascularisation is needed (Dewhirst et al 1995). Most tumours in humans remain restricted in size for months to years until a certain subset of cells acquires an angiogenic phenotype (Gastl et al 1997). The initial step in neovascularisation is formation of a primary vascular network, consisting of tubes of nascent endothelial cells (reviewed in Hungerford & Little 1999). Subsequently, primordial vascular smooth muscle cells (VSMCs) are recruited to the endothelium to form a multilayered vessel wall. During maturation and further development of the vessel, the VSMCs serve as biosynthetic, proliferative and contractile components of the vascular wall.

The tumour induces its own blood supply within a relatively short period of time. While turnover rates for vascular endothelial cells (VECs) are normally in the order of months or even years (Denekamp 1984), VECs supplying the tumour degrade the overlying basement membrane and proximal extracellular matrix, migrate directionally, undergo mitosis and organise themselves into functional capillaries within a matter of days (Polverini 1995). Solid tumour VECs divide at a rate 50- to 200-fold greater than normal VECs (Weiss et al 1988; Folkman 1992). In the clinical context, patient survival decreases once a highly dense tumour vasculature bed is established (Hillen et al 1997).

One molecule that stimulates angiogenesis is angiogenin, which facilitates cell invasion and migration by enhancing the ability of endothelial cells to digest extracellular matrix components and degrade basement membrane (Hu et al 1994). Over-expression of another angiogenesis stimulator, vascular endothelial growth factor (VEGF; also called vascular permeability factor, VPF), which is involved in stimulation of VEC division and growth, is common in tumours. VEGF is associated with tumour progression and poor prognosis (Claffey et al 1996; Inoue et al 1997) and its expression is induced by a number of stimuli.

VECs are stimulated by VEGF synthesised by tumour-infiltrating macrophages, mast cells and tumour cells (Sunderkotter et al 1994). In turn, proliferating VECs secrete growth factors that stimulate growth of the tumour (Folkman 1992; Hamada et al 1992). Moreover, the VEGF receptor (VEGFR) is over-expressed in endothelial cells of tumour blood vessels (Plate et al 1993), enabling these cells to respond to the increasing demands of the tumour.

A blood supply is crucial for growth and tumour cells have the ability to recruit new blood vessels in various ways. Firstly, as stated above, they synthesise angiogenic factors that activate VECs to divide and grow towards the tumour (Polverini 1995). They also stimulate production of cytokines which attract various immune cells including macrophages (Polverini & Leibovich 1984) which in turn secrete angiogenic factors (Plate et al 1994). Thirdly, tumour cells block production of, or overcome inhibition due to, anti-angiogenesis factors (Rastinejad et al 1989). Fourthly, they synthesise enzymes that release angiogenic (Briozzo et al 1991) and other (Taipale & Keski-Oja 1997) growth factors sequestered in the extracellular matrix. Finally, tumour cells stimulate adjacent normal tissues to synthesise enzymes such as collagenase (van den Hooff 1991) that can be activated to promote angiogenesis.

Abnormal Features of Tumour Vasculature

Tumour blood vessels are typically 3–10 times more permeable than normal vasculature (Weindel et al 1994; Yuan et al 1995). A hypoxic state in the tumour increases vascular permeability (Olesen 1986) and, as stated above, provides a potent stimulus for neovascularisation (Adair et al 1990). For instance, in glioblastomas, expression of VEGF is greatest at the necrotic and hypoxic core of the tumour (Shweiki et al 1992). However, not all tumour blood vessels are leaky and permeability may vary both spatially and temporally within the same tumour (Jain 1987a, b).

Like other types of endothelium, cells of tumour venules contain numerous vesicles of 50–70 nm diameter in their cytoplasm, in addition to larger diameter vacuoles (Kohn et al 1992). These vesi-

cles, called vesiculo-vacuolar organelles (VVOs), join up with each other to form transendothelial cell channels allowing extravasation of macromolecules from the vessel lumen into tumour interstitium. Interestingly, even though the frequency of VVOs per length of vessel is not significantly different between tumour and normal venules, extravasation is greater in tumour vessels. This suggests a differential regulatory mechanism for VVO function in normal compared with neoplastic endothelium.

The mechanism of increased tumour vessel permeability complements both discontinuous basement membrane in capillaries and gaps induced between VECs of venules by VEGF (Senger et al 1983). Tumour cells induce adjoining endothelial cells to retract, creating spaces through which malignant cells escape the vascular system (Stetler-Stevenson et al 1993). In addition, tumour vasculature is usually composed of abnormal vascular elements including sinusoidal vessels, large capillaries and blood channels with discontinuous endothelium (Baillie et al 1995; Murray & Carmichael 1995). Compared with blood vessels supplying normal tissue, vessels supplying tumour tissue tend to be arranged in irregular arrays and have dilated lumens (Kan et al 1993). Also, arteriovenous shunts and trifurcations not usually observed in normal tissue are found in tumour vasculature (Jain 1990). Tumour microvessels are tortuous (Jain 1994) and do not respond to vasoactive stimuli (Willmott et al 1991). These chaotic features of tumour vasculature lead to turbulent blood flow with frequent stasis and changes in flow direction (Rak et al 1995).

