Up-regulated Biosynthesis and Expression of Endothelial Cell Vitronectin Receptor Enhances Cancer Cell Adhesion

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ABSTRACT

Extravasation of circulating cancer cells during metastasis is thought to involve adhesion to the vascular endothelium. To characterize this process, we measured the attachment of A549 human lung carcinoma cells to monolayers of cultured human umbilical vein endothelial cells. Pretreatment of the endothelial cells with 10 ng/ml interleukin 1α (IL-1) for 4 h increased cancer cell attachment 2–5-fold. This increase was blocked by 100 μM glycyl-arginyl-glycyl-aspartyl-serine peptide and was decreased 60 ± 10% (SD) by a vitronectin receptor polyclonal antiserum or 56 ± 8% by a vitronectin receptor monoclonal antibody, LM609. Glycyl-arginyl-glycyl-aspartyl-serine or the vitronectin receptor antibodies did not inhibit cancer cell attachment to untreated endothelial cells. A fibronectin receptor antiserum had no effect on attachment to untreated or IL-1-treated endothelial cells. Pretreatment of endothelial cells with IL-1 increased their adhesion to fibronectin and vitronectin and increased the expression of vitronectin receptor and fibronectin receptor as detected by immunofluorescence flow cytometry, quantitative antibody binding, and immunoprecipitation of [32P]methionine-labeled cell extracts. IL-1 pretreatment also increased β1, β3, and α5 integrin mRNA. The A549 cells did not express vitronectin receptor, since LM609 did not inhibit A549 adhesion to vitronectin or bind to A549 cells in flow cytometry, and vitronectin receptor antisera failed to immunoprecipitate vitronectin receptor from A549 cells. Furthermore, the β1 complementary DNA probe failed to hybridize to A549 RNA. A549 cells did express fibronectin receptor, which was increased by IL-1 treatment. We conclude that IL-1 induces the expression of both vitronectin receptor and fibronectin receptor on endothelial cells and that vitronectin receptor, in turn, facilitates A549 cell adhesion to endothelial cells.

INTRODUCTION

Metastasis is a multistep process involving the release of cancer cells from a primary tumor and their entry into the circulation, where they are disseminated to distant organ sites. Subsequently, these cancer cells cross the vessel wall to enter the extravascular tissue and proliferate, forming secondary tumors (1–3). Adhesion of cancer cells to the endothelium appears to be a requisite for their escape from the circulation (4).

It has been demonstrated that cancer cells can attach to intact endothelial cells in vitro and in vivo (5–10). This adhesion can be enhanced by pretreating the endothelium with any of several biological stimuli, including tumor necrosis factor and IL-1 (7–11). This increase in cancer cell adhesion appears to be related, in part, to the expression and de novo synthesis of at least two human endothelial cell-derived molecules (7, 9, 11). INCAM-110 expression is associated with increased melanoma cell adhesion to cultured endothelial cells and appears to be involved in the adhesion of lymphocytes to follicular dendritic cells or endothelial cells (12, 13). Another endothelial cell-derived protein, ELAM-1, facilitates not only leukocyte adhesion to IL-1-, tumor necrosis factor-, or bacterial endotoxin-treated endothelial cells but also promotes the adhesion of some colon carcinoma cells to cytokine-treated endothelial cells (11, 14). We have previously shown that adhesion of some cancer cells to IL-1-treated endothelial cells can be blocked by the GRGDS peptide, presumably by direct competition with a cell surface receptor (10). Since both INCAM-110 and ELAM-1 are GRGDS-independent adhesion molecules (11, 14), our data suggest that adhesion of cancer cells to the perturbed endothelium may also relate to an RGD-dependent endothelial surface adhesion molecule (9, 10).

A large family of adhesion molecules, named the integrins, have recently been described. The integrins are transmembraneous proteins comprising noncovalent heteromers of α and β subunits. These proteins are usually associated with the adhesion of cells to basement membrane components (15–17). Cell adhesion associated with many of the integrin adhesion molecules is dependent upon the RGD peptide, but for others it is not (15–17). Those integrins which are RGD dependent include the fibronectin receptor α5β1 (18, 19) and at least five different receptors for vitronectin, namely the vitronectin receptor α5β3 (20, 21); the platelet glycoprotein Ib/IIIa, αIIbβ3 (22); α5β1 (23, 24); αVβ5 (25–27); and αVβ3 (28). Since the vitronectin receptor and fibronectin receptor are the major RGD-dependent integrins expressed on endothelial cells (19, 21), we performed experiments to determine whether the enhanced cancer cell adhesion to endothelial cells following IL-1 stimulation was dependent upon the expression of these molecules.

