Pergamon

European Journal of Cancer Vol. 32A, No. 14, pp. 2401–2412, 1996
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Printed in Great Britain
0959-8049/96 $15.00 + 0.00

PII: S0959-8049(96)00390-5

Role of Soluble Mediators in Angiogenesis

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INTRODUCTION

FORMATION AND regression of new blood vessels are central events in the development of normal and pathological tissues, inflammation and tissue remodelling. In angiogenesis, endothelial cells, stimulated by angiogenic molecules, change their genetic programme. They produce proteolytic enzymes, migrate, enter the cell cycle and then differentiate again to form new vessels. Integration of growth factors, autacoids, receptors, adhesion molecules, extracellular matrix, proteases and accessory cells is fundamental for the correct outcome of the processes which determine the features of the new vessels.

This review focuses on paradigmatic examples of soluble mediators, including endogenous and exogenous polypeptide growth factors (fibroblast growth factor, hepatocyte growth factor, placental growth factor and HIV-1-Tat) and autacoids (platelet-activating factor and nitric oxide), which regulate functions of endothelial cells related to angiogenesis, and discusses the interlaced work of different Italian laboratories engaged in angiogenesis associated with cancer progression: for more see Internet Web site http://www.unibs.it/~airc/airc1.html.

POLYPEPTIDE GROWTH FACTORS

Angiogenic activity of basic fibroblast growth factor: paracrine and autocrine modes of action

Basic fibroblast growth factor (bFGF) belongs to the family of the heparin-binding growth factors [1]. The single copy human *bFGF* gene encodes multiple bFGF isoforms with molecular weights ranging from 24 to 18 kD. High molecular weight isoforms are collinear NH_2-terminal extensions of the better characterised 18 kD protein [1]. Both low and high molecular weight bFGFs exert angiogenic activity *in vivo* and induce cell proliferation, protease production, and chemotaxis in endothelial cells *in vitro* [2]. Also, bFGF has been shown to stimulate endothelial cells to form capillary-like structures in collagen gels [3] and to invade the amniotic membrane *in vitro* [4]. Moreover, the phenotype induced *in vitro* by bFGF in endothelial cells includes modulation of integrin expression [5], gap junction intercellular communication [6] and urokinase receptor upregulation [7]. Studies with neutralising anti-bFGF antibodies have implicated endogenous bFGF in wound repair [8] and vascularisation of the chorioallantoic membrane during chick embryo development [9].

bFGF is thought to play a role in the growth and/or neovascularisation of solid tumours. Indeed, various tumour cell lines express bFGF *in vitro* [10–13]. *In situ* hybridisation and immunolocalisation experiments have shown the presence of bFGF mRNA and/or protein in neoplastic cells, endothelial cells, and infiltrating cells within tumours of different origin [14–17]. Even though bFGF lacks a leader sequence for secretion [18], data suggest that bFGF is secreted from bFGF-producing cells by an alternative secretion pathway [19, 20] and accumulates in the extracellular matrix (ECM), from where it can be released by ECM-degrading enzymes [21]. Interestingly, the appearance of an angiogenic phenotype correlates with the export of bFGF during the development of fibrosarcoma in a transgenic mouse model [22]. These data suggest that bFGF release may occur *in vivo* and may influence solid tumour growth and neovascularisation. Accordingly, anti-bFGF antibody affects tumour growth under defined experimental conditions [23–25].

Besides experimental evidence for a paracrine mode of action for bFGF, some observations raise the hypothesis that bFGF may also play an autocrine role in endothelial cells. *In vivo*, bFGF expression occurs in the endothelium adjacent to neoplastic cells in several human tumour types [14–17]. Thus, bFGF expression is a common feature of vascular endothelium during tumour angiogenesis. Also, high levels of expression of bFGF are present in endothelial cells during the proliferating phase of human haemangiomas [26] and in spindle cells of endothelial origin in Kaposi's sarcoma [27].

Recent experimental evidence, supporting the possibility that bFGF-dependent tumour angiogenesis may depend on both paracrine and autocrine modes of action on endothelial cells, are summarised in Figure 1.

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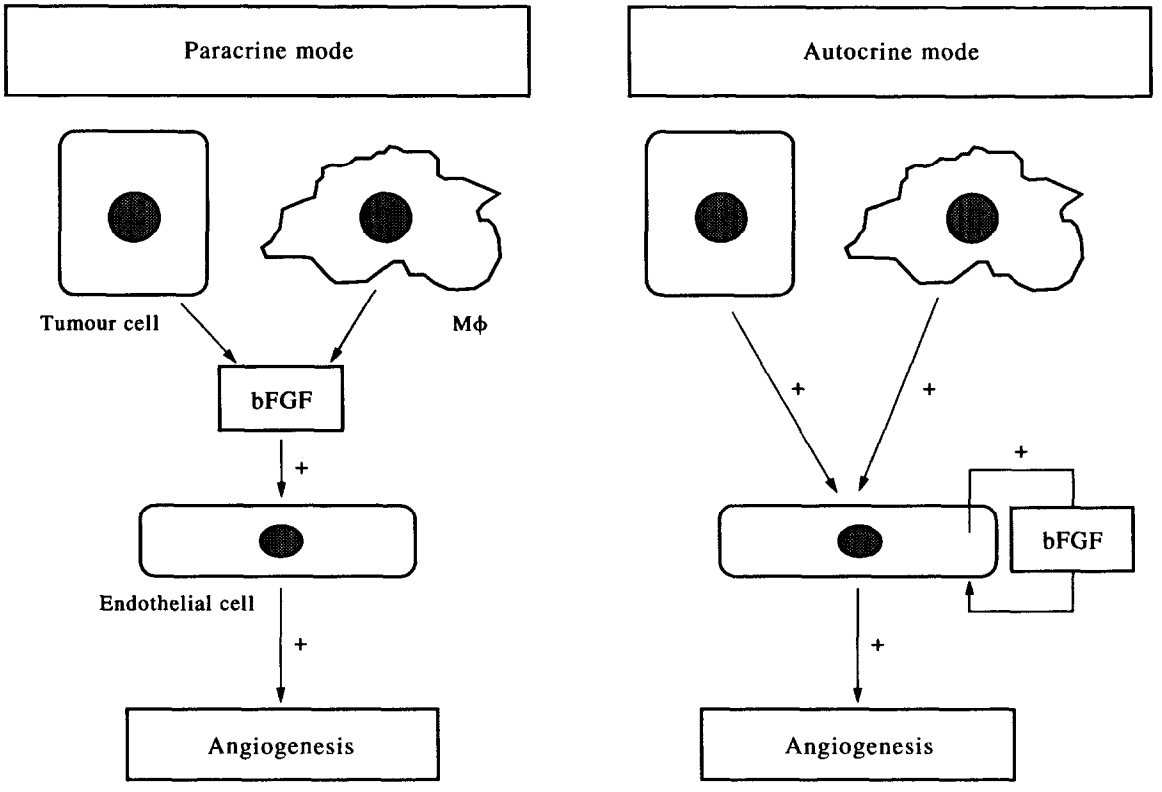