As a tumour grows, its effective capillary density decreases, thereby limiting further growth (Tomisaki et al 1996). Mitosis decreases with increasing distance from the nearest capillary in solid tumours (Folkman 1992). This results in a typical central zone of necrosis surrounded by a layer of viable, proliferating tumour cells with accompanying proliferating VECs (Plate & Mennel 1995). Recent evidence suggests that tumour cell necrosis is partly due to regression of neovasculature within the tumour, perhaps due to a normal host defence mechanism (Holash et al 1999). Based on perfusion rates, four regions within a tumour are recognised (Figure 1; Jain 1996). Due to the relative insufficiency of blood flow to all areas of a tumour, establishment of pockets of hypoxia is common in solid tumours. Hypoxic conditions equate to a highly reducing environment and hence the pH of tumours tends to be lower than that of surrounding normal tissues (Dewhirst et al 1995). This acidity is

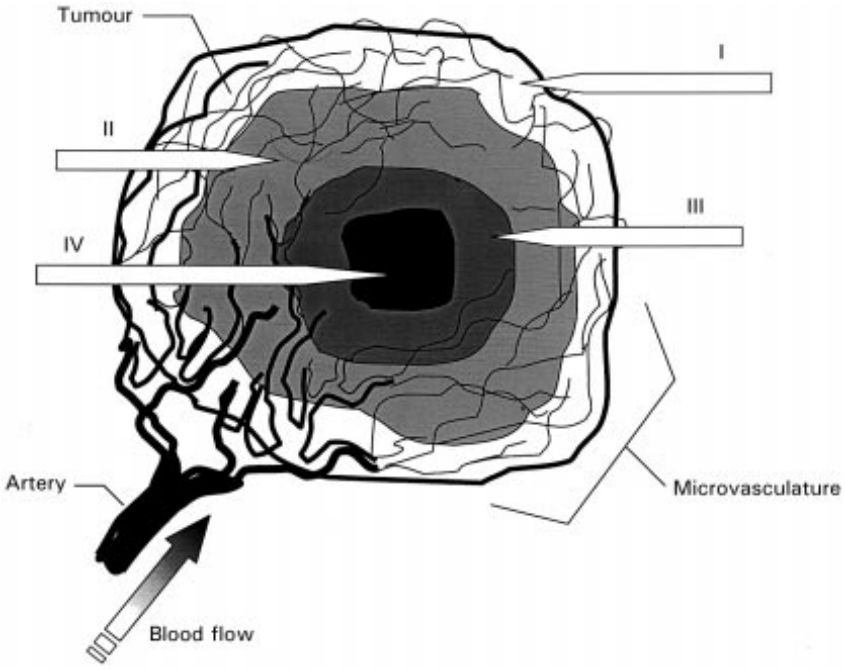

Figure 1. Regions within tumour delineated by vascular supply. I, Advancing front consisting of aggressively-proliferating tumour tissue; II, stabilised region where cell death is equivalent to mitosis; III, semi-necrotic region where the equation is biased in favour of tumour cell death; IV, necrotic zone in which the conditions are not conducive to survival of cells (hypoxic, acidic, scarcity of nutrients).

compounded by inadequate removal of hydrogen ions by the usually restricted tumour blood supply. As stated above, hypoxia may act as an inducer for increased vasculature permeability and neovascularisation.

Endothelial Cells—Brief Biology

Endothelial cells form a single layer of endothelium which lines the entire wall of the cardiovascular system and are one of the most widely distributed cell types in the body, since the vascular system exists in all organs and tissues in the body. The aggregate mass of endothelial cells is about 1000 g, equal to the mass of the liver. Thus, the endothelium is regarded as one of the largest organs in the body (Simionescu 1992).

Endothelial cells differentiate from their precursors, angioblasts, which are induced from the mesoderm, one of the three germ layers (Risau 1995). The cardiovascular system is the first functional organ system that develops in the vertebrate embryo since all the tissues and organs require a nutrition supply via this system. The first step of blood vessel formation is the development and differentiation of VECs. This process is referred to as vasculogenesis, and is regulated by growth factors of the mesoderm (Risau 1997; Yancopoulos et al 1998). A large range of polypeptide growth factors induce endothelial cell proliferation in-vivo and in-vitro (D'Amore 1992; Folkman & D'Amore 1996). The major growth factors include acidic and basic fibroblast growth factors (FGF), VEGF, platelet-derived endothelial cell growth factor (PD-ECGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and transforming growth factor (TGF). Embryonic endothelial cells are able to express growth factor receptors on the plasma membrane, many of which are tyrosine kinases that bind to the growth factors and activate intracellular signal transduction pathways (Mustonen & Alitalo 1995; Korpelainen & Alitalo 1998). As a consequence of cell proliferation, endothelial cells grow into tissues, from which new blood vessels are formed. Many of these growth factors also regulate angiogenesis and vasoregeneration in the fully developed (adult) vertebrate. The former is the formation of new microvessels from existing vessels and the latter is the reparation of damaged endothelial cells (Risau 1997).

Vessel formation is also mediated by cell adhesion molecules including vascular endothelial cadherin (VE-cadherin or cadherin-5; Dejana 1996) and platelet-endothelial cell adhesion molecule-1 (PECAM-1 or CD31; Newman 1997). These molecules are involved in homotype endothelial cell adhesion and the formation of inter-endothelial junctions. These intercellular junctions play an important role in lumen formation, cell polarity generation and vascular permeability regulation (Dejana 1997; Lampugnani & Dejana 1997), and thus, are also capable of determining the efficiency with which drugs extravasate into tumour interstitium.

Morphology and Classification of Endothelial Cells

Histologically, endothelium belongs to the simple squamous epithelium. Endothelial cells are flat with relatively large extended cytoplasm. Most cultured endothelial cells have a cobblestone-like morphology with the nucleus being slightly deviated from the centre of the cell (Jaffe et al 1973; Folkman et al 1979). Endothelial cells are one of the most heterogeneous cell types in the body and based on morphological differences, are divided into three distinct phenotypes: continuous, fenestrated and discontinuous (sinusoidal).

