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Patterns and Emerging Mechanisms of the Angiogenic Switch during Tumorigenesis

Review

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Introduction

Blood vessels are fundamentally composed of endothelial cells, which interconnect to form the tubes that direct and maintain blood flow and tissue perfusion. During embryogenesis, blood vessels develop via two processes: vasculogenesis, whereby endothelial cells are born from progenitor cell types; and angiogenesis, in which new capillaries sprout from existing vessels (reviewed by Risau, 1995; Risau and Flamme, 1995). In the adult, new vessels are produced only through angiogenesis. Notably, the vasculature is quiescent in the normal adult mammal, except for highly orderly processes in the female reproductive cycles (ovulation, menstruation, implantation, pregnancy). The endothelial cells are among the longest-lived in the body outside the central nervous system; in a normal adult vessel, only 1 in every 10,000 endothelial cells (0.01%) is in the cell division cycle at any given time (Engerman et al., 1967; Hobson and Denekamp, 1984), presumably reflecting a process of cell turnover to maintain tissue vitality. In contrast, about 14% of normal intestinal epithelial cells are in the cell division cycle. Thus, the turnover time to replace the cells of the gut is measured in days and that of the endothelium in years. Yet, in response to an appropriate stimulus, the quiescent vasculature can become activated to grow new capillaries. This process of angiogenesis is complex (reviewed by Auerbach and Auerbach, 1994; Cockerill et al., 1995; Folkman, 1995a). The basement membrane surrounding the endothelial cell tube is locally degraded, and the endothelial cells underlying this regional disruption in the barrier change shape and invade into surrounding stroma. This invasion is accompanied by proliferation of the endothelial cells at the leading edge of what becomes a migrating column. A region of differentiation trails behind the advancing front, where the endothelial cells cease proliferating, change shape, and tightly adhere to each other to form a lumen of a new capillary tube. Finally, sprouting tubes fuse and coalesce into loops, circulating the blood into this newly vascularized region. Outside of female reproductive cycles, angiogenesis in the adult is largely controlled by pathological situations, such as wound healing and tumor growth. The complexity of angiogenesis implies the existence of multiple controls, some of which are coming into focus, as will be discussed below.

The importance of angiogenesis for the growth of solid tumors is now well recognized. A considerable body of research spanning almost three decades has documented that tumor growth and metastasis require persistent new blood vessel growth. The classical proof of this principle came from experiments whereby tumor fragments or cultured tumor cells were placed in an avascular site, the cornea of a rabbit eye (Gimbrone et al., 1972). The implants attracted new capillaries that grew in from the limbus to vascularize the expanding tumor mass. If the capillaries were physically prevented from reaching the implant or were inhibited from undergoing angiogenesis, tumor growth was dramatically impaired, restricting the tumor nodule to a diameter of approximately 0.4 mm. Subsequent experiments have confirmed this result and further revealed that in the absence of access to an adequate vasculature, tumor cells become necrotic (Brem et al., 1976) and/or apoptotic (Holmgren et al., 1995; Parangi, 1996), restraining the increase in tumor volume that should result from continuous cell proliferation, the hallmark of cancer.

The principle that ongoing angiogenesis is essential for rapid expansion of a tumor mass has in turn raised another question: when is angiogenesis activated during the development of a tumor, which invariably takes considerable time and appears to progress along a developmental pathway composed of distinctive progenitor stages? Is angiogenesis simply an inevitable consequence whenever nodules of aberrantly proliferating cells become size-limited by a lack of vascularization? Or rather, is controlling the "angiogenic switch" an important part of the repertoire of qualities that a developing tumor must acquire to be successful? The evidence increasingly points to the latter alternative, namely that induction of angiogenesis is a discrete component of the tumor phenotype, one that is often activated during the early, preneoplastic stages in the development of a tumor. The clues have initially come from transgenic mouse models of cancer and now from certain classes of human cancer; in both cases, access to tissue containing the stages in tumor development has been critical to this realization, as is related below.

Transgenic Mouse Models Reveal the Angiogenic Switch in Early Stages of Tumor Development

Transgenic mice carrying dominant oncogenes and/or knockouts of tumor suppressor genes are now being widely utilized to study the process of tumorigenesis (reviewed by Adams and Cory, 1991; Frost et al., 1995; Hanahan, 1988, 1989). In a number of these mouse models, cancers develop through temporally and histologically distinct stages, much as is apparent for many human cancers. The experimental manipulability of the mouse and the reproducibility afforded by genetic predisposition to specific cancers is allowing the individual stages in tumorigenesis to be studied, and perturbed, in efforts to establish functional significance of candidate genes and cellular processes. Specifically, we and our colleagues have investigated the pattern of angiogenesis in three diverse transgenic mouse models: islet cell

