REVIEW

Angiogenesis, thrombospondin, and ductal carcinoma in situ of the breast

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Angiogenesis, the growth of new vessels from existing vasculature, plays an essential role in tumour development. The process involves interaction between a variety of cells, growth factors, and components of the extracellular matrix, regulated by pro-angiogenic and anti-angiogenic factors. This review profiles these factors, outlines the available methods for measuring new vessel formation, and discusses the importance of angiogenesis in breast cancer, with emphasis on ductal carcinoma in situ.

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Angiogenesis is the growth of new vessels from existing vasculature that occurs during development and in vascular remodelling in the adult. It plays an essential role in tumour progression, providing nutrients and growth factors, and a means of tumour cell dissemination. Angiogenesis is a multistep process, which involves changes in the extracellular matrix, and endothelial cell proliferation, migration, and differentiation into capillaries. Studies of tumour biology reveal a complex network of autocrine and paracrine interactions between tumour cells, stromal cells, and endothelial cells, which are in turn influenced by the composition of the extracellular matrix. It has become increasingly apparent over the past few years that new vessel growth depends on the net effect of a variety of pro-angiogenic and anti-angiogenic molecules, including growth factors and components of the extracellular matrix. Not only does the extracellular matrix act as a scaffold for capillary growth, but it can bind and influence the availability of angiogenic molecules and influence the cell cycle through the binding of cell surface receptors. The study of angiogenesis and its role in tumour biology has produced a large body of work in the literature and has resulted in trials of new anti-angiogenic drugs in the treatment of cancer.[1] [2]

ANGIOGENESIS

The microvasculature is a dynamic system that plays an important role in a variety of physiological and pathological processes and it switches between quiescent and activated states. New vessels can arise by several processes: angiogenesis, vascular remodelling, and the recruitment of endothelial precursor cells from bone marrow and blood vessels.[3] Angiogenesis is a multistep process that depends upon cooperation and interaction between a variety of cells, growth factors,

and components of the extracellular matrix. It requires the destabilisation of existing vessels, increased permeability with extravasation of plasma proteins and enzymes into the surrounding stroma, changes in endothelial cell adhesion with endothelial cell migration, proliferation, survival, and stabilisation of newly formed vascular channels. Vascular remodelling describes new vessel formation by the insertion of interstitial tissue columns into the vessel lumen, with subsequent growth of these columns and partitioning of the vessel. This process of intussusception occurs in normal developing organs and has been shown to occur in colonic adenocarcinoma.[4] Co-option of endothelial progenitor cells from the circulation into new vessels is known to occur in development, but may also occur in adults.[5]

Angiogenesis is recognised as a key factor in the progression of invasive tumours, as enunciated in the "angiogenesis progression" hypothesis.[6] The pathways controlling the switch to an angiogenic phenotype in tumours are complex and poorly characterised but include hypoxia, genetic mutation, and stromal and inflammatory cell responses. There is evidence that changes in oncogene and tumour suppressor gene expression influence new vessel growth during tumour progression.[7] For example, mutations in the tumour suppressor genes p53 and VHL result in the loss of expression of the anti-angiogenic factor thrombospondin 1 (TSP-1)[8] and increased expression of the angiogenic factor vascular endothelial growth factor (VEGF),[9] respectively.

In many tumours, including breast cancer, areas of increased tumour cell proliferation are associated with areas of increased microvessel density ("hot spots").[10] Not only do new vessels supply oxygen and nutrients to metabolically active tumour cells, but there is strong evidence for the presence of reciprocal interactions between tumour cells and endothelial cells. In addition to angiogenic factors such as VEGF, activated endothelial cells can produce factors that influence tumour cell growth and invasion—for example, hepatocyte growth factor, basic fibroblast growth factor (bFGF), and extracellular proteases.[6] [11]

Inflammatory and stromal cells are also a source of angiogenic factors and extracellular

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Accepted for publication
19 February 2002