Continuous endothelial cells include the endothelial cells of large blood vessels such as arteries and veins. Most capillaries in connective tissues, muscle and CNS also belong to the continuous endothelial cell type. These endothelial cells form a continuous interface between the lumen and surrounding tissues and rest on a continuous basal lamina. Capillaries such as those in the renal glomerular structures are of the fenestrated endothelial cell type. These endothelial cells contain numerous fenestrae (pores) 70–100 nm in diameter and possess a continuous basal lamina. The discontinuous endothelial cells form the sinusoids of the liver, spleen, bone marrow and some endocrine glands. These endothelial cells are disconnected by gaps and also rest on a non-continuous basal lamina (Clementi & Palade 1969; Palade et al 1979).

Heterogeneity of endothelial cells is also influenced by organ localisation. Endothelial cells from different tissues and organs perform specialised functions (Zetter 1988). For instance, brain capillary endothelial cells are at the interface between the blood and the CNS, the blood–brain barrier (Risau & Wolburg 1990). This unique location requires that the brain endothelial cells develop the tightest intercellular junctions among endothelial cells, have very low pinocytotic activity and a highly selective transcellular transport system. These specialised characteristics of brain endothelial cells allow them to serve as a barrier that

strictly regulates the infiltration of plasma components and circulating cells into the brain.

Unlike brain endothelial cells, some specialised endothelial cells can be highly permeable. The columnar-shaped high endothelium of postcapillary venules of lymph nodes and Payer's patches (lymphoid tissue in the small intestine) are specialised endothelial cells that are mainly involved in the immune response. These endothelial cells have poorly organised tight junctions and are relatively permeable compared with other continuous endothelial cells. The bone-marrow capillary endothelial cells help regulation of proliferation and differentiation of haemopoietic cells of the bone marrow. These capillaries have discontinuities that may facilitate the traffic of haemopoietic and mature blood cells. Thus, depending on the localisation of the tumour, microvasculature permeability to drug molecules may vary. Furthermore, tumour microvessels are generally characterised (Schlageter et al 1999) as continuous fenestrated or discontinuous (with or without fenestrations). The mechanisms by which a tumour alters the behaviour of VECs is a subject of great interest to cancer therapists.

Function of Endothelial Cells

Barrier

In a continuous endothelium, endothelial cells form intercellular tight junctions that seal the lumen of vessels (Simionescu et al 1975; Lampugnani & Dejana 1997). These anatomical structures allow endothelial cells to establish and maintain a barrier that separates the bloodstream from the tissues and organs as well as preventing diffusion of molecules across these cells (Malik et al 1989). The barrier function of endothelial cells is particularly important in neuronal tissue (brain, retina and nerves) that normally requires a highly stable physiological condition. Compared with the non-neuronal tissue, endothelial cells in neuronal tissue, especially in the brain, form well-organised tight junctions, have a remarkable low permeability to molecules, and contain much less endocytotic and transport vesicles (Joo 1996; Staddon & Rubin 1996). Thus, capillary endothelial cells in the neuronal tissue are commonly referred to as the blood–brain barrier, blood–retina barrier or blood–nerve barrier. The barrier function of endothelium is also mediated by other cell types. For example, the astrocytes in the brain enhance the barrier function of endothelial cells (Greenwood 1991). In retina, the retinal pigment epithelium and endothelium together form the blood–retinal barrier that is also maintained by astrocytes (Gardner et al 1997; Rizzolo 1997).

While a compromise in the barrier function may have severe implications for the vitality of healthy organs and tissues, increased permeability in tumour microvasculature enables cancer therapists to deliver drugs selectively to tumours. To further enhance the selectivity of drug delivery, vasoactive agents such as angiotensin II may be used to deliver drug molecules into tumour vessels, since these vessels are immature and lack muscular elements present in normal vasculature (Tvete et al 1981; Heming-
way et al 1990). Angiotensin II has been used to enhance selective delivery of microspheres in liver tumour vasculature in rats (Burton et al 1985), rabbits (Burton et al 1985), sheep (Burton et al 1988) and in patients (Willmott et al 1991; Carter et al 1992). In any case, the physical size of the drug molecule (or its vehicle) and its chemical composition (e.g. hydrophobicity) need to be considered when decisions are made on how to best deliver a drug into a patient.

Transport

The endothelium plays a vital role in the regulation of molecular exchange between bloodstream and tissues to maintain homoeostasis in the body. Transported molecules include ions, nutrients, hormones and a large range of biological factors. Most transport events occur at the level of capillary and venule endothelial cells (Simionescu & Simionescu 1991). For instance, in kidney glomeruli, a large quantity of solute-rich and protein-free liquid passes through the renal glomerular endothelial cells via the fenestrae of the endothelium during filtration of the blood (Marieb 1998).

Transport across the endothelium may occur via the paracellular or transcellular routes. The paracellular route refers to passage of substances between the cells (through intercellular junctions). The transcellular pathway refers to the movement of substances through the cell (through intracellular pathways). The transport of molecules can occur via both pathways, either from the bloodstream to the sub-endothelial tissue or vice-versa. The migration of circulating cells across endothelium is by the paracellular route (Simionescu & Simionescu 1991; Timonen 1997).

As occurs in epithelium, small molecules normally cross endothelium via passive diffusion and specific membrane-transport-protein-mediated active transport. Water and non-polar molecules such as oxygen, carbon dioxide and lipids permeate through the lipid bilayer by simple passive diffu-

sion. For transport of polar molecules such as ions, glucose, amino acids and nucleotides, specific membrane transport proteins are used. These transport proteins are classified as specific membrane transporters (also called carriers) and channel proteins. These transporters are responsible for small molecule uptake by means of facilitated diffusion and active transport mechanisms (ATP dependent). For example, Na^+-K^+-ATPase is a membrane transporter for uptake of K^+ into and at the same time, removal of Na^+ from, cells to maintain a high concentration of intracellular K^+. The movement of ions across the membrane bilayer is against the concentration gradient and requires hydrolysis of ATP to drive the transport. The molecular transfer that is facilitated by transporters and channels is highly selective and more efficient than simple diffusion. In brain capillaries, the plasma membrane transporter proteins of endothelial cells play a vital role in the transport of nutrients, ions and neurotransmitters from the bloodstream into the brain (Takakura et al 1991; Pardridge 1998).

