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Significance of Angiogenesis in Tumour Progression and Metastasis

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Angiogenesis is defined as a vascular neoformation usually of capillary origin. This phenomenon is important during development and under several physiological and or pathological conditions. In recent years, progress has been made to understand this phenomenon at the molecular level. This includes the identification of potent angiogenic factors, the appreciation of the role of proteases, the importance of the extracellular matrix, and the emerging characterisation of signal transduction pathways in endothelial cells. Two important participants in angiogenesis are molecules from the fibroblast growth factor (FGF) and the transforming growth factor-β (TGF-β) family. In our laboratory, we have extensively studied the roles and mechanisms of action of the major FGF prototype, FGF-2 and of the TGF-β member, TGF-β1. Different isoforms of FGF-2 have been previously described, a high molecular weight (HMW) form associated with the nucleus and 18 kDa bFGF that is cytoplasmic. These two forms of FGF-2 also exhibit different functions when expressed endogenously. TGF-β is formed from a latent complex by plasmin-dependent and plasmin-independent pathways. With the exception of macrophages, the plasmin-dependent pathway requires coculture conditions, urokinase, and the concentration of TGF-β on the cell surface by the mannose-6-phosphate receptor and transglutaminase. Other important angiogenic modulators include vascular endothelial growth factor (VEGF) and angiostatin. The nature of the tumour angiogenesis factor is not yet known with certainty, but several identified and not yet identified angiogenic factors may act in concert. It is hoped that an angiostatic treatment for cancer will be derived from these molecular studies.

Key words: tumour angiogenesis, fibroblast growth factor-2, proteases
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INTRODUCTION

ANGIOGENESIS is defined as a process of vascular neoformation that occurs during development, menstruation and several pathological conditions such as neoplasia. Despite the fact that angiogenesis refers to the derivation of blood vessels of all types (micro and macrovessels), the term is usually restricted to the neoformation of capillary blood vessels.

In recent years, a great effort has been made to dissect the molecular steps involved in neovascularisation. Several paracrine or autocrine factors that control the phenomenon have been identified, such as fibroblast growth factor (FGF), transforming growth factor-β (TGF-β) and vascular endothelial cell growth factor (VEGF) [1–3]. In addition, the role of extracellular matrix both as a reservoir of soluble angiogenic factors and as a source of essential signals is now understood [4]. Furthermore, the participation of urokinase type plasminogen activator (uPA), plasminogen activator inhibitor-1 (PAI-1), collagenases or tissue metalloprotease inhibitor (TIMP) [5] in angiogenesis is now appreciated. uPA is not only important for matrix degradation but may also provide signals for endothelial cell movement [6, 7].

The intracellular pathways leading to the formation of capillaries have not yet been elucidated. Several mechanisms for FGF-dependent or TGF-β-dependent signalling are emerging. In our laboratory, the roles of two participants in the control of neovascularisation, basic fibroblast growth factor (bFGF, FGF-2) and TGF-β, have been extensively investigated. In this review, we will summarise recent findings about the mechanisms of angiogenesis and the role of angiogenic and anti-angiogenic molecules in this process, emphasising the roles of FGF-2 and TGF-β.

MOLECULAR MECHANISM OF ANGIOGENESIS

Angiogenesis requires the coordinated activation of genes that are responsible for proliferation, migration and differentiation of endothelial cells to form capillary-like structures. The activation of these genes is thought to occur through paracrine factors. To date, a limited number of these proteins have been identified. The genes activated by these factors encode autocrine/intracrine secondary regulators, proteolytic enzymes, and molecules that are direct downstream substrates of endothelial cytokine receptors.

Basic fibroblast growth factor (FGF-2) is recognised as an important autocrine/intracrine regulator of endothelial cells [8].

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Initially, this protein was identified and cloned as an 18 kDa molecule (18 kDa bFGF) found in the brain, the vasculature and in several cell lines [9]. However, subsequently higher molecular weight FGF-2 forms were described in the placenta, guinea pig brain and in endothelial cells [10–12]. Additional experiments revealed that the cloned mRNA encoded additional 22, 22.5 and 24 kDa forms (HMW bFGF), the translation of which is initiated at CUG codons [13, 14]. HMW bFGF is mainly nuclear and 18 kDa bFGF cytoplasmic [15–17].

