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METASTASIS AND ANGIOGENESIS

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Abstract

The relationship of angiogenesis, the growth of new blood vessels, to the process of tumour metastasis is examined. While the occurrence of angiogenesis generally is a necessary prerequisite for the formation of secondary tumours its presence is not a guarantee that cancer dissemination will occur. Novel approaches to antimetastatic therapy, using the newly formed vascular bed of tumours as the target, have produced successful results in experimental animals though such approaches have yet to be undertaken in the clinic.

Key words: Angiogenesis, angiogenic factors, metastasis, neovascularisation, tumour vasculature

Cancer in its systemic form has been recognised since antiquity, yet an appreciation of the mechanisms underlying discontiguous growth did not emerge until the late 19th century. This appreciation was based upon two important pathological concepts. The first, enunciated by Johannes Müller, was on the cellular character and proliferative nature of all neoplastic growths and the second, established by Rudolf Virchow, was that of the phenomenon of embolism. Although several observers had noticed similarities between widely separated tumour masses in individual patients prior to the establishment of these principles it was only after their promulgation that the pathophysiological basis of tumour dissemination was recognised.

Cancer metastasis is a complex multistep phenomenon which is the exclusive property of malignant tumours. This process, which is outlined in the Figure, generally is divided into a series of discrete stages for descriptive purposes. After the initial transforming event has occurred the neoplastic cells proliferate to form the primary tumour mass. Cells detach from this growth, invade locally by penetrating the interstitial connective tissue matrix and infiltrating the intercellular spaces and cross the continuous basement membrane, which separates organ cells, epithelia and endothelia from underlying connective tissues, to enter the lumina of small blood vessels or lymphatics. Considerable evidence has been accumulated to show that much of this invasive and intravasative behaviour is a consequence of the elaboration and secretion of a variety of degradative enzymes, both by cancer cells and infiltrating host leukocytes, capable of digesting the various protein barriers that have to be crossed in order for cells to reach the circulation (1). Combined with this proteolytic ability disseminating tumour cells possess the capacity for active migration, perhaps under the control of secreted autocrine or paracrine factors which stimulate second messenger signalling systems (2). Once intravasation has occurred cells may grow at the site of penetration or, more commonly, they may be released, either singly or as small embolic clumps, into the vascular/lymphatic channels. Occasionally cells which detach from the main tumour mass may, instead of invading vessels, migrate between local tissue spaces, along nerve sheaths, across the peritoneal cavity or in the cerebrospinal fluid to gain lodgement at remote sites (3). Survival of neoplastic cells in the circulation is an obligatory step to allow continuation of the metastatic sequence but the mere demonstration of viable tumour cells in the blood or lymph is not an inviolable indicator of eventual metastatic development (4). Non-specific trauma during passage round the body certainly is responsible for the demise of the majority of circulating tumour cells (5) but there also is the opportunity for heterotypic interactions with host cells, such as platelets, lymphocytes and monocytes, and many of these interactions are thought to be cytotoxic to the cancer cells (6). Following successful transport in the circulation viable tumour cells are arrested in the microvasculature at a distant site.

It is obvious that detachment of cells from a primary

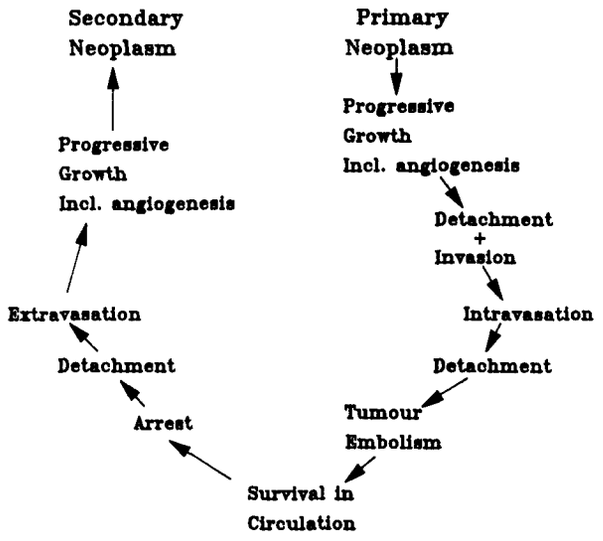

Figure. Steps involved in the metastatic process.