Several pathways are involved in the transendothelial transport of macromolecules (van Hinsbergh 1997). In discontinuous endothelial cells (liver sinusoids and bone marrow), macromolecules as well as circulating cells can cross endothelium via the discontinuities or gaps. In continuous endothelial cells, macromolecules are normally internalised by clathrin-coated or non-clathrin-coated vesicles via a vesicular transcytosis pathway across endothelial cells. The transcytosis pathway that is regulated by membrane receptors is referred to as receptor-mediated transcytosis. Macromolecule transcytosis in capillary and vein endothelial cells are also via fenestrae or VVOs.

Fenestrae are the microdomains of microvascular endothelial cells. Fenestrated endothelial cells are localised in endocrine organs, gastrointestinal tract, kidney glomeruli and in tumours. Fenestrae are circular discontinuities (60 nm diameter) and usually occur in clusters in the most attenuated (the thinnest) cytoplasmic regions (Palade et al 1979). These structures are involved in plasma filtration (in kidney) and a large quantity of solution transport in these tissues and organs. As previously mentioned, VVOs are endothelial cell structures found in tumour microvessels and normal venules (Kohn et al 1992; Dvorak et al 1996). VVOs are grape-like clusters of interconnecting uncoated vesicles and vacuoles which span the entire thickness of an endothelial cell and provide a transendothelial connection between the vascular lumen and the extracellular space for macromolecule extravasation across the endothelium.

Disruption of the intercellular junctions of endothelial cells increases the permeability of the endothelium. This allows macromolecules or even cells (adhered to endothelial cells) to extravasate through these junctions. Thus, VECs may not only function in determining the movement of drug molecules across the vasculature but may also function in transporting drug molecules across the vascular lumen into the tumour interstitium. VECs may transport molecules across vasculature either through a paracellular direction or via a transendothelial pathway. However, the various functions of VECs are closely regulated in keeping with the requirements of the host physiology or the nearby parasitic neoplasm.

Regulatory Factors Involved in Endothelial Transport

Apart from haemodynamic forces (hydrostatic and osmotic pressure), a large variety of biological factors and drugs influence endothelial transport. These factors include the components of blood coagulation, inflammatory cytokines and growth factors (Simionescu 1992). Some drugs that interrupt the cytoskeleton also affect endothelial transport. Most of these factors increase the permeability of the endothelium to molecules either by the paracellular or intracellular routes. Some common mechanisms which result in permeability increases in endothelial cells include the elevation of intracellular calcium levels, decreasing cAMP levels (Moore et al 1998) and activation of protein kinase C activity (Nagpala et al 1996). Factors such as thrombin, tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and VEGF utilise these mechanisms to increase endothelial permeability.

Thrombin is an enzyme responsible for the conversion of fibrinogen to fibrin (Goldsack et al 1998; Mosesson 1998). It has a profound effect on the increase of endothelium permeability to macromolecules by inducing the deposition of fibrin and stimulating the expression of adhesion molecules on endothelial cells (Lo et al 1992; Malik & Lo 1996). Albumin transport can be significantly increased in thrombin-treated endothelial cells both in-vivo and in-vitro (Malik & Fenton 1992; Garcia et al 1995a, b). Since albumin is mainly transported through paracellular pathways in the bovine aortic endothelial cells, the increase in transport is largely due to the disruption of intercellular tight junctions. Thrombin induces an intracellular Ca^{2+} increase in endothelial cells that activates Ca^{2+}/calmodulin. In turn, the Ca^{2+}/calmodulin can activate myosin

light chain kinase that triggers myosin light chain phosphorylation. The phosphorylation of myosin light chain causes the rearrangement of actin filaments and intercellular gap formation in endothelial cells (Shi et al 1998; Verin et al 1998). As a consequence, these intercellular gaps allow macromolecules to pass through thus increasing endothelial permeability.

TNF-α and IL-1 are the principal inflammatory cytokines secreted by various cell types, but mainly by activated macrophages (Mantovani et al 1992; Pober 1998). TNF-α and IL-1 share similar functions in the events of atherogenesis, coagulation, immunoresponse and angiogenesis. TNF-α and IL-1 induce endothelial cells to express adhesion molecules, procoagulation factors and growth factors. TNF-α and IL-1 also increase permeability of VECs by inducing endothelial F-actin (fibrous actin) depolymerisation and intercellular gap formation (Goldblum et al 1993). TNF-α can also cause a decrease of cAMP concentration in endothelial cells (Koga et al 1995) and PECAM-1 phosphorylation (Ferrero et al 1996), events that result in dysfunction of the intercellular junctions.

Growth factors, particularly VEGF (as already mentioned), can increase permeability due to endothelial cells. VEGF is a major vasogenesis factor and is secreted by a large range of cells. VEGF binds to receptors on endothelial cells and activates signal transduction pathways. VEGF induces cadherin (adherens junction protein) phosphorylation that results in disruption of intercellular junctions (Esser et al 1998). VEGF also induces fenestrae formation that enhances the permeability of endothelial cells (Roberts & Palade 1995, 1997). Endothelial cells form fenestrae when co-cultured with VEGF-expressing epithelial cells (Esser et al 1998).

Permeability Studies Using Endothelial Cell Monolayers

The endothelial cell monolayer culture system has been in use since the early 1980s for various studies, most of which have focused on the permeability of endothelial monolayer models. Based on the cell types, the accumulated data has been derived from microvascular and macrovascular endothelial cell monolayers. The former is represented by the brain capillary endothelial cells and the latter by venous or aortic endothelial cells.

