Drug Resistance and the Solid Tumor Microenvironment

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Resistance of human tumors to anticancer drugs is most often ascribed to gene mutations, gene amplification, or epigenetic changes that influence the uptake, metabolism, or export of drugs from single cells. Another important yet little-appreciated cause of anticancer drug resistance is the limited ability of drugs to penetrate tumor tissue and to reach all of the tumor cells in a potentially lethal concentration. To reach all viable cells in the tumor, anticancer drugs must be delivered efficiently through the tumor vasculature, cross the vessel wall, and traverse the tumor tissue. In addition, heterogeneity within the tumor microenvironment leads to marked gradients in the rate of cell proliferation and to regions of hypoxia and acidity, all of which can influence the sensitivity of the tumor cells to drug treatment. In this review, we describe how the tumor microenvironment may be involved in the resistance of solid tumors to chemotherapy and discuss potential strategies to improve the effectiveness of drug treatment by modifying factors relating to the tumor microenvironment.

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The limitations of clinical chemotherapy have been ascribed primarily to mechanisms that mediate drug resistance at the cellular level. Functional gene mutations or other changes that affect the expression of genes encoding proteins that influence the uptake, metabolism, and export of drugs from a single cell are important determinants of drug resistance, as are epigenetic changes that can lead to transient drug resistance. However, substantial evidence suggests that mechanisms that involve the tumor microenvironment also mediate resistance of solid tumors to chemotherapy. For an anticancer drug to kill a high proportion of cancer cells in a solid tumor, it must be distributed throughout the tumor vasculature, cross vessel walls, and traverse the tumor tissue. However, the distribution of many drugs within tumors is heterogeneous, such that only a proportion of the target tumor cells is exposed to a potentially lethal concentration of the cytotoxic agent. The tumor microenvironment is characterized not only by marked gradients in drug concentration but also by gradients in the rate of cell proliferation and by regions of hypoxia and acidity (Fig. 1) (1), all of which can influence tumor cell sensitivity to drug treatment. Also, cells that are sensitive to drugs in tissue culture may be resistant when grown as a tumor in contact (2,3).

In this review, we advance the hypothesis that the tumor microenvironment may contribute substantially to resistance to drug therapy and discuss potential strategies that might modify drug resistance and thereby improve the effectiveness of treatment.

The Tumor Microenvironment

Solid tumors are organ-like structures that are heterogeneous and structurally complex. They comprise cancer cells and stromal cells (i.e., fibroblasts and inflammatory cells) that are embedded in an extracellular matrix and nourished by a vascular network; each of these components may vary from one location to another in the same tumor.

The Extracellular Matrix and Cellular Interactions

Compared with normal tissues, the tumor stroma is associated with an altered extracellular matrix and an increased number of fibroblasts that synthesize growth factors, chemokines, and adhesion molecules (4). The extracellular matrix can vary greatly among tumors, both in amount and in composition (5). The tumor stroma can influence malignant transformation (6,7), plays an important role in the ability of tumors to invade and metastasize (7,8), and affects the sensitivity of tumor cells to drug treatment.

The composition and structure of stromal components in tumors also contribute to an increase in interstitial fluid pressure (see below), which hinders the penetration of macromolecules through tissue (9,10). Also, the three-dimensional structure of tissue itself can influence the sensitivity of constituent cells to both radiation and chemotherapy (11,12). For example, cells grown in contact with each other, either as multicellular tumor spheroids in culture or as tumors in animals, are more resistant to alkylating agents and cisplatin than the same cells after disaggregation (2,3). The mechanisms underlying this observation are unclear, but it implies that drug screening based on assays of dispersed cells in tissue culture is limited in predicting the responsiveness of solid tumors.

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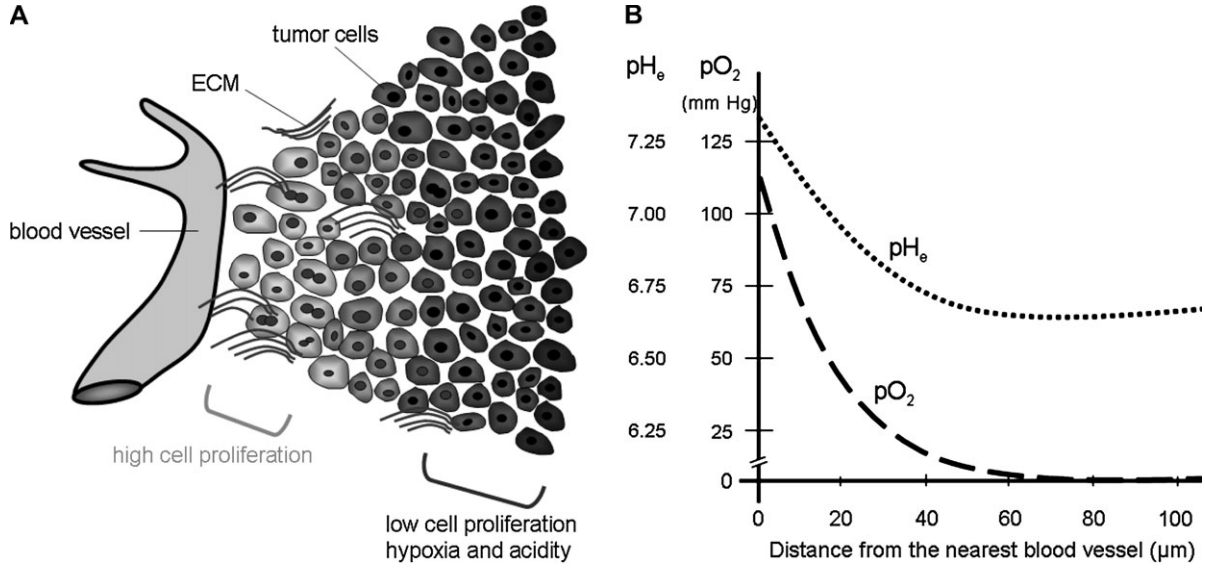

Fig. 1. The tumor microenvironment in relation to blood vessels. **A**) Diagrammatic representation of tumor cells and the extracellular matrix (ECM) surrounding a capillary. **B**) Schematic representation of the gradient of oxygen concentration (pO_2: **dashed line**) and of pH (**dotted line**) in relation to the nearest tumor blood vessel. The relationship of pO_2 and pH with distance from the nearest blood vessel is similar to that reported by Vaupel (1).

Tumor Vasculature and Blood Flow

Tumor responsiveness to chemotherapy is influenced both directly and indirectly by the vasculature, which is abnormal in solid tumors. The vasculature influences the sensitivity of the tumor to drugs because anticancer drugs gain access to tumors via the blood (13) and because the limited supply of nutrients in tumors leads to metabolic changes (including hypoxia) and to gradients of cell proliferation that influence drug sensitivity.

