

Tumor Stem Cell Niches: A New Functional Framework for the Action of Anticancer Drugs

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Abstract: Newer treatments of advanced human cancer increasingly rely on combinations of drugs that have quite different actions yet unexpectedly potentiate each other's effects. Recent research in stem cell biology suggests a model for tumors in which tumor growth is governed by the generation of cells from tumor cell niches rather than from the population as a whole. Each niche contains a population of tumor stem cells supported by a closely associated vascular bed comprising mesenchyme-derived cells and an extracellular matrix. Division of tumor stem cells is asymmetric in the sense that some daughter cells are always retained within the niche while others leave the niche to proliferate further and eventually die. One important potential difference between normal and tumor stem cell niches is that while most normal stem cells are in a non-proliferating or G_0 -state, tumor stem cells are continuously in cycle. Combinations of cytotoxic drugs and antagonists of survival factors to reduce the stem cell population may require the addition of vascular disrupting agents to compromise the function of the tumor cell niche. As well as providing opportunities for new drug discovery, this model of tumor growth also presents challenges as to how the contributions of individual drugs in a combination might be assessed in individual patients.

Keywords: Stem cells, stroma, niche, cytokinetics, survival factors, cytotoxic drugs, signal transduction inhibitors, vascular disrupting agents.

INTRODUCTION

Recent research on cancer has generated a new model for cancer growth which, while still speculative, has important implications for therapy. This model postulates that tumor tissue, like normal tissue, is continuously repopulated from pools of self-renewing stem cells growing in a "niche", which is made up of a specialized vascular bed of endothelial cells, associated cells of mesenchymal origin and extracellular matrix components [1]. Although tumor stem cell niches may be difficult or impossible to identify histologically, they could be essential for the maintenance of the tumor population, implying that cancer treatment should be aimed not at the general ablation of the total tumor cell population but with the elimination of tumor cell niches. The stromal elements that form an integral part of the niche are therefore important targets in addition to the tumor cells. Clues to possible strategies for targeting tumor cell niches may be provided by recent clinical data on drug administration strategies combining cytotoxic drugs with drugs acting on signaling pathways of both tumor and vascular endothelial cells. This review commences with a short description of stem cells in normal tissues to provide a background for discussion. Principles by which tumor stem cell niches might be eliminated are then developed with reference to some of the more recent drugs and antibodies that target interactions between tumor cells and their supporting stroma.

STEM CELLS IN NORMAL TISSUES

Stem cells in hematopoietic tissue were postulated many years ago to explain how the wide variety of blood cells in the circulation appeared to originate from common precursors [1]. Advances in cell sorting led to the morphological identification of such multipotent cells and subsequent research has demonstrated that these cells exist in a highly protective microenvironment within the bone marrow, where vascular endothelial cells and associated "nurse" cells produced both the factors and the supporting extracellular matrix required for their survival. The term "niche" was coined to describe this microenvironment [2] and the main features are shown in (Fig. 1). The niche might typically contain a population of several thousand stem cells, most of which are in a non-proliferating or G_0 state. At intervals, individual cells are stimulated to enter the cell cycle, apparently in response to mitogens released by stromal microenvironment. The reason as to why only individuals of a large population are selected at any time to enter the division cycle is not known.

The difference between G_0 -phase and cycling cells in a population is now beginning to be understood [3]. Mitogens that induce an individual G_0 -phase cell to enter the cell cycle do so by activating surface receptors. This leads through internal signaling pathways to the activation of a number of transcriptional activators and synthesis of many different proteins. One of these, cyclin D1, binds to the CDK4 and/or CDK6 cyclin-dependent kinases, allowing them to phosphorylate and remove the retinoblastoma protein from members of the E2F family of transcriptional activators. This then allows the production of a range of further proteins including the minichromosomal maintenance (MCM)

