Vascular Permeability Factor: A Tumor-derived Polypeptide that Induces Endothelial Cell and Monocyte Procoagulant Activity, and Promotes Monocyte Migration

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Summary

Systemic infusion of low concentrations of tumor necrosis factor/cachectin (TNF) into mice that bear TNF-sensitive tumors leads to activation of coagulation, fibrin formation, and occlusive thrombosis exclusively within the tumor vascular bed. To identify mechanisms underlying the localization of this vascular procoagulant response, a tumor-derived polypeptide has been purified to homogeneity from supernatants of murine methylcholanthrene A–induced fibrosarcomas that induces endothelial tissue factor synthesis and expression (half-maximal response at ~300 pM), and augments the procoagulant response to TNF in a synergistic fashion. This tumor-derived polypeptide was identified as the murine homologue of vascular permeability factor (VPF) based on similar mobility on SDS-PAGE, an homologous NH_2-terminal amino acid sequence, and recognition by a monospecific antibody to guinea pig VPF. In addition, VPF was shown to induce monocyte activation, as evidenced by expression of tissue factor. Finally, VPF was shown to induce monocyte chemotaxis across collagen membranes and endothelial cell monolayers. Taken together, these results indicate that VPF can modulate the coagulant properties of endothelium and monocytes, and can promote monocyte migration into the tumor bed. This suggests one mechanism through which tumor-derived mediators can alter properties of the vessel wall.

Tumor vasculature, derived from the ingrowth of normal vessels, is in continuous contact with host mediators and metabolic/regulatory products of the tumor. Since the endothelium is the actual interface between host and tumor, studies into the properties of tumor vascular endothelium provide insights into the effects of local factors on vascular function. One striking example of the altered responsiveness of tumor vasculature is observed after the systemic infusion of a low concentration of tumor necrosis factor/cachectin (TNF) into mice that bear TNF-sensitive tumors, such as methylcholanthrene A–induced fibrosarcomas (meth A sarcomas)[1] (1–3). Under these conditions, intravascular fibrin deposition, progressing to occlusive thrombosis and associated with a fall in blood flow, is observed in tumor vessels, but not in the normal vessels. An important potential mechanism through which TNF can initiate this localized thrombotic process involves enhanced focal induction of endothelial synthesis and expression of tissue factor, a cell surface procoagulant cofactor (4–6). Tissue factor is believed to be the major initiator of coagulation in vivo (6).

To understand mechanisms that could account for selective or enhanced induction of tissue factor in endothelium of tumor vasculature, we have examined the role of tumor-derived mediators. The results indicate that cultured meth A sarcoma cells elaborate at least two distinct polypeptides that induce tissue factor in endothelium and enhance the procoagulant response to TNF. In a previous report (7), we described the properties of one of these polypeptides, referred to as meth A factor. This report describes the second of these proteins. The biochemical properties, NH_2-terminal sequence, and reactivity with monospecific antisera indicate that this second tumor-derived polypeptide is the murine homologue of vascular permeability factor (VPF) (8), a protein previously shown to promote endothelial growth and angiogenesis, as

[1] *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; meth A sarcomas, methylcholanthrene A–induced fibrosarcomas; VEGF, vascular endothelial growth factor; VPF, vascular permeability factor.

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well as vascular leakage (9). VPF is closely related, if not identical, to vascular endothelial growth factor (VEGF) (10–12). Murine VPF is also shown here to stimulate tissue factor expression in monocytes, and to cause monocyte chemotaxis, through collagen membranes and through endothelial monolayers. These findings suggest that tumor-derived VPF can modulate the vascular procoagulant response to TNF. Furthermore, these results suggest that VPF could attract monocytes to the tumor bed where they, in turn, could further promote activation of coagulation and contribute to fibrin deposition.

Materials and Methods

Cell Culture and Assays. Human umbilical vein endothelial cells were prepared by the method of Jaffe (13), as modified by Thornton et al. (14). Experiments were carried out within 48 h of the cells achieving confluence, and cultures were characterized as described previously (7). Meth A cells, provided by Drs. Hoffman and Old (Memorial Sloan-Kettering Cancer Center, New York) (15), were grown in RPMI 1640 containing 10% calf serum, as previously described (7, 16). In brief, they were immobilized in a continuously perfused 3-liter bioreactor (Bellco Glass, Inc., Vineland, NJ). Conditioned serum-free medium was collected from the bioreactor at a rate of 416 ml/h, and concentrated 20-fold by ultrafiltration before purification.

Expression of tissue factor in endothelial cells was assessed by incubating cultures with column fractions or purified VPF in medium 199 containing 10 mM Hepes (pH 7.4), polymyxin B (1 μg/ml) in the presence/absence of TNF, antibodies, or other reagents (e.g., cycloheximide). These studies were carried out in 35-mm diameter wells with $\sim 1.2 \times 10^5$ cells/cm^2 at confluence. Samples of murine or guinea pig VPF were incubated with either endothelium alone or in the presence of human rTNF for 6 h (or for the indicated times) at 37°C. Assays were carried out with whole cells obtained in suspension after scraping from the dish with a rubber policeman (cell viability was >90% based on trypan blue exclusion) or on intact monolayers. Tissue factor activity was determined by a two-stage coagulant assay and, in a limited number of experiments, by measuring the formation of factor Xa in the presence of purified factor VIIa (8 nM) and X (1.5 μM) using a synthetic substrate assay to assess factor Xa formation, as described previously (17). Further characterization of endothelial procoagulant activity included preincubation of cultures with a blocking mAb to human tissue factor (5 μg/ml) (18) for 30 min at 37°C before carrying out the tissue factor assay (either coagulant or amidolytic). Tissue factor equivalents were determined by using a standard curve of purified human tissue factor (19). To construct the standard curve, tissue factor was reconstituted into phosphatidylserine/phosphatidylcholine vesicles (20:80) (19), and factor Xa formation was determined by the two-stage coagulant assay. Purified human tissue factor reconstituted into phospholipid vesicles and blocking mAb to human tissue factor was generously provided by Dr. R. Bach (Mt. Sinai School of Medicine, New York) (18, 19).

Human monocytes were isolated from the mononuclear fraction of peripheral blood by a discontinuous Ficoll gradient (histopaque 1077; Sigma Chemical Co., St. Louis, MO) using gradient centrifugation with Percoll (Sigma Chemical Co.), as described by Fluks (20). Monocyte preparations were assessed morphologically with Wright's stain (>90), and their viability was >90% by trypan blue exclusion. Induction of monocyte tissue factor was studied by incubating the cells (10^5/assay) for 6 h at 37°C in RPMI 1640 containing 10 mM Hepes, pH 7.4, penicillin, streptomycin (50 U/ml; 50 μg/ml), polymyxin B (5 μg/ml), and autologous serum (10%) in the presence of varying concentrations of VPF or other factors. Tissue factor was determined as described above, and the same protocol was followed with the antibody to tissue factor.

