Characterization of the Receptor to Vasculotropin on Bovine Adrenal Cortex-derived Capillary Endothelial Cells*

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Recently a new growth factor was purified to homogeneity, and its bioactivity seemed to be restricted to vascular endothelial cells. As it was also angiogenic in vitro, it was provisionally named vasculotropin (VAS). As an iodination procedure used to label VAS did not damage the molecule, it was possible to undertake binding studies. The binding of iodinated vasculotropin to bovine adrenal cortex-derived capillary endothelial cells was quantitative at 250 pm, and half-maximal binding occurred at 47 pm. Scatchard’s analysis of the data demonstrated two apparent classes of binding sites with apparent dissociation constants of 2 and 82 pm displaying 280 and 3400 binding sites, respectively. The binding was specific; half-displacement was observed with a 2-fold excess of unlabeled VAS. The structurally related platelet-derived growth factor did not compete in a radioreceptor assay. $^{125}$I-VAS was displaced by suramin and not by heparin. $^{125}$I-VAS was covalently cross-linked to its cell surface receptor on intact bovine adrenal cortex-derived capillary endothelial cells using the homobifunctional agents ethylene glycol bis(succinimidy1 succinate) or disuccinimidyl tartarate. A major macromolecular species with an apparent molecular mass of 230,000 Da was labeled under reducing and nonreducing conditions. These data demonstrate the existence of a specific binding protein for VAS and an estimation of the size at 185,000 Da.

A new angiogenic growth factor was recently purified from the conditioned medium of the tumoral cell line AtT20 derived from mouse anterior pituitary (1). This secreted growth factor is a homodimer of 45 kDa whose amino terminus sequence showed no significant homology with any other known protein. It stimulates the proliferation of bovine vascular endothelial cells derived from adrenal cortex (ACE). 1 or brain microvessels, or human umbilical vein. However, it failed to elicit any mitogenic response in cells of various origins such as corneal endothelium, granulosa, adrenal cortex, keratinocytes, vascular smooth muscle, and even the baby hamster kidney clone 21, a cell line known to respond to a wide variety of growth factors. This growth factor also promotes new capillary formation when added on the chick chorioallantoic membrane (1). Since this new biological activity appeared to be restricted to vascular endothelium it was provisionally named vasculotropin (VAS). This vascular endothelium growth factor was also purified from the conditioned medium of normal folliculo-stellate cells cultured from bovine anterior pituitary (2). Concomitantly, three other groups purified a similar growth factor from the conditioned medium of various cell lines (3–5). It was also shown that this molecule enhanced the vascular permeability (6). The identity between these two biological entities was formally confirmed by the cloning of the gene. The sequence data depicted a structural homology between VAS and the B and A chains of PDGF (7–10). By analogy to other growth factors and hormone systems, the cellular action of VAS is presumably exerted through interaction with specific cell surface receptors. To understand the mode of action of VAS and its relation with other growth factors it is important to provide information on the functional properties of these receptors. This report describes the characteristics of the binding of VAS and provides information on the biochemical properties of the specific cell surface binding proteins. The possible interactions of the structurally related PDGF toward specific binding of VAS to its receptors were examined.

EXPERIMENTAL PROCEDURES

Materials—EGS, DST, and IODO-GEN were from Pierce Chemical Co. Aprotinin, benzamidin, leupeptin, and pepstatin were purchased from Sigma. Electrophoresis reagents were from Bio-Rad. PD 10 columns and heparin-Sepharose were from Pharmacia LKB Biotechnology Inc. Na$^{125}$I was from Amersham Corp. Cell culture reagents were from Gibco. Cell culture trays were from Costar. VAS was purified from the conditioned medium of the mouse AtT20 cell line as described (1). For Linbro BB-PDGf was from Gibco. Sumin was obtained from Mobay Inc. and heparin from Chosy.

Growth Factor Bioassays—Routinely, ACE cells were grown in DMEM supplemented with 10% calf serum, 2 mM glutamine, 0.25 µg/mL fungizone, 50 µg/mL gentamicin, 100 /g/mL penicillin, and 100 µg/mL streptomycin. Stock cultures received 1 ng/mL bFGF every other day (11). For growth factor bioassays, ACE cells were seeded at 5000 cells/well in 12-well cluster plates in 1 mL of culture medium. Increasing concentrations of growth factors were added every other day and cells trypsinized and counted on day 4.