Here we demonstrate that there is both increased expression and de novo synthesis of the vitronectin receptor (α5β3) and the fibronectin receptor (α5β1) on endothelial cells following treatment with IL-1 and that the increased expression of vitronectin receptor contributes to the enhanced adhesion of human A549 lung adenocarcinoma cells to the endothelial cells.

MATERIALS AND METHODS

Materials. Cell culture media were obtained from Gibco (Burlington, Ontario). Pooled human sera (type AB) were supplied by the Canadian Red Cross (Hamilton, Ontario). The GRGDS and GRGES peptides were obtained from Peninsula Laboratories, Inc. (La Jolla, CA). The rabbit anti-human vitronectin receptor polyclonal antiserum and the two related mouse monoclonal anti-human vitronectin receptor ascites fluids (LM609 and LM142) were gifts from Dr. D. A. Cheresh (Scirpp Clinic, La Jolla, CA). LM142 recognizes a specific epitope on the α chain, and LM609 interferes with the RGD binding site of the dimer (21, 29, 30). The rabbit anti-human fibronectin receptor polyclonal antiserum (31), purified human vitronectin, human fibronectin, additional rabbit anti-human vitronectin receptor polyclonal antiserum (32), and β1, β3, and α5 (32) DNA probes were obtained from Telios Biochemicals (San Diego, CA). Normal rabbit serum, normal mouse ascites fluid, rabbit anti-β2-microglobulin polyclonal antiserum, and anti-HLA mouse monoclonal (W6/32) ascites fluid were purchased.
from Cedarlane Laboratories (Hornby, Ontario). Goat anti-rabbit IgG-FITC conjugate and goat anti-mouse IgG-FITC conjugate were obtained from Bethesda Research Laboratories (Burlington, Ontario). Recombinant human IL-1 was supplied by Dr. P. Lomedico (Hoffmann-La Roche, Nutley, NJ). [4,5-3H(N)]Leucine (53 Ci/ml), [5-3H]iododeoxyuridine (2200 Ci/ml), goat anti-rabbit IgG-AlF(ab2) fragment (9.14 µCi/µg), sheep anti-mouse IgG-AlF(ab2) fragment (5.19 µCi/µg), and [35S]methionine (1075 Ci/mmol) were obtained from Flow/ICN Biomedicals, Inc. (Mississauga, Ontario). Protein A-Sepharose was obtained from Pharmacia (Baie d'Urfe, Quebec).

Cell Culture. Endothelial cells, obtained by collagenase digestion of umbilical cord veins, were grown in medium (M199 supplemented with 20% pooled human serum, 100 µg/ml streptomycin, 100 units/ml penicillin, 1% L-glutamine, and 100 µg/ml pituitary-derived endothelial cell growth factor) (33). When confluent, the cells were isolated by trypsinization, reseeded (1:3 dilution) onto 177-mm² fibronectin-coated plastic dishes (Teflar) in 24-well Costar culture dishes, and grown to confluent monolayers. A549 human lung adenocarcinoma cells (American Type Culture Collection, Parkville, MD) were grown in Dulbecco's medium supplemented with 10% fetal calf serum, 100 µg/ml streptomycin, 100 units/ml penicillin, and 1% L-glutamine (10). A549 cell binding, labeling and adhesion assay. Subconfluent A549 cultures were labeled for 24-48 h with 0.5 µCi/ml [3H]-deoxyuridine. The A549 cells were detached from the culture dish by adding 5 mM EGTA in HBSS, pH 7.4, washed with M199, and resuspended in M199 supplemented with 20% human serum to a concentration of 10⁵ cells/ml (10). Aggregates were dispersed by passage through an 18-gauge needle. The specific activity of the radiolabeled A549 cells was 1.9 ± 0.4 (SD) cells/cpm.

