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Feature Articles

Novel Growth Regulatory Factors and Tumour Angiogenesis

Roy Bicknell and Adrian L. Harris

INTRODUCTION

RESEARCH OVER the past 20 years in many laboratories has established that angiogenesis is an essential component of tumour growth. A variety of experimental systems have shown that tumours do not grow beyond a size of 2–3 mm^3 unless they are able to attract the growth of new capillaries from the existing vascular network. The evidence that growth of solid tumours is angiogenesis dependent has been reviewed by Folkman [1]. In addition, several clinical studies have shown that neovascularisation is a poor prognostic factor in breast [2], cervical [3] and bladder [4] cancer. Apart from their necessity for growth of the tumour, the new blood vessels provide an essential entry route to the vasculature for metastasis of tumour cells. The last 2 years has seen a surge in the number of factors known to stimulate or inhibit angiogenesis. It has become clear that many well characterised growth factors for epithelium are not active on endothelium. Angiogenesis involves proliferation of capillary endothelium. In the healthy adult, endothelial cells are normally held in a quiescent state (an exception occurs during the menstrual cycle), and proliferate only in response to unusual circumstances for example wound healing and in disease states such as tumour vascularisation. It follows that the proliferating capillary endothelial cell offers a unique target for antiangiogenesis therapy [5]. In addition the endothelium may provide a drug resistance barrier that protects the tumour cells from anticancer drugs. Several resistance mechanisms found in tumours are present in endothelial cells, one example is expression of the multidrug resistance gene [6].

This paper reviews results of the last 2 years that are of relevance to tumour angiogenesis and assesses the possibility of antiangiogenic therapy.

ANGIOGENIC FACTORS

Angiogenesis is a complex, multistep process that involves not only endothelial cell proliferation but also digestion of the extracellular matrix surrounding intact capillaries by collagenases and related proteases, endothelial cell migration and differentiation into functioning capillaries.

Several quite different assays of angiogenesis have been used. In addition the component steps of angiogenesis, e.g. cell proliferation, migration or tube formation are often studied separately *in vitro*. Few factors have been examined for activity in each assay, leaving a somewhat complex picture of the precise role of different factors in tumour angiogenesis. Table 1 lists the known polypeptide angiogenic factors and endothelial growth factors, a list which is rapidly increasing. The most studied factor in the context of angiogenesis is fibroblast growth factor (FGF), now known to be a member of a family of at least seven sequence related growth factors (basic FGF, acidic FGF, hst/KS3, int-2, FGF5, FGF6 and keratinocyte growth factor or FGF7) [7], all of which are potentially angiogenically active. Other angiogenic polypeptides which are mitogens for endo-

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Table 1. Peptide angiogenic and endothelial cell growth factors

Angiogenic factors	Mitogens
Also mitogens	G-CSF
FGF	GM-CSF
EGF/TGF-α	IL-4
PdECGF	Erythropoietin
VEGF	Endothelin
Substance P	Hepatocyte growth factor
Non-mitogens	Calcitonin gene-related peptide
Angiogenin	Human growth hormone
Angiotensin II	
Inhibitors of endothelial mitogenesis	
TGF-β	
IL-1	
TNF-α	
IL-6	