Figure 1. Paracrine and autocrine activity of bFGF on the endothelium. Tumour cells and inflammatory cells, including macrophages (Mϕ), release bFGF which acts on endothelial cells to induce angiogenesis in a paracrine mode of action. Alternatively, endothelial cells are stimulated to upregulate the levels of endogenous bFGF which, in turn, induces angiogenesis via an autocrine loop of stimulation.

Paracrine angiogenic activity of basic fibroblast growth factor

bFGF is present in the extracts of biopsies of normal human cycling endometrium at levels higher than those found in other tissues, including myometrium, brain and placenta [28]. bFGF also has been isolated from uterus [29] and human endometrium [30].

Recently, the presence of a biologically active, immunoreactive bFGF-like protein has been reported in biopsies of well-differentiated adenocarcinomas of the endometrium from cycling and postmenopausal patients [31], and bFGF immunostaining has been observed in the glandular epithelium in complex hyperplasia and carcinoma of endometrium [31]. These findings raise the hypothesis that bFGF may be involved in the growth and/or neovascularisation of endometrial cancer. Data to support this hypothesis have been obtained by transfection of the human endometrial adenocarcinoma HEC-1-B cell line [32] with an expression vector harbouring the human bFGF cDNA under the control of the human β-actin gene promoter [33]. When bFGF transfectants were screened for the capacity to release the growth factor, significant amounts of bFGF were present in conditioned medium and ECM of the bFGF-B9, but not the bFGF-A8 clone, even though both cell lines produced similar levels of intracellular bFGF. Compared with parental cells, bFGF-B9 cells showed downregulation of tyrosine kinase FGF receptors together with upregulation of urokinase-type plasminogen activator expression, which was abolished by incubation of the cell cultures with neutralising anti-bFGF antibody. *In vivo*, bFGF-B9 cells formed highly vascularised tumours growing faster than parental cells when injected subcutaneously into nude mice. Also, they were more potent than non-transfected cells in inducing an angiogenic response in the rabbit cornea assay. In contrast, bFGF-A8 cell phenotype was indistinguishable from parental cells both *in vitro* and *in vivo* [33]. NIH 3T3 cell transfectants expressing very high levels of bFGF fused with a signal peptide sequence under the control of a viral promoter produce vascularised tumours in nude mice [34]. Similar results are observed for human breast carcinoma MCF-7 cells transfected with FGF-4 [35], a member of the FGF family endowed with a signal peptide sequence [1]. The data described above indicate that wild type bFGF, devoid of a signal peptide sequence, enhances the angiogenic and tumorigenic potential of cell transfectants when expressed at levels close to those observed under natural conditions. The effects induced by the growth factor are observed only in those cells that export significant amounts of bFGF, while bFGF-producing cells that do not release increased amounts of the growth factor are indistinguishable from the parental cell line. Therefore, clonal heterogeneity in bFGF production and secretion may have important consequences for tumour development. An autocrine loop of stimulation is activated by bFGF only in those tumour cells which are able to secrete the growth factor in significant amounts. Also, as predicted for a paracrine mechanism of tumour neovascularisation, the potentiation of the angio-

genic capacity of transfected cells is restricted to bFGF-releasing cells. Both the increased fibrinolytic activity and angiogenic potential of bFGF-releasing cells, together with other possible autocrine and paracrine effects consequent to bFGF overexpression and export, may contribute to determining the capacity of these cells to induce the early appearance of fast-growing neovascularised tumours when injected into nude mice. Similar conclusions have been drawn for MCF-7 human breast carcinoma cells transfected with the angiogenic vascular endothelial growth factor [36].

Extrapolation of the autocrine and paracrine properties of a neoplastic tissue from the data obtained *in situ* on bFGF production and localisation in tumour biopsies must be carefully considered. However, even though the evidence for increased production of bFGF from immunohistochemical and/or *in situ* hybridisation studies does not provide information on the capacity of tumour cells to secrete the growth factor, some experimental data indicate that bFGF export does occur *in vivo*. For instance, bFGF is detectable in the urine of patients with a wide spectrum of cancers [37] and in cerebrospinal fluid of children with brain tumours [38]. These data suggest that bFGF overexpression and release from neoplastic cells may occur in human cancer and contribute to its neovascularisation and progression.

Autocrine angiogenic activity of bFGF

As mentioned above, several observations support the hypothesis that bFGF may also play an autocrine role in endothelial cells. Neovascularisation may be triggered by molecule(s) released by tumour cells and/or infiltrating inflammatory cells that induce endogenous bFGF upregulation in the quiescent endothelium. In keeping with this hypothesis is the observation that tumour cells of different origin release molecule(s) able to interact with the endothelium and to upregulate the expression of bFGF which, in turn, stimulates the fibrinolytic potential of the endothelial cell in an autocrine manner [38]. In addition, bFGF itself, thrombin, and interleukin-2 induce bFGF production in endothelial cells [40, 41]. Finally, we have observed that nitric oxide-donors stimulate bFGF upregulation in coronary venular endothelial cells (M. Ziche, University of Florence, Italy).

The biological consequences of endothelial cell activation by endogenous bFGF have been investigated in mouse aortic and brain capillary endothelial cell lines transfected with a retroviral expression vector harbouring a human bFGF cDNA [42, 43]. Transfected clones were characterised by a transformed morphology and an increased saturation density. bFGF transfectants showed invasive behaviour and sprouting activity in three-dimensional fibrin gels, and formed a complex network of branching cord-like structures connecting foci of infiltrating cells when seeded on laminin-rich basement membrane matrix (Matrigel). The invasive and morphogenetic behaviour was prevented by anti-bFGF antibody, revealing the autocrine modality of the process [42, 43]. Finally, bFGF-transfected mouse aortic endothelial cells gave rise to highly vascularised lesions resembling Kaposi's sarcoma when injected into nude mice, and induced angiogenesis in avascular rabbit cornea. Injected into the allantoic sac of the chick embryo, they caused an increase in vascular density and formation of haemangiomas in the chorioallantoic membrane [42].

