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Review

The Role of Vascular Permeability Factor and Basic Fibroblast Growth Factor in Tumor Angiogenesis

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In the last decade a considerable amount of research has been dedicated to studying the process of angiogenesis. In the field of tumor biology angiogenesis is a relevant subject of investigation as well, since newly formed blood vessels are required for the growth of tumors and provide an exit route for metastasizing tumor cells. In this review we discuss some aspects of tumor angiogenesis with emphasis on the role that growth factors bFGF and VPF play in this process. A number of biochemical characteristics and biological properties of the two factors and their receptors are reviewed, and the expression of bFGF and VPF in both normal tissues and in tumors is discussed. Finally, we speculate on the use of bFGF and VPF expression as a diagnostic parameter and on possible clinical applications.

Key words: Angiogenesis / bFGF / VPF

Introduction

The major role of the vasculature is its transport function: the supply of oxygen and nutrients to cells and the removal of waste products from cells. In higher organisms, blood vessels are essential to every living tissue, including tumor cells, that are as dependent on the vasculature as cells in normal tissues. Avascular tumors do not grow beyond the size of 1–2 mm^3, while after vascularization quick expansion to much larger sizes is observed (Folkman, 1990). Mitotic rates of tumor cells decrease with increasing distance from capillaries and tumors with a high blood supply have a higher fraction of dividing cells and lower necrosis rates than tumors with a low blood supply (Folkman, 1990; Lyng *et al.*, 1992). Thus, tumors are truly dependent on angiogenesis (the formation of new blood vessels from existing vessels) for their own growth. Furthermore, angiogenesis is essential for metastasis, the most life-threatening aspect of cancer, as blood vessels provide an escape route for disseminating tumor cells (Folkman, 1990; Blood and Zetter, 1990; Liotta *et al.*, 1991; Folkman, 1987). Recently, a correlation between the degree of tumor angiogenesis and malignancy has been reported for melanoma (Srivastava *et al.*, 1988; Smolle *et al.*, 1989; Barnhill *et al.*, 1992; Barnhill and Levy, 1993; Graham *et al.*, 1994; reviewed in Denijn and Ruiter, 1993), breast cancer (Weidner *et al.*, 1991; Horak *et al.*, 1992; Bosari *et al.*, 1992; Toi *et al.*, 1993; Vartanian and Weidner, 1994), prostate carcinoma (Bigler *et al.*, 1993; Wakui *et al.*, 1992; Weidner *et al.*, 1993), non-small-cell lung cancer (Macchiarini *et al.*, 1992; Macchiarini *et al.*, 1994), testicular germ cell tumor (Olivarez *et al.*, 1994), and brain tumor (Li *et al.*, 1994), stressing the importance of angiogenesis as a relevant factor in tumor biology.

Tumor Angiogenesis

It is important to realize that angiogenesis is a multistep process involving more than endothelial cell proliferation alone. Breakdown of the basal lamina surrounding the endothelial cell layer is an important (and initial) step in migration. In order to form new vessels, endothelial cells have to migrate in a certain direction. Migration takes place preferentially through a provisional matrix, formed for instance by fibrin deposits that are generated by leakage of fibrinogen from the vessel (Folkman and Shing, 1992b; Liotta *et al.*, 1991; Senger *et al.*, 1993; Liotta and Stetler Stevenson, 1991). Finally, migrated endothelial cells have to differentiate, and form a basal membrane around the newly formed vascular sprout. Sprouts formed from different parental vessels eventually have to merge to form a vessel along which transport of blood can occur, and pericytes have to be recruited to assume their perivascular position (Schlingemann, 1990).

Tumor angiogenesis has properties somewhat different from normal angiogenesis (reviewed in Folkman, 1992; Steeg, 1992; Mahadevan and Hart, 1991; Herlyn and Malkowicz, 1991; and Ruiter *et al.*, 1993). The fast growth of tumor cells requires that angiogenesis takes place at a high rate in order to keep up with the needs of an expanding tumor. Although different types of tumor vasculature exist (Dvorak *et al.*, 1988), a series of structural abnormalities is often found in tumor vessels if compared with blood vessels in normal tissues. In general, tumor vasculature is considered to be of an 'immature' nature. Some of the characteristics often found in tumor vasculature are: a high turnover rate of endothelial cells, gaps in the en-

dothelial lining and basement membrane, low amounts of pericytes; a heterogeneous, sometimes chaotic organization and distribution of the tumor vasculature characterized by the existence of e.g. non-functional capillary sprouts or arteriovenous anastomoses; an increased vascular permeability, and the occurrence of intravascular coagulation (reviewed in Jain, 1988; Vaupel *et al.*, 1989; Blood and Zetter, 1990; Dvorak *et al.*, 1988; and Rickles *et al.*, 1983). In tumors blood flow, oxygen pressure, and tissue pH values are generally very variable, but on the average mostly lower than in normal tissues (Vaupel *et al.*, 1989). Because of the absence of lymphatic vessels, interstitial pressure is often high in tumors, leading to further transport problems (Jain, 1987). It is not surprising therefore that necrosis is a general phenomenon, especially in larger tumors (Vaupel *et al.*, 1989; Lyng *et al.*, 1992).

Angiogenic Factors

Positive regulation of angiogenesis is mediated by molecules referred to as angiogenic factors. In order to induce the growth of blood vessels, tumor cells produce such angiogenic factors, that, in general, belong to the classes of growth factors or cytokines. These factors not only induce mitosis and/or migration of endothelial cells, but can also be involved in the induction of specific proteases like the collagenases and constituents of the plasminogen activator complex. These enzymes are required for the initial step in angiogenesis, degradation of the extracellular matrix (reviewed in Klagsbrun and D'Amore, 1991; Liotta *et al.*, 1991; Folkman and Shing, 1992b; and Folkman and Klagsbrun, 1987). Some angiogenic factors do not stimulate cell division or even inhibit division of endothelial cells, like TNFα, TGFβ, and angiogenin. These factors are thought to stimulate angiogenesis indirectly, by e.g. attracting monocytes and activating them to produce other, directly acting angiogenic factors (Frater Schroder *et al.*, 1987; RayChaudhury and D'Amore, 1991; Blood and Zetter, 1990; Folkman and Shing, 1992b; Sunderkotter *et al.*, 1991; Folkman and Shing, 1992b).

Recently, synergistic effects of combinations of different growth factors on several angiogenic actions have been described. The mode of cooperation of the many angiogenic factors *in vivo* is very complex. Some angiogenic factors may not be able to initiate all steps required for angiogenesis, but in concert with other factors they can contribute to the completion of the whole process. Furthermore, the action of angiogenic factors is counteracted by several anti-angiogenic factors (Klagsbrun, 1991), in normal situations leading to a balance between angiogenesis and anti-angiogenesis. This balance can shift towards angiogenesis if this is imposed by the situation, like after wounding, during the menstrual cycle, or during tumor growth.

