Anti-Cancer Drugs 1995, **6**, pp. 3–18

Review paper

Consequences of angiogenesis for tumor progression, metastasis and cancer therapy

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The growth of solid tumors to a clinically relevant size is dependent upon an adequate blood supply.[1] This is achieved by the process of tumor stroma generation where the formation of new capillaries is a central event.[1,2] Progressive recruitment of blood vessels to the tumor site and reciprocal support of tumor expansion by the resulting neovasculature are thought to result in a self-perpetuating loop helping to drive the growth of solid tumors.[3] The development of new vasculature also allows an 'evacuation route' for metastatically-competent tumor cells, enabling them to depart from the primary site and colonize initially unaffected organs.[4] Several molecular and cellular mechanisms have been identified by which tumor parenchyma may exert its angiogenic effect on host endothelial cells.[1–3,5–7] As a result of this paracrine influence, tumor-associated endothelial cells acquire an 'immature' phenotype[1] manifested by rapid proliferation, migration, release of proteases and expression of cytokines, endothelial-specific tyrosine kinases (e.g. *flk-1*, *tek* and others) as well as numerous other molecular alterations.[3] Consequently a network of structurally and functionally aberrant blood vessels is formed within the tumor mass.[8] There is also evidence that endothelial cells themselves, and likewise other stromal cells, may act reciprocally to alter the behavior of adjacent tumor cells in a paracrine or cell contact mediated fashion.[3] For example, production of interleukin 6(IL-6) by endothelial cells may have a differential effect on human melanoma cells expressing different degrees of aggressiveness.[9] In this manner endothelial derived cytokines could conceivably contribute to tumor progression by suppressing the growth of the less aggressive tumor cells and promoting dominance of their malignant counterparts in 'strategic' perivascular zones. Distinct biological features expressed by tumor-associated vasculature may serve as potential prognostic markers of disease progression as well as novel targets for therapeutic intervention.

Key words: Angiogenesis, cancer therapy, metastasis, tumor progression.

Introduction

The rapid progress made in molecular methodology over the last two decades has resulted in an impressive series of discoveries regarding the genetic events involved in tumor development and progression. Activation of dominantly acting oncogenes or loss of recessively acting tumor- or metastasis-suppressor genes have been frequently detected in various types of cancer.[10,11] These remarkable advances in our understanding of the nature of malignant transformation have promoted the reductionist notion that cancer—an otherwise multicellular and extremely complex disease—can be understood primarily if not exclusively on the basis of a detailed analysis of the growth control abnormalities expressed by various clonal tumor cell lines in tissue culture.

Until recently most models describing and explaining the course of tumor progression focused on aspects of tumor cell autonomy, and put much less emphasis on the role of tumor–host interactions as an essential part of the process.[11,12] Recent findings, however, suggest that several genes, although fulfilling criteria of tumor or metastasis suppressors, do not necessarily affect tumor cell proliferation directly. Rather their activity seems to be necessary to restrain tumor growth promoting cell–cell or cell–extracellular matrix interactions. This mode of action has been suggested for several different molecules including tissue inhibitor of metalloproteinases (TIMP),[13] E-cadherin,[14] DCC,[11] connexin 32,[15] a suppressor gene that controls thrombo-

The work in RSK's laboratory is supported by grants from the National Cancer Institute of Canada, Medical Research Council of Canada and National Institutes of Health, USA (CA4-1233). RSK is a Terry Fox Cancer Research Scientist of the National Cancer Institute of Canada.

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spondin expression[16] and a number of other molecules.[17,18]

Studies on the role of oncogenes and tumor suppressor genes in the pathology of tumor stroma generation are clearly gaining momentum in current efforts to understand basic principles of tumor growth and metastasis.[5] For example, loss of a tumor suppressor gene controlling the oncogenic behavior of BHK cells results in conversion to a tumorigenic phenotype and is strictly coordinated with a shut off in the expression of an angiogenesis inhibitor homologous to thrombospondin (TSP).[5] Similarly in fibroblasts from patients with Li–Fraumeni (L–F) syndrome, spontaneous loss of the remaining single copy of the wild-type p53 tumor suppressor gene leads to a down-regulation of TSP expression,[19] supporting the notion that p53 regulates TSP, which in turn regulates angiogenesis and endothelial cell growth. Interestingly p53 gene 'knock-out' in mice leads to a high frequency (30%) of endothelial neoplasia.[20] In another study, transgenic mice carrying the *neu* oncogene driven by an MMTV promoter were found to develop mammary tumors, which were not only highly aggressive, but also highly angiogenic.[21] (Muller, personal communication.) It has also been reported that progression of benign hyperplasia to rapidly growing fibrosarcomas in mice carrying an oncogenic transgene is associated with the onset of secretion of the highly angiogenic cytokine, basic fibroblast growth factor (bFGF).[22] Similarly, in isolated pancreatic islets from transgenic mice carrying a viral oncogene driven by the insulin gene promoter, the acquisition of angiogenic potential preceded tumorigenic conversion by these pancreatic multicellular aggregates.[23] Some of the generic mechanisms found to be involved in the 'angiogenic switch' during tumor progression are listed in Table 1. Overall, such findings are consistent with the view that the nature of the growth control defect in cancer comprises molecular mechanisms operating within transformed tumor cells but also includes intercellular interactions at the interface between tumor and host.[3]

Reciprocal interactions between tumor cells and host stromal cells, in addition to affecting tumor growth,[24,25] may also contribute to tumor cell invasion of the surrounding tissues and metastatic dissemination to distant organs. In this respect, recent evidence suggests that some of the proteolytic enzymes generally associated with tumor cell invasion and metastasis are in fact produced by 'activated' stromal cells rather than by tumor cells *per se*. These stromal cell associated enzymes include, e.g. stromyelysin 3,[26] collagenase type IV[27] and plasminogen activator (uPA).[28]

Thus, interactions between transformed parenchymal tumor cells and 'activated' host stromal cells are likely to contribute to a self-accelerating chain of events driving tumor progression as well as tumor growth.[3] Consequently, therapeutic strategies aimed at disrupting such tumor–host interactions could facilitate stabilization of the disease and possibly lead to improvements in long-term survival rates, if not cure. In this regard targeting host-derived blood vessels within solid tumors offers a unique therapeutic opportunity based on the absolute dependence of solid tumor growth on this single stromal component.[29] The remainder of this review will focus on some of the interactions between tumor cells and endothelial cells as well as the consequences these interactions may have on tumor behavior and success or failure of anti-cancer therapy.