Endothelial cells express all four forms of FGF-2. The expression of FGF-2 varies according to the origin of the endothelial cells; i.e. microvascular endothelial cells usually express more FGF-2 than macrovascular endothelial cells. It has been demonstrated that endogenous FGF-2 affects the cell phenotype. This view derives from several experiments. First, the movement of bovine capillary endothelial cells is blocked with anti-FGF-2 antibodies [11]. This suggests a role of endogenous FGF-2 in migration as well as an extracellular localisation of FGF-2. Second, NIH 3T3 cells expressing all FGF-2 forms display enhancement in migration that is abrogated with anti-FGF-2 antibodies [18]. We recently examined which FGF-2 form is responsible for a given phenotype, i.e. migration or proliferation, by establishing transfected cell lines expressing different bFGF isoforms and that are supertransfected with truncated FGF receptors [19]. Cells expressing 18 kDa bFGF demonstrated enhanced migration whereas cells expressing HMW bFGF did not. Cells expressing HMW bFGF grew in low serum conditions, whereas cells expressing 18 kDa bFGF did not. Cells expressing HMW bFGF transfected with a cDNA encoding the 18 kDa bFGF form displayed an increase in migration, but had unchanged growth properties. The properties related to 18 kDa bFGF could be reverted by the supertransfection of a dominant negative FGF receptor, but those properties related to HMW bFGF expression could not. These results indicate that 18 kDa bFGF and HMW bFGF have different properties when expressed in cells, and act through different pathways, i.e. receptor-dependent for 18 kDa bFGF and receptor-independent for HMW bFGF.

Another potential autocrine growth regulator for endothelial cells is TGF-β. Endothelial cells express TGF-β as an inactive precursor consisting of the active homodimer plus the two propeptides (latent TGF-β: LTGF-β). This high molecular weight complex is unable to interact with its receptor. In addition, LTGF-β contains another protein, termed the latent TGF-β binding protein (LTBP), to form a complex of approximately 210 kDa. In order to become active, TGF-β must be activated in the extracellular milieu. The mechanism of this activation has been extensively studied in our laboratory [2]. Cocultures of bovine endothelial cells and either pericytes or smooth muscle cells convert LTGF-β to TGF-β. Activation is species-specific, requires cell to cell contact, proteases, and interaction of LTBP with the cell surface. TGF-β activation can be divided into two mechanisms, one plasmin-dependent, the other plasmin-independent. The plasmin-dependent pathway involves binding of TGF-β to mannose-6-phosphate receptors, crosslinking by transglutaminase and cleavage by plasmin. Plasmin is generated by cell surface-associated uPA from plasminogen. This activation occurs only in heterotypic cocultures when endothelial cells are cocultured with smooth muscle cells or pericytes. The second mechanism involves activation of TGF-β by thrombospondin. This activation does not require plasmin.

The proteolytic system that has been the most intensely investigated with regard to its role in angiogenesis is the plasminogen activator system [5]. Endothelial cells stimulated by angiogenic factors, such as FGF-2, display increased expression of urokinase-type plasminogen activator (uPA). TGF-β also increased uPA expression, but PAI-1 levels are also dependent upon extracellular TGF-β concentration. This response to TGF-β also correlates with the bifunctional regulatory properties of this molecule in angiogenesis. It is an inhibitor at high concentrations and a stimulator at low concentrations. Receptor-bound uPA may trigger cell migration. Pro-uPA, which is proteolytically inactive, is able to stimulate migration of bovine aortic endothelial cells [6]. Recently, an interesting study of the role of uPA in cell migration of epithelial cells was performed [7]. The authors showed that pro-uPA triggers migration by activating protein kinase ϵ which phosphorylates cytokeratins. It is likely that this mechanism is also involved in the migration of endothelial cells.

Several downstream substrates of receptors for angiogenic factors have been identified. For example, the FGF-R1 associates with PLC-γ, and the src kinase. Src kinase, in turn, phosphorylates cortactine. The vascular endothelial growth factor (VEGF) receptor (Flk-1) also phosphorylates downstream substrates [3]. How these signalling molecules affect the phenotype of endothelial cells is not yet understood.

The extracellular matrix provides additional signals to promote angiogenesis. The extracellular matrix is a storage site for endothelial cell growth factors that can be mobilised by heparin, heparinase, plasmin or phospholipase C or D [20, 21]. In addition, the matrix acts by physical tensional forces that allow the differentiation programme to proceed [4]. In addition, β1 integrins are induced by FGF-2 either added exogenously or when overexpressed in cells [22, Klein *et al.*, unpublished results).