Chemotaxis of monocytes and other peripheral blood cells was investigated using the method of Quinn et al. (21). Briefly, cells isolated as described above were placed in the upper chamber, and a test substance was placed in the lower chamber. In certain experiments, mediators were also placed in the upper chamber. Chemotactic assays were performed after 3 h of incubation, and cells in 10 fields were counted for each condition assayed. Chemotaxis of monocytes across endothelial monolayers was studied by growing endothelium to confluence on Cellagen (ICN Biomedicals, Costa Mesa, CA), and VPF was placed in the outer (lower) well. At the end of the incubation period, monolayers were fixed for electron microscopy in glutaraldehyde (2.5%)/sodium cacodylate (0.1 M, pH 7.2), post-fixed in osmium tetroxide (2%), washed, en bloc stained in uranyl acetate (0.5%), dehydrated in ethanol, and embedded in Epon 812. Sections were observed in an electron microscope (model 300; Philips Electronic Instruments, Inc., Mahwah, NJ).

Purification of VPF and Other Reagents. Murine VPF was purified from serum-free medium conditioned by cultured meth A cells by sequential chromatography on S-Sepharose, Mono S, FPLC and reversed-phase HPLC (C_4). Conditioned medium was acidified to pH 5.5 with hydrochloric acid, diluted 1:1 with 50 mM MES (pH 5.5), and applied to the cation exchange resin S-Sepharose Fast Flow (5 ml resin/liter) (Pharmacia Fine Chemicals, Piscataway, NJ). The resin was washed extensively in buffer containing MES (50 mM; pH 5.5), NaCl (50 mM), octyl-β-glucoside (0.1%), and PMSF (0.2 mM), and step-eluted with the same buffer containing 1 M NaCl. Fractions with OD_{280} >0.05 were pooled and dialyzed extensively against phosphate buffer (50 mM; pH 5.5) containing NaCl (50 mM) and octyl-β-glucoside (0.1%). These procedures were performed at 4°C. The pool from Fast S was then applied to a Mono S FPLC column (HR 5/5; Pharmacia Fine Chemicals) equilibrated in the same buffer. The column was eluted with an ascending salt gradient (0–1 M NaCl), the active fractions were pooled, the pH was adjusted to 2.2 with trifluoroacetic acid, and the sample was applied to a reversed-phase HPLC column (C_4 reversed phase; 4.6 × 20 mm, Supellco, Inc., Bellefonte, PA). The column was eluted with a linear acetonitrile gradient (0–100%), aliquots of the fractions were concentrated by lyophilization, and then processed as follows. To assess tissue factor inducing activity, samples were solubilized in endothelial cell culture medium (see above), and for SDS-PAGE according to the method of Laemmli (22), samples were solubilized in sample buffer. To elute samples from SDS-PAGE, gel slices (0.5 cm) were incubated in elution buffer (Tris/HCl, 0.1 M, pH 7.5; NaCl, 0.3 M; EDTA, 2 mM; octyl-β-glucoside, 0.2%; BSA, 0.1 mg/ml) with agitation overnight at 4°C. Samples were centrifuged, and aliquots of the supernatants were tested for tissue factor–inducing activity on endothelial monolayers as described. For sequence analysis, purified murine VPF was subjected to reduced SDS-PAGE, transferred to polyvinylidene difluoride membranes, by the method of Matsudaira (23), and the band corresponding to M_r ~23,000 was cut out and sequenced (model 470A; Applied Biosystems, Inc., Foster City, CA).

Purified guinea pig VPF and monospecific blocking antibody to guinea pig VPF (made in a rabbit) were prepared as described previously (10). Immunoadsorption of VPF was accomplished using the IgG fraction of the anti-VPF antiserum bound to protein A–Sepharose (Pharmacia Fine Chemicals), as described in the legend

to Fig. 4. In pilot studies, each VPF-containing sample was always adsorbed at least twice to protein A–coated beads with adsorbed anti-VPF IgG to be certain that maximum removal of VPF had been accomplished. Murine meth A factor was also prepared by a previously described procedure (7). Purified, human rTNF-α (~10^8 U/mg) was generously provided by Dr. P. Lomedico, Hoffman-La Roche, Inc., Nutley, NJ.

Results

Purification of a ~46 kD Polypeptide from Murine meth A Sarcoma Cells that Induces Endothelial Procoagulant Activity. The localized vascular response that follows systemic infusion of low concentrations of TNF into mice that bear meth A fibrosarcomas could result, at least in part, from the concerted action of tumor-derived mediators and TNF acting on the endothelial cell. In a previous study, a polypeptide factor of M_r ~56,000 on reduced SDS-PAGE (meth A factor), isolated from cultured meth A fibrosarcoma cells, induced endothelial tissue factor and enhanced the procoagulant response to TNF (7). During the course of these studies, another mediator, distinct from meth A factor, was identified in the conditioned medium of meth A cells, which was also a potent inducer of endothelial procoagulant activity (which has been characterized as tissue factor, see below), and is the subject of this report.

After batch adsorption and elution of serum-free medium conditioned by meth A cells from S-Sepharose chromatography on heparin-ultragel separated the procoagulant-inducing activity into two pools (Fig. 1): activity I eluted at ~0.15 M NaCl, whereas activity II required ~0.8 M NaCl for elution. Using our previously described procedure (7), meth A factor was purified from the low salt pool (Fig. 1, *inset*). The material in the second activity peak with higher affinity for

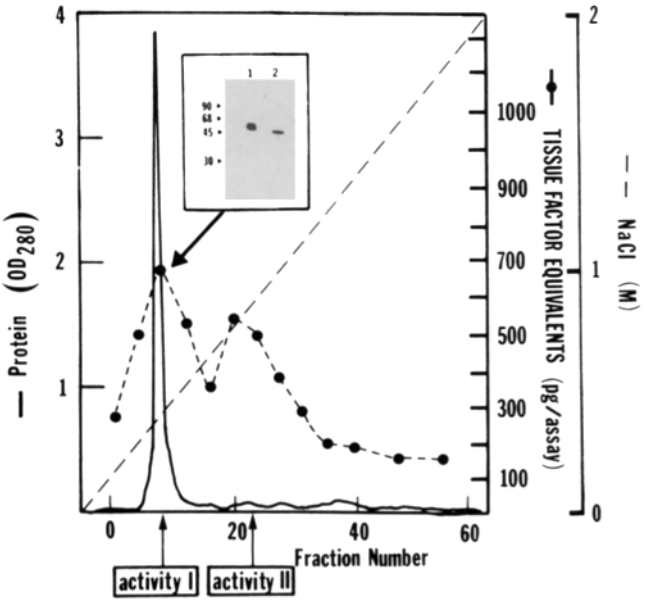

Figure 1. Chromatography of partially purified meth A–conditioned medium on heparin-ultragel. Meth A–conditioned medium was subjected to batch adsorption on S-Sepharose (as described in the text), step eluted with 1 M NaCl, dialyzed against phosphate (50 mM; pH 7.4), and then applied to heparin-ultragel. The heparin column was eluted with an ascending salt gradient (0–2 M NaCl), and fractions were assayed for their ability to induce tissue factor activity in endothelial cells as described. The two separated peaks of activity (I and II) are indicated with arrows. (*Inset*) Procoagulant inducing activity peak I from the heparin column was pooled, and subjected to further purification using the procedure previously described for meth A factor (see text). (Lane *1*) Purified meth A factor on reduced; (lane *2*) nonreduced SDS-PAGE. Arrowheads indicate migration of standard proteins run simultaneously: phosphorylase (90 kD), albumin (68 kD), OVA (45 kD), and carbonic anhydrase (30 kD).