Table 1. Molecular mechanisms of the 'angiogenic switch'

Generic mechanism	Molecule involved (example)	Tumor type	Observation	Reference
Onset of TAF[a] production	? bFGF	pancreatic islets in transgenic mice; human melanoma	transformation is associated with the onset of TAF production	23 96
Onset of TAF secretion	bFGF	fibrosarcoma in mice carrying BPV trans gene	onset of the bFGF export during tumor progression	22
Loss of angiogenesis inhibitor expression	thrombospondin, (p53)	BHK cells; human breast cancer cells; trans formed L-F fibroblasts	loss of tumor suppressor gene is associated with loss of TSP expression	19, 154
'Masterswitch effect'	*ras*; genes regulated by *ras* (TGFa, VPF?)	intestinal cancer; colon cancer?	*ras* transformed cells acquired angiogenic phenotype	J Rak, J Filmus and RS Kerbel, unpublished

[a]TAF, tumor angiogenesis factor.

Tumor progression and metastasis

The fundamental observation that tumor growth is not only associated with an increase in size of the lesion but, more importantly, with qualitative changes in its characteristics, led to the concept of 'tumor progression'.[30] It is generally believed that during this process tumor cells acquire features of greater malignancy. Some examples include hypersensitivity to growth factors resulting in increased growth potential, invasiveness, metastatic competence as well as resistance to normal regulatory cytokines and therapeutic agents.[30–32] The pace of these changes varies between individual cases, ranging from slow and continuous to stepwise, rapid and sometimes fulminant. For example, rapid, probably one-step acquisition of a highly malignant phenotype is observed in what is referred to as 'type II progression'. This type of progression is frequently linked with cases of so-called 'unknown primary tumors' (UPT).[33] By definition, the first manifestation of UTP is an advanced and usually deadly metastatic disease, *without* the preceding development of an apparent primary lesion.[33] Another aspect that may vary is the pathway of progression of a particular tumor, i.e. the type, seqence and final outcome of changes observed in the phenotype of transformed cells over time.[30,34,35]

It has been postulated that multiple genetic changes observed during human tumor progression may result from either activation of a 'master switch' gene followed by coordinated expression of a particular set of 'responder' genes[36] or separate genetic alterations may occur independently.[11] In this regard a breakthrough came from studies on genetic correlates of colon carcinoma progression.[11] Studies by Vogelstein and colleagues identified sequential chromosomal and genetic abnormalities accompanying clinical steps of disease progression.[11] The sequence with which all these alterations occur is not inviolate and it appears to be their accumulation rather than order which is of biological significance.[11] Similar studies have also been conducted on many other human malignancies, including glioblastoma and melanoma.[37,38]

The discovery of cumulative genetic alterations in colon carcinoma and other tumor types in conjunction with the fact that the vast majority of tumors were found to be clonal in origin[39] understandably highlighted the 'cellular' dimension of the tumor progression process. This is consistent with the notion that the process can essentially be described as a 'clonal evolution' of the transformed cellular lineage.[12,31] According to this concept, the genetic instability of tumor cells and selection by the microenvironment constitute the two main forces driving this cellular 'evolution'. Consequently several cycles of tumor cell diversification and selection could lead to the emergence of a rare 'species', a highly malignant cellular variant, capable of invading the surrounding tissue and metastasizing to distant organs. The execution of the metastatic program would therefore naturally require the expression of a highly specialized phenotype in order to avoid destruction while traversing the many steps and physiological barriers of the 'metastatic cascade'.[40] For example, tumor cells would have to leave the primary site, survive the highly traumatic voyage in the blood stream, extravasate and then expand in the foreign tissue microenvironment of various distant organ sites.[40,41] As predicted by the 'decathlon champion' model of metastasis, even a small minority (a clone) of metastatic cells could eventually kill the host by virtue of their high efficiency in colonizing vital organs outside the primary tumor.[40] Indeed highly metastatic variants have been isolated from various animal and human tumor cell lines or surgical specimens using sequential *in vivo* selection protocols.[32,40] Based on these findings it was anticipated that cells isolated from primary tumors should express entirely different phenotypes than cells that are already present in the metastatic foci. Somewhat surprisingly this was infrequently found to be the case.[32] On the contrary, cells representative of advanced primary tumors and their metastases were often found to be similar or even identical with respect to multiple features, including metastatic competence in experimental animals.[32] This is now thought to be a result of the fact that along with the other necessary components required for metastatic competence, tumor cels are co-selected for progressive growth properties which endow the metastatic subpopulation with a selective growth advantage. The latter feature may eventually lead to partial or complete 'clonal dominance' of metastatically competent tumor cells at the primary tumor site.[32] Quantitative enrichment in those aggressive cellular variants is likely to increase the probability of invasion and formation of secondary tumor deposits in distant organs.[3,32] Thus in addition to intracellular genetic changes, the process of tumor progression also appears to rely on changes occurring at the whole cell population level. Again, several studies have pointed out the fact that many properties of tumors, including growth rate, drug resistance, metastatic capacity and survival, cannot always be predicted by a simple analysis of separated tumor cell clones. Rather, formation of

multicellular functional 'units', 'community effects' or other types of cellular interactions operating in this cellular 'society' are believed to be highly relevant for the expression of the respective tumor properties.[42–48] The recruitment of host stromal cells in general, and angiogenesis in particular, add another level of complexity to the cellular interactions accompanying tumor progression.