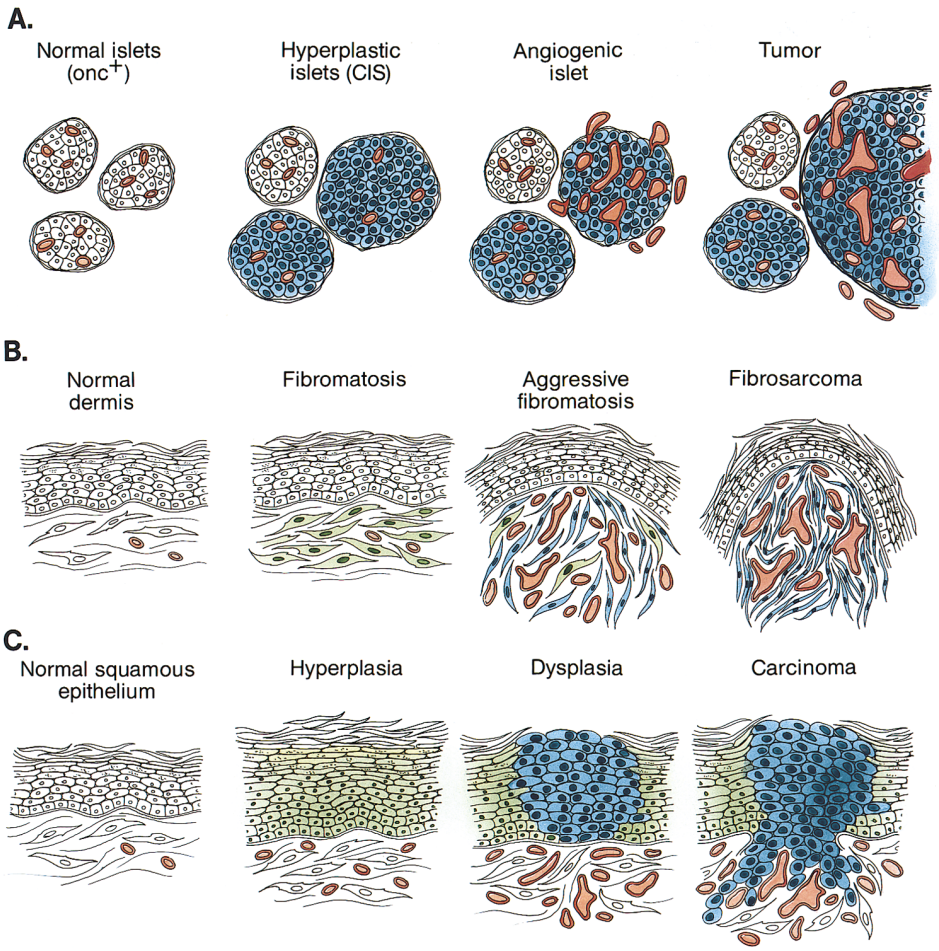

Figure 1. The Angiogenic Switch Occurs Prior to Tumor Formation in Three Transgenic Mouse Models of Tumorigenesis

(A) Expression of the Tag oncogene in the pancreatic islets elicits four sequential stages in tumor development: normal, oncogene-expressing islets; hyperplastic islets, populated by proliferating cells with the histological hallmarks of CIS; angiogenic islets, in which new blood vessel growth has been activated; and solid tumors, which are islet cell carcinomas.

(B) In transgenic mice carrying the BPV-1 oncogenes, the normal dermis is initially converted into a state of mild fibromatosis, revealed as focal accumulation of dermal fibroblasts. Angiogenesis is first evident in the next stage, aggressive fibromatosis, which is also marked by dense arrays of proliferating fibroblastic cells; both hyperproliferation and angiogenesis persist in the subsequent stage, protuberant fibrosarcoma.

(C) Targeted expression of the HPV-16 oncogenes to basal cells of the epidermis induces multistage development of squamous cell carcinoma, beginning as hyperplasia of keratinocytes, with a mild increase in vessel density; which progresses to dysplasia, marked by morphologically aberrant keratinocytes with a high proliferation index and by abundant neovascularization; finally, two classes of squamous carcinoma arise, both evidencing extensive angiogenesis.

carcinoma, dermal fibrosarcoma, and epidermal squamous cell carcinoma. In all three models, extensive vascularization and ongoing angiogenesis has been apparent in the end-stage tumors. Remarkably, in each case, an angiogenic switch could also be visualized during early stages preceding the appearance of the solid tumors, suggesting that activation of angiogenesis is a discrete event in tumor development. These models are summarized briefly, in turn; the results are schematized in Figure 1.

The so-called "RIP-Tag" transgenic mice express the SV40 T antigen (or Tag) oncogene in the insulin-producing β cells (Hanahan, 1985), which are localized in approximately 400 islet nodules scattered throughout the pancreas. In several independent lines of mice, a few (2–10) of these 400 islets develop into solid tumors by 12–16 weeks of age. The distribution of this cell type into these natural focal nodules has allowed histological, temporal, and statistical analysis of tumorigenesis. A set of four discrete stages in the pathway to cancer is apparent by these criteria. Initially, every islet contains β cells, which express the oncogene but otherwise appear normal, with a low proliferation index. Then, focal activation of hyperproliferation occurs in individual islets (Teitelman et al., 1988). This stage, while historically referred to as "hyperplastic," is composed of cells with the histological properties of a carcinoma-in-situ (CIS), wherein more than 20% of the cells are in S phase at any given time. Insulin-like growth factor (IGF)-II is activated at this stage and probably modulates apoptosis that accompanies the aberrant proliferation (Christofori et al., 1994, 1995a; Naik et al., 1994). Yet,

these hyperplastic islet nodules, while morphologically transformed in appearance, are not competent to proceed directly to a rapidly growing islet cell carcinoma, since 50% of the islets switch to the hyperproliferative (CIS) stage, while only 1%–2% progress to solid tumors. We have identified a discrete stage, referred to as "angiogenic islet," that appears to be an intermediate between these two stages, both statistically and temporally. We defined the angiogenic switch using an in vitro bioassay for angiogenic activity, in which capillary endothelial cells were cocultured in a three-dimensional collagen gel with islets isolated at different ages from the RIP-Tag mice (Folkman et al., 1989). At early ages, none of the oncogene-expressing islets were angiogenic. Then, in older mice, individual islets scored as angiogenic, attracting a starburst of endothelial cells converging on the angiogenic islet. Over time, about 10% of the total islets scored as angiogenic. The in vitro bioassay was substantiated by histological analysis, which revealed two hallmarks of angiogenesis, capillary sprouting and endothelial cell proliferation, in a subset of the hyperplastic islets and in all solid tumors in these transgenic mice (Folkman et al., 1989). The provocative result was that neither oncogene expression nor hyperproliferation appeared to be sufficient to activate the angiogenic switch, which rather appeared as a discrete, temporally separate step in this multistage pathway (Figure 1A).

A second transgenic mouse model of multistage tumorigenesis, in which the bovine papillomavirus oncogenes elicit dermal fibrosarcomas (Lacey et al., 1986; Sippola-Thiele et al., 1988), has also been analyzed for the appearance of angiogenesis (Kandel et al., 1991). Dense vascularization and evidence of new blood vessel growth first became apparent in a late preneoplastic stage, aggressive fibromatosis; angiogenesis was also evident in all end-stage fibrosarcomas (Figure 1B). Even though the fibrosarcoma model is very different from the islet cell system, the results suggest again that angiogenesis is a rate-limiting step in tumor development. Moreover, the pattern persisted when a third transgenic model was investigated, wherein basal keratinocytes were progressively transformed into squamous cell carcinomas by the human papillomavirus type 16 oncogenes (Arbeit et al., 1994, 1996; Arbeit, 1996; Coussens et al., 1996; Hurlin et al., 1995). Here again, the angiogenic switch was evident prior to the emergence of the squamous cancers (Figure 1C). In this multistage model of squamous carcinogenesis, weak angiogenic activity could be detected in the hyperplastic stage in the form of modestly increased vessel density in the underlying dermis. Angiogenesis became pronounced in the dysplastic stage, with abundant capillaries juxtaposed to the basement membrane separating stroma from dysplastic cells. Intense vascularization persisted in the invasive squamous cancers that arose out of such dysplastic lesions (Smith-McCune et al., submitted).

In summary, all three of the diverse transgenic mouse models of tumorigenesis analyzed to date have revealed an angiogenic switch that becomes activated during the early stages of tumor development, suggesting that regulation of angiogenesis is a discrete, potentially rate-limiting step in the pathway to many solid tumors. One important test of the value of mouse models is the applicability of observations made therein to the human condition. In two case studies, angiogenesis has been detected in preneoplastic stages, consistent with identification of the angiogenic switch as a step in the pathway to human cancer, as the following section elaborates.

