The Unique Physiology of Solid Tumors: Opportunities (and Problems) for Cancer Therapy

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Review

The Unique Physiology of Solid Tumors: Opportunities (and Problems) for Cancer Therapy[1]

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Abstract

The physiology of solid tumors differs from that of normal tissues in a number of important aspects, the majority of which stem from differences between the two vasculatures. Compared with the regular, ordered vasculature of normal tissues, blood vessels in tumors are often highly abnormal, distended capillaries with leaky walls and sluggish flow. Tumor growth also requires continuous new vessel growth, or angiogenesis. These physiological differences can be problems for cancer treatment; for example, hypoxia in solid tumors leads to resistance to radiotherapy and to some anticancer drugs. However, these differences can also be exploited for selective cancer treatment. Here we review four such areas that are under active investigation: (*a*) hypoxia-selective cytotoxins take advantage of the unique low oxygen tension in the majority of human solid tumors. Tirapazamine, a drug in the final stages of clinical trials, is one of the more promising of these agents; (*b*) leaky tumor blood vessels can be exploited using liposomes that have been sterically stabilized to have a long intravascular half-life, allowing them to selectively accumulate in solid tumors; (*c*) the tumor microenvironment is a stimulus to angiogenenesis, and inhibition of angiogenesis can be a powerful anticancer therapy not susceptible to acquired drug resistance; and (*d*) we discuss attempts to use gene therapy activated either by the low oxygen environment or by necrotic regions of tumors.

Introduction

Tumor Physiology: A Relatively Unexplored Target for Cancer Therapy. Nonsurgical methods of cancer treatment, primarily radiation therapy and chemotherapy, rely almost exclusively on agents that kill cells. The main problem with these current treatments, however, is that they do not, in general, have specificity for cancer cells. In the case of radiation therapy, a degree of specificity is achieved by localizing the radiation to the tumor and its immediate surrounding normal tissue. For anticancer drugs, it is primarily the rapid proliferation of many of the cancer cells that makes them more sensitive to cell killing than their normal cellular counterparts. However, both modalities are limited by their cytotoxic effects on normal cells. In the case of radiotherapy, normal tissue surrounding the tumor limits the radiation dose, whereas for anticancer drugs, it is usually the killing of rapidly dividing normal cells, such as those in the bone marrow, hair follicles, and epithelial cells lining the gastrointestinal tract, that limit the dose that can be given.

To achieve greater efficacy with present day treatments, investigators are attempting to exploit differences between normal and malignant cells at the cellular and molecular level. However, there is a second critical difference between normal and malignant tissues that has the potential for exploitation to produce more specific anticancer therapy. As this review will detail, the physiology of solid tumors at the microenvironmental level is sufficiently different from that of the normal tissues from which they arise to provide a unique and selective target for cancer treatment. To date, targeting tumor physiology for anticancer therapy has received considerably less attention than approaches based on the cellular and molecular differences between transformed and untransformed cells. Table 1 lists the principal differences in physiology between normal and malignant tissues that can be exploited (and can also be a problem) in cancer treatment.

The Vasculature: Basis for the Unique Physiology of Solid Tumors. The underlying differences between the physiology of normal and tumor tissues stems from the tumor vasculature. This is composed of two types of vessels: the existing vessels in normal tissues into which the tumor has invaded; and tumor microvessels arising from neovascularization resulting from increased expression of proangiogenic factors produced by tumor cells. Both types of vessels develop structural and physiological abnormalities that have become a hallmark of the tumor microvasculature. Although early studies of tumor blood flow described marked heterogeneity and often sluggish flow (1), it was the later studies of vascular casting techniques and window chamber preparations that identified the structural basis for these flow inhomogeneities (2–4). These studies showed that tumor blood vessels are highly irregular, tortuous, have arterio-venous shunts, blind ends, lack smooth muscle or enervation, and have incomplete endothelial linings and basement membranes (Fig. 1). As a result, blood flow is often sluggish, highly irregular, and the vessels much "leakier" than those in normal tissues. These characteristics of tumor vasculature lead to the physiological differences described in the following sections that present both problems and opportunities for treatment of solid tumors.

Hypoxia-selective Cytotoxins

Tumor Hypoxia. The pioneering work of Gray *et al.* (5) demonstrated that the sensitivity to radiation damage of cells and tissues depended on the presence of oxygen at the time of irradiation. The histological studies of human lung adenocarcinomas by Thomlinson and Gray (6) provided a mechanism by which cells could be hypoxic in tumors. They postulated that because of their unrestrained growth, tumor cells would be forced away from vessels beyond the effective diffusion distance of oxygen in respiring tissue, thereby becoming hypoxic and eventually necrotic. Given typical values for intracapillary oxygen tensions and oxygen consumption rates, the oxygen diffusion distance would be approximately 150 μm (Fig. 2).

Fig. 2 shows two important further consequences of reducing oxygen concentration: (*a*) the fraction of proliferating cells and/or the rate of cell proliferation decreases as a function of distance from the vasculature (7, 8), a phenomenon that, at least *in vitro*, is largely, or wholly, the result of decreasing oxygen levels (9). An important consequence of this hypoxia-induced inhibition of proliferation is that because most anticancer drugs are primarily effective against rapidly dividing cells, their effectiveness would be expected to fall off as a

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Table 1 *Physiological characteristics of malignant tissues that can potentially be exploited for cancer therapy, how these differ from those of normal tissues, and how these characteristics may also be detrimental to therapy*

Characteristics	Normal tissue	Tumor	Detrimental aspects for therapy	Method of exploiting for therapy
Microvasculature	Developed with ordered, regulated flow	Constant new vessel growth; vessels leaky, tortuous, often sluggish and irregular flow	Poor delivery of some therapeutic agents due to irregular flow and high interstitial pressure	Antiangiogenic agents Stealth liposomes
Oxygenation	Heterogenous, but rarely hypoxic regions	Highly heterogeneous with hypoxic regions common	Reduces tumor sensitivity to radiation and anticancer drugs; predisposes to increased malignancy (e.g., metastasis)	Selective cytotoxins; gene therapy targeted by hypoxia
Necrosis	Not present	Present	Not known if any	Gene therapy targeted to necrosis

function of distance from blood vessels. This has been shown experimentally (10); and (*b*) because hypoxic cells must be the ones most distant from blood vessels, they will be exposed to lower concentrations of drug than those adjacent to blood vessels, primarily as a result of the metabolism of such agents through successive cellular layers.