Tumor angiogenesis

Solid tumor growth *in vivo* beyond 1–2 mm in diameter is almost without exception associated with recruitment of new blood vessels.[1,29,49] This is a conseqence of the release of angiogenic factors by tumor cells themselves or by infiltrating inflammatory cells.[50,51] There are a number of angiogenic factors which are known to induce the process of new blood vessel formation.[1,3,6,7] Resulting angiogenesis is a multistep process that involves sprouting and subsequent capillary loop formation of endothelial cells, most likely originating from local post-capillary venules.[1,6] The angiogenic stimulus is believed to trigger several functional responses in endothelial cells, including local basement membrane dissolution, endothelial cell migration, proliferation and microvessel morphogenesis.[1,6] The process appears to be regulated by a number of polypeptide cytokines,[7] but also by fibrin,[2] various extracellular matrix molecules, integrins and other adhesion molecules,[52,53] proteolytic enzymes,[54,55] various carbohydrates including hyaluronan,[56] carbohydrate binding proteins,[57,58] steroid hormones,[59,60] lipids, metal ions, and a variety of different small molecular weight substances.[61] An updated list of angiogenic factors and molecules identified thus far can be found in some of the review articles recently published on the subject.[1,5–7,62] Alternative mechanisms of blood vessel formation which do not involve sprouting have also been described recently.[63,64]

In order to measure the angiogenic activity of tumor cells and their angiogenic factors, a number of angiogenesis assays have been developed.[65] Efforts to use these assays as a means of accurately quantitating angiogenic activity as a function of tumor type, disease progression or response to therapy have been largely unsuccessful. It is conceivable that difficulties in quantitating the stimulatory ('afferent') component of the angiogenic reaction may be due to the fact that the response of endothelial cells to angiogenic cytokines is not always linear. For example, endothelial cells respond in a somewhat biphasic fashion to increasing concentrations of angiogenic polypeptides such as acidic or basic fibroblast growth factor (aFGF and bFGF) or tumor necrosis factor (TNF)-α, both *in vivo* and *in vitro*[66,67] (Rak, unpublished observations). Interestingly, a number of angiogenesis stimulators, including bFGF, are produced by endothelial cells themselves, either constitutively or upon stimulation, and may act on these cells in an autocrine manner.[68,69] The latter situation may conceivably lead to an 'explosive' self-amplifying event rather than a typical dose-dependent process limited by the availability of the stimulant. On the other hand, the aforementioned angiogenesis assays have been used successfully for quantitation of angiogenesis *inhibitors*.[66] This may be explained by the fact that angiosuppressive agents may interfere predominantly with the rate limiting, effector ('efferent') arm of the angiogenic process.

The course of angiogenesis probably depends on multiple factors including the nature of the inducing stimulus and characteristics of local endothelial cells in a particular target organ. Nevertheless, there seem to be two generic differences between angiogenesis taking place in normal as opposed to tumor tissue. First, tumor expansion is continuous and this usually triggers chronic vascular growth and remodeling rather than finite repair processes.[2,8,70] Second, the tumor neovascularization leads to a wide range of structural and functional blood vessel abnormalities, including tortuosities, dilatations, arterial-venous (A-V) shunts and trifurcations,[8] that are normally not found during the course of 'normal' angiogenesis. In addition, tumor-associated blood vessels also have poorly developed basement membranes and lack collaterals, innervation and pericytes. Some of these factors may contribute to blood vessel leakiness. Blood vessel permeability may also be regulated by certain cytokines such as the angiogenic polypeptide vascular permeability factor (VPF), also known as vascular endothelial growth factor (VEGF),[71,72] which is secreted by various types of tumor cells.[71–74]

These multiple abnormalities in blood vessel function and architecture are likely to result in disturbances in blood flow, with frequent stasis and changes in flow direction, leading to impairment in metabolite exchange.[8,75] In the absence of functional lymphatic drainage of the tumor tissue, the resulting high interstitial pressure within solid tumors may further compromise the penetration of nutrients into the deeper layers of the tumor and ultimately lead to local necrosis.[8] Thus tumor perfusion

appears to be inefficient, despite an abundant and continuously expanding neovasculature.

Interactions between heterogenous tumor cell subpopulations and the vasculature

As discussed at the outset, it has been well documented that even within a single tumor lesion tumor cells are highly heterogenous.[40,44,76] It is also believed that continuous generation of new cellular variants leads to the acquisition of more invasive, metastatic or drug resistant properties.[32,76] Consequently, various tumor cell subpopulations may also differ in their ability to elicit neovascularization.[3,44,77–79] For example, different types of angiogenic signals may be released by different subpopulations of tumor cells due to variable expression of a number of genes encoding potentially angiogenic cytokines. Some of these 'tumor angiogenesis factors' may be expressed constitutively or may be switched on in response to local microenvironmental stimuli. For example, recent studies suggest that under hypoxic conditions, glioblastoma cells switch on expression of VEGF/VPF, which otherwise is usually not expressed by this tumor type.[73,74] It is also possible that angiogenic signals may be generated by host stromal or inflammatory cells present at the tumor site as a result of stimulation by paracrine factors elaborated by various subpopulations of tumor cells.[3,21,50,80] Conversely, as will be discussed later, some endothelial cell products may also selectively alter the behavior of adjacent tumor cells. Thus tumor–endothelial cell interactions may generally be thought of as reciprocal, dynamic and selective (Figure 1).