Blood vessels in tumors are often dilated and convoluted and, compared with normal tissues, have branching patterns that feature excessive loops and arteriolar–venous shunts (Fig. 2) (14). The vessels in some tumors are not organized into arterioles, capillaries, and venules but instead share features of all of these structures. The walls of tumor vessels may have fenestrations, discontinuous or absent basement membranes, and fewer pericytes than walls of normal vessels and may lack perivascular smooth muscle (15,16). In addition, cancer cells may be integrated into the vessel wall (17). These abnormalities tend to make tumor vessels leaky, although their permeability varies both within and among tumors (17–19).

Blood flow in many tumors is disorganized and variable (16). In a vascular network, flow rate is directly proportional to the difference in pressure between the arteries and the veins and inversely proportional to the viscous and geometric resistance. In tumors, the difference in pressure between arterioles and venules is reduced and viscous and geometric resistance is increased (20,21). These abnormalities, as well as the compression of blood vessels by cancer cells (22), increase resistance to blood flow and impair blood supply to the tumor. In addition, vascular morphology and rates of blood flow may vary with location and with time, even in the same tumor (1,23). As a result, there is reduction in delivery of nutrient metabolites and in the clearance of breakdown products

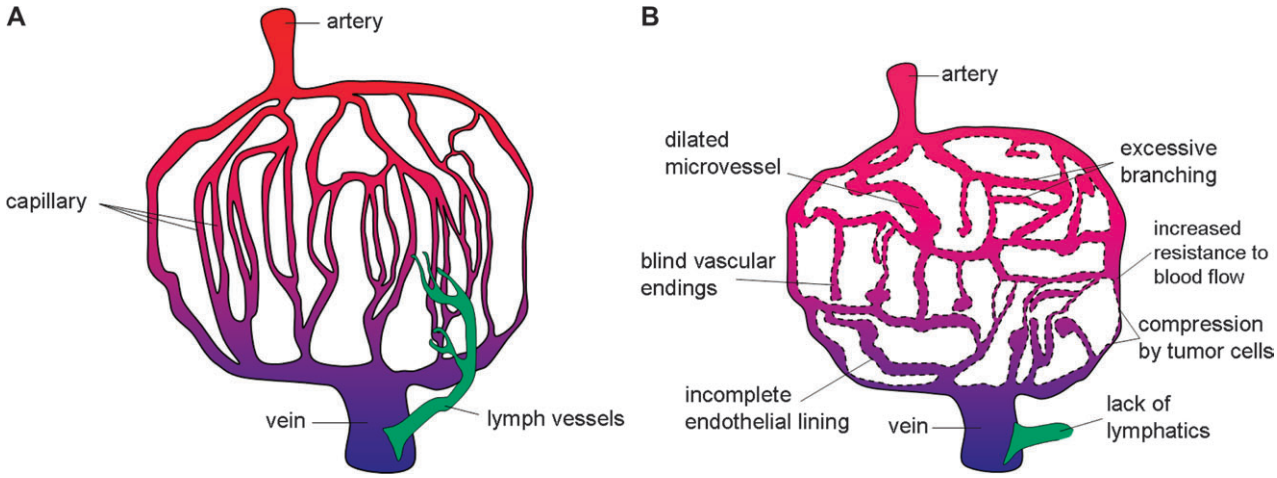

Fig. 2. Diagrammatic representation of the vascular system. **A**) Normal tissue. **B**) Solid tumor. **Red** represents well-oxygenated arterial blood, **blue** represents poorly oxygenated venous blood, and **green** represents lymphatic vessels.

of metabolism, leading to hypoxic and acidic regions in tumors (Fig. 1) (1,24–26). Delivery of anticancer drugs is similarly compromised (27).

In normal tissues, fluid is removed through a network of lymphatic vessels as well as through the veins. Solid tumors may lack or have fewer functional lymph vessels than normal tissues (28), which contributes to the increased interstitial fluid pressure within them (29–31). Increased interstitial fluid pressure inhibits the distribution of larger molecules by convection (31–34) and compresses blood vessels such that blood is diverted away from the center of the tumor toward the periphery.

Tumor Hypoxia and Acidity

Most solid tumors contain regions of hypoxia. Such regions are typically identified with the use of oxygen-sensitive electrodes that are inserted into the tumor (35–37) or by monitoring the cellular uptake of agents such as pimonidazole or EF5 that are reduced under hypoxic conditions (38,39) or the expression of endogenous markers of hypoxia (40). The limited vasculature of tumors results in chronic or diffusion-limited hypoxia because tumor cells are typically farther from the nearest capillary than cells in normal tissues (e.g., more than ~100 μm), so that the oxygen concentration may fall to zero (Fig. 1, B). The distance from blood vessels at which hypoxia occurs varies because of the variable delivery of oxygen within tumor blood vessels and the variable consumption of oxygen by cancer cells. Cells in hypoxic regions may be viable, but they are often adjacent to regions of necrosis. Cells that are produced in regions proximal to blood vessels can migrate into hypoxic areas and become necrotic, presumably because of nutrient deprivation (41,42). If cells close to blood vessels are killed by treatment, the nutrient supply to previously hypoxic cells may improve, allowing those cells to survive and regenerate the tumor. Transient hypoxia is also common in tumors and results from the temporary shutdown of blood vessels (43,44).

Hypoxic regions of tumors are likely to have a decreased supply of nutrients such as glucose and essential amino acids. This is because tumor cells often use glycolysis—the conversion of glucose into lactate to produce ATP—to obtain the energy they need to survive and proliferate rather than oxidative metabolism, a more efficient pathway that leads to production of CO_2 and carbonic acid (45,46). Decreased clearance of these acidic products of metabolism leads to low interstitial pH, another characteristic of solid tumors (24,25,45).

The Tumor Microenvironment and Drug Activity

Tumor Cell Proliferation and the Microenvironment

Nutrient deprivation induces cell cycle arrest, and the rate of proliferation of tumor cells therefore decreases with increasing distance from tumor blood vessels (Fig. 1, A) (41,47,48). Most chemotherapeutic drugs, including, possibly, biologic agents that target cell proliferation, are more effective against proliferating than quiescent cells (49). Consequently, slowly proliferating cells at increasing distances from tumor blood vessels are likely to be resistant to therapy.

