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Expression of Vascular Permeability Factor (Vascular Endothelial Growth Factor) and Its Receptors in Adenocarcinomas of the Gastrointestinal Tract[1]

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ABSTRACT

Vascular permeability factor (VPF) is one of the most potent known inducers of microvascular hyperpermeability; in addition, it is a selective endothelial cell growth factor, hence its alternate name, vascular endothelial growth factor. VPF exerts its actions on the microvasculature by interacting with specific endothelial cell receptors. VPF is expressed by many transplantable animal tumors, by tumor cell lines in culture, and by certain normal cells *in situ*. The purpose of the present investigation was to determine whether and with what consistency VPF and its endothelial cell receptors are expressed in primary autochthonous human tumors of gastrointestinal tract origin, as determined by *in situ* hybridization and immunohistochemistry. Twenty-one primary adenocarcinomas (17 colon, 2 stomach, 1 small bowel, and 1 pancreas) were studied. The malignant epithelial cells expressed VPF mRNA strongly, in contrast to normal epithelium, hyperplastic polyps, and adenomas, which expressed little or no VPF mRNA. VPF expression was further increased in tumor cells immediately adjacent to zones of tumor necrosis; in such areas, occasional stromal cells also expressed VPF mRNA. In the ten colon carcinomas studied, tumor cells stained for VPF protein by immunohistochemistry. The endothelial cells of nearby stromal blood vessels also stained for VPF by immunohistochemistry and in addition expressed mRNAs encoding the VPF receptors *flt-1* and *kdr* as determined by *in situ* hybridization. Endothelial cells away from the tumor did not stain for VPF and no definite mRNA expression for *flt-1* or *kdr* was detected by *in situ* hybridization. The ganglion cells of the myenteric plexus of normal bowel expressed VPF mRNA and protein. These data indicate that primary autochthonous human tumors of gastrointestinal origin regularly express both VPF mRNA and VPF protein and that adjacent stromal vessels express mRNAs for both known VPF receptors. VPF is likely to contribute to tumor growth by promoting angiogenesis and stroma formation, both directly, through its action as an endothelial cell growth factor, and indirectly, by increasing vascular permeability, thereby leading to plasma protein extravasation, fibrin deposition, and the eventual replacement of the resulting matrix with vascularized stroma.

INTRODUCTION

Solid tumors are composed of two distinct but interdependent compartments, the malignant cells themselves and the vascular and connective tissue stroma that they induce and in which they are dispersed. Stroma provides the vascular supply that tumors require for obtaining nutrients, gas exchange, and waste disposal. Therefore, if they are to grow beyond minimal size, tumors must acquire stroma (1).

The mechanisms by which tumors induce stroma have received considerable attention in recent years. Most work has focused on one aspect of stroma generation, angiogenesis, and has called attention to a variety of tumor-secreted "angiogenesis factors," particularly basic fibroblastic growth factor, but also transforming growth factor β, tumor necrosis factor α, etc., that induce new blood vessel formation in a variety of *in vitro* and *in vivo* assays (1). Another mediator that is likely to play an important role in both angiogenesis and other aspects of tumor stroma generation is VPF.[3] VPF is synthesized and secreted by a variety of tumor cells in tissue culture and by several transplantable animal tumors *in vivo*, and elevated expression of VPF has been found in tumors of the central nervous system in humans (2–7). VPF was originally recognized for its capacity to increase the permeability of the microvasculature to circulating macromolecules (*i.e.*, plasma proteins) (8). On a molar basis, VPF is some 50,000 times more potent than histamine at increasing microvascular permeability (9, 10). More recently, VPF has also been shown to be a selective endothelial cell mitogen, hence its alternative name, vascular endothelial growth factor or VEGF (11–15). The actions induced by VPF apparently result from a direct interaction between VPF and specific endothelial cell surface receptors, two of which, *flt-1* and *kdr*, have recently been identified (16, 17). Signal transduction involves an increase in free cytosolic calcium in target endothelial cells, likely through phospholipase C activation (18).

The goal of the present investigation was to determine whether and to what extent VPF has a role in the biology of autochthonous human tumors. In a prior study, Northern analyses of mRNAs isolated from a small number of human tumors provided support for the possibility that VPF expression is elevated in at least some human tumors, including those arising in the gastrointestinal tract (19). However, those studies suggested large variations in VPF expression among different tumors, even among those of the same type and tissue of origin. The differences could have reflected genuine variations in tumor cell mRNA levels; on the other hand, tumors vary widely in stromal content and the differences observed might just as well have been attributed to varying proportions of tumor and stromal cells in different samples. Therefore, to examine VPF mRNA expression in human tumors in greater detail, we performed ISH and IH for VPF on a series of primary adenocarcinomas arising in the gastrointestinal tract. In addition, we used ISH to determine whether mRNAs encoding the VPF receptors *flt-1* and *kdr* were expressed in tumor blood vessels differently than in normal vessels.

MATERIALS AND METHODS

Tumors and Control Tissues. Twenty-one primary adenocarcinomas were examined as soon as possible after resection and portions of tumors, polyps (when present in the same specimen), and normal tissue were fixed and processed for ISH and IH (see below). Twenty of the adenocarcinomas arose in the gastrointestinal tract (17 colorectal, 2 stomach, 1 duodenum) and all had invaded deeply into or through the muscularis propria (earlier stage tumors could not be studied since all tissue was used for diagnostic purposes); the remaining malignancy arose in the pancreas. In addition, benign polyps and grossly normal appearing bowel were obtained from a single case of familial adenomatous polyposis.