Endothelial cells derived from the brain microvasculature have been widely used for blood–brain barrier studies. Bovine brain endothelial cell monolayers grown on collagen-coated nylon meshes were first described by Bowman et al (1983). These cells formed monolayers on filters and developed tight junction complexes that restricted ^{14}C-sucrose movement across the monolayers. The TEER (transendothelial electrical resistance) values registered from brain endothelial cell model systems ranged from 140 to 600 cm^2. When the endothelial cells were co-cultured with astrocytes, the TEER value was elevated by 60%. The permeability to ^{14}C-inulin and ^{14}C-sucrose was reduced when cell monolayers reached their peak TEER (Dehouck et al 1990; Rubin et al 1991). Endothelial cells derived from retinal capillaries have been used for blood-retinal barrier studies, and the average TEER in these cell monolayers has been reported to range from 129 to 140 cm^2 (Gillies & Su 1995; Gillies et al 1995; Gardner et al 1997).

Endothelial cells isolated from peripheral capillaries have lower TEER values than endothelial cells from neuronal tissues (Furie et al 1984). The mean TEER of endothelial cells from bovine adrenal microvessels was 69 cm^2 when the cells were cultured on human amniotic membrane for eight days. These cells were used as a model system for a study of neutrophil migration across endothelial monolayers (Furie et al 1987).

The early monolayer systems of large blood vessel endothelial cells were established by using aortic endothelial cells from bovine and rabbit tissues and have been widely used for paracellular permeability studies (Hennig et al 1984; Navab et al 1986). These cells also formed tight junction complexes when grown on filters. The TEER values in these cells ranged from 14 to 20 cm^2, much lower than the TEER generated by brain capillary endothelial cells (Albelda et al 1988). Monolayers of these large vessel cells are also able to restrict albumin permeability through intercellular junctions (Siflinger-Birnboim et al 1987). Such permeability studies may be extended to include VECs isolated from within tumours, or those around the neoplastic growth, in an effort to better understand the interaction between the tumour and the VECs within reach of its chemical signalling. Such studies may also allow the screening of novel agents that increase vasculature permeability of tumour VECs or restrict permeability of VECs from healthy organs and tissues. These issues are pertinent to both conventional forms of cancer drug therapy and novel ones such as genotherapy.

Table 1. Modes of genotherapy for cancer.

Administration of ribozymes/DNAzymes that degrade oncogene RNA (e.g. K-*ras*)
Administration of antisense strands that specifically block expression of oncogene (e.g. c-*myc*)
Insertion of a wild-type tumour suppressor gene into tumour (e.g. *p53*)
Insertion of a sensitivity (suicide) gene into tumour (e.g. HSV-thymidine kinase gene)
Insertion of a gene encoding a foreign antigen to enhance immunological recognition
Insertion of a cytokine gene (e.g. interleukin-12) into immune cells to enhance anti-tumour activity
Insertion of a gene encoding the multidrug resistance protein (MRP) into stem cells for protection against toxic effects of cytotoxic anti-tumour drugs

Cancer Genotherapy

Genotherapy is steadily gaining recognition alongside the older modes of tumour management such as chemo- and radiotherapy. More than 300 clinical trials for genotherapy have been initiated worldwide, 50% of these for cancer involving more than a thousand patients. The various modes of genotherapy for cancer are shown in Table 1. A look at the clinical results hitherto primarily in patients with very advanced cancers that are refractory to conventional therapies, reveals that genotherapy may effect tumour regression with a concomitant acceptable low toxicity.

However, present tools for gene delivery are inapt, and results from early clinical trials (from 1996) pointed out that a lesser emphasis on clinical trials and a greater emphasis on improving existing technology and an injection of radical and novel ideas of gene delivery are required. There is need for targeting genes to tumours as it would prevent side-effects to normal cells while maximising gene dosage to their cancerous counterparts. Discussed below are the prospects for targeting vectors to tumours via the vascular route.

Using the Vascular Route to the Tumour for Gene Delivery

While there exist various mechanisms for targeting genes into tumours (Table 2), administration of genetic constructs through an artery feeding a tumour facilitates localisation of the anticancer agent. Administration through this route depends on exploitation of vascular differences between tumour and normal tissue. These differences include the increased permeability of tumour blood vessels, their tortuosity and disarray and occlusion of their lumens. In fact, the more rapidly growing and poorly differentiated a tumour, the more chaotic its vasculature.

Table 2. Mechanisms for targeting genetic constructs into tumours.

Delivery injection into the tumour
Administration via artery upstream of tumour
Targeting to tumour vascular endothelial cells
Use of antibodies or ligands
Use of specific promoters

Increased permeability may be attributed to factors secreted by the tumour cells inducing upregulation of VVOs in the tumour compared with normal tissue venules (Kohn et al 1992). VVOs are present in all venules but are concentrated at the tumour-host interface. These transendothelial channels can ferry complexes as large as 50 nm within 10 s of delivery into the vessel. Otherwise, the venules have intact (continuous) endothelium. Even though the tumour-to-normal ratio of VVOs is not different in and around the tumour vicinity, there is a 4-fold higher permeability in the tumour venules and this has been attributed to an upregulation of function of these vesicles in tumour venules. Increased permeability may also be due to an increased frequency of pores along capillaries of tumour beds. These capillaries and newly formed larger vessels supplying the tumour tend to have incomplete (discontinuous) basement membranes.

Targeting to Tumour Vascular Endothelial Cells

As stated above, the primary requirement for tumour growth is an adequate blood supply. For most solid tumours, growth beyond 2 mm is only possible when a new vascular supply is established around and within the tumour. VECs in the tumour proliferate up to 200 times faster than in normal tissue (Folkman 1992). VECs are directly accessible to intravascular administration of genes. Each millimetre of capillary is believed to support at least 100 tumour cells, and it is suggested that the death of one VEC should eradicate 100 tumour cells. VECs are genetically stable, thus less heterogeneous than their tumour cell counterparts. All these factors are responsible for an interesting shift in studies aimed at severing the blood supply to the tumour rather than attacking the growth directly. Thus, instead of targeting the foreign therapeutic gene to tumour cells, delivering it specifically into

VECs surrounding tumours should theoretically have a greater antitumour effect.