Interactions Between Tumor Cells and Their Microenvironment

Interactions among cancer cells and between cancer cells and various cytokines, hormones, growth factors, and the extracellular matrix can affect the sensitivity of the cells to apoptosis and their response to chemotherapy. This phenomenon, known as cell adhesion–mediated drug resistance, has been observed in a variety of cancer types (50,51). For example, insulin-like growth factor I was observed to protect a mouse colon cancer cell line against several cytotoxic agents (52); integrins (receptors that mediate attachment and spreading of extracellular matrix proteins) have been reported to inhibit the apoptotic response of small-cell lung cancer to chemotherapy-induced DNA damage (53); and interactions between cancer cells and the basement membrane have been shown to confer resistance to apoptosis (54). Although the phenotype of cell adhesion–mediated drug resistance is complex and highly variable from one tumor to another, agents that modify cell adhesion might enhance the effects of chemotherapy (55).

Tumor Hypoxia

The presence of hypoxia in tumors is known to lead to the activation of genes that are associated with angiogenesis and cell survival, and this effect is mediated by the transcription factor hypoxia-inducible factor 1 (56,57). Expression of these genes may result in the expansion of populations of cells with altered biochemical pathways that may have a drug-resistant phenotype. For example, hypoxia selects for cells that have lost sensitivity to p53-mediated apoptosis and for cells that are deficient in DNA mismatch repair (which may, in turn, be resistant to platinum-based chemotherapeutic agents) (58–60). Transient hypoxia has been reported to cause amplification and increased expression of the genes encoding P-glycoprotein and dihydrofolate reductase, which induce drug resistance to substrates of P-glycoprotein and to folate antagonists, respectively (61–63). Transient hypoxia that is associated with glucose deprivation can also disrupt protein folding in the endoplasmic reticulum (64); this effect may confer resistance to topoisomerase II–targeted drugs (65–67) and enhance P-glycoprotein expression and multidrug resistance (68).

In the presence of oxygen, many anticancer drugs generate free radicals that damage DNA. These drugs accept electrons from biologic sources and then transfer the electrons to oxygen (69). For example, doxorubicin undergoes chemical reduction to a semiquinone radical, which in turn reduces oxygen to a superoxide that may contribute to cytotoxicity (70). Thus, at low oxygen concentrations the cytotoxicity of drugs whose activity is mediated by free radicals is decreased (71). By contrast, mitomycin C and several experimental drugs require reduction under hypoxic conditions for their activation (72,73). Agents under development that are activated under hypoxic conditions have the potential to reduce drug resistance that is related to the microenvironment (see below).

Tumor Acidity

The pH in the tumor microenvironment can influence the cytotoxicity of anticancer drugs. Molecules diffuse passively across the cell membrane most efficiently in the uncharged form. Because the extracellular pH in tumors is low and the intracellular pH of tumor cells is neutral to alkaline, weakly basic drugs that have an acid

dissociation constant of 7.5–9.5, such as doxorubicin, mitoxantrone, vincristine, and vinblastine, are protonated and display decreased cellular uptake (24,74). Alkalinization of the extracellular environment enhances the uptake and cytotoxicity of some of these drugs (e.g., doxorubicin and mitoxantrone) (75,76). By contrast, weakly acidic drugs such as chlorambucil or cyclophosphamide concentrate some in the relatively neutral intracellular space (74,77). The acidic microenvironment may also inhibit active transport of some drugs, including methotrexate (78).

Drug Distribution in Solid Tumors

Determinants of Drug Distribution

Drugs must leave tumor blood vessels efficiently and then penetrate tumor tissues to reach all of the cancer cells (79,80); both processes depend on convection and/or diffusion. Convection depends on gradients of pressure (both hydrostatic and osmotic) between the vascular space and the interstitial space; vessel permeability and the surface area for exchange; and the volume and structure of the extracellular matrix. Drug diffusion is determined by concentration gradients. Another determinant of drug distribution within tissues is the half-life of the drug in the circulation; a drug that has a long are half-life will establish a more uniform distribution in tissues even if its extravasation and penetration of tissues are relatively slow, whereas a drug that has a short half-life will have a nonuniform distribution.

Drug distribution in tumors is influenced by gradients in pressure within them. In tumors, the oncotic pressure gradient is almost zero and the interstitial fluid pressure is often elevated and approximately the same as the microvascular pressure (30,32). These conditions lead to decreases in the extravasation of macromolecules, particularly in central regions of tumors, where the interstitial fluid pressure may be similar to the microvascular pressure (81–83). Vessels in some regions of tumors may have fenestrations that increase extravasation of drugs (17); an increase in extravasation of a drug can increase its effectiveness if the drug exits from tumor capillaries (84) but can decrease its effectiveness if the drug is lost from large vessels at the tumor periphery.

After a drug leaves the vascular compartment, it must penetrate a human tumor for distances up to 200 μm to reach all viable cells in the tumor. High interstitial fluid pressure has been associated with poor drug penetration (31) and, in one study of patients with lymphoma or melanoma (85), response to chemotherapy. In that study (85), the authors showed that only patients who had low interstitial fluid pressure (either initially or during treatment) responded to treatment. However, another study (86) found no association between reduction of interstitial fluid pressure and tumor response. Thus, it is not known if lowering the interstitial fluid pressure would, in general, result in a better response to chemotherapy.

The composition and organization of the extracellular matrix, cell–cell interactions, and the tumor cell architecture also affect drug penetration (87). For example, tumors that have a well-organized and richly interconnected collagen network display lower penetration by high–molecular-weight agents than those with a poorly organized collagen network (10). Tumors with high packing density of the constituent cells and a reduced interstitial space and volume of the extracellular matrix have lower drug penetration than tumors with a low packing density (88–90).

Because many tumors have an elevated interstitial fluid pressure and lack a functional lymphatic system, their penetration by most drugs probably relies more on diffusion than convection (32,91). Diffusion of a drug is determined by its concentration gradient in the tumor tissue; by various properties of the drug, including its molecular size and shape and its solubility in water and lipids; by the composition and structure of the extracellular matrix; and by drug consumption, which includes both metabolism and binding of the drug to tissue components (79,80). Sequestration of drugs in tumor cells and/or their binding to components of the extracellular matrix or at the target site inhibit drug penetration to deeper regions of the tumor (92). Impaired drug penetration due to binding in tissue might apply to antibodies that bind to targeted antigens (e.g., trastuzumab binding to HER2 on cancer cells) (93,94), to basic drugs (e.g., doxorubicin and mitoxantrone) that are sequestered in acidic organelles such as perinuclear endosomes (95,96), and to drugs that bind avidly to DNA (97,98). Sequestration in acidic organelles and avid binding to DNA have been implicated in the poor tissue penetration of doxorubicin, epirubicin, and mitoxantrone (96,98).