Some of the angiogenic factors (like TGFα and TGFβ) are expressed as latent precursor molecules which are activated by specific proteolytic cleavage(s) (Lee *et al.*, 1985; Massague, 1990; Barnard *et al.*, 1990). Other angiogenic factors (the members of the FGF family and some variants of VPF/VEGF) are immobilized by binding to heparin-like molecules in extracellular matrix, but are released by proteolytic processes as well (Saksela and Rifkin, 1990; Houck *et al.*, 1992). Thus, by inducing extracellular proteolysis angiogenic factors may activate or mobilize themselves or each other. Table 1 comprises a number of angiogenic molecules that have been implied in both normal and pathological neovascularization, with their respective *in vitro* activities on endothelial cells. The relevance of two of these factors in tumor angiogenesis, vascular permeability factor (VPF) and basic fibroblast growth factor (bFGF), was shown by inhibition of angiogenesis and tumor growth by specific antibodies (Hori *et al.*, 1991; Kim *et al.*, 1993). Therefore, we will confine ourselves here to discussing the involvement of these two growth factors in the angiogenic process.

Table 1 Growth Factors and Cytokines with Angiogenic Activity. Known effects on endothelial cell mitosis, migration, and proteolytic activity are also indicated. Adapted in part from Blood and Zetter (1990) and Folkman and Shing (1992b) with additional information taken from Maglione *et al.* (1991), Koch *et al.* (1992), Forsberg *et al.* (1993), Beitz *et al.* (1991), Koch *et al.* (1994), Ishikawa *et al.* (1989), Heimark *et al.* (1986), Pepper *et al.* (1990), Unemori *et al.* (1992), Pepper *et al.* (1991) and RayChaudhury and D'Amore (1991). + stimulation; – inhibition; 0 no effect.

Factor*	Effect on endothelial cells		
	Proliferation	Migration	Proteolytic activity
aFGF	+	+	+
bFGF	+	+	+
TGFα	+		
TGFβ	–	–	–
EGF	+		
VPF/VEGF	+	+	+
PlGF	+		
PD-ECGF	+/0	+	
IL-8	+	+	
PDGF	+/0		
TNFα	–	+	
angiogenin	0	0	

* aFGF acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; IL, interleukin; PD-ECGF, platelet derived-endothelial cell growth factor; PDGF, platelet derived growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor; PlGF, placenta growth factor; VPF/VEGF, vascular permeability factor/vascular endothelial growth factor.

VPF

In recent years, interest in the angiogenic molecule vascular permeability factor (VPF) has increased tremendously. VPF was initially isolated as a proteinous factor secreted by guinea pig tumors growing in the peritoneal cavity,

where it caused accumulation of ascites fluid rich in serum proteins. The isolated factor induced also rapid and transient leakage of serum proteins from the dermal vasculature into guinea pig skin without leading to endothelial cell damage (Senger *et al.*, 1983). Later this factor was found to be identical to vasculotropin or vascular endothelial growth factor (VEGF), which had been isolated and cloned independantly by several groups as an endothelial cell-specific mitogen and an inducer of angiogenesis *in vivo* (Tischer *et al.*, 1989; Plouet *et al.*, 1989; Leung *et al.*, 1989; Keck *et al.*, 1989; Conn *et al.*, 1990).

VPF has later been shown to induce rapid von Willebrand factor release from endothelial cells (Brock *et al.*, 1991), to be chemotactic for endothelial cells (Koch *et al.*, 1994), and to increase the expression of GLUT-1 glucose transporter (Pekala *et al.*, 1990), tissue factor (Clauss *et al.*, 1990), interstitial collagenase (Unemori *et al.*, 1992), plasminogen activators and plasminogen activator inhibitor-1 (Pepper *et al.*, 1991) in endothelial cells. VPF thus induces multiple processes directly involved in angiogenesis, but it also stimulates activation of the coagulation cascade by the endothelium, and it enhances vascular permeability. The latter phenomena are often considered to be specific properties of tumor vasculature (Jain, 1987; Vaupel *et al.*, 1989), but angiogenesis during wound healing is associated with hyperpermeable microvessels and formation of fibrin as well (Brown *et al.*, 1992).

VPF is often referred to as an endothelial cell-specific factor, but this specificity is not absolute, as VPF also induces tissue factor expression on monocytes and is chemotactic for monocytes (Clauss *et al.*, 1990). In all other cell types tested, VPF did not elicit a response, so it is probably correct to say that VPF has a very restricted target cell specificity, with endothelial cells being the main target.

The VPF translation product is provided with a secretory signal peptide that is cleaved off during secretion from the producer cells (Leung *et al.*, 1989; Keck *et al.*, 1989). It is, however, not self-evident that VPF can reach its target cells, since some variants of VPF are retained by the producer cells at extracellular sites (see below).

Several molecular variants of the VPF protein exist due to alternative splicing of its mRNA. The pre-mRNA for VPF contains 8 exons, of which exons 6 and 7 can either be present or absent in the mature mRNA (Tischer *et al.*, 1991). The VPF variants generally found in human cells have a length of 189 amino acids (encoded by all 8 exons), 165 amino acids (lacking the amino acids from exon 6) and 121 amino acids (lacking the amino acids from exons 6 and 7), respectively. Two other rare forms of human VPF mRNA have been described; a variant encoding a 206 amino acid protein (containing all exons and part of the 7th intron) was found in liver (Houck *et al.*, 1991) and a variant encoding a 145 amino acid protein (lacking exon 7 only) was found in uterine tissue (Charnock Jones *et al.*, 1993).

Exon 6 of the VPF messenger codes for a stretch of basic amino acids, due to which the VPF splice variants containing these residues (VPF_{189}, VPF_{206}, and probably also VPF_{145}) have a high affinity for heparin. Therefore, these variants are retained bound by the producer cells on extracellular heparin-like molecules, probably proteoglycans. VPF_{189} was only released from the cells after addition of suramin or heparin to the medium, or by heparinase treatment of the producer cells. Moreover, plasmin cleavage resulted in the release of the N-terminal half of VPF_{189}, which was still biologically active (Houck *et al.*, 1992). VPF_{165} has a lower affinity for heparin and is only partly retained by the producer cells, while VPF_{121} has no significant affinity for heparin and is completely released from the producer cells (Houck *et al.*, 1992). These two soluble VPF variants are the only species readily found in conditioned media from mammalian cells, generally migrating as bands of 15–24 kDa on SDS-PAGE under reducing