Iodination of VAS—1 µg of purified VAS was mixed with 19 µL of 0.5 M phosphate buffer, pH 7.8, and transferred into a glass tube on ice. Iodination was performed according the procedure of Hunter and Greenwood (12); 1 mL of Na$^{125}$I and 10 µL of chloramine T (0.2 mg/mL) were added in a final volume of 150 µL, and a later the reaction was stopped by the addition of 10 µL of saturated acetyltyrosine and 10 µL of 10% trichloroacetic acid.

The purity of the iodinated product was estimated to be superior to 90% as determined by gel electrophoresis. Free $^{125}$I was less than 3% in the final preparation. Bioactivity of the iodinated VAS was measured by comparison with the bioactivities of native VAS. This procedure allowed a 50% yield with a specific activity of 5000 cpm/µg. In separate experiments, VAS was radiolabeled using the IODO-
Vasculotropin Receptor

Gen reagent according to Ref. 13. This procedure allowed a 20% yield with a specific activity of 60,000 cpm/ng and was therefore not currently used.

Binding Assays—ACE cells were seeded at 200,000 cells/35-mm dishes in DMEM medium supplemented with 10% calf serum and antibiotics. After 3 days of subconfluent culture ACE cells in 6-well cluster dishes (108 cells) were transfected at 4°C, and all subsequent operations were done in the cold. The cells were washed 3 times with binding buffer (DMEM medium containing 20 mM Hepes, pH 7.3, and 1 mg/ml gelatin) and then incubated with the desired concentrations of iodinated VAS in a final volume of 0.5 ml. Nonspecific binding was determined in the presence of an excess (500 ng) of VAS purified by Mono S fast pressure liquid chromatography (1). Both total and nonspecific binding were determined in duplicate. The dishes were shaken on an oscillating platform rotating at 1 cycle/s. Preliminary experiments showed that the equilibrium was reached within 2 h. After 2 h the cells were washed three times with cold PBS and one more time with PBS containing 2 mM NaCl according to Ref. 14, then lysed with 0.5 ml of 1% sodium dodecyl sulfate, 1 mM EDTA in water. Solubilized material was counted in a Kontron γ counter. At saturating concentrations, the nonspecific binding was less than 20%. Values were analyzed according to Scatchard’s procedure (15) with the use of the Ligand fitting program version 2.3.1.1 (16). For radioreceptor assay, 12-well cluster plates were used and the competitors were added in 0.25 ml of the same medium containing 0.8 ng/ml iodinated VAS. Down-regulation of VAS and PDGF receptors was achieved by incubating the plates 3 h at 37°C with 100 ng/ml growth factors.

Cross-linking of 125I-vasculotropin—ACE cells were grown in 6-cm dishes, and just before the assay, cells were washed and incubated with 5 ng/ml iodinated vasculotropin and various iodinations of competitors as described above in a final volume of 1.5 ml. After 2 h, dishes were washed with cold PBS, and the cross-linking was immediately performed at room temperature according to Ref. 17. Dishes were allowed to stand for 15 min on an oscillating platform in 2 ml of PBS containing 0.2 mM either diisocynimidyl tartarate or ethylene glycol bis(succinimidyl succinate) freshly dissolved in dimethyl sulfoxide. The reaction was stopped by the addition of 200 μl of quenching buffer containing 10 mM Tris-HCl, pH 7.5, 200 mM glycine, and 2 mM EDTA, and the dishes were transferred on ice. Each dish was washed 3 times with 10 ml of cold PBS, and 1 ml of PBS containing 10 μg of leupeptin, pepstatin, aprotinin, benzamidin, and 2 mM EDTA was added. The cells were scraped off with a rubber policeman, and the suspension was sedimented by centrifugation for 30 s in a microcentrifuge, and then lysed by 200 μl of 10 mM Tris-HCl, pH 7.0, 0.5% Nonidet P-40, 1 mM EDTA, and 10 μg of the antiproteases mentioned above for 30 min at 4°C. The suspension was centrifuged for 15 min in a microcentrifuge, and the supernatant was taken for analysis by gel electrophoresis.

Electrophoresis and Autoradiography—Electrophoresis was performed according to Laemmli (15). Iodinated samples were boiled 3 min in a sample buffer containing 1% NaDodSO4, 0.05% bromphenol blue, 10% glycerol, 75 mM Tris-HCl, pH 6.9, and applied on a 7% polyacrylamide gel with a 3% stacking gel. Cell extracts cross-linked with 125I-VAS (106 cells/lane) were run for 6 h at 20 mA. The gels were fixed and stained with 0.1% Coomassie Blue in 50% trichloroacetic acid for 15 min, destained with 7% acetic acid, dried, and subjected to autoradiography at -70°C using Kodak X-Omat R films.

