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Laminin-10 and Lutheran blood group glycoproteins in adhesion of human endothelial cells

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Vainionpää, Noora, Yamato Kikkawa, Kari Lounatmaa, Jeffrey H. Miner, Patricia Rousselle, and Ismo Virtanen. Laminin-10 and Lutheran blood group glycoproteins in adhesion of human endothelial cells. *Am J Physiol Cell Physiol* 290: C764–C775, 2006. First published October 19, 2005; doi:10.1152/ajpcell.00285.2005.—Laminin α5-chain, a constituent of laminins-10 and -11, is expressed in endothelial basement membranes. In this study we evaluated the roles of α5 laminins and Lutheran blood group glycoproteins (Lu), recently identified receptors of the laminin α5-chain, in the adhesion of human dermal microvascular and pulmonary artery endothelial cells. Field emission scanning electron microscopy and immunohistochemistry showed that the endothelial cells spread on laminin-10 and formed fibronectin-positive fibrillar adhesion structures. Immunoprecipitation results suggested that the cells produced fibronectin, which they could use as adhesion substratum, during the adhesion process. When the protein synthesis during the adhesion was inhibited with cycloheximide, the formation of fibrillar adhesions on laminin-10 was abolished, suggesting that laminin-10 does not stimulate the formation of any adhesion structures. Northern and Western blot analyses showed that the cells expressed M_r 78,000 and 85,000 isoforms of Lu. Quantitative cell adhesion assays showed that in the endothelial cell adhesion to laminin-10, Lu acted in concert with integrins β_1 and $\alpha_v\beta_3$, whereas in the adhesion to laminin-10/11, Lu and integrin β_1 were involved. In the cells adhering to the α5 laminins, Lu and the integrins showed uniform cell surface distribution. These findings indicate that α5 laminins stimulate endothelial cell adhesion but not the formation of fibrillar or focal adhesions. Lu mediates the adhesion of human endothelial cells to α5 laminins in collaboration with integrins β_1 and $\alpha_v\beta_3$.

integrin; cycloheximide

INTERACTIONS BETWEEN ENDOTHELIAL CELLS and ECM are crucial for the formation of blood vessels and maintenance of endothelial integrity. Information about the mechanisms involved in these interactions is essential for the understanding of normal development and growth, as well as the pathogenesis of various diseases, such as vascular diseases and cancer. Furthermore, the components of these interactions are potential targets in the development of new treatments for pathological conditions (23, 31, 42, 55).

Basement membranes (BMs) are specialized sheets of ECM found in intimate contact with endothelia and epithelia, as well as with certain individual cells, such as adipose, muscle, and Schwann cells. In addition to the function of BMs as structural scaffolds for cells and tissues, the components of BMs are ligands for cell surface receptors and have effects on the adhesion, differentiation, migration, proliferation, and survival of the cells. Laminins (Ln), which belong to the main constituents of BMs, are a family of heterotrimeric molecules, each composed of α-, β-, and γ-chains. To date, five α-, three β-, and three γ-chains have been identified, and they form at least 15 laminin isoforms. All laminin chains share structural similarities, but the laminin α-chains in particular possess many receptor binding sites, are differently recognized by the cells, and are expressed in a tissue-specific and developmentally regulated manner (6, 38, 47).

Laminin α5-chain is a component of Ln-10 ($\alpha_5\beta_1\gamma_1$), Ln-11 ($\alpha_5\beta_2\gamma_1$), and the recently identified Ln-15 ($\alpha_5\beta_2\gamma_3$) (6, 38). Ln-10 has the broadest expression pattern of laminins and is a constituent of most BMs of fetal and adult tissues (34, 37, 54). Ln-11 has a more restricted distribution and is expressed, for example, in the glomerular BM of kidney, neuromuscular synaptic cleft, and BMs of arterial smooth muscle cells (36). The laminin α5-chain is produced by rodent endothelial cells and found in endothelial BMs (37, 54). Lack of the laminin α5-chain in knockout mice leads to several developmental defects, including defective vascularization of placenta and kidney glomeruli (33, 35), which suggests an important role for this laminin chain in endothelial development and function.

The effects of laminins on cell behavior are mediated by cell surface receptors. Among the integrins, $\alpha_2\beta_1$ (48), $\alpha_3\beta_1$ (26, 56), $\alpha_6\beta_1$ (25, 56), $\alpha_6\beta_4$ (25, 28), and $\alpha_v\beta_3$ (18, 51) have been reported to mediate the adhesion of various cell types to α5 laminins. Also, dystroglycan glycoprotein complex binds to laminin α5-chain (28, 67).

Lutheran blood group glycoproteins (Lu) have recently been shown to function as adhesion receptors specific to the laminin α5-chain (24, 43). They include two proteins of 78 and 85 kDa (7, 46), which are products of alternatively spliced RNA transcripts of a single gene (11, 44, 50). The smaller protein is also known as basal cell adhesion molecule (1, 16). The role of Lu as a laminin receptor was recognized in studies of normal and sickle red blood cells (10, 60, 68), Lu-transfected human erythroleukemia cells (10, 43), and Lu-transfected murine fibroblasts (10). Kikkawa et al. (27) recently proposed that Lu

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alone would be unable to mediate the adhesion of human mesangial cells to Ln-10/11.

Only a few studies have focused on the interaction of human endothelial cells and α5 laminins (9, 15). These studies have evaluated the role of integrins in this interaction, and the role of other potential receptors, such as Lu, has remained elusive. In this study we evaluated the roles of α5 laminins and Lu in the adhesion of human endothelial cells.

MATERIALS AND METHODS

Cell culture. Human pulmonary artery endothelial (HPAE) cells were obtained from PromoCell (Heidelberg, Germany), and human dermal microvascular endothelial (HDME) cells were obtained from both PromoCell and Cambrex Bioscience (Walkersville, MD). The cells were cultured in endothelial cell growth medium-2 (EGM-2; PromoCell) and endothelial cell growth medium-2MV (EGM-2MV; PromoCell), respectively.

Northern blot analysis. Total RNA was isolated from HDME and HPAE cells by acid phenol-guanidinium thiocyanate-chloroform extraction using standard methods (5). Poly(A)$^+$ RNAs were enriched using DynaBeads oligo(dT)$_{25}$ beads (Dynal, Oslo, Norway) according to the manufacturer's instructions. The RNA was then separated according to size in a denaturing 1.2% agarose gel and transferred onto Hybond membranes (Amersham Biosciences, Uppsala, Sweden) by upward capillary transfer. The Northern hybridization was performed using the DIG High Prime DNA labeling and detection starter kit II (Roche, Mannheim, Germany) according to the manufacturer's instructions. A cDNA expression plasmid containing the full-length human Lu coding region was purchased from Invitrogen (Carlsbad, CA) and modified as previously described (24). A 700-bp probe was created by restriction with *Sma*I (Promega, Madison, WI). Prehybridization was carried out at 55°C for 30 min, and the hybridization was carried out at 55°C for 24 h. The blots were exposed to Hyperfilm MP (Amersham Biosciences). As size markers, a 0.24- to 0.5-kb RNA ladder (Invitrogen) and a 0.28- to 6.58-kb RNA marker (Promega) were used.