mass, clumping of cells to each other or to distinct cell types and cellular adhesion to microvascular endothelium or exposed basement membranes in distant organs are all processes dependent upon cell surface moieties on both host and tumour cells. The past few years have seen an explosion of knowledge regarding such cell adhesion molecules and substrate adhesion molecules (7) and it is likely that variations in expression or regulation of these molecules dramatically influences metastatic success while, in leukaemia at least, it is apparent that patterns of metastasis are also a consequence of specific molecule expression (8).

Subsequent to their arrest cells which will give rise to secondary tumour growths must extravasate, a step which necessitates penetration of the vascular basement membrane and dissolution of, and movement through, the extravascular connective tissue stroma. In order to form a clinically overt secondary neoplasm, it is then essential that such cells proliferate and grow (Figure). Once established the metastatic tumour can itself generate the metastatic sequence so that initial secondary growths may act as generalising sites (4).

It is clear that the pathobiology of cancer spread is enormously complex and depends upon an interplay between host and tumour cell properties. These forces of selection applied to an heterogeneous starting population of cells are thought to allow the emergence of pre-existent populations of metastatic cells suggesting that, in large measure, metastasis is a selective process (9, 10).

What role though does the process of angiogenesis or neovascularisation play in metastasis? Angiogenesis is defined as the generation and development of capillary blood vessels. Originally the phrase was coined to describe the growth of new vessels in the placenta (see ref. 11), but it is clear that angiogenesis occurs in several physiological and pathological situations, including generation of the vascular tree during embryonic development, regeneration of the endometrium after menstruation, wound repair, certain chronic inflammatory conditions and tumour growth (12). Folkman and his colleagues showed conclusively that development of a tumour mass beyond approximately 2 mm in size could only proceed if a vascular supply was established (12–14). Since the occurrence of metastasis frequently, though not invariably, is a correlate of increasing size of the primary tumour (15) and since metastatic deposits of 2 mm diameter size are clinically inapparent, it is clear that angiogenesis must play an integral role in metastasis. Indeed from the Figure it can be seen that, of the discrete steps which constitute metastasis, angiogenesis is likely to be involved in two stages of secondary tumour development.

The vascularisation of tumours

It has been known since the 19th century that tumour stroma contains a rich network of capillary vessels (for review see 16). However, these static observations gave few clues as to the source of the vessels and it was not until the development of the transparent chamber technique (17) which was then adapted for implantation into the back of mice (18, 19) that it was shown that growing tumours elicited the continuous ingrowth of new capillaries from surrounding host tissue and that this vascular proliferation was considerably greater than that evoked by mere inflammation. These observations suggested that a tumour-derived factor was responsible for the enhanced vascularisation. However, it was not until Greenblatt and Shubik interposed a millipore filter between a melanoma transplant and a transparent chamber implanted in the hamster cheek pouch that it was shown that such a diffusible factor truly existed (for review see 16). A few years later Folkman and co-workers (13) were able to isolate a soluble factor from the Walker 256 tumour, partially purify it to show it was polypeptide in nature and coined the term 'Tumor Angiogensis Factor' (TAF). Since that time it has become apparent that a variety of polypeptide factors produced both by neoplastic and normal tissues have angiogenic activity and, since the cDNAs which code for these proteins have been isolated, it is now possible to dissect out the role that each of these factors plays in elicitation of an angiogenic response (see below).

Though the vessels which supply developing tumours are derived from host vasculature there may be substantial differences in their architecture from that observed in normal tissue. Thus tumour vessels often are dilated, saccular and tortuous and may contain tumour cells within the endothelial lining of the vessel wall (20). Blood flow through such vessels may be sluggish relative to that in adjacent normal tissues and the tumour microvasculature may be hyperpermeable to plasma proteins. Somewhat