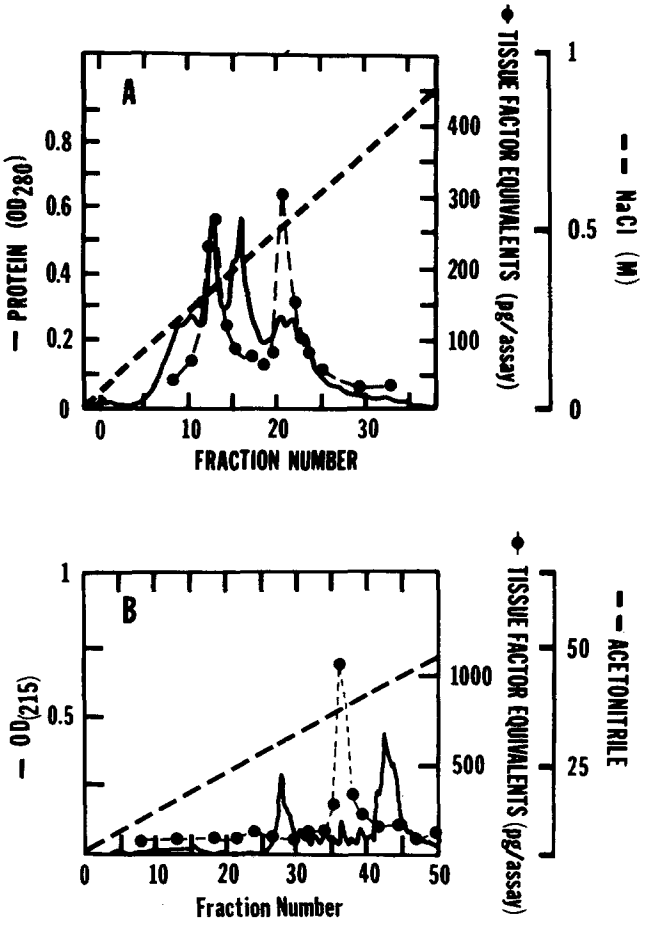

Figure 2. Purification of murine VPF from meth A–conditioned medium. (*A*) Chromatography of meth A–conditioned medium on Mono A. Meth A–conditioned medium was prepared for chromatography on Mono S as described in the text, and the column was eluted with ascending salt gradient (0–1 M NaCl). Tissue factor activity induced in endothelial cell cultures was assayed after a 6-h incubation with aliquots from each fraction. (*B*) Chromatography on reversed-phase HPLC. The pool of fractions containing peak procoagulant-inducing activity from the Mono S column was applied to a reversed-phase C_4 column (4.6 × 20 mm) in the presence of 0.1% trifluoroacetic acid and eluted with an ascending acetonitrile gradient (0–50%). Tissue factor activity induced in endothelial cultures was assayed as described in the text and in *A*. Peak activity eluted at an acetonitrile concentration of ~38%. The mean of duplicate determinations of tissue factor activity is shown in *A* and *B* (only the results of peak fractions are shown).

Table 1. *Purification of Murine VPF*

Step	Protein	Total activity*	Neutralized VPF activity‡	Yield	Specific VPF activity§	Purification for each step
	mg	*× 10³*	*× 10³*	*%*	*× 10³*	*fold*
Meth A–conditioned medium	800.00	1,200	750	100	0.9	1.0
S-Sepharose	180.00	980	412	55	2.3	2.4
Mono S	2.00	340	338	45	169	73.5
Reversed-phase	0.005	125	125	17	25,000	148.0

Depicted is the purification of 10 liters of meth A conditioned by sequential batch adsorption to S-Sepharose, Mono S FPLC, and reversed-phase HPLC, as described in the text.

* Total activity denotes tissue factor activity induced in the endothelium by column fractions after a 6-h incubation period. The numbers represent picograms of tissue factor induced in the endothelium per 10^6 cells.

‡ Tissue factor induction due to VPF (neutralized VPF activity) was determined by adsorption of samples with immobilized anti-VPF IgG, as described in the text.

§ Neutralized VPF activity divided by the amount of protein (milligrams).

immobilized heparin was studied in detail. First, tumor-conditioned medium was subjected to sequential chromatography on cation exchange resins, first by batch adsorption on Fast S and then, after dialysis, on Mono S FPLC. This led to the identification of a major peak of endothelial procoagulant-inducing activity that eluted at 0.5 M NaCl from Mono S (Fig. 2 *A*), and proved to have an affinity for heparin comparable with activity pool II from the heparin ultragel column in Fig. 1. The other peak of activity, which eluted at 0.3 M, was the previously identified meth A factor and corresponded to activity peak I from the heparin-ultragel column (Fig. 1). The material that eluted at a higher salt concentration from the Mono S column was pooled and subjected to reversed-phase HPLC chromatography (C_4), eluting at an acetonitrile concentration of ~38% (Fig. 2 *B*). Using the sequence of steps outlined above, ~2.5×10^4-fold purification was obtained with a yield of ~17% (see Table 1).

When fractions with peak activity from the reversed-phase column were subjected to SDS-PAGE, a single major band was observed under reducing conditions with M_r corresponding to ~23 kD (Fig. 3 *a*). Elution of material from the gel using the same sample prepared under reducing conditions, but not subjected to the fixation and staining procedure, produced a peak of endothelial procoagulant inducing activity (Fig. 3 *b*) that comigrated with the ~23-kD band. Thus, even after reduction, activity was retained by the purified polypeptide, probably consequent on refolding of the protein during the incubation in nondenaturing buffer after gel elution. Nonreduced, the same sample applied to in Fig. 3 *a* migrated as a single band, with M_r corresponding to ~46