***In Situ* Hybridization.** Tissues were fixed for 4 h in 4% paraformaldehyde in phosphate buffered saline, pH 7.4, at 4°C and were then transferred to 30% sucrose in phosphate buffered saline, pH 7.4, overnight at 4°C. Tissue was then

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[3] The abbreviations used are: VPF/VEGF, vascular permeability factor/vascular endothelial growth factor; ISH, *in situ* hybridization; IH, immunohistochemistry.

frozen in OCT compound (Miles Diagnostics, Elkhart, IN) and stored at −70°C. Six-μm frozen sections were subjected to ISH as described previously (20). The antisense single-stranded RNA probe for localizing VPF mRNA and its sense control have been described previously (20); the antisense probe hybridizes with a region of VPF mRNA coding sequence common to all of the four known VPF splicing variants (21, 22).

PCR primers for *flt-1* and *kdr* were derived from sequences within the kinase insert regions of the respective molecules (23, 24), adding *Bam*HI and *Eco*RI sites for cloning. The following primers were synthesized by David Gonzales at Beth Israel Hospital using an Applied Biosystem machine:

(1) For *flt-1:*
UP 5′ CTAGGATCCGTGACTTATTTTTTCTCAACAAGG 3′
DN 5′ CTCGAATTCAGATCTTCCATAGTGATGGGCTC 3′

(2) For *kdr:*
UP 5′ CGTGGATCCACCAAAGGGGCACGATTCCGTC 3′
DN 5′ CTCGAATTCTGTAACAGATGAGATGCTCCAAGG 3′

Pairs of *flt-1* and *kdr* primers were run using the PCR hot start Ampli Wax technique according to the Perkin Elmer Cetus (Norwalk, CT) protocol (35 cycles; annealing temperature, 60°C; extension for 2 min at 72°C), using human fetal liver cDNA obtained from Clontech (Palo Alto, CA) as a template. The resulting products were precipitated with ethanol, digested with *Bam*HI and *Eco*RI, electrophoresed on 1% agarose gels (FMC, Rockland, ME), and intercepted on NA45 (Schleicher and Schuell, Keene, NH) according to the manufacturer's instructions. The fragments were cloned into pGem 3 (ProMega, Madison, WI) and clones were prepared and sequenced (Sequenase 2; United States Biochemicals, Cleveland, OH). Correct sequence clones of *flt-1* and of *kdr* (225 and 209 base pairs, respectively) were used to prepare ^{35}S-labeled antisense and sense probes for ISH using SP6 and T7 polymerases and materials from ProMega (19).

Immunohistochemistry. Preparation of a rabbit antibody to an NH_2-terminal peptide (amino acid residues 1–26) of human VPF and its affinity purification have been described previously (2, 25). This antipeptide antibody bound VPF in enzyme-linked immunosorbent assays and on immunoblots (25) and has been found useful for IH (2, 20). IH was performed on 6-μm frozen sections of the same blocks used for ISH, using the affinity-purified antibody to the VPF NH_2 terminus with an avidin-biotin peroxidase conjugate protocol (2, 20). Normal rabbit IgG diluted to an equivalent protein concentration served as a control in place of the primary antibody.

RESULTS

***In Situ* Hybridization for Localizing Sites of VPF mRNA Expression.** Seventeen primary adenocarcinomas of the colon were studied by ISH with both antisense and sense (control) probes to VPF. All had invaded into or through the muscularis propria. The malignant epithelium of 13 of these tumors labeled strongly for VPF mRNA when hybridized with the antisense probe (Figs. 1, 2, 3); the remaining