Thus, tumor hypoxia would be expected to be an important factor leading to resistance to radiotherapy (because of hypoxia, *per se*, affecting cellular radiation sensitivity) and to chemotherapy (because of lower proliferation and lower drug concentrations in the hypoxic cells). The consequent reduction of cell kill to anticancer treatment as a function of distance from tumor blood vessels is shown in Fig. 2.

What is the evidence for this model? Hypoxia is a common feature of both human and animal tumors (11, 12). The vast majority of human solid cancers have median pO_2 levels lower than their normal tissue of origin (12, 13). In animal tumors, it can be shown that these hypoxic cells are also viable and contribute to the resistance of transplanted tumors to both radiation (14) and to some anticancer drugs (15, 16). In human tumors, there is direct evidence from measurements of oxygen levels that hypoxia contributes to resistance to radiotherapy (17–19). Similar studies have not been performed with chemotherapy, although the evidence of a strong correlation between the response of head and neck cancers to chemotherapy and to radiotherapy implicates hypoxia as a cause of drug resistance (20, 21).

Hypoxia in solid tumors, however, has an important consequence in addition to conferring a direct resistance to radiation and chemotherapy. Graeber *et al.* (22) showed recently that low oxygen levels caused apoptosis in minimally transformed mouse embryo fibroblasts and that this apoptosis depended to a large extent on wild-type p53. They further showed, using these same cells growing as solid tumors in immune-deprived mice, that apoptosis colocalized with hypoxic regions in tumors derived from p53 wild-type mice. In tumors derived from p53 −/− cells, there was much less apoptosis and no colocalization with tumor hypoxia. These findings provide evidence that hypoxia, by selecting for mutant p53, might predispose tumors to a more malignant phenotype. Clinical data support this conclusion. Studies both with soft tissue sarcomas (23) and with carcinoma of the cervix (24) have shown that hypoxic tumors are more likely to be metastatic.

The model shown in Fig. 2 of hypoxic cells occurring at the diffusion distance of oxygen is the classic model of tumor hypoxia generally attributed to Thomlinson and Gray (6). However, we and others have proposed that tumor hypoxia can occur in a second way, by temporary obstruction or cessation of tumor blood flow–the so-called acute hypoxia model (25, 26). Definitive evidence for this type of acute hypoxia arising from fluctuating blood flow has come from elegant studies with transplanted tumors in mice using diffusion-limited fluorescent dyes (27, 28). Because fluctuating blood flow has also been demonstrated in human tumors (29, 30), it is likely that this type of hypoxia is also present in human tumors. The consequences of acute hypoxia will be similar to those of the diffusion-limited hypoxia.

