

Distribution of Vascular Permeability Factor (Vascular Endothelial Growth Factor) in Tumors: Concentration in Tumor Blood Vessels

By Harold F. Dvorak, Tracy M. Sioussat, Lawrence F. Brown, Brygida Berse, Janice A. Nagy, Ana Sotrel, Eleanor J. Manseau, Livingston Van De Water, and Donald R. Senger

From the Departments of Pathology, Beth Israel Hospital and Harvard Medical School, and the Charles A. Dana Foundation, Beth Israel Hospital, Boston, Massachusetts 02215

Summary

Vascular permeability factor (VPF) is a highly conserved 34–42-kD protein secreted by many tumor cells. Among the most potent vascular permeability-enhancing factors known, VPF is also a selective vascular endothelial cell mitogen, and therefore has been called vascular endothelial cell growth factor (VEGF). Our goal was to define the cellular sites of VPF (VEGF) synthesis and accumulation in tumors in vivo. Immunohistochemical studies were performed on solid and ascites guinea pig line 1 and line 10 bile duct carcinomas using antibodies directed against peptides synthesized to represent the NH₂-terminal and internal sequences of VPF. These antibodies stained tumor cells and, uniformly and most intensely, the endothelium of immediately adjacent blood vessels, both preexisting and those newly induced by tumor angiogenesis. A similar pattern of VPF staining was observed in autochthonous human lymphoma. In situ hybridization demonstrated VPF mRNA in nearly all line 10 tumor cells but not in tumor blood vessels, indicating that immunohistochemical labeling of tumor vessels with antibodies to VPF peptides reflects uptake of VPF, not endogenous synthesis. VPF protein staining was evident in adjacent preexisting venules and small veins as early as 5 h after tumor transplant and plateaued at maximally intense levels in newly induced tumor vessels by ~5 d. VPF-stained vessels were also hyperpermeable to macromolecules as judged by their capacity to accumulate circulating colloidal carbon. In contrast, vessels more than ~0.5 mm distant from tumors were not hyperpermeable and did not exhibit immunohistochemical staining for VPF. Vessel staining disappeared within 24–48 h of tumor rejection. These studies indicate that VPF is synthesized by tumor cells in vivo and accumulates in nearby blood vessels, its target of action. Because leaky tumor vessels initiate a cascade of events, which include plasma extravasation and which lead ultimately to angiogenesis and tumor stroma formation, VPF may have a pivotal role in promoting tumor growth. Also, VPF immunostaining provides a new marker for tumor blood vessels that may be exploitable for tumor imaging or therapy.

Vascular permeability factor (VPF) is thought to be responsible for the vascular hyperpermeability and consequent plasma protein-rich fluid accumulation that occurs in solid and ascites tumors (1–3). In addition, VPF exerts a number of other effects on vascular endothelial cells (EC); e.g., VPF increases intracellular calcium, stimulates inositol triphosphate formation, provokes von Willebrand factor release, and stimulates tissue factor expression (4, 5). VPF is also a specific EC mitogen and has been alternatively called vascular endothelial cell growth factor (VEGF) (6–9).

In the present study, we used in situ hybridization and immunohistochemistry to define the distribution of VPF in

tumors in vivo. Not unexpectedly, abundant VPF mRNA and protein were present in tumor cells. Of great interest, however, staining for VPF protein was selectively concentrated in tumor-associated blood vessels, the putative target of VPF action in angiogenesis and tumor stroma generation.

Materials and Methods

Animals, Tumors, and Antipeptide Antibodies. Ascites variants of chemically induced guinea pig line 1 and line 10 bile duct carcinomas were passaged in the peritoneal cavities of inbred strain 2 guinea pigs (1). To form solid tumors, 3×10^6 washed ascites

tumor cells were injected subcutaneously into 400 g strain 2 guinea pigs (1). To identify leaky blood vessels, colloidal carbon was injected intravenously 30 min before tumor harvest (10). Autochthonous human tumors were obtained after surgery.

The NH₂-terminal 25-amino acid sequence of guinea pig VPF, here designated peptide 1A and expressed in single letter code, is APMAEGEQKPREVVVKFMDVYKRSYC (11). The 26-amino acid NH₂ terminus of human VPF (peptide 1B) differs from that of guinea pig VPF by the six underlined amino acids: AP-MAEGGGQNHHEVVVKFMDVYQRSYC (8). These and an internal peptide (CECRPKKDRARQEKKSVR, peptide 6) corresponding to amino acids 102–119 of the 189-amino acid form of human VPF (8) were synthesized, coupled to a protein carrier, and used to immunize rabbits (11).