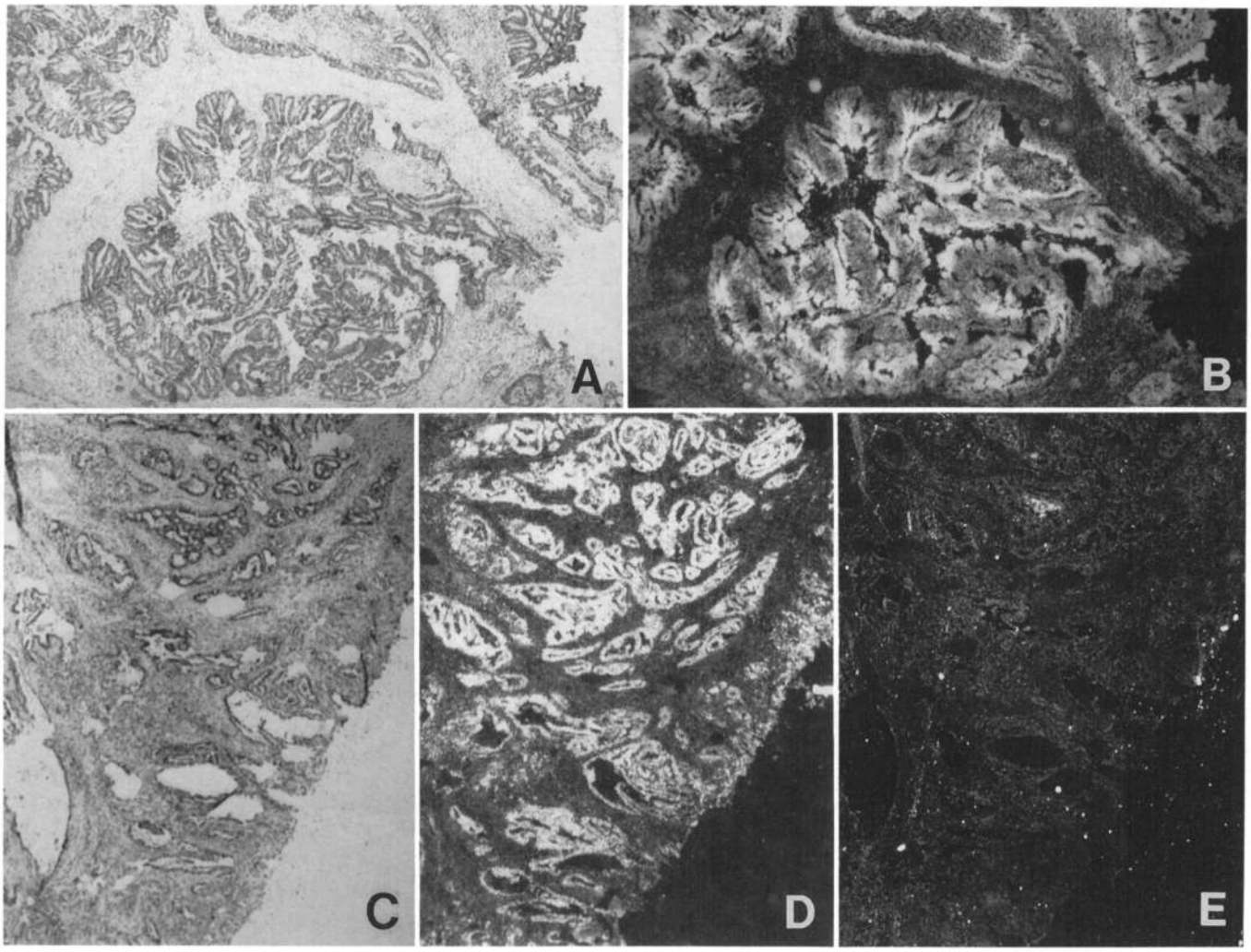

Fig. 1. Low power photomicrographs: ISH of colonic (*A, B*) and gastric (*C-E*) adenocarcinomas probed with antisense (*A-D*) and sense (*E*) riboprobes to VPF. *A*, *B* and *C*, *D*, bright-field and dark-field photomicrographs of the same field; *E*, dark-field photomicrograph of a serial section of the gastric adenocarcinoma. Note intense labeling (bright white) of tumor cells in both colonic and gastric tumors with the antisense VPF probe, best visualized in dark field (*B, D*). In contrast, the sense probe did not label the gastric (*E*) or colonic (not shown) tumors. × 24.

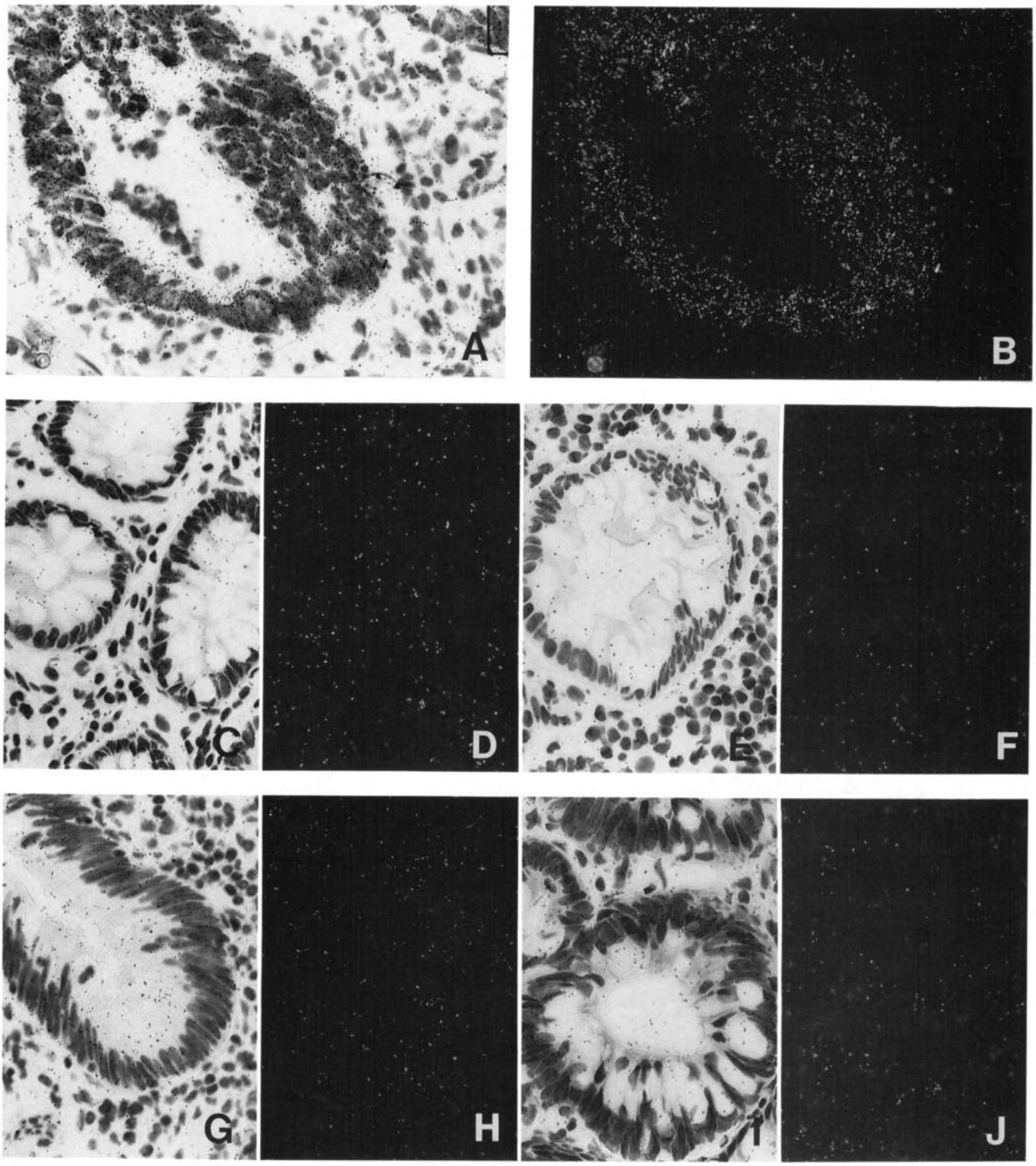

Fig. 2. High power photomicrographs. ISH with antisense probe to VPF of a colonic adenocarcinoma (*A, B*), normal colonic epithelium (*C, D*), a hyperplastic polyp (*E, F*), and a colonic adenoma (*G, H*), all from a single colonic resection specimen. Each pair of photomicrographs represents the same microscopic field photographed in bright field and dark field, respectively. Note the intense labeling of malignant epithelial cells (*A, B*) but not of normal, hyperplastic, or adenomatous epithelium (*C-H*). *I* and *J*, ISH of a colonic adenoma removed from a patient with familial polyposis hybridized with the antisense VPF probe, photographed in bright and dark field, respectively. No distinct labeling is seen. × 288.