The endothelial cell monolayers were incubated with 10 ng/ml IL-1 or with suspending medium at 37°C for 4 h, when the medium was replaced with 0.5 ml fresh supplemented M199. Five × 10⁵ [3H]-deoxyuridine-labeled A549 cells (0.5 ml) were added and incubated at 37°C. Thirty min later, nonadherent A549 cells were aspirated, and the endothelial cell monolayer was washed three times with unsupplemented medium. The radioactivity associated with each monolayer was determined in a Packard gamma counter, and the number of cpm was determined in duplicate for each sample.

Immunoprecipitations were carried out as described by Ignozzi et al. (35). Briefly, an aliquot of the labeled cell suspension (normalized for cpm) was made up to 50 µl with octylglucoside lysis buffer containing 0.1% Triton X-100 and incubated with 5 µl of the appropriate primary antibody for 18 h. One hundred µl of 10% protein A-Sepharose beads were added and incubated for 2 h. The protein-A Sepharose beads were washed once with 1 ml/g bovine serum albumin, 0.1% Triton X-100 in Tris-buffered saline, pH 7.4, and then lysed with 200 µCi/ml bovine serum albumin, 0.1% Triton X-100 in 150 mM NaCl, 25 mM Tris-HCl, pH 7.2, 1 MEGS, 1 mM MgCl₂, 1 mM CaCl₂, 3 mM phenylmethylsulfonyl fluoride, 1 mg/ml soybean trypsin inhibitor and 1 mg/ml aprotinin. The radioactivity of trichloroacetic acid precipitates and the amount of protein were determined in duplicate for each sample.

Immunoprecipitations were carried out as described by Ignozzi et al. (35). Briefly, an aliquot of the labeled cell suspension (normalized for cpm) was made up to 50 µl with octylglucoside lysis buffer containing 0.1% Triton X-100 and incubated with 5 µl of the appropriate primary antibody for 18 h. One hundred µl of 10% protein A-Sepharose beads were added and incubated for 2 h. The protein-A Sepharose beads were washed once with 1 ml/g bovine serum albumin, 0.1% Triton X-100 in Tris-buffered saline, pH 7.4, and then lysed with 200 µCi/ml bovine serum albumin, 0.1% Triton X-100 in 150 mM NaCl, 25 mM Tris-HCl, pH 7.2, 1 MEGS, 1 mM MgCl₂, 1 mM CaCl₂, and then resuspended in 150 mM NaCl, 25 mM Tris-HCl, pH 7.2, 1 MEGS, 1 mM MgCl₂, 1 mM CaCl₂. The washed beads were boiled in 100 µl of sodium dodecyl sulfate sample buffer (2% sodium dodecyl sulfate, 10% glycerol, 0.5 mg/ml bromophenol blue in 0.0625 mM Tris-HCl, pH 6.8) and then electrophoresed on a 7.5% polyacrylamide gel containing sodium dodecyl sulfate (36). For reducing conditions, the samples were boiled following the addition of 5% β-mercaptoethanol before electrophoresis. Gels were soaked in Amplify and dried before autoradiography. The autoradiographs were analyzed by densitometry on a Biorad model 620 densitometer.

RNA Isolation and Northern Blotting. RNA from untreated or IL-1-treated endothelial cells or A549 cells was prepared by the guanidine isothiocyanate-CsCl centrifugation procedure of Chirgwin et al. (37). The total RNA (20 µg/lane) was electrophoresed on 1% agarose gels.
cell monolayers were washed three times in M199, and the number of A549 cells was performed by densitometry. The RNA was transferred to nitrocellulose membranes and hybridized to \( \beta_1, \beta_2 \), or \( \alpha \) cDNA probes labeled with \[^{32}P\]dCTP by the random primer labeling method (39). Hybridizations were conducted in \( 5\times \) SSC (200 mm NaCl, 20 mm sodium citrate), 4.5\( \times \) Denhardt’s solution, 0.1% sodium dodecyl sulfate, and 0.2 ng/ml salmon sperm DNA at 62°C and were washed in 2\( \times \) SSC and 0.1% sodium dodecyl sulfate at 62°C. The blots were exposed to XOMAT X-ray film at \(-70°C\) and developed, and quantitative analysis was performed by densitometry.