Quantifying Drug Distribution

In vitro and in vivo approaches have been used to examine how anticancer drugs penetrate and distribute within tumors. Solid tumor models in tissue culture that have been useful in studying drug distribution include multicellular tumor spheroids (Fig. 3, A) and multilayered cell cultures (Fig. 3, B; Table 1) (79). The penetration of anticancer drugs into spheroids, which has been studied by fluorescence microscopy and by autoradiography (for radiolabeled drugs) applied to histologic sections, was found to be poor for doxorubicin (11,89,99,100), methotrexate (101), vincristine (102), vinblastine (103), and some other drugs (11) but better for 5-fluorouracil (11,103). A better model is provided by multilayered cell cultures, which have a linear geometry that facilitates the quantification of drug transport through tumor tissue (Fig. 3, C and D). Studies with multilayered cell cultures have confirmed very slow tissue penetration of drugs that bind avidly to DNA, such as doxorubicin and mitoxantrone (90,98,104), and relatively slow penetration of several other drugs with different modes of action (105–108).

Several methods have been used to study drug distribution in tumors grown in experimental animals. Window chambers have been used to study the distribution of naturally fluorescent or colored drugs in tumors of living animals. The tumor tissue is implanted in a chamber that is usually embedded in a skin fold of a rat or mouse and covered with a translucent "window," and drug distribution is photographed in the living animal as a function of time after administration (25,84,91). The window chamber method can be applied to drugs that are labeled with a fluorophore or chromophore, although such labeling might change their properties. An alternative in vivo method is to quantify the concentration of naturally fluorescent drugs in tissue sections, analogous to methods used with spheroids (109,110). This approach uses computer methods to map the distribution of drug (via its fluorescence) in relation to the nearest blood vessel in the section. Such methods

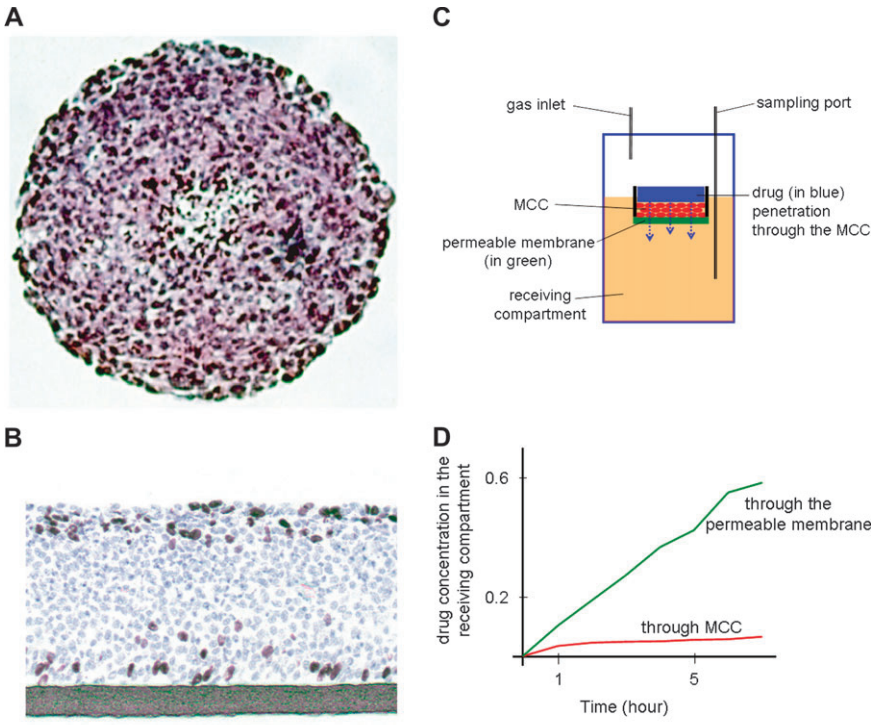

Fig. 3. In vitro models used to study the penetration of anticancer drugs through tumor tissue. Photomicrographs of (**A**) a multicellular tumor spheroid and (**B**) a multilayered cell culture (MCC) on a permeable membrane support. Proliferating cells labeled with bromodeoxyuridine (**black**) are located predominantly in the peripheral areas. Figure provided by A. I. Minchinton and reprinted from (79). (**C**) Schematic representation of experimental method used to quantify drug penetration. A drug is added on the top of the MCC (blue) and sampled as a funtion of time in the receiving compartment below the MCC. (**D**) Time-dependent penetration of a drug through an MCC compared with penetration through the permeable support membrane alone. The *y*-axis represents the drug concentration in the receiving compartment as a ratio of that expected when equilibrium has been established; profiles are based on data for mitoxantrone obtained by Tannock and colleagues (104).

have shown that high concentrations of doxorubicin are localized around blood vessels in several experimental tumors, including human tumor xenografts, with minimal concentrations achieved in more distal regions (Fig. 4, A and Fig. 5, A) (110). Similar concentration gradients of doxorubicin have been reported in relation to blood vessels of human breast cancer (109). We also found decreasing concentration with increasing distance from blood vessels for mitoxantrone (Fig. 4, B) and topotecan (Trédan O and Tannock IF: unpublished observations). Other drugs can be recognized in tissues by antibodies to them, including therapeutic monoclonal antibodies such as cetuximab (Fig. 4, C) (Lee CM and Tannock IF: unpublished observations).

Only a few anticancer drugs can be identified in tissues by their natural fluorescence or by antibodies to them. The activity of nonfluorescent drugs can be identified by their effects on cells, such as inhibition of cell proliferation (which is detected using fluorescently labeled antibodies against markers of cell proliferation such as cyclin D1, Ki67, or bromodeoxyuridine incorporation into DNA), or by induction of cell death (recognized by fluorescently labeled antibodies against molecules expressed in cells that are triggered to undergo apoptosis, such as activated caspase-9). Identification in tissue of the effect of a drug on cells has been used to show that gemcitabine exerts toxic effects in regions close to tumor blood vessels, while surviving cells in more distal regions can repopulate the tumor (111).

The distribution of drugs in tumors is heterogeneous. As a result, a large fraction of apparently viable tumor cells in solid tumors is not exposed to a lethal, or for some drugs (e.g., doxorubicin), even a detectable, concentration following a single injection (Fig. 5, A). Heterogeneity of drug distribution in tumors also limits the value of classical pharmacokinetics in predicting tumor response to therapy. Pharmacokinetics involves studying the time dependence of drug concentrations in plasma, tumor, and critical normal tissues but usually assumes that the drug concentration within a tumor is uniform. Such an assumption may give misleading predictions about tumor response to treatment because a drug might have a mean concentration in a tumor that is highly effective against cells in culture; however, if perivascular cells are exposed to a high concentration of drug and more distal cells to a very low concentration, the overall therapeutic effect of the drug will be small (Fig. 5, B). By contrast, the highly ordered vasculature in most normal tissues leads to rather uniform drug distributions (Fig. 4, D), thus giving tumors, with their disordered vasculature, a therapeutic disadvantage.