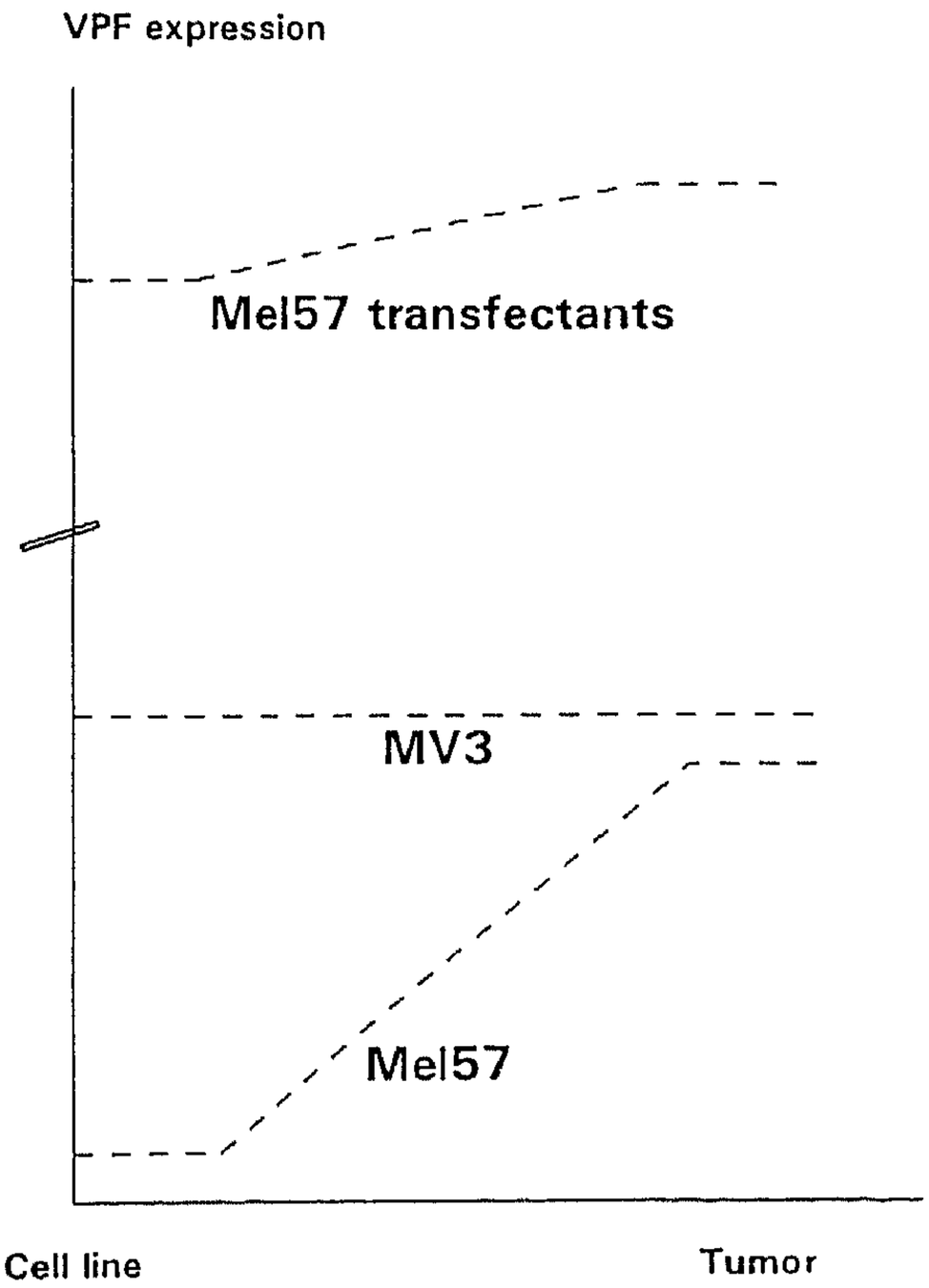

Fig. 1 Schematic Illustration of the VPF Expression Patterns in Several Human Melanoma Cell Lines.
The MV3-type of lines expresses high levels of VPF messenger and protein in culture and the VPF mRNA levels remain equally high in the xenografts. The Mel57 cell line expresses only 5 to 10% of the amount of VPF mRNA found in MV3, but the tumors resulting from these lines have VPF mRNA levels comparable to those in tumors from line MV3. Hence, during the development of Mel57 tumors, VPF expression is up-regulated. To change cell line Mel57 into a line constitutively expressing VPF, it was stably transfected with a VPF expression construct. The resulting transfectant cell line expressed recombinant VPF RNA at a level of approximately 2-fold the VPF mRNA level in cell line MV3. In tumors from the transfectant line the endogenous VPF mRNA was up-regulated, but its contribution was relatively small compared to the level of recombinant VPF RNA that remained being expressed *in vivo* (Pötgens *et al.*, 1995).

conditions (Yeo *et al.*, 1991). Under non-reducing conditions, however, VPF migrates as a broad band between 34 and 43 kDa (Senger *et al.*, 1983) indicating that in its native form VPF is a dimeric protein.

The amino acids encoded by exons 6 and 7 do not appear to be essential for biological activity (Houck *et al.*, 1992), but as demonstrated above, they are important for the bio-availability of VPF. The smaller VPF variants are diffusible molecules probably able to reach their target cells easily. The variants containing the exon 6-encoded amino acids, on the other hand, are stored extracellularly, thereby reaching a higher local concentration, but can be recruited for instance by plasmin cleavage.

In initial searches for VPF receptors, there were always two classes of VPF binding sites found on different types of endothelial cells, with varying dissociation constants. (Vaisman *et al.*, 1990; Bikfalvi *et al.*, 1991; Myoken *et al.*, 1991; Olander *et al.*, 1991; Barleon *et al.*, 1994). Some experimental data indicate that the binding of VPF to its receptors is dependent on the presence of heparin or heparin-like molecules on the cell surface. Binding of VPF to its receptors was enhanced by heparin at concentrations between 0.1–10 μg/ml. Pretreatment of endothelial cells with heparinase abolished binding of VPF, but binding was restored by the addition of soluble heparin (Gitay Goren *et al.*, 1992).

Recently, two receptors for VPF have been cloned. The product of the *fms*-like tyrosine kinase gene *(flt)*, isolated from a human placenta cDNA library (Shibuya *et al.*, 1990), was characterized as a high affinity VPF receptor (de Vries *et al.*, 1992) forming a 205 kDa complex with VPF. The *flt* receptor might be the high affinity binding site on endothelial cells reported in earlier studies. Another putative tyrosine kinase gene, fetal liver kinase 1 *(flk-1)* isolated from a mouse cDNA library (Matthews *et al.*, 1991), was also found to code for a VPF receptor (Quinn *et al.*, 1993). The human homologue of the *flk-1* gene was isolated independently from an endothelial cell cDNA library and designated kinase insert domain-containing receptor *(KDR)* (Terman *et al.*, 1991; Terman *et al.*, 1992). VPF formed complexes with the *flk-1/KDR* receptor of molecular masses between 235 and 195 kDa with a dissociation constant comparable with the lower affinity receptor type in earlier studies (Terman *et al.*, 1992; Quinn *et al.*, 1993; Millauer *et al.*, 1993).

