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New Perspectives in Clinical Oncology from Angiogenesis Research

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TUMOUR BURDEN AFFECTS TUMOUR ANGIOGENESIS

SINCE THE early 1970s, the conventional wisdom in angiogenesis research was that the neovascularisation in a given tumour bed was an isolated event, unrelated to other tumours. In the past 2 years, it has been recognised that total tumour burden can affect angiogenesis in remote metastases. This recognition came about because of the demonstration by O'Reilly and associates [1] that a primary tumour could suppress angiogenesis in its metastases and that this would lead to inhibition of growth of the metastases. This process was found to be mediated by a novel endogenous angiogenesis inhibitor, angiostatin, generated by the primary tumour [1]. Angiostatin is a 38 kD protein with homology to the first four kringle structures of plasminogen. It is a specific inhibitor of endothelial cell proliferation.

Three converging lines of experimental work led up to O'Reilly's discovery of angiostatin. The first was that, during the 1980s, my laboratory had been trying to understand how certain primary tumours could suppress the growth of their metastases. Since the turn of the century, clinicians and laboratory investigators have recognised that certain human and animal primary tumours could suppress the growth of their metastases [2–8]. Furthermore, Prehn argued with compelling data that the rate of tumour growth was inversely proportional to total tumour burden, regardless of how the tumour was distributed, e.g. single tumour versus multiple implants or metastases [9, 10]. Oncologists have observed that very large tumours may grow very slowly and that 'debulking' of a large tumour mass may render the residual tumour more susceptible to cytotoxic chemotherapy. Many explanations have been offered to explain these phenomena, including mechanisms based on immunity (concomitant immunity), hormonal interactions and metabolic changes (accumulation of catabolites).

The second line of experiments was a collaboration with Douglas Hanahan (of the University of California, San Francisco). We were studying the mechanism of the switch to the angiogenic phenotype by beta cell carcinomas arising *de novo* in the pancreatic islets of transgenic mice [11, 12]. Two angiogenic proteins, aFGF (acidic fibroblast growth factor) and VEGF (vascular endothelial growth factor) revealed similarly high levels of expression by these islets, before and after the angiogenic switch [13, 14]. This provocative result forced us to think that the angiogenic switch could not be explained entirely by positive regulators of angiogenesis, but could also involve some role by negative angiogenic regulators.

The third line of experiments was reported in 1989, from Noel Bouck's laboratory at Northwestern University Medical School, Chicago, Illinois, U.S.A. [15]. Thrombospondin was identified as a negative regulator of angiogenesis that was downregulated during the switch to the angiogenic phenotype in transformed fibroblasts. Subsequently, thrombospondin was shown to be upregulated in human tumour cells by the restoration of p53 tumour suppressor function, thus negating the angiogenesis inducers that the cells themselves produced as well as added bFGF, leading to blocked angiogenesis [16]. It immediately occurred to us that a primary tumour elaborating both angiogenic stimulators and 'left-over' inhibitor(s) could generate accumulating levels of the putative inhibitor in the circulation, if such an inhibitor had a longer half-life in the circulation than the stimulator. Thus, when O'Reilly found that angiostatin accumulated in the serum and urine of tumour-bearing mice, but disappeared from serum and urine after removal of the tumour, this suggested a role for angiostatin as a mediator of the suppression of metastases by the primary tumour. It became clear that a primary tumour could influence the growth of a metastasis by inhibiting angiogenesis in the metastasis, without communicating directly with its tumour cells.

Clinical implications

This result provides a conceptual framework to explain the rapid tumour recurrence or the explosive growth of metastases that may occur within a few months after removal of a primary tumour in some patients. Brem and Cawley studied 1300 patients whose primary tumours were excised at a time when there were no detectable metastases; approximately 3% developed metastases within 2–12 months of the surgical resection (Harold Brem, James Cancer Center, Ohio State University, Columbus, Ohio, U.S.A.).