surprisingly it has recently been found, by the use of macromolecular tracers, that immature tumour-penetrating vessels did not leak, whereas the leaky vessels were mature veins or venules lined by continuous endothelium (21). It is conceivable that these variations in new vessel morphology and function may facilitate tumour dissemination. Neoplastic cells lining sinus-like spaces may not require the elaboration of specific proteases, such as collagenase IV, in order to degrade host tissue, but instead may be liberated directly into the bloodstream with no requirement for the digestion of a basement membrane. Similarly, the sluggish flow existing within the first vessels entered may dictate that released cells have greater opportunity to produce potentially protective heterotypic clumps before reaching the more turbulent general circulation. Exactly when the transition to the angiogenic state occurs during tumour development has long been a matter of some uncertainty. However, in a recent series of exceptionally elegant experiments, Folkman and colleagues (22) have addressed this issue. Using transgenic mice, expressing an oncogene in the β-cells of pancreatic islets where a consistent temporal movement from normal to hyperplastic to neoplastic was discernible, these workers were able to show that induction of angiogenic activity preceded tumour formation and correlated with the transition from hyperplasia to neoplasia (22). Thus, for a tumour to become metastatic requires angiogenesis to occur, but it should be noted that the mere existence of this phenomenon is not alone sufficient to ensure the development of secondary tumours since in the transgenic model cited above metastasis is an infrequent event (23). Nonetheless there is a strong correlation between the occurrence of angiogenesis and the incidence of metastasis. Accordingly, Liotta et al. (24) showed that in mice bearing fibrosarcomas implanted in the thigh the first appearance of malignant cells in the efferent circulation of the tumour coincided with the ingrowth of new vessels in the tumour; while the number of cells released and the number of pulmonary metastases developing six days later both correlated with the number of tumour blood vessels (24). A striking clinical example of this association between neovascularisation and the propensity of a tumour to spread is provided by the progression of superficial spreading melanoma. While confined to the epidermis thin superficial melanoma is not vascularised and shows no tendency to metastasise even though its behaviour may be typically invasive. Disruption of the basement membrane underlying the epidermis however, with extension of tumour cells into the dermis, correlates with local vascular proliferation and the formation of systemic metastases (25).

The process of capillary formation

It has been shown by time-lapse cinemicroscopy and light and electron microscopy that the formation of new capillaries is an invasive process which represents a consistent and predictable sequence of events performed by vascular endothelial cells in response to angiogenic stimuli (26–29). The same programme of events is reproduced whether the stimulus is physiological, immunological or tumour-derived. New capillaries always arise from pre-existing capillaries or venules, never from arteries, arterioles or veins. The pre-existing parent vessel (or vessels) in the vicinity of the angiogenic stimulus engorge and increase their permeability. Endothelial cells lining the vessel on the side facing the stimulus undergo a change in morphology, appearing thickened, with increased endoplasmic reticulum, exhibiting prominent ribosomal studding and greater numbers of intracellular organelles (26, 28, 29). Subsequently, the vascular basement membrane adjacent to the stimulus undergoes a patchy loss of integrity. Since endothelial cells *in vitro* can respond to angiogenic factors by releasing collagenases, including type IV, and plasminogen activators (30–32) this disruption may be due to proteolytic digestion by the endothelial cells. Protrusion of pseudopodial processes through gaps in the basement membrane is a prelude to movement into the perivascular stroma toward the stimulus. Continuation of this process leads to the formation of the bipolarly aligned column of cells extending toward the stimulus. Mitosis, which only occurs in cells at the back of the cord of cells nearer the parent vessel and never at the tip, adds to the length of the column. Branching at, or near, the tips occurs and branches from adjacent columns make contact, forming loops. Loop formation is followed by the appearance of a lumen within the loop after which blood flow commences (26). A newly formed capillary loop may generate further sprouts of endothelial cells and, eventually, fresh capillary loops so that several tiers of loops may form resulting in a network of capillaries. Deposition of basement membrane on the abluminal side of the endothelial cells follows the establishment of blood flow in the capillary loop (26, 29).

It is clear from the described sequence of events that there is in the development of new capillary sprouts a way in which angiogenesis may further contribute to metastatic development. That is the broaching of an intact basement membrane, so important in transition from carcinoma *in situ* to frank carcinoma, need not be a consequence of tumour invasion per se. Rather the movement of new vessels through this structure, under the influence of an angiogenic stimulus, using many of the same enzymes as those used by invading tumour cells, could lead just as readily to membrane dissolution and the possibility for neoplastic dissemination as could movement in the opposition direction by tumour cells (25).