The sequences of the cDNAs encoding the VPF receptors *flt* and *flk-1/KDR* predict two homologous transmembrane proteins, which both have 7 extracellular immunoglobulin-like (IgG-like) domains and a tyrosine kinase domain interrupted by a socalled kinase insert. The tyrosine kinase domain is shared between many growth factor receptors, and IgG-like domains and a kinase insert sequence are also found in e.g. the PDGF- and the FGF-receptor family (Fantl *et al.*, 1993). The VPF receptors thus have many features in common with other growth factor receptors.

Expression of VPF receptors is often regarded to be restricted to endothelial cells (Peters *et al.*, 1993; Millauer *et al.*, 1993; Barleon *et al.*, 1994). However, *flk-1* transcripts have been identified in murine umbilical vein stroma and in mouse hematopoietic stem cells as well (Quinn *et al.*, 1993; Matthews *et al.*, 1991). VPF binding sites have been identified on monocytes which were also responsive to VPF (Shen *et al.*, 1993; Clauss *et al.*, 1990). High-affinity binding sites for VPF have even been found on HeLa cells, mouse NIH-3T3 fibroblasts, bovine granulosa cells, and human melanoma cells (Gitay Goren *et al.*, 1992; Gitay Goren *et al.*, 1993), but no response of these cells to VPF has as yet been demonstrated.

bFGF

In contrast to VPF, bFGF has been the subject of investigation for many decades. Already in 1939 mitogenic activity towards fibroblasts present in brain extracts was reported. Continuing research resulted about 20 years ago in the isolation from bovine pituitary extracts of two factors that were mitogenic for fibroblasts. The purification of these factors was facilitated by their high affinity to heparin-Sepharose; this quality of binding to heparin or other glycosaminoglycan (GAG) or proteoglycan molecules turned out later to be of paramount importance for the activity of both factors (see below). Based on their different pI values and their main *in vitro* effects, the two growth factors were designated acidic and basic fibroblast growth factor (aFGF and bFGF). Soon afterwards, amino acid and nucleotide sequences were determined and more growth factors with homologous protein and DNA sequences were identified (Klagsbrun, 1989; Burgess and Maciag, 1989).

bFGF is a member of the fibroblast growth factor family that comprises today at least nine members. Their actions are pleitropic and have been the subject of a number of excellent reviews (Klagsbrun, 1989; Thomas, 1987; Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989). bFGF induces DNA synthesis and proliferation in cells from mesodermal and neuroectodermal origin such as fibroblasts, smooth muscle cells, astrocytes and myoblasts. In endothelial cells, bFGF induces proliferation and affects cell motility, migration, and differentation. Furthermore, endothelial cells are stimulated by bFGF to express specific proteases involved in basal membrane degradation (Flaumenhaft *et al.*, 1992). In both *in vivo* and *in vitro* model systems (Montesano *et al.*, 1986) bFGF is a potent inducer of angiogenesis.

Different species of bFGF molecules exist, which all have comparable or even identical biological activities. The predominant form consists of 155 amino acids (aa), but shorter (146 aa) and longer (196, 201, 210 aa) forms have been reported as well. The different bFGF species arise by alternative splicing, removal of amino terminal aa residues, and/or the use of alternative initiation codons for translation. On SDS-PAGE, protein bands ranging from 16.5 to 24.2 kDa can be observed (Callard and Gearing, 1994; Aggarwal and Gutterman, 1992).

One of the puzzling questions regarding the biology of bFGF is how it is released from producing cells. Since it lacks a classical leader sequence, it cannot be secreted in the usual way. Hypotheses range from release of bFGF from damaged cells only to bFGF not being released at all (Mignatti and Rifkin, 1991; Rifkin and Moscatelli, 1989; Mason, 1994). Recent experiments indicate that release of growth factors lacking leader sequences may employ alternative secretory pathways. A heat shock has been reported to release aFGF (also lacking a leader sequence) from 3T3 fibroblasts (Jackson *et al.*, 1992), whereas the complement membrane attack complex (C5b–C9) releases bFGF from endothelial cells (Benzaquen *et al.*, 1994). Interestingly, normal fibroblasts and cells derived from mild and aggressive fibromatosis and from fibrosarcoma all expressed bFGF in an cell-associated form, but only the latter two stages were highly vascularized and found to export bFGF to extracellular compartments (Kandel *et al.*, 1991). Other indirect evidence indicating that the presence or absence of a leader sequence is not the only factor that determines the release of a protein was obtained from two other FGF family members, FGF3 and FGF9. FGF9, lacking a signal peptide, is readily secreted from the producing cells, whereas FGF3, which does contain this sequence, is hardly secreted (Mason, 1994).

Two receptor classes (with low and high affinity for their ligand) have been reported for the FGF family of growth factors. The high-affinity receptors are members of the family of transmembrane receptor tyrosine kinases, just like the two VPF receptors cloned to date. Four FGF receptors (FGFR-1 to -4) have been described; bFGF can interact with FGFR-1 *(flg)* and with FGFR-2 *(bek)*. These receptors contain 1 to 3 immunoglobulin-like extracellular domains and an intracellular kinase domain (reviewed in Jaye *et al.*, 1992; and Fantl *et al.*, 1993). The low affinity receptor group consists of GAGs such as heparin or heparan sulfate proteoglycans (HSPGs) such as perlecan. Both GAGs and HSPGs can occur in a soluble form, or immobilized on the cell membrane or in the extracellular matrix (Klagsbrun, 1992; Folkman and Shing, 1992a). It is generally accepted that bFGF cannot interact with a high-affinity receptor unless it interacts simultaneously with a low-affinity receptor (Klagsbrun and Baird, 1991; Kan *et al.*, 1993; Vlodavsky *et al.*, 1988). The mechanism of this receptor binding by means of simultaneous interaction with both receptor classes has not yet been fully clarified. HSPG may alter the conformation of either the high-affinity receptor or the bFGF molecule, resulting in a higher affinity of the ligand-receptor interaction. Alternatively, dimerization of high-affinity receptors, a prerequisite for signal transduction, may be facilitated by HSPG molecules (reviewed in Jaye *et al.*, 1992). An additional effect of bFGF binding to extracellular matrix HSPGs may be storage of bFGF in the basal lamina on sites where it is not currently needed. Heparinases or proteases may release the stored bFGF (Saksela and Rifkin, 1990; Vlodavsky *et al.*, Vlodavsky *et al.*, 1990), thereby providing a mechanism for quick bFGF recruitment when stimulation of angiogenesis is required, for instance in wound healing processes, similar to the mechanisms described above for the larger forms of VPF.