**RESULTS**

**RGD-dependent A549/Endothelial Cell Adhesion Following IL-1 Treatment.** Pretreatment of the endothelial cells with 10 ng/ml IL-1 for 4 h increased A549 cell adhesion 1.7-fold (Fig. 1). The GRGDS peptide caused a concentration-dependent decrease in A549 cell adhesion to the IL-1-treated endothelial cells (EC50 ≈ 8 \( \mu \)M GRGDS). One hundred \( \mu \)M GRGDS completely blocked the IL-1-induced adhesion. Adhesion of A549 cells to untreated endothelial cells was not affected by the GRGDS peptide. The GRGDS peptide also blocked adhesion of A549 cells to fibronectin-coated disks, but the EC50 was 65 \( \mu \)M (data not shown). A control peptide, GRGES, had no effect on A549 cell adhesion to untreated or IL-1-treated endothelial cell monolayers.

**Vitronectin Receptor-dependent A549/Endothelial Cell Adhesion Following IL-1 Treatment.** In other experiments, IL-1 pretreatment of the endothelial cells increased A549 cell adhesion by 3.2-fold. Polyclonal vitronectin receptor antiserum (Fig. 2A) and LM609 ascites fluid (Fig. 2B) blocked A549 cell adhesion to IL-1-treated endothelial cells in a concentration-dependent manner. The vitronectin receptor antiserum blocked up to 65% of the IL-1-induced adhesion, while LM609 blocked up to 56% of the induced adhesion. Adhesion of A549 cells to untreated endothelial cells was not affected. Neither the fibronectin receptor antiserum (Fig. 2A) nor the LM142 ascites fluid (Fig. 2B) blocked A549 cell adhesion to either treated or untreated endothelial cells. Similarly, normal rabbit serum and normal mouse ascites fluid had no effect (data not shown).

**Adhesion of Endothelial Cells and A549 Cells to Defined Substrates.** IL-1 pretreatment of endothelial cells resulted in a significant increase in their adhesion to both immobilized vitronectin and fibronectin substrates (Table 1). Adhesion to both substrates was blocked by 100 \( \mu \)M GRGDS peptide but was unaffected by 100 \( \mu \)M GRGES peptide. The vitronectin receptor antiserum inhibited endothelial cell adhesion to vitronectin but not to fibronectin, and the fibronectin receptor antiserum inhibited adhesion to fibronectin but not to vitronectin. Normal rabbit serum had no effect. LM609 blocked adhesion of endothelial cells to immobilized vitronectin, while LM142 did not. Neither monoclonal ascites fluid affected the adhesion of endothelial cells to fibronectin.

**Indirect Immunofluorescence Flow Cytometry.** Untreated endothelial cells demonstrated a significant vitronectin recep-
Adhesion to a vitronectin-coated or fibronectin-coated surface was increased by a maximum of 3.9 ± 0.5-fold, and the binding of fibronectin receptor antisera increased by 2.9 ± 0.4-fold following 8 h of stimulation with IL-1. Binding of β2-microglobulin antisera to the endothelial cell monolayers was un-

Table 1 Effect of RGD-related peptides and antisera on endothelial cell and A549 cell adhesion to vitronectin and fibronectin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage adherent cells*</th>
<th>Endothelial cells</th>
<th>A549 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesion to a vitronectin-coated surface</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>39 ± 6</td>
<td>60 ± 3</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>GRGDS</td>
<td>12 ± 2ª</td>
<td>22 ± 4</td>
<td>8 ± 2ª</td>
</tr>
<tr>
<td>GRGES</td>
<td>41 ± 6</td>
<td>57 ± 5</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>Anti-vitronectin receptor</td>
<td>21 ± 5ª</td>
<td>25 ± 5</td>
<td>18 ± 2ª</td>
</tr>
<tr>
<td>Anti-fibronectin receptor</td>
<td>38 ± 4</td>
<td>64 ± 3</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>36 ± 5</td>
<td>60 ± 7</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>LM609</td>
<td>20 ± 2ª</td>
<td>21 ± 4ª</td>
<td>27 ± 4</td>
</tr>
<tr>
<td>LM142</td>
<td>36 ± 6</td>
<td>54 ± 4</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>Adhesion to a fibronectin-coated surface</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>62 ± 7</td>
<td>85 ± 6</td>
<td>34 ± 2</td>
</tr>
<tr>
<td>GRGDS</td>
<td>37 ± 3ª</td>
<td>67 ± 4ª</td>
<td>25 ± 3ª</td>
</tr>
<tr>
<td>GRGES</td>
<td>65 ± 4</td>
<td>78 ± 7</td>
<td>35 ± 3</td>
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<tr>
<td>Anti-vitronectin receptor</td>
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<tr>
<td>Anti-fibronectin receptor</td>
<td>39 ± 7ª</td>
<td>64 ± 5ª</td>
<td>23 ± 4ª</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
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<td>77 ± 6</td>
<td>36 ± 1</td>
</tr>
<tr>
<td>LM609</td>
<td>60 ± 7</td>
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<td>34 ± 1</td>
</tr>
<tr>
<td>LM142</td>
<td>56 ± 6</td>
<td>79 ± 8</td>
<td>31 ± 2</td>
</tr>
</tbody>
</table>