Immunohistochemistry and In Situ Hybridization. Guinea pig tissues were fixed in formalin or carbodiimide and embedded in paraffin. Fresh frozen sections of human tumors were fixed in acetone. Immunohistochemistry was performed on 5–7 μ m paraffin or frozen sections, using affinity-purified first antibodies to guinea pig or human VPF peptides and an avidin-biotin peroxidase procedure. Controls included substitution of the first antibody with an unrelated rabbit antipeptide antibody, preabsorption of first antibody with specific peptide, and on-the-slide competition for first antibody with specific peptide.

In situ hybridization was performed using ³⁵S-labeled single-stranded RNA probes prepared by in vitro transcription of a 204-bp guinea pig VPF cDNA fragment cloned in pGem3Zf(+) (12; B. Berse et al., manuscript submitted for publication).

Results and Discussion

Localization of VPF (VEGF) in Tumors by Immunohistochemistry and In situ Hybridization. Initial immunohistochemical studies were performed on growing, 5–8-d solid tumors. The most striking finding was that the new blood vessels induced by both line 1 and line 10 tumors stained consistently and intensely with antibodies to VPF peptides 1A and 6 (Fig. 1). Vessel staining was circumferential and involved EC and vascular basement membranes; sometimes, adjacent extracellular matrix also stained weakly (Fig. 1, A and D). Line 1 tumors underwent cell-mediated immune rejection (3) which was accompanied by a striking and rapid (within 24–48 h) loss of vessel staining (not shown). The rapid depletion of VPF that accompanied tumor destruction suggests rapid turnover of blood vessel-associated VPF. Preliminary studies with antibodies to VPF peptide 1B indicate that human tumors also exhibit VPF staining and in a pattern similar to that of guinea pig tumors (Fig. 1 G).

In addition to the tumor microvasculature, vessels coursing through immediately adjacent normal tissues also exhibited specific antibody staining (Fig. 1 F). Immunoreactive vessels were venules and small veins within \sim 0.5 mm of tumors; arterioles, capillaries, and vessels of any type at greater distances from tumor or in other tissues and organs exhibited no staining. Consistent with these findings, antibodies to VPF peptide 1A also stained immediately adjacent, preexisting host venules and small veins as early as 5 h after tumor cell transplant. These preexisting blood vessels, as well as induced tumor

vessels, were hyperpermeable as judged by their concentration of circulating colloidal carbon (10).

A minority of tumor cells stained with antibody to VPF peptide 1A, but nearly all stained with the antibody to peptide 6 (Fig. 1, A, B, and H), suggesting that VPF is abundantly present in nearly all tumor cells. The poor tumor cell staining with antipeptide 1A probably represents failure of fixation to preserve cytoplasmic VPF in a conformation accessible to this antibody. Using in situ hybridization (Fig. 2), nearly all line 10 solid or ascites tumor cells reacted with the antisense RNA probe for guinea pig VPF mRNA; however, tumor blood vessels were negative for VPF transcript.

Relation of VPF (VEGF) Synthesis and Accumulation to Tumor Stroma Generation. Our data clearly indicate that VPF is abundantly synthesized by tumor cells but not detectably by EC; therefore, the VPF identified in tumor-associated vessels reflects selective binding of tumor cell-secreted VPF. We did not unequivocally identify the precise vascular structures to which tumor cell-secreted VPF bound. EC have receptors for VPF (4, 13, 14), and it is likely that some of the staining represents VPF that has bound to the EC surface or that has been subsequently internalized. However, VPF also binds to heparin (2), and some of the staining may reflect binding of VPF to heparin-like proteoglycans present either in the vascular basement membrane or associated with EC. Indeed, cell surface heparin-like molecules are required for binding bFGF, another growth factor implicated in angiogenesis, to its high affinity receptor (15).

The intense tumor vessel labeling observed with anti-VPF peptide antibodies was unexpected. VPF exerts its effects on vascular endothelium at low nM to sub-pM concentrations, well below those detectable by immunohistochemistry; e.g., staining could not be demonstrated when VPF was injected into normal guinea pig skin in amounts that greatly increased local vascular hyperpermeability. Therefore, VPF accumulates in tumor blood vessels in amounts much greater than those necessary to trigger vascular responses. Tumor vessel binding may provide a mechanism for retaining and concentrating VPF, thereby maximizing its activity locally while preventing its spread to more distal sites.

