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SPECIFIC BINDING OF VASCULAR PERMEABILITY FACTOR TO ENDOTHELIAL CELLS

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SUMMARY: Vascular permeability factor (VPF), also known as vascular endothelial cell growth factor, has recently been purified from guinea pig, human, and bovine sources. We show that various fetal or adult endothelial cell strains originating from either capillary or large vessels possess specific high affinity and saturable binding sites for guinea pig tumor-derived [^{125}I]VPF. Two classes of sites with K_Ds of approximately 10 pM and 1 nM were detected for all endothelial cell types examined. Guinea pig [^{125}I]VPF binding to endothelial cells was inhibited by human VPF (ID_{50} = 0.8 ng/ml) and by suramin (ID_{50} = 75 μg/ml) but not by heparin. Cross-linking experiments revealed specific [^{125}I]VPF-receptor complexes of two types. Most of the complexes migrated very slowing in SDS-PAGE, indicating that they were of very high molecular weight and probably highly cross-linked. A portion of the molecules migrated as 270 kDa complexes, indicating that the molecular weight of the endothelial cell VPF receptor is about 230 kDa.

Vascular permeability factor (VPF) is a multi-functional 40 kDa protein known to be secreted by some tumors and by pituitary follicular cells (1-8). VPF acts as a potent and fast acting mediator of vascular permeability (9,10), as an endothelial cell specific growth factor (3,5,8,10) and as an angiogenic agent *in vivo* (4,8,10). Initially, the permeability factor was identified in the media of tumor cells (9,11) and has since been purified from conditioned media of the human histiocytic lymphoma cell line U937 (1), bovine pituitary follicular stellate cells (3,5), and a mouse pituitary cell line AtT-20 (8). A role for VPF in

The abbreviations used are: aFGF, acid fibroblast growth factor; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; EGF, epidermal growth factor; ELISA, enzyme linked immunosorbant assay; FBS, fetal bovine serum; gVPF, guinea pig vascular permeability factor; HUVEC, human umbilical vein endothelial cells; hVPF, human vascular permeability factor; PAGE, polyacrylamide gel electrophoresis; PDGF, platelet derived growth factor; SDS, sodium dodecyl sulfate.

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the ascites fluid accumulation caused by certain tumors has been suggested (9) and it is likely that VPF also participates in non-tumor associated processes involving increased vessel permeability and angiogenesis, such as during inflammation or wound healing.

Although the mechanism of VPF's action is unknown, its activities suggest that it could interact directly with endothelial cells. We recently reported preliminary evidence for the presence of high affinity receptors for VPF on endothelial cells (10). Cross-linking experiments showed that the binding of [^{125}I]VPF to human umbilical vein endothelial (HUVEC) cells was inhibited by unlabeled VPF and prevented by polyclonal anti-VPF antiserum, while various other growth and permeability factors were without effect. These included acidic fibroblast growth factor (aFGF), basic FGF (bFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), tumor necrosis factor, histamine, serotonin, bradykinin, platelet-activating factor and interleukin-2. In this report we characterize the VPF receptor on various types of endothelial cells and present evidence that *in vitro* binding can be inhibited by suramin but not by heparin.

MATERIALS AND METHODS

Cell Culture. Human umbilical vein endothelial cells (HUVEC) were isolated by published procedures (12,13), established in MCDB 131 medium (14) and subcultured in MCDB 107 medium (15) supplemented with 10% fetal bovine serum (FBS), 90 μg/ml Na heparin, 30 μg/ml endothelial cell growth supplement, 10 ng/ml (EGF), and 1 μg/ml hydrocortisone on human fibronectin-coated tissue culture vessels. The ECGS, hydrocortisone, EGF, and human fibronectin were obtained from Collaborative Research. FBS was from HiClone Laboratories Inc. and sodium heparin from Sigma Chemical Co. Fetal bovine aortic endothelial (FBAE) and bovine calf adrenal capillary endothelial cells (BAC) were isolated and maintained as described in Olander et. al. (16). Bovine calf pulmonary artery endothelial cells (CPAE) were obtained from the ATCC and carried in DMEM supplemented with 10% FBS while the adult human aortic endothelial cells (HAEC) were a kind gift of Dr. P. DiCorleto (Cleveland Clinic Foundation) and were maintained in MCDB 107 with the same supplements as the HUVEC.