A NOVEL FORM OF TUMOUR DORMANCY: BALANCED PROLIFERATION AND APOPTOSIS OF TUMOUR CELLS IN THE PRESENCE OF BLOCKED ANGIOGENESIS

Tumour dormancy may be defined as clinically undetectable, microscopic disease, in which there is no expansion of total tumour mass. Stable disease may be defined as a clinically detectable tumour mass which is not undergoing expansion. Explanations of the cellular mechanism of tumour dormancy have been based on assumptions that tumour cells may enter a prolonged quiescent state removed from the cell cycle (G_0) [17]. Other hypotheses involve immune control of tumour size [18–20], or hormone withdrawal in hormone-dependent tumours [21]. However, the demonstration, in mice, that a primary tumour inhibits angiogenesis in remote metastases [1], led Holmgren and colleagues to propose a new hypothesis to explain tumour dormancy [22]. He found that during complete or nearly complete suppression of angiogenesis in metastases which became dormant, the proliferation rate of tumour cells remained as high (up to 40%) as in the growing metastases where angiogenesis was not inhibited. In contrast, the apoptotic rate of tumour cells was generally low (2–3%) in angiogenic growing metastases, but increased by approximately 2–3-fold when angiogenesis was blocked. This pattern, in which inhibition of angiogenesis appears to limit tumour growth by elevating the incidence of tumour cell apoptosis, has also been observed in primary tumours made dormant by therapy with angiostatin [23]. Some transforming oncogenes increase proliferation, but also induce apoptosis [24–26]. These transformed cells can be rescued from apoptosis by survival factors such as insulin-like growth factor 1 (IGF-1) [27]. Holmgren proposed that blocked angiogenesis limits access to these survival factors and leads to increased rates of apoptosis. During robust angiogenesis, these survival factors could become accessible to tumour cells not only from plasma perfusing through the tumour, but also by release from endothelial cells directly, a paracrine route [28].

CERTAIN COMMON CLINICAL PATTERNS OF PRESENTATION OF METASTASES MAY HAVE AN ANGIOGENIC BASIS

The demonstration, in mice, that a primary tumour may suppress the growth of a metastasis by inhibiting its angiogenesis [1], and the model of tumour dormancy based on blocked angiogenesis, have raised the question of whether other common clinical presentations of metastases may be explained by an angiogenic process. Four common patterns and one rare pattern of metastatic presentation are depicted in Table 1.

The first pattern, in which metastases grow after removal of the primary tumour, is analogous to a mouse model of Lewis lung carcinoma in which lung metastases remain microscopic while the primary is present (and generating angiostatin), but grow rapidly within 5 days after the primary tumour is removed [1, 29]. The second pattern, in which metastases grow concomitantly with the primary tumour, is analogous to a mouse model of a subclone of Lewis lung carcinoma in which the primary tumour does not suppress its lung metastases and does not generate detectable levels of angiostatin in the circulation [1, 29]. The third pattern, known to oncologists as the 'occult primary' [30] could be based on metastatic cells that outgrow the primary tumour and suppress its angiogenesis. This is speculation and there are no animal models, with the possible exception of one report, in which metastatic cells were seeded in the lungs, followed later, by implantation of a subcutaneous tumour, whose growth was inhibited [31].

The fourth pattern, in which metastases remain dormant for years following removal of the primary tumour, is observed in breast cancer, colon cancer, Ewing's sarcoma and in many other tumour types. Again, the mechanism of this prolonged dormancy is unknown. However, recent reports indicate that once metastases become clinically detectable, they display a similar rate of growth which is independent of the number of years of dormancy [32]. This observation is consistent with a model of microscopic dormant metastases which do not expand until sometime within a year of becoming clinically detectable [32]. It can

Table 1. Common clinical patterns of metastasis

First clinical presentation	Metastases	Possible angiogenic mechanism
I. Primary tumour	Metastases appear a few months after removal of the primary	Primary tumour inhibits angiogenesis in vascular beds of metastases
II. Primary tumour plus metastases	Metastases already present when primary tumour is diagnosed	Primary tumour does not suppress angiogenesis of metastases
III. Occult or 'unknown' primary tumour in presence of metastases	Metastases only are detected	Metastases shed from small primary tumour soon after it becomes neovascularised. Primary not large enough to suppress angiogenesis in remote metastases (or metastases suppress angiogenesis in the primary)
IV. Primary tumour	Metastases do not appear until years after removal of primary tumour	Metastatic cells may not be angiogenic during dormant period
V. Primary and metastases detectable	Rare cases of renal cell carcinoma in which metastases regress after primary tumour is removed	Metastases possibly dependent on high production of angiogenic factors (or other growth factors from primary tumour)