Synergism between VPF and bFGF

In endothelial cells both bFGF and VPF/VEGF induce the expression of specific proteases involved in extracellular matrix degradation, e.g. collagenases (Presta *et al.*, 1986; Moscatelli *et al.*, 1986; Unemori *et al.*, 1992) and constituents of the plasminogen activator pathway: bFGF induces both urokinase plasminogen activator (uPA) and its inhibitor PAI-1, thereby increasing the uPA/PAI-1 ratio (Pepper *et al.*, 1990; Pepper *et al.*, 1991), while VPF/VEGF is most potent in the induction of tissue plasminogen activator, tPA (Pepper *et al.*, 1991). In connection with these activities, bFGF and VPF/VEGF are capable of inducing invasion and capillary-like tube formation of endothelial cells in collagen gels ('*in vitro* angiogenesis') (Pepper *et al.*, 1993). At least bFGF does so for fibrin gels as well (Pepper *et al.*, 1990). However, the effect on *in vitro* angiogenesis if only one of these factors is added is relatively small. If both factors are added simultaneously the biological effect is much more pronounced. VPF/VEGF and bFGF therefore act synergistically in inducing *in vitro* angiogenesis in collagen gels (Pepper *et al.*, 1992).

VPF and bFGF Expression in Normal Tissues

Several adult organs and tissues in guinea pig and man have been found to contain VPF mRNA, particularly lung, kidney, adrenal gland, liver, stomach, heart (especially the cardiac myocytes), as well as peritoneal macrophages (Berse *et al.*, 1992; Ladoux and Frelin, 1993). In healthy organs with generally non-proliferating blood vessels VPF may play a role in maintaining the existing density of the vasculature or a basal level of vascular permeability necessary for transport of, for instance, nutrients.

Expression of VPF mRNA during mouse brain development was highest during the periods of active vascularization (prenatal and postnatal) and was reduced in adult brain in which angiogenesis had ceased (Breier *et al.*, 1992). These data suggest that VPF is involved in embryonic and fetal angiogenesis.

Recurring angiogenesis takes place in the female reproductive organs of mammals coinciding with the oestrous cycle. In rat corpus luteum, in murine ovarian and uterine tissues with an expanding vasculature, and in human endometrium and myometrium expression of VPF mRNA was found (Phillips *et al.*, 1990; Shweiki *et al.*, 1993; Charnock Jones *et al.*, 1993). Spatial distribution of VPF messengers changed during the course of the oestrous cycle (Charnock Jones *et al.*, 1993) and expression was seen especially in steroid-responsive cell types (Shweiki *et al.*, 1993) suggesting that VPF expression is hormonally regulated in these tissues.

Both the presence of bFGF protein and mRNA have been reported in a wide variety of tissues including skin, brain, liver, components of the male and female reproductive tract, several components of the gastrointestinal tract, the circulatory system, endocrine tissues, and lymphoid tissues (Hughes and Hall, 1993; Cordon Cardo *et al.*, 1990; Schulze Osthoff *et al.*, 1990). Immunoreactivity with anti-bFGF antibodies is observed both in cells and in the extracellular matrix, especially in the basal lamina of blood vessels. In almost all cultured cells bFGF protein and/or mRNA can be detected. Whether this broad expression pattern reflects the *in vivo* situation or is an artifact induced by the culture conditions or fixation is not always clear. The observed production of bFGF by cultured endothelial cells (Schweigerer *et al.*, 1987; Yu *et al.*, 1993) may be an especially relevant phenomenon to investigate, since it suggests that an autocrine mechanism for angiogenesis may exist (Sato *et al.*, 1991; Folkman, 1984).

The expression of FGFR-1 has a much more confined distribution pattern than bFGF itself. Staining with specific antibodies was observed mainly on microvascular endothelial cells, whereas FGFR-1 and -2 mRNA expression was reported amongst other tissues in brain, liver, skin and growth plates of developing bones (Callard and Gearing, 1994).

VPF and bFGF Expression in Tumors

Apart from its presumed role in wound healing (Brown *et al.*, 1992) and in diseases such as diabetic retinopathy (Adamis *et al.*, 1993) and rheumatoid arthritis (Koch *et al.*, 1994; Fava *et al.*, 1994), the role of VPF in tumor angiogenesis has been most intensively studied. VPF expression levels in tumors are often higher than in surrounding normal tissue. One of the earliest observations leading to this hypothesis was that guinea pig line 10 carcinoma cells and a series of human tumor cells (osteogenic sarcoma, bladder carcinoma, cervical carcinoma, and fibrosarcoma cells) produced and secreted high levels of VPF activity (Senger *et al.*, 1986). Most interestingly, two of these tumorigenic cell lines were derived from non-tumorigenic parental cell lines which secreted much less VPF activity. An increased level of VPF mRNA was also found in colonic adenocarcinoma, renal cell carcinoma, human tumorigenic fibrosarcoma and osteosarcoma cell lines, and in transformed pheochromocytoma cells, as compared to less malignant or normal tissues or cells (Berse *et al.*, 1992; Brown *et al.*, 1993; Takahashi *et al.*, 1994; Claffey *et al.*, 1992).

Many observations have led to the idea that hypoxia may be an important VPF-inducing mechanism in tumors. Locally reduced oxygen pressure due to insufficient vascularization might induce VPF expression in an attempt to stimulate further angiogenesis and thereby to improve oxygen supply. Different groups have demonstrated an up-regulation of VPF transcript levels adjacent to necrotic (presumably hypoxic) areas in human and rat glioblastoma (Shweiki *et al.*, 1992; Plate *et al.*, 1992). This up-regulation was likely to be caused by hypoxia, as VPF mRNA levels were also increased in glioma cell lines cultured under low oxygen tension (Shweiki *et al.*, 1992; Plate *et al.*, 1993). In human adenocarcinomas increased VPF messenger levels were also found around necrotic sites, although this was only observed in tumor stroma but not in tumor cells (Brown *et al.*, 1993). Furthermore, a number of human melanoma cell lines had much higher expression levels of VPF when growing as (highly necrotic) tumors in nude mice than in cell culture. Again, hypoxia is a likely cause for this difference as VPF expression in these melanoma lines could also be induced in a hypoxic environment *in vitro* (Pötgens *et al.*, 1995).

VPF secreted by tumor cells is believed to accumulate on nearby tumor blood vessels. In solid guinea pig line 10 tumors and in human lymphomas, VPF mRNA was found to be synthesized in tumor cells, but upon immunohistochemistry using an anti-VPF antibody staining was observed mainly on the endothelium of tumor blood vessels and blood vessels immediately adjacent to the tumor (Dvorak *et al.*, 1991). No staining for VPF was found in more distant blood vessels. Other studies have revealed that transcripts for the VPF receptors *flt* and *flk-1/KDR* are present in higher amounts in tumor endothelial cells than in endothelial cells outside of the tumors (Brown *et al.*, 1993; Plate *et al.*, 1992; Plate *et al.*, 1993). This not only suggests that VPF accumulates in tumor blood vessels because many binding sites for VPF are present there, it also suggests that tumor blood vessels are more responsive to VPF than normal blood vessels.