Western blot analysis. For Western blot analysis, samples of HPAE and HDME cells were made by boiling the detached cells in nonreducing sample buffer. SDS-PAGE was performed according to Laemmli's procedure with 8% gels. The proteins were transferred onto nitrocellulose filters and blocked with 5% dry milk in PBS. The filters were exposed to MAb BRIC221 against Lu (Serotec, Oxford, UK). Immunoreactive bands were visualized using peroxidase-coupled goat anti-mouse IgG (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) with nickel intensification and diaminobenzidine as substrate (Sigma, St. Louis, MO) according to the manufacturer's instructions. Sigmamarker with high-molecular-weight range (Sigma) was used as a size marker.

Radioactive metabolic labeling and fluorography. Endothelial cell cultures were incubated in methionine-deficient culture medium with or without cycloheximide (10 μg/ml; Sigma) for 1 h. Next, 25 μCi/ml S^{35}-labeled methionine (Amersham Biosciences) was added to the culture medium, and the cells were incubated for 2–3 h. The culture medium of the cells was collected, cleared by centrifugation, supplemented with normal mouse serum and 0.5% Triton X-100, preabsorbed with uncoupled GammaBind Plus Sepharose beads, and applied to the GammaBind Plus Sepharose beads precoupled with MAb 4C7 to laminin α5-chain (12, 58) or with MAb 52DH1 to fibronectin (61). The bound proteins were eluted with Laemmli's sample buffer, and SDS-PAGE was performed according to Laemmli's procedure with 5% gels under reducing conditions. Fluorography was performed using Hyperfilm MP (Amersham Biosciences) according to standard methods.

Quantitative cell adhesion assay. Quantitative cell adhesion assays were performed using a method based on intracellular acid phosphatase (49, 56). Human Ln-10 was purified from the culture medium of PANC-1 pancreatic adenocarcinoma cells by using immunoaffinity chromatography as previously described (56). Human placental Ln-10/11 and mouse Ln-1 from Engelbreth-Holm-Swarm tumor (EHS-Ln) were obtained from Sigma. Human Ln-5 was immunopurified from the culture medium of SCC25 cells as previously described (62). Fibronectin was purified from outdated human plasma (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) by performing gelatin-Sepharose affinity chromatography (Amersham Biosciences) according to the method of Engvall and Ruoslahti (13). The following function-blocking MAbs to integrins were used: PIE6 to integrin α_2-subunit (Chemicon, Temecula, CA), PIB5 (Chemicon) and 3G8 (Ref. 25; a kind gift from Prof. K. Sekiguchi, Institute for Protein Research, Osaka University, Osaka, Japan) to integrin α_3-subunit, GoH3 to integrin α_6-subunit (Chemicon), LM609 to integrin $\alpha_v\beta_3$-subunit (Chemicon), and 13 to integrin β_1-subunit (Ref. 65; kindly provided by Prof. K. M. Yamada, Craniofacial and Developmental Biology and Regeneration Branch, National Institute for Dental and Craniofacial Research, Bethesda, MD). The concentration of the MAbs in the experiments was 2 μg/ml, with the exception of MAb 3G8, which was used at a concentration of 6 μg/ml.

Wells of 96-well plates were coated with laminins or fibronectin (4 μg/ml) at room temperature (RT) for 1 h and washed twice with PBS. Thereafter, part of the wells was exposed to soluble recombinant protein (10 μg/ml in PBS) corresponding to the extracellular domain of Lu (Sol-Lu; Ref. 24) at RT for 1 h, whereas part of the wells was exposed only to PBS. The wells were then washed twice with PBS, subsequently treated with 3% BSA in PBS at RT for 1 h, and washed again twice with PBS. Cycloheximide was added (10 μg/ml) to the culture medium of the cells 1 h before the cells were plated, as well as to the adhesion medium (EGM-2 or EGM-2MV without fetal calf serum). The cells were detached with trypsin and EDTA, exposed to trypsin-neutralizing solution (PromoCell), and washed with the adhesion medium. Thereafter, the function-blocking MAbs were added to the cell suspensions. The cells were plated at 2×10^4 cells/well, and the plates were incubated at 37°C in 5% CO_2 for 1 h. The wells were carefully washed to remove nonadherent cells, whereas the control wells showing the amount of the cells originally plated were not washed. The plate was centrifuged with a Hermle Z 400 K centrifuge (Hermle Labortechnik, Wehingen, Germany) at 500 rpm for 5 min to minimize loss of the cells from the control wells, showing the amount of cells plated, and the adhesion media of these wells were carefully removed. Substrate solution (Sigma 104 phosphatase substrate, 6 mg/ml in 50 mM sodium acetate buffer with 0.1% Triton-X100, pH 5) was added to each well, and the plates were incubated at 37°C for 1 h. The reaction was stopped with 1 M NaOH, and the absorbances were measured at 405 nm in an ELISA reader. BSA-coated wells were used as controls showing unspecific adhesion. Experiments were performed in triplicate, and the amount of adhered cells is expressed as a percentage of the cells originally plated (±SD of 3 wells). The difference between two variables was tested with a two-sided, unpaired *t*-test with a significance level of $\alpha = 0.05$.

Morphological adhesion assays. For visualization of the morphology and adhesion structure formation of the adhering cells, we also performed morphological adhesion assays. For this purpose, cell culture dishes with glass coverslips were coated with Ln-10, Ln-10/11, or Ln-10 combined with fibronectin at RT for 1 h. After two washes with PBS, the dishes were subsequently coated with 3% BSA in PBS at RT for 1 h and washed again twice with PBS. The cells were detached with trypsin and EDTA, exposed to trypsin-neutralizing solution, washed with the adhesion medium (EGM-2 or EGM-2MV without fetal calf serum), plated onto the coverslips in the adhesion medium, and incubated at 37°C in 5% CO_2 for 2 h. For certain experiments, cycloheximide (10 μg/ml) was added to the culture medium 1 h before the cells were plated, as well as to the adhesion medium. The nonadherent cells were removed by washing the samples twice carefully with PBS, and the morphology of the cells was