Adapted from [60].

be speculated that these metastases were originally derived from cells that were not angiogenic, but underwent the switch to the angiogenic phenotype prior to becoming clinically detectable. No animal model has been reported. However, Michael O'Reilly in our laboratory has demonstrated a B16 melanoma in C57BI/6 mice in which dormant, non-angiogenic lung metastases of less than 0.1–0.2 mm diameter are found as late as 6 months after removal of the primary tumour in otherwise healthy mice. This is a quarter the normal life-span of these animals (M. O'Reilly, Harward Medical School, Massachusetts, U.S.A.). A study of non-angiogenic tumour cells that escape from the primary tumour, and the mechanism by which their metastases switch to the angiogenic phenotype, would be a fruitful area of research.

The fifth pattern of metastatic presentation is occasionally observed, for example, when removal of a renal cell carcinoma is followed by regression of lung metastases. One can speculate that the metastases may have been dependent upon high production of circulating angiogenic factors [33] (and possibly other growth factors) from the primary tumour. In renal cell carcinomas, high tissue levels of bFGF correlate with high mortality [33]. In fact, we found that 10% of a group of patients with a wide spectrum of malignancies had abnormally elevated levels of the angiogenic polypeptide bFGF in their serum and 37% of 950 patients had abnormally elevated levels of bFGF in their urine [34]. Currently, there is no animal model.

The clinical presentations of metastases are discussed here in terms of angiogenic mechanisms, because this approach offers a plausible unifying explanation for the different patterns which oncologists see in their cancer patients. The similarity of animal models to human patterns of metastasis presentation does not prove that angiogenic control of metastatic growth is responsible for the behaviour of metastases in cancer patients. These mechanisms have yet to be demonstrated in human cancer. However, clinicians who are aware of them may be able to uncover evidence which supports or rejects the hypothesis.

DORMANCY THERAPY

Systemic administration of angiostatin to tumour-bearing mice potently inhibits the growth of three murine tumours and three human tumours [23]. Human breast cancer, prostate cancer and colon cancer growing in SCID immunodeficient mice, treated with angiostatin after they attain a size of up to 1% of body weight, all regress to microscopic dormant foci and did not recur during therapy. Tumour cell proliferation is balanced by a high rate of apoptosis in the presence of blocked angiogenesis (Table 2). There is no toxicity or drug resistance. All tumours recur when treatment is discontinued, except for the prostate carcinomas. After discontinuation of therapy in the prostate carcinomas, four mice were killed and examined histologically. Of the remaining three, one died of recurrent tumour and two are currently tumour-free and healthy at 1 year.

With previously reported angiogenesis inhibitors, such as the synthetic analogue of fumagillin, TNP-470 (AGM-1470) [35], tumour regression has not been possible, although large tumours such as Lewis lung carcinomas have been reproducibly inhibited by approximately 65% (i.e. treated tumour volume/control tumour volume [T/C] of 0.35) [36]. Because TNP-470 is among the more potent of the angiogenesis inhibitors currently in clinical trial [37, 38], we had always assumed that once such inhibitors were approved for use in cancer patients, they would be employed only as adjuncts to conventional chemotherapy or radiotherapy. However, the greater anti-angiogenic activity of angiostatin and its ability to produce tumour dormancy, suggests that, in the future, anti-angiogenic therapy may also be continued after the completion of chemotherapy or radiotherapy, to maintain dormancy in any residual tumour. Thus, anti-angiogenic 'dormancy therapy' may eventually be used for prolonged periods of time, analogous to therapy with tamoxifen. This will depend on the availability of angiogenesis inhibitors that have little or no toxicity and that do not induce drug resistance.