Angiogenesis in Premalignant Stages of Two Human Cancers

The use of immunological markers to visualize the vasculature has become an important new tool in assessing the histopathology of cancerous lesions, as seen for example in the murine fibrosarcoma study discussed above (Kandel et al., 1991). Both von Willebrand's factor (vWF) and CD31 are expressed widely, albeit variably, in the endothelium, which has allowed these and other markers to be used as tools to assess the density and character of capillaries in tissue sections. In human oncology, an important application of this capability to visualize capillaries has been the development of new prognostic tests for probability of relapse (or disease-free survival) following surgical resection of an invasive cancer. Vessel density in invasive cancers has been demonstrated to be a significant prognostic indicator, both for breast (Weidner et al., 1991) and prostate (Weidner et al., 1993) cancers; if the vessel density was low, the prognosis was good, whereas the prognosis fell with increasing density of blood vessels, the mark of a potent angiogenic response. In addition to evaluating malignant carcinomas, immunostaining with endothelial cell markers has been used to detect angiogenesis in preneoplastic lesions associated with cancers of the mammary duct and the uterine cervix. The results are schematized in Figure 2. Briefly, histopathological evaluation of biopsies taken from cancerous breasts has revealed distinctive preneoplastic lesions, including hyperplastic ducts, dysplastic ducts, and CIS. Using vWF immunostaining, a subset of the CIS lesions in the human breast can be seen to be angiogenic (Brown et al., 1995; Guidi et al., 1994; Weidner et al., 1992), in loose analogy to the subset of hyperproliferative ("CIS-like") islets in the RIP-Tag mice that are angiogenic. In contrast to the mouse, it has not been possible to perform comprehensive temporal and histological analyses on the early development of these presumptive stages in the human breast, but the patterns are remarkably similar. Thus, we infer that angiogenic CIS lesions in the breast may represent an intermediate stage that separates preangiogenic CIS and invasive cancer.

The human cervix has been extensively characterized through the routine collection of PAP smears, which has revealed a series of aberrant proliferative stages, ranging from varying grades of dysplasia to CIS (histologically graded as CIN I–III) and invasive cancer; about 80% of cervical carcinomas contain fragments of an HPV genome (typically HPV16/18) and express viral oncogenes. When biopsies of cervical lesions were analyzed by immunostaining with vWF to reveal the capillaries, an angiogenic switch was readily apparent in the mid–late dysplasias (CIN II–III), wherein new vessels became densely apposed along the basement membrane underlying the dysplastic epithelium (Guidi et al., 1995;

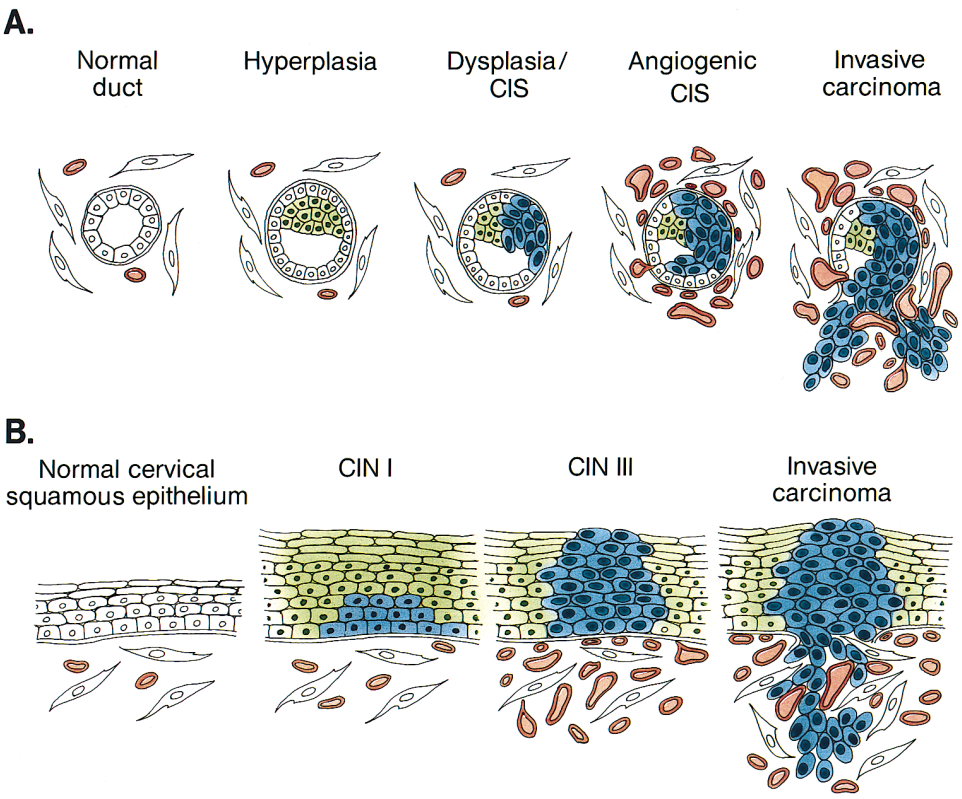

Figure 2. The Angiogenic Switch Can Be Visualized in Neoplastic Lesions Inferred to Be Progenitor Stages to Human Breast and Cervical Cancer

(A) Breast cancers arise from the ductal epithelium of the breast; ductal lesions that are presumptive progenitors can be ordered into a pathway of increasing aberrancy, beginning with hyperplasia and progressing to dysplasia and CIS. Of these, a subset of CIS lesions have switched on angiogenesis, as evidenced by abundant new capillaries, suggesting that angiogenic-CIS is an intermediate stage between CIS and invasive cancer.

(B) The squamous cervical epithelium evidences premalignant lesions graded as cervical intraepithelial neoplasia I–III, which are inferred to represent a pathway to cervical cancer. A modest increase in new vessel density is evident in CIN I/II lesions, while CIN III lesions (analogous to advanced dysplasia/CIS) show abundant new vessels, indicative of the angiogenic switch from vascular quiescence to sustained neovascularization.

Smith-McCune and Weidner, 1994). An initial mild increase in vessel density has been detected in the early dysplastic (CIN I) stage (Guidi et al., 1995; Smith-McCune et al., submitted). This pattern is remarkably similar to that seen in the epidermis of K14–HPV16 transgenic mice, which presents a similar multistage pathway in another squamous epithelium (of the skin) under the influence of the HPV16 oncogenes. Thus, we infer that the angiogenic switch is a very early event in the pathways to invasive squamous cell cancers, perhaps occurring in two regulatory phases, of mild and intense neovascularization. Clues from cell culture bioassays also support this notion that the switch can have several "on" settings, which progressively ratchet up the intensity of neovascularization.