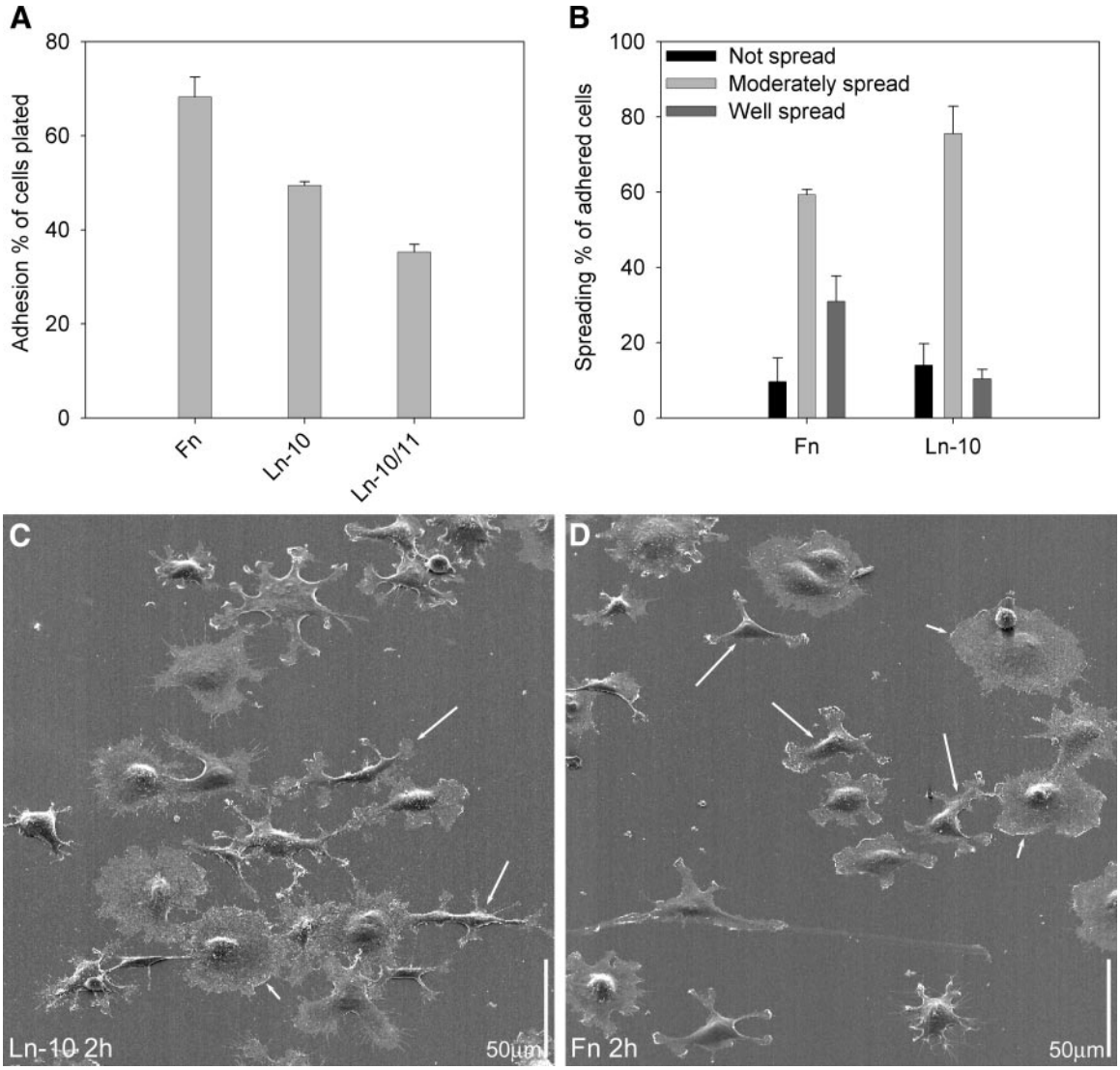

Fig. 1. Endothelial cell adhesion to laminin (Ln)-10, Ln-10/11, and fibronectin (Fn). *A*: of the plated human pulmonary artery endothelial (HPAE) cells, 50% adhered to Ln-10, 35% adhered to Ln-10/11, and 70% adhered to Fn. The morphology of the attached cells was visualized using field emission scanning electron microscopy (FESEM). Of the human dermal microvascular endothelial (HDME) cells plated on Ln-10 (*B* and *C*), 15% of the attached cells did not spread and 75% of the cells spread moderately and formed multiple protrusions (long arrows), whereas 10% of the cells spread well and formed some lamellipodia (short arrow). Of the HDME cells plated on Fn (*B* and *D*), 10% of the cells did not spread, 60% of the cells formed multiple short protrusions (long arrows), and 30% of the cells spread well and formed lamellipodia (short arrows).

visualized with either field emission scanning electron microscopy (FESEM) or indirect immunofluorescence microscopy.

Field emission scanning electron microscopy. For FESEM, the cells cultured on glass coverslips were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at RT for 45 min. The specimens were coated with 20 nm of chromium with Emitech K575X sputter, and the cells were studied with a Jeol JSM 6335F microscope at a 5-kV operating voltage and 40° inclination.

For comparison of the cell morphology on different adhesion substrata, the adhered cells were counted in three independent visual fields and divided according to their morphology into three groups of nonspread (round), moderately spread (protrusion forming), and well-spread (lamellipodia forming) cells. The results for each group are expressed as percentages of the all adhered cells (±SD of 3 visual fields).

Immunofluorescence microscopy. For indirect immunofluorescence microscopy, the following antibodies were used: MAb 102DF5 to integrin β_1-subunit (66), MAb LM142.69 to integrin α_v-subunit (Ref. 4; a kind gift from Prof. D. A. Cheresh, Scripps Clinic and Research Foundation, La Jolla, CA), rat MAb BIE5 to integrin α_5-subunit (Ref. 64; a kind gift from Prof. Z. Werb, Department of Anatomy, University of California, San Francisco, CA), MAb BRIC221 to Lu (Serotec), MAb TA205 to talin (Serotec), MAb FB11 to vinculin (Biohit, Helsinki, Finland), rabbit antiserum to vinculin (30), MAb 52DH1 to fibronectin (61), and rabbit antiserum to fibronectin (Dako, Glostrup, Denmark).