A MODEL OF ENDOTHELIAL CELL AND TUMOUR CELL COMPARTMENTS IN MALIGNANCY

Virtually all current methods of chemotherapy and radiotherapy are designed to treat the tumour cell population in a solid tumour or in leukaemia. The endothelial cell population is rarely considered. Yet, a cubic centimeter of tumour containing 10^8–10^9 tumour cells will also contain approximately 20×10^6 endothelial cells [58], i.e. approximately one endothelial cell for every 100 tumour cells. Furthermore, in most tumours, tumour cells are arranged as perivascular cuffs. Microscopic histological sections (4 μm thick) show capillaries or small venules encircled by approximately 3–6 (or more) concentric layers of tumour cells. Three-dimensional reconstructions and confocal microscopy reveal that these configurations are in fact, microcylinders of tumour cells surrounding each vessel [29]. This configuration is similar from one tumour to another, regardless of whether the tumour appears to be 'highly vascularised' or 'poorly vascularised' when the gross tumour specimen is

Table 2. Tumour cell proliferation and apoptosis in human tumours in SCID immunodeficient mice treated with human angiostatin subcutaneously or vehicle alone. Prostate cancer, colon cancer and breast cancer were treated for 28 days, 32 days and 58 days, respectively

		Proliferative index (Ki67)	Apoptotic index (TUNEL)
Colon cancer	Angiostatin	84.4 ± (0.92)%	5.30 ± (0.70)%
	Saline	80.3 ± (2.20)%	1.15 ± (0.12)%
Breast cancer	Angiostatin	44.1 ± (0.40)%	2.50 ± (0.15)%
	Saline	46.3 ± (0.60)%	0.55 ± (0.06)%
Prostate cancer	Angiostatin	–	6.50%
	Saline	–	1.20 ± (0.10)%

Adapted from [23]. Values show are means and standard errors.

observed. Small differences in intercapillary distance at the microscopic level can give the macroscopic appearance of rich or poor neovascularisation. Nevertheless, in both tumour types, it should be emphasised that the capillary blood vessels are new and proliferating. This small point is important because of a misconception held by some oncologists that 'only highly vascular tumours would be candidates for anti-angiogenic therapy.' Experimental evidence indicates that a wide variety of tumours respond to anti-angiogenic therapy regardless of their vascular appearance.

The close configuration of tumour cells and endothelial cells permits a form of paracrine communication between these two cell populations [39–41]. Tumour cells release endothelial mitogens, such as bFGF and VEGF (and others), while endothelial cells produce proteins which act as growth factors or survival factors for tumour cells. These include platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), interleukin-6, granulocyte monocyte colony stimulating factor (GM-CSF), and heparin-binding epithelial growth factor (HB-EGF) (for review see Bussolino and colleagues [28, 42]). This model is illustrated in Figure 1.

Because anti-angiogenic therapy alone (with angiostatin, a specific inhibitor of endothelial cells) can cause tumour dormancy in mice, a result equivalent to or better than that accomplished with chemotherapy in mice (Timothy Browder, Children's Hospital, Boston, Massachusetts, U.S.A.), the endothelial compartment needs to be considered as a therapeutic target. It has the advantage that endothelial cells undergo little or no mutation, and thus anti-angiogenic therapy directed at the endothelial population has not resulted in drug resistance in experimental animals. Kerbel has proposed anti-angiogenic therapy as a strategy to bypass drug resistance [43]. Furthermore, combinations of anti-angiogenic therapy (not as potent as angiostatin, e.g. TNP-470) and conventional chemotherapy, are curative in mouse tumours, where either agent alone is not [44].

This model may be useful for understanding certain current practices with conventional chemotherapy. For example, paclitaxel is usually administered for metastatic breast cancer once every 3 weeks for approximately 6–7 such cycles. However, there are scattered reports of patients treated for 20 cycles or more, who have stable disease. Why have these patients not developed drug resistance? What if the tumour compartment has become drug resistant, but the endothelial cell compartment has not? At least two reports show that paclitaxel has some anti-angiogenic activity in addition to its cytotoxicity [45, 46]. This could explain its long-term effect in some cases. The majority of chemotherapeutic drugs do not have anti-angiogenic activity [47]. In fact, Timothy Browder in my laboratory found that systemically administered cyclophosphamide or doxorubicin, but not 5-fluorouracil or others, could inhibit neovascularisation in the mouse cornea. Klauber and associates found that paclitaxel administered systemically also inhibits corneal vascularisation [61].