4 also labeled unequivocally but less intensely.

The majority of individual tumor cells expressed VPF mRNA. Several of these colon carcinomas contained focal areas of overt necrosis, and labeling with the VPF antisense probe was distinctly intensified in tumor epithelium that was immediately adjacent to necrotic foci (Fig. 3, *A* and *B*). Background labeling with the antisense probe was consistently low and in no case was specific hybridization observed with the sense (control) probe (Fig. 1*E*).

In addition to the intense labeling found overlying epithelial carcinoma cells, occasional cells of the tumor stroma also labeled for VPF mRNA. Stromal labeling was distinctly focal compared to the labeling regularly observed over tumor cells and usually occurred in stroma

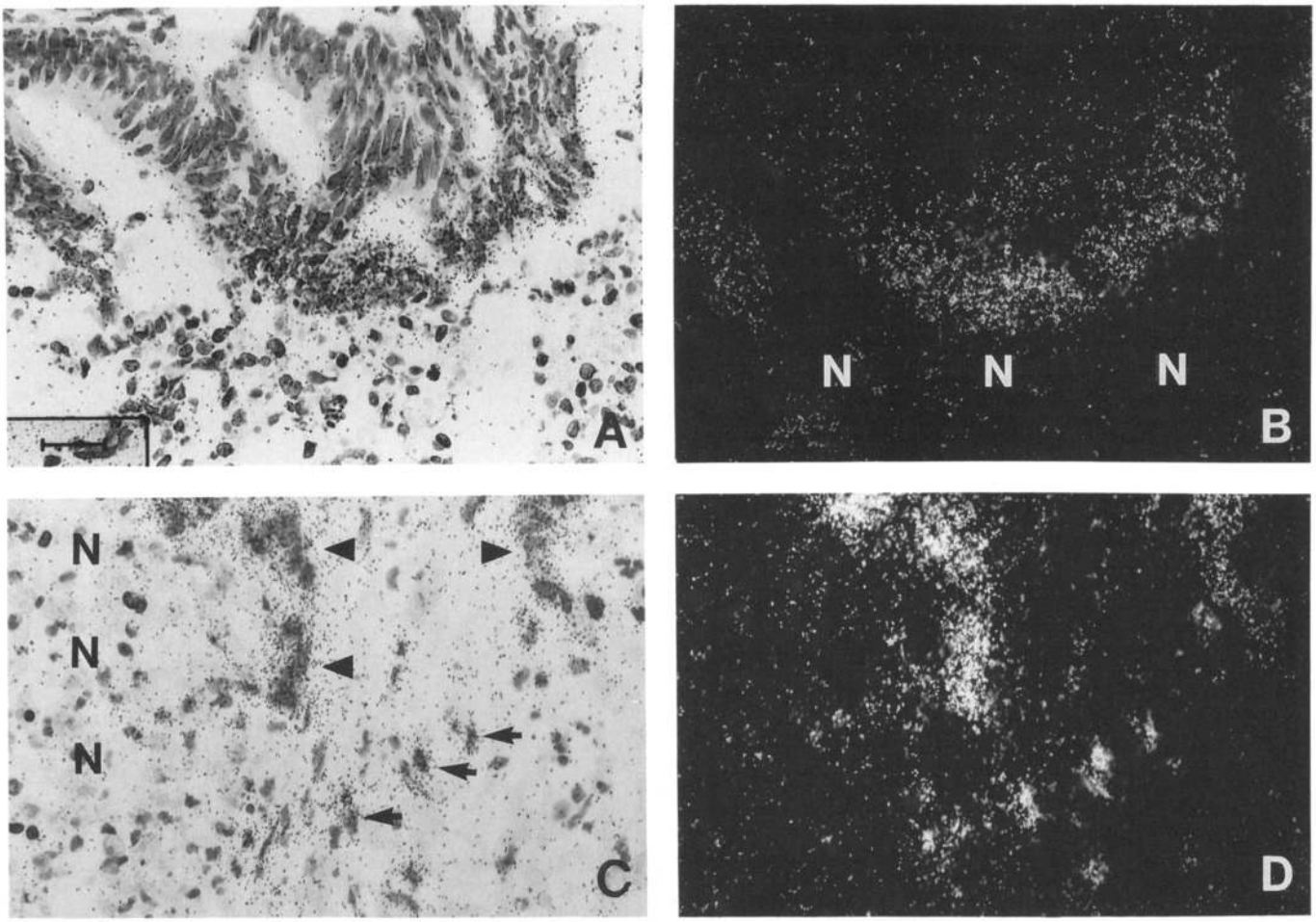

Fig. 3. ISH of a colonic adenocarcinoma, illustrating enhanced labeling of malignant epithelium and of stromal cells adjacent to microscopic foci of necrosis. *A, B,* bright and dark field views, respectively, of the same microscopic field hybridized with the VPF antisense probe. Note enhanced labeling of malignant epithelial cells immediately adjacent to zones of necrosis (N). *C, D,* bright and dark field views, respectively, of the same field illustrating intense labeling of clusters of tumor cells with large, irregular nuclei (*arrowheads*) as well as individual stromal cells with smaller, regular, elongate nuclei (*arrows*) immediately adjacent to necrotic zones (N) with VPF antisense probe. × 288. *Bar,* 30 μm.

immediately adjacent to foci of overt tissue necrosis (Fig. 3, *C* and *D*). Labeled stromal cells included fibroblasts and smooth muscle cells, but in some cases the identity of labeled stromal cells could not be determined with certainty; however, vascular endothelium did not express detectable VPF mRNA.