PARACRINE FACTORS CONTROLLING ANGIOGENESIS

Fibroblast growth factors

The FGF family is a family of pleiotropic molecules comprising nine members [1]. In the previous section, we discussed several features of FGF-2. In addition, a variety of tumours have been shown to express FGFs, including the two major prototypes, FGF-1 and FGF-2 [1, 23]. FGF-1 and FGF-2 do not contain signal sequences and are not released through the classical signal sequence pathway [18, 23]. This has been viewed as an argument against a role for these growth factors in tumour angiogenesis. Nevertheless, two models of FGF release have been proposed. In the first model, FGF is released after cell damage or wounding. Several tumours undergo cell death and necrosis at a certain stage of their development, and this might be a way by which FGF-1 or FGF-2 reach neighbouring endothelial cells. Another mechanism, the release by an alternate secretory pathway, has also been proposed. This hypothesis is based on the fact that the migration of single cells transfected with the cDNA encoding 18 kDa bFGF is inhibited by anti-FGF-2 antibodies [18]. FGF-1 is released by heat shock from cells transfected with the FGF-1 cDNA [23]. Dimerisation has been postulated to be important for this release. Fibrosarcoma cells release high amounts of FGF-2 at the onset of the angiogenic switch. Taken together, these data suggest that FGF might act as a tumour cell-derived paracrine factor.

Vascular endothelial growth factor (VEGF)

VEGF is an angiogenic growth factor thought to be specific for endothelial cells [3]. This molecule is produced by a variety

of tumour cells, and has been recently closely linked to tumour angiogenesis [3]. Indeed, gliomas produce VEGF and the VEGF receptor has been localised in neighbouring endothelial cells. In addition, dominant negative mutants of the VEGF receptor Flk-1 inhibit glioblastoma growth *in vivo*. VEGF possesses a classical signal sequence, and is released efficiently from cells. It is, therefore, an attractive candidate for a paracrine tumour angiogenesis factor.

Transforming growth factor β

TGF-β is a biphasic regulator of angiogenesis [24]. Subcutaneous injection of TGF-β induces fibrotic lesions and a marked angiogenic response. From these data, it has been concluded that TGF-β is a positive regulator of angiogenesis. However, TGF-β inhibits endothelial cell proliferation *in vitro*. Pepper and associates [25] have demonstrated that TGF-β exhibits a biphasic effect on endothelial cell differentiation. At low concentrations, TGF-β stimulates endothelial cell cord formation whereas at higher concentrations it is inhibitory.

A large number of tumour cells produce TGF-β. It is, however, unclear whether or even how TGF-β can be activated by these cells. Thus, a role of TGF-β as a paracrine tumour angiogenic factor or angiogenic inhibitor remains to be established.

Angiostatin

It has been observed that removal of primary tumour can be followed by a rapid onset of metastasis. This would suggest that a primary tumour can inhibit its metastatic growth. Recently, a new angiogenic inhibitor, named angiostatin, has been identified in the urine of mice. This inhibitor is present in tumour-bearing mice, but not in control mice [26] and its activity is identical to a 32 kDa plasmin fragment. The inhibitory activity is specific for endothelial cells and blocks neovascularisation *in vivo*. The cellular origin of angiostatin is unclear at present. It is possibly derived either from the tumour cells or from neighbouring stroma cells.

Thrombospondin

Many tumour cells secrete thrombospondin. During tumour progression, thrombospondin production is downregulated in tumour cells at the onset of the angiogenic switch. Thrombospondin is a potent inhibitor of endothelial cell growth and angiogenesis.

Angiogenin

Angiogenin has been initially isolated from colon carcinoma cells [27]. It is a protein of 14 kDa and belongs to the pancreatic ribonuclease family. This factor is angiogenic *in vivo*, but does not stimulate the growth of endothelial cells or angiogenesis *in vitro*. The mechanism of action of this molecule appears, therefore, to be indirect.

Platelet-derived growth factor (PDGF)

The role of PDGF in angiogenesis is not clear at present. Early investigators did not find PDGF receptor in endothelial cells. Subsequently, it was reported that capillary endothelial cells, but not large vessel endothelial cells, have PDGF type β receptors. PDGF-β receptors are also present in glioma-derived microvascular endothelial cells. The *in vivo* angiogenic effects of PDGF are still controversial. Tumours such as gliomas produce PDGF. Whether this tumour-derived PDGF can act as an paracrine angiogenic factor remains to be established.