The pattern of new blood vessel growth, described here in five cancers spanning two species and five cell types, argues, first, that the angiogenic switch is a discrete process, subject to specific regulatory controls; and second, that the switch is an essential part of the phenotypic repertoire that characterizes a successful tumor. Figure 3 presents a histological picture of the angiogenic switch in these five models. A similar stage-specific switch is evident during development of cutaneous melanoma in humans (reviewed by Rak et al., 1995).

Therefore, having argued that regulation of the switch is important and potentially rate-limiting for initial tumor development as well as for the expansion and metastasis of an established tumor, we now wish to discuss the emerging picture of how the angiogenic switch may be controlled.

Emerging Mechanisms of the Angiogenic Switch

The Angiogenic Inducers

The observation that tumors could be implanted either into an avascular region, such as the cornea, or on a characteristically vascularized surface, such as the chick chorioalantoic membrane, and in each case elicit the ingrowth of new capillaries, suggested that tumors released diffusible activators of angiogenesis that could signal a quiescent vasculature to begin capillary sprouting. This hypothesis motivated a search for so-called tumor angiogenesis factors. Indeed, inducers of angiogenesis have been identified. A number of in vitro and in vivo bioassays have been developed to mimic the complex process of angiogenesis (reviewed by Cockerill et al., 1995). Among these, two assays in particular have been widely used to screen for angiogenic regulatory factors, each mimicking an aspect of angiogenesis;

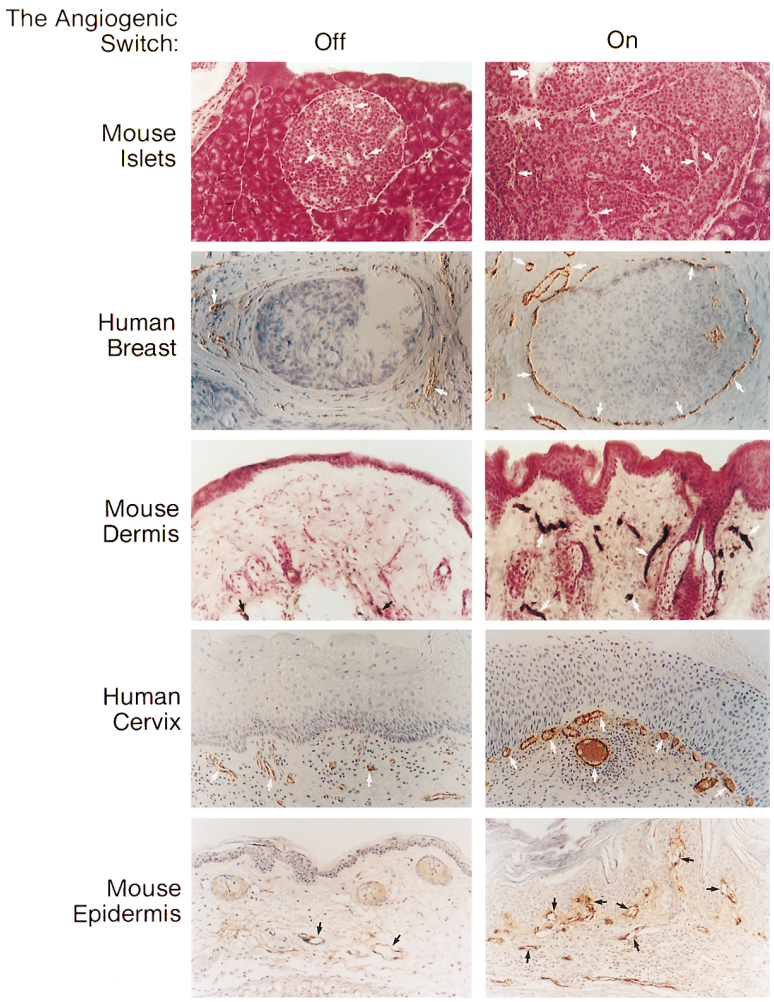

Figure 3. A Histological View of the Angiogenic Switch

Tissue sections illustrate the situation before and after the angiogenic switch in progenitor lesions to tumors in the indicated tissues, whose multistage developmental pathways were summarized in Figures 1 and 2. In each panel, the arrows highlight a few representative vessels in the quiescent (left column) or angiogenic (right column) states. The top row shows a hyperplastic mouse islet, full of proliferating β cells (left), which contains a quiescent vasculature (as do normal islets), and another islet nodule (right) that has switched on new blood vessel growth (a few of which are highlighted with arrows), with the hallmark appearance of vascular lacuna (large arrow). The human breast panels show a CIS (left), with normal duct-associated vasculature, and an angiogenic-CIS lesion (right), with extensive neovascularization. The mouse dermis panels show a prevascular mild fibromatosis lesion (left), with a few vessels deep in the dermis, and an aggressive fibromatosis (right), with large capillaries spread throughout the thickened dermal layer. In the human cervix, a normal tissue (left) has vessels deep in the stroma, whereas the CIN III lesion (right) has attracted a dense array of new capillaries underlying the basement membrane. In the normal mouse ear, a few vessels are present in the stroma (left), whereas in HPV-16–induced epidermal dysplasia, capillaries are distributed throughout the dermis, in particular closely juxtaposed to the basement membrane underlying the transformed keratinocytes. The islet panels were stained with hematoxylin and eosin, while the remainder were first immunostained with antisera to the blood vessel marker vWF and then counterstained with hematoxylin alone or with hematoxylin and eosin (mouse dermis). All magnifications are approximately 62×.