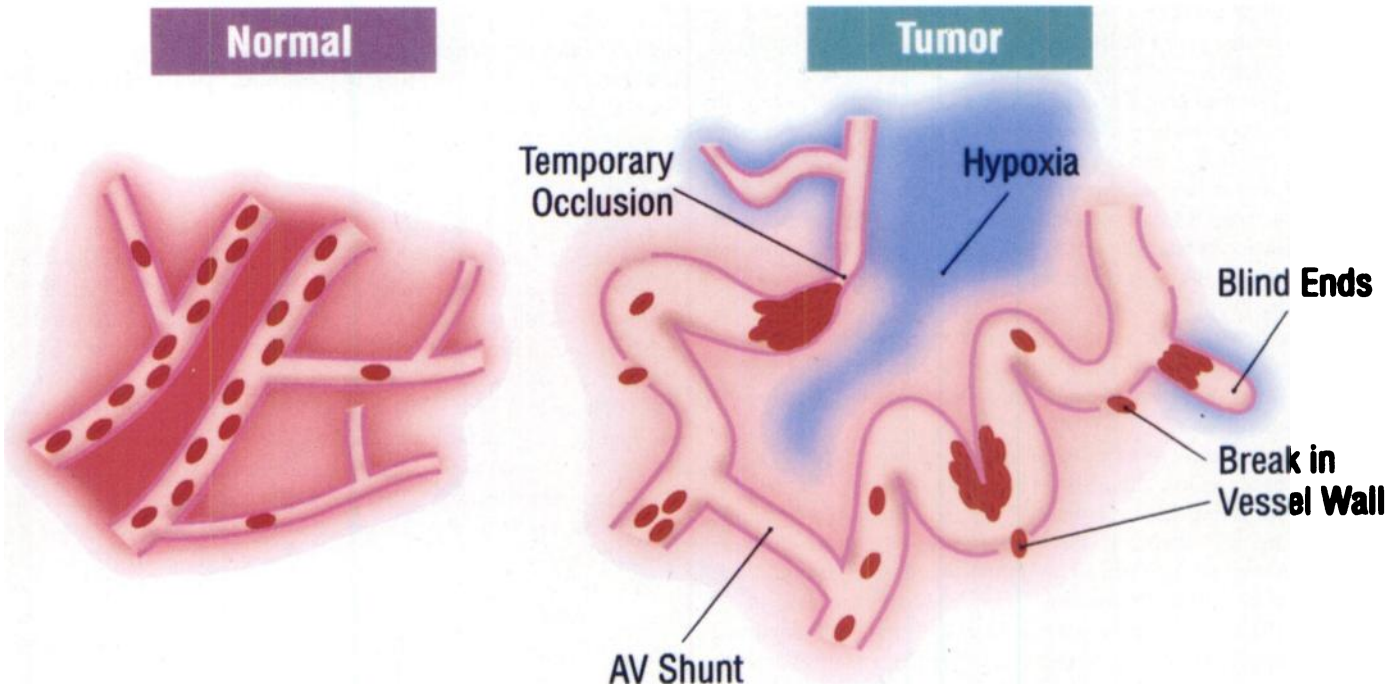

Fig. 1. Diagram showing the principal differences between the vasculature of normal and malignant tissues. Whereas normal tissues have relatively uniform and well-ordered blood vessels that are sufficiently close together to oxygenate all of the tissue, blood vessels in tumors are tortuous, have incomplete vessel walls, have sluggish and irregular blood flow, and have regions of hypoxia between the vessels.

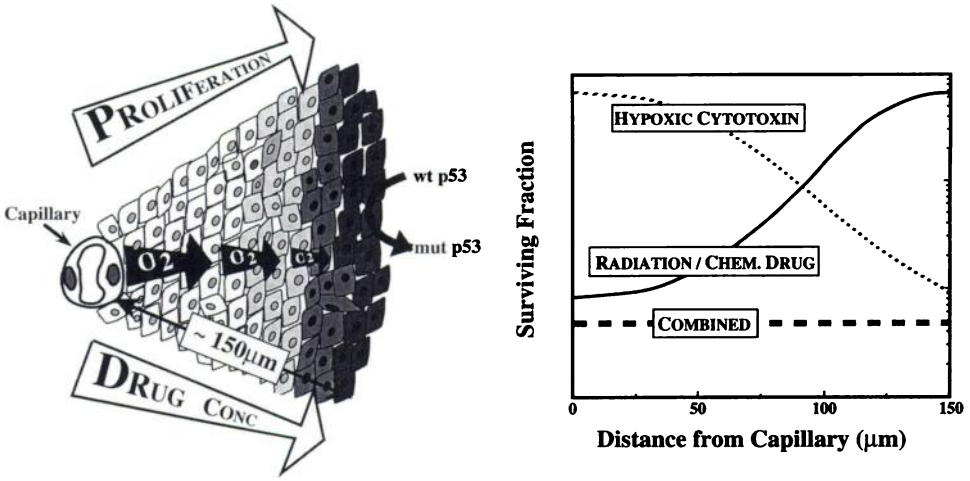

Fig. 2. A diagrammatical representation of a portion of a tumor cord surrounding a capillary. As the oxygen concentration decreases with increasing distances from the capillary, both cell proliferation rates and drug concentration (*Conc.*) also decrease. The hypoxic environment also can select against cells expressing wild-type p53. Shown on the *right* is how the level of cell kill to radiation and to many anticancer (*CHEM.*) drugs decreases with increasing distance from the capillary. It also shows how a drug with specificity for killing hypoxic cells would give a complementary profile of cell kill compared with radiation and chemotherapy, and that the combined toxicity would be greater than that achievable by either agent alone. Redrawn from Brown and Siim (40).

Any cells surrounding a closed blood vessel will be resistant to radiation killing because of their lack of oxygen at the time of radiation and will be exposed to lower levels of anticancer drugs than those surrounding blood vessels with normal flow. This would be expected to lead to inhomogeneities in response to anticancer agents, as has been observed in experimental tumors (31).

Hypoxia-selective Cytotoxins. Can the low oxygen levels in tumors be turned from a disadvantage to an advantage in cancer treatment? Such a possibility was proposed over 20 years ago by Lin *et al.* (32), who reasoned that compounds based on the quinone structure of mitomycin C might be more active in hypoxic tumors. It was known at that time that mitomycin C required metabolic reduction of the benzoquinone ring to produce the cytotoxic bifunctional alkylating agent. Lin *et al.* (32) reasoned that a lower oxidation reduction (redox) potential for tumor tissue relative to most normal tissues could increase reductive activation of these quinone derivatives in tumors. Although this was not the correct mechanism for the increased cytotoxicity of mitomycin C and certain analogues toward hypoxic cells (much lower levels of hypoxia are needed to change cellular redox potential), these studies were important in suggesting the potential of hypoxia-activated drugs and led to the concept of selectively killing the hypoxic cells in solid tumors (33–37).

It is important to note that specifically killing the hypoxic cells in tumors has greater therapeutic potential than oxygenating the cells or chemically sensitizing them to radiation or chemotherapy. Not only is the killing tumor specific (hypoxia is tumor specific), but the cells killed are the ones resistant to conventional therapy. This principle of "complementary cytotoxicity" is illustrated in Fig. 2. The combined killing of two agents with complementary cytotoxicity is potentially much greater than that of two agents acting on the same cell population. The other major advantage of hypoxia-selective cytotoxins is their potential for providing enhancement to the killing of standard anticancer drugs. This cannot be done by temporarily oxygenating the hypoxic tumor cells.