Taken together, our data support a model of tumor stroma generation that is initiated by tumor cell secretion of VPF (1, 3). VPF acts directly on EC, initially to induce vascular hyperpermeability and later to maintain hyperpermeability and to promote endothelial cell growth, perhaps in concert with other growth factors such as bFGF (16). Tumor vessel hyperpermeability results in extravasation and clotting of fibrinogen to form an extravascular fibrin gel that provides a favorable substrate for invasion by fibroblasts and EC (3, 10); together, these transform the fibrin gel into granulation tissue and, later, into desmoplastic scar. Fibrin gels alone can replicate this entire response without the need for tumor cells or exogenous growth factors (3). Thus, with its dual actions on EC permeability and proliferation, VPF initiates and perpetuates a chain of events that produces tumor stroma by a process analogous to normal wound healing (3).

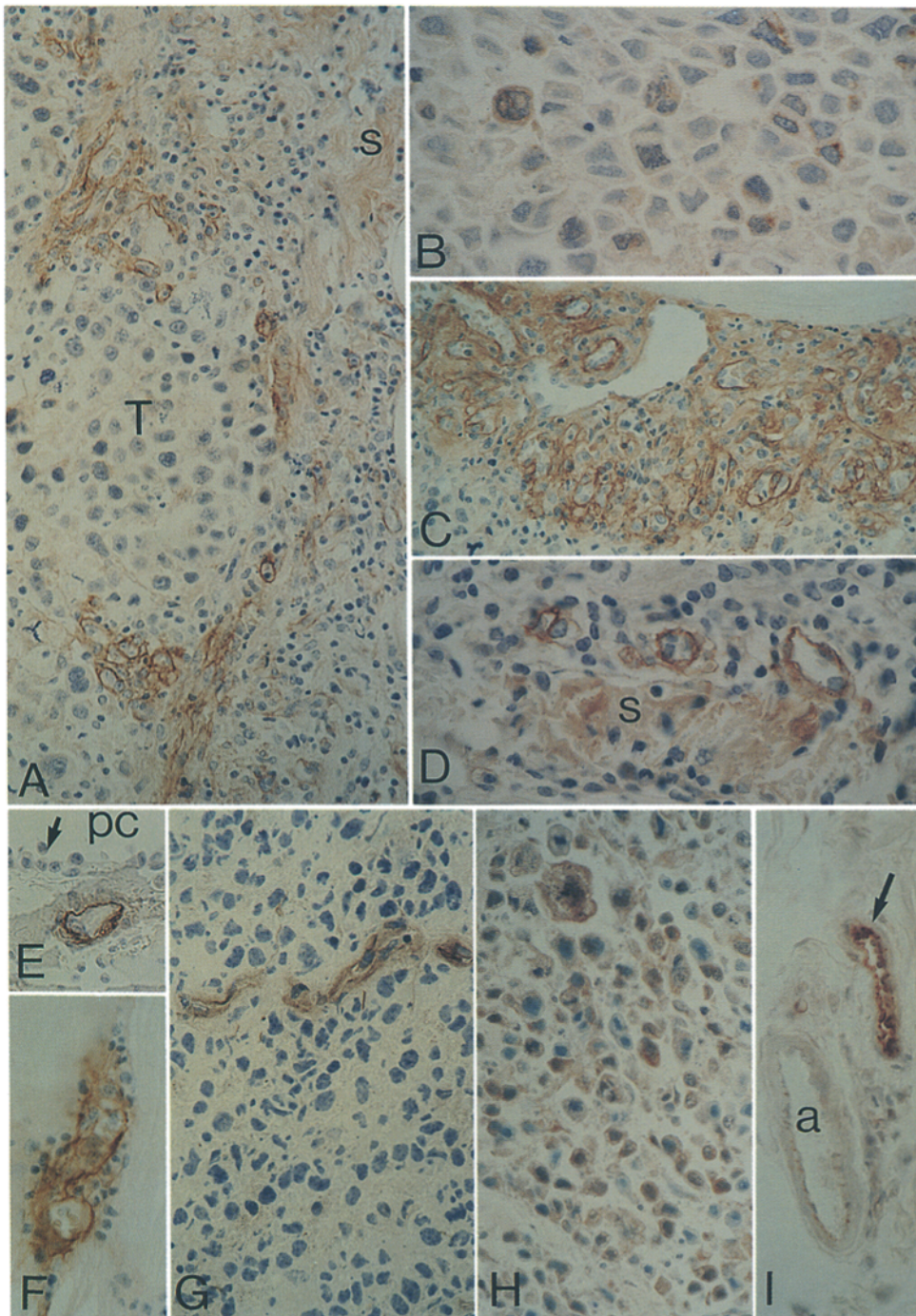


Figure 1. Immunohistochemistry of line 10 (A-C, E, F, H and I) and line 1 (D) guinea pig bile duct carcinomas and a human large cell lymphoma (G) demonstrated with antibodies directed against guinea pig or human VPF peptides: (A-F) peptide 1A; (G) peptide 1B; (H and I) peptide 6. Immunohistochemical reaction product appears yellow-brown. (A) Overview showing minimal staining of line 10 tumor cells (T) with antibody to peptide 1A but intense staining of tumor-associated blood vessels 8 d after transplant. Focally, adjacent stroma (s) is also stained. (B-D) Higher magnification photographs illustrating variable line 10 tumor cell staining (B) and intense staining of line 10 (C) and line 1 (D) tumor vessels; (s) staining of adjacent stroma. (E) New, ascites tumor-induced blood vessel of the peritoneal wall stains intensely; attached tumor cells (arrow) are unstained; (pc) peritoneal cavity. (F) Preexisting venules of normal skeletal muscle