Table 1

<table>
<thead>
<tr>
<th>Binding</th>
<th>Native</th>
<th>Preincubation with VAS</th>
<th>Preincubation with PDGF</th>
<th>Bioactivity</th>
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<td>cpm/dish x 10^-5</td>
<td>cells/dish x 10^-5</td>
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RESULTS

Characteristics of the Binding—The preparations of purified mouse VAS had previously been shown to contain a single molecular species as analyzed by NaDodSO4-PAGE (1, 19). Growth-promoting activity on ACE cells of iodinated VAS was also compared with that of the native one (Table I). It appeared that the maximal stimulation of ACE cells proliferation provided by labeled and unlabeled VAS was almost identical indicating that the iodination of VAS resulted in the recovery of the fully active growth factor. The yield was about 30% with specific activity on average of 300,000 cpm/ng. To determine the parameters of the binding, various amounts of iodinated VAS were added at 4°C on confluent cells in the presence or the absence of 500 ng of the native growth factor. Half maximal and maximal binding occurred at 47 and 250 pM, respectively (Fig. 1). The dissociation constants calculated according to the Ligand fitting program from the slopes obtained on a Scatchard’s representation (Fig. 2, inset) were 2 and 82 pM, respectively, for the two binding sites, assuming an apparent molecular mass of 45,000 Da for VAS. The abscissa intercepts in this representation indicate the presence of increasing concentrations of 125I-labeled VAS. The nonspecific binding was determined in parallel dishes containing 500 ng of unlabeled VAS and subtracted from the values. It represented 20% of the total binding at the maximum. Scatchard’s plot of the data is represented in the inset.

Specificity of the Binding—Various amounts of cold VAS were incubated for 2 h at 4°C in the presence of 3 ng/ml tracer. High salt washes did not remove any significant iodinated according to the Ligand fitting program from the slopes obtained on a Scatchard’s representation (Fig. 2, inset) were 2 and 82 pM, respectively, for the two binding sites, assuming an apparent molecular mass of 45,000 Da for VAS. The abscissa intercepts in this representation indicate the presence of increasing concentrations of 125I-labeled VAS. The nonspecific binding was determined in parallel dishes containing 500 ng of unlabeled VAS and subtracted from the values. It represented 20% of the total binding at the maximum. Scatchard’s plot of the data is represented in the inset.

FIG. 1. Concentration dependence on the binding of 125I-labeled VAS on ACE cells. Subconfluent ACE cells in 36 mm dishes were incubated for 2 h at 4°C in the presence of increasing concentrations of 125I-labeled VAS. The nonspecific binding was determined in parallel dishes containing 500 ng of unlabeled VAS and subtracted from the values. It represented 20% of the total binding at the maximum. Scatchard’s plot of the data is represented in the inset.
cultures of ACE cells were incubated at 4 °C with 5 rig/ml iodinated VAS and various amounts of cold VAS in the binding buffer. After 2 h cells were rinsed with PBS, lysed (○), or processed for cross-linking (●) as described in the text. Aliquots of cell lysates were then counted in a γ counter.

Similar experiments performed by replacing VAS by PDGF did not affect the binding of iodinated VAS. By contrast, suramin, known to interfere with the binding of PDGF or FGF to their respective receptors, inhibited both the binding and the mitogenicity of VAS. Heparin had no effect.

**Affinity Cross-linking—**Bound 125I-VAS was cross-linked to ACE cells with EGS, and cell extracts were further analyzed by NaDodSO₄-PAGE and autoradiography (Fig. 3, lane 1). The product of the cross-linking reaction revealed one major macromolecular species of M, 230,000. Assuming that this apparent molecular mass includes that of VAS, then the corresponding labeled native molecules prior to cross-linking would have a Mᵣ of 185,000. The specificity of the binding was demonstrated by the decrease of the intensity M, 230,000 band when increasing amounts of unlabeled VAS were added prior to the cross-linking (lanes 1–5). Aliquots of the cell lysates were counted, and it appeared that the dose-response curve obtained was parallel to that of the radioreceptor assay performed in the absence of cross-linking. Half-inhibition was also obtained with a 2-fold excess of unlabeled VAS (Fig. 2). A smear was detected in the ~145,000-Da range, but it seemed to represent a proteolytic degradation product since its intensity increased when protease inhibitors were omitted during the lysis reaction procedure (data not shown). When the gel was run in reducing conditions, a similar pattern was observed (lane 6).

Although the products of the reactions appeared to be different, treatment with EGS resulted in the formation of aggregates which did not enter the gel (lanes 1–5) whereas no aggregates were seen when DST was used (lane 7), but the yield was significantly lower.

