Clustering of Vitronectin and RGD Peptides on Microspheres Leads to Engagement of Integrins on the Luminal Aspect of Endothelial Cell Membrane

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In previous work (Conforti et al, Blood 80:437, 1992), we have shown that integrins in endothelial cells (EC) are not polarized to the basal cell membrane, but are also exposed on the apical cell surface, in contact with blood. Therefore, endothelial integrins might be available for binding circulating plasma proteins. However soluble plasma vitronectin (vn) bound very poorly to EC apical surface and this interaction was unaffected by Arg-Gly-Asp (RGD) peptides or an anti-avβ3 serum. In contrast, beads (diameter, 4.5 μm) coupled with plasma vn associated to EC apical surface in a time- and concentration-dependent way. Addition of antibodies directed to vn, avβ3, and RGD-containing peptides blocked the interaction of vn beads with EC. In contrast, integrin receptors not only on their abluminal, but also on their luminal surface. The distribution of integrins on the endothelial/blood interface might be an important mechanism for binding plasma proteins and circulating cells on their surface. However, direct evidence that apically located endothelial integrins are functionally active is still lacking.

To address this issue we investigated vitronectin (vn) binding to EC. Vn can bind to cells through at least two domains; the Arg-Gly-Asp (RGD sequence) located close to the aminoterminal domain of the molecule serves as binding site for integrins during cellular adhesion to immobilized vn, whereas the heparin-binding domain within the carboxyterminal portion mediates binding of soluble vn complexes to cells. Vn occurs in at least two conformational configurations: The plasma form that is intramolecularly stabilized and does not expose the heparin-binding domain, and the multimeric form that, after treatment with urea or low pH, undergoes a conformational transition and exposes the cryptic heparin-binding site. The RGD sequence is apparently exposed on both forms and is not affected by conformational changes of vn.

In the present report, we present evidence that integrins on the luminal aspect of EC can bind soluble vn neither in its plasma nor in the multimeric form. However, integrin binding could be detected when vn was present in a highly aggregated state or immobilized on beads. Thus, because specific binding of the circulating plasma form of vn to EC is very weak, if existent at all, EC integrins might discriminate between soluble and clustered forms of RGD ligands.

MATERIALS AND METHODS

Materials. Chemical reagents were purchased from the following sources: bovine serum albumin (BSA), unfraccionated heparin sodium salt from porcine intestinal mucosa, insulin-transferrin-sodium selenite media supplement for cell culture, antimouse polyclonal IgG-peroxidase conjugate, crystal violet, o-phenylenediamine dihydrochloride, TRIS (hydroxymethyl) aminomethane, 2-deoxy-D-glucose, Tween-20 (polyoxyethylene sorbitan monolaurate) (Sigma Chemical Co., St Louis, MO); heparan sulfate was a kind gift from Dr L. A. Fransson, University of Lund, Lund, Sweden; culture reagents (GIBCO, Paisley, UK); tissue culture plates and flasks (Falcon, Becton Dickinson Labware, NJ, and Costar, Cambridge, MA).

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Submitted October 15, 1993; accepted April 11, 1994.

Supported by The Italian National Research Council (P. F. Appliance Cliniche della Ricerca Oncologica), Telethon Project No. A.03, Associazione Italiana per la Ricerca sul Cancro, and by Commission of the European Communities (BRIDGE: BIOT-CP79-0195). I.M.P. is a recipient of a Science Plan of European Communities fellowship.

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0006-4971/94/8404-0022$3.00/0

carrier-free Na\textsuperscript{125}I (New England Nuclear, Boston, MA); paraformaldehyde, sucrose and urea (Merck, Darmstadt, Germany) Diff-Quick carrier-free NaI\textsuperscript{125}I (New England Nuclear, Boston, MA); paraformal-fixing solution (Men M-450, tosyl activated magnetic beads and Dynal magnetic device (Dynal AS, Oslo, Norway); IODO-GEN (Pierce Europe BV, Oud-Beijerland, The Netherlands); column PD-10 (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Antibodies. Some of the antibodies used in this study were kindly donated by the following investigators: anti-av\textbeta\textbeta 3 monoclonal antibody (MoAb) LM609 and anti-av MoAb LM142 under the form of ascitic fluid\textsuperscript{11} by Dr D. A. Cheresh (The Scripps Research Institute, La Jolla, CA); anti-\beta\textbeta 3 MoAb 7E3 purified immunoglobulins (IgGs) by Dr B. S. Coller (State University of New York at Stony Brook); anti-\beta 1 MoAb Lia \textfrac{1}{2} supernatant\textsuperscript{12} by Dr F. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain); anti-\textgamma\textnu MoAb 16A7 purified IgGs by Dr P. Declerck, (University of Leuven, Leuven, Belgium). Rabbit sera raised by injecting placenta-purified \alpha\textnu 3 and \alpha\textbeta 1 receptors and anti-av\textgamma\textnu MoAb (clone P1F6)\textsuperscript{14} under the form of ascitic fluid were from Telios. (Pharmaceuticals Inc, San Diego, CA). Irrelevant MoAbs (ascitic fluid or purified IgG) and preimmune rabbit serum were used as control.