HPAE and HDME cells cultured on glass coverslips were fixed in methanol at −20°C for 15 min. The specimens were first exposed to MAbs at RT for 30 min, followed by either FITC-coupled goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) or Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR) at RT for 30 min. For double-labeling experiments, the specimens were subsequently exposed to rat MAbs or rabbit polyclonal antisera, followed by tetramethylrhodamine isothiocyanate (TRITC)-coupled goat anti-rat IgG (Jackson Immunoresearch), TRITC-coupled goat anti-rabbit IgG (Jackson Immunoresearch), or Alexa Fluor 594 goat

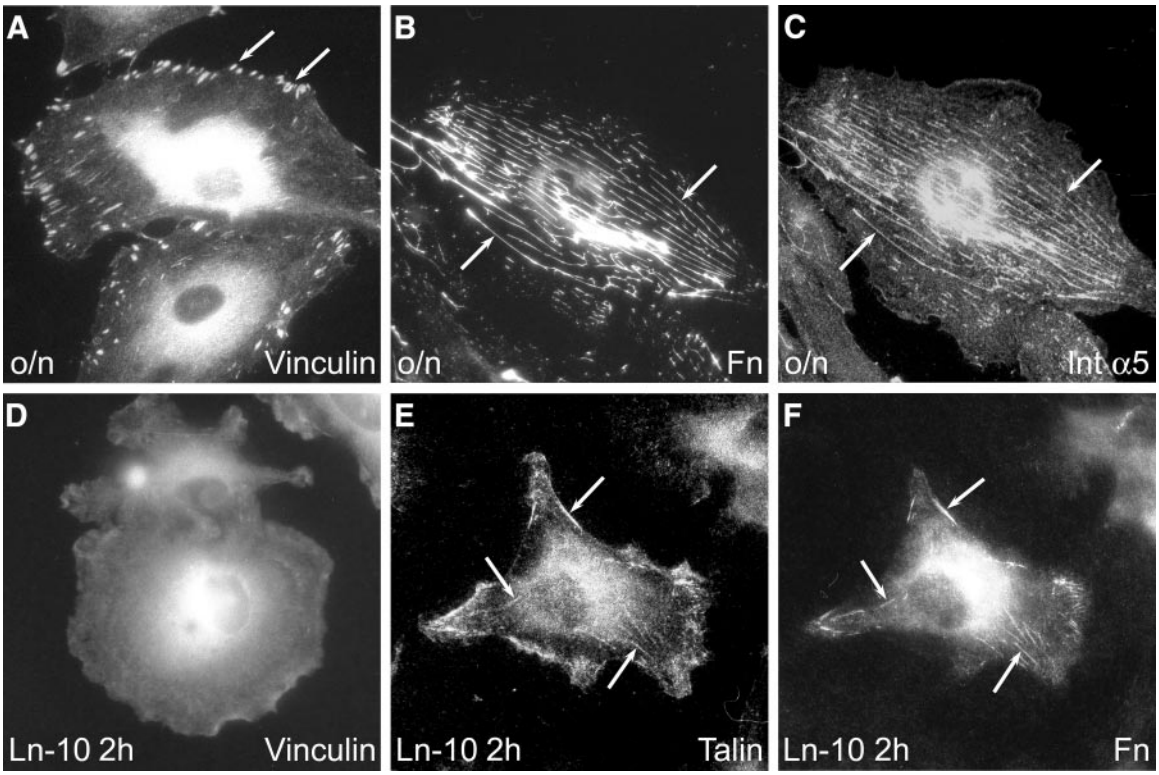

Fig. 2. Adhesion structure formation of human endothelial cells determined with immunofluorescence technique. *A*: in overnight (o/n) cultures of HDME cells, immunoreactivity for vinculin shows a nail-like distribution (arrows) in the periphery of the cells. Immunoreactivities for Fn (*B*, HDME) and integrin α_5 (*C*, HDME) were in colocalization in fibrillar structures (arrows) in the center of the cells. In the 2 h of adhesion to Ln-10, the cells showed diffuse immunoreactivity for vinculin (*D*, HPAE). Immunoreactivities for talin (*E*, HDME) and Fn (*F*, HDME) were found in colocalization in fibrillar structures (arrows).

anti-rabbit IgG (Molecular Probes). The specimens were embedded in sodium veronal-glycerol buffer (1:1, pH 8.4) and examined using a Leica Aristoplan microscope equipped with appropriate filters.

RESULTS

Morphology and adhesion structure formation in the endothelial cell adhesion to Ln-10. The role of Ln-10 in endothelial cell adhesion was evaluated by studying its ability to stimulate cell adhesion, spreading, and formation of adhesion structures in quantitative and morphological adhesion assays.

In a quantitative cell adhesion assay, 50% of the plated cells adhered to Ln-10 and 35% adhered to Ln-10/11, whereas 70% adhered to fibronectin (Fig. 1*A*). The morphology of the attached cells was visualized using FESEM. To compare the adhesion-promoting properties of the different substrata, we counted the attached cells in three individual visual fields in each sample and divided them into three groups of nonspread (round), moderately spread (protrusion forming), and well-spread (lamellipodia forming) cells (Fig. 1*B*). On Ln-10, 15% of the adhered cells did not spread and showed a round morphology and 75% of the cells spread moderately (the cell body was round, and cells formed multiple protrusions), whereas 10% of the cells spread well and showed some lamellipodia (Fig. 1, *B* and *C*). On fibronectin, 10% of the adhered cells showed a round morphology and 60% of the cells spread moderately and formed multiple protrusions, whereas 30% of the cells spread well and formed some lamellipodia (Fig. 1, *B* and *D*).

The formation of cell-substratum adhesion structures was studied using the indirect immunofluorescence technique. In overnight cultures, the endothelial cells showed immunoreactivity for vinculin (Fig. 2*A*) in nail-like plaques in the periphery of the cells, as well as immunoreactivities for both fibronectin (Fig. 2*B*) and integrin α_5 (Fig. 2*C*) in colocalization in fibrillar structures in the center of the cells. On the other hand, within 2 h of adhesion to Ln-10, the cells showed diffuse cytoplasmic immunoreactivity for vinculin (Fig. 2*D*). Immunoreactivities for talin (Fig. 2*E*) and fibronectin (Fig. 2*F*) were colocalized in fibrillar structures in the cells.

The detection of fibronectin-immunoreactive adhesion structures in the cells adhering to Ln-10 suggested that endothelial cells secrete endogenous proteins during the adhesion assay. The production of endogenous proteins by the cells within 2–3 h was studied using radioactive metabolic labeling and immunoprecipitation. Immunoprecipitation with MAb 52DH1 showed that the cells synthesized a polypeptide with M_r ~220,000 (Fig. 3*A*), corresponding to the size of fibronectin (61), and immunoprecipitation with MAb 4C7 showed that the cells synthesized two polypeptides of M_r ~380,000 and ~390,000 (Fig. 3*B*), corresponding to laminin α5-chain (2). Exposure of the cells to cycloheximide prevented the synthesis of the proteins (Fig. 3, *A* and *B*).