In ten cases, grossly normal colonic tissue away from the carcinoma was also studied. Equivocal or very light labeling of normal colonic epithelium was noted focally in several cases with the antisense VPF probe but never approached the intensity observed in even the most lightly labeled carcinoma. In two cases we were able to study hyperplastic and adenomatous polyps and normal mucosa present in the same resection specimen as an invasive carcinoma and only the carcinomatous epithelium labeled convincingly with the VPF antisense probe (Fig. 2, *A-H*). In the single case of familial adenomatous polyposis that we studied, unequivocal labeling was not detected in either the adenomatous or the intervening, normal appearing epithelium (Fig. 2, *I* and *J*).

Several other carcinomas of gastrointestinal origin were also studied by ISH with the VPF antisense probe. The malignant epithelium of a primary duodenal adenocarcinoma, a gastric adenocarcinoma (Fig. 1, *C* and *D*; Fig. 4, *A* and *B*) and a poorly differentiated adenocarcinoma of the pancreas (Fig. 4, *E* and *F*) labeled strongly for VPF mRNA. A second gastric adenocarcinoma labeled less intensely but unequivocally. In contrast, normal epithelium of the small intestine, pancreatic acini, and gastric mucosa from these same cases labeled weakly or not at all with the VPF antisense probe (not shown), nor did tumors label with the VPF sense probe (Figs. 1*E* and 4, *C* and *D*).

There was, however, one additional finding; occasional ganglion cells in normal colon, stomach, and small bowel labeled strongly for VPF mRNA.

***In Situ* Hybridization for Localization of Sites of VPF Receptor mRNA Expression.** Five colonic adenocarcinomas with accompanying grossly normal colonic tissue from the same resection specimens were also studied with riboprobes for the VPF receptors *flt-1* and *kdr.* Endothelial cells lining small blood vessels of the tumor stroma labeled strongly for both *flt-1* and *kdr* mRNAs with these probes (Fig. 5, *A,B,E,* and *F*). By contrast, the endothelium lining small blood vessels of normal colon away from carcinomas did not label convincingly for either flt-1 or kdr mRNA (Fig. 5, *C, D, G,* and *H*). Apart from endothelial cells, no other cell types labeled for *flt-1* or *kdr* mRNA. Background labeling with the antisense probes was low and no specific labeling was found with either sense probe.

Immunohistochemical Staining for Localization of VPF Protein. Immunohistochemical staining for VPF was performed on ten colonic adenocarcinomas. Malignant epithelial cells of these invasive tumors, along with the endothelial cells of adjacent small blood vessels in tumor stroma, stained strongly with the affinity-purified antibody to the VPF NH_2-terminal peptide (Fig. 6). In contrast, normal colonic epithelium stained very weakly or not at all and the endothelial cells of small blood vessels of normal colon did not stain. How-