In blocking experiments we used antibody concentrations able to induce maximal effect as reported in the pertinent references.

Anti-\textgamma\textnu MoAb BV1 and MoAb BV2, supernatants and purified IgGs, were prepared in our laboratory by standard hybridoma techniques using purified human denatured \textgamma\textnu as immunogen. The MoAbs were characterized by their ability to bind purified \textgamma\textnu in the enzyme-linked immunosorbent assay (ELISA) technique\textsuperscript{15} (see Fig 1, A and B) and by immunoprecipitation\textsuperscript{16} (not shown). The ELISA technique was performed essentially as described\textsuperscript{16}. Briefly, microtiter wells were coated with 30 \textmu L of 7 \mu g/mL solution of either plasma \textgamma\textnu and multimeric \textgamma\textnu in carbonate bicarbonate buffer pH 9.6 and incubated overnight at 4°C in humidified chamber. Plates were washed three times with 0.05% Tween 20 in phosphate-buffered saline (PBS) containing 0.5% BSA and then incubated with the same buffer containing 1% BSA (100 \mu L/well) for 1 hour at 37°C. After wash, the plates were incubated with different concentrations of anti-\textgamma\textnu: MoAbs BV1, BV2, and nonimmune mouse IgGs (0.001 to 50 \mu g/mL) in PBS 0.05% Tween 20 containing 0.5% BSA for 1 hour at room temperature.

The wells were washed three times with PBS, incubated with peroxidase-conjugate goat antimouse IgGs (0.15 \mu g/mL, 100 \mu L/well) for 1 hour at room temperature. Binding of IgGs was quantified by addition of chromogenic substrate as described below for soluble \textgamma\textnu binding to EC. As reported in Fig 1, A and B, MoAbs BV1 and BV2 recognized \textgamma\textnu both in its plasma and denatured form. Recognition of plasma \textgamma\textnu was slightly better than that of multimeric \textgamma\textnu. MoAbs BV1 and BV2 showed a comparable activity.

Both MoAbs BV1 and BV2 inhibited EC adhesion and spreading on \textgamma\textnu-coated (7 \mu g/mL) substrates (Fig 1C). These assays have been previously described in detail\textsuperscript{17}. Briefly, wells were precoated with 7 \mu g/mL of multimeric \textgamma\textnu (2 hours at 37°C) residual protein binding sites on wells were saturated by further incubation with 1% BSA in PBS (30 minutes at 37°C). EC (18 \times 10\textsuperscript{3}/well) in suspension were added to the wells in the presence of increasing concentration of MoAbs BV1, BV2, and nonimmune mouse IgGs. After 2 hours at 37°C unbound cells were removed by washing twice in PBS-Ca\textsuperscript{2+} Mg\textsuperscript{2+} and adherent cells were fixed and stained with crystal violet as described.\textsuperscript{17} The number of adherent and spread cells was evaluated by phase-contrast microscopy (at least 10 fields per well were counted). As reported in Fig 1C, MoAb BV2 was more effective than MoAb BV1 in inhibiting cell adhesion and spreading.

By ELISA, MoAb BV1 selectively recognized human \textgamma\textnu, but not bovine \textgamma\textnu (measured by coating the wells with undiluted bovine serum, not shown).