In children with acute lymphoblastic leukaemia, bone marrow biopsies taken before therapy show intense neovascularisation [48]. Microvessel counts of histological sections stained with the antibody to Von Willebrand factor are 7–10 times higher than non-leukaemic bone marrows. Confocal microscopy of these biopsies reveals that leukaemic cells also cluster around capillary vessels in the form of perivascular microcylinders, similar to their configuration in solid tumours. It remains to be demonstrated whether angiogenesis inhibitors can be added to conventional therapy.

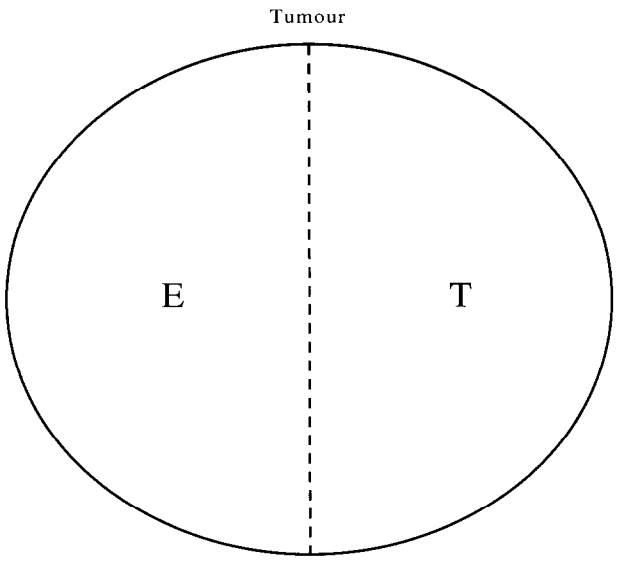

Figure 1. Model of endothelial cell and tumour cell compartments. Tumour cells can stimulate endothelial cell proliferation (and migration) by production of angiogenic proteins such as bFGF, VEGF, platelet-derived endothelial cell growth factor (PD-ECGF) and others. Endothelial cells can stimulate growth of tumour cells by production of platelet-derived growth factor (PDGF-BB), insulin-like growth factor I (IGF-1), bFGF, heparin-binding-epithelial growth factor (HB-EGF), granulocyte colony stimulating factor (G-CSF) and interleukin-6 (IL-6) (based on Folkman) [28]. This is the paracrine effect of neovascularisation, which together with the 'perfusion' of nutrients and oxygen and removal of catabolites, permits expansion of a tumour or its metastasis. This two-compartment model is important in understanding the value of adding anti-angiogenic therapy to conventional chemotherapy. For example, in experimental animals, long-term inhibition of angiogenesis can control tumour growth with little or no development of drug resistance. This may be due, in part, to the fact that the mutation rate of endothelial cells is very low compared to the high mutation rate of tumour cells. In one recent analysis, a gram of tumour contained 20 million endothelial cells [58]. A gram of tumour would contain approximately 10^8–10^9 tumour cells. Therefore, the ratio of endothelial cells to tumour cells may be in the range of $1{:}10^2$ to $1{:}10^3$. Reprinted with permission from *Nature Medicine* 1996, Vol. 2, p. 167.

THE INDOLENT TUMOUR — A POSSIBLE ANGIOGENIC MECHANISM

Some human tumours, e.g. prostate cancer, grow slowly for years and then seem to shift to a faster rate of expansion of tumour mass. Other tumours, e.g. glioblastoma and certain breast cancers, appear to slow their expansion at a late stage in their existence, especially if they have become very large masses. These tumours are called indolent. While it is widely assumed that the slow growth rate of the indolent tumour is due to a low mitotic rate, it is also possible that angiogenesis plays a role. In some tumours, the switch to the angiogenic phenotype may not be maximal initially, but may increase incrementally over time. This could limit the

rate of tumour growth, independent of the mitotic rate of its tumour cells.