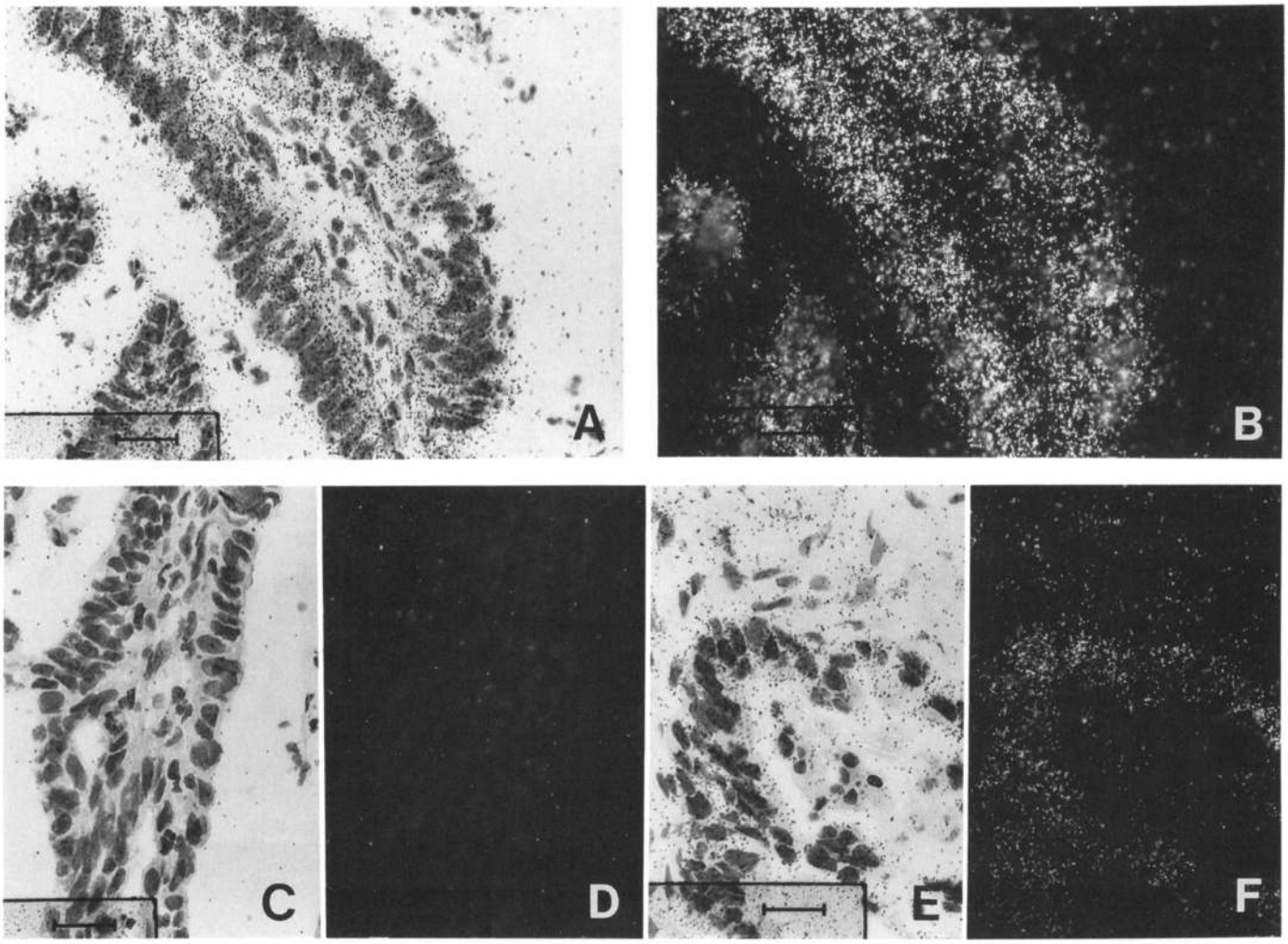

Fig. 4. ISH of gastric (*A-D*) and pancreatic (*E,F*) adenocarcinomas hybridized with antisense (*A, B, E, F*) and sense (*C, D*) probes to VPF. Successive figure pairs represent the same microscopic field photographed in bright and dark field, respectively. Note strong labeling of malignant epithelium with antisense probes (*A, B, E, F*) whereas sections hybridized with the sense probe (*C, D*) show no epithelial labeling and low background. × 288. *Bar,* 30 μm.

ever, ganglion cells and nerves of normal bowel did stain strongly for VPF. Smooth muscle cells of the muscularis mucosa, the muscularis propria and also the muscular walls of small and medium sized arteries and veins sometimes stained weakly for VPF; however, we are uncertain as to whether this staining is specific since very weak smooth muscle staining was also seen occasionally with our control antibody. No specific staining of tumor cells, endothelial cells, or ganglion cells was seen when control IgG was substituted for the primary antibody.

DISCUSSION

All of the 21 primary autochthonous human adenocarcinomas of the gastrointestinal tract that we studied expressed VPF mRNA as judged by ISH. In 16 of these cases, labeling of malignant epithelium was strong. In the remaining 5 cases labeling was less intense but still distinctly stronger than that of the corresponding normal epithelia. In two resected colons, hyperplastic polyps and adenomas were present in addition to invasive adenocarcinomas. In contrast to the adenocarcinomas, which hybridized strongly with the antisense VPF riboprobe, the hyperplastic polyps and adenomas did not label above the near background levels of normal epithelium. Similarly, polyps from a patient with familial adenomatous polyposis labeled with no greater intensity than normal epithelium. Taken together, these findings suggest that elevated expression of the VPF gene is a consistent feature of adenocarcinomas that arise in the gastrointestinal tract. Apparently, however, up-regulation of VPF expression occurred fairly late in tumor progression, being associated with overt malignancy but not with premalignant adenomas that exhibited little atypia. To define more precisely the point in malignant progression at which transcription of the VPF gene is up-regulated will require a larger series of cases that includes adenomas with varying degrees of dysplasia and carcinoma *in situ*.

In all of the 21 adenocarcinomas that we studied, the majority of individual tumor cells expressed VPF mRNA as judged by ISH. However, VPF expression was increased still further in tumor cells that were located immediately adjacent to zones of tumor necrosis (Fig. 3); moreover, in such zones, stromal cells, including at least fibroblasts and smooth muscle cells (but not vascular endothelium), also expressed VPF mRNA strongly, whereas they did not do so in other areas of tumor stroma or in the connective tissue of normal bowel. Tissues adjacent to zones of necrosis are likely to be hypoxic, as well as to exhibit other properties that result from faulty or uneven blood flow such as acidosis, nutrient deprivation, and accumulation of metabolites (26). Therefore, because many malignant tumors have a faulty blood supply (27, 28), and because hypoxia has in fact been reported to increase VPF mRNA expression by cultured cells (7), it has been suggested that hypoxia or some other property of underper-