To avoid the effects of endogenously secreted proteins on the results of the adhesion assays, we studied the cell adhesion, spreading, and formation of cell-substratum adhesion structures in the presence of cycloheximide. In quantitative cell

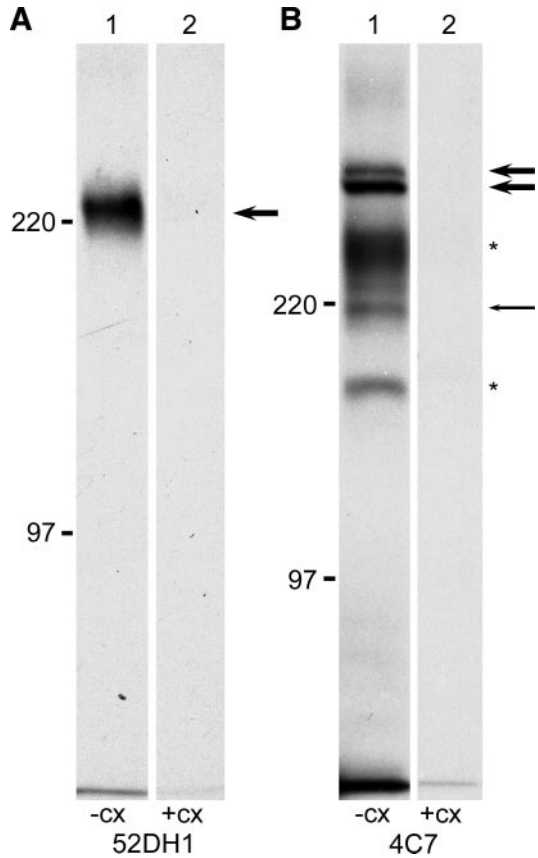

Fig. 3. Endothelial cells were grown with (+) or without (−) cycloheximide (cx), and synthesis and secretion of proteins were detected using metabolic labeling with [S^{35}]methionine and immunoprecipitation. *A*: after 2 h of incubation without cx, MAb 52DH1 precipitated a polypeptide of M_r ~220,000 (*lane 1*, arrow) from the culture medium of HPAE cells. When the cells were cultured with cx, no proteins were detected (*lane 2*). *B*: after 3 h of incubation without cx, MAb 4C7 precipitated 2 polypeptides of M_r ~380,000 and ~390,000, corresponding to laminin α5-chain (*lane 1*, thick arrows) from the culture medium of HDME cells. In addition, polypeptides of M_r ~220,000, corresponding to the sizes of laminin β1- and γ1-chains (*lane 1*, thin arrow) were precipitated. Two unspecific bands (*lane 1*, asterisks) also were detected in the control precipitation without a MAb. When the cells were cultured with cx, no proteins were detected (*lane 2*).

adhesion assay, 30% of the plated cells adhered to Ln-10 and 25% of the cells adhered to Ln-10/11, whereas 55% of the cells adhered to fibronectin (Fig. 4*A*). The morphology of the attached cells was visualized using FESEM. On Ln-10, 5% of the adhered cells did not spread and showed a round morphology and 90% of the cells spread moderately (the cell body acquired a spindle-shaped form, and the cells formed multiple long extensions), whereas 5% of the cells spread well and formed lamellipodia (Fig. 4, *B* and *C*). On fibronectin, 10% of the adhered cells showed a round morphology and 75% of the cells spread moderately (the cell body was round, and cells formed multiple short protrusions), whereas 15% of the cells spread well and formed lamellipodia (Fig. 4, *B* and *D*). The cells acquired a more spindle-shaped form, and their extensions were thinner and longer in the adhesion to Ln-10 than in the adhesion to fibronectin (Fig. 4, *C* and *D*).

Upon 2 h of adhesion to Ln-10, the cells showed diffuse cytoplasmic immunoreactivities for talin (Fig. 5*A*) and vinculin (Fig. 5*B*). When the coverslips were coated with both fibronectin and Ln-10, the cells showed nail-like structures immunoreactive for both talin (Fig. 5*C*) and vinculin (Fig. 5*D*). The results for endothelial cell adhesion to Ln-10/11 were comparable to the results for adhesion to Ln-10 (not shown).

Receptors mediating the adhesion of endothelial cells to Ln-10. Among potential receptors of α5 laminins, endothelial cells express integrins $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_v\beta_3$ (8, 9). Garin-Chesa et al. (16) suggested that human umbilical vein endothelial cells express a Lu isoform of M_r 90,000. Before evaluating the role of these receptors in the adhesion of endothelial cells to Ln-10, we studied whether HPAE and HDME cells express Lu.

Northern blot analysis of HPAE (Fig. 6*A*) and HDME (not shown) cells showed two Lu transcripts of ~2.5 and ~4.0 kb, of which the smaller one was more pronounced than the larger one. Western blot analysis of endothelial cell lysates with MAb BRIC 221 to Lu showed two polypeptides of M_r ~78,000 and ~85,000 (Fig. 6*B*), of which the larger one was more prominent than the smaller one. In immunohistochemistry of overnight cultures of HPAE (Fig. 6*C*) and HDME cells (Fig. 6*D*), Lu showed a uniform punctate immunoreactivity on the cells.

The roles of Lu and integrins in the adhesion were studied using quantitative cell adhesion assay in the presence of cycloheximide. Recombinant protein Sol-Lu was used to inhibit Lu function in the adhesion assays by saturation of Lu binding sites of laminin α5 chain (27). The specificity of the inhibitory effect of Sol-Lu in the assays was tested using fibronectin, Ln-5 ($\alpha_3\beta_3\gamma_2$), and EHS-Ln ($\alpha_1\beta_1\gamma_1$) as adhesion substrates. The cells adhered to these proteins, but the adhesion was not inhibited substantially with the preincubation of the proteins with Sol-Lu (Fig. 7, *A–D*).

The experiments were then performed with HPAE cells and native human Ln-10 as an adhesive substrate (Fig. 7*C*). Of the plated cells, 45% adhered to this laminin. With Sol-Lu, the adhesion diminished to 20%. With MAb to integrin β_1, the adhesion diminished to 15%, whereas with the combination of Sol-Lu and MAb to integrin β_1, the adhesion was <10%. With the combination of Sol-Lu and MAb to integrin $\alpha_v\beta_3$, the adhesion was <5%. On the other hand, in the adhesion of HDME cells (Fig. 7*D*), 30% of the plated cells adhered to Ln-10. With Sol-Lu, the adhesion decreased to 10%. With MAb to integrin β_1, the adhesion was diminished to 5%, whereas with the combination of Sol-Lu and MAb to integrin β_1, as well as with Sol-Lu and MAb to integrin $\alpha_v\beta_3$, the adhesion was even less. The inhibitory effects of MAbs to integrins α_2 (not shown), α_3, and α_6 (Fig. 7, *C* and *D*) were negligible as single agents, as well as in various combinations (not shown).