Thus, newly synthesised endogenous bFGF triggers an angiogenetic programme in endothelial cells and causes the recruitment of more quiescent endothelial cells, thus amplifying the angiogenic stimulus. These data support the notion that bFGF overexpression has an autocrine role for endothelial cells and that tumour neovascularisation and angioproliferative diseases can be triggered by stimuli that induce vascular endothelium to produce its own autocrine factor(s).

Placental growth factor: structure and function

Potential tumour angiogenic factors include a small family of direct angiogenic growth factors, structurally related to platelet-derived growth factor, called vasculotrophins. Currently, four angiogenic growth factors have been isolated: vascular endothelial growth factor (VEGF)-A, -B, -C [44], and placental growth factor (PlGF) [45]. These factors are dimeric glycoproteins that display a high amino acid similarity in the platelet-derived growth factor-like domain, with complete conservation of the characteristically spaced eight cysteine residues.

The *PlGF* gene maps on chromosome 14 [46]. The PlGF coding sequence is encoded by seven exons spanning approximately an 8 kb-long DNA interval [46]. Two forms of PlGF are generated by skipping the 63 bp-long exon 6, which codes for a highly basic 21-amino acid stretch at the carboxyl end of PlGF-2 [46]. After removal of an 18-amino acid-long signal peptide, the PlGF-1 and PlGF-2 precursors are secreted as glycosylated dimeric proteins 131- and 152-amino acid long, $PlGF_{131}$ and $PlGF_{152}$, respectively [46–48]. $PlGF_{131}$ is readily secreted into conditioned medium, whereas $PlGF_{152}$ is most likely sequestered on the cell surface or in the ECM since $PlGF_{152}$ but not $PlGF_{131}$ has been shown to bind to heparin [45–49]. However, it remains unclear whether such alternative splicing may have important consequences for physiological and pathological processes.

The biological activity of PlGF is still unclear, since several contradictory reports have been published thus far. Similar biological activity for PlGF and VEGF has been suggested [45, 46] based on the similarity between these two molecules. VEGF has been shown to be a potent mitogenic and angiogenic factor for endothelial cells *in vitro*, to elicit angiogenesis *in vitro* and to enhance the permeability of the vascular endothelium [44]. Accordingly, in an initial study, we showed that the conditioned medium from $PlGF_{131}$-overexpressing COS cells was able to stimulate the proliferation of bovine pulmonary endothelial cells [45] *in vitro*. However, a more recent study showed that a concentration greater than 100 ng/ml of purified $PlGF_{131}$ and $PlGF_{152}$ failed to stimulate proliferation of bovine adrenal cortex-derived capillary endothelial cells and only stimulated the growth of human endothelial cells from umbilical cord veins by 10–30% [50]. Conversely, Sawano and coworkers reported that purified $PlGF_{152}$, but not $PlGF_{131}$, was able to stimulate the growth of this cell type, although with a 10-fold lower potency compared with VEGF [49]. *In vivo* experiments demonstrated that $PlGF_{152}$, but not $PlGF_{131}$, showed permeabilising activity in the Miles assay [49], with a potency 10-fold lower than that of VEGF, although previous studies failed to show any permeability activity exerted by up to 500 ng of $PlGF_{152}$ in the Miles vascular per-

meability test [50]. A possible explanation for such discrepant results may be that PlGF may be labile and that the different preparations of PlGF that have been used in such studies may retain variable levels of activity. Overall, it appears that $VEGF_{165}$ is more active as a mitogenic and permeabilising factor compared with PlGF, and that $PlGF_{152}$ is more active than $PlGF_{131}$.

PlGF and VEGF have been shown to interact in several ways. PlGF has been shown to potentiate either the mitogenic activity of suboptimal doses of VEGF on endothelial cells and the permeabilising effects of VEGF in the Miles assay [50]. Moreover, heterodimers between VEGF and PlGF have been isolated from conditioned medium collected from several rat and human tumour cell lines [51, 52]. Interestingly, in all cell lines where VEGF and PlGF are co-expressed, there is a preference for VEGF to exist as a VEGF/PlGF heterodimer with the excess amount of PlGF protein present as PlGF/PlGF homodimer [52]. Mitogenic activity of $PlGF_{131}$ homodimers and $VEGF_{165}/PlGF_{131}$ heterodimers has also been assayed by a modified Boyden chamber test. Heterodimeric $VEGF_{165}/PlGF_{131}$ molecules showed chemotactic activities for human endothelial cells from umbilical cord veins at doses as low as 2.5 ng/ml, whereas $PlGF_{131}/PlGF_{131}$ homodimers failed to induce such a response under the same experimental conditions [52].

Heterodimeric $VEGF_{165}/PlGF_{131}$ molecules appear to have intermediate mitogenic activity compared with $VEGF_{165}/VEGF_{165}$ and $PlGF_{131}/PlGF_{131}$ homodimers, but the same potency as the chemotactic factor [52]. Therefore, it is likely that ligand and/or receptor heterodimerisation might provide a complex means of fine regulation of endothelial functions, suggesting that PlGF might downregulate the proliferative activity exerted by VEGF and instead upregulate other endotheliotrophic functions (i.e. chemotaxis) by formation of heterodimers with VEGF.

VEGF homodimers have been shown to bind to and induce autophosphorylation of both FLT-1 (K_d = 16 pM) and Flk-1/KDR (K_d = 410 pM) high affinity receptors [44], whereas PlGF binds and autophosphorylates only FLT-1 (K_d = 160 pM) [44, 53]. Heterodimeric VEGF/PlGF has been shown to bind to the Flk-1/KDR receptor [52]. Recent studies suggest that Flk-1/KDR receptor mediates proliferative and chemotactic signaliing in endothelial cells, whereas Flt-1 is only involved in the chemotactic, but not in the proliferative response [44]. The differential response elicited in endothelial cells by the activation of the Flt-1 or Flk-1/KDR receptors, and the different binding specificity of the VEGF, PlGF homodimers and PlGF/VEGF heterodimers may partly account for the different endotheliotrophic activities shown by VEGF and/or PlGF.