Does VPF expression actually play a role in the development of the tumor vascular bed? There is indirect evidence that VPF does indeed influence tumor neovascularization. In glioma, the sites of highest VPF production – near necrotic foci – were surrounded by clusters of newly formed microvessels (Shweiki *et al.*, 1992), thus showing a clear spatial correlation between VPF expression and angiogenesis.

Transfection experiments have recently provided direct evidence for the importance of VPF in tumor angiogenesis and tumor growth. By transfection of non-tumorigenic Chinese hamster ovary (CHO) cells with a VPF expression construct, these cells acquired the ability to proliferate in nude mice and to form vascularized lesions (Ferrara *et al.*, 1993). Similarly, transfection of the human tumor cell line HeLa with a VPF expression construct conferred to these cells a growth advantage in nude mice compared to control cells, which was associated with a higher angiogenic activity in the xenografts (Kondo *et al.*, 1993). In our laboratory, we investigated the effect of VPF on the tumor vasculature in a melanoma xenograft model. Human melanoma cell line Mel57, whose VPF expression was hypoxia sensitive (Pötgens *et al.*, 1995), was transfected with a construct containing a VPF cDNA. This resulted in a transfectant with a constitutively high production of VPF (see Figure 1). When injected subcutaneously into nude mice, the vascular architecture of the resulting tumors

showed distinct qualitative changes, whereas growth rates and necrosis rates of the tumors were unaltered. In the VPF-transfected melanoma xenografts staining for endothelium and for extracellular matrix revealed that tumor stroma at the periphery of tumor cell nodules showed a high degree of vascularization. The tumor vasculature manifested itself as a dense network located mainly at the periphery of tumor cell clumps in which, because of the relative absence of penetrating vasculature, necrotic centers were often observed. Melanomas from the parental cell line or from a vector-transfected line had a vasculature with quite a different arrangement: blood vessels appeared as separate units scattered throughout most of the tumors. Some parts of the tumors were poorly vascularized and were also necrotic, but in most parts sufficient numbers of blood vessels were present (Pötgens *et al.*, 1995), see Figure 2. These results demonstrate that VPF not only affects the number of blood vessels as was found in other studies (Kondo *et al.*, 1993; Ferrara *et al.*, 1993), but can influence the patterns in which formation of blood vessels takes place as well. The mechanism responsible for the VPF-induced aberrant vascular phenotype is still a topic under investigation in our laboratory.

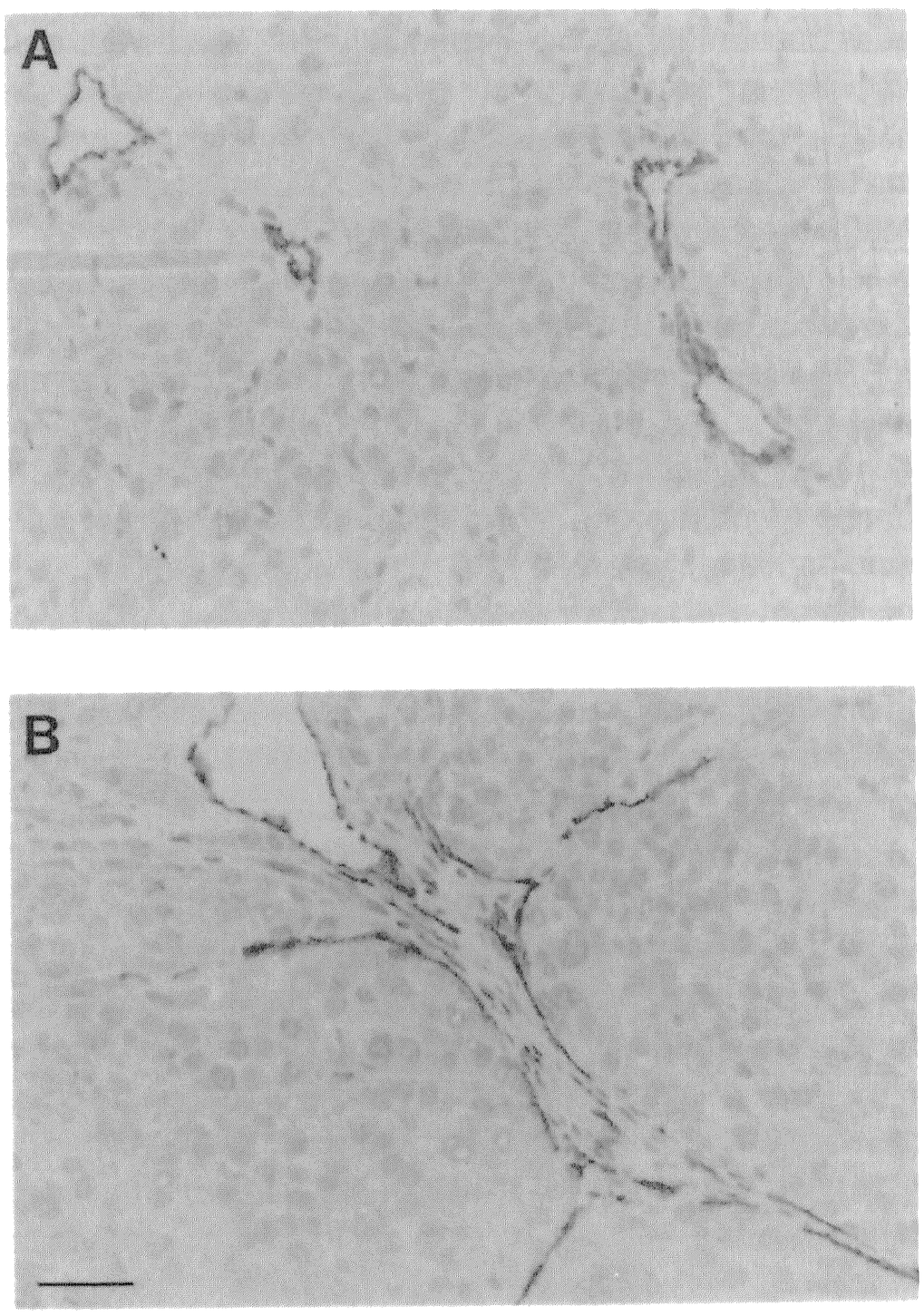

Fig. 2 Sections of Melanoma Xenografts in Nude Mice Stained with a Monoclonal Antibody Specific for Mouse Vascular Endothelium.
(A) Tumor from cell line Mel57 stably transfected with vector DNA only. (B) Tumor from cell line Mel57 stably transfected with a VPF expression construct. Note the organization of endothelial cells/blood vessels in stromal septa between avascular tumor cell nodules. Scale bar = 40 μm.