**DISCUSSION**

This report demonstrates the presence of high affinity VAS binding sites on bovine capillary endothelial cells cultured from adrenal microvessels. Since the iodination procedure did not damage the specific activity of VAS, it was possible to undertake the binding studies. The binding of VAS was time- and concentration-dependent and saturable. At the steady state the binding was maximal at a concentration of 250 pM, and half-maximal binding was obtained with 47 pM. Since half-maximal and maximal stimulation of VAS on ACE proliferation are obtained with concentrations of 5 and 30 pM, respectively, it is possible to calculate that only a small number of receptors must be occupied to provide a proliferative response. Scatchard’s analysis led to the description of two classes of high affinity binding sites with apparent dissociation constants of 2 and 82 pM, respectively. The apparent dissociation constants are within the range observed for the binding of other growth factors such as platelet-derived growth factor (20), transforming growth factor β (21), and FGF (13, 22, 23) to responsive cells. These values are also very similar to those we reported for the binding of VAS to bovine brain-derived capillary endothelial cells (19). Although a single class of binding sites with a Kᵣ of 50 pM was reported...
Vasculotropin Receptor

for the binding of the related mitogen guinea pig vascular permeability factor to bovine aortic endothelial cells (6), this discrepancy might be due to the very low abundance of the binding sites displaying a $K_d$ of 2 pm which would not have been detected.

The binding was also specific since epidermal growth factor, insulin, or other angiogenic growth factors such as bFGF, acidic FGF, or transforming growth factor $\beta$ did not compete. Interestingly, the structurally related growth factor PDGF did not modulate the bioactivity of VAS. Since only B-type PDGF receptors were recently described on microvascular derived endothelial cells (24, 25), it was possible to look into a putative relationship between VAS and PDGF receptors on these cells. For this purpose we used a BB isoform which would bind to the B-type PDGF receptors. No direct competition by BB PDGF nor the down-regulation of the B-type PDGF receptors modified the binding of VAS. These results taken together with the lack of overlapping of the bioactivities of these two growth factors strongly suggest that the structural homology existing between these two growth factors is not correlated with a biological cross-reactivity.

One cross-linked complex with VAS at $M_1$ 230,000 was identified. After the subtraction of the mass of the cross-linked VAS, the VAS binding protein would have a molecular mass of 185,000. Similar results were obtained by the use of DST or EGS which represent 6- and 16-Å arms, respectively. The main difference between these two reagents was that EGS cross-linked at higher yields than DST but contributed to the formation of higher molecular mass aggregates which would mask possible higher molecular mass complexes such as those described for aortic endothelial cells (6). This 300,000 Da complex was not observed on capillary endothelial cells. Although another band of cross-linked material was detected in the 145,000 Da range it did not seem to represent a second binding protein, since its intensity was related to the absence or presence of protease inhibitors during the cell lysis reaction (data not shown).

Meanwhile the presence of two apparent classes of binding sites remains unclear. The purity of the molecule used was ascertained by several criteria (1), but isolectric forms of VAS were also described (6); they might interact differently with the cell membrane receptor. It might also be due to a heterogeneity of iodinated molecules of growth factor under-detected by gel electrophoresis and by biosassays, which would lead to different interactions. However, the VAS iodinated with the IODO-GEN method (currently preserving the biological properties of the molecules) bound to ACE cells in the same manner that the chloramine T labeled. Although the detection of a single band by cross-linking experiments does not rule out the existence of two cell membrane binding proteins, bFGF was reported to bind to a receptor and to a lower affinity binding site (14, 26-28). In the bFGF-cell interaction, the low affinity binding site has an average $K_d$ in the nanomolar range, is sensitive to high salt concentrations, and is presumably represented by heparan sulfate proteoglycan of the extracellular matrix secreted by the cell. Such a low affinity binding site to VAS on ACE cell membrane does not seem to exist since high salt washes did not remove any significant iodinated material. It was also unlikely that VAS binding proteins would not have been cross-linked since unlabeled VAS provided a similar competition pattern toward iodinated VAS when examined before or after the cross-linking procedure. Alternatively, two apparent classes of binding sites might correspond to two or three binding proteins of different molecular masses as detected by similar cross-linking experiments as was reported for bTGF (29). Effectively VAS is involved in at least two different biological pathways such as mitogenicity and vascular permeability. Nothing is yet known about whether these activities are mediated through the interaction of VAS with one or several receptors.

One approach to test this hypothesis might be represented by the recent finding that VAS is a mitogen for nonendothelial cells such as the retinal pigmented epithelial cell (30). A comparative study of the binding proteins on these two target cells might constitute a useful tool for this purpose.

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REFERENCES