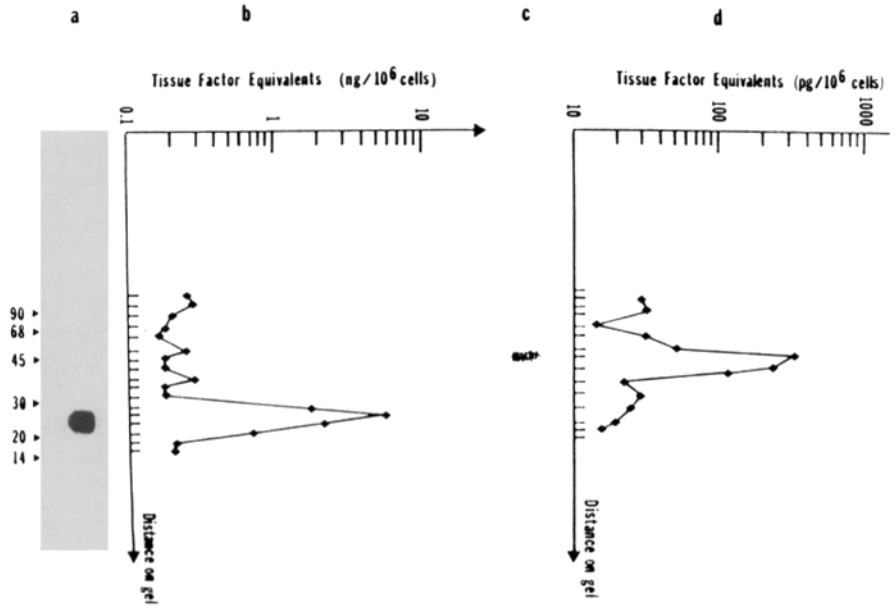

Figure 3. SDS-PAGE (12.5%) and gel elution of murine VPF. (*a*) Reduced SDS-PAGE of purified murine VPF (after reversed-phase chromatography) visualized by silver staining. (*b*) Activity profile of material eluted from the indicated slice of a reduced SDS gel run identically as in *a* (see text). Data are expressed as tissue factor equivalents, as described in the text. (*c*) Nonreduced SDS-PAGE of purified murine VPF visualized by silver staining. (*d*) Elution of tissue factor–inducing activity from slices of the nonreduced SDS gel corresponding to *c*. The mean of duplicate determinations are shown. Apparent molecular weights, shown by the arrows, were interpolated from semi-logarithmic plots based on the migration of standard proteins run simultaneously (phosphorylase, 90,000; BSA, 68,000; OVA, 45,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,000; lysozyme, 14,000).

kD (Fig. 3 *c*). Elution of material from nonreduced samples identical to those applied in Fig. 3 *c* demonstrated that the peak of procoagulant-inducing activity had also migrated in parallel with the ~46-kD band (Fig. 3 *d*).

Further properties of the tumor-derived ~46-kD polypeptide included resistance to heat (98°C for 10 min) and acidic conditions (pH 2.0 for 1 h), but sensitivity to protease treatment (protease K, 0.4 ng/10 ng substrate for 2 h at 37°C). Although this material could induce procoagulant activity in endothelium, it had no direct, intrinsic ability to shorten the clotting time of recalcified plasma (data not shown).

Identification of the Murine meth A Sarcoma–derived ~46-kD Polypeptide as the Murine Homologue of VPF. VPF is a polypeptide mediator produced constitutively by certain tumor cells and cell lines, e.g., guinea pig line 10 cells, bovine pituitary cells, and human U937 cells (8–12), that increases vascular permeability and stimulates endothelial proliferation. On SDS-PAGE, VPF migrates with M_r ~34,000–46,000 nonreduced and ~18,000–23,000 reduced, similar to the polypeptide isolated from the meth A cells described in Figs. 1 and 2. Furthermore, VPF has been reported to have an affinity for immobilized heparin comparable with that observed for activity II in Fig. 1 (10). Based on these findings, it seemed possible that the murine ~46-kD polypeptide with higher affinity for heparin was similar to VPF.

NH_2-terminal sequence analysis showed that five of the first seven residues of the murine ~46 kD polypeptide were identical to that reported for guinea pig VPF. The sequence of the murine ~46 kD factor was Ala-Pro-Thr-Thr-Glu-Gly-Glu, whereas the sequence of guinea pig VPF (8) is Ala-Pro-Met-Ala-Glu-Gly-Glu (in the case of bovine VPF/VEGF, four of the first seven residues are identical) (12).

The identity of the 46-kD polypeptide with VPF was confirmed using a monospecific, neutralizing antibody to guinea pig VPF. Fig. 4 *A* shows that anti–guinea pig IgG could quantitatively prevent induction of endothelial tissue factor by the murine ~46-kD polypeptide, coincident with immunoadsorption of this polypeptide from samples (data not shown). The anti-VPF IgG did not interact with the previously described meth A factor (7), confirming our impression that VPF and meth A factor are distinct. Furthermore, purified guinea pig VPF also induced procoagulant activity in endothelium (Fig. 4 *A*). Taken together, these data indicate that the murine ~46-kD polypeptide with high affinity for heparin derived from meth A sarcoma cells is most likely the murine homologue of VPF (termed murine VPF).

The procoagulant activity induced by VPF in the endothelium was identified as tissue factor, based on its inhibition when cultures were pre-incubated with a neutralizing antibody to tissue factor (Fig. 4 *B*).

Modulation of Endothelial Cell Procoagulant Activity by VPF. When purified murine or guinea pig VPF was incubated with endothelial cultures, induction of tissue factor activity occurred in a time-dependent manner (Fig. 5, *insets*). Cellular procoagulant activity was detectable by 4 h of incubation, maximal by 6–9 h, and began to fall off by 10 h after the exposure to either purified murine or guinea pig VPF. Expression of tissue factor in response to VPF resulted from

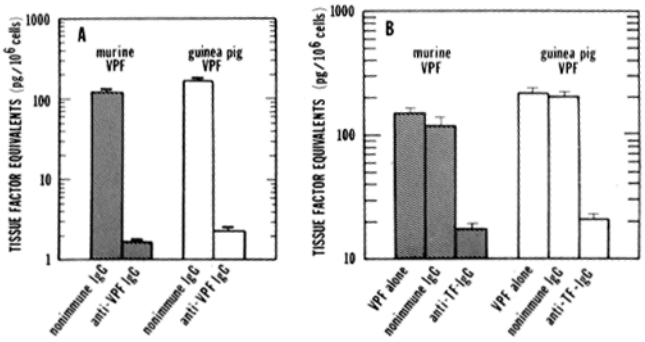

Figure 4. Effect of VPF and antibody to VPF on endothelial cell tissue factor activity. (*A*) Induction of endothelial tissue factor by VPF. Murine VPF (5 ng/ml) or guinea pig VPF (5 ng/ml) was incubated with polyclonal IgG to VPF (anti-VPF; 10 μg) or nonimmune IgG (10 μg/ml) adsorbed to protein A–Sepharose (0.1 ml), and the resin was removed by centrifugation. Samples were then incubated with endothelial monolayers for 6 h at 37°C, and tissue factor activity was assessed as described in the text. The mean of triplicate determinations is shown. (*B*) Identification of the endothelial procoagulant activity induced by VPF as tissue factor. Murine VPF (5 ng/ml) or guinea pig VPF (VPF; 5 ng/ml) was incubated with endothelial monolayers for 6 h at 37°C, and then after washing the cultures, anti–tissue factor IgG (anti-TF IgG; 5 μg/ml) or the same amount of nonimmune IgG was incubated with the cultures under serum-free conditions for 30 min at 37°C. Then, tissue factor activity was assessed as described in the text. The mean ± SD of triplicate determinations is shown. For certain points, where error bars are difficult to see, the error was smaller than the symbol size.

de novo protein synthesis as indicated by inhibition in the presence of cycloheximide. When TNF was present along with VPF in the incubation mixture, tissue factor expression was enhanced at each time point after 2 h. Induction of endothelial tissue factor by murine VPF also depended on the concentration of mediator, being half-maximal at ~300 pM, and maximal by ~1 nM (Fig. 5, *A* and *B*). This is somewhat higher than the half-maximal concentration of VPF reported for stimulation of endothelial growth (60–100 pM), although it is lower than levels of VPF that increase vascular permeability in vivo (8–12). When endothelial cells were incubated with VPF and TNF, induction of tissue factor by the two agents was greater than that observed with either agent alone, and appeared to be more than additive. Furthermore, in the presence of TNF (5 pM), the concentration of VPF required for the half-maximal response decreased to ~150 pM with murine VPF and ~90 pM with guinea pig VPF.