There are presently three different classes of hypoxia-specific cytotoxins that are in use clinically or are being developed for clinical use. They are the quinone antibiotics, the nitroimidazoles, and the benzotriazine di-*N*-oxides. In the quinone class, the three principal agents of current clinical interest are mitomycin C, porfiromycin, and E09. All are structurally similar and require reductive metabolism for activity. Each is converted on reductive metabolism to a bifunctional alkylating agent and probably produce their major cytotoxic activity through the formation of DNA interstrand cross-links. Reviews of the mechanism of action, pharmacology, and preclinical and clinical activities of these drugs have been published (38–40).

Mitomycin C, justifiably considered to be the prototype bioreductive drug (37), was introduced into the clinic in 1958 and has demonstrated efficacy toward a number of different tumors in combination with other chemotherapy drugs and with radiation. However, its selective toxicity toward hypoxic cells is modest, with values for hypoxic cytotoxicity ratios (the ratio of drug concentration to produce equal cell kill for aerobic and hypoxic cells) of 1 (no preferential toxicity) to approximately 5 (37, 41–43). However, based on this activity, mitomycin C has been combined with radiotherapy in two randomized trials of head and neck cancer (44, 45), the pooled results of which gave a statistically significant disease-free survival benefit. Whether this promising finding is the result of preferential cytotoxicity of mitomycin C toward hypoxic cells or to cytotoxicity to both aerobic and hypoxic cells is, however, open to debate (40). Nonetheless, encouraged by this success, the Yale group is now testing porfiromycin, a drug that has a somewhat greater hypoxic-selective toxicity than mitomycin C.

The third drug in this series, E09, is a much more efficient substrate for DT-diaphorase than either mitomycin C or porfiromycin and shows high toxicity to both aerobic and hypoxic cells in cells with high DT-diaphorase levels. Cells with low DT-diaphorase levels are much less susceptible to killing by E09 under aerobic conditions but show a high (up to 50-fold) preferential toxicity toward hypoxic cells. However, the pharmacokinetics of this agent work against its clinical utility, and Phase I clinical studies have shown little activity of this drug (46).

A second class of bioreductive agents is the nitroimidazoles, the first two of which, metronidazole and misonidazole, were extensively tested as hypoxic radiosensitizing agents (47). Further drug development by Adams *et al.* (48) produced a compound, RSU1069 (1(2-nitro-1-imidazolyl)-3-(1-aziridinyl)-2-propanol), which has been shown to be a highly efficient cytotoxic agent with activity both *in vitro* and *in vivo* (48, 49). RSU1069 has a hypoxic cytotoxicity ratio of some 10–100 for different cell lines *in vitro*, and it, or its prodrug, RB6145, has shown excellent activity with mouse tumor models when combined with irradiation or agents that induce hypoxia (50). Unfortunately, however, clinical testing of RB6145 has been aborted due to irreversible cytotoxicity toward retinal cells (51).

TPZ[3] is the first, and thus far, only representative of a third class of hypoxia-selective cytotoxins. This drug has a high selective hypoxic cytotoxicity (20–300 for different cell lines) and maintains its differ-

[3] The abbreviations used are: TPZ, tirapazamine; RES, reticular endothelial system; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor; HRE, hypoxia-responsive element; 5-FC, 5-fluorocytosine.

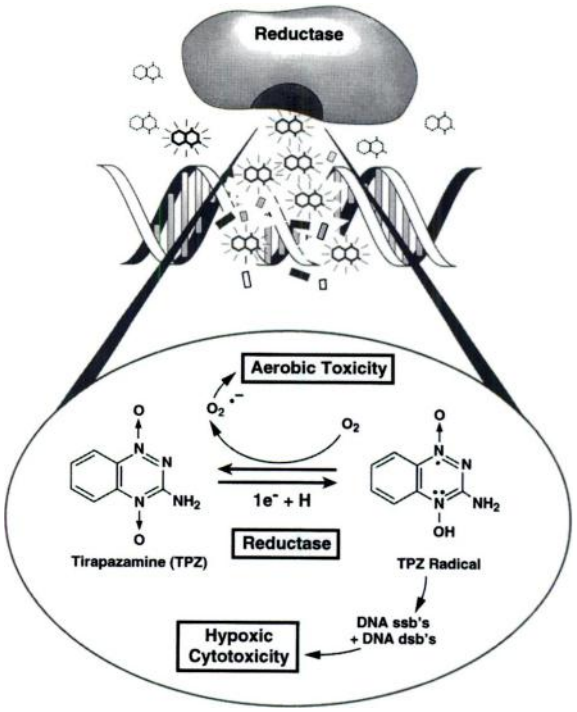

Fig. 3. A schematic showing the metabolism of TPZ to its active free radical moiety causing preferential toxicity to hypoxic cells by producing DNA damage. The postulated close association of the reductase enzyme(s) to DNA is shown. *ssb*, single-strand break; *dsb*, double-strand break.

ential toxicity relative to aerobic cells at oxygen concentrations ~10-fold higher than do other bioreductive drugs (52). This could be an important reason for its excellent efficacy in preclinical models, both with radiation and some anticancer drugs, because cells at "intermediate" oxygen levels may be more important than the extremely hypoxic cells in governing tumor response to fractionated irradiation (53).

The mechanism of the selective toxicity of TPZ is illustrated in Fig. 3. The toxic species has been identified as the radical formed by the 1-electron reduction of TPZ, with DNA double-strand breaks, leading to chromosome breaks as the principal mechanism of cell killing under hypoxia (54). Recent work has shown that the activating enzyme(s) leading to DNA damage is located in the cell nucleus, probably associated with the nuclear matrix (55, 56). Under aerobic conditions, oxygen can remove the additional electron from the TPZ radical, thereby back-oxidizing it to the nontoxic parent with the concomitant production of superoxide radical (57). In preclinical testing, TPZ has shown itself to be effective in sensitizing tumors to fractionated radiation (58) with no increase of sensitivity of normal tissues (59). TPZ is also effective in increasing tumor cell killing without increasing the toxicity of a number of commonly used anticancer drugs, particularly cisplatin and carboplatin (60, 61). This potentiation can be quite large, up to the equivalent of giving five times more cisplatin to the tumor for no increase in the systemic toxicity of cisplatin (60, 62).