Radioiodination of VPF. Highly purified guinea pig VPF (gVPF) (10) was stored frozen in 0.1% trifluoroacetic acid and approximately 30% acetonitrile. A sample containing 2-4 μg was dried in a microfuge tube using a Savent SpeedVac and redissolved in 50 μl 0.1M potassium phosphate pH 8.2, 1.0% Triton X-100. One mCi [^{125}I]diiodo Bolton-Hunter reagent (DuPont-NEM) was used to radiolabel the VPF as described by Bolton and Hunter (17). Reaction products were separated from the labeled protein by passage over a Sephadex G-25 column. The trichloroacetic acid

(TCA) precipitability of each batch of radiolabeled ligand was determined and was approximately 80%. Analysis on 10-15% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) PhastGel (Pharmacia) and autoradiography confirmed that the 40 kDa VPF band was labeled. The permeability activity of the radioiodinated VPF was tested using the Miles assay (18) and found to be essentially the same as starting material. Additionally, its mitogenic activity or fetal bovine aortic endothelial cells was unchanged from that of unlabeled gVPF ($EC_{50} \approx$ 18 pM). Either 1% bovine serum albumin (BSA) or 2.5 mg/ml gelatin was added to protect the VPF from radiation damage and from non-specific loss on surfaces. The VPF concentration of the pool was determined by enzyme linked immunosorbent assay (ELISA). Specific activity ranged between 10 - 49 μCi/μg or between 0.1 - 0.4 moles Bolton-Hunter label per mole VPF.

Binding Experiments. Monolayers of near confluent endothelial cells were washed 3 times with warm serum-free medium or Binding Buffer (Hank's Balanced Salt Solution with 25 mM HEPES and 0.1% BSA) before bringing the cells to the temperature of the assay. Then radiolabeled VPF was added in minimum volume and the binding allowed to proceed for the designated time on a rotating platform. Unlabeled VPF that had been partially purified by heparin-Sepharose chromatography and concentrated by ultrafiltration was added in 100-fold excess to some culture wells to estimate the amount of non-specific binding. Binding was terminated by removing unbound radiolabeled gVPF by washing each plate 4 times with cold Tyrode's buffer containing 25 mM HEPES and 0.5% BSA. Bound counts were solubilized by addition of Lysing buffer (0.01 M Tris pH 7.4, 0.001 M EDTA 0.5% SDS) and counted along with one wash of the plate with the Lysing buffer in a Packard γ-counter. Each determination was performed in duplicate. The specific conditions for each experiment are given in the figure legends.

RESULTS AND DISCUSSION

A total of seven endothelial cell lines were tested for the ability to bind [^{125}I]gVPF. All of the lines bound between 0.2 - 1.2 ng/10^6 cells when incubated with 0.1 nM [^{125}I]gVPF (Table I), regardless of whether the cells were of human or bovine origin, or whether they originated from capillaries, large vessels, or from fetal or adult tissue. The HUE6 strain of umbilical endothelial cells consistently bound more VPF than other endothelial cell strains examined. This strain, which expresses Factor VIII antigen, exhibited somewhat more elongated and refractile morphology than the other cell lines and could be passaged for more generations in culture (about 25 passages) than other umbilical cell isolates. Even though there was some variability among the strains tested, it is clear from these data that the ability to bind VPF is a common property of all of the endothelial cells examined.