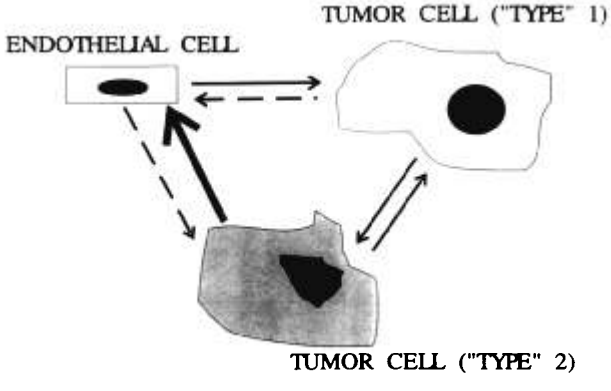

Figure 1. Interactions of heterogenous tumor cells with endothelial cells. Endothelial cells not only receive angiogenic stimuli but may also deliver regulatory signals to tumor cells. Tumor cell subpopulations (types) may differ in their capacity to generate and respond to those regulatory signals. Interactions between heterogenous tumor cells and endothelium are likely to be: 1, reciprocal; 2, dynamic; 3, selective; 4, direct and indirect.

Tumor cell subpopulations are likely to emerge and expand within the tumor mass in certain microdomains that would require a distinct blood vessel supply for their continued growth. If tumor cell subpopulations occupying adjacent microdomains differ in their angiogenic capacity one would anticipate finding greater blood vessel densities in some microregions than in others.[3] Indeed such vascular 'hot spots' have been found in tissue specimens from breast and prostate cancer.[81–84] More importantly, the dense vascularity of these 'hot spots' appears to correlate with an unfavorable prognosis in these tumor types,[81] suggesting that tumor cells occupying these regions are likely to be both highly angiogenic and metastatic.[3] It is also possible that blood vessels attracted by a more angiogenic tumor cell subpopulation may facilitate growth, and even metastasis, of adjacent and relatively less angiogenic tumor cells.[44,77] Although demonstrated in some cases,[77] this 'co-operative mode' of tumor–endothelial cell interaction stands in contrast to work which has shown that more malignant (and presumably more angiogenic) tumor cells sometimes overgrow ('dominate') their less aggressive counterparts *in vivo*.[32] We postulate therefore that more often than not, different tumor cell subpopulations 'compete' rather than 'co-operate' during tumor neovascularization and that a greater angiogenic capacity may endow tumor cells with a selective growth advantage[3] (Figure 2).

The role of angiogenesis during tumor progression: cutaneous malignant melanoma as a paradigm

Blood vessels recruited by angiogenic stimuli contribute to solid tumor growth by maintaining perfusion and hence viability of tumor parenchyma.[1,85] During tumor development the 'switch to the angiogenic phenotype' was shown to be strictly correlated with the acquisition of progressive growth properties by the tumor cells.[22,23,29,85] Furthermore, the increase in blood vessel counts in hypervascular 'hot spots' was shown to be associated with a poor prognosis in breast or prostate cancers.[84] Vascularity has also been associated with tumor progression in cutaneous melanoma.[35,49,78,86–89]

Cutaneous malignant melanoma progression recently attracted much experimental attention for a

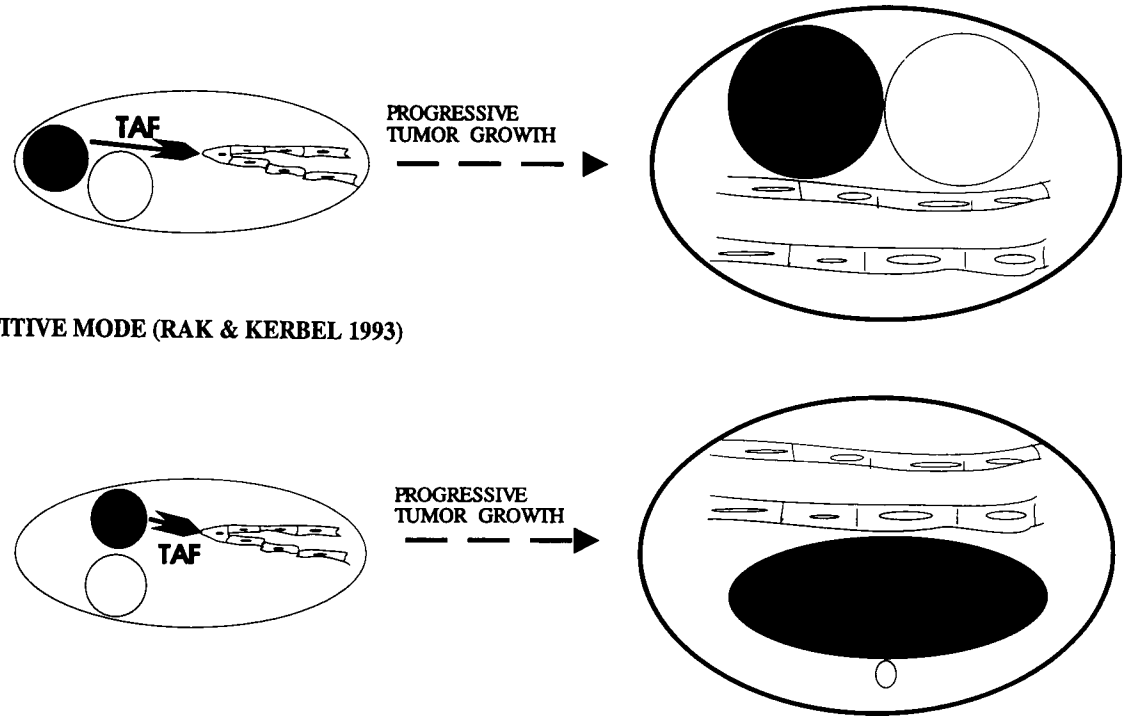

Figure 2. Two current hypotheses predicting that either the competitive or the co-operative mode of interaction governs tumor subpopulation expansion. Tumor cells producing tumor angiogenesis factor (TAF) may either facilitate expansion of the adjacent less angiogenic counterparts or successfully compete with the latter for blood vessels and thereby acquire a selective growth advantage.