Angiogenic factors

The initial isolation and partial purification of the first nominated soluble angiogenic factor by Folkman et al. (13) in the 1970s had proved difficult because of, in part at

least, the lack of suitable assays. With the availability of cultured capillary endothelial cells and the realisation that the stages of endothelial cell proliferation and migration could be monitored *in vitro*, there was fresh impetus into the search for angiogenic factors. By the early 1980s several polypeptide factors from a variety of normal, as well as tumour, tissues were shown to be capable of stimulating endothelial cell proliferation. Such factors include: heparin-binding endothelial cell growth factors, as exemplified by acidic and basic fibroblast growth factors (33), the transforming growth factors (TGF's), TGF-α and TGF-β (34), the polypeptide angiogenin (11), which was first isolated from conditioned medium of the HT29 human colon carcinoma line and has been shown to have sequence homology with pancreatic ribonucleases, tumour necrosis factor α (TNF-α), a major secretory product of activated macrophages (35), interleukin-1 α (IL-1 α) (36) another cytokine released by macrophages, platelet-derived endothelial cell growth factor (PD-ECGF) a protein of relative molecular mass of about 45 000 which seems to be the sole endothelial cell growth factor in human platelets (37), heparin which, though in itself does not seem to be angiogenic per se, is capable of enhancing tumour-induced angiogenesis and a variety of miscellaneous factors such as prostaglandins E_1 and E_2. Future years certainly will see additions to this rather incomplete list but at present it seems likely that the scheme outlined by Folkman & Klagsbrun (11) will generally hold true. These authors pointed out that the various angiogenic factors either did or did not have an effect on the proliferation and motility of capillary endothelial cells *in vitro*. Those which stimulated motility or mitosis of such cells *in vitro*, they proposed, had the vascular endothelial cell as their primary target *in vivo*, whereas factors which had no effect on these cells *in vitro* probably acted via some indirect pathway *in vivo* (11). This indirect pathway they felt could be via an activation and stimulation of infiltrating host leukocytes, could be via release of endothelial mitogens from storage in the extracellular matrix or could be by the release of intracellular stores of endothelial growth factors (11).

This concept of the possible duality of action of angiogenic factors, which need not be exclusive in particular tumours, raises an important point. Solid tumours are not composed solely of neoplastic cells. Rather there is a complex infiltrating stroma, frequently induced by the malignant cells themselves, containing many host cells. This stroma is composed of blood vessels, interstitial collagens, proteoglycans, non-collagenous proteins and cellular infiltrates containing, amongst other cell types, lymphocytes, macrophages, mast cells, fibroblasts and histiocytes. Dvorak (38) has suggested that the deposition of a fibrin and fibronectin matrix containing enmeshed platelets and leukocytes is an early event in tumour development which helps to produce a complex tumour growth from a simple aggregate of neoplastic cells. It is believed that the normally vascularly-contained fibrinogen and fibronectin are able to attain an extravascular position because tumour cells are capable of secreting a factor, termed vascular permeability factor, which causes normal microvasculature to become extra permeable to macromolecular proteins (21, 38). It is interesting to speculate whether metastatic tumours of unknown origin, where metastasis is evident but where no primary can be found, secrete large amounts of this factor which could permit the early migration of tumour cells into the permeabilised vessels. Naturally proving such a speculation is bound to be difficult to achieve since the source material is, by definition, impossible to find!