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Abbreviations: bFGF, basic fibroblast growth factor; CIN, cervical intraepithelial neoplasia; DCIS, ductal carcinoma in situ; MMP, matrix metalloprotease; TF, tissue factor; TSP, thrombospondin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

proteases. For example, macrophages can synthesise VEGF, tumour necrosis factor α, bFGF, interleukin 8 and the anti-angiogenic factor TSP-1. In addition, they produce extracellular proteases and elements of the coagulation system, such as tissue factor (TF) and thrombin, which play a role in vessel growth.[12][13]

Two studies investigated the expression of multiple growth factors and their receptors in the tumour, endothelial, and stromal compartments of breast cancer, and have identified potential autocrine and paracrine loops in vivo. In addition, correlations between the presence of these loops and tumour proliferation were found.[14][15] These data highlight the importance of studying multiple factors within the tumour milieu and suggest a possible role for computer modelling of these complex processes.

ANGIOGENIC AND ANTI-ANGIOGENIC FACTORS

Growth factors specific to angiogenesis include VEGF and the angiopoietins. VEGF expression is induced by hypoxia and a variety of oncogenes, including ras and erbB, and is inhibited by the tumour suppressor gene VHL. VHL is structurally related to other growth factors including VEGF-C (a subtype of VEGF) and placental growth factor. There are three VEGF receptors, two of which are present on endothelial cells (VEGFR-1 and VEGFR-2) and are activated by VEGF. The third is present on lymphatic endothelium (VEGFR-3), binds VEGF-C, and is involved in lymphangiogenesis. Increased VEGF expression has been described in several tumours and has been associated with increased microvessel density in oesophageal squamous carcinoma,[16] renal cell carcinoma,[17] non-small cell lung cancer,[18] and prostatic adenocarcinoma.[19] Angiopoietin 1 is one of a family of four related molecules, and it binds the Tie 2 receptor present on endothelial cells, resulting in the stabilisation of vessels. Angiopoietin 2 acts as an antagonist to angiopoietin 1 at this receptor, resulting in the destabilisation of microvessels that is required in the early stages of angiogenesis. Increased angiopoietin 2 expression has been found in non-small cell lung cancer,[20] uveal melanoma,[21] and hepatocellular carcinoma,[22] and this molecule is coexpressed with VEGF.[20][21]

In addition to specific angiogenic factors, there are several multifunctional growth factors that have angiogenic activity. Thymidine phosphorylase (also known as platelet derived endothelial cell growth factor) is an intracellular enzyme that regulates thymidine metabolism and has angiogenic activity, stimulating both endothelial cell proliferation and chemotaxis. It can also confer resistance to hypoxia induced apoptosis. It is expressed by both tumour and stromal cells in a variety of tumours, including breast cancer.[23] bFGF is produced by tumour, stromal, and endothelial cells, and stimulates endothelial cell proliferation, protease production, chemotaxis, and vascular tube formation.[24]

Extracellular proteases including the matrix metalloproteases (MMPs) and cathepsins are also produced by tumour, stromal, and endothelial cells, resulting in the degradation of the basement membrane and the extracellular matrix.[25] Not only does this facilitate capillary formation, it also releases angiogenic and anti-angiogenic factors bound to the stroma and exposes integrin binding sites involved in cell migration. For example, b-FGF binds the extracellular matrix, so providing an inactive pool of b-FGF, which is released and activated by proteolytic enzymes such as MMP-2.[26]

Finally, elements of the coagulation system, including thrombin, plasmin, and TF have also been shown to encourage new vessel growth.[27] TF is expressed by tumour cells[28] and tumour associated vasculature,[29] where it can activate the extrinsic coagulation pathway, resulting in thrombin production. Thrombin catalyses the deposition of fibrin, which aids endothelial cell migration and also has a mitogenic effect.[30]

Urokinase-type plasminogen activator, present in the extracellular matrix and on the surface of endothelial cells, converts plasminogen to plasmin, which in turn activates MMPs.[31]