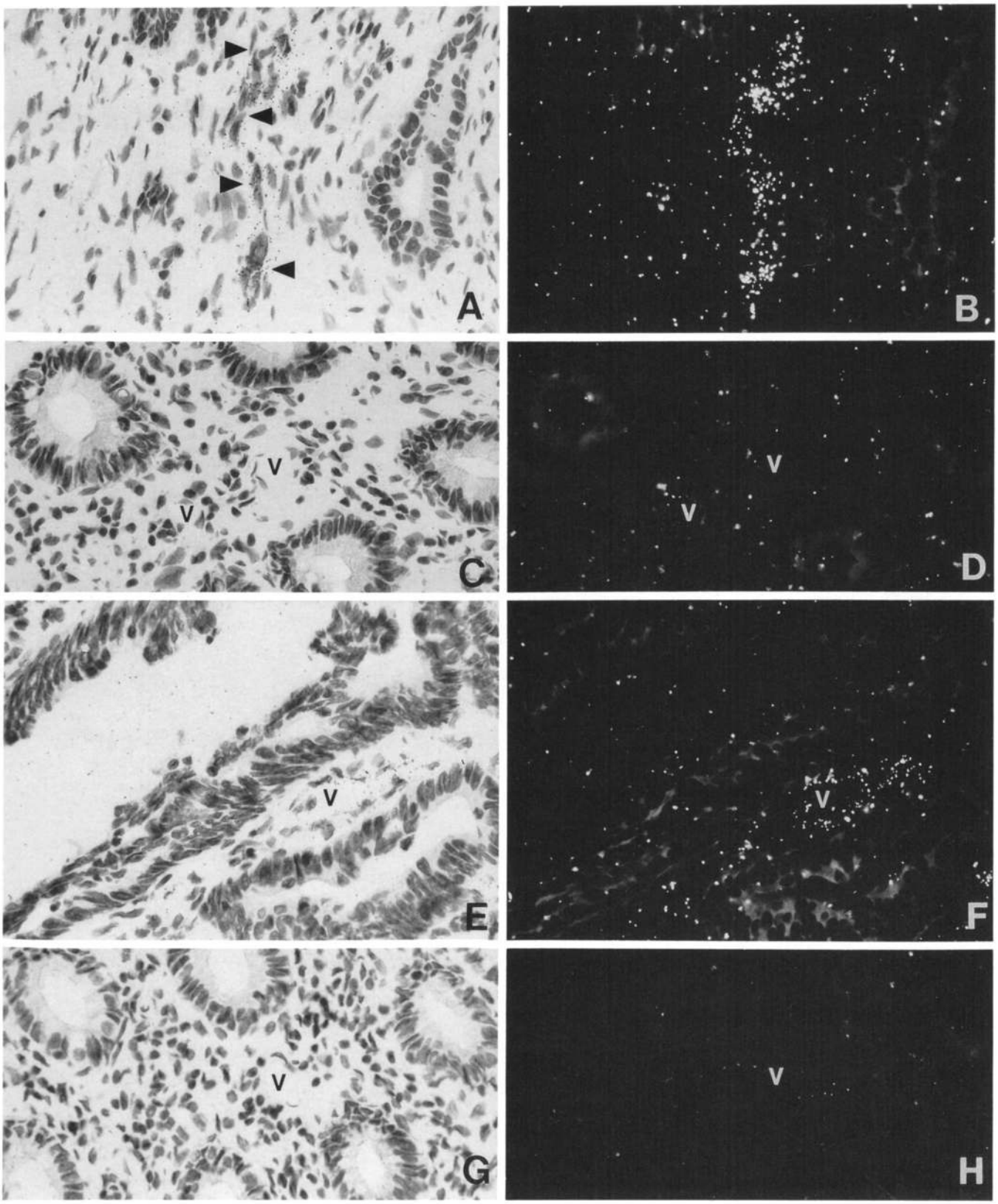

Fig. 5. ISH of a colonic adenocarcinoma (*A, B, E, F*) and of normal colon (*C, D, G, H*) hybridized with antisense riboprobes to the VPF receptors *flt-1* (*A-D*) and *kdr* (*E-H*). Paired micrographs of the same fields were photographed in bright field (*A, C, E, G*) and dark field (*B, D, F, H*), respectively. Note intense labeling of microvessel endothelium (outlined by *arrowheads* in *A* and by luminal *v* in *E* and *F*) in tumor stroma whereas comparably sized vessels in normal colonic stroma (luminal *v* in *C, D, G, H*) have no distinct labeling. × 288.