Since in in vivo situations endothelium is likely to be exposed to several tumor-derived mediators over longer times, it was important to examine the combined effects of VPF and meth A factor in the presence and absence of TNF. For these experiments, endothelial cells were pre-incubated with one mediator for 24 h (VPF or meth A factor), and then the second mediator was added with or without TNF for an additional 6 h (Fig. 6). Because of this difference in incubation times between the experiments in Fig. 6 and Fig. 5, *A* and *B* (in the latter the total incubation time was only 6 h), the absolute amount of tissue factor induced in endothelium in the two experiments is not closely comparable.

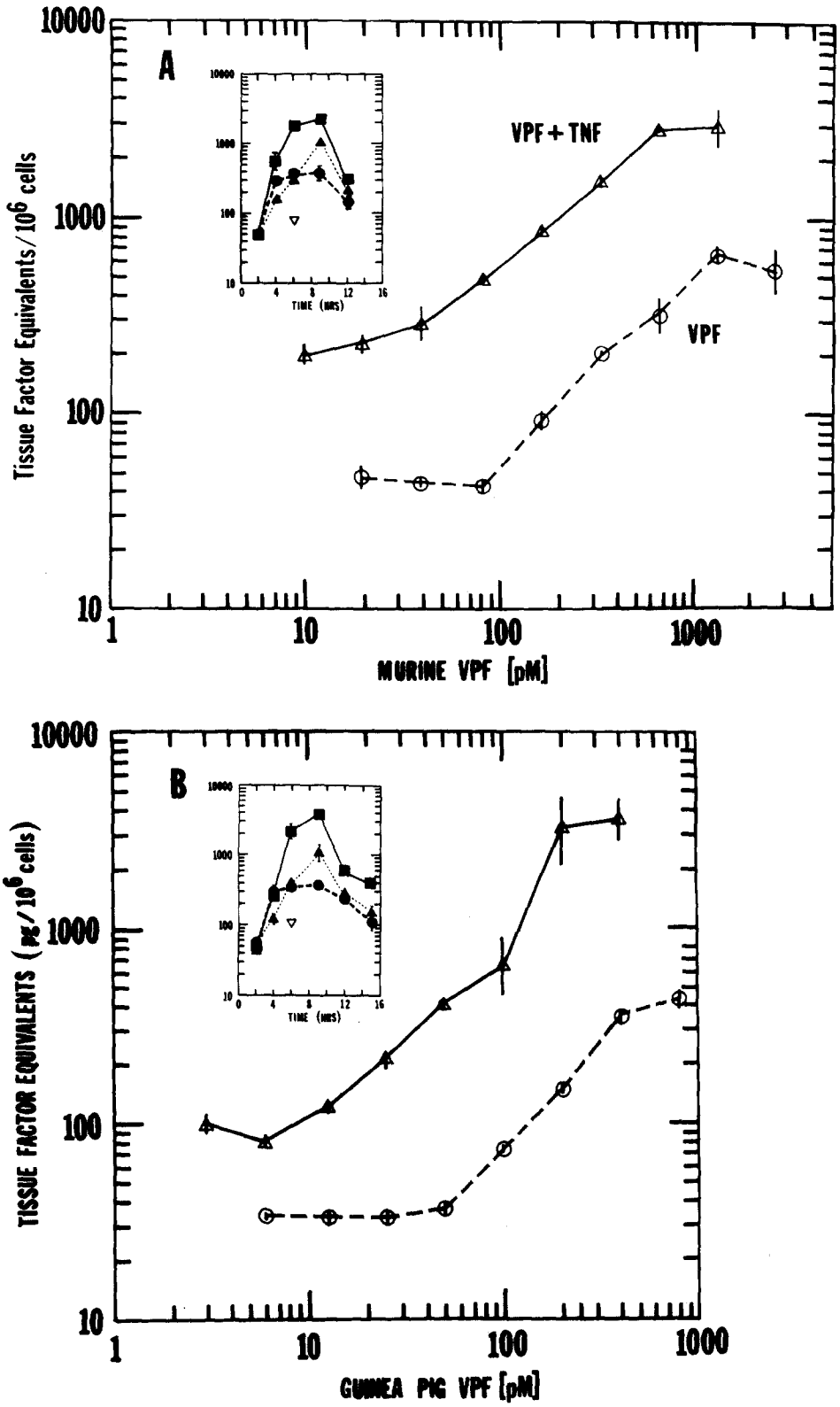

Figure 5. Dose dependence and time course of endothelial cell tissue factor induction by murine VPF and guinea pig VPF. Endothelial monolayers were incubated for 6 h at 37°C with the indicated concentrations of either murine VPF (*A*) or guinea pig VPF (*B*) either alone (*dotted line*) or in the presence of TNF (5 pM; *solid line*). When endothelial cells were incubated under the same conditions with TNF alone (5 pM), ~40 pg of tissue factor per 10^6 cells was induced (*Insets*) Time course for induction of tissue factor by VPF. (*Inset A*) Purified VPF (200 pM) was incubated for the indicated times with endothelial monolayers either alone (*filled circles*) or in the presence of TNF (5 pM; *filled squares*). TNF alone (5 pM) is shown by the filled triangles. The effect of cycloheximide (5 μg/ml) on VPF-induced expression of endothelial procoagulant activity (endothelium was incubated with VPF [200 pM] and cycloheximide) is shown by the open triangle. (*Inset B*) The same study as in *inset A*, except purified guinea pig VPF was used in place of murine VPF. In each inset, incubation time (*x*-axis) is plotted vs. tissue factor equivalents (pg/10^6 cells; *y*-axis). The mean ± SD of triplicate determinations is shown. In certain cases, where the error bars are difficult to see, the error was smaller than the symbol size.

This was expected from the time course of tissue factor expression induced by VPF (Fig. 5, *insets*). When endothelium was preincubated with a low concentration of either VPF or meth A factor alone, induction of tissue factor activity was observed, and later, addition of TNF enhanced the response. Simultaneous exposure of endothelial cells to VPF and meth A factor together did not enhance the procoagulant response compared with that observed with either mediator

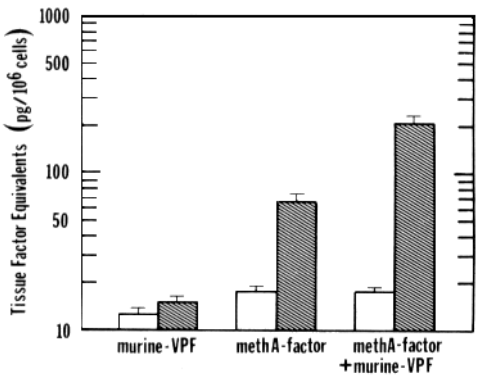

Figure 6. Effect of VPF, meth A factor, and TNF on endothelial tissue factor. Endothelial monolayers were incubated with either murine VPF (150 pM) alone (*open bar*) or in the presence of TNF (5 pM; *hatched bar*); with meth A factor (30 pM) alone (*open bar*) or in the presence of TNF (5 pM; *hatched bar*); or with meth A factor + murine VPF (at the same concentrations as above) alone (*open bar*) or in the presence of TNF (5 pM; *hatched bar*). For these studies, endothelial cultures were preincubated for 24 h at 37°C with VPF, and then meth A factor, in the presence or absence of TNF, was added for an additional 6 h. At the end of the incubation period, tissue factor activity was determined as described in the text. The mean ± SD of triplicate determinations is shown.