thelial cells include epidermal growth factor/transforming growth factor-α (EGF/TGF-α) [8], and the more recently reported platelet derived endothelial cell growth factor (PdECGF) [9] and vascular endothelial growth factor (VEGF) [10] (also known as follicular stellate-derived growth factor [11], vascular permeability factor [12] and glioma-derived vascular endothelial cell growth factor [13]). VEGF is of particular interest in that it appears to be a specific growth factor for endothelium in contrast to the FGF and EGF families which are mitogenic for a wide range of cells. Granulocyte colony stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF) [14] and interleukin-4 (IL-4) [15] are mitogenic for endothelial cells and thus probably angiogenic *in vivo*. Angiogenin [16] is not a mitogen for endothelial cells, although it does activate capillary endothelial cells and augment a primary mitogenic stimulus [17–19]. Tumour necrosis factor α (TNF-α) [20] and TGF-β [21, 22] paradoxically inhibit endothelial proliferation *in vitro* and yet are angiogenic *in vivo*. Angiogenesis in the rabbit cornea in response to TGF-β and TNF-α (in contrast to that of FGF) is accompanied by an inflammatory response [23, 24], and secondary factors (possibly G-CSF and GM-CSF) no doubt mediate the angiogenic signal here. Interleukin-6 (IL-6) is both angiogenic and yet an inhibitor of endothelial cell growth *in vitro* [25, 26]. Interleukin-1 (IL-1) also inhibits growth of endothelial cells [27, 28]; however, reports concerning its angiogenic activity are conflicting. One report has claimed strong angiogenic activity in the rabbit corneal assay [29], whereas another has claimed that this effect is due to contaminants active in the assay and that IL-1 has no intrinsic angiogenic activity but actually blocks the angiogenic response to FGF in the rabbit cornea [28]. Other reported angiogenic peptides include angiotensin II [30] and substance P [31]. Growth factors for endothelium which have yet to be tested in angiogenic assays include erythropoietin [32], endothelin [33], hepatocyte growth factor [34], calcitonin gene-related peptide [35] and human growth hormone [36]. In addition to the peptide angiogenic factors, other low molecular weight angiogenic factors include nicotinamide and derivatives [37] and lipid derived factors such as prostaglandins E_1 and E_2 [38, 39] 1-butyrylglycerol [40], stable prostacyclin analogues [41], and long chain fatty acids and their amides such as erucamide [42], all of which may be shed by tumours. The release of these factors, together with the increasing number of polypeptide angiogenic factors indicates the difficulty in attempting to prevent tumour angiogenesis by targetting angiogenic factors. An example of the failure of anti-FGF antibodies to inhibit angiogenesis is seen in the growth of hybridomas secreting large amounts of anti-FGF antibodies which, despite blocking the *in vitro* activity of FGF and the appearance at high concentrations in the plasma of hybridoma grafted nude mice, still formed highly vascularised tumours of a histiocytoma appearance [43]. This contrasts with the success of anti-FGF monoclonals in blocking FGF induced angiogenesis where FGF is the only angiogenic factor present (FGF was incorporated into an inert sponge, followed by implantation in a mouse and subsequent assessment of the vascularity of the sponge, with and without monoclonal treatment, several days later) [44]. Clearly the implications of successful inhibition of the angiogenic activity of a single purified factor must be regarded with some caution when considered in the wider context of tumour angiogenesis.

ANGIOGENESIS INHIBITORS

Angiogenesis antagonists (Table 2) fall into two categories, namely: (i) those that inhibit production of angiogenic factors by tumours, and (ii) those that inhibit endothelial cell proliferation. Interferons α and β have been shown to inhibit the angiogenic signal produced by both a bladder carcinoma and a hepatoma [45]. Maximal inhibition was 50% of controls, higher concentrations of interferon result in no further reduction in angiogenic activity. Clearly other factors may depress angiogenic factor production by tumours. However, in terms of therapy, the diversity in behaviour of solid tumours and all the problems inherent in targetting tumour cells (other than by DNA damaging agents) are present here, i.e. different tumours and subpopulations of tumours may or may not respond to the factor.

Reports of polypeptide inhibitors of angiogenesis are rare. As far back as 1982, Taylor and Folkman reported that platelet factor 4 (PF4) produced avascular zones on the chorioallantoic membrane of the chick [46]. This observation was reported again in 1990 [47] in a paper which in addition showed that small peptides (12 aminoacids) from the carboxyl-terminal region were as potent as the complete PF4 molecule. In addition, PF4 was shown to directly inhibit human umbilical vein endothelial cell proliferation. Nevertheless, somewhat high concentrations (2–5 μmol/l) are required to see significant inhibition. These concentrations would probably be difficult to obtain *in vivo* without side-effects.

Another angiogenesis inhibitor recently isolated from cartilage is a collagenase inhibitor [48] that was previously isolated, sequenced and cloned from several sources [49, 50] and called TIMP (tissue inhibitor of metalloproteinases). A second tissue derived metalloproteinase inhibitor called TIMP-2 has also been characterised [51]. TIMP-1 is the most potent inhibitor of angiogenesis yet identified. Thrombospondin, a 140 kD glycoprotein, is also an inhibitor of angiogenesis [52] and is secreted when an active cancer suppressor gene is present in hamster and hamster-human hybrid cells [53].