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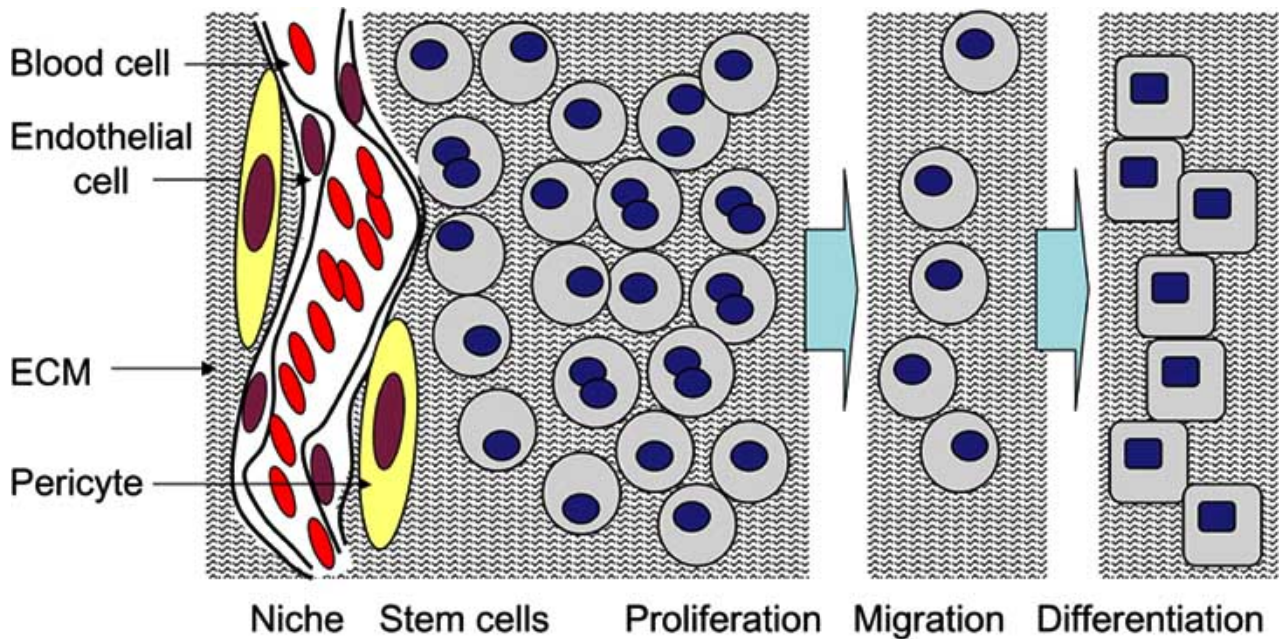


Fig. (1). Diagram of a normal stem cell niche. It contains capillaries, vascular endothelial cells, pericytes and the fibrous proteins of the extracellular matrix. Other stromal cells, immune cells and nerves may also be present. Most of the stem cells are in a non-proliferating state and when one stem cell undergoes a transition to the cell cycle and to cell division, one daughter cell remains as a stem cell in the niche while the other leaves to initiate a proliferating progenitor cell population. These cells migrate to their target tissue, exit from the cell cycle and differentiate.

proteins, the origin of DNA replication (ORF) proteins and cyclin E [3]. The presence of such proteins defines entry into the cell cycle (G_1 -phase) and under appropriate conditions the cells are able to progress through S-phase, G_2 -phase and cell division. Stem cells have an additional, essential feature, which is the ability to undergo “asymmetric” division, whereupon one of the daughter cells remains as a stem cell in the niche while the other proliferates further, migrates and differentiates [2]. Differentiation is associated with eventual reversion to G_0 -phase, where the synthesis of MCM, ORF and other proteins ceases.

Based on current evidence, it is likely that cells in all tissues of the body are replenished from repositories of stem cells. The bone marrow contains a large number of stem cell niches, containing not only hematopoietic stem cells but also mesenchymal stem cells [4]. The gastrointestinal tract contains large number of stem cell niches associated with crypts [5]. Cell turnover in these crypts is often rapid with cell replacement every few days. The brain contains stem cell niches in the subventricular zone and other areas [6]. A large number of stem cell niches are located in the “bulge” of hair follicles provide precursors of keratinocytes, melanocytes and other cells that populate the skin. When removed and cultured under appropriate conditions, these stem cells can produce cells of a wide variety of lineages [7]. It is possible that each organ of the body contains large numbers of stem cell niches responsible for the maintenance of the organ, and that stem cells can migrate from one niche to another.

The stem cell niche, as well as containing stem cells, vascular endothelial cells, associated pericytes and other stromal cells, is likely to contain both immune cells and a

nerve supply. A continuous exchange of signals is thought to occur among the different cells of the niche. Signals from stromal components to the stem cells might be expected to include not only survival factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF), but also those acting on developmental pathways such as wingless (wnt), notch and hedgehog (Hh) [8]. Such signals might differentiate the niches from other parts of the tissue.