number of reasons. Poor prognosis and rapid development of clinically malignant disease were found to correlate with increasing thickness of primary lesions, especially beyond a threshold level of 0.76 mm.[90–92] Because lesions from different phases of tumor development are readily accessible for observation and sampling, it became possible to describe distinct stages of melanoma progression and sometimes establish corresponding cell lines in tissue culture.[91–93] It is believed that early, curable melanoma usually begins as a so-called radial growth phase (RGP) lesion which is thin, often confined to the epidermis and avascular. Expansion of melanoma into the dermal mesenchyme is associated with the vertical growth phase (VGP) of the tumor. During this phase the lesion is evolving towards an invasive, thick, vascular and eventually incurable metastatic tumor.[89,91,92,94,95]

Melanoma cell lines representative of sequential stages of tumor progression express a host of differential characteristics reminiscent of their in situ phenotype.[92] Differences in production of various cytokines, some of which possess angiogenic activity, have been noted between cells from 'early' versus 'late' melanoma lesions.[78,96,97] Differences also exist between early and late stage melanoma cells in their responsiveness to a number of growth inhibitory cytokines including TGF-β, IL-1, IL-6, OSM and others. Progression of early phase melanoma cells to a state of so-called 'multicytokine resistance' appears to parallel the increase in clinical aggressiveness of the respective lesions.[94,98–101] Interestingly, endothelial cells are also capable of producing a number of these potentially growth inhibitory cytokines.[3,9] In fact, it has recently been documented that during angiogenesis, endothelial cells overexpress at least one of these cytokines, i.e. IL-6.[102] It is also known that, although RGP melanomas usually remain avascular, the tumor cells, even at this early phase of disease progression, produce some potentially angiogenic polypeptides, including bFGF.[96,97,103] One would anticipate then, that once these angiogenic factors are released, the resulting onset of angiogenesis in an early stage melanoma may actually have a somewhat 'suicidal' effect on the tumor cells themselves, in that it would expose these cytokine-sensitive cells to endothelium derived growth inhibitors.[3] For further tumor growth it would seem necessary that this effect be overcome by selection of cytokine

resistant tumor cell variants. Indeed, we have recently demonstrated that melanoma cells isolated from different stages of the disease progressively lose their sensitivity to endothelial cell derived growth inhibitors, including IL-6.[9] That is consistent with results reported recently by Barnhill *et al.*[89] These investigators found that transition from RGP to VGP melanoma is frequently associated with a sudden burst of angiogenesis. Paradoxically the high blood vessel count of the 'progressive' RGP tumors coincided with histological 'regression' of the tumor and with a greater risk of metastasis at the later stages of disease progression.[89,104] The most recent evidence indicates that high vascular counts detected in thin primary melanomas, which otherwise carry a favorable prognosis, is predictive of recurrent, fatal metastatic disease.[105] Such a predictive association was also observed in thicker, more advanced primary lesions by some investigators[49,86–88] but not by others.[105,106] Thus a causal relationship may exist between the initial 'switch to the angiogenic state' and histological regression followed by progression of RGP melanoma toward metastatic disease (Figure 3). Tumor progression in this case may be driven by selection of more malignant cellular variants that can withstand the exposure to inhibitory cytokines associated with the presence of an abundant vasculature, whereas their cytokine-sensitive counterparts may undergo complete or partial extinction.[3,9,91,107,108] It is not clear whether the regression observed in cases of hypervascular RGP melanoma can be entirely attributed to endothelial derived IL-6 or if other inflammatory cytokines are involved as well.[89]

Also of interest is the fact that IL-6 was shown to stimulate the motility of breast cancer cells *in vitro*.[109] It is tempting to speculate that in breast cancer greater motility (invasiveness) of cancer cells may be induced by the presence of adjacent endothelial cells in vascular 'hot spots' as observed in