Table I

Binding of VPF to Various Endothelial Cell Strains

CELL STRAIN	ng VPF/10^6 cells
HUE6 (p.10)	1.21
BAEC17 (p.12)	0.21
BAC10A (p.12)	0.31
HUE6 (p.18)	1.38
HAEC (p.8)	0.94
CPAE (p.17)	0.31
HUE2A (p.7)	0.40
HUE2A (p.9)	0.36
HUE173 (p.4)	0.41
HUE6 (p.10)	1.61

0.1 nM [^{125}I]gVPF in 2.5 ml Binding Buffer was incubated with subconfluent monolayers of various human and bovine endothelial cells in 60mm tissue culture plates for 18 hours at 4°C. Non-specific binding, which was approximately 20% in this experiment, was determined in the presence of 100-fold excess unlabeled VPF, subtracted and the ng/10^6 cells calculated. HUE6, HUE2A, and HUE173 are strains of human umbilical vein endothelial cells. HAEC are human adult aortic endothelial cells. BAEC17 are fetal bovine aortic endothelial cells; BAC10A are bovine calf adrenal capillary endothelial cells; and CPAE are calf pulmonary artery endothelial cells. Passage number is designated as (p.#).

The kinetics of [^{125}I]gVPF binding to HUVEC cells was examined at different temperatures. Specific binding reached equilibrium after 3-4 hours at 4°C and after 1-2 hours at room temperature or 37°C. The total amount of [^{125}I]VPF bound at equilibrium (approximately 0.7 +/- 0.1 ng/10^6 cells) was the same at all three temperatures.

Figure 1A shows that the binding of [^{125}I]VPF to HUV6 cells was high affinity, saturable and that the amount of non-specific binding varied between 9% and 23%. Scatchard analysis (Fig. 1B) indicated the presence of two classes of binding sites, with K_Ds of 9 pM (25,000 sites/cell) and 1.5 nM (180,000 sites/cell). Similar experiments were carried out for two other HUVEC lines (Table II). The K_Ds for both classes of sites were similar amongst the cell types, but the HUE6 cells had significantly higher numbers of both high affinity and low affinity receptors. Bovine brain capillary endothelial cells were recently reported to bind an endothelial cell specific growth factor from mouse AtT-20 pituitary cells termed "vasculotropin" which is homologous to gVPF (8). Two classes of vasculotropin binding sites were detected with dissociation constants of 4 pM (900 sites/cell)

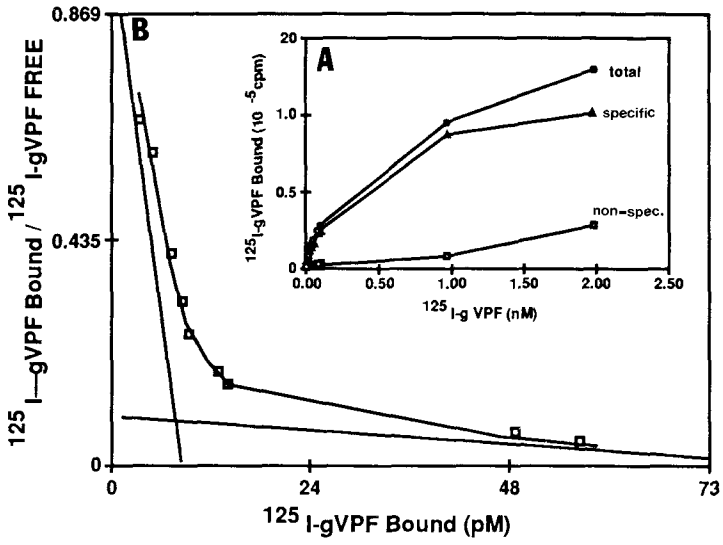


Fig. 1. The saturation binding curve and Scatchard plot of VPF binding to HUE6 cells. **A.** Binding was carried out on subconfluent HUE6 cells in 60 mm plates for 18 hours at 4°C in the presence of increasing concentrations of [^{125}I]gVPF in 2.5 ml Binding Buffer. Non-specific binding (9-23% of the total binding) was determined at each concentration in the presence of 100-fold excess unlabeled VPF. **B.** Scatchard plot of data in **A** using the "LIGAND" program developed by Munson and Rodbard (19) to fit the data to a two-site model.