ª 3H-labeled endothelial cells or A549 cells were treated with IL-1 or suspending media, harvested, and then incubated with vitronectin- or fibronectin-coated wells for 1 h in the presence of the indicated peptide (100 μg/ml) or antibody solutions (titre, 1:100). Weakly adherent cells were washed away, and adherent cells were lysed in 2% sodium dodecyl sulfate in HBSS, pH 7.4. The data show the percentage of added cells ± SE, which remained on the substratum.

* Adhesion of IL-1-treated cells was significantly greater than that of untreated cells, (P < 0.05).

Adhesion was significantly inhibited compared to the corresponding positive control (P < 0.05).

**tor-dependent fluorescence (Table 2) which was increased by 1.6 ± 0.1-fold after IL-1 treatment (mean ± SE from three independent experiments). The fluorescence intensity associated with fibronectin receptor antisera binding increased by 1.3 ± 0.1-fold. Normal rabbit serum and β2-microglobulin antisera binding did not change with IL-1 pretreatment. Untreated endothelial cells demonstrated significant binding of LM142 and LM609 above the normal mouse ascites control. IL-1 pretreatment increased the mean channel fluorescence detected with LM142 by 1.6 ± 0.1-fold and that detected with LM609 by 1.5 ± 0.1-fold (mean ± SE for three independent experiments). Neither normal mouse ascites nor W6/32 binding was increased by IL-1 treatment.

For A549 cells, the mean channel fluorescence associated with fibronectin receptor antisera binding was significantly greater than for normal rabbit serum (Table 2). Pretreatment of the A549 cells with IL-1 increased fibronectin receptor antisera binding by 1.3 ± 0.1-fold. However, the binding of the vitronectin receptor antisera to both untreated and IL-1-pretreated cells was weak. None of the monoclonal antibodies demonstrated increased binding to A549 cells following IL-1 pretreatment. LM609 did not bind to A549 cells, but LM142 binding was significantly greater than that associated with normal mouse ascites binding.

**Direct Quantitative Binding of Antisera to Endothelial Cell Monolayers.** Increased expression of endothelial cell vitronectin receptor and fibronectin receptor antigens in response to IL-1 treatment was also shown by direct quantitative binding of antisera (Fig. 3). Binding of the vitronectin receptor antisera was determined using 125I-labeled sheep anti-vitronectin IgG as the secondary reagent. B, shows the binding of the monoclonal ascites fluids LM609 (•), LM142 (•), and W6/32 (A) to endothelial cell monolayers as determined in quadruplicate using 125I-sheep anti-mouse IgG as the secondary reagent. Points, mean from three independent experiments; bars, SEM.