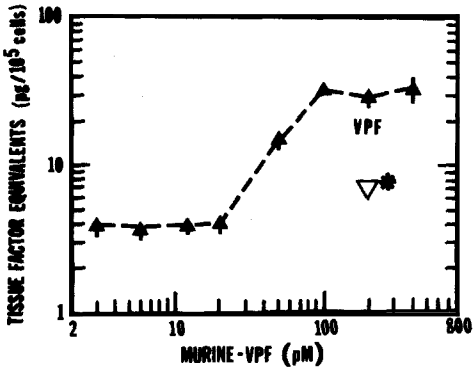

Figure 7. Induction of monocyte tissue factor by VPF. Peripheral blood monocytes (10^5/assay) were incubated for 6 h at 37°C with the indicated concentrations of murine VPF. When VPF was adsorbed from samples with specific antibody, as in Fig. 4, VPF-mediated induction of tissue factor was blocked (*open triangle*). When antibody to tissue factor (5 μg/ml) was incubated with monocytes (30 min at 37°C), after pre-incubation of cultures with VPF for 6 h, procoagulant activity was blocked (*). The mean ± SD of triplicate determinations is shown. In certain cases, where the error bars are difficult to see, the error was smaller than the size of the symbol.

alone, but when TNF was added in the presence of both tumor-derived factors, the greatest tissue factor induction of all was observed.

Stimulation of Monocyte Procoagulant Activity by VPF. Monocyte-macrophages are a recognized source of inducible tissue factor activity (24–26), both within the vasculature, and in the extravascular space. This led us to examine if VPF could induce monocyte tissue factor. The addition of murine VPF to monocytes led to the production of monocyte procoagulant activity that could be blocked by neutralizing antibody to human tissue factor (Fig. 7). This occurred in a time-dependent manner, and could be blocked by the simultaneous addition of cycloheximide to cultures (data not shown). The effect of murine VPF on monocyte tissue factor was also dependent on the concentration of mediator, being half-maximal at ~60 pM (Fig. 7). Pre-incubation of VPF-containing samples with the monospecific anti-VPF antibody linked to a solid support blocked VPF-mediated induction of monocyte tissue factor, indicating that VPF was the active species for the induction of tissue factor.

Stimulation of Monocyte Chemotaxis by VPF. For VPF-stimulated monocytes to exert their effect locally, a mechanism promoting their migration was required. This led us to examine if VPF could selectively induce monocyte migration. VPF induced migration of monocytes isolated from human blood. Checkerboard analysis indicated that this was chemotaxis and not chemokinesis (Table 2). Chemotaxis was blocked when samples of VPF were pre-absorbed with anti-VPF IgG (data not shown). In contrast to induction of monocyte migration, VPF was not a chemoattractant for human polymorphonuclear leukocytes or lymphocytes. When endothelial cells were grown to confluence on collagen mem-

Table 2. *Checkerboard Analysis of Monocyte Chemotactic Response to VPF*

VPF (lower compartment)	VPF (upper compartment)			
	0 pM	10 pM	30 pM	100 pM
pM				
10	4.7 ± 1.2*	4.3 ± 0.5	5.0 ± 1.6	4.0 ± 1.2
30	8.3 ± 1.2	6.0 ± 0.8	5.7 ± 1.6	4.5 ± 1.4
100	27.0 ± 2.9	13.0 ± 2.1	3.3 ± 0.5	3.4 ± 0.8

Checkerboard analysis was carried out by adding the indicated concentration of murine VPF in balanced salt solution to the upper or lower well of the chemotactic chamber, as described in the text. The positive control, consisting of the chemotactic response to FMLP (10^{-7} M), resulted in 40 cells per high-powered field. No VPF in the lower chamber resulted in <4 cells per high-powered field.
* Number of cells per high-powered field ± SEM, $n = 4$.

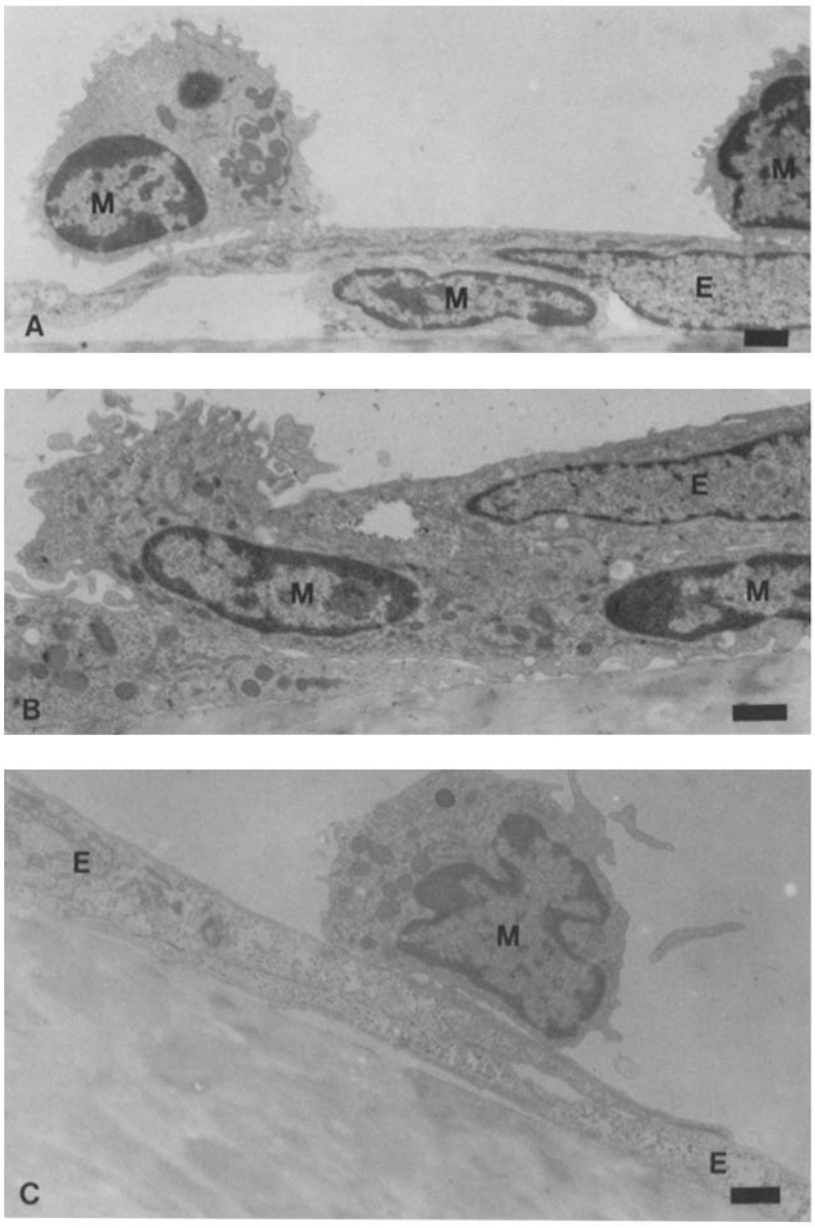

Figure 8. Induction of monocyte migration across endothelial cell monolayers by VPF. Confluent endothelial monolayers grown on collagen membranes were incubated with human peripheral blood monocytes (10^6/cm^2) for 3.5 h at 37°C in the presence (*A* and *B*) or absence (*C*) of VPF (100 pM) in the compartment beneath the monolayer. Cultures were washed and prepared for electron microscopy as described in the text. Monocytes (*M*) can be seen beneath the endothelial (*E*) monolayer and also attached to the endothelial surface. The scale bar denotes 1 μm in each case.