ROLE OF ANGIOGENESIS IN TUMOUR PROGRESSION

It has been demonstrated that a blood supply is critical for tumour development and progression [28]. At the beginning of their development, tumours are generally non-angiogenic. After a variable latency period, several tumours, such as breast carcinomas, become vascularised and switch to a more aggressive phenotype [28]. The switch to an angiogenic phenotype depends upon the net balance of angiogenic stimulators and inhibitors. The development of metastasis is also greatly influenced by angiogenesis. Metastases, which are less than 0.3 mm in diameter, have a high death rate and are not vascularised [26]. The switch to an angiogenic phenotype in the Lewis lung carcinoma is dependent upon the removal of an angiogenic inhibitor, angiostatin. However, this it not a general rule, and the growth of other tumour metastases, such as melanoma B16, is not affected by the removal of an angiogenic inhibitor produced by tumour cells. This type of metastases is most likely due to the activation of an intrinsic programme within the metastatic cells. Thus, the initiation of angiogenesis within a tumour or within its metastases depends upon a critical balance of angiogenic activators and inhibitors that are turned on or off at specific stages of tumour development.

ANGIOSTATIC THERAPY AS ANTICANCER THERAPY

Anti-angiogenic anticancer therapy may act at several points of the angiogenic cascade. It may (1) interfere with receptor binding or activation of a particular angiogenic factor, (2) inhibit the release of a particular angiogenic factor by tumour cells, (3) enhance the production or action of an angiogenic inhibitor, (4) be similar or identical to a particular angiogenic inhibitor, (5) interfere with signal transduction processes or autocrine/intracrine activation of capillary endothelial cells, or (6) inhibit the matrix degradation by protease or matrix degrading enzymes. For example, PF-4 interferes with FGF-2 binding to inhibit biological activity [29].

Attempts have been made to translate the concepts of vascular-driven tumorigenesis into pharmacological tools, able to stop tumour growth or to prevent further tumour progression [28]. There are presently several clinical trials underway testing the efficacy and safety of various anti-angiogenic regimens. The advantages of anti-angiogenic therapy over standard chemotherapy are its low cytoxicity and drug resistance. The combination of an anti-angiogenic agent, such as TNP-470, with classical cytotoxic therapy enhances the effect of either drug alone. The highest efficiency of anti-angiogenic therapy is achieved by treatment over a long period of time as it has been

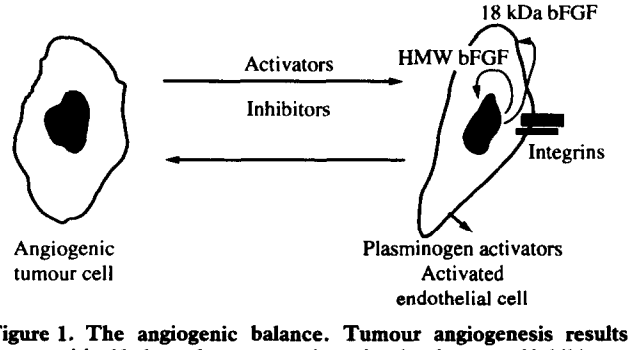

Figure 1. The angiogenic balance. Tumour angiogenesis results from a critical balance between angiogenic stimulators and inhibitors. In addition, the ability of a given subset of endothelial cells to respond to a specific angiogenic factor may determine the outcome of the final angiogenic response.

demonstrated in haemangiomas in infants [30]. It can be anticipated that in the near future an efficient anticancer therapy based on anti-angiogenic principles will be derived from these studies.

CONCLUSION

In this article, I have overviewed the most recent advances in the angiogenesis field, emphasising the regulation of tumour angiogenesis. As depicted in Figure 1, tumour angiogenesis can be viewed as being controlled by a critical balance between tumour-derived angiogenic activators and inhibitors, which will determine the final angiogenic response of endothelial cells. The following conclusions can be derived from these recent studies:

(1) The molecular mechanisms that control angiogenesis are incompletely understood. Several factors important for this phenomenon have been identified. These include soluble autocrine/intracrine factors such as FGF-2, protease systems such as uPA/PAI-1, intracellular signalling molecules, and matrix-dependent tensional forces. How these factors act in concert is unclear at present.

(2) The family of FGFs and TGF-β are important regulators of angiogenesis. Recently, progress has been made in the understanding of the roles, mechanisms of action, and, in the case of TGF-β, the activation from the latent to the active form. Several molecular weight forms of FGF-2 have been identified. HMW bFGF is mainly nuclear and 18 kDa bFGF mainly cytoplasmic. These forms exhibit different biological activities when expressed in cells.

(3) Tumours may be dormant for some time, but may become aggressive by switching to an angiogenic phenotype. The net result of the angiogenic response is determined by the concentration of angiogenic activators over angiogenic inhibitors. In addition, tumours may control the appearance of their metastases by an angiogenesis-mediated mechanism. One such tumour-derived angiogenic factor has been recently identified and has been named angiostatin.

(4) Anti-angiogenic therapy of cancer lies in the near future. It is possible that a combination of cytotoxic therapy and anti-angiogenic therapy represent a promising new therapy of cancer.

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