TUMOR STEM CELLS

Early studies sought to grow disaggregated samples of clinical tumor tissue following removal at surgery. When a single cell suspension was grown on a semisolid matrix such as agar in the presence of appropriate growth factors, an extremely low proportion of cells grew into colonies containing dozens or hundreds of cells [9]. Such assays were termed “stem cell assays” and the clonogenic cells were described as stem cells. The currently accepted view is that although some cells are capable of proliferation in the presence of combinations of growth factors, tumor stem cells *in vivo* are really defined by their ability to grow in a niche microenvironment.

An important potential property of tumor stem cells has emerged from experiments in which gastric cancer was induced in mice by chronic infection with *Helicobacter pylori* bacteria [10]. By genetically labeling bone marrow cells it was found that gastric tumors could originate from circulating cells derived from the bone marrow. The model proposed is that individual crypts in gastric epithelium each contain a niche and an associated stem cell population, and that in the presence of a chronic inflammatory stimulus these

stem cells are progressively depleted. The niche is then engrafted by circulating bone marrow-derived stem cells, which repopulate the crypt. However, some of these bone marrow-derived stem cells contain mutations and develop abnormally, eventually repopulating the whole crypt. Depending on the nature of the mutations, this process may lead to abnormal growth such as metaplasia, dysplasia and intraepithelial cancer. These results also suggest that tumor stem cells, which might be produced at low frequency by a process of successive mutation within a normal niche, might establish themselves by displacing normal stem cells from the niche.

Perhaps the most important functional difference between normal and tumor stem cells is related to their regulation of the cell cycle (Fig. 2). Whereas normal stem cells oscillate between a quiescent (G_0 -phase) and a cycling state, it is likely that tumor stem cells are perpetually in the cell cycle. The nature of the defect in cell cycle regulation varies from one tumor to another but may include mutation of the ras protein, mutation or deletion of the retinoblastoma protein and deletion of the p16^{INK4A} protein that regulates the activity of the cyclin-dependent kinase-4 and -6 (CDK4 and CDK6) [11]. The defect in cell cycle regulation affects the fate of the proliferating tumor population as well by inhibiting its exit from the cycle. The asymmetric cell division is maintained in the niche but daughter cells leaving the niche proliferate, only partially differentiate and then die. It is clear from studies on tumor cell kinetics that the rate of cell death is only slightly less than the rate of cell division [12]. This implies that an individual cell will on average only divide one or two times before dying.

The loss of regulation through the CDK4/6 pathway does not necessarily mean that the tumor stem cells proliferate rapidly, because they have “back-up” cell cycle regulation mechanisms, for instance through cyclin-dependent kinase-2

(CDK2). Two of the main negative regulators of CDK2 are the p21^{WAF1} and p27^{KIP1} proteins, which bind to CDK2 and inhibit its ability to initiate the transition of cells from G_1 -phase to S-phase, as well as to progress through S-phase [11]. A number of proteins potentially produced by stromal cells, such as members of the transforming growth factor- (TGF-) family can act through surface receptors not only to increase the rate of apoptosis, but also to regulate cellular concentrations of p21^{WAF1} and p27^{KIP1}, and thus lengthen the cell cycle time [13].

As mentioned above, tumor stem cell niches within tumor tissue may not be identifiable histologically. However, they might be identified by their spectrum of gene expression since tumor stem cells, like normal tissue stem cells, might express genes associated with self-renewal. For example, expression of the polycomb group *BMI-1* gene has been associated with self-renewal not only in hematopoietic tumor cells but also in cell lines derived from non-small cell lung cancers and breast cancers, and in clinical samples from a number of epithelial malignancies [14].