Table 2 Indirect immunofluorescence flow cytometry of untreated and IL-1-pretreated endothelial cells and A549 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean channel fluorescence*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>Antiserum</td>
<td>Un-treated</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>Anti-vitronectin receptor</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>Anti-fibronectin receptor</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>Anti-β2-microglobulin</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>Normal mouse ascites</td>
<td>4.9 ± 0.2</td>
</tr>
<tr>
<td>LM609</td>
<td>20.5 ± 0.2</td>
</tr>
<tr>
<td>LM142</td>
<td>31.5 ± 0.2</td>
</tr>
<tr>
<td>W6/32</td>
<td>66.0 ± 0.2</td>
</tr>
</tbody>
</table>

ª Untreated or IL-1-treated endothelial cells or A549 cells were incubated with the antibody solutions for 30 min at 4°C. The cells were washed and incubated with an FITC-conjugated secondary antibody for an additional 30 min. The cells were then fixed with 1% paraformaldehyde, and the fluorescent profiles were determined over a log scale of fluorescence. The data report the mean channel fluorescence ± SD for the fluorescent profile.

* Mean channel fluorescence on IL-1-pretreated cells was significantly greater than on untreated cells.

![Fig. 3. Binding of anti-integrin polyclonal antisera and vitronectin receptor monoclonal ascites fluids to untreated and IL-1-pretreated endothelial cells.](image-url)
changed by IL-1 treatment. IL-1 treatment of the endothelial cell monolayers increased the binding of the vitronectin receptor monoclonal ascites fluids LM609 and LM142 by 2.5 ± 0.4-fold after 8 h, but binding of the control, W6/32, was unchanged.

Immunoprecipitation Analysis of Antibody Binding. De novo synthesis of vitronectin receptor and fibronectin receptor were examined by the immunoprecipitation of [35S]methionine-labeled endothelial cells and A549 cells. The specific activities of the endothelial cell lysates were similar between cultures, independent of IL-1 treatment (not shown). The vitronectin receptor antiserum specifically immunoprecipitated bands at $M_r$ 95,000 and 150,000 ($M_r$ 105,000 and 130,000 reduced), corresponding to the reported molecular weights of the $\alpha_5$ and $\alpha_1$ subunits (20, 21, 35) (Fig. 4). The density of the $M_r$ 150,000 band ($\alpha_1$) increased 1.8 ± 0.3-fold after 4 h of IL-1 treatment and 4.0 ± 0.4-fold after 12 h. Similarly, the density of the $M_r$ 95,000 band ($\beta_1$) increased 1.9 ± 0.3-fold in response to 4 h of IL-1 treatment and 3.5 ± 0.4-fold after 12 h. The fibronectin receptor antiserum specifically precipitated bands at $M_r$ 120,000 and 140,000, corresponding to the expected positions of the $\alpha_5$ and $\beta_1$ subunits (19, 40). Treatment of the endothelial cells with IL-1 for 8 h increased the density of the $M_r$ 140,000 band ($\alpha_5$) 3.2 ± 0.3-fold and the density of the $M_r$ 120,000 band ($\beta_1$) 2.9 ± 0.2-fold.

A549 cells labeled for 4 h with [35S]methionine in the presence or absence of IL-1 showed no significant differences in specific activity. Immunoprecipitation of [35S]methionine-labeled A549 extracts with the vitronectin receptor antiserum showed no bands in addition to those obtained after immunoprecipitation with normal rabbit serum (Fig. 5). In contrast, immunoprecipitation with the fibronectin receptor antiserum revealed bands at $M_r$ 140,000 and 120,000, corresponding to the expected positions of the $\alpha_5$ and $\beta_1$ subunits. IL-1 treatment of the A549 cells correlated with an increased density of the $M_r$ 120,000 band.

Northern Blotting. We performed Northern blot analysis to determine if the IL-1-dependent increases in vitronectin receptor and fibronectin receptor protein were due to increased steady-state mRNA. Twenty $\mu$g of total RNA were electrophoresed on 7.5% polyacrylamide gels containing sodium dodecyl sulfate. Ordinate, positions of molecular weight markers.

![Fig. 4. Immunoprecipitation of [35S]methionine-labeled endothelial cell extracts treated with IL-1. Endothelial cells were treated with suspending buffer only (Lanes 1, 6, and 9) or with IL-1 for 4 h (Lanes 2, 5, 7, 8, and 10), 8 h (Lanes 3 and 11) or 12 h (Lane 4) and labeled with [35S]methionine for the final 4 h of treatment. Extracts were immunoprecipitated with vitronectin receptor polyclonal antiserum (Lanes 1-4 and 6-7), normal rabbit serum (Lanes 5 and 8), and fibronectin receptor polyclonal antiserum (Lanes 9-11). Some samples were reduced by treatment with 5% $\beta$-mercaptoethanol (Lanes 6-8). Immune complexes were purified with protein-A Sepharose and electrophoresed on 7.5% polyacrylamide gels containing sodium dodecyl sulfate. Ordinate, positions of molecular weight markers.](image)