adjacent to a line 10 tumor are strongly stained with antibody to peptide 1A. (G) Vessels in a human B cell lymphoma react strongly with antibody to peptide 1B. (H and I) Staining of nearly all line 10 tumor cells (H) and a nearby venule (arrow in I) with antibodies to peptide 6. As with antibodies to the VPF NH₂ terminus, adjacent venules and small veins (arrow in I) stain intensely, but arteries (a in I) stain faintly or not at all; (A) $\times 245$; (B and D) $\times 405$; (C) $\times 313$; (E, F, and H) $\times 260$; (G) $\times 320$; (I) $\times 410$.

Finally, it should be noted that VPF immunostaining distinguishes tumor blood vessels from those found elsewhere in tumor-bearing or control animals. This finding stands in contrast to the widespread distribution of bFGF in vascular

basement membranes of normal tissues (17). VPF provides a new marker for tumor blood vessels and may offer a useful target for tumor imaging and therapy.

This work was supported by National Institutes of Health grants CA-28471, CA-50453, and CA-43967 and by American Cancer Society postdoctoral fellowships PF-3648 and PRTF 68.

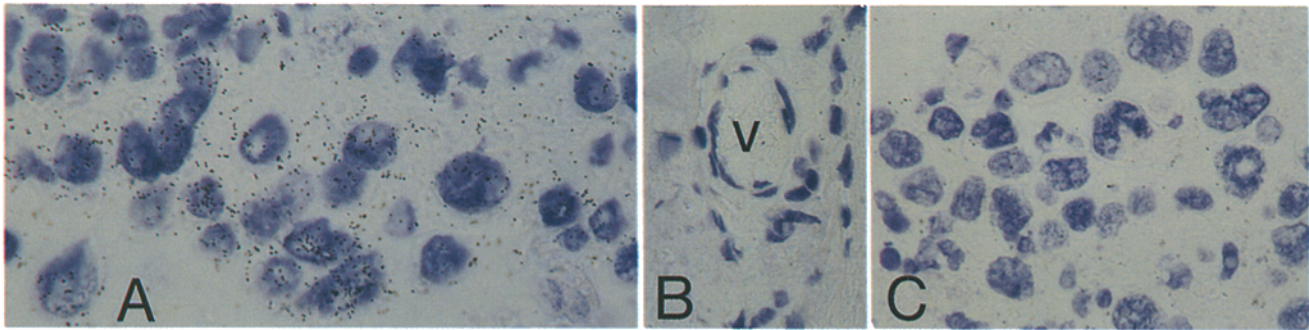


Figure 2. Autoradiographs representing in situ hybridization performed on paraffin sections of solid line 10 tumors with ^{35}S -labeled antisense (A and B) and sense (C) RNA probes to guinea pig VPF mRNA. With antisense probes, labeling is observed over nearly all tumor cells (A) but not over adjacent microvessels (v in B). Autoradiograph exposure times in A were selected to permit clear visualization of cells underlying grains. With the corresponding sense probe (C), tumor cells are not labeled, even after lengthy exposures; (A) $\times 500$; (B and C) $\times 385$.

Address correspondence to Harold F. Dvorak, Department of Pathology, Beth Israel Hospital, Boston, MA 02215.

Received for publication 20 June 1991.

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