In normal tissues, angiogenic inhibitors exist to prevent exuberant new vessel growth. These inhibitors have been described and studied more recently and include circulating factors, such as angiostatin and endostatin, and the extracellular protein TSP-1. Angiostatin is a cleavage product of plasminogen and is produced by some primary tumours, either directly or via enzymatic cleavage of plasminogen. It has been shown to inhibit endothelial cell proliferation and the growth of metastases, presumably by inhibiting angiogenesis, a phenomenon described as tumour dormancy.[32] Removal of the primary tumour results in the loss of circulating angiostatin and unfettered growth of the metastases. Endostatin is a protein fragment of collagen XVIII that also inhibits endothelial cell proliferation.[33]

The TSPs are a family of five extracellular proteins that show varied tissue distribution. TSP-1 expression has been reported in squamous cell carcinoma,[34] melanoma,[35] and breast carcinoma.[8][35][36] It is localised to the cell surface and to the extracellular matrix.[37] There is a close relation between the expression of TSP-1 and wild-type p53, loss of wild-type p53 being associated with loss of TSP-1 expression.[38] TSP-1 has been shown to have both angiogenic and anti-angiogenic effects. The molecule possesses an integrin recognition site that recognises a specific amino acid sequence (RGD) present within integrin molecules including αV β3. This integrin is expressed by newly formed vessels and facilitates endothelial cell survival[39][40] and adhesion to components of the extracellular matrix, including TSP-1, fibronectin, and fibrin. However, TSP-1 can bind heparin sulfate on the cell surface and in the extracellular matrix, thereby inhibiting the action of heparin dependent growth factors, such as bFGF.[41] TSP-1 also binds transforming growth factor β, which itself has both pro-angiogenic and anti-angiogenic effects.[42]

MEASUREMENT OF TUMOUR ANGIOGENESIS

The study of angiogenesis in tumour biology is fraught with difficulty, particularly in the assessment of vascularity in histological sections from archival tissue. In this instance one is essentially measuring a dynamic three dimensional process in a static two dimensional preselected section and there has been much debate in the literature as to which counting method most accurately measures tumour microvessel density.

A variety of markers for the identification of microvessels in histological sections are available, the most commonly used being factor VIII, CD31, and CD34. E-9 is reported to be a marker of activated/proliferating endothelial cells,[43] and may more accurately reflect angiogenic activity within a tumour.

Methods of counting include manual counting of vascular hotspots as described by Weidner et al,[44] assessment with a Chalkey graticule, and computerised image analysis,[45] with reported good correlation between these methods.[45–47] The relative merits of the various counting methods and available antibodies have been well reviewed in the literature,[48][49] and there are published recommendations for the assessment of tumour microvessel density in histological sections.[50]

A fully automated method of counting microvessel density has been described by Belien et al.[51] Using image analysis, each vessel in a whole tumour section was mapped to create a microvessel map of the tumour. This was analysed by a program that automatically searched for microvessel "hotspots", as defined by the programmer, and then proceeded to count the vessels in that hot spot. They found that although for the same hotspots the correlation between manual and automated counts was good, the hotspots selected by the automated system had a greater microvessel count than those selected manually. These results suggest that

full automation could result in greater accuracy, objectivity, and reproducibility of microvessel counts.

Comparison between studies in which counting protocols vary is difficult. Some would argue that the expression of angiogenic factors is a better measure of tumour angiogenesis than tumour microvessel density. However, new vessel growth depends on the net effect of multiple growth factors and inhibitors, and interactions between tumour cells, inflammatory cells, stromal cells, and endothelial cells. Tumour microvessels are the visible manifestation of this activity and quantitation of microvessels remains a valid measure of tumour angiogenesis.