IMPLICATIONS OF THE TUMOR STEM CELL HYPOTHESIS FOR CANCER TREATMENT

The concept that tumor stem cells compete with normal stem cells for niches and eventually repopulate them to become “powerhouses” of the tumor has a number of implications. One is that the majority of tumor cells within cancer tissue may be inevitably destined for apoptosis once they have left the niche, whatever the treatment. Thus, treatments that lead to tumor shrinkage must be interpreted with care because they do not necessarily imply an effect on the tumor stem cell population, from which the cancer repopulates. A mathematical model for chronic myeloid leukemia has been used to address the action by which the drug imatinib (Gleevec, Glivec), which targets the Bcr-abl

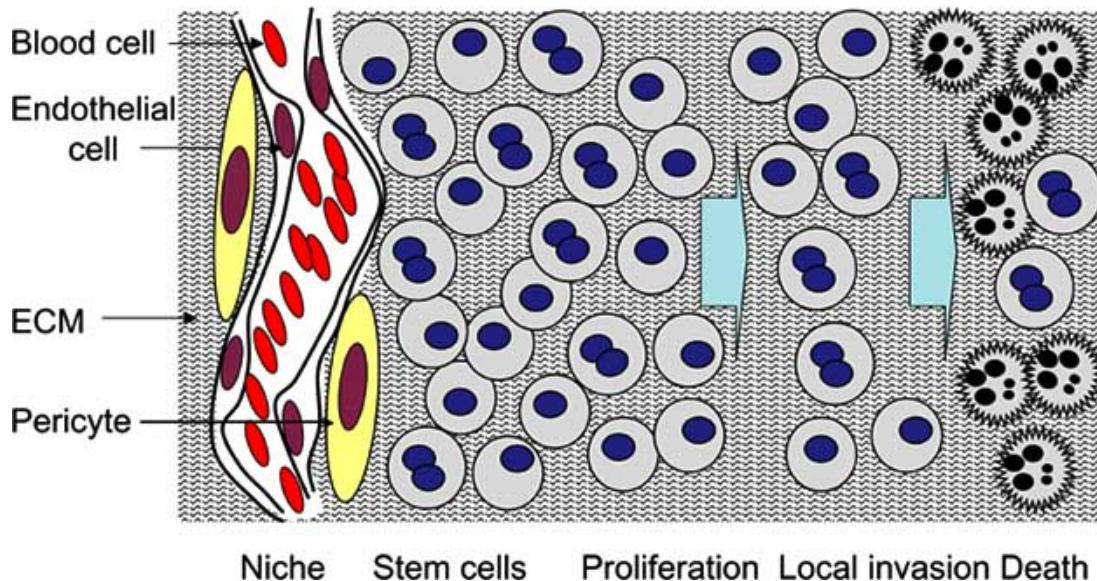


Fig. (2). Diagram of a tumor stem cell niche. As with the normal stem cell niche, it contains vascular endothelial cells, pericytes and the extracellular matrix, but the tumor stem cells are all in cycle rather than in G_0 -phase. As each stem cell undergoes cell division, one daughter cell remains as a stem cell while the other leaves the niche to proliferate further and to migrate locally into tissue. The proliferating cells cannot revert to a non-cycling state, are therefore unable to undergo complete differentiation, and eventually die, probably by apoptosis.

kinase in these cells [15], acts on tumors. The data suggest that although imatinib is a potent inhibitor of the production of partially differentiated leukemia cells, it is ineffective in reducing the small stem cell population from which they are derived [16].

Tumor stem cells, like normal tissue stem cells, may have both intrinsic, multidrug resistance mechanisms that protect them from apoptosis [17] as well as extrinsic mechanisms mediated by survival factors generated within the niche. Although tumor stem cells may have lost the ability to shuttle between cell cycle and quiescence, they may still have long cycle times and these can modify the efficacy of cytotoxic drugs currently used in cancer treatment. When human tumor tissue is partially disaggregated to preserve, as much as possible, the microenvironmental features, the cell cycle times of the resulting cultures vary according to the tumor from three days to several weeks, suggesting that genetic and/or microenvironmental mechanisms regulate cell cycle length [18].