Role of placental growth factor in neoangiogenesis in human diseases

The role of VEGF in tumour-associated angiogenesis has been clearly demonstrated in many solid tumours [44, 54], suggesting that VEGF upregulation may be a general feature of highly vascularised neoplasms independent of their histological derivation. Conversely, few studies have been carried out on the role of PlGF in tumour angiogenesis. Previous studies have shown that, PlGF expression is detectable in some mammary and colon carcinoma cell lines, and in human renal clear cell carcinomas [46, 55, 56], suggesting that PlGF might play a role in tumour neoangiogenesis. However, consistent with the reported lower bioactivity shown by PlGF in comparison with VEGF, PlGF expression does not appear to be necessary for the hypervascularisation of glioblastomas [57], germ cell-derived (GCTs) [54] and thyroid tumours [58] (Table 1). Moreover, we have shown that whereas VEGF expression in germ cell tumours consistently correlates with the extent of tumour vascular density in all histological types of human GCTs, the correlation between the amount of PlGF mRNA and the degree of tumour vascularisation is weak ($r = 0.265$, $P < 0.05$; $n = 24$) [54]. Interestingly, our recent studies suggest that two different angiogenic pathways characterise thyroid malignant tumours and hyperplastic goiters. We have demonstrated that, in the majority of human thyroid tumours, VEGF but not PlGF expression is upregulated in association with malignant progression [58]. Conversely, by analysis of human Graves' patients and of an animal model of TSH (thyroid-stimulating hormone)-dependent thyroid goitrogenesis (propylthiouracil-fed rats), we have shown that hypervascularity in goiters may result from co-ordinated TSH-induced expression of growth factors PlGF and VEGF in thyrocytes, and enhanced expression in thyroid capillaries of their receptors Flt-1 and Flk-1/KDR. Interestingly, PlGF and VEGF expression occurs in parallel with the development of abnormally branched, hyperpermeable vasculature, suggesting a causal role in goitre hypervascularisation [59]. However, during goitre development, vascular remodelling does not involve sprouting, as postulated for VEGF-dependent tumour-associated neoangiogenesis [44], but occurs by initial proliferation of endothelial cells followed by enlargement and fusion of pre-existing vessels. This essentially results in enhancement of vascular permeability more than cell growth [44], and suggests that factors other than VEGF homodimers may be involved in goitre hypervascularisation. Since co-expression of VEGF and PlGF preferentially lead to VEGF/PlGF heterodimer formation [52], it is likely that TSH stimulation induces the production of high levels of VEGF/PlGF heterodimers. Thus, it is reasonable to speculate that the co-ordinated TSH-dependent expression of PlGF and VEGF, during goitre hypervascularisation, may determine a fine-tuned modulation of VEGF activity driven by PlGF, which would result in enhancement of chemotaxis and permeabilisation, and in suppression of endothelial cell growth, thus indicating a pivotal role for PlGF in goitre vascularisation.

Such a hypothesis has also been put forward for the role of PlGF in the placenta [49]. Unlike VEGF, which is expressed in most normal tissues, we have shown that high

Table 1. PlGF and VEGF expression in human tumours

	PlGF	VEGF
Breast carcinomas	0/39	20/39
Neuroblastomas	0/5	5/5
Colon carcinomas	1/7	5/5
Non-seminomas	8/26	23/26
Seminomas	5/18	13/18
Oesophageal	0/8	7/8
Lung carcinomas	0/8	6/8
Thyroid carcinomas	2/25	12/25
Ovarian adenocarcinomas	10/39	30/39

levels of PlGF expression are present only in a limited number of normal adult human and mouse tissues such as term placenta and thyroid (although in lung, heart and kidney low levels of PlGF mRNA are detected) [46, 60]. This observation has led to the suggestion that PlGF may play a key role in organs where a highly permeable vascular system is required as in the case of kidney, thyroid and placenta. In placenta, PlGF expression has been postulated to play a crucial role in permeability of the blood vessels rather than in cell growth [49], since both VEGF and PlGF are simultaneously expressed at a high level in the early and middle stages of placenta development, when both extracellular growth and high levels of vascular permeability between the fetal and the maternal systems are considered important. Conversely, at later stages of placental development, when the maintenance of vascular permeability appears to be more important than endothelial cell proliferation, PlGF mRNA expression remains constant whereas VEGF expression declines [49].

However, a definite insight into the role of PlGF on tumour angiogenesis will be gained only by determining the effects on the tumour vascular system and/or on the *in vivo* growth of tumour xenografts caused by the inhibition of PlGF activity with monoclonal antibodies. To this aim, a monoclonal anti-PlGF antibody has recently been developed (unpublished data).

Regulation of placental growth factor expression

We have shown that TSH induces an increase in the steady-state level of PlGF mRNA in rat cultured thyrocytes (G. Viglietto, Instituto dei Tumori "Fondazione G. Pascale", Naples, Italy). In the same cells, as with VEGF [61], the activation of the protein kinase-A-dependent pathway with forskolin or of the protein kinase C pathway with phorbol esters, also induced a dose- and time-dependent increase in the PlGF mRNA expression, suggesting that in thyroid these growth factors may be regulated in part by the same pathways. However, in contrast to VEGF [62], in a recent study we have shown that PlGF expression is not upregulated by hypoxia either in normal thyroid cells or in tumour-derived cell lines [54]. Following exposure to hypoxic conditions, tumour cells upregulate VEGF, perhaps as an ultimate attempt to deal with the lack of vascularisation of the tissue. The finding that PlGF is not modulated by hypoxia could indicate that, unlike VEGF, which may be regarded as an 'emergency' angiogenic factor, PlGF may not be a component of the mechanism governing the revascularisation of poorly oxygenated tissues. These observations suggest that VEGF and PlGF may play different roles in physiological and/or pathological vascularisation, and thus they are not simply redundant molecules.

Hepatocyte growth factor

Hepatocyte growth factor (HGF), a potent mitogen for cultured rat hepatocytes, is a pleiotropic growth factor originally isolated from serum and platelets. The sequence analysis and the cDNA cloning studies have shown that HGF is the same molecule as Scatter Factor originally described as a secretory product of fibroblasts able to increase, *in vitro*, the motility and the invasiveness of normal and malignant epithelial cells [63]. Mature HGF is an 82 kDa, 647 amino acid residue heterodimeric glycoprotein [63] which acts on target cells through binding to its high affinity receptor encoded by the *C-MET* proto-oncogene [63]. The HGF receptor is a widely expressed 190-kDa heterodimer composed of a 50-kDa α chain, covalently linked to a 145-kDa β chain. The α chain is extracellular, whereas the β chain has an extracellular domain involved in HGF binding, a transmembrane domain and a cytoplasmic tyrosine kinase domain [63]. Ligand binding induces kinase activation and autophosphorylation of tyrosine residues located in the β chain.