ANGIOGENESIS AND INVASIVE BREAST CARCINOMA

Notwithstanding these limitations, there is a large body of evidence in the literature that angiogenesis has prognostic relevance in certain tumours, including invasive breast cancer. Weidner and colleagues[44] demonstrated a correlation between tumour microvessel density in breast cancer and the presence of axillary lymph node and distant metastases. Further studies have confirmed these results and have shown that tumour microvessel density in invasive breast carcinoma is an independent prognostic factor for disease free and overall survival.[52–55] Some studies have failed to show an association between microvessel density and prognosis, possibly because of deviation from recommended counting methods.[56]

The angiogenic factors studied in invasive breast cancer include VEGF and thymidine phosphorylase; both in relation to microvessel density and as independent prognostic indicators. Increased expression of VEGF by breast cancer cells has been correlated with increased microvessel density[57 58] and poorer prognosis in node positive[59] and node negative patients.[60] The expression and activation of one of the VEGF receptors, VEGFR-2, is increased in invasive breast cancer.[15 61] One study showed the association between VEGF and microvessel density to hold only for invasive ductal carcinoma and not for invasive lobular carcinoma,[58] and others have not found an association between VEGF expression and vascularity.[14] Thymidine phosphorylase is also upregulated in invasive breast carcinoma and is expressed by both tumour cells and inflammatory cells in the surrounding stroma. Expression of thymidine phosphorylase is associated with increased microvessel density and has been reported to have prognostic value.[62] Other angiogenic factors that are increased in invasive ductal carcinoma include bFGF,[63] MMPs,[64] and the integrin $\alpha V \beta 3$, which shows increased expression on tumour vessels.[65]

There has been less work on anti-angiogenic factors in breast carcinomas. Invasive ductal and lobular carcinomas show different patterns of TSP-1 expression. TSP-1 protein has been consistently localised to the desmoplastic stroma around invasive ductal carcinoma, with relatively little staining of tumour cells.[36 66–70] The converse is seen in invasive lobular carcinoma.[36] Although TSP-1 is reported to have an anti-angiogenic effect in breast cancer cell lines,[8] studies of TSP-1 expression in tissue sections from invasive breast cancer show both positive[66 70] and negative[35 71] associations between TSP-1 and microvessel density.[72] Interestingly, one study by Pazouki et al found that human breast cancers fell into two groups when TSP-1 and microvessel density were assessed, one group showing a positive association between TSP-1 and microvessel density and the other a negative association.[73]

ANGIOGENESIS AND IN SITU CARCINOMA

It is becoming increasingly apparent that the angiogenic phenotype may be acquired early in tumour evolution and increased angiogenic activity has been reported in preinvasive neoplastic lesions of the cervix,[74–76] vulva,[77] bladder,[78] oral mucosa,[79] and lung.[80 81] Angiogenesis in in situ tumours may differ from that seen in invasive tumours. The increase in vessels is seen in the normal stroma around or beneath the in situ tumour, rather than between clusters of tumour cells, as in invasive disease. Therefore, in in situ tumours, angiogenic factors produced by inflammatory and stromal cells may play a greater role than those derived from the tumour cells themselves. Two patterns of vascularity have been described in several in situ tumours. Studies of cervical intraepithelial neoplasia (CIN) have demonstrated an increase in vessels lying immediately adjacent to the basement membrane, at the interface between the abnormal squamous epithelium and the underlying stroma, and an increase in vessels within this underlying stroma.[75] Similar patterns have been described in ductal carcinoma in situ (DCIS), as discussed below. In parallel with invasive disease, increased microvessel density is associated with the transition from low grade to high grade in situ lesions, suggesting that angiogenesis may play a role in tumour progression at this stage. For example, in the cervix, both vascular patterns were significantly increased in high grade CIN as compared with low grade lesions and benign squamous epithelium.[74] This increase was also correlated with increased expression of VEGF mRNA by the neoplastic cells.[75] In the lung, dysplastic bronchial mucosa and carcinoma in situ had significantly higher microvessel counts than did normal, hyperplastic, or metaplastic epithelium.[80 81]

ANGIOGENESIS IN DCIS
Morphological patterns and relation to histological characteristics