Based on these preclinical studies, Phase I clinical trials were initiated in 1992, and at this time, Phase II trials with radiation (63), with chemotherapy (64), as well as two large Phase III trials combining TPZ with cisplatin in stage IIIB and IV nonsmall cell lung cancer, have been completed. The results of these trials will be available in mid 1998.

In summary, TPZ is the first drug introduced into the clinic as a specific hypoxia-activated drug. The potential of targeting drug cytotoxicity and/or anticancer drug potentiation to the hypoxic cells in solid tumors is an exciting one and exploits what is clearly a difference between the physiology of tumors and normal tissues. Further drug development of new hypoxia-selective cytotoxins is clearly warranted, because it is unlikely that TPZ is the optimum hypoxia-selective cytotoxin.

Liposome Delivery

Tumor vessels are often leaky, demonstrating increased permeability to circulating large molecules, even up to sizes of 400 nm in diameter (65–67). This leakiness of the vasculature results from an incomplete or missing endothelial cell lining and basement membrane (65, 68). Increased permeability of tumor vasculature has allowed specific targeting of anticancer drugs to tumors using small liposomes of ~100-nm diameter that have been specially modified to remain in the circulation for long periods of time.

The discovery that drugs could be encapsulated in fatty vesicles or liposomes in the 1960s led to high enthusiasm that this system would fill the role of the "magic bullet" for drug delivery. However, animal experiments soon proved these early expectations to be misguided, because liposomes, when injected i.v., are rapidly recognized and removed by the RES. An elegant solution to this problem was found by coating lipids with carbohydrate groups to have them resemble erythrocytes (69–71). These developments gave rise to today's sterically stabilized, or Stealth™, liposome which is coated with polyethylene glycol to provide a steric barrier to recognition and destruction by the RES (72). Such liposomes with encapsulated epirubicin or doxorubicin exhibit improved antitumor efficacy compared with conventional liposomes or to free drug (73). Stealth liposomes remain in the blood circulation for extended periods of time, with most of the liposomes remaining in the blood and only 10–15% ending up in the liver, a major improvement over conventional liposomes, the majority of which are rapidly captured by the RES in the liver (74).

Despite the impressive preclinical activity of both doxorubicin and vincristine encapsulated in sterically stabilized (Stealth) liposomes, none of the studies to date have attempted to determine the extent to which the increased antitumor activity is the result of increased tumor concentrations of liposomes carrying the drug or to prolonged exposure time of the tumor cells to drug. Presumably, both are important, although the benefit of prolonged exposure time is very likely to be dependent on tumor proliferation rate and the drug in question.

Clinical studies of doxorubicin encapsulated in Stealth liposomes (Doxil) began a number of years ago, and the results to date confirm the prolonged circulation time (plasma $t_{1/2}$ of 45 h compared with ~10 h for free doxorubicin) and increased concentrations of free drug in pleural effusion of a variety of tumors in those patients treated with doxil compared with free doxorubicin (75). These Phase I/II studies have also identified a novel toxicity of doxorubicin in Stealth liposomes, *i.e.*, a prolonged desquamating painful dermatitis primarily affecting hands and feet, possibly due to liposome-mediated deposition of drug in the skin. However, the results to date for a variety of solid tumors support the preclinical findings that increased antitumor efficacy will be likely with doxorubicin encapsulated in Stealth liposomes *versus* free drug.

Despite the obvious advantage provided by leaky blood vessels allowing high tumor concentrations of Stealth liposomes, it has to be recognized that the distribution of the liposomes is highly heterogeneous and concentrated in the perivascular interstitial regions (67, 76). Presumably, the antitumor activity of these heterogeneous deposits is the result of a slow release of drug throughout the tumor from these hot spots of high drug concentrations.

Antiangiogenesis

In the early 1970s, Folkman (77, 78) introduced what was then the controversial hypothesis that the growth of solid tumors was absolutely dependent upon new blood vessel formation, or angiogenesis, developing from outside the growing tumor mass. He also suggested that, this being the case, therapy aimed specifically at the angiogenic process or "antiangiogenesis" could be effective if tumor growth is angiogenesis dependent. Although slow to gain momentum in the midst of the promise for selective therapies based on the identification and functional characterization of tumor oncogenes and suppressor genes, work on antiangiogenesis as a therapy for solid tumors has accelerated enormously in the past 5 years, with several lead compounds that now show great promise in preclinical testing. Because a comprehensive review of the literature in this field is beyond the scope of the present review, we will focus instead on the most promising targets and particularly those controlled by the tumor microenvironment.

At first sight, the large number of angiogenic factors that have been implicated in tumor vascularization, including basic and acidic fibroblast growth factor, VEGF, transforming growth factors α and β, TNF-α, interleukin 8, and angiogenin, to name a few of the more important (79, 80), would make it seem unlikely that to target a single or a few angiogenic factors would be successful. Based on the rapidity with which tumor cells can adapt and select for mutants resistant to common anticancer drugs, it would seem probable that inhibition of one or more of these proangiogenic factors would cause the tumors to switch their angiogenic dependent growth to other cytokines. However, strategies aimed at one of these factors, VEGF and/or its two high-infinity receptors expressed in endothelial cells, flt-1 and KDR/Flk-1, have been remarkably promising (81–83). Indeed, VEGF is rapidly emerging as the dominant angiogenic factor in solid tumor development.