Fig 1. Characterization of MoAbs BV1 and BV2. Binding of MoAb BV1 and BV2 to multimeric \textgamma\textnu (A) and plasma \textgamma\textnu (B). Increasing concentrations of MoAbs BV1 (△) and BV2 (□) or nonimmune mouse IgGs (○) were added to \textgamma\textnu- (7 \mu g/mL) coated plates for 1 hour at room temperature. The amount of antibody bound was then evaluated by ELISA technique as described in Materials and Methods. Values expressed in optical density (OD) are means ± SD of three replicates. (C) Effect of MoAbs BV1 and BV2 on EC adhesion and spreading to \textgamma\textnu-coated substrate. EC adhesion assay was performed as described previously.\textsuperscript{17} EC in suspension were added to the wells in the presence of increasing concentration of BV1 (△, △), BV2 (□, □) and nonimmune mouse IgGs (○, ○). The number of adherent (△, △, □, □) and spread (△, □, ○, ○) cells was evaluated by phase-contrast microscopy. Values (means of two experiments performed in triplicate) are expressed as percentage of the number of cells adherent and spread in absence of antibodies.
Proteins and peptides. Human plasma Vn was purified under nondenaturing conditions as previously described. Multimeric Vn was prepared by incubating plasma Vn for 1 hour at 37°C in 6 mL L urea followed by extensive dialysis against PBS. Plasma Vn was radiolabeled with [125I] by the iodogen procedure reaching specific radioactivity of 1.4 μCi/μg protein. Synthetic hexapeptides Gly-Arg-Gly-Asp-Ser-Pro-(GRGDSP) and Gly-Arg-Gly-Glu-Ser-Pro-(GRGESP) were either synthesized in our laboratory as described or purchased from Telios. The synthetic (cyclical) peptide Gly-Pen-Gly-Gly-Asp-Ser-Pro-Cys-Ala (GPenGRGDSPCA) was obtained from Telios. The cyclic peptides Gly-Pen-Gly-Arg-Ala-Arg-Gly-Asp-Ser-Pro-Cys-Ala (GPenRARGDNPCA) and Arg-Pmc-Gly-His-Lys-Gly-Glu-Leu-Arg-Cys-Arg-Pmc-GHKGELRCR were a kind gift of Dr J. F. Tschopp (Telios Pharmaceuticals Inc, San Diego, CA). GRGDSP and GRGESP coupled to albumin were prepared as described.

EC culture. EC were isolated from normal-term umbilical cord veins by collagenase perfusion as previously described in detail. Cells were grown in tissue culture dishes coated with gelatin (0.5%) in 20% newborn calf serum-medium 199 supplemented with endothelial cell growth supplement (prepared from bovine brain) (50 μg/mL), heparin (100 μg/mL), and penicillin (100 U/mL)streptomycin (100 μg/mL) and used between the second and the third passage. For binding assays, cells were seeded in gelatin-coated 96-microtiter wells and used at confluency (2 × 10^4 cells/well).

Gelatin was used because preliminary experiments showed that under these conditions, RGD peptides or anti-avb3 serum (within the time frame and concentrations used in this study) did not induce a detectable detachment of the cells from the wells. Soluble Vn binding to EC. Microtiter wells of confluent EC were incubated overnight with 100 μg/well of medium 199 containing 4% BSA, and 0.08% insulin-transferrin-sodium selenite supplement. Medium was three washes in PBS, MoAb BVI (25 pg/mL) or the same concentration of irrelevant MoAb IgGs in PBS (60 μL/well) were added for 2 hours at room temperature. After rinsing in PBS, peroxidase-conjugated goat-antimouse IgGs (0.15 pg/mL) were applied for 90 minutes by slow end-over-end rotation at room temperature. The beads adhering to the cell surface were counted by phase contrast microscopy. At least 10 fields per well were counted (corresponding to about 300 cells at confluency).

RESULTS

We first measured the binding of soluble plasma Vn to EC monolayers. To more selectively evaluate Vn binding to the apical cell surface an ELISA set-up was used. Binding of Vn to receptors located on the basal aspect of the cell membrane would be poorly detected by this assay because preliminary data showed that, because of their high molecular weight IgG cannot effectively traverse EC cell-to-cell junctions. As reported in Fig 2, purified soluble Vn bound very little to confluent EC monolayers. This association was not dependent on Vn concentration in the medium (Fig 2) or time of incubation (in the range of 5 to 120 minutes, not shown). Heparin (up to 570 μg/mL), GRGDSP peptides (up to 800 μmol/L), and anti-avb3 serum (up to 0.02 dilution) added during the binding assay did not affect Vn binding (not shown). For comparison, we tested the binding of multimeric Vn, the heparin binding form of the adhesive protein. As reported in Fig 2, multimeric Vn associated to EC in a concentration-dependent way. Time-course experiments showed that at 30 μg/mL, the binding of multimeric Vn was already apparent at 5 minutes and reached the equilibrium at 1 hour (not shown). As reported in Fig 2, heparin blocked binding of multimeric Vn to the cells, but similar to plasma Vn, no effect was found by GRGDSP peptides (up to 800 μmol/L) and anti-avb3 serum (up to 0.02 dilution) (not shown).
These results were in agreement with previously published data\textsuperscript{24,25} and did not support the hypothesis that integrins on EC luminal surface can bind soluble vn.