Because the diffusion coefficient of macromolecules decreases with increasing molecular weight, the delivery to tumor cells of large-molecule therapeutic agents, such as monoclonal antibodies, liposomes, nanoparticles, or gene vectors, might be particularly compromised, although delivery will also depend on their half-lives in the circulation and on their distribution by convection. For example, our unpublished studies of the therapeutic monoclonal antibody cetuximab suggest that it displays good tissue penetration, although its distribution in tumors is time- and dose-dependent (Lee CM and Tannock IF: unpublished observations). The long half-life of cetuximab allows the drug to penetrate tissue before it is cleared from the circulation (Fig. 4, C).

Why Chemotherapy Is Sometimes Effective When Drug Distribution Is Poor

Given that some drugs, including doxorubicin, show very poor distribution in solid tumors, it is important to consider why bolus injections of these agents are sometimes effective in shrinking solid tumors. There are several possible explanations. First, drugs administered as bolus injections are preferentially distributed

Table 1. Experimental methods for studying drug penetration*

	Model system	Description	Characteristics	Methods for studying the penetration of anticancer drugs	References
In vitro	Multicellular spheroids	Spherical aggregates of tumor cells	Supports development of: ECM Cell contact Gradient of cell proliferation from the surface to the center Gradients of nutrient concentration Hypoxia and central necrosis	Semi-quantitative method Spheroids incubated in medium that contains drug Drug detection in cross sections of spheroids by: Fluorescence (e.g., doxorubicin) Autoradiography (using radiolabeled drugs)	11, 12, 89, 99–103
	Multilayered cell cultures	Tumor cells grown on a collagen-coated semipermeable membrane (form a disc of tissue)		Quantitative method Drug is added to compartment on one side of the MCC Time-dependent drug penetration by sampling from compartment on other side of the MCC Comparison of rate of penetration through the MCC and through the support membrane alone Drug detection by: Fluorescence (e.g., doxorubicin) Autoradiography (using radiolabeled drugs)	90, 96, 98, 104–108, 153, 168, 169
In vivo	Window chambers	Growing tumors are observed directly in the living animal	Takes into account the varying physical conditions and especially the temporal changes in the vascular network	Direct assessment of penetration of fluorescent or colored molecules from tumor blood vessel into the surrounding tumor tissue	25, 84, 91, 117, 118
	Sections of tumor tissue	Cryosections of human and animal tumors	Direct visualization of blood vessels by antibody to CD-31 (expressed on endothelial cells) and/or of blood flow by injection of fluorescent marker (e.g., lectin)	Direct quantification of fluorescent drugs (e.g., doxorubicin) in tumor microregions Detection of an effect that drugs have on the cells using fluorescently-labeled antibodies (e.g., markers of cell proliferation or apoptosis)	89, 109–111, 171

* ECM = extracellular matrix; MCC = multilayered cell culture.

close to blood vessels, as are the proliferating cells that are most sensitive to them (49,110,111). These are the cells that are responsible for tumor growth at the time of treatment and are killed most efficiently by treatment. However, tumor regrowth may take place as surviving cells distal to blood vessels that were originally nonproliferating reenter the cell cycle as their nutrition improves after lysis of cells closer to blood vessels (111). Second, cancer patients usually receive several courses of treatment. Sequential injections of a drug might lead to deeper penetration through tumor tissue as cells proximal to blood vessels are removed and to subsequent killing of cells at increasing distances from blood vessels (79,103). Third, only a proportion of the cells in a tumor are stem cells that have the ability to regenerate the tumor (112). Successful outcomes of chemotherapy, such as sustained complete remission, require eradication of stem cells. The location of stem cells in tumors is unclear, but recent work suggests that they might be preferentially located near blood vessels (113). If that is the case, a homogeneous distribution of drug within tumors would not be needed to eradicate tumor stem cells. However, there is also evidence that hypoxic cells can repopulate human tumors after radiotherapy (114–116) and that cells distal to blood vessels can repopulate experimental tumors after chemotherapy (111,114), which suggests that at least some tumors have target stem cells located far from blood vessels.

Strategies to Overcome Drug Resistance due to Microenvironmental Factors

Methods to Increase Delivery of Drugs to Tumor Cells

Table 2 summarizes strategies that have been used to improve drug penetration in tumors. One strategy involves pretreating tumors with antiangiogenic therapy, an approach that may appear to be counterintuitive given that destruction of the tumor's vasculature might be expected to impair drug delivery. However, several studies (117–120) have shown that treatment of animals with DC101, an antibody to the vascular endothelial growth factor (VEGF) receptor, can lead to a transient increase in oxygenation