It seems reasonable to assume that other polypeptide inhibi-

Table 2. Inhibitors of angiogenesis

Interferons α and β	Penicillamine
Platelet factor-4	Vitamin D3 analogues
TIMP-1 and TIMP-2	Herbimycin A
Thrombospondin	Minocycline
Steroid/β-cyclodextrin tetradecasulphate	Fumigallin and derivatives

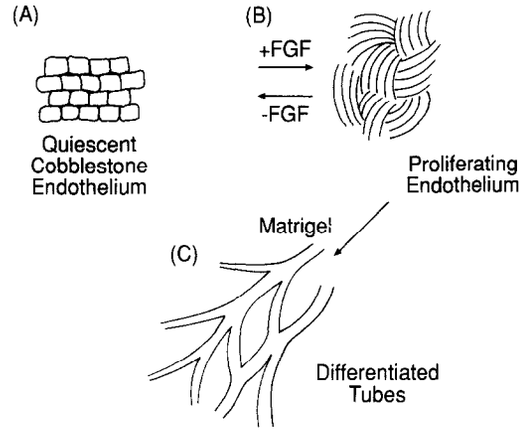

Fig. 1. Morphology of capillary endothelial cells in different environments. (A) Quiescent cobblestone on a gelatin matrix. (B) Fibroblast-like morphology on treatment with FGF; the effect of FGF is reversible. (C) Differentiated tubes on matrigel.

tors of angiogenesis will be identified. Whether they have a future in therapy will depend firstly upon their ability to inhibit the growth of tumours in nude mice xenografts. A real problem with polypeptide inhibitors is targetting. Systemic treatment is likely to be expensive if effective concentrations are to be achieved in the tissues, whereas local administration is useless in metastasised tumours (except where there exists good reason to believe the metastases are localised).

Reports of low molecular weight inhibitors of angiogenesis are also scarce. Inhibition of angiogenesis by combinations of heparin and various steroids has lacked reproducibiltiy. A more consistent inhibition of angiogenesis has been claimed with combinations of steroid and a synthetic heparin substitute of defined structure, namely, β-cyclodextrin tetradecasulphate [54]. Penicillamine [55], vitamin D3-analogues [56], the fungal metabolite herbimycin A [57] and the anticollagenase minocycline [58] have all been reported to possess anti-angiogenic properties, but no data on their effect on the growth of tumours in nude mice is yet available. Recently derivatives of the fungal metabolite fumigallin (termed angioinhibins) have been reported as potent inhibitors of angiogenesis and suppressors of tumour growth in xenografted nude mice [59, 60]. Suramin may partly antagonise angiogenesis by blocking binding of growth factors (e.g. FGF) to their receptors on endothelial cells and there is evidence of antitumour effects in man [61].

ROLE OF THE EXTRACELULLAR MATRIX

Aside from the identification of new polypeptide angiogenic factors another recent development in the field has been the realisation of the importance of the extracellular matrix in the development of a vascular bed. Capillary endothelial cells are unusually plastic (a property not shared with large vessel endothelium). Figure 1 illustrates how diverse morphological changes may be induced in capillary endothelium either by addition of a soluble mitogen/angiogenic factor or by variation of the matrix on which the cells are cultured. Thus, while capillary cells have long been known to form "tubelike" structures when maintained either in prolonged culture [62] or by stimulation with phorbol esters when cultured on a gelatin matrix [63], the cells that participate in tube formation under these conditions represent only a small fraction of the total population. In contrast, by plating on "matrigel" (an extracellular matrix extract from Engelbreth–Holm–Swarm mouse tumour) a 100% differentiated population of tubes is obtained [64], a situation which lends itself to study of the molecular biology of the differentiation process. Laminin appears to be the major active component of matrigel, although induction of tubes by pure laminin is weaker than that by matrigel and other factors such as minor components of the matrigel (e.g. collagen IV) and spacial distribution and density of laminin on the surface presented to the endothelial cell are probably of importance [65].