However, modification of vn by heating resulted in aggregated forms of the protein (reaching mw greater than 10\(^6\)) that could associate to EC monolayers in an RGD-dependent way. As reported in Fig 3, unlike multimeric vn, heparin or heparan sulfate were ineffective in competing for the aggregated vn binding. In contrast, the RGD containing cyclic peptides were able to substantially reduce binding of the aggregated form of vn to about 20\% of control suggesting major involvement of integrins in this interaction. These data were also supported by the concentration dependence of RGD peptides with half maximal inhibition at about 7 \(\mu\)mol/L. Under these conditions, cells did not detach from their substratum. Compared with 37°C, less than 20\% total binding of aggregated vn was observed at 4°C, suggesting that active metabolism was required for integrin-mediated recognition (data not shown). Overall, these data suggested that integrins could be involved in vn binding when the density of the ligand was sufficiently high.

To further investigate this point, plasma vn was linked to beads and the bead suspension added to confluent EC monolayers. The beads (diameter, 4.5 \(\mu\)m) were large enough to be counted directly using phase-contrast microscopy when attached on EC surface (Fig 4).

As reported in Fig 5, vn-bead binding to EC was dependent on (1) time of incubation, reaching a plateau at 60 minutes (A); (2) the number of beads added (B); and (3) the concentration of vn present on the bead surface (C). The minimal vn concentration able to give a detectable binding was in the range of 0.3 to 0.5 \(\times\) 10\(^{-13}\) g/bead.

Irrelevant binding of albumin-coated beads was observed (less than 6 beads per 10\(^2\) cells adding 5 \(\times\) 10\(^3\) beads/mL for up to 120 minutes incubation, Fig 4C).

The mechanism of vn-bead binding to EC was further established by competition assays.

As reported in Fig 6, anti-\(\alpha\)v\(\beta\)3 serum blocked vn-bead binding to EC, whereas anti-\(\alpha\)5\(\beta\)1 serum was ineffective. Table 1 reports the effect of other agents on vn-bead binding. Two anti-vn MoAbs able to inhibit EC adhesion to vn (16A7 and BV2) blocked vn-bead binding. Two inhibitory MoAbs, LM609 directed to \(\alpha\)v\(\beta\)3 and 7E3 directed to \(\beta\)3\textsuperscript{11,17} inhibited vn-bead binding, whereas the nonblocking MoAbs LM142, directed to \(\alpha\),\textsuperscript{11} P1F6 (blocking MoAb, directed to \(\alpha\)v\textsuperscript{14,14} and Lia \(1/2\) (blocking MoAb, directed to \(\beta\)1)\textsuperscript{15} were inactive. Finally, the cyclic GPenGRGDSPCA (but not GRGESP) peptide inhibited vn-bead binding to EC (see also Fig 4), whereas heparin, up to 570 \(\mu\)g/mL was without effect.

To further investigate the presence of recognition sites for RGD ligands on the apical surface of EC, binding of albumin-linked GRGDSP and GRGESP peptides coated to beads was studied. GRGDSP, but not GRGESP-coated beads significantly bound to EC in a time-dependent manner reaching
a plateau at 2 hours (Fig 7A). The binding was blocked by anti-αvβ3 serum, LM609 and 7E3 MoAbs (directed to αvβ3 and β3, respectively), and GRGDSP and GPenGRGDSPCA peptides, whereas MoAbs P1F6 (directed to αvβ5) and Lia ½ (directed to β1) were inactive (Fig 7B).

**DISCUSSION**

The major finding of this paper is that apically located EC integrins (and in particular αvβ3) retain their biologic function and bind surface-linked vn and RGD peptides. In
The reason why avo3 recognizes vn only when it is immobilized on surfaces is intriguing. This phenomenon could be caused by a change in the ligand conformation and/or to an increase in its local density. Other integrin ligands present on EC are able to bind to the luminal surface of EC when bound to circulating cells. Fibrinogen of particular interest because it contains two times three puta-

different types of cell-binding mechanisms depending on their conformation. For instance, only when it is coated on a surface and treated by reducing agents, thrombospondin can bind αvβ3, presumably by exposure of its otherwise hindered RGD domain. Thrombin can be bound by αvβ3 through its RGD domain only when the molecule is modified by different treatments. In addition, integrins bind more effectively to their specific ligands when they are allowed to cluster or bind to a surface, and this appears to be correlated with ligand density.