VEGF is synthesised by the tumour under conditions of hypoxia. The factor acts, via VEGF-receptors (VEGFRs), on VECs lining the tumour and results in neovascularisation in which the vessels supplying normal tissue are re-orientated to supply the tumour instead. Once the vessel bed has been established, the tumour grows exponentially. In one study where anti-VEGF antibodies were delivered into nude mice tumour vasculature, the vessels were inhibited and growth of human tumour xenografts was inhibited completely (Burrows & Thorpe 1993). Another study in rats demonstrated similar reductions in tumour growth (Ohizumi et al 1997). VECs supplying the tumour could be targeted with a therapeutic gene by putting a VEGFR promoter in front of the gene and delivering it thus. This should enable expression in only VECs that are rapidly dividing, namely those leading into the tumour. Another way of restricting the size of a tumour is by use of genetically-modified macrophages (that normally infiltrate tumours) with anti-angiogenic factors. The use of antisense strands, ribozymes or DNAzymes to silence VEGF or VEGFR genes is also a possibility.

Major Hindrances to Genotherapy of Tumours

In general, a major concern in targeting therapeutic agents to tumours is the inherent heterogeneity of tumours together with the fact that the vascular supply to a tumour is not consistent throughout. There are sites that are well-vascularised, while other regions tend to be poorly supplied with a vascular bed.

Another form of hindrance is the biological and physiological barriers to intravascular delivery. Firstly, an anticancer agent that is administered via the blood supply encounters the vastness of the vascular space and the possibility of non-specific interactions with the numerous components in blood. While gross systemic distribution may be counteracted by delivery through an artery upstream of a tumour, additional mechanisms of tumour cell-specific targeting are needed to enhance the targeting effect. Secondly, the agent must extravasate into the appropriate region. Here, exploitation of the tumour vascular bed abnormalities (e.g. greater permeability) may aid specific delivery to the tumour site.

Perhaps the greatest obstruction is transport through interstitial space. Due to reduced lymphatic fluid drainage in most tumours, fluid pressure tends to build up, and thus further extravasation of fluid and carried agents is hindered. Lastly, genetic constructs must enter tumour cells to produce the therapeutic effect. Furthermore, with therapeutic genes, the construct has to gain entry into the nucleus for the transcriptional process to set in motion expression of the therapeutic protein. Failure to satisfy any of these steps equates into a non-therapeutic result.

There are properties that the gene vector has to have as it travels towards the tumour via the bloodstream (if delivered such). Even with delivery directly into the tumour, these features are desirable. Firstly, the agent must be resistant against metabolism and degradation. Secondly, it should avoid non-specific binding to proteins and other biomolecules (e.g. binding of proteins to the surface of liposomes affects their transfection rate). Finally, the vector must not elicit an immunological response. This is quite relevant when using viral vectors, as some studies reveal that administration of adenoviral vectors leads to inflammation. Lipoplexes (discussed below) are a safer alternative to viral vectors for delivery of genetic constructs to solid tumours. While the aforementioned features of tumours may seem discouraging to cancer therapists, the fact that a tumour's survival critically depends on its blood supply provides a common mechanism for the destruction of solid tumours.

Lipoplexes and Tumour Genotherapy

Lipoplexes are formed by the interaction of anionic nucleic acids binding to the surface of cationic liposomes, eventually forming multilamellar lipid-nucleic acid complexes. In the case of DNA, the nucleic acid molecules persist glued to lipidic molecules with a lipid bilayer surrounding the compacted nucleolipidic particles in one of several different moieties: a cylindrical form, where the DNA is coated by a curved lipid bilayer (Radler et al 1997); a flat lamellar form, where DNA is sandwiched between lipid layers (Dan 1998); and a form where DNA is condensed as parallel helices between lipid bilayers (Battersby et al 1998). These discrepant observations may be attributed to the lipidic formulation of the vesicles, the manner in which the complexes were formed, the size of the nucleic acid, the lipid:nucleic acid ratio and the technique used to treat and visualise these complexes. Most observations of lipoplexes are made using electron microscopy but erythrosine dye, which has affinity for the cationic lipids, may be employed to enhance visibility of complexes with conventional light microscopy (Dass et al 1996; Dass 2000). In addition to electrostatic attraction, hydrophobic interactions are believed to aid com-

Table 3. Intravascular administration of lipoplexes.

Route	Species	References
Intra-arterial injection	Human	Nabel et al 1994
	Rat	Dass et al 1997a, 2000; Dass 1998; Rainov et al 1999; Schmid et al 1998
	Rabbit	Leclerc et al 1992; Nabel et al 1992; Losordo et al 1994; Takeshita et al 1994
	Pig	Nabel et al 1990, 1992, 1993a, b, c
	Dog	Lim et al 1991; Chapman et al 1992
Intravenous injection	Mouse	Stewart et al 1992; Zhu et al 1993; Lesoon-Wood et al 1995; Liu et al 1995; Parker et al 1995; Thierry et al 1995; Clarke et al 1996; Stephan et al 1999; Xu et al 1997; Bei et al 1998; Song & Liu 1998; Song et al 1998; Barron et al 1999a, b; Li et al 1999; Liu et al 1999; Schughart et al 1999; Tan et al 1999; Xu et al 1999; Dass, unpublished results
	Rat	Leibiger et al 1991; Tsan et al 1995; Jeschke et al 1999
	Rabbit	Nabel et al 1992; Conary et al 1994; Canonico et al 1994
	Monkey	Parker et al 1995
	Pig	Nabel et al 1992

plex formation between lipids and nucleic acids (Wong et al 1996).