Recent data suggest that the spectrum of HGF activities is not restricted to epithelial cells [63], and recently it has been reported that HGF is a powerful activator of growth and motility of mouse and human endothelial cells [64–68]. Endothelial cells express a high affinity binding site (K_d = 0.35 nM) corresponding to the HGF receptor which is phosphorylated on tyrosine residues of the β chain upon HGF binding. A second, low affinity binding site (K_d = 2 nM) relates to its binding to ECM [64]. HGF-induced receptor phosphorylation triggers migration and proliferation of endothelial cells [64, 65] as shown by the agonist effect elicited by a monoclonal antibody against the extracellular domain of the receptor. Furthermore, inhibitors of tyrosine kinases, effective on HGF receptors, inhibited HGF-induced activation of endothelial cells. Furthermore, HGF induces the *in vitro* organisation of endothelial cells into capillary-like structure [64]. The *in vitro* effects of HGF have, as a counterpart, an angiogenic *in vivo* effect [64, 65, 69]. By using deletion mutants or site-directed mutagenesis, we have demonstrated that *in vivo* angiogenesis and *in vitro* activation of endothelial cells are elicited only by a fully processed heterodimeric HGF molecule containing both α and β chains, in contrast to that demonstrated in epithelial cells, that migrate after stimulation with HGF mutants containing only the first two or three kringle motifs of α chain [70, 71].

In contrast to VEGF and PlGF, which induce angiogenesis almost exclusively by direct activation of endothelial cells, HGF represents an example of an angiogenic molecule also able to trigger accessory cells (i.e. monocytes/macrophages) to release angiogenic molecules. In the Matrigel model [65], HGF recruits macrophages and induces the expression of other angiogenic polypeptides (VEGF, PlGF) [65] or the synthesis of platelet activating factor (PAF), an angiogenic autacoid (G. Camussi, University of Pavia at Varese, Italy). In this model, a neutralising anti-VEGF antibody and PAF receptor antagonists reduce significantly the angiogenic response to HGF [65]. From *in vitro* studies, we have demonstrated that murine endothelial cells are unable to produce PAF after HGF stimulation. In contrast, murine macrophages synthesised PAF after stimulation with HGF, thus suggesting a macrophage origin of PAF detected within Matrigel containing HGF. Furthermore, macrophages were induced to migrate after HGF challenge. Mouse leucocyte depletion prevented the infiltration of macrophages, the synthesis of PAF and the angiogenesis induced by HGF. These results suggest that the angiogenic effect of HGF is *in vivo*, at least in part, mediated by PAF synthesised from macrophages infiltrating the Matrigel plug and by VEGF. Recent results suggest that HGF can be produced by endothelial cells themselves, and indeed seems to be implicated in an

Biological activities:

Transcription:

(a) HIV genome
(b) Other viral genes (EBV, JCV)
(c) Cellular genes (TNF-β, TGF-β1, IL-2, IL-4R, IL-6, IL-8...)

Cytokine-like:

(a) Growth stimulation
(b) Angiogenesis
(c) Cell survival
(d) Tumorigenicity
(e) Involvement in apoptosis

Localisation:

(a) Nuclear
(b) Extracellular?
(c) Cell surface?

Figure 2. Schematic structure and biological activities of HIV-1 Tat protein. N-term, NH_2 terminal region; Cys-rich, cysteine rich domain; Core, core domain; Basic, basic domain; C-term, COOH terminal region containing RGD sequence.

autocrine pathway [72–74], as demonstrated for bFGF (see above).

Currently, a direct involvement of HGF in tumour angiogenesis has not been reported, except for Kaposi's sarcoma [75, 76]. However, the relevance of HGF-HGF receptor in malignant transformation of epithelial cells and in organogenesis [63] matched to the angiogenic properties, point to a critical role of HGF in cancer progression.

HIV-1 Tat protein: an example of an exogenous angiogenic molecule

Tat is 'officially' the HIV-1 transactivating protein, responsible for viral transcription. However, this remarkable protein has a number of both intracellular and extracellular activities (Figure 2). Recent studies have demonstrated that HIV-Tat represents a novel exogenous angiogenic mediator, relevant for pathogenesis of vascular disorders associated with AIDS, including Kaposi's sarcoma [27].

Our interest in the possible angiogenic potential of Tat started when early studies indicated that mice transgenic for HIV-Tat could develop angiogenic, Kaposi's sarcoma-like lesions [77]. This phenomenon was later confirmed by Corallini and associates [78]. In 1990 the laboratory of Robert Gallo demonstrated that recombinant Tat was, in fact, able to enhance the growth of Kaposi's sarcoma-derived spindle cells [79]. In collaboration with Gallo's group, we discovered that Tat was able to induce chemotactic and invasive behaviour of Kaposi's sarcoma cells and of endothelial cells treated with inflammatory cytokines [80]. Tat also proved to act as a morphogen on endothelial cells plated on Matrigel, increasing their capability of organising into a capillary-like network.

Tat stimulates endothelial cell-induced basement membrane digestion, measured by degradation of radiolabelled collagen type IV. The enzyme most probably involved is urokinase-type plasminogen activator; Tat expression is strongly correlated with high levels of secreted urokinase-type plasminogen activator in cells obtained from Tat transgenic mice [81]. In contrast, we could not detect induction of the gelatinolytic activity characteristic of type IV collagenases by Tat [82].

All these properties described for Tat are characteristic of angiogenic growth factors. However, in contrast to bFGF,

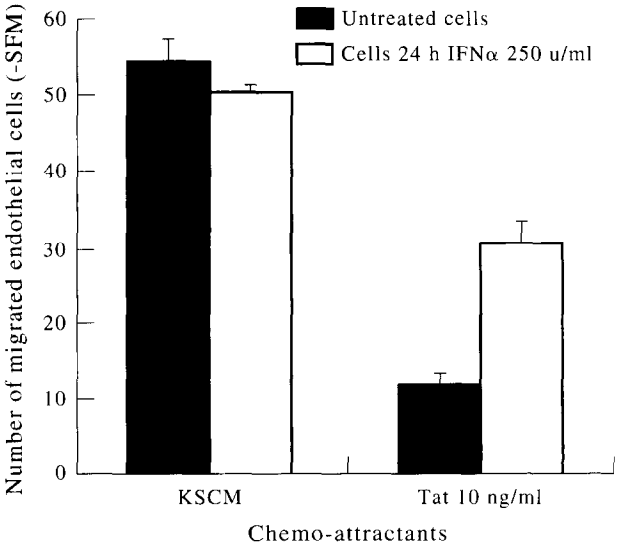

Figure 3. Effect of interferon α on the migration of endothelial cells stimulated by conditional medium of Kaposi's sarcoma cells (KSCM) or recombinant Tat. Human endothelial cells were treated for 24 h with the cytokine and then the migratory response was studied by Boyden's chamber technique [88].

MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALG
ISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKE

Figure 4. HIV-1 Tat amino acid sequence.

the action of Tat on growth and migration of endothelial cells is detectable *in vitro* only after treating the endothelium with a cocktail of inflammatory cytokines [83]. Interferon gamma, interleukin-1 and tumour necrosis factor-α have been demonstrated to activate endothelial cells to 'spindle cell'-like phenotype [83]. Our laboratory has shown that interferon alpha can alone induce, at low doses, endothelial cells to migrate to Tat in the Boyden chamber assay (Figure 3). It is, therefore, evident that activation of the endothelium can enhance the response to Tat, although the molecular mechanisms of this enhancement have not yet been investigated.

Looking at the protein sequence of Tat, attention was immediately drawn by an RGD in the carboxyl terminal of the molecule, encoded by the second exon of the gene (Figure 4) [84]. RGD sequences are characteristic of several ECM proteins and are involved in cell adhesion and interaction with integrin receptors [85].

Inflammatory cytokines are able to induce expression of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins, putative Tat receptors, on endothelial and KS cells. *In vitro* experiments have shown that these cell types are effectively able to adhere on Tat-coated multiwell plates, thus Tat–endothelial cell interactions could be dependent on an integrin-mediated mechanism [86].

Another possibility is evident from *in vivo* studies of our laboratory, where we noticed that heparin was able to increase Tat-induced angiogenesis in mice [87]. We speculated that Tat could mimic a heparin-binding angiogenic growth factor [88]. Basic domains are commonly found in angiogenic growth factors (VEGF, spliced PlGF, HGF, bFGF, angiogenic). Sequence analysis indicates analogous sequences in the basic Arg, Lys-rich domain of Tat (Figure 4). Heparin/heparan sulphates are necessary for the proper activation of tyrosine-kinase receptors by heparin-binding growth factors. Growth responses to these factors are highly potentiated by the addition of heparin *in vitro* [89]. We found Tat to be angiogenic in the rabbit cornea in combination with heparin. Using a heparin-sepharose affinity column, we demonstrated that Tat has the ability to bind heparin, the elution profile confirming a strong binding similar to that of bFGF. In contrast, cytochrome-C, a protein with a similar pI and size, showed weak binding to heparin. Addition of heparin allowed Tat to induce the growth and migration of endothelial cells without prestimulation with inflammatory cytokines. Using a synthetic peptide containing the basic domain of Tat (amino acids 46–60), we found biological activity both *in vivo* and *in vitro* on endothelial cells. The RGD sequence present on the second exon of Tat (amino acids 65–80) was active *in vitro*, but was not able to induce an angiogenic response *in vivo* [86].

The angiogenic growth factors having heparin-binding properties can be stored in the ECM, anchored to the heparan-sulphates of basement membranes. This possibility would allow Tat secreted by infected cells to accumulate and be released during inflammation, following the action of heparanases. As found with other angiogenic growth factors, heparin/heparan sulphates appear to be involved in the angiogenic properties of Tat protein, and Tat is able, via its basic domain, to bind heparin and to act at the extracellular level as a heparin-binding growth factor.

AUTACOIDS

Platelet activating factor (PAF)

Platelet activating factor (PAF) is a phospholipid mediator with a wide spectrum of biological activities relevant for the development of inflammation, embryogenesis and cell differentiation [90]. Many cell types that synthesise PAF, including endothelial cells, express PAF-binding sites on their surface [91] and are targets for PAF action [92–95]. PAF directly enhances the permeability of endothelial cell monolayers and induces changes of the cell cytoskeleton, characteristic of cells undergoing translational movements [92–95]. These observations prompted us to evaluate whether PAF may stimulate the migration of endothelial cells *in vitro*.

Indeed, PAF, added to the lower well of the Boyden's chamber, induced a dose-dependent migration of endothelial cells across the 5 μm pore size gelatin-coated polycarbonate filters [93]. PAF-induced migration of endothelial cells was a receptor-dependent phenomenon, as pretreatment with PAF receptor antagonists inhibited the migration and the D-stereoisomer of PAF was ineffective [93]. PAF receptor, the cDNA of which has been recently cloned, belongs to the family of 'serpentine' receptors containing seven α-helical domains that weave in and out of the plasma membrane [96], and are expressed in endothelial cells [93]. The *in vitro* observations prompted us to study the angiogenic effect of PAF *in vivo*. In fact, the directional migration of endothelial cells within the ECM is a prerequisite for neoangiogenesis.

By using the Matrigel model, we observed in mice that PAF induced *in vivo* the formation of canalised vessels [93], evident after only 16–24 h. The minimal concentration of PAF able to induce angiogenesis was 10 nM, an amount compatible with levels of bioactive PAF produced *in vivo* within inflamed tissues. PAF was found to induce an *in vivo* angiogenic response either in a heparin-independent or a heparin-dependent manner, depending on the dose used [93]. At pharmacological concentrations (1–5 μM), PAF induced a heparin-independent angiogenesis. In contrast, at physiological concentrations of PAF (10–50 nM), heparin was required suggesting that the angiogenic effect of PAF may depend on the secondary involvement of heparin-binding endothelial cell growth factors with angiogenic properties. Indeed, several studies have suggested that PAF may promote transcription of genes coding for cytokines and growth factors. PAF has been implicated as a mediator of angiogenesis, induced by tumour necrosis factor, since it is actively synthesised *in vivo* during the angiogenic process and PAF-receptor antagonists significantly reduce the angiogenic response to this cytokine which sustains *in vivo* angiogenesis [97]. Kaposi's sarcoma cells possess specific high

affinity binding sites for PAF and express mRNA for PAF receptor. At nanomolar concentrations, PAF stimulates chemotaxis and chemokinesis of these cells [98]. The *in vivo* neoangiogenesis induced by the conditioned medium of Kaposi's sarcoma cells is inhibited by the PAF receptor antagonist WEB 2170 suggesting that PAF may play a relevant role in the formation of new vessels. However, the angiogenic effect of PAF is amplified by other factors, such as basic and acid FGF, PlGF, VEGF and HGF [98]. PAF receptor antagonist WEB 2170 abolishes the expression of the transcripts of these factors within Matrigel containing the conditioned medium of Kaposi's sarcoma cells, suggesting a role of PAF in the induction of angiogenic factor transcription [98].