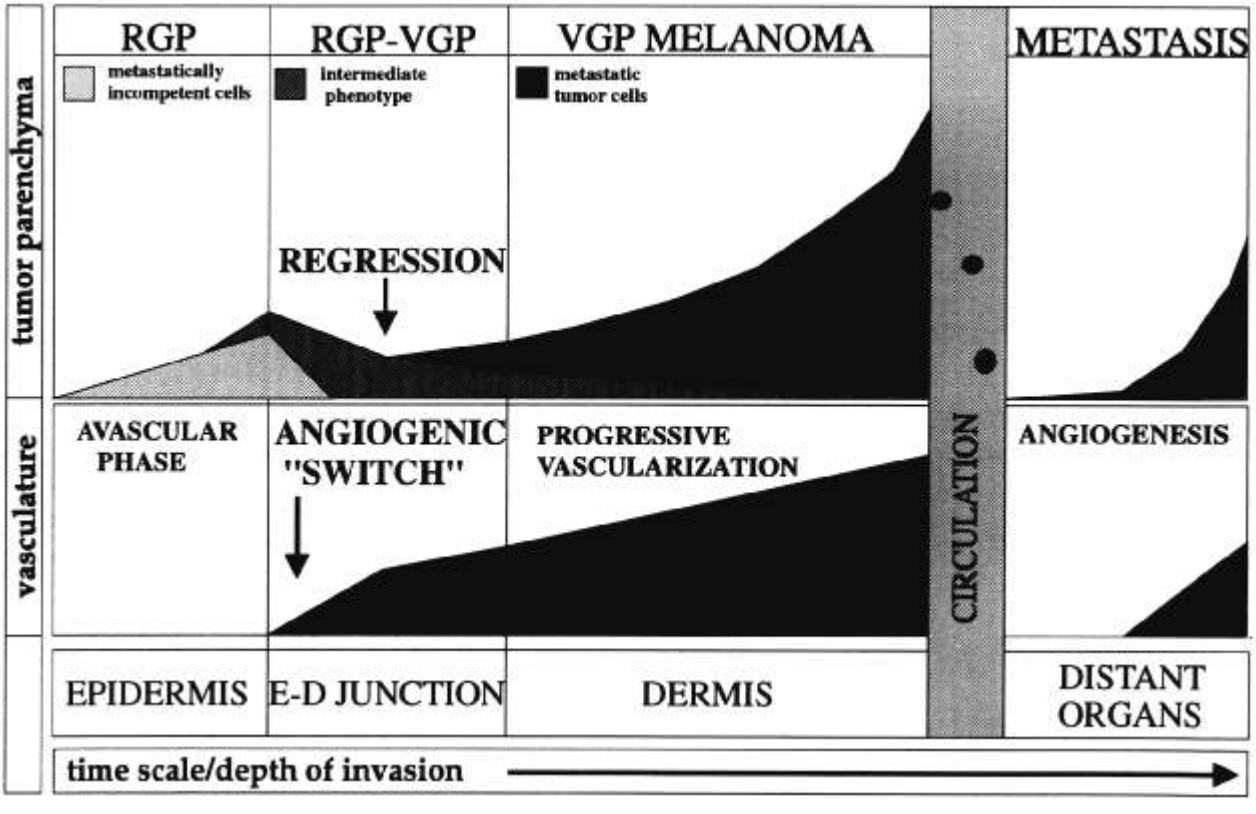

Figure 3. Interrelationship between angiogenesis and the stage of progression in malignant melanoma. Transition from RGP to VGP is frequently associated with histological regression within the lesion. This regression coincides with a 'switch' to the highly angiogenic state. At this stage of tumor progression abundant blood vessels are frequently observed. In the presence of endothelial cells and inflammatory cells the more aggressive melanoma cell populations (black) may acquire a growth advantage over their non-metastatic counterparts (light gray; dark gray represents intermediate phenotypes). These effects may be mediated by IL-6 and other cytokines that can selectively inhibit the growth of early stage melanoma cells. Further increase in tumor vascularity, a secondary 'angiogenic switch', is frequently observed during the later stages of disease progression. This cascade of events may facilitate tumor cell dissemination and metastasis.

breast cancer.[84] This may be another explanation for why a high blood vessel count in these 'hot spots' is thought to be a correlate of subsequent metastatic disease.[84]

Characteristics of tumor-associated endothelial cells

Cells from the endothelial lineage express multiple levels of heterogeneity.[110] For example, even endothelial cells in the same vessel have been shown to display different turnover rates.[111] Moreover, endothelium from large vessels was shown to be phenotypically and functionally different from microvascular endothelial cells.[112] It is also known that isolated endothelial cells in tissue culture can be highly diverse in terms of their growth and tubule forming capacity, as well as other properties.[110,113,114] It may be that some kind of stem cell-like hierarchy exists in the endothelial cell lineage that allows a self renewal of these cells upon injury.[3] Furthermore, organ- or vascular bed-specific features have been found in different types of endothelium.[110,115] Some of these features have been implicated in organ-specific homing of lymphocytes.[116] In addition, organ preference can apparently play an important role in the phenomenon of organ-specific metastasis.[117] The differential expression of adhesion molecules and growth or motility factors by endothelial cells in different vascular beds was implicated in this so called 'seed and soil effect'.[117,118]

Although it is conceivable that certain properties of tumor-associated endothelial cells are to some extent site-dependent,[115] there is also evidence that distinct phenotypic features are induced by the tumor microenvironment itself. Recent animal studies have shown that the labeling index of tumor-associated endothelial cells is up to 50-fold higher than their quiescent counterparts from healthy tissues.[119] This high proliferation rate is likely to be due to the imbalance between angiogenesis stimulators and inhibitors at the tumor site.[54]

At the molecular level, the 'activated' (and functionally immature) endothelial cell phenotype is associated with a unique pattern of gene expression.[120] Some of the molecules expressed by tumor-associated (but not by normal quiescent) endothelial cells have been identified using monoclonal antibodies, e.g. the difference in expression of the antigen recognized by the EN7/44 monoclonal antibody.[121] This antibody was raised against highly vascular fragments of human breast carcinoma tissue, and was found to preferentially react with budding blood vessels in breast cancer and mammary hyperplasia.[121] Another antibody, designated FB5, originally raised against cultured fetal fibroblasts, was shown to react with endosialin molecules expressed specifically by blood vessels associated with several types of human cancer.[122] Similarly, the recently described antibody 4A11 preferentially reacts with endothelial cells in adrenal tumors, Kaposi's sarcoma and a variety of inflammatory lesions.[123] Two monoclonal antibodies called TEC4 and TEC11 were shown to recognize endoglin, a cell surface proteoglycan that is expressed selectively by tumor-associated endothelial cells.[124]