namely, endothelial cell proliferation and migration. The proliferation assay uses cultured capillary endothelial cells and measures either increased cell number or the incorporation of radiolabeled or modified nucleosides to detect cells in S phase. In contrast, the chemotaxis assay separates endothelial cells and a test solution by a porous membrane disc (a Boyden Chamber), such that migration of endothelial cells across the barrier is indicative of a chemoattractant present in the test solution. Both assays have inherent strengths, biases, and limitations; each is often complemented by the use of an in vivo assay, such as implants into the normally avascular cornea of rabbits or rodents (the "corneal pocket" assay). A number of inducers of angiogenesis have been detected using these assays. The first to be discovered (in 1982) was basic fibroblast growth factor (bFGF, or FGF-2), followed shortly by its relative, acidic FGF (aFGF, or FGF-1; reviewed by Christofori, 1996; Friesel and Maciag, 1995; Folkman and Shing, 1992). Both proteins are members of a family of growth factors that are characterized by high affinity binding to heparin; each is unusual in its lack of a traditional signal sequence for secretion. Both, however, can be released from cells under certain circumstances (Christofori, 1996; Friesel and Maciag, 1995; Kandel et al., 1991). At about the same time, a secreted protein was identified by its ability to elicit vascular permeability (Senger et al., 1983); subsequently, this factor, called vascular permeability factor or vascular endothelial growth factor (VPF/VEGF), was shown to be a potent inducer of angiogenesis (reviewed by Brown et al., 1996; Ferrara, 1993). Recently, two related endothelial growth factors, VEGF-B and VEGF-C, have been identified (Olofsson et al., 1996; Joukov et al., 1996; Lee et al., 1996; Grimmond et al., 1996). All three VEGF genes as well as acidic and basic FGF are widely expressed in normal adult organs of mice and humans, suggestive of roles in tissue homeostasis as well as angiogenesis, since the later is such a rare event in most tissues. Both a/bFGF and VEGF bind to receptors on endothelial cells that are transmembrane tyrosine kinases and thus are coupled through the signal transduction cascade to the cellular regulatory network. The three VEGF receptors, flk, flt-1, and flt-4 (VEGFR-1–3), are specifically expressed on endothelial cells (reviewed by Brown et al., 1996; Risau and Flamme, 1995), whereas the four FGF receptors, FGFR1–4, are more widely expressed (reviewed by Christofori, 1996; Friesel and Maciag, 1995; Rak et al., 1995).

During the past decade, an increasing number of angiogenic inducers has been identified (reviewed by Bouck

et al., 1996; Folkman, 1995a, 1995b). Concurrently, however, a pattern has emerged, in that bFGF and VEGF are commonly expressed in a wide variety of human and animal cancers; moreover, bFGF (and now VEGF) is being detected at elevated levels in the urine and serum of a significant fraction of tumor-bearing patients (as discussed in Folkman, 1995a, 1995b). Interestingly, VEGF and bFGF have been shown to synergize using in vitro angiogenesis assays (Pepper et al., 1992; Goto et al., 1993), indicating that they can serve complementary functions, consistent with their common association in tumors. Indeed, the tumor development pathways of all three transgenic mouse models presented in Figure 1 evidence an involvement of VEGF and either aFGF, bFGF, or both. Thus, these two classes of angiogenic inducer certainly represent a frequent component of the angiogenic switch. Notwithstanding this consensus, the diversity and number of angiogenic inducers identified to date argues that there will also prove to be tissue-specific and perhaps transformation-specific regulators associated with particular cancers and with the normal physiological regulation of angiogenesis. Moreover, convergent on this perspective regarding angiogenic inducers has been the realization that there is an equally important component to the switch, one governed by negative regulatory factors, called angiogenesis inhibitors.

The Angiogenic Inhibitors

The first clues to the existence of endogenous angiogenesis inhibitors came with the observations that α interferon (Brouty-Boye and Zetter, 1980; Sidky and Borden, 1987) and platelet factor-4 (Taylor and Folkman, 1982; Sato et al., 1990; Sharp et al., 1990) could inhibit endothelial cell chemotaxis and proliferation, respectively. But the significance of negative regulators of angiogenesis first became apparent through a series of experiments by Bouck and colleagues (Good et al., 1990; Rastinejad et al., 1989). A nontumorigenic hamster cell line became tumorigenic concomitant with a mutation that inactivated a tumor suppressor gene. Comparison of cell lines containing or missing the suppressor gene revealed that the nontumorigenic line released high levels of a potent angiogenesis inhibitor, both of endothelial chemotaxis in the Boyden Chamber assay and of in vivo neovascularization using the corneal pocket assay; by comparison, the tumorigenic cell lines had much lower levels of inhibitor (Rastinejad et al., 1989). The inhibitory activity was purified and shown to be a truncated form of thrombospondin-1 (TSP-1), a secreted glycoprotein. Subsequently, the intact TSP-1 molecule was shown to be an angiogenesis inhibitor (Good et al., 1990), as were small peptides contained within it (Tolsma et al., 1993). Further, TSP-1 has been shown to be expressed at high levels in a number of normal rodent and human cells and at appreciably lower levels in many tumor cells. Provocatively, TSP-1 has recently been shown to be regulated by the wild-type p53 tumor suppressor protein in fibroblasts (Dameron et al., 1994) and mammary epithelial cells (Volpert et al., 1995), such that upon loss of p53 function in their transformed derivatives, the levels of this angiogenesis inhibitor dropped precipitously. Restoration of p53 function upregulated TSP-1 and impaired the angiogenic capability of the tumor cells. Furthermore, p53 has been shown in other cell types, such as astrocytes (Van Meir et al., 1994), to control angiogenesis-inhibitory activities distinct from TSP. The bottom line of these experiments is, first, that endogenous angiogenesis inhibitors can serve to counteract inducer signals to grow new capillaries; and second, that these angiogenesis inhibitors can be controlled by tumor suppressor genes, consistent with the functional definition of such genes as interfering with the tumor phenotype, of which angiogenesis is a key component.

A New Paradigm of Cryptic Angiogenesis Inhibitors within Larger Proteins

The fact that the endothelium is quiescent for long periods and yet can be induced to sprout new capillaries in a matter of hours in response to, for example, a wound, has long suggested that angiogenesis regulators might be stored for expedient use. In addition, one can expect that regulators will be synthesized for persistence of an angiogenic response. It is known that the FGFs and other growth and angiogenic factors can be sequestered in the extracellular matrix of many cell types, including endothelial cells, presumably to be released by proteolytic degradation of the matrix (Baird and Ling, 1987; Folkman et al., 1988). It now appears that angiogenesis inhibitors are also stored, but in a much different fashion, as cryptic parts of larger molecules that are not themselves inhibitors of angiogenesis. The prototype came from the discovery that a 29 kDa fragment of fibronectin inhibited endothelial cell proliferation in vitro (Homandberg et al., 1985). Notably, the intact fibronectin molecule, an abundant component of the circulatory system, was not itself an inhibitor. Subsequently, a fragment of prolactin, the 16 kDa fragment, was shown to be an inhibitor of endothelial cells, whereas the intact molecule was not (Clapp et al., 1993; Ferrera et al., 1991). More recently, a potent inhibitor of angiogenesis, called angiostatin, has been identified as a fragment of plasminogen (O'Reilly et al., 1994). Circulating angiostatin is able to maintain the dormancy of metastases and primary tumors by blocking blood vessel growth, resulting in small nests of tumor cells that cycle through proliferation and apoptosis, restrained by their inability to induce angiogenesis to support their growth (Holmgren et al., 1995; O'Reilly et al., 1994, 1996). The fact that abundant components of the circulatory system such as fibronectin and plasminogen can be converted into potent angiogenic factors suggests a new form of regulation, whereby proteases specifically release 29 kDa fibronectin or angiostatin from their intact parental molecules to limit an angiogenic response, for example in the transient wound healing process. Recently, platelet factor-4, itself a weak angiogenesis inhibitor, has been shown to contain a fragment that is 50 times more potent at inhibiting angiogenesis (Gupta et al., 1995). The pattern of protease fragments as angiogenesis inhibitors continues to expand to other molecules, including the propeptides of type 1 collagen (Tolsma et al., 1993) and a peptide fragment of epidermal growth factor (Nelson et al., 1995).