In a series of breast carcinomas, Weidner and colleagues[44] described a ring of small vessels around ducts involved by DCIS. Two patterns of vascularity around DCIS were subsequently described by Guidi et al, resembling those seen in CIN. The first of these comprised a cuff or necklace of microvessels immediately adjacent to the basement membrane of ducts with DCIS and the second a diffuse increase in stromal microvessels surrounding involved duct spaces.[82] The presence of microvessels was assessed both semiquantitatively and quantitatively with good correlation. Increased stromal microvessel density was associated with high histological grade[83] and with the presence of comedo necrosis.[82 84 85] Engels and colleagues[83] also reported an association between the presence of a cuff of vessels and the grade of DCIS, but this has not been confirmed by other studies.[82 86]

Angiogenic factors

Increased VEGF mRNA expression has been demonstrated in tumour cells of DCIS compared with neighbouring benign epithelium, and was associated with an increase in stromal microvessel density around DCIS ducts.[87] The same group reported increased VEGF mRNA expression in high grade DCIS and in DCIS with a cuff of microvessels, although these associations were not significant. Increased expression of VEGF receptors has also been found on endothelial cells around DCIS.[88] Other groups have failed to find an association between VEGF expression by tumour cells in DCIS and stromal microvessel density.[58 84] However, hypoxia inducible factor 1α is increased in DCIS and associated with increased stromal microvessel density.[89] Under hypoxic conditions hypoxia inducible factor 1α is stabilised in the nucleus and transactivates various genes, including the VEGF gene.

In DCIS, thymidine phosphorylase is expressed by both tumour and stromal cells, in contrast to VEGF, which is primarily of tumour origin. Studies to date have produced varied results. Engels et al reported the expression of thymidine phosphorylase by DCIS tumour cells, which showed a significant association with the presence of a cuff of vessels, but was not associated with stromal microvessel density.[90] Other studies have failed to confirm the association between thymidine phosphorylase expression in DCIS tumour

cells and a cuff of microvessels.[84][91] Lee and colleagues[91] investigated the expression of thymidine phosphorylase by inflammatory cells in the stroma around DCIS. They found an association between the expression of thymidine phosphorylase by inflammatory cells and stromal microvessel density, complimenting their previous findings of an association between perivascular inflammation and increased stromal vascularity in DCIS.[86] These data highlight the potential importance of inflammatory cells as a source of angiogenic factors in DCIS.

TF is expressed by DCIS tumour cells and on vascular endothelial cells in the surrounding stroma.[92] This may be secondary to VEGF, which is also expressed by endothelial cells around DCIS, and which is known to induce endothelial cell expression of TF.[93] Thus, the coagulation system, elements of which are implicated in new vessel growth (for example, thrombin and fibrin), probably plays a role in angiogenesis around DCIS. In addition, MMP mRNAs are expressed in in situ and in invasive breast carcinomas. The MMP mRNAs expressed include MMP-2, MMP-7, MMP-9, and MMP-11, and expression has been described variably in tumour cells and stromal fibroblasts.[64][94][95]

Anti-angiogenic factors

The expression of both TSP-1 protein and mRNA has been described in several studies of between two and 18 cases of DCIS.[36][66][70] TSP-1 protein was localised to the basement membrane around involved duct spaces and the immediately adjacent collagen. In one study, the TSP-1 mRNA signal was localised to the myoepithelial cells bordering DCIS ducts, suggesting that these cells are the source of TSP-1.[68]

In a larger series of 58 patients with DCIS, we confirmed the pattern of TSP-1 protein expression as described, and also saw a pattern of stromal staining between involved ducts similar to that seen in invasive carcinoma.[96] Our results are supported by the study of Brown et al, in which TSP-1 mRNA was identified in stromal cells in the vicinity of ducts involved by DCIS.[67] We failed to find an association between TSP expression (both stromal and periductal) and either the presence of a cuff of vessels or stromal microvessel density. These data suggest that other factors have a more influential role in angiogenesis around DCIS.

As in studies of invasive carcinomas, studies of angiogenic factors in DCIS have produced conflicting results. This may be the result of differing sample size, lack of correlation between mRNA and protein expression, or variation in vessel counting methods. Study results may also be influenced by difficulties in distinguishing new or remodelled vessels from pre-existing vessels and the evaluation of angiogenic factors in isolation, which does not take account of synergistic/antagonistic interactions between these factors.