In addition to its potent and specific vascular endothelial mitogenic activity, VEGF also increases vascular permeability. Indeed, it was originally discovered as a tumor-secreted protein that rendered microvasculature hyperpermeable and was named vascular permeability factor (84). One possible reason for the major importance of VEGF as an angiogenic agent is that it is the only one of the angiogenic factors that also produces vascular permeability, and there is a considerable body of evidence suggesting that the microvascular hyperpermeability is an essential factor in angiogenesis favoring the migration of endothelial cells through the extravascular matrix (85).

Numerous strategies are presently being used to inhibit VEGF activity in tumors. They include antisense VEGF mRNA, monoclonal antibodies, and farnesyltransferase inhibitors. A proof of concept of this approach is that monoclonal antibodies against VEGF have been shown to inhibit the growth of human tumors in nude mice with a concomitant reduction in vascularity, although the same antibodies produced no effect on the growth of the tumor cells *in vitro* (86).

A variation on the strategy of inhibiting VEGF (or any angiogenic factor) is to inhibit its receptor. The proof of principle of the effectiveness of this was shown by Millauer *et al.* (81), who demonstrated that infection of the vasculature surrounding implanted glioblastoma cells with viruses expressing a dominant negative mutant form of Flk-1 suppressed glioblastoma tumor growth. Also, the tumors that arose had a large central necrosis surrounded by a thin layer of tumor cells with no invasion of the tumor by vasculature. Recently, high potency small molecule inhibitors of the Flk-1 receptor for VEGF have been isolated and shown to inhibit angiogenesis (82), to markedly inhibit the growth of s.c. tumors, and to prevent metastatic spread (83). These data provide evidence that VEGF expression can have a profound influence on tumor growth and metastatic spread.

As with many biological processes, there are natural antagonists to angiogenesis, such as angiostatin and endostatin, that could be used in conjunction with inhibitors of angiogenic factors or their receptors (87). Angiostatin is a peptide formed from the cleavage of plasminogen, which, when secreted by tumors, inhibits the growth of metastases in the same host (87). It acts by selectively inhibiting endothelial cells to respond to angiogenic signals, and when given to mice bearing transplanted murine or human tumors, causes marked regression of the tumors to microscopic dormant foci (88–90). Recently, a second natural inhibitor of angiogenesis, endostatin, with similar potent activity against established transplanted tumors has been described (91).

Although angiogenesis is a highly attractive tumor-specific target for therapy, it is nonetheless a normal process occurring in embryonic and placental development, wound healing, ovulation, and chronic inflammation (92). The signal linking these different processes could, in many instances, be hypoxia. Intuitively, this makes sense. A cell that is low in oxygen responds to this stress by secreting a cytokine that will increase blood vessel growth toward that cell. In tumors, there is compelling evidence that hypoxia stimulates angiogenesis by increasing VEGF production. One of the most elegant early demonstrations of this came from the work of Shweiki *et al.* (93), who showed that in human glioblastomas, VEGF message was highly expressed adjacent to areas of focal necrosis (where hypoxia would be expected) and that new capillary bundles were localized alongside VEGF-producing cells. Further evidence that tumor hypoxia is an important signal for angiogenesis, and a determinant of tumor growth, is provided by the recent work of Maxwell *et al.* (94), who showed that tumors growing from cells deficient in the hypoxia-activated transcription factor, hypoxia-inducible factor 1, did not show increased VEGF induction adjacent to necrosis, were poorly vascularized, and grew much slower than tumors with competent hypoxia-inducible factor 1.

Because VEGF can be induced in normal cells by hypoxia (95, 96), the question arises as to the relative contributions to tumor angiogenesis from the microenvironment (*i.e.*, hypoxia) and from malignancy *per se* (97). Mazure *et al.* (98) have shown recently that it is probably both: oncogenic transformation (at least by certain oncogenes) and hypoxia act synergistically to induce VEGF. Therefore, the use of agents that inhibit oncogenic *ras* activity may act as antitumor agents in part by inhibiting signaling pathways in the angiogenic activation cascade.

Antiangiogenesis therapy has several important advantages over standard anticancer treatment: (*a*) it targets a process that, under most circumstances, is tumor specific and therefore likely to have little systemic toxicity; (*b*) it has the advantage that with an endothelial cell target, there is not the problem of the drug having to reach tumor cells, often many cell layers from the vasculature; and (*c*) one of the most important advantages of antiangiogenic therapy is that the genetic stability of endothelial cells (as opposed to tumor cells) should prevent the development of drug resistance on repeated administration of the agent. This was first proposed by Kerbel (99) and recently dramatically demonstrated by Boehm *et al.* (100), who induced multiple regressions in transplanted mouse tumors by repeated administration of endostatin. This concept is illustrated in Fig. 4.

In summary, antiangiogenetic strategies are now showing a great deal of promise in murine tumor models. The responses of such transplanted tumors to inhibitors of VEGF or its receptor Flk-1, or to the natural antiangiogenetic peptides, angiostatin and endostatin, are extremely impressive. We believe it would not be overly optimistic to conclude that if human spontaneous tumors are as dependent on continued angiogenesis as are the more rapidly growing transplanted tumors in rodents (and this is a major unknown), then these strategies could constitute a very important advance in cancer therapy.