Thus, a paradigm is emerging which suggests that a class of angiogenesis inhibitors is contained within other proteins that are not themselves inhibitors. The capability to release inhibitor fragments from storage as cryptic segments of abundant proteins may contribute to maintaining the normal quiescence of the vasculature and

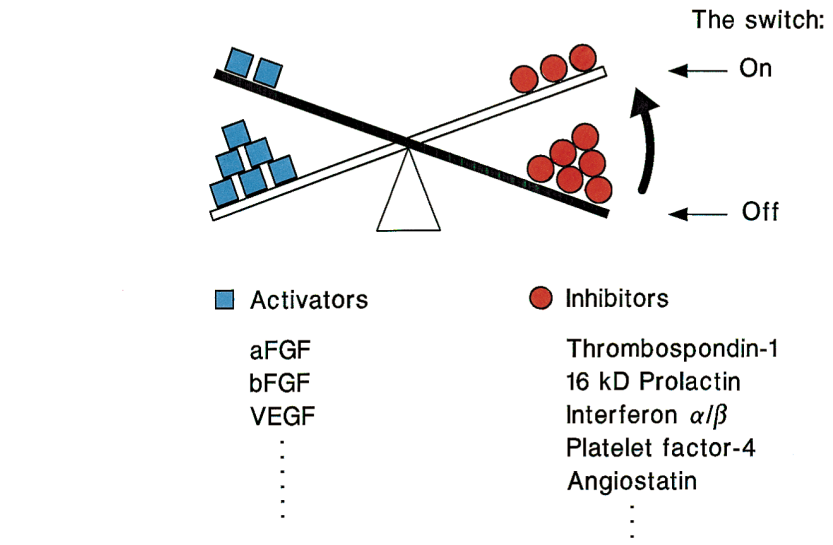

Figure 4. The Balance Hypothesis for the Angiogenic Switch

The normally quiescent vasculature can be activated to sprout new capillaries (angiogenesis), a morphogenic process controlled by an angiogenic switch mechanism. The prevailing evidence suggests that changes in the relative balance of inducers and inhibitors of angiogenesis can activate the switch. In some tissues, the absence of angiogenesis inducers may keep the switch off, while in others the angiogenesis inducers are present but held in check by higher levels of angiogenesis inhibitors. Thus, either reducing the inhibitor concentration, e.g., for TSP-1, by loss of a tumor suppressor gene; or increasing the activator levels, e.g., for induction of VEGF, by hypoxia, can each change the balance and activate the switch, leading to the growth of new blood vessels.

to turning off transitory angiogenic processes such as ovulation and wound healing; perhaps this strategy also serves the organism by suppressing tumorigenesis and other diseases with an angiogenic component. A major question for the future relates to the source and regulation of the proteases that release the cryptic inhibitors. In one case, that of angiostatin, certain clones of Lewis lung tumor paradoxically induce its release from circulating plasminogen (O'Reilly et al., 1994). Beyond that, it is not clear what cell types normally serve to release cryptic inhibitors and under what circumstances. Elucidation of mechanisms and regulation of cryptic angiogenic inhibitor release could well present therapeutic opportunities.

The Balance Hypothesis for the Angiogenic Switch

The above sections have summarized the evidence that angiogenesis can be regulated both by inducers and inhibitors of endothelial cell proliferation and migration. But which class of angiogenic regulators is most important? Increasingly, there are provocative clues suggesting that the balance of inhibitors and inducers governs the angiogenic switch; the hypothesis is schematized in Figure 4. A set of three clues summarizes the basis for this hypothesis. The first clue comes from in vitro bioassays. In both proliferation and chemotaxis assays, bFGF and VEGF can elicit a positive response from capillary endothelial cells. If increasing amounts of an inhibitor such as TSP-1 are added, the response becomes blocked, suggesting that when the levels of inhibitors are sufficiently high, the signals of the positive activator(s) are overruled, keeping (or turning) the angiogenic switch off (Good et al., 1990; Rastinejad et al., 1989). The second clue comes from studying human tumor cells, in which the restoration of p53 tumor suppressor function results in up-regulation of TSP, overruling both the angiogenesis inducers that the cells themselves synthesize as well as added bFGF, thereby blocking angiogenesis (Dameron et al., 1994). The third clue comes from the pancreatic islet model for the angiogenic switch (see Figure 1), in which two potent angiogenic factors, aFGF and VEGF, are expressed consitutively in normal islet cells (of nontransgenic mice) as well as throughout tumorigenesis in the transgenic mice (Christofori, 1996; Christofori et al., 1995b). Remarkably, there is no new blood vessel growth in normal pancreatic islets, despite the expression of these potent angiogenesis inducers. It seems likely that angiogenesis inhibitors keep the switch off in normal islets expressing VEGF and aFGF. If so, then loss of these inhibitors would shift the balance and trigger the switch during tumorigenesis. Indeed, genetic evidence exists for an angiogenesis suppressor gene located on mouse chromosome 16; this locus frequently shows loss of heterozygosity both in the angiogenic islet stage and in end-stage tumors of this tumorigenesis pathway (Parangi et al., 1995). The predictions are, therefore, first, that an angiogenesis suppressor gene encodes or controls expression of an angiogenesis inhibitor that maintains the quiescence of the vasculature in normal islets constitutively expressing VEGF and aFGF; and second, that this angiogenic inhibitor is down-modulated during tumorigenesis, thus activating the angiogenic switch.