Because of the limited availability of native human Ln-10, a mixture of Ln-10 and -11, produced by pepsin digestion from human placenta, has been used as adhesion substrate in many cell adhesion studies. For comparison, we also performed the quantitative cell adhesion experiments with the mixture Ln-10/11. In the adhesion of HPAE cells, 45% of the plated cells adhered to this substrate (Fig. 8*A*). With Sol-Lu, the adhesion diminished to 35%. With MAb to integrin β_1, the adhesion diminished to 30%, and with the combination of Sol-Lu and MAb to integrin β_1, the adhesion was <20%. On the other hand, 35% of the plated HDME cells adhered to Ln-10/11 (Fig. 8*B*). With Sol-Lu, the adhesion diminished to 25%. With MAb to integrin β_1, the adhesion was 10%. When combined with

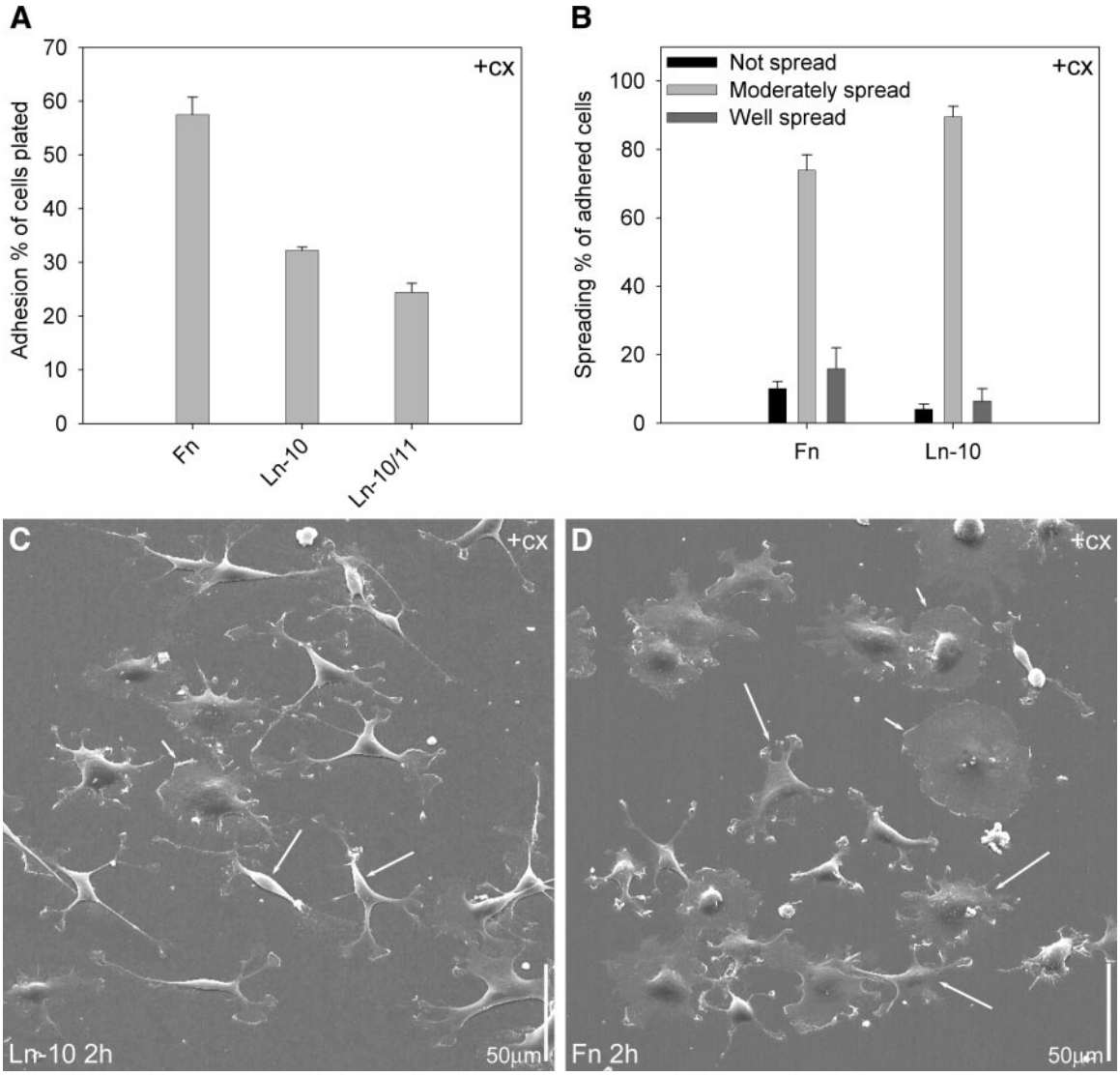

Fig. 4. Endothelial cell adhesion to Ln-10, Ln-10/11, and Fn in the presence of cx. Of the plated cells, 30% adhered to Ln-10, 25% to Ln-10/11, and 55% to Fn (*A*, HPAE). FESEM showed that on Ln-10 (*B* and *C*, HDME), 5% of the attached cells did not spread, 85% of the cells acquired a spindle-shaped form with multiple long, thin projections (long arrows), and 5% of the cells formed some lamellipodia (short arrow). On Fn (*B* and *D*, HDME), 10% of the attached cells did not spread, 75% of the cells had a round cell body and formed multiple short protrusions (long arrows), and 15% of the cells formed some lamellipodia (short arrows).

Sol-Lu, the inhibitory effect of MAb to integrin β_1 did not increase substantially. MAbs to integrins α_2 (not shown), α_3, α_6, and $\alpha_v\beta_3$ (Fig. 8, *A* and *B*) had smaller inhibitory effects on the adhesion as single agents, as well as in various combinations (not shown).

In contrast to our results, earlier studies have presented evidence that endothelial cells would adhere to Ln-10 predominantly via integrin $\alpha_3\beta_1$ (9, 15). We repeated the experiments, shown above with the MAb PIB5 to integrin α_3, using the function-blocking MAb 3G8 to integrin α_3, but it did not inhibit the cell adhesion to Ln-10 or Ln-10/11 alone or in combination with Sol-Lu (not shown). Therefore, using quantitative cell adhesion assay, we also studied whether the endothelial cells have a functional α_3-integrin in their adhesion to native human Ln-5. Of the plated HDME cells, 20% adhered to this laminin. With the MAb PIB5 to integrin α_3, the adhesion diminished to 10%, and MAb to integrin β_1 prevented the adhesion totally (Fig. 8*C*).