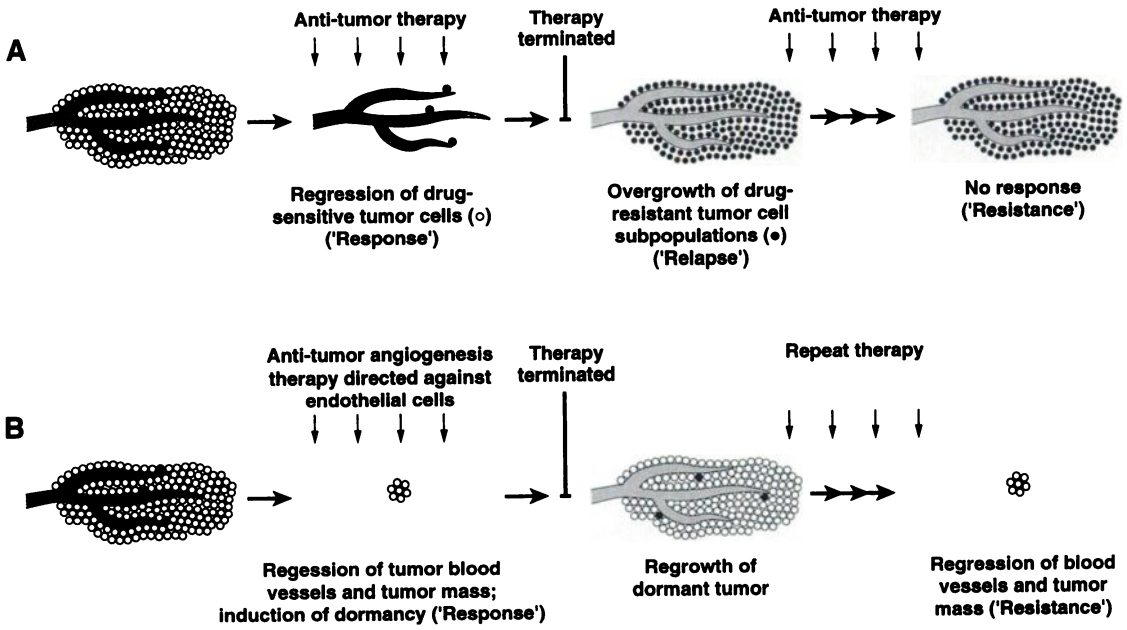

Fig. 4. The mechanism by which resistance to many standard anticancer drugs develops by outgrowth of drug-resistant tumor cell subpopulations, contrasted with the lack of development of resistant tumors when antiangiogenesis therapy is directed against endothelial cells [redrawn from Kerbel (112)].

Gene Therapy

Activated by Hypoxic Stress Response. The newest direction for exploiting tumor physiology is aimed toward the evolving field of gene therapy. In this novel approach to anticancer therapy, genetic material is transferred into cells by a variety of techniques with the ultimate goal of selective killing of cancer cells and the sparing of normal cells. At present, this selective killing of tumor cells can be achieved either by controlling vector delivery and activity or by transgene expression. Previous studies by Hallahan *et al.* (101) have tested the feasibility of intratumoral control of transgene expression using a radiation-inducible promotor ligated to the *TNF-α* gene. They were able to demonstrate radiation increased TNF-α production intratumorally after a 50-Gy single dose of ionizing radiation. Furthermore, the radiation-induced increase in TNF-α production resulted in increased tumor cell killing. These experiments on radiation-inducible gene expression establish the proof in principle that a strategy to control gene expression by stress-responsive promotors is feasible *in vivo*.

A direct test of the ability of the low oxygen conditions to selectively control gene expression and increase cell kill was performed by Dachs *et al.* (102), who linked a HRE from the mouse *phosphoglycerate kinase-1* gene to the bacterial cytosine deaminase-encoding gene and stably transfected it into HT1080 cells. To test the hypothesis that only under low oxygen conditions would the expression of the bacterial cytosine deaminase gene be increased, the HT1080 cells that were stably transfected with the HRE-cytosine deaminase gene construct were exposed either to 5-fluorouracil or to 5-FC under aerobic and hypoxic conditions. Only hypoxic cells were sensitive to 5-FC, the inactive prodrug, which requires enzymatic conversion to 5-fluorouracil by cytosine deaminase to be cytotoxic. The increased sensitivity of HT1080 transfectants to 5-FC suggested that *in vitro* HREs could be used to control the expression of a prodrug activating enzyme. At present, we do not have functional data on the ability of HREs to control the activation of nontoxic prodrugs into a tumoricidal form *in vivo*. However, the ability of HREs to induce the activity of a reporter gene has been evaluated intratumorally, and HREs seem to be able to transcriptionally regulate gene expression under low oxygen conditions *in vivo* (102).

There are numerous implications of these studies for cancer gene therapy: (*a*) the use of HREs will provide a selective means of controlling gene transcription in a wide variety of solid tumors based on the lower oxygen levels of tumors compared with normal tissues; (*b*) the use of enzymes under the control of an HRE adds a safeguard (compared with expression of a toxic substance), because increased expression of the prodrug activating enzyme is itself nontoxic; (*c*) the expanding list of inactive cytotoxic prodrugs and prodrug activating enzymes increases the possibility of finding the most efficacious combinations for different tumor types; and (*d*) continued research on the regulation of gene induction by hypoxia should offer new transcriptional regulatory elements as well as transcriptional stabilizing elements that will increase the dynamic range and specificity of transcriptional responses to a low oxygen environment. Potentially this approach, therefore, would allow the activation of a nontoxic prodrug to its toxic metabolite selectively in solid tumors. However, as with most forms of gene therapy, targeting the constructs to the tumor remains a major hurdle.