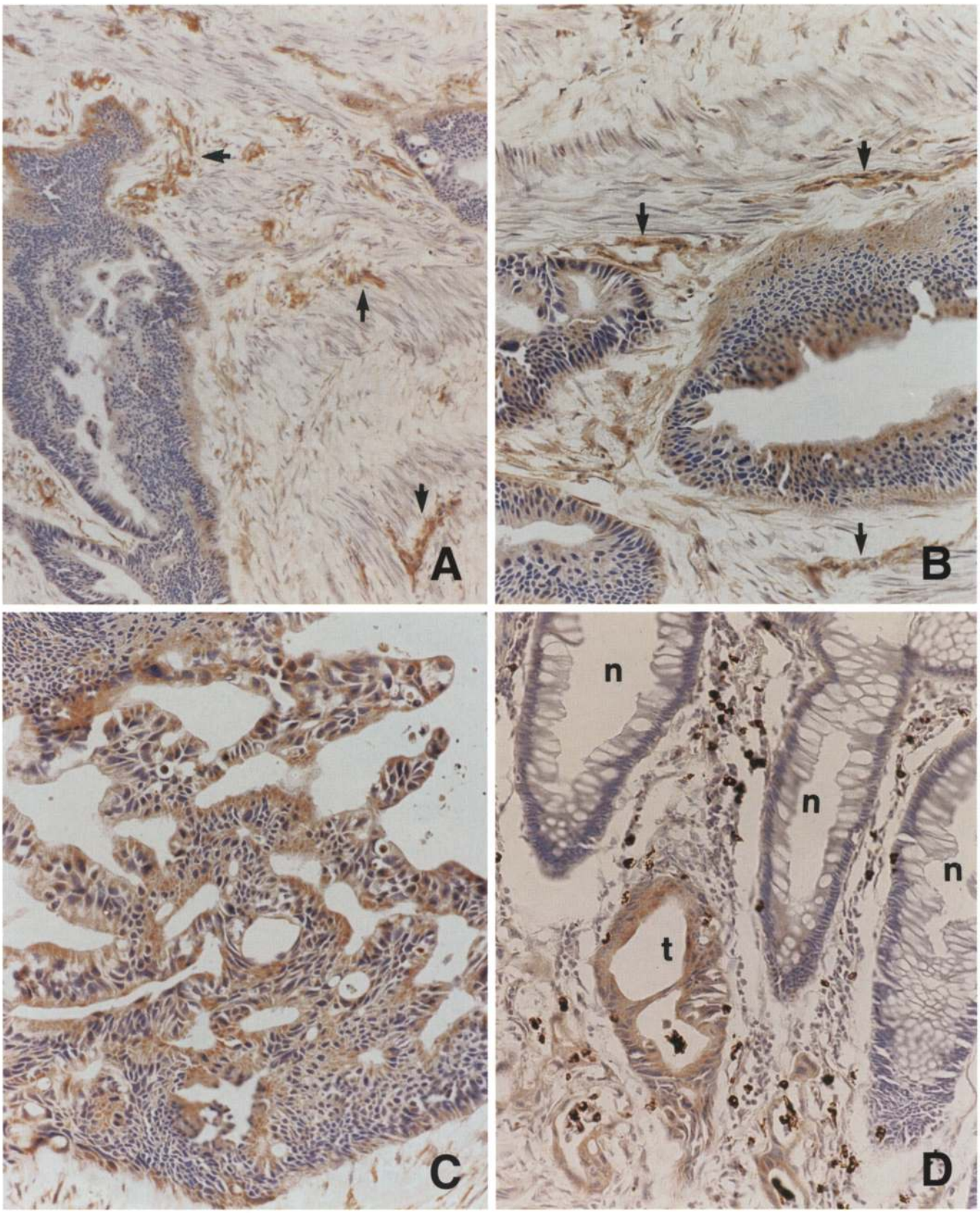

Fig. 6. Immunohistochemical staining of a typical colonic adenocarcinoma with affinity-purified anti-peptide antibody to VPF. Low (*A*) and higher (*B-D*) power views show immunospecific staining of malignant glands and of adjacent tumor stromal blood vessel endothelium (*arrows, A, B*). Malignant epithelium stained with variable intensity (compare *A-D*). In *D*, several positive staining tumor glands (lower part, with the lumen of the largest marked *t*) are situated immediately adjacent to three nonstaining normal glands (*n*). Also in *D*, mast cells exhibit strong staining which was also present with control IgG and is a common artifact of avidin-biotin immunohistochemistry. *A*, × 107; *B-D*, × 279.

fused tissues provides the stimulus that up-regulates VPF transcription in malignancy (29).

However, several of our findings suggest that hypoxia, low pH, and other features of the microenvironment do not provide the entire explanation for VPF expression by tumors. (*a*) In all of the 21 carcinomas that we studied, nearly all tumor cells expressed relatively abundant VPF mRNA. VPF mRNA expression was not confined to limited portions of tumors that may have been poorly vascularized or underperfused; rather, all parts of these tumors expressed VPF mRNA and protein at much higher levels than corresponding normal epithelium. This point was made most dramatically in histological sections of tumors in which malignant glands intermingled with normal epithelium and therefore were subjected to a similar microenvironment; nonetheless, in this mixed population of cells only the carcinoma cells expressed detectable VPF mRNA (Fig. 6*D*). (*b*) Many transformed cell lines grown in well oxygenated tissue cultures at appropriate pH and with adequate nutrients nonetheless synthesize and secrete relatively large amounts of VPF, in striking contrast to their nontransformed counterparts cultured under identical conditions (3, 4, 19). Finally, VPF is expressed in readily detectable amounts by certain normal cells *in situ* (*e.g.,* renal glomerular epithelium, pulmonary alveolar cells, cardiac myocytes) that are perfused by an abundant blood supply (19, 20, 30, 31). Thus, while poor vascular perfusion leading to necrosis may have accentuated VPF expression focally in tumors, it is likely that other, as yet undefined factors are also important in determining the increased VPF expression that, at least in the gastrointestinal tract, seems to be a characteristic of the malignant phenotype.

In a previous IH study of guinea pig bile duct carcinomas and of a human brain tumor, we reported strong VPF staining of tumor stromal blood vessels but weak or equivocal staining of tumor cells (2). Similar findings were recently reported in gliomas where small tumor vessel endothelial cells stained for VPF whereas the tumor cells themselves did not (6). In our present study, colonic adenocarcinomas studied by IH for VPF exhibited staining of both epithelial tumor cells and of the endothelial cells lining nearby small blood vessels (Fig. 6). It seems certain that in all of these studies the VPF protein staining observed in tumor vessel endothelium was not attributable to endothelial cell VPF synthesis since these endothelial cells did not express detectable VPF mRNA as judged by ISH. Therefore, the VPF staining of tumor vessel endothelial cells observed must have resulted from an accumulation of VPF protein that had been synthesized by other (presumably tumor) cells. In agreement with this interpretation, endothelial cells that stained for VPF also expressed readily detectable amounts of mRNAs coding for the VPF receptors *flt-1* and *kdr;* a similar finding for the mRNA of *flt-1* has been reported in highly malignant glioblastomas (6). Neither VPF protein nor the mRNAs coding for either known VPF receptor were detected in the endothelial cells of normal colon.

The increased expression of VPF by tumor cells and the binding of tumor cell-secreted VPF to endothelial cells are likely to have important biological implications for the generation of vascularized tumor stroma. Local vascular hyperpermeability, possibly induced by VPF in both tumors and wounds (32), leads to the extravasation of plasma proteins including fibrinogen; once extravasated into tissues, fibrinogen clots, forming a gel in the extravascular space that may be expected to incorporate other extravasated proteins such as fibronectin (33, 34). Appropriately prepared fibrin gels provide an extracellular matrix which favors the migration of fibroblasts and macrophages (35, 36). These and other cells (*e.g.,* endothelial and tumor cells) probably interact with the provisional matrix containing fibrin, fibronectin, and other proteins by means of surface integrins which bind these matrix components (37, 38). As shown in animal models, fibrin, with or without covalent linkage to other stromal proteins, provides a highly favorable matrix for the inward migration of new blood vessels and fibroblasts; these, in turn, deposit mature connective tissue components such as interstitial collagens, resulting in a connective tissue that closely resembles the granulation tissue of healing wounds (39).

VPF is also a selective endothelial cell mitogen (11, 12, 40) and therefore may also be expected to stimulate the growth of new blood vessels directly. In addition, VPF exerts other direct effects on endothelial cells that are likely to affect the extracellular matrix: *e.g.,* release of von Willebrand factor (18); induction of procoagulant activity (41); and plasminogen activator (42). Also, VPF may be chemotactic for macrophages (41) and in this way may modulate the inflammatory response.

Finally, we have reported a potentially interesting incidental finding, unrelated to cancer, namely that both VPF mRNA and protein are found in neurons of the normal gastrointestinal myenteric plexus. This source of VPF has not been described previously and its function is unknown. It will be interesting to determine whether VPF is found in other autonomic neurons and whether neuronal VPF might regulate microvascular permeability or other endothelial cell functions.

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