CONCLUSION

The angiogenic phenotype of breast carcinoma, as in other tumours, is acquired early in tumorigenesis while the tumour is still in situ. It is likely that multiple angiogenic factors are involved and the two vascular patterns in DCIS may arise by different mechanisms. For example, the cuff of vessels may arise in response to an angiogenic factor produced by tumour cells that diffuses only a short distance or is inactivated by factors in the surrounding stroma. The stromal vessels may be induced by angiogenic factors produced by tumour and stromal cells (for example, tumour associated macrophages), in response to some as yet unknown factor produced by the tumour. Invasive tumours arising from highly vascular in situ lesions are likely to be highly angiogenic, although this will depend not only on tumour phenotype, but also on the influence of the surrounding stroma. It may be that the full angiogenic potential of a tumour is not realised until it

becomes invasive, thereby permitting interaction with stromal cells and the extracellular matrix.

Considering the invasive potential of high grade DCIS, the reported association between increased microvessel density, high histological grade, and comedo necrosis suggests that angiogenesis may influence the progression of DCIS to invasive carcinoma. The acquisition of invasive properties by in situ tumour is a complex process, involving interactions between tumour cells, endothelial cells, stroma, and the extracellular matrix. The expression of MMPs by both tumour and endothelial cells is upregulated by several growth factors including VEGF, epidermal growth factor, and platelet derived growth factor. Paracrine interactions between endothelial and tumour cells may play an important role in tumour progression—for example, in the transition of radial growth phase melanoma to thick vertical growth phase.[6] Skobe et al showed that the invasiveness of a human squamous carcinoma cell line transplanted into mice was associated with ongoing angiogenesis, and that halting angiogenesis (using antibodies to VEGFR-2 expressed on endothelial cells) resulted in reversion of the tumour cells to a non-invasive phenotype.[97] They also found that overexpression of VEGF by these tumour cells resulted in an accelerated rate of tumour growth, with the development of an invasive border. In contrast, cell lines that did not overexpress VEGF showed slower growth, with a pushing tumour border.[98] Similarly, Brooks et al found that in human breast cancer implants, inhibition of angiogenesis using antibodies to the integrin $\alpha V \beta 3$ (which like VEGFR-2 is expressed on endothelial cells) resulted in reduced tumour invasiveness.[99]

Our knowledge of DCIS and approach to histological assessment have advanced considerably in the past two decades, and it is known that patients with large, high grade lesions are at greater risk of developing invasive carcinoma. It is not possible to predict which lesions will progress, and all patients with DCIS require surgery and many undergo radiotherapy. The possibility that angiogenesis may be important in the transition to invasive disease is exciting. Currently, the available information is incomplete and there is a need for detailed profiling of angiogenic factors expressed in both DCIS and invasive carcinoma, possibly with computer modelling of the multiple possible pathways and paracrine interactions that govern angiogenesis. Knowledge of the angiogenic profile of an individual tumour may improve our ability to prognosticate and the development of specific anti-angiogenic therapeutic regimens may provide an alternative to current treatment options both for in situ and invasive disease.

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Take home messages

- Angiogenesis, the formation of new vessels from existing vasculature, is essential for tumour growth and metastasis
- Increased angiogenic activity has been documented in a variety of pre-invasive neoplastic conditions, suggesting that the angiogenic phenotype may be acquired early in tumour evolution
- The angiogenic process relies on a complex interaction between tumour, stromal, endothelial and inflammatory cells, growth factors, and the extracellular matrix, which is regulated by pro-angiogenic and anti-angiogenic factors
- Tumour microvessels are the visible manifestation of this complex interaction and quantitation of microvessels in tissue sections is a valid measure of tumour angiogenesis
- Microvessel density is an independent prognostic indicator in invasive breast cancer
- A variety of angiogenic factors are produced by ductal carcinoma in situ, which are variously associated with changes in vascularity, and may facilitate the transition to invasive carcinoma
- The development of specific anti-angiogenic regimens may provide an alternative to current treatment protocols for breast cancer

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