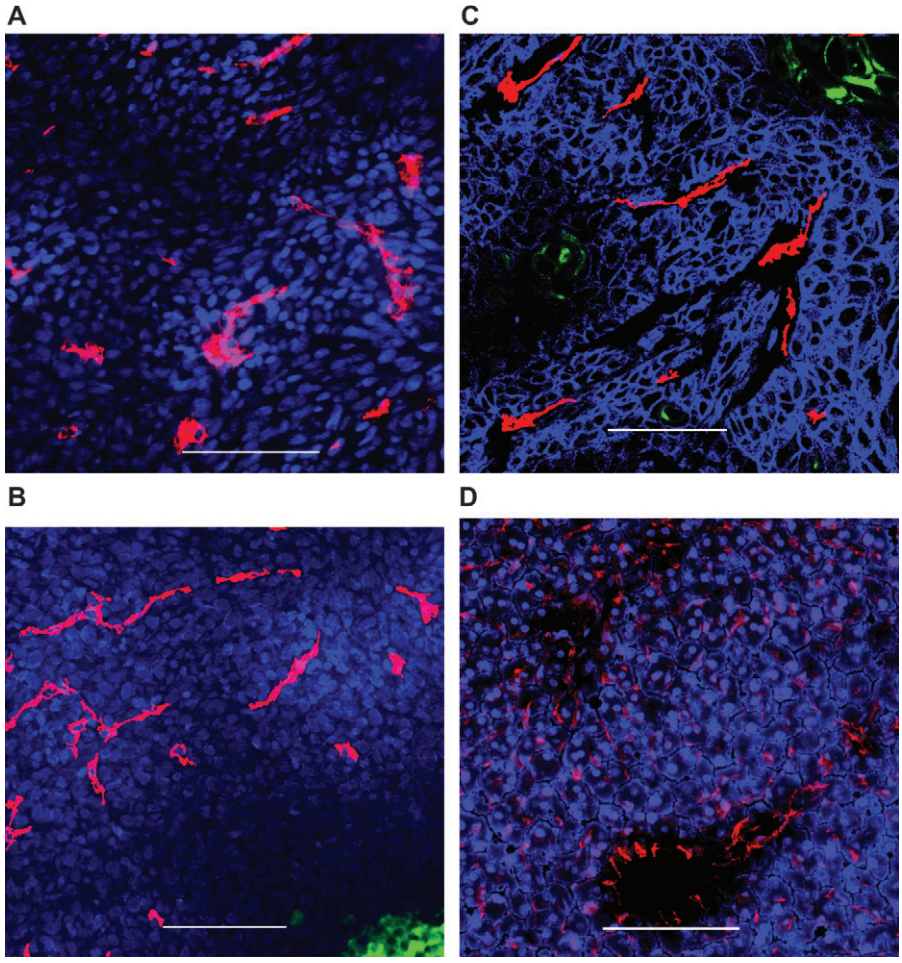

Fig. 4. The distribution of anticancer drugs in relation to blood vessels and to regions of hypoxia in experimental tumors. Distribution of (**A**) doxorubicin in the EMT6 murine breast sarcoma and (**B**) mitoxantrone in a human breast cancer xenograft 10 minutes after intravenous injection; both drugs are distributed mainly around blood vessels. (**C**) Distribution of cetuximab in a human cervical cancer xenograft 2 hours after intraperitoneal injection. This tumor expresses high level of epidermal growth factor receptor. (**D**) Distribution of doxorubicin in normal mouse liver. Drugs are pseudocolored in **blue**, vessels in **red**, and hypoxic regions in **green**. Scale bar = 100 μm.

and deeper penetration of molecules into experimental tumors. This effect is thought to be due to pruning of immature and abnormal blood vessels (or "normalization" of the tumor vasculature), which leads to a reduction in the interstitial fluid pressure (118–121). Other studies (122,123) have reported increased responses of experimental tumors to combined treatment with chemotherapy and antiangiogenic agents, and clinical trials of patients with metastatic colorectal cancer (124) and non–small-cell lung cancer (125) have shown prolonged survival when bevacizumab (a humanized monoclonal antibody directed against VEGF) was added to conventional chemotherapy. In another clinical trial (126), functional computed tomography and positron emission tomography scans of 12 patients with locally advanced rectal cancer performed 12 days after the administration of bevacizumab suggested improved function of the residual tumor vasculature. In a phase I trial (127), bevacizumab also led to an increase in tumor cell proliferation, suggesting that tumor cells might be more sensitive to chemotherapy both because of better drug delivery and better response to cell cycle–active agents. Several preclinical (118,119,128,129) and clinical (130) studies have shown a substantial reduction in vascular permeability after angiogenesis-inhibiting treatment that leads to a decrease in the interstitial fluid pressure. However, these effects appear to depend on the type of tumor being treated. For example, antiangiogenic therapy combined with capecitabine did not improve survival of women with metastatic breast cancer (131). Other experimental studies have demonstrated that antiangiogenic therapy can 1) decrease the overall distribution of large macromolecules such as antibodies (132,133), 2) decrease blood perfusion (127,134), and 3) modify the metabolic characteristics of the tumor microenvironment and lead to an increased level of tumor hypoxia (135).

Agents that damage existing blood vessels in tumors might also influence response to chemotherapy. Vascular-disrupting agents (such as tumor necrosis factor, flavone acetic acid and its derivatives, and tubulin-binding agents such as combretastatin A-4 disodium phosphate) directly damage the established tumor endothelium and have been shown to increase vessel permeability and drug delivery (128,136). For example, combretastatin A-4 disodium phosphate increases vessel permeability and reduces tumor blood flow, which in turn decreases cisplatin clearance from experimental tumors, consequently increasing the net amount of drug within them (137). However, injecting vascular-disrupting agents before chemotherapy may be problematic because it might result in reduced blood flow and increased interstitial fluid pressure, which together could impair delivery of drugs to tumors (128).

Another possible method for improving drug delivery is to modulate the muscle tone of blood vessels with, for example, the use of histamine (138) or a selective endothelin receptor A antagonist (139,140), which would increase tumor blood flow. Botulinum neurotoxin type A induces relaxation of tumor vessels and has been

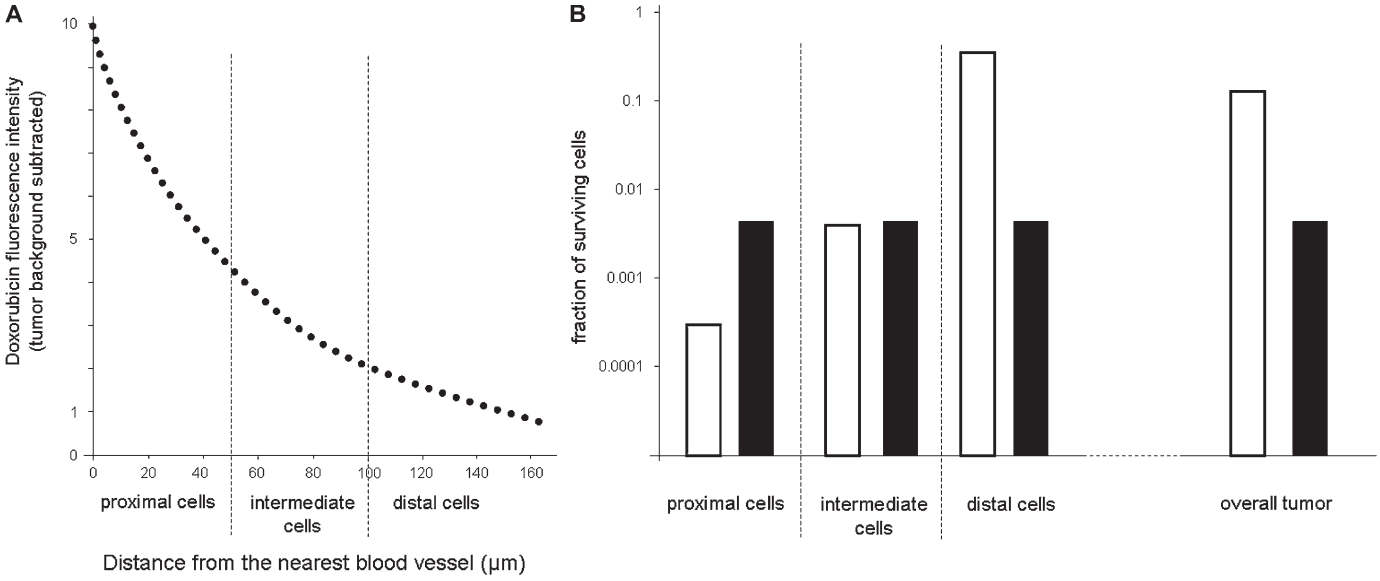

Fig. 5. Heterogeneity in the distribution of doxorubicin in an experimental tumor and its effect on overall survival of tumor cells. (**A**) Doxorubicin fluorescence intensity (quantified in a mouse breast adenocarcinoma cross section) as a function of distance from a tumor blood vessel, based on data from Primeau et al. (110). (**B**) Surviving fractions for three tumor cell populations characterized by their proximity to a blood vessel and for the overall tumor cell population, estimated for the experimentally determined distribution of doxorubicin shown in panel A (**open bars**) or by assuming a homogeneous distribution (**solid bars**). The estimate of cell survival assumes that the three subpopulations are equally represented in the overall population of tumor cells and that cells are equally sensitive to a given concentration of doxorubicin. The false assumption of a uniform distribution leads to a marked overestimate of drug effects to kill cancer cells.

shown to promote in vivo tumor perfusion and to delay tumor growth when combined with cyclophosphamide (141).