Treatment of tumor-bearing mice with neutralizing anti-VPF antibodies also demonstrated the role of this growth factor in tumor angiogenesis. The enhanced growth of VPF-transfected HeLa cells was suppressed by anti-VPF antibodies (Kondo *et al.*, 1993). In xenografts of rhabdomyosarcoma and glioblastoma cells in nude mice, treatment with a neutralizing antibody against VPF also inhibited tumor growth and decreased the vascular density in the lesions. The antibody did not affect the growth rate of the tumor cells *in vitro* (Kim *et al.*, 1993). In other experiments VPF activity was inhibited by virus-mediated gene transfer into endothelial cells *in vivo* of a sequence encoding a dominant-negative mutant of the *flk-1* VPF receptor. The growth of glioblastomas in nude mice was inhibited by this treatment which resulted in a non-vascularized and highly necrotic tumor cell mass (Millauer *et al.*, 1994).

VPF expression by tumors may also enhance the permeability of tumor blood vessels. This was demonstrated by the fact that the relative expression levels of VPF in a series of tumor cell lines correlated with the *in vivo* vessel permeability in tumors developing from these lines after injection into nude mice (Roberts and Hasan, 1993). Furthermore, the accumulation of VPF on the endothelium of tumor blood vessels was associated with hyperpermeability of these vessels for macromolecules (Dvorak *et al.*, 1991). A VPF-induced enhancement of vascular permeability may facilitate tumor vascularization as vessel permeability can lead to the deposition of a fibrin matrix around existing vessels, which in its turn is a substrate for developing tumor stroma and ingrowing capillaries (reviewed in Senger *et al.*, 1993).

Since angiogenesis is an important step in metastasis, VPF expression might also influence this process. However, no reports have as yet been published which directly correlate VPF expression with metastasis. This relation will also be addressed in future studies with the aforementioned VPF-overproducing melanoma line.

bFGF expression has been demonstrated in a number of neoplastic cell types, both in cultured cells and in tissue. bFGF expression by tumor cells is not only significant in view of a possible increase in tumor angiogenesis, but may also serve as an autocrine growth stimulation for the tumor cells themselves. bFGF has been detected in adrenal pheochromocytoma and chemodectoma (Statuto *et al.*, 1993), renal cell carcinoma (Singh *et al.*, 1994), bladder carcinoma (Allen and Maher, 1993), brain tumors and astrocytomas (Brem *et al.*, 1992; Zagzag *et al.*, 1990; Li *et al.*, 1994), hepatocellular carcinoma (Li *et al.*, 1994), and carcinoma of the digestive tract (Ohtani *et al.*, 1993; Li *et al.*, 1994). bFGF mRNA was detectable in metastatic and primary invasive melanoma, but not in melanoma *in situ* and in benign melanocyte nevi (Reed *et al.*, 1994). Cultured melanocytes do not express bFGF, but are dependent on this factor for their growth. Cells derived from all other stages of melanoma progression (dysplastic nevus, primary melanoma, melanoma metastasis) all express bFGF

and show a decreasing dependency on exogenously added bFGF for their growth *in vitro* (Shih and Herlyn, 1993; Rodeck *et al.*, 1991). Cell lines derived from primary melanoma and melanoma metastasis do not, in fact, require any exogenous bFGF at all for their growth thereby illustrating the capacity of bFGF to serve as a an autocrine growth factor (Rodeck *et al.*, 1991). We studied the expression level of bFGF protein in a panel of melanoma cell lines. These cell lines all give rise to tumors when injected subcutaneously in nude mice, but differ in their ability and/or speed with which the primary tumors (xenografts) give rise to lung metastases. All tested cell lines produced bFGF mRNA as determined by RT-PCR. bFGF protein levels were determined in lysates of the cell lines by Western blot employing specific anti-bFGF monoclonal antibodies developed in our laboratory (Westphal *et al.*, manuscript in preparation). Two cell lines (MV3 and BLM) which gave rise to high numbers of lung metastases very quickly after take of the primary tumor showed high bFGF protein levels, whereas slower metastasizing lines (MV1 and M14) were negative in this assay (Figure 3). In cultured human umbilical vein endothelial cells and cultured melanocytes we could not detect bFGF as well, but melanoma line 530, which forms slowly growing tumors and no lung metastases in nude mice, was positive for bFGF protein expression. We are currently determining the vascularization level of the different melanoma xenografts to investigate a possible correlation between bFGF expression level and vascular density or tumor angiogenesis.

Evidence for a possible involvement of different FGFRs in malignant progression was derived from data obtained with human astrocytomas. Malignant progression in this tumor type was accompanied by an increased expression of FGFs. Although expression of FGFR-2 was abundant in normal white matter and in low grade astrocytomas, it was absent in malignant astrocytomas. Expression of FGFR-1, on the other hand, was observed to shift from the FGFR-1α form to the alternatively spliced FGFR-1β form in tumors progressing from relatively benign to more malignant phenotypes (Yamaguchi *et al.*, 1994).