A distinct pattern of gene expression in tumor-associated endothelial cells was also detected at the mRNA level. For example, overexpression of mRNA for the c-*ets* transcription factor (and oncogene) was detected in tumor-associated blood vessels as well as in exponentially growing or cytokine-stimulated endothelial cells in culture.[125] This gene is believed to be involved in regulating the expression of extracellular matrix degrading enzymes which are thought to play a role in angiogenesis.[125] Endothelial cell specific receptor tyrosine kinases (see Table 2) encoded by the genes *tie-1*,[126] *flk-1*[127,128] or *tek*[129–131] have been recently described. Both *flk-1* and *tek* receptor tyrosine kinases are expressed by endothelial cell precursors during embryogenesis in the mouse[128,129,132,133] and also in blood vessels associated with xenotransplants of human malignant melanoma cells in nude mice (Yamaguchi and Rak, 1991, unpublished). No such expression of *flk-1* or *tek* was observed in surrounding healthy tissue in tumor-bearing mice[128] (Yamaguchi and Rak, 1991, unpublished). The similarity between 'embryonal' and 'tumor associated' endothelial cells with respect to *flk-1* and *tek* gene expression suggests that tumor microenvironment may *induce* or *select* some sort of 'embryonal' or 'stem cell' phenotype in endothelial cells recruited to the tumor site. Since *flk-1* acts as a functional receptor for vascular permeability factor VPF/VEGF,[132,134] interference with its function would be expected to produce some kind of anti-angiogenic and antitumor effect. Indeed, treatment of tumor-bearing mice with a neutralizing antibody to VEGF was found to produce such an effect.[135] Moreover co-injection of C6 mouse glioblastoma cells with cells producing a retrovirus encoding a defective mutant of the *flk-1* gene resulted in a dominant-negative effect on VEGF binding capacity of the *flk-1* gene product. As a result, both angiogenesis and tumor growth were profoundly inhibited.[136] The significance of

Table 2. Endothelial RTKs

RTC	Other designations	Ligand	Expression	References
flg	*fms*-like gene, FGFR1	bFGF	endothelial cells in culture, tumor associated endothelium	156, 157
bek	FGFR2	bFGF	endothelial cells in culture	157
flk-1	KDR	VEGF/VPF	angioblasts, tumor associated endothelial cells, kidney, brain	73, 74, 132, 134, 137
flt (1–4)	*fms*-like tyrosine kinase	VEGF	embryonal blood vessels, human fetal lung, kidney, liver	127, 139, 141
tie-1		?	early embryonal vascular system	126, 158
tie-2		?	angioblasts, sprouting endothelial cells, embryonal organs	130, 131, 158
tek	*tie-2* truncated form	?	embryonal blood vessels, tumor associated endothelial cells	129, Yamaguchi and Rak, unpublished (1992)

this finding stems from the fact that the expression of VPF/VEGF by tumor cells and its corresponding receptor by endothelial cells has been described in at least two other clinical and experimental tumors including glioblastoma multiforme, as well as cancers of the urinary tract and kidney.[73,74,137,138] Interestingly, proliferating endothelial cells in tissue culture overexpress another VEGF receptor tyrosine kinase (*flt*) the role of which is not fully understood.[139] In addition, hemangioma-like endothelial outgrowths have been observed in transgenic mice overexpressing a different receptor tyrosine kinase, *flp*.[140] Again, it is not known whether the latter tyrosine kinase is expressed and plays a role in solid tumor-induced angiogenesis. A potential role in this process was, however, recently implicated for tyrosine kinase receptors binding bFGF. In these experiments overexpression of the high affinity receptor transcript and bFGF receptor protein was demonstrated in blood vessels of transplantable murine tumors, (J Gross-Dzubow, personal communication). Somewhat paradoxically, however, the *flg* gene product, another fibroblast growth factor receptor with tyrosine kinase activity, was shown to be upregulated when endothelial cells exit the phase of rapid proliferation.[141]

Altered expression of several molecules unrelated to cytokine receptors has also been recently reported in tumor-associated endothelium. For example, abnormal expression of the fibronectin receptor on the luminal surface of rapidly growing endothelial cells or MHC class II antigen expression on interferon (IFN)-γ activated tumor associated endothelium was observed.[142,143] It is noteworthy that some unique characteristics of vascular endothelium may change with tumor progression and such alterations could be explored as potential targets for anti-vascular or anti-angiogenic cancer therapy.

Targeting of tumor-associated blood vessels as a novel anti-cancer treatment strategy

Several considerations make targeting the tumor vasculature an attractive possibility as a form of anti-cancer therapy.[144–148] First, tumor cell genetic instability and resulting heterogeneity are thought to be two of the main factors causing the rapid development of tumor cell resistance to natural growth control mechanisms as well as therapeutic agents. By contrast, normal stromal cells should be genetically stable and hence more likely to remain susceptible to exogenous therapeutic influences, i.e. their capacity to develop resistance should be minimal, in comparison to tumors.[148] Second, the accessibility of endothelial cells to blood borne pharmacological manipulations makes them particularly attractive for such stromal cell targeting. Targeting the tumor vasculature is also a strategy that might circumvent the problem of limited penetration frequently encountered when delivering high molecular weight drugs to solid tumors with elevated interstitial pressures.[8] Such penetration would not be necessary when targeting the vasculature. It

is thought that antivascular therapy would simultaneously obliterate the capacity of the tumor to continue to grow and metastasize. Third, the effect of vascular damage on tumor growth is likely to be amplified by the fact that the viability of numerous tumor cells depends on a much smaller number of functional capillaries. Finally, tumor-associated endothelial cells express characteristics which are not normally found in the vasculature of tissues outside the tumor, and this may allow the design of highly specific and non-toxic treatment strategies.

Possible specific strategies for executing an antivascular attack have been suggested by several investigators and have led to experiments with a variety of anti-angiogenic agents (Table 3). It would seem reasonable to attempt to obliterate the angiogenic circuit driving blood vessel formation in a particular tumor. This could be achieved by targeting the relevant angiogenic factor, its receptor or, alternatively, properties of activated endothelial cells such as growth, migration, proteolysis or morphogenesis using specific antibodies, inhibitors or receptor antagonists.[145,149,150] One difficulty in executing this 'anti-angiogenic' approach is that the exact mechanism of angiogenesis operating in any given tumor is usually unknown and multiple angiogenic factors are likely to be involved. It is conceivable that the angiogenic capacity could perhaps be 'exhausted' by interference with intracellularly acting cytokines regulating growth, survival and senescence of endothelial cells. Potential target molecules for this kind of intervention would likely include IL-1, FGF[151] or IL-6 (Rak, unpublished).