A common molecule used in cationic liposome synthesis is the neutral lipid dioleoylphosphatidyl ethanolamine (DOPE). The role of DOPE is to facilitate membrane fusion or aid in the destabilisation of the plasmalemma or endosome (Felgner et al 1994). Helper lipids such as DOPE are also required to stabilise the cationic liposome suspension as cationic lipids repel each other (Zuidam & Barenholz 1998). Liposomes formulated without neutral lipid(s) have inferior rates of transfection (Lasic & Pearlman 1996), while varying rates of transfection may result from varying ratios of cationic:neutral lipid used to formulate the liposomes (Farhood et al 1995). Cationic-lipid-coated encapsulated albumin increases extravasation across the blood–brain barrier (Fenart et al 1999). This may be quite important for gene delivery to the brain and its parasitic tumours. Success of cationic-liposome-mediated DNA transfer is, however, dependent on numerous factors (Dass & Burton 1999). This plethora of factors may explain the inherent variability of lipofection (lipoplex-mediated transfection), especially in-vivo (Dass et al 2000). Further enhancement in tumour gene delivery may be attained by using other vesicles such as fusogenic or pH-sensitive liposomes (reviewed in Dass et al 1997c). Nevertheless, the fact that lipoplexes may be administered in-vivo via the vascular system (Table 3) highlights the usefulness of these gene transfer vehicles.

Biological and Physiological Considerations for Lipoplex Gene Transfer into Tumours

In many tumours, the vascular bed is well-developed and, in some cases, better than normal tissues (Jain 1996). For such tumours, liposomal delivery of genetic material holds great promise (Lasic 1996), since large liposomes are retained in the first capillary bed they encounter. For instance, tail vein injections result in retention of the majority of liposomal material in the lung vasculature. Small unilamellar vesicles (SUVs; diameter $\leq$200 nm) escape the capillary bed and are predominantly taken up by the organs of the reticulo-endothelial system (RES), lungs, liver and spleen. Upstream intra-arterial delivery (Figure 2) of cationic large unilamellar vesicles (LUVs; diameter $> 1\,\mu$m) rather than intermediate lamellar vesicles (IUVs) may be more feasible. Thus, targeting may be achieved by delivering therapeutic genetic constructs as close as possible to the site via a catheter (Shi et al 1994; Dass et al 1997a, 2000; Dass 1998). This would ensure delivery of a maximum dose of therapeutic DNA since enzymatic degradation, interaction of the nucleic acid with the biological surroundings such as the vessel wall, and dilution in the blood would be minimised (reviewed in Dass & Jessup 2000).

As stated above, tumour vasculature is more permeable than normal tissue vasculature. Also, neovascularisation of tumours usually leads to newly formed vessels that are leaky due to fragile basement membranes (Liotta et al 1976). Tumour cells in culture and in-vivo secrete VEGF which, apart from increasing the permeability of tumour vessels, aids in the accumulation of excess fluid commonly associated with tumours (Brock et al 1991). It also aids the process of metastasis (Meister et al 1999). Apart from VEGF, other factors inducing vascular permeability in tumours include nitric oxide (Jadeski & Lala 1999), bradykinin (Martin-Chouly et al 1999) and interleukin-2 (Ettinghausen et al 1988). Increased permeability would facilitate movement of lipoplexes from vessels into tumour interstitium.

Interestingly, Thurston et al (1998) showed that angiogenic VECs in murine tumours internalised