Probably a concept which is more likely to be amenable to investigation is the possibility that differences in tumour infiltrate may contribute to variations in degree of angiogenesis in specific neoplasms and therefore to differences in metastatic behaviour. Many of the angiogenic factors listed above, such as TGF-β, TNF-α and IL-1 α, are known to be produced by leukocytes. It is conceivable that heavy infiltrates of such cells may contribute markedly to the angiogenesis of certain tumours. Equally, since heparin is known to be a potent enhancer of angiogenic activity, it may be of interest that collections of mast cells often are found at the invasive edge of certain tumours (39). It need not only be the cellular component of the tumour stroma which has an effect on angiogenesis. The extracellular matrix components of the stroma, which include collagens, proteoglycans, glycosaminoglycans and the non-collagenous glycoprotein adhesion-promoting molecules like laminin and fibronectin, may have a profound effect on microvascular endothelial cells (40). These molecules exert a strong chemotactic effect upon endothelial cells and, since *in vivo* endothelial cell migration precedes proliferation in the process of capillary formation, it is possible that such a response plays an important part in determining vessel ingrowth. The ability of endothelial cells to respond to a variety of molecules has been shown to be influenced by the nature of the substratum to which they adhere. Thus, endothelial cells cultured on collagen substrata (type I/III or type IV) adopt a differentiated phenotype and form capillary-like structures whereas on a laminin substratum they proliferate rapidly. Recently we have shown that polyurethane sponge discs pre-soaked in laminin or fibrinogen solutions, prior to implantation in the subcutis of rats, induced a more rapid onset of capillary formation in the sponge than did sponges pre-soaked in type IV collagen solution (36). It seems likely, therefore, that not only the nature and degree of host infiltrating cells but also the composition of the extracellular matrix can have a profound effect on the rate and extent of the angiogenic response. Since this composition varies considerably from tumour type to tumour type, it is conceivable that it will influence the biological behaviour of specific or individual neoplasms. For example, laminin *in vitro* has been shown

to exert a strong chemotactic effect on certain tumour cells as well as promoting new cell growth (40). Furthermore, tumour cells grown on a laminin substrate have been shown to exhibit an up-regulation of type IV collagenase synthesis (41). Deposition of laminin by endothelial cells in the vicinity of new vessel growth might therefore confer increased invasive, and subsequently metastatic, potential on adjacent tumour cells.

Inhibition of angiogenesis as an anti-metastatic strategy

A number of studies have, as indicated earlier, shown that growth of a tumour beyond a critical mass is only possible if there is a parallel increase in the number of new, functional capillary units to ensure adequate nutrition of the tumour and efficient removal of potentially toxic metabolites. Growth therefore is marked by two distinct phases with angiogenesis being the crucial determinant in the transition from one phase to the other; an initial avascular slow growth phase is followed by a vascular phase of rapid development (12, 14, 22).

An appreciation of the importance of angiogenesis to tumour growth led to the idea that inhibition of neo-vascularisation might prove an effective way of containing tumour development. Given the strong correlation between the occurrence of angiogenesis and the incidence of metastasis, it was further hoped that such an approach could succeed in limiting the number and size of secondary tumours.

Because mast cell heparin is capable of potentiating angiogenesis induced by other soluble factors protamine, an arginine rich basic protein capable of specifically antagonising heparin, was tested for its ability to inhibit angiogenesis (42). Mice bearing subcutaneously implanted tumours were treated with systemic protamine. Although this treatment reduced the mean volume and number of pulmonary tumours, and indeed caused some of the metastases to remain avascular and dormant, it did not cause significant regression of the primary tumour or appear to be directly cytotoxic to the tumour cells (42).

Whilst attempting to reduce background inflammation in the chick chorio-allantoic membrane during assessment of the angiogenic-potentiating effect of heparin Folkman et al. (43) found serendipitously that the combination of heparin and cortisone was a potent inhibitor of angiogenesis. Tested against a variety of spontaneously metastasising tumours implanted subcutaneously in mice, systemic administration of cortisone and a hexasaccharide fragment of heparin (devoid of anti-coagulant properties) brought about a highly significant reduction in the incidence of metastases (43). Such metastases as remained tended to be small and avascular, while the majority of animals showed a marked reduction in primary tumour size with regression being maintained long after cessation of treatment (43). While similar results have been reproduced in some other laboratories they have not in others; a difference that may reflect the non-uniformity of heparin preparations. As pointed out recently, heparin preparations are heterogeneous with regard to composition, molecular size and structure; a disadvantage which might be offset by using synthetic heparin substitutes like β-cyclodextrin tetradecasulphate (44). Unfortunately the heparin/cortisone regimen would be a hard one to transpose to clinical practice because of the glucocorticoid and mineralocorticoid side effects of cortisone. However, the use of 'angiostatic steroids', lacking glucocorticoid activities, in combination with synthetic heparin fragments may offer potential therapeutic utility.