branes, and VPF was placed in the compartment below the monolayer, it induced monocytes to cross the monolayer (Fig. 8). In the presence of VPF, there was increased association of monocytes with the endothelial cells, and monocytes were observed migrating between cell–cell junctions and beneath the endothelial monolayer (Fig. 8, *A* and *B*), compared with controls without VPF (Fig. 8 *C*). Over several experiments, VPF appeared to induce an approximately threefold increase in the number of monocytes migrating across endothelial monolayers.

Discussion

In view of the potential importance of perturbations of endothelial coagulant properties, much work has focussed on cytokines, including mediators produced by tumor cells, and their ability to change the endothelium from a surface promoting blood fluidity to one that could support clot formation (27–31). In a previous study, we described a polypeptide produced by meth A sarcoma cells that induced tissue factor in endothelium and enhanced the procoagulant response of these cells to TNF (7). The experiments described in the current study demonstrate an additional, distinct polypeptide that appears to be the murine homologue of VPF. The present data suggest that elaboration of VPF by meth A sarcoma cells can induce tissue factor production by the endothelium. VPF has been characterized in previous studies as an agent that increases vascular permeability, and stimulates endothelial growth in vitro and angiogenesis in vivo (8–12). The growth-promoting property of VPF has led to the independent

purification and cloning of a group of proteins known as VEGF (8, 12). One of the cDNA variants of VEGF has the same amino acid sequence as VPF, and the other two are smaller versions that could arise from differential mRNA splicing (12). Our isolation of VPF using an assay reflecting induction of tissue factor in endothelial cells represents the third independent isolation of the same factor using three different types of assays, and suggests a new and unexpected role for VPF in the regulation of coagulation.

Since growing cells have been reported to express more tissue factor than cells in quiescent cultures (32), it was important to determine whether VPF-mediated production of tissue factor was secondary to induction of cell growth. This would not seem to be the case, however, since the experiments were carried out on confluent cultures, in which there was no increase in cell number after addition of VPF. Furthermore, we have also observed in pilot studies that basic fibroblast growth factor (bFGF), another potent mitogen for endothelial cells (33, 34), did not induce tissue factor expression, although the characteristic change in morphology of the monolayer reported with bFGF was observed (35). This indicates that induction of tissue factor is a specific effect of VPF, not of any endothelial mitogen.

When TNF was infused into mice bearing sensitive tumors, an intense and localized vascular reaction occurred that led to decreased tumor blood flow and clot formation in the tumor vasculature (3). This observation suggested that endothelial cells in the tumor bed may have been specifically primed to respond to the infused TNF. One mechanism that could account for this is enhancement of TNF-mediated induction of tissue factor by tumor products such as VPF and meth A factor. The experiments presented in Fig. 6 lend support to this hypothesis. Furthermore, experiments in which endothelial cells were incubated for longer times (several days) with VPF, to mimic the in vivo situation, and then challenged with TNF, also showed an enhanced procoagulant response to TNF. If one compares generation of endothelial tissue factor in unfractionated tumor-conditioned medium, it would appear that VPF and meth A factor each contribute ~50% towards the induction of procoagulant activity (Table 1). This is consistent with the concept that both factors contribute to induction of tissue factor in the tumor vascular bed.

Although the current work has focused on induction of procoagulant activity by VPF and TNF, the interaction of these factors may be at least as important for altering other endothelial properties, such as barrier function of the cell monolayer. For example, both VPF and TNF have been shown to increase vascular permeability, though, presumably, by independent and potentially different mechanisms (9, 36–38). Thus, an exaggerated and rapid decrease in barrier function of endothelium in tumor vasculature after TNF infusion could be a hemodynamically significant factor in determining alterations in tumor blood flow. In terms of mechanisms through which VPF and TNF bring about changes in cellular properties, we considered the possibility that VPF could enhance endothelial binding sites for TNF, by analogy with studies indicating that certain mediators and induction of the growth/migratory state in endothelium can lead to enhancement of TNF binding (39, 40). However, pilot radioligand binding studies with radioiodinated TNF did not demonstrate a significant effect of VPF on the binding of TNF to the endothelial cell surface, suggesting that VPF and TNF may reinforce each other's actions at an intracellular site. In this context, VPF has recently been shown to induce a rise in endothelial cytosolic calcium (41), suggesting a possible common locus in intracellular signal transduction mechanisms at which TNF and VPF could act.

Solid tumors are dependent on both growth of the stroma and angiogenesis (27). H. Dvorak (28) has proposed that increased vascular permeability in the tumor leads to the escape of plasma components, including fibrinogen, and can promote formation of a fibrin gel that serves as a substrate for tumor and blood vessel growth. Since endothelium has a central role in regulating both the coagulation mechanism and barrier function (29), we have examined the effects of tumor-derived mediators on these properties of endothelial cells. Alterations in these homeostatic functions are closely linked, since activation of coagulation leads to formation of proteases, such as thrombin and fibrin, which have been shown to change endothelial shape and cause gaps to form between hitherto contiguous cells, leading to increased vascular permeability (30, 31). Conversely, increased permeability or leakage of the endothelial monolayer could bring plasma proteins or formed elements of the blood into contact with the collagenous basement membrane and initiate clotting.

VPF-mediated monocyte chemotaxis and activation may provide a mechanism explaining the presence of activated macrophages in certain neoplasms. Based on our data demonstrating that VPF can induce tissue factor and chemotaxis in monocytes/macrophages, it is possible that VPF produced by a tumor contributes to the in vivo observations. Although further experiments will be required to test this hypothesis, attraction of macrophages to the periphery of neoplastic lesions and their participation in formation of a fibrin network about the lesion could diminish access and function of immune effector cells in the host response to the tumor (27, 28, 42).

These studies contribute to an emerging picture in which the altered properties of tumor vasculature result from the effects and interactions of tumor-derived and host response mediators. Furthermore, they point to a potentially important role of VPF in the modulation of endothelial and monocyte procoagulant activity, and migration of monocytes into the tumor bed.