Angiostatin therapy of murine tumours [1, 23] provided the first clue for this idea. Untreated tumours that were growing rapidly or tumours that were held dormant by anti-angiogenic therapy revealed the same high proliferation rate of their tumour cells. Angiogenesis was inversely related to tumour cell apoptosis, but not to mitosis. A second clue came from the experiments of Yihai Cao in my laboratory. He transfected angiostatin into murine fibrosarcoma cells and selected transfectants for different levels of expression of the inhibitor (Cao *et al.*, unpublished data). Angiostatin transfection did not affect the proliferation rate of tumour cells *in vitro*. However, the growth of primary tumours arising from injections of the transfected cells into mice was slowed by up to 77% of the control tumour cells. The transfected tumours thus became indolent. The rate of tumour growth was governed mainly by the level of overall angiogenic activity, itself a result of the level of expression of angiostatin.

CERTAIN MISCONCEPTIONS ABOUT TUMOUR ANGIOGENESIS MAY AFFECT THE DESIGN OF CLINICAL TRIALS FOR ANTI-ANGIOGENIC THERAPY

As angiogenesis inhibitors move from the laboratory to clinical trial, certain misconceptions about these novel agents may interfere with the optimum design of a clinical study.

For example, the presence of angiogenesis does not distinguish between a benign and a malignant tumour [49]. Adrenal adenomas are highly vascularised benign tumours, but apparently lack the growth potential to take advantage of the new blood vessels they have induced. Thus, the onset of angiogenesis permits expansion of a tumour mass, but does not guarantee it. In fact, the switch to the angiogenic phenotype occurs independently of other events in tumorigenesis. In most tumours, angiogenesis appears after the expression of the malignant phenotype. However, in carcinoma of the cervix, the preneoplastic stage of dysplasia becomes neovascularised before the malignant tumour appears [50]. This sequence of events also occurs in certain spontaneously arising tumours in animals [51].

Angiogenesis may not be necessary for certain tumour cells that can grow as a flat sheet between membranes, i.e. gliomatosis in the meninges.

It is widely believed that the blood vessels of a large tumour are 'established'. The argument follows that anti-angiogenic therapy could never reduce tumour size or cause tumour regression because 'established' vessels would, by definition, be refractory to such treatment. Anti-angiogenic therapy can, however, cause growing blood vessels to involute [52], and can bring about regression of growing tumours [23, 53, 54]. Furthermore, the replication rate of endothelial cells in tumour vessels is significantly greater than in the endothelial cells of normal tissue [55]. Obviously, a few feeder vessels, usually arteries, may be observed in the midst of a histological cross-section of a tumour, and could be considered as established, but these are not the new microvessels which tumour cells have induced.

A commonly stated axiom is that 'tumours outgrow their blood supply'. This is inaccurate. Growing tumours can gradually compress their blood supply because of increasing interstitial pressure [56]. These compressed areas become ischaemic, but they are not avascular. Necrosis follows. Vessel compression also interferes with the optimal delivery of therapeutic agents [56]. Paradoxically, anti-angiogenic therapy can decrease ischaemia apparently due to its ability to 'unpack' a tumour and to decrease interstitial pressure.

CONCLUSION

Recently reported studies of the effect of tumour burden on angiogenesis and of the identification of at least one endogenous protein which mediates the inhibition of angiogenesis in metastases by a primary tumour have been described. These experiments convey an important lesson, namely that we may have underestimated the power of the endothelial cell to control tumour growth, perhaps because angiogenesis inhibitors available until now have been unable to block completely or virtually completely endothelial proliferation (or migration) in a tumour bed. The induction of prolonged tumour dormancy in a large primary tumour, without toxicity or drug resistance in tumour-bearing mice, has become possible because of the increased potency of endogenous angiogenesis inhibitors. These results from angiogenesis research have also changed our thinking about other aspects of human cancer.

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