Razoxane (ICRF-159) appears to have a unique mode of action. Systemic administration of this drug to mice bearing Lewis lung carcinomas which had been implanted subcutaneously led to a very significant decrease in the subsequent incidence of pulmonary metastases (45). Histology of tumours from treated animals showed this was related to a 'normalisation' of the structure and integrity of capillary vessels within the cancer leading to a reduced rate of escape of malignant cells into the bloodstream; a mechanism postulated to underlie this drug's anti-metastatic effects (45).

Most recently we have shown that the flavonoid, flavone acetic acid (FAA), acts to shut down the blood supply of tumours growing in the subcutaneous tissue of mice. This inhibition of blood flow, which is very rapid in onset with significant diminution in clearance of radiolabelled tracer occurring as early as 1 h post-injection of FAA, appears to be selective only for tumour vasculature (Mahadevan et al. Unpublished data). While this compound has proven to be ineffectual against the human malignancies it has so far been tested against this seems to not totally preclude its potential usefulness since it has not yet been tried, for example, to close down tumour vasculature subsequent to the administration of more conventional cytotoxic agents; possibly a way of potentially achieving high levels of drugs within neoplastic tissues.

These examples suggest that the newly formed vascular bed of tumours may provide a potential target for the systemic therapy of tumours. It has been estimated that at least 50% of patients with solid malignancies have developed occult metastases at the time of presentation. Currently non-specific cytotoxic chemotherapy is the mainstay of treatment for such metastatic disease and, with a few notable exceptions, has offered only marginal benefits for most tumours. It is not surprising, therefore, that the theoretical basis of systemic therapy which only produces an effect via an anti-angiogenic response in neoplastic tissue is an attractive one. However, a major clinical problem associated with such possible treatments is the likelihood that any agent capable of interfering with tumour angiogenesis also could compromise the ability of the body to mount a physiological or useful angiogenic response, such as in wound healing.

Concluding remarks

Once an aggregate of tumour cells reaches a size such that simple diffusion of nutrients from the interstitium alone can no longer sustain all the cells, then the aggregate may attain a steady state at which there is no net increase in the size of the cell mass; any addition of cells is balanced by the death of others. Growth beyond this stage requires an increase in the number of new, functional capillaries to provide adequate supplies of nutrients. The process of angiogenesis is therefore a critical determinant of primary tumour development. This association between tumour development and angiogenic activity is underlined by the recent finding that several fibroblast growth factor genes have transforming potential (46), since the ubiquitous FGFs have strong angiogenic activity, and the demonstration that inhibition of neovascularisation has been tightly linked to the presence of an active cancer suppressor gene (47).

However, beyond regulation of primary tumour size, angiogenesis has a significant effect on the ability of neoplasms to metastasise. Generally, in order for a cancer to spread it must initiate an angiogenic response though, because metastasis is such a complex process, the mere existence of this phenomenon is not sufficient to ensure that secondary spread will occur successfully. The nature and mode of the ingrowing vessels is thought to facilitate the release of cells into the circulation, while the penetrative capacity of endothelial cells is thought to be due to many of the mechanisms used by invasive cancer cells. Breakdown of tissue barriers which constrain growing tumours could, therefore, be due to endothelial cell migration, rather than neoplastic cell migration per se. It should, however, be pointed out that actual penetration by the ingrowing blood vessels is not totally necessary for cancer dissemination. To the best of our knowledge the existence of lymphatic vessels within tumours has not been documented and yet metastatic spread via lymph channels, particularly by carcinomas, is a common event.

The hope is that the phenomenon of provision of new blood vessels to tumours may provide a potential target for therapeutic approaches which are equally successful against both primary and secondary growths. At the time of writing, such a hope has little other than a few preliminary animal experiments to suggest its feasibility but, with increasing knowledge of the molecular basis of angiogenesis, this direction of research is sure to expand in future years. Anti-angiogenic therapy could prove to be an effective way of dealing with disseminated disease.

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