In conclusion, PAF may directly provide the signal for migration of endothelial cells, which is a prerequisite for the formation of new vessels. However, PAF itself is unable to stimulate directly the proliferation of endothelial cells [93, 98]. Therefore, angiogenesis induced *in vivo* by PAF needs the co-operation of other angiogenic factors which are able to stimulate the proliferation of the endothelium.

Nitric oxide

Three experimental observations have attracted our attention to study the role of nitric oxide (NO), the most potent mediator of vasodilation, at the cardiovascular level, in angiogenesis [99]. Firstly, in experimental models where angiogenesis can be directly monitored, vasodilation and hyperaemia of the pre-existing capillaries and the persistence of a dilation state of the newly formed vessels are typical findings. Similarly, VEGF is also a vasodilating agent [44]. Secondly, several angiogenesis factors/mediators promote relaxation in vascular preparations, and peptides known to induce endothelium-mediated vasorelaxation are angiogenic. Thirdly, persistent vasodilation is a specific feature in tumour vasculature and in the surrounding tissue. Based on these considerations, we hypothesised that an endothelial derived relaxing factor could actively participate in angiogenesis.

Endothelium-dependent relaxation has been clearly demonstrated to be caused by an endothelium-derived relaxing factor identified as NO [99]. NO is an inorganic free radical gas synthesised from L-arginine by a family of coenzymes called NO-synthases. Two of these are constitutively expressed and a third is inducible by immunological stimuli. The NO released by the constitutive enzyme acts as an important signalling molecule, whilst NO released by the inducible NO synthase is generated for long periods and has been shown to be cytostatic/cytotoxic for tumour cells. The release by endothelial cells of NO can be blocked by L-arginine analogues, such as N^G-mono-methyl-L-arginine (L-NMMA) and L^G-nitro-L-arginine methyl ester (L-NAME), while D-NMMA and D-NAME are ineffective.

We thus investigated whether NO could act as an angiogenesis effector and as a modulator of the activity and/or the production of angiogenesis factors. The role of NO was assessed by testing the activity of NO donor drugs and vasodilating peptides that increase intracellular NO on the proliferation and migration of cultured capillary endothelium. By using the vasodilator sodium nitroprusside, which provides an exogenous source of NO, and the neuropeptide substance P, which induces NO production in endothelial cells, we could show that NO generating compounds promote endothelial cell proliferation and migration [100, 101]. NO signal transduction in target cells involves activation of the soluble guanylate cyclase and elevation of intracellular cGMP cyclic guanosine monophosphate. The growth promoting effect of NO on cultured endothelium appeared to be linked to cGMP generation, suggesting the existence of an autocrine/paracrine loop in the NO effect.

The relevance of NO in angiogenesis *in vivo* was assessed in rabbit corneas receiving angiogenesis effector during systemic treatment with NO-synthase inhibitors. NO-synthase inhibitors suppress angiogenesis induced by vasodilating effectors, such as substance P and prostaglandin E1

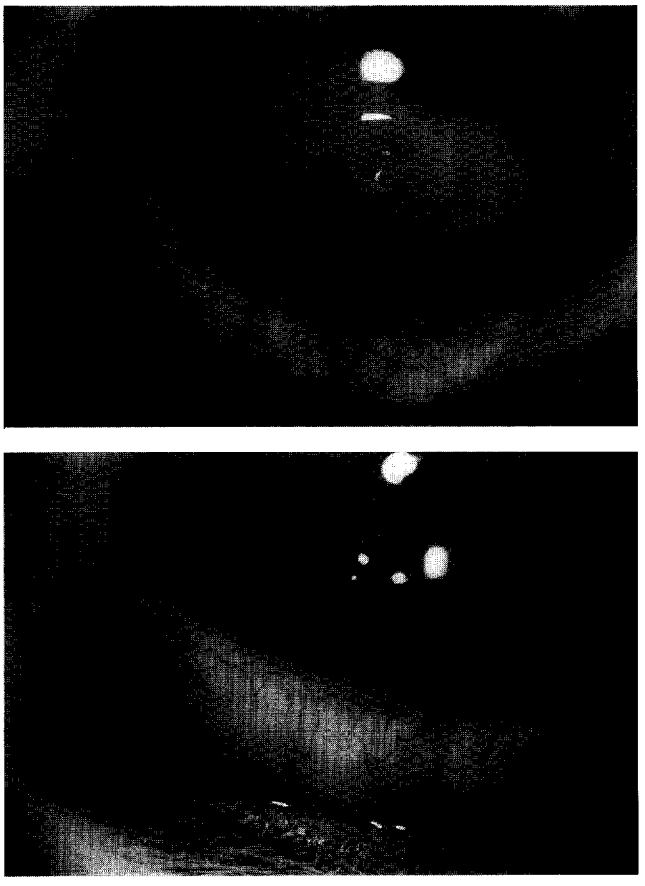

Figure 5. Effect of nitric oxide inhibition in corneal angiogenesis. Corneal assays were performed in New Zealand white rabbits as described in [101, 109]. The slow-release pellets incorporating prostaglandin E1 were implanted into the micropocket. Subsequent daily observation of the implants was made with a slit lamp stereomicroscope without anaesthesia. An angiogenic response was scored positive when budding of vessels from the limbal plexus occurred after 3–4 days and capillaries progressed to reach the implanted pellet within the next 10 days. The number of positive implants over the total implants performed was scored during each observation.The effect of systemic NO-synthase inhibition produced by L-NAME treatment was evaluated on angiogenesis produced by prostaglandin E1. L-NAME was given in the drinking water *ad libitum* (0.5 g/l) 1 week before surgery and 10 days after corneal implant. Note the dense vascular network induced by prostaglandin E1 0.25 μg/pellet (P) in control untreated animals (a); in animals receiving L-NAME treatment, prostaglandin E1 failed to produce angiogenesis (b). The photographs (×18) were taken at day 10 after surgical implant through a slit-lamp stereomicroscope.

(Figure 5) [101]. Conversely, exogenous bFGF can elicit angiogenesis *in vivo* despite the block of NO production by capillary endothelium. These observations indicate that bFGF production or bFGF receptor expression by endothelial cells might be secondarily activated during NO-dependent vasodilation.