The distributions of Lu and integrins α_v and β_1 in the endothelial cells adhering to Ln-10 were studied using immunofluorescence microscopy. In the absence of cycloheximide, immunoreactivity for integrin α_v was uniformly distributed on most of the cells, but in some cells it was located to tiny fibrillar structures (Fig. 9*A*). Immunoreactivity for integrin β_1 was located to small fibrillar structures, which often appeared in circular formation near the periphery of the cells (Fig. 9*B*). Immunoreactivity for Lu had a uniform punctate distribution on the endothelial cells (Fig. 9*C*). In the presence of cycloheximide, immunoreactivities for integrins α_v (Fig. 9*D*) and β_1 (Fig. 9*E*) were diffusely distributed on the cells, and immunoreactivity for Lu showed a uniform punctate distribution on the cells (Fig. 9*F*).

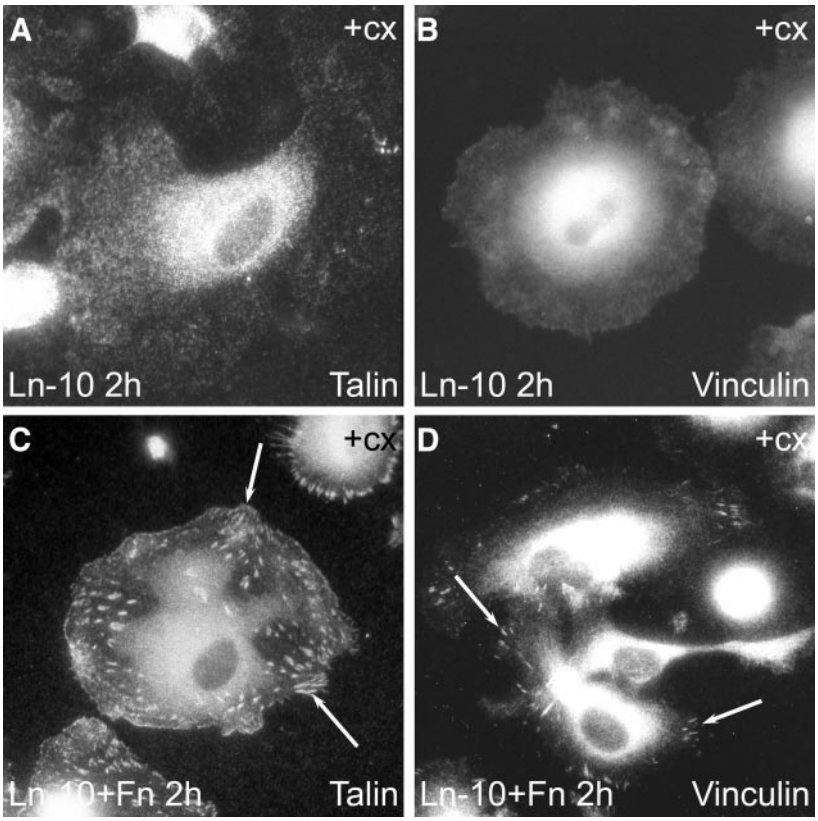

Fig. 5. Adhesion structure formation in the presence of cx determined using an immunofluorescence technique. In the cells adhering to Ln-10, diffuse immunoreactivities for talin (*A*, HPAE) and vinculin (*B*, HPAE) were found. In the adhesion to the combination of Ln-10 and Fn, immunoreactivities for talin (*C*, HDME) and vinculin (*D*, HDME) were detected in nail-like structures at the periphery of the cells (arrows).

DISCUSSION

To evaluate the role of α5 laminins in the adhesion of endothelial cells, we first studied the morphology and adhesion structure formation of these cells in their adhesion to Ln-10. The results show that in overnight cultures, endothelial cells formed typical nail-like focal adhesions immunoreactive for vinculin, as well as fibrillar adhesions immunoreactive for fibronectin and integrin α_5. In their 2-h adhesion to Ln-10, on the other hand, the cells spread and formed tiny fibrillar adhesions (also known as ECM adhesions) immunoreactive for talin and fibronectin, which are related to the reorganization of ECM fibronectin into fibronectin fibrils (17), but no typical focal adhesions, which are considered to be devoid of fibronectin (3).

The fibronectin immunoreactivity of the adhesion structures formed in the adhesion to Ln-10 suggested to us that the cells produced endogenous proteins that they could use as adhesion substrates during the adhesion assay. The immunoprecipitation results supported these findings by showing that the cells secreted within 2–3 h were ECM proteins such as fibronectin and laminin α5-chain, and the production of these proteins could be prevented with cycloheximide. To avoid the effects of

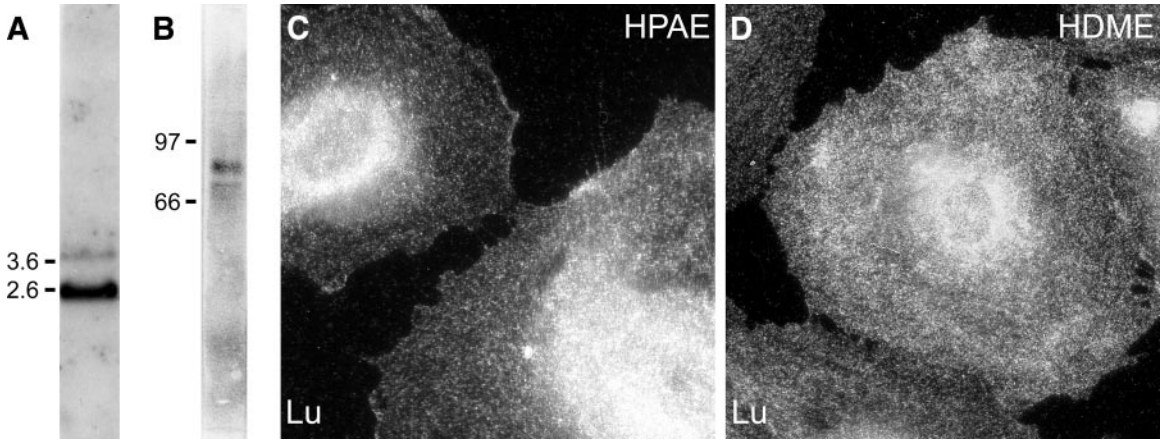

Fig. 6. Lutheran blood group glycoprotein (Lu) expression in human endothelial cells. *A*: in Northern blot experiments with RNA of HPAE cells, the Lu probe detected 2 transcripts of ~2.5 and ~4.0 kb, of which the smaller was more prominent. *B*: Western blot analysis of HPAE lysates with MAb 221 against Lu detected 2 polypeptides of M_r ~78,000 and ~85,000, of which the larger was pronounced. Under immunofluorescence microscopy, Lu showed a punctate distribution on HPAE (*C*) and HDME cells (*D*).