Interestingly, whereas heparin was most effective in inhibiting soluble multimeric/denatured vn binding, it did not change the interaction of vn-coated beads with EC. This suggests that if a change in conformation occurs, the heparin-binding domain remains hindered or not available for promoting adhesion. Thus, vn linked to beads presents different features than multimeric soluble vn.

The biologic relevance of the binding of vn beads to EC outlined in this work remains to be elucidated. Circulating cells, including tumor cells and bacteria can bind vn, other RGD containing proteins (such as fibrinogen, von Willebrand factor or fibronectin) can associate to the endothelium when bound to circulating cells. Fibrinogen is of particular interest because it contains two times three puta-

Table 1. Effect of Competitors on vn-Bead Binding to EC

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Concentration</th>
<th>Beads/10^6 Cells</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>NA</td>
<td>103 ± 10</td>
<td>NA</td>
</tr>
<tr>
<td>BV2</td>
<td>0.1 (dil)</td>
<td>8 ± 1</td>
<td>92</td>
</tr>
<tr>
<td>16A7</td>
<td>50 (μg/mL)</td>
<td>31 ± 3</td>
<td>70</td>
</tr>
<tr>
<td>LM 609</td>
<td>0.05 (dil)</td>
<td>61 ± 7</td>
<td>41</td>
</tr>
<tr>
<td>LM 142</td>
<td>0.05 (dil)</td>
<td>172 ± 50</td>
<td>0</td>
</tr>
<tr>
<td>7E3</td>
<td>20 (μg/mL)</td>
<td>55 ± 7</td>
<td>47</td>
</tr>
<tr>
<td>Lia 1/2</td>
<td>0.1 (dil)</td>
<td>103 ± 27</td>
<td>0</td>
</tr>
<tr>
<td>P1F6</td>
<td>0.005 (dil)</td>
<td>106 ± 15</td>
<td>0</td>
</tr>
<tr>
<td>GRGDSPCA</td>
<td>50 (μmol/L)</td>
<td>73 ± 9</td>
<td>30</td>
</tr>
<tr>
<td>200</td>
<td>45 ± 6</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>55 ± 6</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>GRGESP</td>
<td>800 (μmol/L)</td>
<td>114 ± 12</td>
<td>0</td>
</tr>
<tr>
<td>Heparin</td>
<td>570 (μg/mL)</td>
<td>96 ± 9</td>
<td>7</td>
</tr>
</tbody>
</table>

Vn-coated beads (3.3 x 10^5/mL) were added to confluent EC monolayers for 60 minutes at 37°C in the presence or absence of inhibitors. BV2, anti-vn MoAb; 16A7, anti-vn MoAb; LM609 anti-αvβ3 MoAb; LM142 anti-vn MoAb; 7E3, anti-β3 MoAb; Lia 1/2 anti-β1 MoAb; P1F6 anti-αvβ5 MoAb. Blocking antibodies were used at concentrations able to produce maximal inhibitory activity in appropriate assays. Percent inhibition was calculated by comparing binding of beads in the absence and presence of competitors. Data are means ± SEM of three experiments.

Abbreviations: NA, not applicable; dil, dilution.
tive available integrin recognition domains and has been shown to act as bridging molecule in the adherence of bacteria to EC. In addition, fibrinogen mediates platelet binding to αvβ3-transfected Chinese Hamster Ovary cells by bridging αIIb-β3 and αvβ3. Therefore, fibrinogen bound to activated platelets or to other cell types could be recognized and bound by endothelial αvβ3 and help to localize circulating cells to the intact vascular surface.

In addition to EC integrins, ICAM-1 has been found to act as a fibrinogen receptor that thereby acts as bridging molecule between leukocytes and EC. Interestingly, ICAM-1 antibodies only partially inhibited fibrinogen-mediated adhesion of leukocytes to resting EC suggesting that additional adhesive mechanisms contribute to this effect.

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BINDING OF PLASMA VN-COATED BEADS

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Clustering of vitronectin and RGD peptides on microspheres leads to engagement of integrins on the luminal aspect of endothelial cell membrane

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