Drug penetration into tumor tissue is inhibited by high interstitial fluid pressure; thus, reduction in tumor interstitial fluid pressure might improve drug distribution (31). Some agents, including corticosteroids that are used routinely for prevention of nausea such as dexamethasone, reduce interstitial fluid pressure (142). The reduction in tumor cell density caused by chemotherapy itself could decompress blood vessels, reduce microvascular pressure, and decrease interstitial fluid pressure. For example, low-dose paclitaxel induces tumor cell apoptosis, which has been shown to reduce interstitial fluid pressure and to enhance the delivery of paclitaxel to solid tumors (143,144).

The concept that low-dose chemotherapy might cause limited cell killing but lead to reductions in tumor cell packing density and interstitial fluid pressure sufficient to enhance the distribution of subsequent doses has been applied in the clinic. One randomized phase II study (145) demonstrated that paclitaxel, but not doxorubicin, reduced interstitial fluid pressure and increased partial pressure of oxygen (pO_2) in breast cancer patients treated

Table 2. Reversal strategies used in vivo to improve drug penetration*

Reversal strategy	Method used	Possible mechanism of action	References
Improvement of tumor blood flow	Inhibiting neoangiogenesis	Pseudonormalization of the tumor vasculature	117–123, 126, 128–130
	Modulating the vessels muscular tone	Inhibiting the neurogenic contractions of tumor vessels	138–141
Increased tumor blood permeability	Damaging tumor endothelium	Altering endothelial barrier function	128, 136, 137
Reduction in IFP	Targeting VEGF	Decreasing vessel permeability	118–121, 128–130
	Induction of apoptosis by pretreatment with paclitaxel or other drug	Reducing the tumor cell density	143–145
	Using prostaglandin E1	Decreasing stromal cell contraction	34, 148
	Agonizing bradykinin	Increasing pore size of the tumor vasculature and total vascular surface area	149
	Targeting PDGFβ	Decreasing stromal cell contraction and interactions between these cells and ECM	150, 151
Inhibition of drug sequestration	Decreasing uptake of basic drugs into acidic endosomes (by raising their pH)	Decreasing net uptake of drug into cells and thereby increasing quantity of drug in the interstitial space	78, 96, 153
Modification of ECM	Degrading ECM: collagenase or relaxin	Remodeling ECM with antiadhesive effect	6, 158, 159

* VEGF = vascular endothelial growth factor; IFP = interstitial fluid pressure; PDGFβ = platelet-derived growth factor-beta; ECM = extracellular matrix.

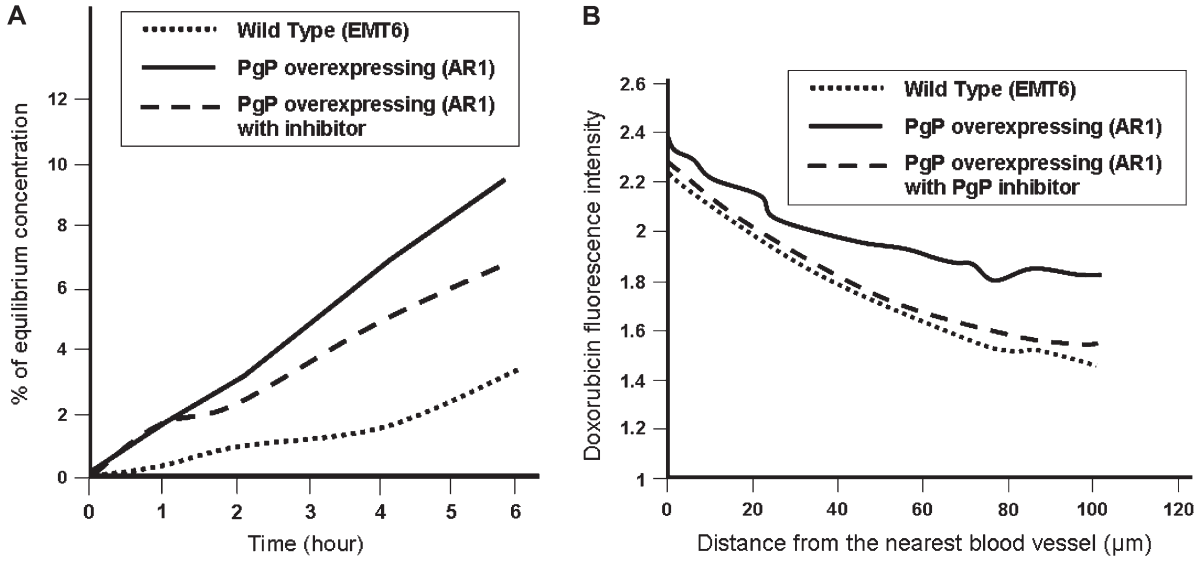

Fig. 6. Effect of high levels of P-glycoprotein (PgP) on tumor cells and of inhibition of PgP on the penetration of doxorubicin through tissue. (**A**) Time course of the penetration of doxorubicin (represented by the concentration of the drug in the receiving compartment as a ratio of the concentration expected at equilibrium) in multilayered cell cultures derived from EMT6 murine breast sarcoma cells. (**B**) Concentration of doxorubicin (represented by its fluorescence intensity) as a function of distance from blood vessels generated from wild-type EMT6 murine breast sarcomas (**dotted line**), from the AR1 murine breast sarcomas that overexpress PgP without (**solid line**) and with the presence of verapamil, an inhibitor of PgP function (**dashed line**).

with neoadjuvant chemotherapy. The impact of this strategy on clinical outcome has not been evaluated and remains unclear.

Pharmacologic agents (e.g., hydralazine) have been used to induce decreases in tumor blood flow and to lower tumor interstitial fluid pressure (146,147), and consequently some agents (e.g., prostaglandin E1-methyl ester and bradykinin receptor agonist) might enhance uptake of anticancer drugs (34,148,149). The platelet-derived growth factor-beta receptor also mediates high tumor interstitial fluid pressure, and imatinib, an antagonist of this receptor, might decrease interstitial fluid pressure in tumors and thus enhance the therapeutic effects of chemotherapy (150,151). However, lowering interstitial fluid pressure might cause undesirable effects, such as fluid accumulation in normal tissues (152).