STRATEGIES FOR THE THERAPEUTIC ELIMINATION OF TUMOR STEM CELLS

Strategies for reduction of the stem cell population must take into account the importance of targeting tumor cell niches while sparing normal stem cell niches. Tumor stem cells may be extremely well protected from apoptosis by survival factors provided by the stromal cells in the niche. The transition of individual tumor cells from cell cycle to apoptosis appears to be a random event and might be best modeled by a probability function [19]. It is known that the expression of some proteins associated with the induction of apoptosis, such as p53, oscillates with time as a result of a positive feed back mechanism on transcription [20] and such oscillations may control the probability of apoptosis. Although tumor cell death is controlled by several mechanisms, one of the dominant mechanisms involves mitochondria [21]. A permeability change in the outer mitochondrial membrane leads to the release of cytochrome c, Smac/DIABLO and other proteins that activate the caspase cascade leading to cell death. The permeability change is in turn regulated by the relative concentrations of the large family of interacting proteins called the Bcl2 family, which includes a balance of pro-apoptotic and anti-apoptotic members. The situation is summarized in (Fig. 3) and the challenge for the elimination of tumor stem cells is to increase the ratio of pro-apoptotic and anti-apoptotic signals by chemotherapeutic means.

Both radiation and cytotoxic drugs induce DNA damage, which can result an increased probability of apoptosis. The induction of double stranded DNA breaks leads to the activation of the Ataxia-telangiectasia mutated (ATM), while the stalling of DNA replication forks leads to the activation of ATM- and Rad3-related (ATR) kinase [22], resulting in long-lasting effects which include an increased rate of apoptosis and a reduced rate of proliferation. The ATM and ATR kinases activate many cellular signaling pathways including those involving the p53 and p73 transcription factors. These in turn direct the synthesis of pro-apoptotic members of the Bcl2 family such as bax and PUMA [23, 24]. DNA damaging agents, as well as drugs such as paclitaxel,

also cause activation of ceramide synthase and a corresponding increase in the cellular concentrations of the lipid messenger ceramide [25, 26]. Ceramide, by activating phosphatases and other processes, decreases the activity of the serine/threonine kinase AKT, which protects cells from apoptosis through several pathways including the inactivation of BAD, a pro-apoptotic member of the Bcl2 family [27] (Fig. 3). Inhibition of AKT thus increases the chances of apoptosis [28].

While radiation and cytotoxic drugs generally change the balance of members of the Bcl2 family towards apoptosis, external survival factors such as epidermal growth factor (EGF), insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF), acting through their corresponding receptor tyrosine kinases and the enzyme phosphoinositol-3-kinase (PI3K), shift the balance towards survival. One pathway involves the phosphorylation and activation of AKT, which leads to the inactivation of BAD, a pro-apoptotic member of the Bcl-2 family (Fig. 3). External signals acting on receptors regulating developmental pathways, which may be active in tumor stem cells, may also lead to increased survival. For example, the hedgehog developmental signaling pathway leads to the increased concentrations of the transcriptional activator gli-1, which is up-regulates Bcl2 expression and protects against apoptosis [29].

Inhibition of the binding of such survival factors, or the inhibition of downstream kinases and other signaling pathways, might add to the effects of DNA damage outlined above. The clearest example is provided by the humanized antibody trastuzumab (Herceptin) [30] which acts on one of the receptors of the epidermal growth factor receptor pathway and has activity against a number of tumors but particularly of breast cancer. Trastuzumab is in clinical use and it is highly effective in combination with cytotoxic therapy [31]. Many other inhibitors of the EGF pathway are undergoing development and evaluation at present. Gefitinib (Iressa) [32] acts on the tyrosine kinase associated with the EGF receptor. It has been registered for clinical use and has shown single-agent activity against some lung cancer patients, particularly those that have a mutated EGF receptor, but has not so far been shown to combine well with cytotoxic therapy [33]. Although a full survey is outside the scope of this review, there are many other inhibitors of the growth receptor-PI3K-AKT pathway, including cetuximab and elotinib, acting on the EGF receptor [33], and imatinib (Glivec, Gleevec) acting on the platelet-derived growth factor (PDGF) and KIT receptors [34]. A further area of potential interest concerns developmental gene regulated pathways such as Hh, Wnt and Notch. The alkaloid cyclopamine, identified natural product on the basis of its teratogenicity to developing lambs, is an interesting example of a drug that inhibits Hh signaling and might therefore be expected to reduce Bcl2 synthesis and to increase the likelihood of apoptosis. The Hh pathway has been identified in small cell lung cancer, in various aspects of gastrointestinal development and in pancreatic cancer tumorigenesis [35]. Experimental studies have demonstrated that cyclopamine inhibits growth of a mouse medulloblastoma line both *in vitro* and *in vivo* [36] and patents have been issued for analogs [37].