These and other clues suggest a regulatory mechanism that integrates the cumulative levels of inducer

and inhibitor signals to maintain the endothelial cell in alternative states of quiescence or angiogenesis. Thus, changes in the balance of positive and negative signals mediate the angiogenic switch (Figure 4), a point Bouck and colleagues have also made recently (Bouck et al., 1996). A net balance of inhibitors over activators would maintain the switch in the off position, whereas a shift to an excess of activating stimuli would turn on angiogenesis. There are now ample tools to address this hypothesis, and indeed, new observations seem likely to refine it. For example, aFGF and bFGF are selectively exported by a number of tumor cell lines but not by many normal cells (Christofori, 1996; Friesel and Maciag, 1995; Kandel et al., 1991), suggesting that another feature of the switch mechanism may be the capability to sequester angiogenesis inducers such that despite being synthesized they are unavailable to stimulate angiogenesis until the sequestration is relaxed. In regard to endogenous inhibitors, a major objective will be to assess the proteolytic mechanisms and the generality by which cells can process abundant circulatory proteins such as fibrinogen and plasminogen to release cryptic angiogenesis inhibitors. Local processing of circulatory proteins may serve to produce levels of inhibitory activity sufficient to maintain the angiogenic switch in the off position. If the endothelium is normally bathed in locally or systemically processed cryptic inhibitors, the regulatory balance could be shifted and the angiogenic switch activated either by reducing the rates of inhibitor processing or by increasing the levels of the inducers seen at the endothelial cell, for example by up-regulating VEGF synthesis or by releasing sequestered FGF. It seems likely that the alternative strategies of increasing activator levels or reducing inhibitor levels to activate the angiogenic switch will each be utilized by different tissues according to their physiological characteristics. Thus, tissues such as the epidermis, which are well separated from the vasculature by a basement membrane and a stromal support, may favor mechanisms that increase inducer levels to elicit angiogenesis. In contrast, endocrine organs such as the pancreatic islets that are intimately vascularized may utilize negative regulators to maintain a quiescent endothelium and therefore require down-regulation of inhibitor production to activate the angiogenic switch.

The realization that increasing numbers of both angiogenic activators and inhibitors are being discovered (currently, there are at least a dozen of each), most with demonstrable activity directly on endothelial cells, raises an interesting question: how are all of these complementary and opposing signals integrated by the signal transduction network in endothelial cells, so as to control the complex dynamics of maintaining quiescence, activating angiogenesis, and then completing differentiation to produce functional new capillaries? Beyond the manifest goal of further clarifying the regulatory mechanisms of the angiogenic switch and the complex process of angiogenesis, an important opportunity now exists to apply this new knowledge in clinical oncology to restrict angiogenesis by altering the balance of angiogenic regulators.

Apoptosis, Clinical Applications, and the Goal to Control the Switch

The growing appreciation of the fact that angiogenesis is an integral component of the tumor phenotype has fueled considerable efforts to identify angiogenesis inhibitors that can be used as part of therapeutic strategies to interfere with tumor growth and metastasis. In addition to endogenous inhibitors such as interferon α/β and platelet factor-4, synthetic compounds have been identified using cell culture assays. These include the fungal-derived compound AGM1470 (TNP470), thalidomide, a number of metalloproteinase inhibitors, and others (reviewed by Auerbach and Auerbach, 1994; Bouck et al., 1996; Folkman, 1995a). Currently, there are nine compounds in clinical trials as angiogenesis inhibitors, with perhaps two dozen others in various stages of research and development as potential antiangiogenic therapeutics.

Looking beyond this first generation of angiogenesis inhibitors identified in cell-based assays, we envision the capability to control the angiogenic switch through specific knowledge both of the regulatory mechanism underlying the switch and the cellular details of the process of new blood vessel morphogenesis. One approach will likely involve altering the balance of endogenous inhibitors and activators by controlling synthesis or affecting sequestration and release. The beginnings of this approach are apparent. Monoclonal antibodies that bind and block the actions of VEGF are in development, as are compounds that interfere with signal transduction from its receptors. Knowledge of the morphogenesis of new vessels is also providing opportunities. For example, sprouting capillaries have been shown to express a specific type of cell–matrix interaction molecule, the integrins $\alpha_v\beta_3$ or $\alpha_v\beta_5$; abrogation of the contacts of these integrins to matrix evokes programmed cell death (apoptosis) of the new endothelial cells and dramatically impairs neovascularization of tumors (Brooks et al., 1994; Friedlander et al., 1995). Both antiintegrin antibodies and interfering RDG peptides are in development as candidates for blocking capillary morphogenesis via induction of apoptosis.

One emerging biological principle is the interrelationship between tumor angiogenesis and modulation of cell death. Classically, it has been apparent in certain tumors that necrotic death accompanies inadequate blood flow; for example, necrosis has been reported in glioblastomas and attributed to hypoxia resulting from inadequate vascularization. In turn, this clue led to the discovery that hypoxia can induce VEGF synthesis (Shweike et al., 1992; Shweiki et al., 1995; Stein et al., 1995). One study in which VEGF receptor function was inhibited in a transplantable glioblastoma with a dominant-negative VEGF receptor resulted in impaired tumor growth, which was ascribed to widespread necrotic cell death (Millauer et al., 1994). In addition, however, to necrotic cell death, it is now becoming clear that rapid expansion of a tumor mass can be significantly affected by the incidence of apoptosis.

To exemplify the interplay of apoptosis and angiogenesis, consider the transplantable Lewis lung carcinoma model (Figure 5A). Lewis lung tumors growing subcutaneously in mice seed distant metastases in the lung, but

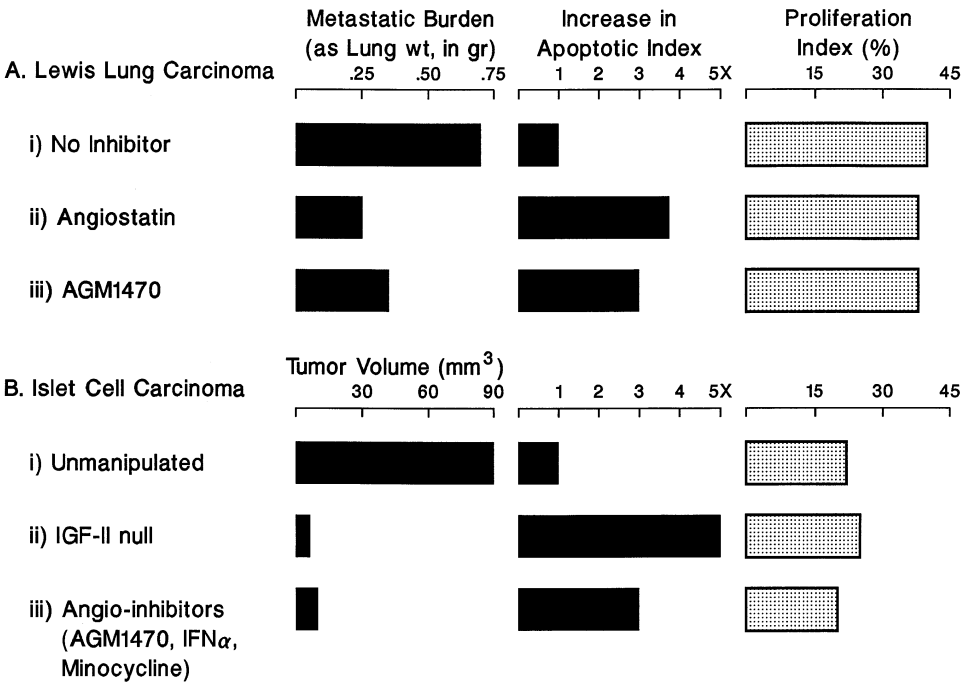

Figure 5. Tumor Growth Can Be Restricted by Apoptosis of Tumor Cells Elicited by Inhibition of Angiogenesis