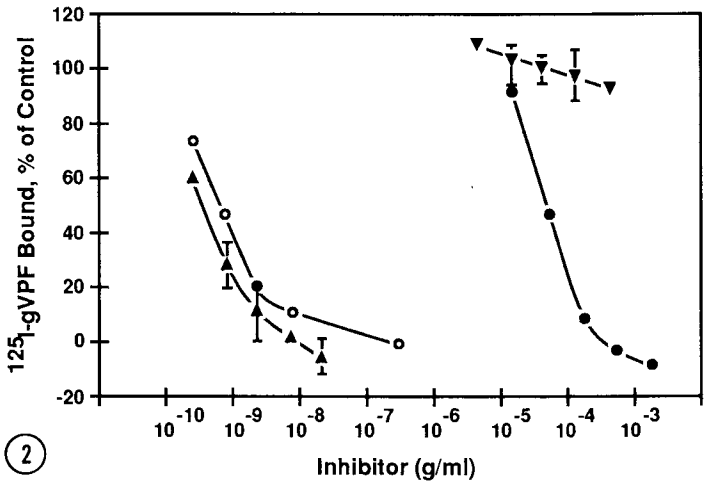
and 41 pM (4600 sites/cell) (20). While the high affinity constant was essentially the same as reported here, the lower affinity constant was two orders of magnitude higher. Also, significantly fewer vasculotropin receptors of both classes were detected on the bovine brain capillary endothelial cells . Similar results were obtained by Vaisman et al. (24) with murine VPF binding to bovine aortic endothelial cells except that the number of receptors they report are the same order of magnitude as the number of receptors we found on HUVECs. Further experiments will be required to determine if these differences

Table II

Parameters of [^{125}I]gVPF Binding to Various HUVEC Strains

	Dissociation Constants		Receptors/Cell	
HUVEC STRAIN	**K_{D1}(pM)**	**K_{D2}(nM)**	**B_{M1}**	**B_{M2}**
HUE2A (p.8)	8 ± 3	3.4 ± 2.0	5,040 ± 1,430	13,800 ± 3,500
HUE6 (p.10)	19 ± 5	1.7 ± 2.0	24,000 ± 5,040	112,500 ± 56,300
HUE6 (p.8)	8 ± 2	0.9 ± 0.2	25,000 ± 3,800	250,000 ± 24,000
HUE173 (p.4)	2 ± 3	0.1 ± 0.1	1,610 ± 1,000	13,700 ± 2,900

These were determined as described for Figure 2.



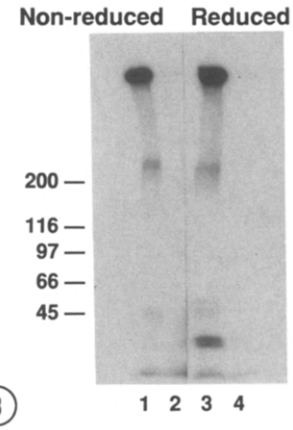


Fig. 2. Effect of gVPF, hVPF, Suramin, and Heparin on the Binding of [^{125}I]gVPF to HUE6 cells. Binding experiments were performed on subconfluent HUE6 cells in 24-well plates. Competitors were always added to the monolayers first in 0.5 ml Binding Buffer and equilibrated for 1 hour at room temperature. Then 0.1 nM [^{125}I]gVPF was added and the binding continued for two hours longer. Since the off rate is very slow at room temperature (>6 hours; data not shown), the binding in these competitive reactions does not reach equilibrium but favors the competitor binding. Data represent competition by unlabeled gVPF O --- O, unlabeled hVPF ▲ --- ▲, and suramin ● --- ●, Na Heparin ▼ --- ▼.