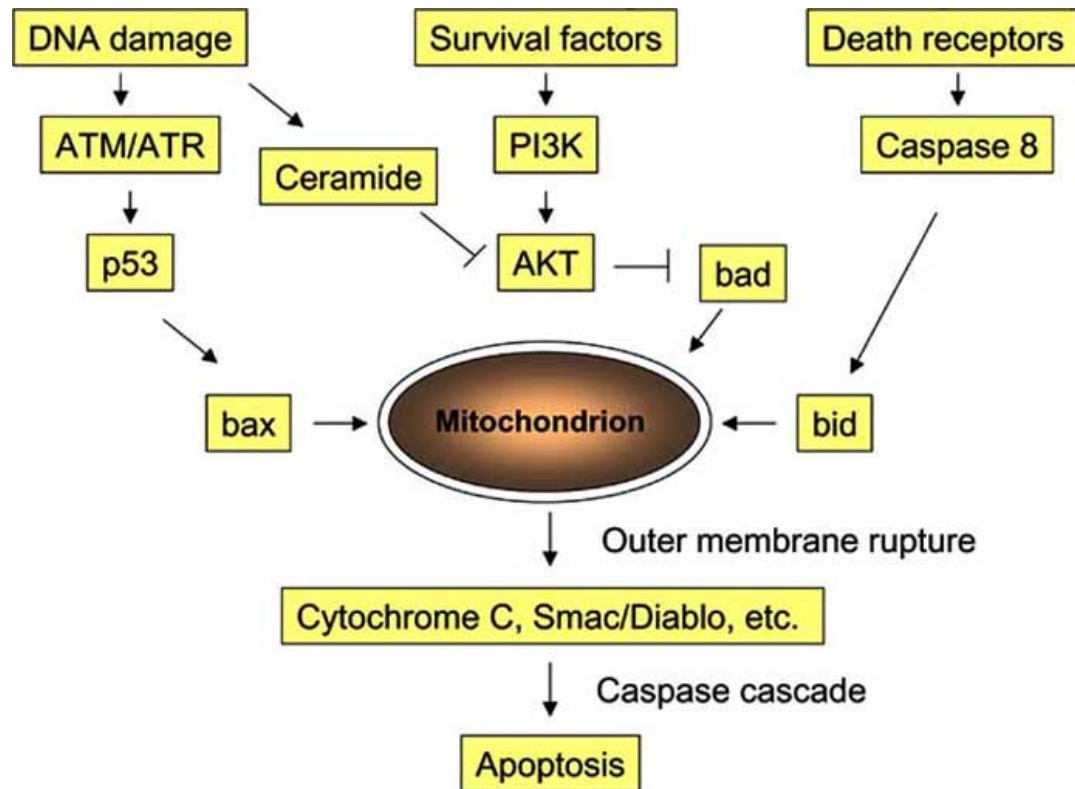


Fig. (3). Control pathways regulating the transition of stem cells to apoptosis. The scheme is conceptual only, since many pathways are omitted. A stream of survival signals from the stroma act through the PI3K-AKT pathway to produce anti-apoptotic signals including the inactivation of BAD, a pro-apoptotic member of the Bcl-2 family. Drugs inhibiting the action of these survival signals increase the action of BAD. Cytotoxic drugs that damage DNA activate the ATM and ATR kinases, which produce, among other mediators, bax, a pro-apoptotic member of the Bcl-2 family. Cytotoxic drugs and mitotic poisons also induce ceramide synthesis, leading to dephosphorylation and inactivation of AKT and to increased amounts of BAD. Death receptors, activated by DNA damage, induce several pathways including bid, a pro-apoptotic member of the Bcl-2 family. The mitochondrion sums the effects of pro- and anti-apoptotic members of the Bcl-2 family and appropriate conditions, the outer mitochondrial membrane ruptures, leading to activation of the caspase cascade and to apoptosis.