![Fig. 5. Immunoprecipitation of [35S]methionine-labeled A549 cell extracts treated with IL-1. A549 cells were labeled with [35S]methionine for 4 h in the presence or absence of IL-1. Untreated (Lanes 1, 3, and 5) and IL-1-treated (Lanes 2, 4, and 6) A549 cells were immunoprecipitated with normal rabbit serum (Lanes 1 and 2), vitronectin receptor polyclonal antisera (Lanes 3 and 4), and fibronectin receptor polyclonal antiserum (Lanes 5 and 6). Immune complexes were isolated with protein-A Sepharose and electrophoresed on 7.5% polyacrylamide gels containing sodium dodecyl sulfate. Ordinate, positions of molecular weight markers.](image)

![Fig. 6. Northern blotting of total RNA from endothelial cells and A549 cells treated with IL-1. Total RNA was isolated from endothelial cells which were not treated (Lane 1) or were treated with IL-1 for 2 h (Lane 2), 4 h (Lane 3), or 8 h (Lane 4) or from A549 cells which were not treated (Lane 5) or were treated with IL-1 for 4 h (Lane 6). A, ethidium bromide-stained RNA run on 1% agarose gels containing sodium dodecyl sulfate. B, hybridization of the $\alpha_5$ cDNA probe; C, hybridization of the $\alpha_1$ probe. D, hybridization of the $\beta_1$ probe; E, hybridization of the $\beta_5$ probe. Hybridizations were conducted in 5x SSC (200 mM NaCl, 20 mM sodium citrate), 4x5 Denhardt's solution, 0.1% sodium dodecyl sulfate, and 0.2 mg/ml salmon sperm DNA at 62°C. The blots were washed in 2x SSC and 0.1% sodium dodecyl sulfate at 62°C.](image)
α, cDNA probe hybridized to an RNA species at 6.8 kilobases, and the intensity of this band increased 1.4-fold after 2 h and 3.0-fold after 8 h of IL-1 treatment (Fig. 6D).

RNA isolated from untreated and IL-1-treated A549 cells was similarly hybridized to the β1, β3, and αc cDNA probes. The β1 probe hybridized to two RNA species at 4.2 and 4.0 kilobases, and the intensity of these bands increased 2.4-fold after IL-1 treatment (Fig. 6B). The β3 probe failed to hybridize to A549 RNA (Fig. 6C), while the αc probe hybridized to bands at 6.8 and 4.5 kilobases, which did not change after IL-1 treatment (Fig. 6D).

**DISCUSSION**

Our previous investigations suggested that an RGD-dependent, integrin-like adhesion molecule is involved in cancer cell/endothelial cell adhesion following cytokine stimulation of the endothelium (8–10). The present study provides direct evidence that this adhesion is mediated, in part, through the vitronectin receptor (αcβ3). We have shown that the EC50 for GRGDS competition is approximately 8 μM, consistent with the involvement of vitronectin receptor (23). Furthermore, a polyclonal antiserum against the vitronectin receptor and a vitronectin receptor monoclonal ascites fluid, LM609, partially blocked the IL-1-induced increase in A549/endothelial cell adhesion. LM609, in particular, is specific for the αcβ3 dimer and does not suppress adhesion mediated through other related molecules such as gpIIb/IIIa, αcβ5, or αcβ1 (23–26, 29, 41). Our data also suggest that the fibronectin receptor is not involved in mediating A549/endothelial cell adhesion since the EC50 for GRGDS competition of A549/fibronectin adhesion was 65 μM and since the fibronectin receptor antiserum did not block adhesion. This supports the possibility that, in addition to ELAM-1 and ICAM-110 (11, 14), the vitronectin receptor (αcβ3) contributes to cancer cell/endothelial cell adhesion.