Fig. 3. Autoradiograph of SDS-PAGE analysis of affinity labeled HUE6 cells with [^{125}I]gVPF. HUE6 cells grown to near-confluency in 60-mm plastic tissue culture dishes were incubated with 0.1nM [^{125}I]gVPF at 4°C for 4 hours (lanes 1 and 3) or in the presence of [^{125}I]gVPF plus 10 nM unlabeled gVPF (lanes 2 and 4). At the end of the binding period, the cell layers were washed 3 times and then crosslinked with 0.25 mM disuccinimidyl suberate for 15 minutes. The reaction was quenched with 200 µl 0.2M glycine and the cells scraped into 1 ml PBS plus protease inhibitors (1mN EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin A and 20 KIU/ml aprotinin). Cell membranes were spun down and solubilized into 75 µl PBS with inhibitors plus 1% Triton X-100, separated under non-reducing (lanes 1 and 2) and reducing (lanes 3 and 4) conditions on 4-12% gradient SDS-PAGE and autoradiographed for 5 days at -70°C. The top of the gel coincides with the very dark bands.

are a reflection of species differences, endothelial cell differences, or both.

A competition assay was used to test the effects of potential inhibitors on [^{125}I]gVPF binding (Figure 2). Human VPF competed as effectively as guinea pig VPF for binding to the receptor. Suramin, an anionic anti-trypanosomal drug reported to inhibit the binding of growth factors such as bFGF, PDGF and EGF to their receptors (21,22) blocked [^{125}I]gVPF binding at a concentration of approximately 75 µg/ml (ID_{50} = 50 µM). Heparin,

another anion with high charge density, was tested in the radioreceptor assay and did not compete for [^{125}I]gVPF binding.

Our previous results indicated that when [^{125}I]VPF was chemically crosslinked to HUVEC, aggregates were formed which barely entered a 7.5% gel. We have repeated these experiments using the HUE6 strain of endothelial cells which have an exceptionally high number of receptors per cell. While the majority of the radiolabeled material was still present in large aggregates which remained near the top of the gel, an additional band of approximately 270 kDa was seen under both nonreducing and reducing conditions (Fig. 3). This was the result of specific binding of [^{125}I]VPF since no radiolabeled band was present when the binding took place in the presence of excess unlabeled gVPF. Since the molecular weight of VPF is 40 kDa, the receptor is estimated to be approximately 230 kDa. Possibly, the receptors aggregate upon binding VPF, thus making it possible for disuccinimidyl suberate to crosslink multiple receptor-ligand complexes together into large aggregates. Alternatively the high molecular weight complex, also specifically tagged by [^{125}I]VPF, represents the second class of binding sites. A similarly large, proteoglycan receptor has been reported for tumor growth factor-β (23). Cross-linking experiments performed by Vaisman et al. (24) indicate a molecular weight of 180 kDa for the VPF receptor on bovine aortic endothelial cells.

The present data show that endothelial cells of different origins can bind VPF. Analysis of the binding data revealed two distinct apparent classes of binding sites. One interpretation of these data is that the endothelial cell VPF receptors are heterogeneous. Alternatively, multiple binding affinities could be the result of heterogeneity in VPF itself. As many as 12 isoforms of gVPF have been detected using 2-dimensional electrophoresis (10), thus the [^{125}I]VPF used in the present experiments contained multiple isoforms. Even though permeability enhancing and growth promoting activities are associated with all of the isoforms (10), it is not known if the specific activities are identical. This issue will not be resolved until homogeneous isoforms of VPF are available. Regardless of the underlying mechanism, the heterogeneity of binding observed here raises the interesting possibility that quantitative differences in binding of VPF to endothelium are physiologically relevant. For example, half-maximal growth stimulation of endothelial cells

by VPF occurs in vitro in the 1.6-50 pM range (3,5,10) whereas permeability enhancement does not occur until the VPF concentrations in solutions used for intradermal injections are above 1 nM (1,10). Even though the assay systems that measure these two responses are quite different, it is reasonable to hypothesize that these events are quantitatively regulated by VPF-receptor interactions involving specific isoforms of VPF and its receptor(s).