An intrinsic problem in the approach of combining cytotoxic therapy with inhibition of survival pathways is that little is known of the survival pathways favored by individual human tumors. Most chronic granulocytic cancers respond dramatically to imatinib because the bcr-abl fusion kinase in these cells provides major survival stimulus [38]. A small proportion of non-small cell lung cancers respond to gefitinib because a mutant EGF receptor delivers a strong survival stimulus [39]. How can one tell whether an individual's tumor will be dependent for survival on a given factor? Gene expression profiling may provide information, but this will of necessity use total tumor material rather than tumor stem cells from a niche. One approach considered in this laboratory has been to culture surgical material for a short time in the presence of an inhibitor of EGFR. The material is only partially disaggregated to preserve the cellular architecture as much as possible. The period of incubation (7 days) is long enough to detect proliferating cells that may include stem cells. The results show that a proportion of non-small cell lung cancers and of ovarian cancers are strongly inhibited, that a proportion do not respond, and a small proportion are actually stimulated by the drug [18, 40].

STRATEGIES TARGETING THE TUMOR CELL NICHE

One might expect that chemotherapeutic reduction of the tumor stem cell population would be followed by repopulation by surviving tumor stem cells. It is therefore pertinent to consider whether it might be possible to disable the tumor stem cell niche to hinder such repopulation. Since the niche is totally dependent on the presence an efficient blood supply, one therapeutic strategy would be to target the niche endothelium. Tumor tissue is generally characterized by chaotic tissue organization and consequent poor vascular access [41], but it is quite possible that tumor stem cell niches are well endowed with a blood supply and that delivery of both low molecular weight drugs, antibodies and other therapeutic molecules to the niche can be quite efficient. This strategy requires an element of selectivity for the tumor cell niche as opposed to a normal niche, and although we can only speculate at this stage, there are several ways in which this may occur. Tumor stem cells may modify the physiology of the niche, for instance by excessive production of signaling molecules such as vascular endothelial cell growth factor (VEGF), and this may confer selective sensitivity to some drugs or antibodies. Tumor

tissue may also be more immunologically reactive than normal tissue and signaling molecules such as cytokines might modify the physiology of the niche.

Blockade of the action of VEGF represents a direct method of inhibiting VEGF signaling. Bevacizumab (Avastin) is a humanized antibody that binds to VEGF and reduces its extracellular concentration. Although bevacizumab could act to antagonize the activity of VEGF as a mitogen, it might also antagonize its action as a survival factor, acting through the PI3K-AKT pathway. In fact, a study in rectal carcinoma patients showed that bevacizumab decreased both tumor perfusion and microvascular density, suggesting that this agent could act on established tumor vasculature [42]. Bevacizumab might then complement the action of cytotoxic therapy, as shown in Fig. 3. Clinical trials of bevacizumab in combination with cytotoxic 5-fluorouracil-based therapy have shown considerable promise and applications to the treatment other cancer types are well underway [43].

The concept that bevacizumab could act as a vascular disrupting agent in tumor stem cell niches raises the question of whether other vascular disrupting agents might similarly be deployed in combination treatment. A number of such agents, including 5,6-dimethylxanthenone-4-acetic acid (DMXAA) [44] and combretastatin A-4 phosphate (CA-4P) [45], are now undergoing clinical trial [46]. DMXAA is known to induce selective tumor endothelial cell apoptosis [47], although its mechanism of action is still unclear. CA-4P acts on the cytoskeleton of tumor endothelial cells to distort their shape. Even "classical" antiangiogenic drugs such as endostatin are known to exert an antivascular action in addition to their other effects on endothelial cells [48]

CURRENT AND FUTURE DEVELOPMENTS

Early approaches to the development of chemotherapeutic agents for cancer were concerned almost exclusively with the cancer cell as a target and many of the cytotoxic drugs in use today were first shown to be active in a murine leukemia ascites assay, such as the L1210 or P388 models [49]. The move from an *in vivo* to an *in vitro* model using a panel of cell lines [50] reinforced the focus on the tumor cell. The concept of the vascular environment as a potential target for chemotherapy [51] led to a change in thinking about cancer, as well as to the development of antiangiogenic drugs designed to prevent the expansion of the tumor's blood supply [52]. Although the principle that tumors are populated by stem cells is not new, the concept of tumor stem niches as targets provides many challenges for new therapeutic approaches.

It is clear from the issues discussed in this review that many, perhaps most, aspects of this concept are speculative and require further experimental substantiation, and the approach considered here of combining cytotoxic agents with inhibitors of signal transduction in both tumor and endothelial cells is only one of several that can be envisaged. There is an urgent need for improved assay systems in both experimental and clinical settings that will monitor the effects of individual drugs and of drug combinations.

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