EXPLOITATION OF ABNORMAL TUMOUR VASCULATURE AS A TARGET FOR THERAPY

We have emphasised that proliferating endothelium offers a unique target for antiangiogenic therapy. To this end we have developed a procedure for the large scale isolation of pure human capillary endothelium [66]. By treating these cells with FGF to induce the proliferating phenotype, isolating plasma membranes and immunising nude mice we hope to isolate monoclonals specific to proliferating endothelium. These monoclonals could then be used in targetting tumour endothelium where strategies such as ADEPT [67] (antibody-dependent enzyme-activated prodrug therapy) linked antibodies may be particularly effective. Other approaches that attempt to exploit abnormal tumour vasculature are being investigated. Some cytotoxic drugs such as mitomycin C are more active under hypoxia [68] and their use in combination with drugs and techniques that damage endothelium could well have synergistic antitumour effects. The macromolecular drug delivery vehicle SMANCS [69] [styrene-maleic acid copolymer-conjugated antitumour protein (neocarzinostatin) non-covalently bound to albumin] was developed to exploit the increased permeability of tumour endothelium to macromolecules linked to potent anticancer drugs. One final observation that should be noted is that differences exist between the response of murine and human tumours to therapeutic agents that act via the endothelium, in particular, tumour necrosis factor and flavone acetic acid are much more effective in murine [70] than human cancer [71].

CONCLUSIONS

The number of factors known to affect endothelial growth has increased markedly. However, many of these are much less potent than FGF and it will be important to establish which are relevant *in vivo*. Analysis of human tumour biopsies to determine which particular angiogenic factors are produced by given tumour types and studies with human capillary endothelium may be particularly helpful in this regard. Capillary endothelium from different organs appears to respond to a different repertoire of growth factors, for example, lung capillary endothelium is stimulated by EGF [8] but not that from adrenals (A. Moghaddam and R.B.). Nevertheless, it is likely that mechanisms of angiogenesis are common to many tumour types and the hope is that antiangiogenic therapies may be widely applicable.

In several tumour types angiogenesis is associated with a worse prognosis and it may be possible to apply antiangiogenic therapies early in clinical trials. Synergy between antiangiogenic therapies and other treatments such as hypoxic sensitisors and ADEPT therapy could be of benefit. Rapid progress in endothelial cell biology is revealing new opportunities for the development of antiangiogenic strategies.

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Morphological Studies Using *in situ* Hybridisation

Giorgio Terenghi and Julia M. Polak

INTRODUCTION

THE UNDERSTANDING of cellular processes requires investigation of translated peptides and proteins, and of their mRNA transcription. Immunohistochemistry is a powerful tool in identifying peptide storage in well defined anatomical structures, and it has been used successfully in a variety of investigative and diagnostic situations [1, 2]. Detection of mRNA can be achieved using northern blot analysis on tissue extracts. However, this technique has certain limitations, particularly in the analysis of heterogeneous tissues with mixed cellular populations, where a dilution effect of the mRNA under investigation might offset the sensitivity of the technique.

The recent development of reliable *in situ* hybridisation methodologies has allowed the identification of mRNA to specific cell types. This technique is based on the use of labelled nucleic acid probes which are able to link with complementary RNA or DNA target sequences to form a hybrid molecule. Because of the specificity of complementary base pairing between nucleic acids, *in situ* hybridisation allows to identify specific gene expression with anatomical accuracy.

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TISSUE PREPARATION

To obtain optimal results with *in situ* hybridisation it is necessary to achieve nucleic acid retention combined with preservation of tissue morphology; hence the use of appropriate fixation is indispensable. Different authors favour a variety of fixatives, and several studies have tried to demonstrate that a specific fixative might be better than others [3–6]. Although the final choice is dictated by the type of tissue and the method of processing, paraformaldehyde appears to be the most widely used reagent for peptide mRNA detection.

The rate of mRNA degradation varies considerably for different sequences, and endogenous nucleases, particularly RNase, appear to be the major contributors in the degradation process. Because the mRNA breakdown is generally rapid, it is recommended to carry out the fixation with a minimum delay. A 10–20 minutes delay from tissue collection is considered acceptable as longer delays can have an adverse effect on hybridisation results [7]. Interestingly, successful results for *in situ* hybridisation have been seen on tissue collected up to 10 hours postmortem [8, 9]. This might be explained by a slow degradation process in cooled untouched tissues [10, 11], as it has been observed that dehydration, manipulation and cutting of unfixed samples during routine dissection accelerate the degradation process, most probably by release of lysosomal content and of endonucleases [10].