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REFERENCES

1. Connolly, D.T., Olander, J.V., Heuvelman, D., Nelson, R., Monsell, R., Siegel, N., Haymore, B.L., Leimgruber, R., Feder, J. (1989a) J. Biol. Chem. **264**, 20017-20024.
2. Keck, P.V., Hauser, S.D., Krivi, G., Warren, T., Feder, J., and Connolly, D.T. (1989) Science **246**, 1309-1312.
3. Ferrara, N., and Henzel, W.J. (1989) Biochem. Biophys. Res. Commun. **161**, 851-858.
4. Leung, D.W., Cachianes, G., Kuang, W-J., Goeddel, D.V., Ferrara, N. (1989) Science **246**, 1306-1309.
5. Gospodarowicz, D., Abraham, J.A., Schilling, J.(1989) Proc. Natl. Acad. Sci. USA **86**, 7311-7315.
6. Tischer,E., Gospodarowicz, D., Mitchell, R., Silva, M., Schilling, J., Lau, K., Crisp, T., Fiddes, J.C., Abraham, J.A. (1989) Biochem. Biophys. Res. Commun. **165**, 1198-1206.
7. Conn, G., Soderman, D.D., Schaffer, M-T., Wile, M., Hatcher, V.B., and Thomas, K.A. (1990) Proc. Natl. Acad. Sci. USA **87**, 1323-1327.
8. Plouet, J., Schilling, J., Gospodarowicz, D.(1989) EMBO J. **8**, 3801-3806.
9. Senger, D.R., Galli, S.J., Dvorak, A.M., Perruzzi, C.A., Harvey, V.S., Dvorak, H.F. (1983) Science **219**, 983-985.
10. Connolly, D.T., Heuvelman, D.M., Nelson, R., Olander, J.V., Eppley, B.L., Delfino, J.J., Siegel, N.R., Leimgruber, R.M., Feder, J. (1989b) J. Clin. Invest. **84**, 1470-1478.
11. Senger, D.R., Perruzzi, C.A., Feder, J., Dvorak, H.F. (1986) Cancer Research **46**, 5629-5632.
12. Jaffe, E.A. (1980) Transplant. Proc. (Suppl. 1) **12**, 49-53.
13. Gimbrone, M.A., Jr. (1976) in Progress in Hemostasis and Thrombosis (Spaet, T.H., ed.) pp.1-28, Grune and Stratton, Inc., NY..
14. Knedler, A., and Ham, R.G. (1987) In Vitro Cell. Dev. Biol. **23**, 481-490.

15. Hoshi, H., and McKeehan, W.L. (1986) In Vitro Cell. Dev. Biol. **22**, 51-56.
16. Olander, J.V., Marasa, J.C., Kimes, R.C., Johnston, G.M., Feder, J. (1982) In Vitro **18**, 99-107.
17. Bolton, A.E., and Hunter, W.M. (1973) Biochem. J. **133**, 529-539.
18. Miles, A.A., and Miles, E.M. (1952) J. Physiol. (London) **118**, 228-257.
19. Munson, P.J. and Rodbard, D. (1980) Anal. Biochem. **107**, 220-239.
20. Plouet, J., and Moukadiri, H. (1990) Biochimie **72**, 51-55.
21. Betsholtz, C., Johnsson, A., Heldin, C-H., Westermark, B. (1986) Proc. Natl. Acad. Sci. **83**,6440-6443.
22. Moscatelli, D., and Quarto, N. (1989) J. Cell Biol. **109**, 2519-2527.
23. Cheifetz, S., Andres, J.L., Massague, J. (1988) J. Biol. Chem. **263**, 16984-16991.
24. Vaisman, N., Gospodarowicz, D. Neufeld, G. (1990) J. Biol. Chem. **265**, 19461-19466.