Targeted by Tumor Necrosis. It has been known for several decades that certain species of anaerobic bacteria of the genus *Clostridium* can selectively germinate and grow in the hypoxic/necrotic regions of solid tumors after i.v. injection of spores. This was first dramatically demonstrated by Malmgren and Flanagan (103) with *C. tetani,* the causative agent of tetanus. Mice, when injected i.v. with spores of this bacteria, remained healthy unless they had tumors, in which case death by tetanus resulted within 48 h. This was caused by germination of the bacteria in the tumors and release of toxins systemically. Mose *et al.* (104) later isolated a nonpathogenic strain of *C. sporogenes* (later renamed *C. oncolyticum*), which germinated in tumors after i.v. injection of the spores, causing tumor lysis and shrinkage of the tumors (104). Extensive preclinical testing was followed by clinical trials of this agent, particularly with patients with glioblastoma who received injections of up to 10^{10} *C. oncolyticum* spores (105, 106). Lysis was demonstrated in the tumors, with no evidence of clostridial germination or tissue destruction in the surrounding normal tissue. With the exception of mild to moderate fever, the patient suffered no ill effects from the injection of these organisms. However, no clinical benefit was demonstrated, presumably

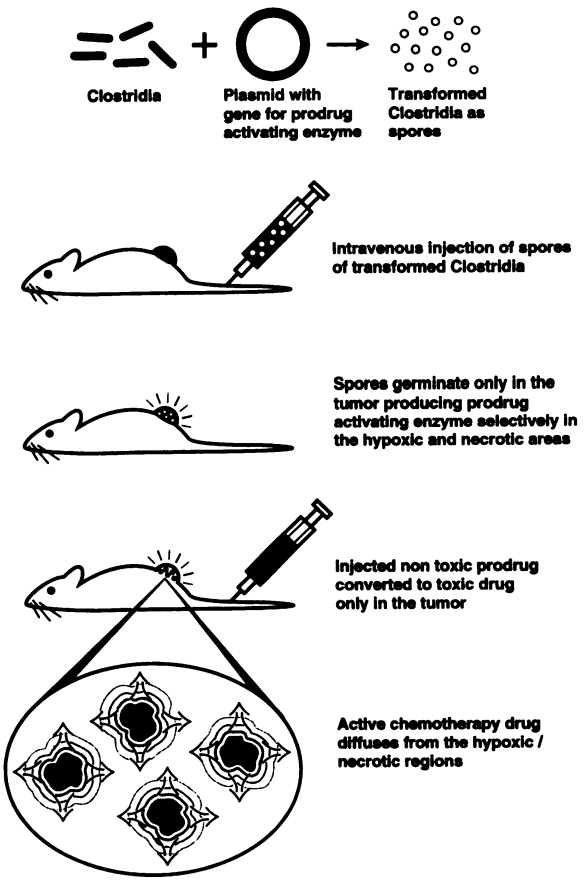

Fig. 5. A schematic of gene therapy using genetically transformed *Clostridia*, an obligate anaerobe. The transformed *Clostridia* are injected i.v. as spores, which distribute throughout the body, but only germinate in the hypoxic/necrotic areas of the tumor, thereby generating the prodrug-activating enzyme in the tumor, which can subsequently activate a prodrug to a toxic drug in these areas. Toxic drug will diffuse from the necrotic/hypoxic areas with the highest concentrations of drug in these areas. This would be expected to produce cytotoxicity complementary to that of conventional therapy in a manner similar to that shown in Fig. 2.

because of rapid regrowth of tumor from oxygenated tissue; therefore, clinical trials were discontinued.

We have proposed that these tumor-targeting clostridia could be genetically manipulated, thereby exploiting tumor hypoxia for a new form of gene therapy (107). Although it might be possible to obtain an antitumor effect with the bacteria expressing a toxic protein, we believe a more effective and potentially safer approach would be for the bacteria to express an enzyme that could convert a nontoxic prodrug into a toxic drug. Because the enzyme would only be expressed in the tumor, the conversion of a systemic prodrug to a toxic anticancer drug would occur only in the tumor (Fig. 5).

We have recently demonstrated the proof of this principle using genetically engineered *C. beijerinkii,* a species that also colonizes hypoxic areas in tumors (although not to the same extent of *C. oncolyticum*) (108). These clostridia were transformed with a plasmid expressing the *E. coli* enzyme, nitroreductase, which can convert the monofunctional alkylating agent, CB1954, into a potent bifunctional alkylating agent (109). This drug, originally synthesized over 25 years ago (110), has potent activity against Walker 256 rat carcinoma cells due to efficient metabolism to the bifunctional agent by rat (but not human) DT-diaphorase (111). We showed that both nitroreductase protein and activity could be detected in tumors from mice injected with clostridia carrying the nitroreductase gene but not in tumors derived from mice injected with control clostridia. Furthermore, no detectable nitroreductase protein was found in any of the normal tissues from mice expressing this protein in the tumors (108).

These studies establish the possibility of using tumor necrosis and/or hypoxia to target specific proteins to tumors and have these proteins expressed solely in the tumors. Future work is needed to achieve high enough stable expression of activating enzymes in the tumors to achieve antitumor activity by metabolism of prodrugs.

Conclusions

The vasculature and cellular environment, often termed the "microenvironment," of solid tumors is different from that of normal tissues in several important respects. We have outlined in this review what we believe are the most important of these for cancer therapy. Several of these differences, *e.g.,* high interstitial pressure and tumor hypoxia, have been recognized for some time as having detrimental effects on some types of cancer treatment. More recently, however, microenvironmental differences between normal and malignant tissues are being appreciated as opportunities for tumor-selective therapy. We review here four such opportunities that are in, or approaching, clinical testing: hypoxia-selective cytotoxins, sterically stabilized liposomes, antiangiogenesis, and gene therapy activated by hypoxia or by tumor necrosis. Each of these exploits a unique feature of solid tumors, thereby providing an exciting opportunity for tumor-selective cancer treatment.

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