Drug penetration is impaired by drug uptake and retention in cells close to blood vessels and might be improved by inhibiting these processes. Unfortunately, inhibiting drug uptake and retention frequently also leads to decreased toxicity for the tumor cells near blood vessels. Tunggal et al. (153) have shown that increased expression of the membrane-based export pump P-glycoprotein (which decreases the net cellular uptake of several substrate anticancer drugs) is associated with improved penetration of doxorubicin in multilayered cell culture and in experimental tumors. P-glycoprotein inhibitors such as verapamil and PSC 833 can decrease such penetration (Fig. 6, A and B; Patel K and Tannock IF: unpublished observation). This effect might, in part, explain the failure of inhibitors of P-glycoprotein to improve outcome in clinical trials (154,155).

A more effective strategy to improve both drug penetration and the efficacy of drug treatment might be to inhibit sequestration of drugs in subcellular compartments that do not convey toxicity to cancer cells. For example, basic drugs such as doxorubicin and mitoxantrone are sequestered in acidic endosomes, and this sequestration might be inhibited by proton pump inhibitors such as omeprazole (which raise endosomal pH) or by chloroquine (a basic drug that is also sequestered in endosomes, where it raises the pH); the decrease in net drug uptake into cells that results from inhibiting drug sequestration in acidic endosomes has been shown to increase drug penetration through multilayered cell culture (96). Manipulation of tumor pH has also been shown to modify the cellular uptake of weakly acidic drugs such as melphalan (156) and weakly basic drugs such as mitoxantrone (157). None of these strategies has yet demonstrated efficacy in vivo.

Modification of the tumor extracellular matrix might also facilitate the penetration of drugs into tumors. Treatment of tumors with the extracellular matrix–dissolving enzyme collagenase enhances the interstitial diffusion rate and the intratumoral delivery of macromolecules (158). Relaxin (a hormone secreted by women during pregnancy) has also been shown to degrade the tumor extracellular matrix and to improve macromolecular diffusion in tumors (159). However, agents that modify the extracellular matrix or its interactions with tumor cells might increase the probability of metastatic spread, so that their clinical applicability is unclear.

Perhaps the simplest method to improve drug distribution in tumors is the use of protracted continuous infusion. A relatively short half-life in blood prevents most drugs from establishing a good tumor distribution after a single injection. Continuous infusion can maintain diffusion or convection for prolonged periods and is likely to achieve a more uniform distribution than a single injection of drug. Drug distribution after a bolus injection is likely to be more uniform in well-vascularized normal tissues than in the tumor (Fig. 4), so that continuous drug infusion might provide a therapeutic advantage as compared with a bolus injection. For example, the therapeutic index of 5-fluorouracil is better when it is administered as a continuous infusion rather than as a bolus

injection (160,161), although this benefit might also be due to modification of mechanisms leading to cytotoxicity, when the drug is given as a continuous infusion (160).

One method to modify the pharmacokinetic properties of anticancer drugs is to incorporate them into macromolecular carriers such as liposomes or nanoparticles. In addition to the complex having a longer half-life than free drug in plasma, these large macromolecules are able to pass through fenestrations in the tumor blood vessels and release drug molecules into the interstitial space (80,84,162). This strategy for transporting low–molecular-weight drugs can lead to higher efficacy than injection of the free drug (163). Furthermore, coating the drug-carrying liposomes with antibodies to specific tumor antigens can facilitate the targeting of these macromolecular drug carriers to malignant cells (164).

Drugs With Toxicity for Cancer Cells in Nutrient-Deprived Regions of Tumors

Some drugs have been developed to exert selective toxicity against cells in nutrient-deprived regions of tumors. Nontoxic prodrugs can be activated under hypoxic conditions; such agents might have an improved therapeutic index by complementing the selective activities of radiotherapy for well-oxygenated cells and of chemotherapy for cells closer to tumor blood vessels. Tirapazamine, a drug that selectively kills hypoxic cells (165), has been investigated in combination with chemotherapy, with conflicting results. One phase III trial (Cisplatin and Tirapazamine in Subjects with Advanced Previously Untreated Non–Small-Cell Lung Tumors [CATAPULT] I), which randomly assigned 446 patients with advanced non–small-cell lung cancer to receive either tirapazamine and cisplatin or cisplatin alone, found better response rates and survival for patients who received the combined treatment (166). However, a second phase III trial (Southwest Oncology Group [SWOG] S0003) that randomly assigned 367 patients with advanced non–small-cell lung cancer to receive carboplatin and paclitaxel with or without tirapazamine did not show an improvement in response rate or survival for patients who received the combined treatment (167). Moreover, tirapazamine increased the toxic effects of the carboplatin–paclitaxel regimen. The limited capacity of tirapazamine to penetrate tumor tissue to reach the sensitive (i.e., hypoxic) tumor cells might explain these conflicting clinical results (108,168,169).

Other hypoxia-activated agents might have greater clinical potential if they have a greater capacity than tirapazamine to penetrate tissues. For example, AQ4N, a prodrug that is reduced and activated to AQ4, an agent similar to mitoxantrone, in hypoxic regions of tumors, has been evaluated in a clinical phase I study (170). Our ongoing studies [(171), Trédan O and Tannock IF: unpublished observations] suggest that AQ4N (or its reduced form AQ4) penetrates deep within experimental tumor tissue and selectively accumulates in hypoxic tumor cells, and that the combination of mitoxantrone to oxygenated tumor regions and AQ4 to hypoxic regions results in effective drug exposure over the entire tumor following combined treatment.

Some anaerobic organisms have the ability to preferentially colonize and replicate within the hypoxic tumor microenvironment (172). Anaerobic bacteria might therefore be used to exert an anticancer effect against cells far from blood vessels (173) and to thereby complement the effects of conventional chemotherapy (174). These bacteria also have potential as vectors for gene delivery (175), and in preclinical models anaerobic bacteria with membrane-disrupting properties have demonstrated anticancer activity by enhancing the release of membrane-encapsulated doxorubicin within tumors (176).

Conclusions and Perspectives

The effectiveness of drug therapy is impaired by limited delivery of drugs to some regions of tumors and by effects of the tumor microenvironment on drug activity and on the metabolism and proliferation of tumor cells. Agents that improve drug delivery or activity by targeting the tumor microenvironment, especially in hypoxic regions of tumors, represent an important future direction for cancer therapy. Adding vascular-disrupting agents that increase the extent of the hypoxic/acidic region might enhance the anticancer activity of various drugs that show increased efficacy against acidic cells, hypoxia-activated prodrugs, or bacteriolytic therapies. The development of methodologies to characterize causes of drug resistance related to the tumor microenvironment has considerable potential to improve the outcomes of patients following systemic treatment of solid tumors.

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