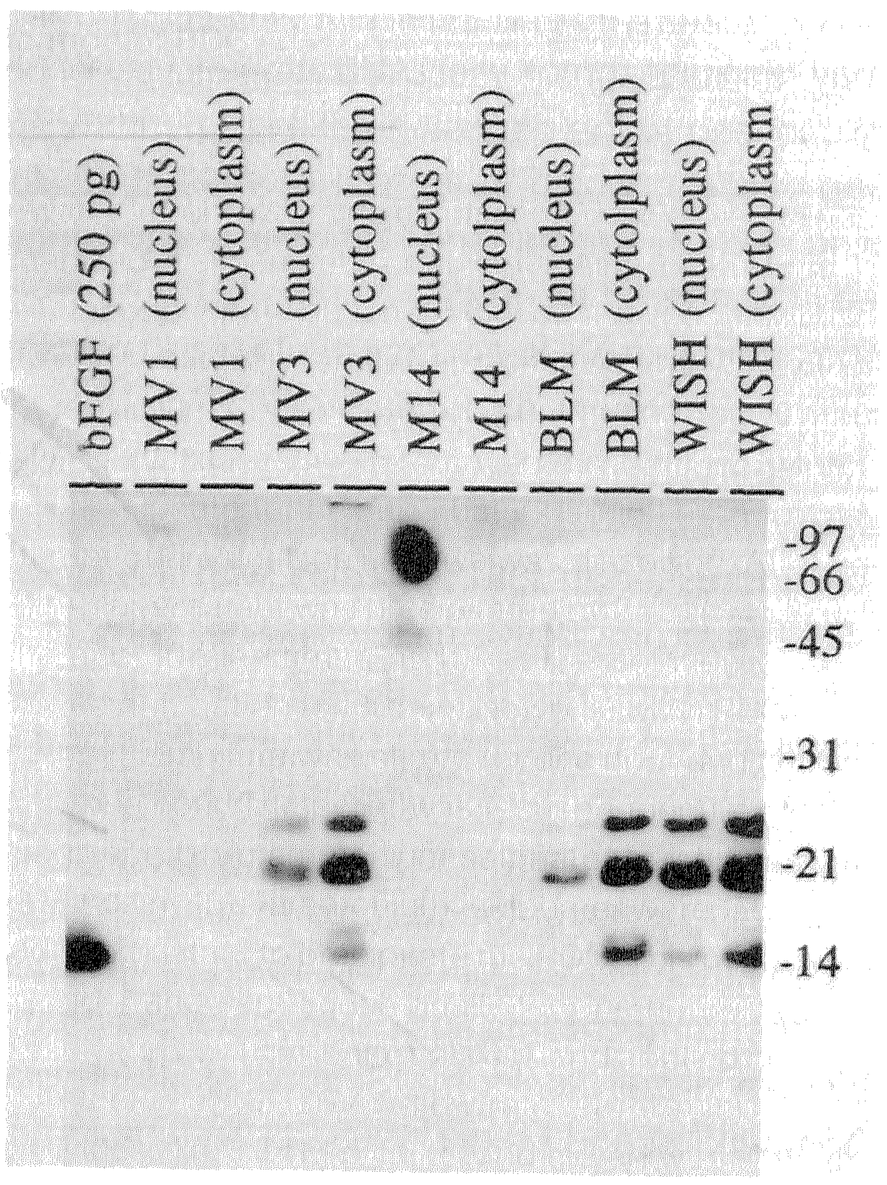

Fig. 3 Expression of bFGF Protein in Melanoma Cell Lines MV1, MV3, M14 and BLM and in Amnion Cell Line WISH.
Cultured cells were lysed with 1% Triton-X100, and nuclear and cytoplasmic fractions were subjected to SDS-PAGE. After protein transfer to nitrocellulose, bFGF was detected by anti-bFGF monoclonal antibody 4E3 developed in our laboratory. Recombinant human bFGF was used as a positive control (left lane). Molecular weight markers are indicated at the right of the figure in kDa. bFGF species of 15, 20 and 25 kDa were detected with the 20 kDa species as the dominant form.

VPF and bFGF in Tumor Diagnosis and Their Potential Clinical Applications

The many observations on VPF expression in tumors indicate that the level of VPF expression in suspect cancers might be a parameter for/with prognostic value. On the other hand, VPF expression was also found in normal tissues (presumably in lower quantities) and was associated with other diseases than cancer as well as discussed earlier. The level of VPF expression may therefore be more important than assessment of VPF expression *per se*. Methods determining VPF protein or messenger levels are being developed and may become important tools in tumor diagnosis in the future. For the measurement of VPF mRNA levels in tumors Northern blotting techniques might be useful, but given the small amounts of material generally available from biopsies a quantitative RT-PCR method would probably be more practical. As yet, few studies have compared VPF messenger levels between malignant and less malignant or normal tissues (Berse *et al.*, 1992; Brown *et al.*, 1993; Takahashi *et al.*, 1994), but these studies do not answer the question whether VPF mRNA determinations are useful in tumor diagnosis or prognosis.

For the determination of VPF protein in the lesions themselves or in body fluids specific immunoassays are required. A time-resolved immunofluorometric assay (IFA) for VPF was shown to be potentially useful as a diagnostic test for malignancy: the VPF levels in human ascitic tumor effusions correlated with the presence of malignant cells in the lesions (Yeo *et al.*, 1992; Yeo *et al.*, 1993). No VPF was detected with this method in plasma and urine from tumor-bearing animals or from patients with malignant cancers.

The spatial distribution of VPF expression as assessed by *in situ* hybridisation and/or immunohistochemistry might also be indicative for the degree of tumor malignancy and therefore a candidate for diagnostic or prognostic parameter. In gliomas the highest levels of VPF mRNA were found around necrotic foci, and there was also evidence that induction of VPF expression is mediated by hypoxia in these tumors (Shweiki *et al.*, 1992;

Plate *et al.*, 1992; Plate *et al.*, 1993). In other tumor types VPF expression was more homogeneous (Brown *et al.*, 1993). From a panel of human melanoma lines a subset which was most malignant in nude mice showed both *in vitro* and *in vivo* (xenografts) a constitutively high expression level of VPF. A less malignant group of melanoma lines had a low expression of VPF in culture which was, however, up-regulated *in vivo* and by low oxygen tension *in vitro* (Pötgens *et al.*, 1995). It would be interesting to study the spatial distribution of VPF expression in the tumors from these melanoma lines to see if the distribution differs with the grade of malignancy.

The importance of bFGF in tumor diagnosis, prognosis and treatment is doubtful. Although an elevated expression level of bFGF has in a number of neoplasms been correlated with a more malignant phenotype, the almost omnipresence of the factor in healthy tissues does not make bFGF a diagnostic or prognostic parameter with a high discriminative value. Measurement of the exact level of either bFGF protein or mRNA could be a more informative parameter, although our preliminary experiments in the melanoma xenograft model do not indicate a relation between bFGF protein levels and malignancy. Considering the dual effect of bFGF on tumor growth (by increasing tumor angiogenesis and tumor growth as an autocrine mechanism), anti-bFGF may be effective in the treatment of tumors. In an experimental setting this approach has yielded both positive (Hori *et al.*, 1991) and negative (Matsuzaki *et al.*, 1989) results. We are currently determining whether our anti-bFGF mAbs can block bFGF activity, to test their action in the melanoma xenograft model.

Concluding Remarks

Anti-angiogenic strategies may be therapeutically beneficial in blocking both tumor growth and metastasis (D'Amore, 1988; Folkman, 1985; Denekamp, 1993; Denekamp, 1990; Teicher *et al.*, 1992). Both tumor-expressed VPF and bFGF are considered as major factors in shaping the tumor vasculature and, consequently, as components of the metastatic phenotype of a tumor. Blocking of VPF and/or bFGF activity therefore may inhibit both tumor progression and metastasis and might have clinical applications. Among the many agents recently tested for their anti-angiogenic and tumor-inhibiting activity (D'Amato *et al.*, 1994; Pepper *et al.*, 1994; O'Reilly *et al.*, 1994; reviewed in Klagsbrun, 1991), VPF/bFGF inhibitors might also find a place.

A future approach in anti-angiogenesis therapy might be the design of VPF or bFGF receptor antagonists which should be able to bind to and occupy their receptors without activating them. Also dominant-negative VPF analogues might be designed which should dimerize with and thereby inactivate normal VPF polypeptide chains. Attempts to produce such analogs of other growth factors, for instance of the related factor PDGF, have shown that this can be rather difficult (Vassbotn *et al.*, 1993). Knowledge about the structure of the protein and about the receptor-binding domains of the factor is certainly a prerequisite for success.

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