IL-1 pretreatment increased the amount of vitronectin receptor and fibronectin receptor antigens on the endothelial cell surface. This was identified by flow cytometry using the vitronectin receptor antiserum, the monoclonal ascites fluids LM142 and LM609, and fibronectin antiserum, all of which showed significant shifts in fluorescence corresponding to increased vitronectin receptor and fibronectin receptor expression in response to IL-1 pretreatment. Increased endothelial cell surface vitronectin receptor and fibronectin receptor were also detected by quantitative antibody binding with all of these antibodies. IL-1 treatment increased the rate of de novo synthesis of the vitronectin receptor subunits and fibronectin receptor subunits as detected by immunoprecipitation of [35S]methionine-labeled extracts and by Northern blotting to detect αc, β3, and β1 mRNA. Similar increases have been reported for fibroblasts and cancer cells in response to transforming growth factor β pretreatment (35, 42, 43) but, to our knowledge, have not been reported previously in endothelial cells.

This study indicated that A549 cells do not express vitronectin receptor. IL-1 pretreatment did not alter A549 adhesion to vitronectin. While others have shown that adhesion of many cell types to vitronectin, fibrinogen, and von Willebrand factor via αcβ3 are inhibited by LM609 (21, 29, 30), adhesion of A549 cells to vitronectin (and fibrinogen) was not inhibited by this antibody in our hands. The polyclonal vitronectin receptor antiserum did inhibit A549 adhesion to vitronectin, perhaps due to cross-reactivity with αcβ3, αcβ5, or αcβ1, which also mediate adhesion to vitronectin (25–28, 44, 45). Indirect immunofluorescence flow cytometry demonstrated that A549 cells were deficient in αcβ3, since they were unable to bind LM609. LM142 binding was associated with a small amount of fluorescence, suggesting the presence of an αc-like surface antigen. This low level of antigen, however, was not detectable by immunoprecipitation with the vitronectin receptor antiserum. Furthermore, the β3 mRNA was not detected by Northern blot analysis of the A549 total RNA. The αc probe hybridized weakly to A549 RNA species of 6.8 and 4.5 kilobases, but the intensity of these bands did not change after IL-1 treatment. These techniques also showed that A549 cells express fibronectin receptor and that fibronectin receptor expression was increased after IL-1 treatment.

Our results demonstrated that vitronectin receptor is involved in A549 cell adhesion to IL-1-treated endothelial cells but not to untreated endothelial cells. Since vitronectin receptor was not detected on the A549 cells, we postulate that adhesion is dependent on the expression of endothelial cell vitronectin receptor. The kinetics of both the increased expression of vitronectin receptor on the endothelial cell surface and induced A549 cell adhesion, in response to IL-1 treatment, are similar. Since the induction of cell adhesion is also dependent on protein synthesis (10), it appears that the increase in adhesion is due to increased vitronectin receptor synthesis. Untreated endothelial cells express vitronectin receptor, which is involved in endothelial cell/subendothelial matrix adhesion via abluminal adhesion plaques (40, 46–48). The observation that modest increases in total cell surface vitronectin receptor (approximately 2–3-fold) caused substantial increases in A549 adhesion might be related to the subcellular location of vitronectin receptor in response to IL-1 treatment. It would appear that IL-1 treatment must be able to promote the expression of vitronectin receptor on the luminal surface of the endothelial cells. Experiments to test this hypothesis are currently under way.

We conclude that pretreatment of endothelial cells with IL-1 increased the expression of cell surface vitronectin receptor, which, in turn, facilitated A549 cell/endothelial cell adhesion. Expression of the vitronectin receptor by activated endothelium, in response to localized or systemic inflammatory reactions, may account for adhesion of intravascular cancer cells to the vessel wall at sites of arrest. In vivo, sites of inflammation are more susceptible to cancer cell metastasis, consistent with the in vitro increases in cancer cell adhesion following cytokine treatment (49). Experimental metastasis is greater in IL-1-treated mice than in untreated mice (10, 50). Furthermore, experimental metastasis in both untreated (51, 52) and IL-1-treated mice (50) can be blocked by coinjecting GRGDS peptide with i.v. cancer cells. This suggests that endothelial activation is more susceptible to cancer cell metastasis, consistent with the in vitro increases in cancer cell adhesion following cytokine treatment (49). Experimental metastasis is greater in IL-1-treated mice than in untreated mice (10, 50).

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