Other reports have implicated a role for NO-synthase in angiogenesis. For example, it has been shown that angiogenic activity is only released from bacterial endotoxin treated human monocytes in the presence of L-arginine [102]. Although the source of angiogenic activity was not identified in this study, it was nonetheless blocked by the NO-synthase inhibitors. Similarly, L-arginine was shown to favour healing and angiogenesis in gastric ulcerations, while NO-synthase inhibitors delayed it [103]. The *in vivo* progression of murine haemangiomas, induced by transplantation of endothelial cells immortalised by middle T antigen of polyomavirus, is reduced by canavanine, an NO-synthase inhibitor [104]. Other observations have indicated a cytotoxic/cytostatic effect of NO on the vascular development of the chorioallantoic membrane suggesting a diversity of effects in embryonic versus adult tissue [105].

Most of the cellular components of the tumour mass (the tumour cells themselves and the immune cells infiltrate) have been shown to generate NO *in vitro*. However, the role of NO in tumour biology is still poorly understood. Malignant tumours exert a powerful influence on neighbouring blood vessel. Haemodynamic studies have shown that vasculature associated with tumours is insensitive to vasoactive agents and appears to be in an almost maximal state of vasodilation [106]. When compared to normal arteries *in vitro*, tumour associated vessels were unresponsive to the vasoconstrictor agents, such as phenylelphrine, an effect shown to be linked to increase NO-synthase activity [106]. Transfection of the inducible NO-synthase into a colon adenocarcinoma line gave a cell line that, despite growing more slowly *in vitro*, promoted tumours which grew more rapidly and were more vascularised than wild type cells [107]. Other observations in agreement with NO being a specific signal for tumour vascularisation include the fact that blocking NO-synthase activity retards the growth of xenografted tumours [108]. Thus, several data exist in support of NO as a signalling molecule in tumour angiogenesis. Based on these considerations, we hypothesise that NO released by capillaries in the proximity of a tumour under the control of a local growth factor can be instrumental in tumour angiogenesis.

To assess the role of NO in tumour angiogenesis, we investigated whether the L-arginine/NO pathway participated in VEGF activity. Moreover, VEGF expression has been detected in several human tumours and correlates with tumour grade, while the transfection of VEGF into human breast carcinoma cells enhances tumour growth and vascular density [109]. We investigated the role of NO in mediating the mitogenic effect of VEGF on cultured microvascular endothelium isolated from coronary post-capillary venules. The role of NO was determined by monitoring proliferation and cGMP levels in the presence and absence of NO-synthase blockers. We found that VEGF, but not bFGF, elevates endothelial cell cGMP [110]. The elevation of cGMP was blocked by the NO-synthase inhibitors, implicating NO in the observed elevation of the cyclic nucleotide.

Table 2. Role of nitric oxide on cGMP accumulation and proliferation of post-capillary endothelial cells induced by VEGF and bFGF

	cGMP accumulation (fmol/mg of protein)	Cell proliferation (Total cell number/well)
Control conditions		
Basal	36.5 ± 5	1287 ± 82
bFGF 10 ng/ml	50.4 ± 5	1917 ± 62
VEGF 10 ng/ml	96.5 ± 15**	2042 ± 96**
NaNP 0.1 mM	68 ± 13**	1930 ± 98**
L-NMMA 200 μM pretreatment		
Basal	42 ± 5	1317 ± 72
bFGF 10 ng/ml	48 ± 10	1883 ± 136
VEGF 10 ng/ml	51.4 ± 8†	1445 ± 70†
NaNP 0.1 mM	63 + 10	ND

cGMP levels were evaluated by radioimmunoassay in coronary post-capillary endothelial cell extracts. Cells, grown to 90% confluence in 100 mm Petri dishes, were stimulated with the growth factors in the presence of 60 U/ml superoxide dismutase for 10 min. Data are reported as fmol/mg of proteins. Numbers represent means ± SEM of 6 determinations.

Coronary post-capillary endothelial cell proliferation was evaluated as total cell number counted/well after 48 h incubation with the stimuli. Briefly, cells were seeded at the density of 1500/well in 96 multiwell plates coated with gelatin in DMEM containing 5% bovine calf serum and allowed to adhere for 5 h. Medium was replaced with DMEM + 1% bovine calf serum containing the test substances and the incubation continued for 48 h. At the end of the incubation period, cells were fixed with methanol and stained with Diff-Quik. Cells were counted at 10× magnification with the aid of an ocular grid. Data represent means ± SEM obtained from two experiments run in triplicate. NaNP = sodium nitroprusside, ND = not done. In both experimental protocols, cells were pretreated with 200 μM of the NO-synthase inhibitor L-NMMA 1 h before the addition of stimuli.

**$P < 0.01$ versus basal condition and †versus VEGF alone, Student's *t*-test.

Moreover, NO-synthase inhibitors blocked VEGF-, but not bFGF-stimulated growth (Table 2) [110]. Based on these observations, we concluded that the NO-synthase/guanylate cyclase pathway of endothelial cells is involved in VEGF-induced signalling of mitogenesis.

CONCLUSION

In summary, there is evidence indicating a role for NO in angiogenesis. Our data support the existence of an autocrine loop exerted by microvascular endothelium in angiogenesis, which involves NO production and cGMP elevation. The NO synthase/guanylate cyclase is an ideal signalling mechanism for integrating both chemical and physical influences. Local chemical mediators, such as substance P and VEGF, activate the signalling pathway via associated surface receptors. By contrast, NO production can be increased in response to elevated shear rate, thereby linking angiogenesis to flow rate. Our data indicate that, in the acquisition of angiogenic phenotype by microvascular endothelium, NO production significantly contributes to the growth-promoting effect of vasodilating peptides and VEGF, but not for that of bFGF. Thus, although the NO pathway integrates several chemical and physical modulators of the angiogenic process, not all angiogenic factors depend on this signalling

cascade. Although more work is needed to elucidate fully the role of NO in angiogenesis, the nitric oxide pathway appears to be a promising target to be considered in pro- and anti-angiogenic therapeutic strategies.

Note added in proof—We recently demonstrated that HIV-1-Tat activates endothelial cells by interacting with the KDR receptor (A. Albini *et al.*). The angiogenesis induced by HIV-1-Tat protein is mediated by the Flk-1/KDR receptor on vascular endothelial cells. *Nature Med* 1996, **6**, 1371–1375.

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Acknowledgements—The experimental work from our Laboratories cited in this review has been supported by grants from the Italian Association for Cancer Research (AIRC): Special Project on Angiogenesis.