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References

1. Beutler, B., and A. Cerami. 1986. Cachectin and tumor necrosis factor as two sides of the same biological coin. *Nature (Lond.).* 32:584.
2. Old, L. 1986. Tumor necrosis factor. *Science (Wash. DC).* 230:630.
3. Nawroth, P., D. Handley, G. Matsueda, R. De Waal, H. Gerlach, D. Blohm, and D. Stern. 1988. Tumor necrosis factor/cachectin-induced intravascular fibrin formation in meth A fibrosarcomas. *J. Exp. Med.* 168:637.
4. Nawroth, P., and D. Stern. 1986. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. *J. Exp. Med.* 164:740.
5. Bevilacqua, M., J. Pober, G. Majeau, W. Fiers, R. Cotran, and M. Gimbrone. 1986. Recombinant TNF induces procoagulant activity in endothelium. *Proc. Natl. Acad. Sci. USA.* 83:4533.
6. Nemerson, Y., and R. Bach. 1982. Tissue factor revisited. *Prog. Hemostasis Thromb.* 6:237.
7. Clauss, M., C. Murray, M. Vianna, R. De Waal, G. Thurston, P. Nawroth, H. Gerlach, M. Gerlach, R. Bach, P. Familletti, and D. Stern. 1990. A polypeptide factor produced by fibrosarcoma cells that induces endothelial tissue factor and enhances the procoagulant response to tumor necrosis factor/cachectin. *J. Biol. Chem.* 265:7078.
8. Keck, P., S. Hauser, G. Krivi, K. Sanzo, T. Warren, J. Feder, and D. Connolly. 1989. Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science (Wash. DC).* 246:1309.
9. Senger, D.R., S.J. Galli, A. Dvorak, C. Perruzzi, V. Harvey, and H. Dvorak. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science (Wash. DC).* 219:983.
10. Connolly, D., D. Heuvelman, R. Nelson, J. Olander, B. Eppley, J. Delfino, N. Siegel, R. Leimbruber, and J. Feder. 1989. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J. Clin. Invest.* 84:1470.
11. Ferrara, N., and W. Henzel. 1989. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 161:851.
12. Leung, D., G. Cachianes, W.-J. Kuang, D. Goeddel, and N. Ferrara. 1989. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science (Wash. DC).* 246:1306.
13. Jaffe, E. 1986. Culture and identification of large vessel endothelial cells. *In* Biology of Endothelial Cells. E. Jaffe, editor. Martinus Nijhoff, Boston. 1–13.
14. Thornton, S., S. Mueller, and E. Levine. 1983. Human endothelial cells: use of heparin in cloning and long-term serial cultivation. *Science (Wash. DC).* 222:623.
15. Old, L., B. Benacerraf, D. Clarke, E. Carswell, and E. Stockert. 1961. Role of the reticuloendothelial system in the host reaction to neoplasia. *Cancer Res.* 21:1281.
16. Familletti, P.C., and J.E. Fredericks. 1988. Techniques for mammalian cell immobilization. *BioTechnology.* 6:41.
17. Nawroth, P., D. Stern, W. Kisiel, and R. Bach. 1985. Cellular requirements for tissue factor generation by perturbed bovine aortic endothelial cells in culture. *Thromb. Res.* 40:677.
18. Carson, S., S. Ross, R. Bach, and A. Guha. 1987. Immunopurification of human tissue factor using an inhibitory monoclonal antibody. *Blood.* 70:490.
19. Bach, R., R. Gentry, and Y. Nemerson. 1986. Factor VII binding to tissue factor in reconstituted phospholipid vesicles: Induction of cooperativity by phosphatidylserine. *Biochemistry.* 25:4007.
20. Fluks, A.J. 1981. Three step isolation of human blood monocytes using discontinuous density gradients of percoll. *J. Immun. Methods.* 41:225.
21. Quinn, M.T., S. Parthasarthy, and D. Steinberg. 1988. Lysophosphatidylcholine: a chemotactic factor for human monocytes and its potential role in atheriogenesis. *Proc. Natl. Acad. Sci. USA.* 85:2805.
22. Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.).* 227:680.
23. Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* 262:10035.
24. Prydz, H., and A. Allison. 1978. Tissue thromboplastin activity of isolated human monocytes. *Thromb. Haemostasis.* 39:582.
25. Brooze, G. 1982. Binding of human factor VII and VIIa to monocytes. *J. Clin. Invest.* 70:526.
26. Lyberg, T., K. Galdal, S. Evensen, and H. Prydz. 1983. Cellular cooperation in endothelial cell thromboplastin synthesis. *Br. J. Haematol.* 53:85.
27. Folkman, J. 1985. Tumor angiogenesis. *Adv. Cancer Res.* 43:175.
28. Dvorak, H. 1986. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N. Engl. J. Med.* 315:1650.
29. Simionescu, N., and M. Simionescu. 1988. Endothelial Cell Biology. Plenum Publishing Corporation. New York, NY. 458 pp.
30. Del Vecchio, P., A. Siflinger-Birnboim, J. Shepard, R. Bizios,

J. Cooper, and A. Malik. 1987. Endothelial monolayer permeability to macromolecules. *Fed. Proc.* 46:2511.
31. Kadish, J., C. Butterfield, and J. Folkman. 1979. Effect of fibrin on cultured vascular endothelium. *Tissue & Cell.* 11:99.
32. Bloem, L., L. Chen, W. Konigsberg, and R. Bach. 1989. Serum stimulation of quiescent human fibroblasts induces the synthesis of tissue factor mRNA followed by the appearance of tissue factor antigen and procoagulant activity. *J. Cell. Physiol.* 139:418.
33. Rifkin, D., and D. Moscatelli. 1989. Recent developments in the cell biology of basic fibroblast growth factor. *J. Cell Biol.* 109:1.
34. Folkman, J., and M. Klagsburn. 1986. Angiogenic factors. *Science (Wash. DC).* 235:442.
35. Tsuboi, R., Y. Sato, and D. Rifkin. 1990. Correlation of cell migration, cell invasion, receptor number, proteinase production, and basic fibroblast growth factor levels in endothelial cells. *J. Cell Biol.* 110:511.
36. Clark, M., M.-J. Chen, S. Crooke, and J. Bomalaski. 1988. Tumor necrosis factor induces phospholipase A_2-activating protein in endothelial cells. *Biochem. J.* 250:125.
37. Henning, B., S. Goldblum, and C. McClain. 1987. Interleukin 1 and tumor necrosis factor/cachectin increase endothelial permeability in vitro. *J. Leukocyte Biol.* 42:551A.
38. Brett, J., H. Gerlach, P. Nawroth, S. Steinberg, G. Godman, and D. Stern. 1989. Tumor necrosis factor/cachectin increases permeability of endothelial cell monolayers by a mechanism involving regulatory G proteins. *J. Exp. Med.* 169:1977.
39. Gerlach, H., H. Lieberman, R. Bach, G. Godman, J. Brett, and D. Stern. 1989. Enhanced responsiveness of endothelium in the growing/motile state to tumor necrosis factor/cachectin. *J. Exp. Med.* 170:913.
40. Ding, A., F. Porteu, E. Sanchez, and C. Nathan. 1990. Downregulation of tumor necrosis factor receptors on macrophages and endothelial cells by microtubule depolymerizing agents. *J. Exp. Med.* 171:715.
41. Brock, T., H. Dvorak, and D. Senger. 1990. Tumor-secreted vascular permeability factor (VPF) increase endothelial cell cytosolic calcium. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:2071.
42. Plow, E., D. Freaney, and T. Edgington. 1982. Inhibition of lymphocyte protein synthesis by fibrinogen-derived peptides. *J. Immunol.* 128:1595.