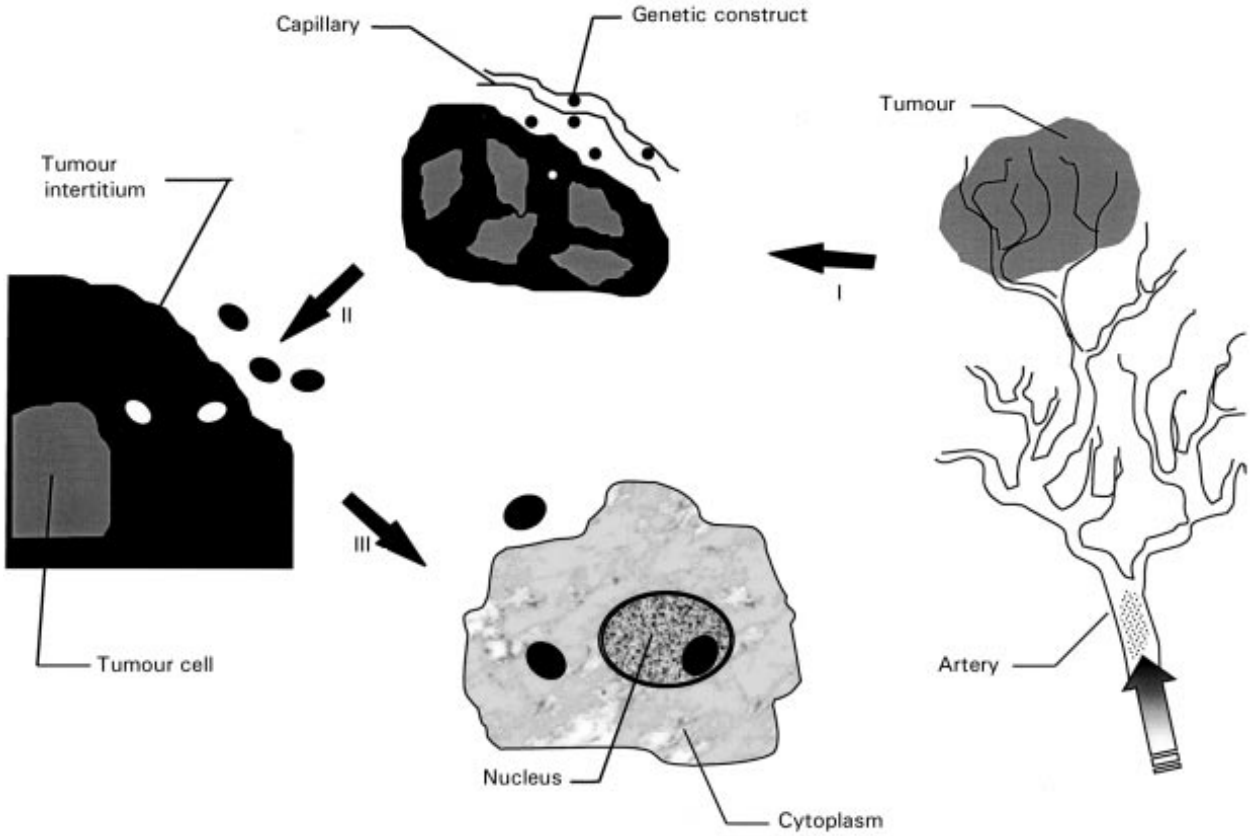

Figure 2. Intravascular delivery of genetic construct to tumour – steps involved for therapeutic effect. I, Extravasation of construct (free or associated with carrier) from tumour vascular bed; II, entry and movement through tumour interstitium; III, entry into target cell (escape into cytoplasm or penetration into nucleus).

lipoplexes at a greater rate than anionic, neutral or sterically stabilised liposomes. Such selectivity in uptake should render it possible to target tumour VECs with anti-angiogenic genes (or antisense strands). If lipoplexes are targeted to the tumour vascular bed using such devices as microspheres (DeCruz et al 1996; Dass et al 1997b) or microplexes (Dass et al 1999, 2000), then possible side-effects of therapeutic genes (such as *p53*) or antisense strands (such as those against *bcl*-2) should be reduced. Highly selective delivery is important particularly when dealing with the vascular system, since generation of new tissue, such as that involved in the menstrual cycle, is dependent on vessel regeneration (reviewed in Ferrara 1999). Once a tumour's blood supply is curbed or completely inhibited, the tumour itself should be eradicated.

It has been noted that uptake of antisense strands is much faster in leukaemic human cell lines that in normal cells from the same patient (Calabretta et al 1991; Zhao et al 1996). Normal brain cells of rats do not permit entry of plasmids as much as brain tumour (glioma) cells (Nishi et al 1996). A transplanted tumour line in the kidneys of rats shows greater expression of a foreign gene than the normal kidney parenchyma (Dass et al 1997b, 2000; Dass 1998), possibly because of the greater division rate of mutated cells. Alternatively, it may be explained by a more demanding blood supply to the tumour. Regardless of the mechanism, these findings have great implications for vascular-based gene delivery to solid tumours.

One limiting factor is that cancerous cells often occupy less than 50% of a tumour volume (Jain 1996). One to ten percent of the volume is made up by the vasculature, the rest consists predominantly of a collagen-rich matrix, the interstitium. To reach a tumour cell, an active agent must traverse the endothelial barrier and through the often-thicker interstitial matrix (O'Connor & Bale 1984). Moreover, in tumours, interstitial pressure is higher than intravascular pressure (Boucher et al 1996). Hence, movement of large molecules such as DNA through vessels occurs mainly by diffusion (Jain 1996). However, in regions of the tumour where

interstitial pressure is low, movement of large molecules occurs via convective transport caused by solvent drag.

It must be borne in mind that tumours seen clinically contain well-supplied, rapidly growing regions interspersed with poorly perfused, often necrotic areas (reviewed in Murray & Carmichael 1995; Dass et al 1998). In solid tumour tissue, blood vessels become tortuous, with variable intercapillary distances and compression and occlusion of lumens. Insufficient perfusion results eventually in necrosis in certain areas and also hypoxic areas containing otherwise viable tumour cells. Hypoxia has also been noted to induce apoptosis in tumour cells (Gee et al 1999). This heterogeneity poses a problem to any sort of drug delivery whether it be from a distance or direct injection into the tumour. Ideally, the drug has to reach the periphery of the tumour, a region that is characterised by vigorous cellular turnover (Figure 1).

There are various limiting factors in the delivery of genotherapeutic agents to solid tumours. While the efficacy of the vector is dependent on matters such as the expression level in cells or resistance against endonucleases, it is largely reliant on the route of delivery to the tumour. At present, the vascular route promises to be the best mode of administration since it provides the therapeutic construct access to the rapidly growing regions of the tumour. While the use of three-dimensional tumour models (Santini et al 1999) may be useful for basic studies, it may fail in truly portraying the natural architecture of a tumour, including the microvascular bed. The issues pertinent to gene therapy are also relevant to other forms of drug therapy such as chemo- and radiotherapy. The importance of the vascular system in targeting genetic constructs to tumours is generally relevant to all these modes of cancer management.

Conclusions

Vascular endothelial cells (VECs) constituting the microvasculature of a solid tumour are a notable subset of cells. As a tumour is heavily reliant on its blood supply and thus on the VECs making the vessels supplying it, a fatal assault on these VECs would result in the eventual death of the dependent tumour. Apart from a direct assault on the VECs, the vascular supply represents the most reliable route for delivery of drugs to the aggressively growing regions of a solid tumour. The relatively permeable vasculature of the tumour has been exploited for selective delivery of chemo-, radio- and genotherapeutic agents to a tumour. In addition, the tortuous and disarrayed nature of a tumour's microvasculature allows selective lodgement of chemo-, radio- and genotherapeutic carriers such as microspheres. These factors highlight the increasing trend for treatment of tumours via the vascular pathway, a route which will be better utilised with increased understanding of the biological and molecular mechanisms regulating the cells making up the tumour microvasculature—vascular endothelial cells.

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