(A) Liver metastases from a subcutaneous growth of Lewis lung carcinoma will grow explosively in the absence (i) of the primary tumor, which produces a circulating endothelial inhibitor (angiostatin). The apoptotic index is low and the proliferation index very high. When the primary tumor is present (ii), resulting in high serum levels of angiostatin, growth of the metastases is impaired, and the apoptotic index is markedly increased in the metastatic nodules. If the primary tumor generating angiostatin is absent, but instead the angiogenesis inhibitory drug AGM1470 (TNP470) is inoculated daily (iii), metastatic growth is again restricted, concomitant with increased apoptosis. Neither angiogenesis inhibitor affects the proliferation index of the metastatic tumor cells (shown); in both cases, the density of new capillaries is significantly reduced (data not shown).

(B) Transgenic mice expressing the SV-Tag oncogene in pancreatic β cells develop islet cell carcinomas with a high proliferation index and a low incidence of apoptosis (i). In the absence of the survival factor IGF-II, tumor volume is dramatically impaired, with a concordant 5× increase in apoptotic index (ii), demonstrating that apoptosis can restrain primary tumor growth. When the transgenic mice are treated with a regimen of angiogenesis inhibitors, including AGM1470, tumor growth is again impaired, also with a concordant increase in the incidence of apoptosis (iii), indicating that the reduced vascularization (data not shown) elicits tumor cell apoptosis. Again, tumor cell proliferation is not affected by inhibition of angiogenesis.

these remain dormant as small nodules of tumor cells that do not elicit angiogenesis, as a consequence of the presence in the blood of high levels of the angiogenesis inhibitor angiostatin, which is released from circulating plasminogen by the primary tumor (O'Reilly et al., 1994.) (The reason that angiostatin does not inhibit angiogenesis in the primary tumor is not clear but is thought to reflect a local balance in favor of angiogenesis inducers.) Examination of the latent metastases revealed a high incidence of S-phase cells (30%) and yet little increase in metastatic tumor mass. The explanation appears to be that the latent metastases evidence a high incidence of apoptosis (8%), balancing rapid cell division with cell death (Holmgren et al., 1995). Release of the angiogenic blockade (by removing the primary tumor) allowed angiogenesis and rapid expansion of lung metastases (Holmgren et al., 1995; O'Reilly et al., 1994). The synthetic angiogenesis inhibitor AGM1470 could similarly hold latent metastases in place; again, the pattern was one of high incidence both of cells in S phase and of cells undergoing apoptosis (Holmgren et al., 1995). Recently, purified angiostatin has been shown to impair significantly the growth of three primary human carcinomas and two additional murine tumors in immunodeficient and syngeneic mice, respectively; tumors in the angiostatin-treated mice also had comparable proliferation indices to saline-treated controls and markedly increased frequencies of tumor cell apoptosis (O'Reilly et al., 1996).

In a second example, that of islet cell carcinomas in RIP-Tag transgenic mice (Figure 1A), the end-stage tumors evidence a low incidence of apoptosis, which appears to be maintained both by intrinsic factors and by intense vascularization of tumors (Figure 5B). When the embryonic growth/survival factor IGF-II, which is activated in this tumorigenesis pathway, was abrogated using gene-knockout mice, the tumor volume was dramatically decreased (Christofori et al., 1994, 1995a; Naik et al., 1994). The S-phase incidence was comparably high (more than 20%) in both normal and IGF-II–deficient tumors (Naik et al., 1994); the difference was in the apoptotic incidence, which rose 5-fold in the IGF-II–null tumors (Christofori et al., 1994). Thus, IGF-II produced by the tumor cells is implicated in the control of apoptosis. Yet the low level of apoptosis seen in the islet cell tumors is also highly dependent on persistent angiogenesis. When the RIP-Tag mice were treated with a regimen of

angiogenesis inhibitors (interferon α, minocycline, and AGM1470), tumor growth was dramatically impaired and vessel density significantly reduced (Parangi et al., 1996). Again, the same pattern was apparent: the S-phase incidence remained high, while the apoptotic incidence increased significantly in the small tumors arising in mice treated with angiogenesis inhibitors.

Together, these two models implicate the vasculature as a paracrine regulator of apoptosis. Therefore, in addition to its long-recognized association with necrotic cell death, inadequate vascularization can also elicit tumor cell apoptosis. And recall that lack of integrin signaling can produce endothelial cell apoptosis. This convergent realization, that apoptosis can significantly modulate tumor growth by its occurrence both in tumor cells and in the supporting endothelium, opens up new opportunities for the design of anticancer therapeutics that enhance apoptosis.

In conclusion, the horizons of angiogenesis research include several opportunities in regard to cancer. First, angiogenesis inhibitors seem likely to become an important component of therapeutic strategies aimed at invasive metastatic tumors. Second, as methods for early detection of certain classes of cancer improve, it may become possible to interfere with initial tumor development by blocking the angiogenic switch that precedes the progression to invasive cancer, for example at the CIS stage. Third, a major issue in oncology is the development of effective adjuvant therapies for use following resection of a primary tumor. In many cases, cancer cells, either local or at remote sites, are left behind with a potential for progression to produce a recurrent cancer; antiangiogenic therapy could restrain such lesions or even drive them into apoptotic catastrophe. For all of these applications, more effective angiogenesis inhibitors will likely need to be developed; knowledge of molecular and cellular mechanism will certainly facilitate that process. Moreover, as this review has documented, the complementarity of mouse models with human diseases will continue to afford powerful opportunities. For example, therapeutic trials using mouse models are amenable to expedient combinatorial testing, as exemplified both by traditional tumor transplantation models (Teicher et al., 1992) and, more recently, by the use of transgenic models of de novo tumorigenesis (Parangi et al., 1996). There is reason to be optimistic that in the foreseeable future, the initial angiogenic switch during tumorigenesis, as well as ongoing neovascularization in tumors, can be controlled and perhaps completely curtailed, contributing thereby to more efficacious cancer therapies.

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