Another strategy, originally proposed by Denekamp, is to target directly a known characteristic of tumor associated endothelial cells thought to be critical, i.e. their high proliferation rate.[146,157] This 'angiosuppressive' effect could be accomplished by using endothelial cell-specific cytotoxic agents including derivatives of known chemotherapeutic drugs. In fact some of the effects of treatment with IFN, certain cytokines, ionizing radiation, hyperthermia and even certain anti-cancer drugs may be attributable, at least in part, to blood vessel damage leading to hemorrhagic necrosis of the tumor.[146] The available drugs studied so far, however, usually lack sufficient endothelial cell specificity and can therefore cause severe toxicity. It seems somewhat paradoxical that although essentially all of the antiproliferative chemotherapeutic agents should cause some degree of vascular damage this has not been noted with any consistency suggesting that some degree of drug resistance may also be expressed (or induced) in tumor-associated endothelial cells.[148] Alternatively, specific immunotoxins, retroviruses or antisense reagents can be designed to target genes expressed by tumor-associated endothelial cells, including KDR (human homolog of *flk-1*) and other tyrosine kinases.[3,136]

It is possible that different types of tumors will require different anti-angiogenic treatment strategies. For example, recent studies show that at least in human breast carcinoma the proliferating fraction of endothelial cells is much smaller than one would have expected based on previously cited rates most of which were obtained using animal models.[152,153] Thus in this disease the expansion of the microcirculation may be brought about by endothelial cell migration and remodeling rather than cell growth *per se*.[152] In such cases antiproliferative agents are likely to be ineffective and should be replaced by protease inhibitors, steroids or other compounds interfering with endothelial cell migration, base-

Table 3. Anti-angiogenic and angiosuppressive agents[144–147,159,160]

Natural angiogenesis inhibitors
platelet factor 4 (PF4)
TIMP-1, TIMP-2
thrombospondin
cartilage derived inhibitor (CDI)
IFN-α and -β
Angiostatin
Antagonists of angiogenic growth factors
suramin and 254 compound
pentosan polysulfate (PPS)
Angiostatic steroids and their co-inhibitors
cortisone
medroxyprogesterone
heparin (fragments)
β-cyclodextrin tetradecasufate
HNT
Anti-inflammatory drugs
indometacin
ibuprofen
Natural antiangiogenic compounds and their derivatives
angioinhibins (fumagilin, AGM-1470)
vitamine D_3 analogs
herbimycin A
retinoids
somatostatin analogs
sulfated chitin derivatives
Anti-cancer drugs
flavone acetic acid (FAA)
bleomycin
metotrexate
mitoxantrone
Other
copper chelators (D-penicillamine)
minocycline
mitotoxins (FGF-saporin)
RGD polymers

ment membrane turnover and morphogenesis.[1] It has also been postulated that blood channels may develop in a tumor mass in areas where the endothelial lining is incomplete or absent. It is unclear how blood flow could be maintained under these highly coagulogenic conditions but certainly in such a situation specific anti-endothelial therapy would be less effective.[6]

Anti-angiogenesis therapy of solid tumors: present problems and future directions

Clearly, there are a number of appealing approaches to treating solid tumors by inhibition of tumor angiogenesis. The main problem will undoubtedly be to achieve selectivity: are there molecular targets associated with new blood vessel formation that are not found on normal, mature blood vessels? In a sense this requires shifting our traditional focus on developing 'magic bullets' specific for tumor cells to designing or discovering similar magic bullets which show selectivity for tumor-associated blood vessels. However, even if such complete selectivity cannot be attained it does not exclude—as chemotherapy and radiotherapy have shown—the possibility of using anti-angiogenesis strategies to treat solid tumors.

A second problem stems from the assumption that complete kill of solid tumor masses may never be attainable since small (e.g. 1 mm) tumors can survive without a blood vessel supply. However, anti-angiogenesis may be an excellent paradigm for the concept of treating cancer by *controlling* progressive tumor growth as opposed to trying to achieve total tumor cell *kill*.[154] This may lead to long-term disease stabilization in some cases.

So what does the future hold? Obviously there will continue to be a great emphasis on finding anti-angiogenic agents or drugs which have the desired magic bullet properties. As such drugs are uncovered, the appropriate phase I and II clinical trials will ensue, and hopefully some of these agents will be found to be clinically promising. This approach, however, raises an interesting problem. If we examine the treatment of cancer by chemotherapy it is of course well known that single chemotherapeutic drugs are almost never used to treat the disease. Rather it is the *combination of different classes of drugs* which is the mainstay of chemotherapy protocols. With this analogy in mind, it would seem obvious that a similar 'combination anti-angiogenic therapy' might be necessary to detect successes in treating solid tumors by the strategy of inhibiting angiogenesis. Given the realities of how phase I clinical trials are conducted, and the issues concerning the commercialization and patents of new drugs, it may be extremely difficult to establish the right combination of circumstances required to detect optimal anti-angiogenic effects in patients with solid tumors. Thus combination anti-angiogenic therapy will have to be explored primarily by basic researchers using appropriate animal models. It would seem worthwhile to consider seriously this type of therapeutic approach given the fact that there are many steps in the angiogenic process which appear susceptible to therapeutic attack. For example, endothelial cell invasion of surrounding basement membranes, adhesion to the subendothelial matrix, migration into the stroma and proliferation may all represent potential targets for anti-angiogenic drugs. Thus optimal antivascular therapy will likely include certain anti-integrin antibodies (e.g. anti-$\alpha v\beta 3$)[53] to inhibit endothelial cell invasion and/or adhesion in combination with other agents which, for example, inhibit endothelial cell proliferation, such as antibodies to VEGF/VPF or bFGF receptor tyrosine kinases.

Acknowledgments

The authors are thankful for valuable critical comments of our colleagues at the Cancer Research Division, and the excellent secretarial help of Terrie Walker and Lynda Woodcock.

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(Received 8 September 1994; accepted 20 September 1994)