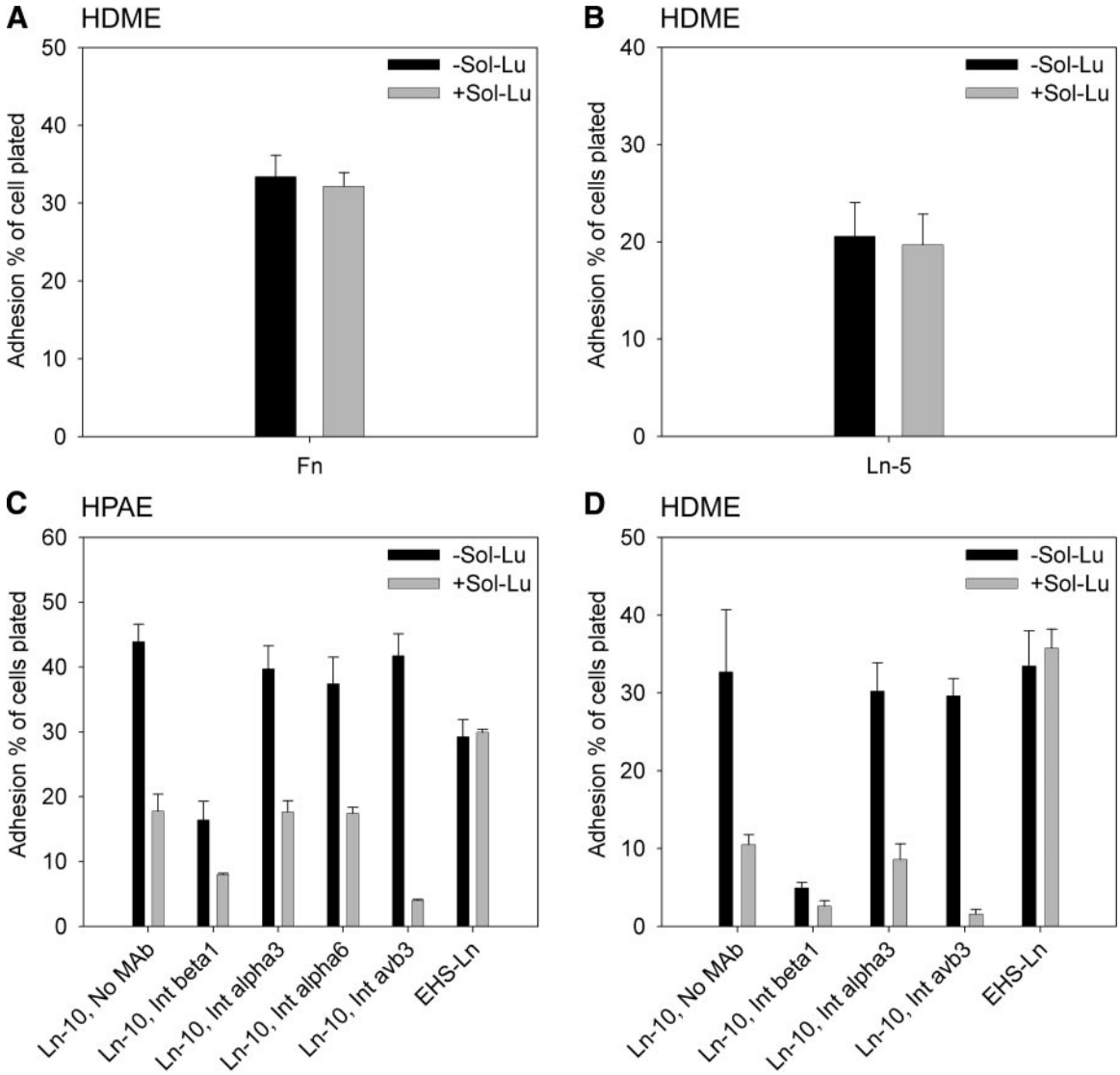

Fig. 7. Effects of soluble recombinant protein corresponding to the extracellular domain of Lu (Sol-Lu) and MAbs to integrins (Int) on the adhesion of endothelial cells. Adhesion of HDME cells to Fn (*A*) or Ln-5 (*B*) was not inhibited with Sol-Lu. *C*: in the adhesion of HPAE cells to Ln-10, 45% of the plated cells adhered to this laminin. Adhesion diminished to 20% with Sol-Lu. With the MAb to integrin β_1, the adhesion diminished to 15%, and with the combination of MAb with integrin β_1 and Sol-Lu, the adhesion was <10%. With the combination of MAb to integrin $\alpha_v\beta_3$ (avb3) and Sol-Lu, the adhesion was <5%. *D*: in the adhesion of HDME cells to Ln-10, 30% of the cells adhered to this laminin. With Sol-Lu, the adhesion was diminished to 10%, whereas with MAb to integrin β_1 it was 5%. Combinations of MAb to integrin β_1 and Sol-Lu, as well as MAb to integrin $\alpha_v\beta_3$ and Sol-Lu, prevented the adhesion nearly completely. MAbs to integrins α_3 and α_6 had minor effects on the adhesion of both cells. Sol-Lu had no effects on the adhesion of the cells to mouse Ln-1 from Engelbreth-Holm-Swarm tumor (EHS-Ln).

these endogenous proteins on the results of adhesion assays, we performed the assays in the presence of cycloheximide. Under these conditions, the cells spread on Ln-10 but formed neither fibrillar nor focal adhesions.

By definition (17, 40), cell adhesion in the absence of cell spreading and focal adhesion formation indicates weak adhesion, a transient phase of attachment failing to support cell survival, whereas adhesion and cell spreading without focal adhesion formation indicates intermediate adhesion considered to support cell survival and motility. Firm adhesion correlates with formation of focal adhesions and actin stress fibers and is crucial for survival, growth, and maintenance of the differentiated phenotype of anchorage-dependent cells. Our findings show that Ln-10 stimulates endothelial cell spreading without focal adhesion formation, suggesting that instead of anchoring the cells firmly to the substratum, this laminin retains the motile phenotype of the cells.

Some immunohistochemical studies have suggested that Lu is found in blood vessel walls (45, 52), and Moulson et al. (39) showed that it is located around the smooth muscle cells of blood vessels. Garin-Chesa et al. (16) proposed that human umbilical vein endothelial cells produce a M_r 90,000 isoform of Lu. In the present study Northern and Western blot analysis showed that the endothelial cells produced two RNA transcripts of ~2.5 and ~4.0 kb and two protein isoforms of M_r ~78,000 and ~85,000, which correspond to the Lu isoforms detected in human red blood cells, tumor cells, and many human tissues (7, 46, 50). The long-tail isoform of Lu, which

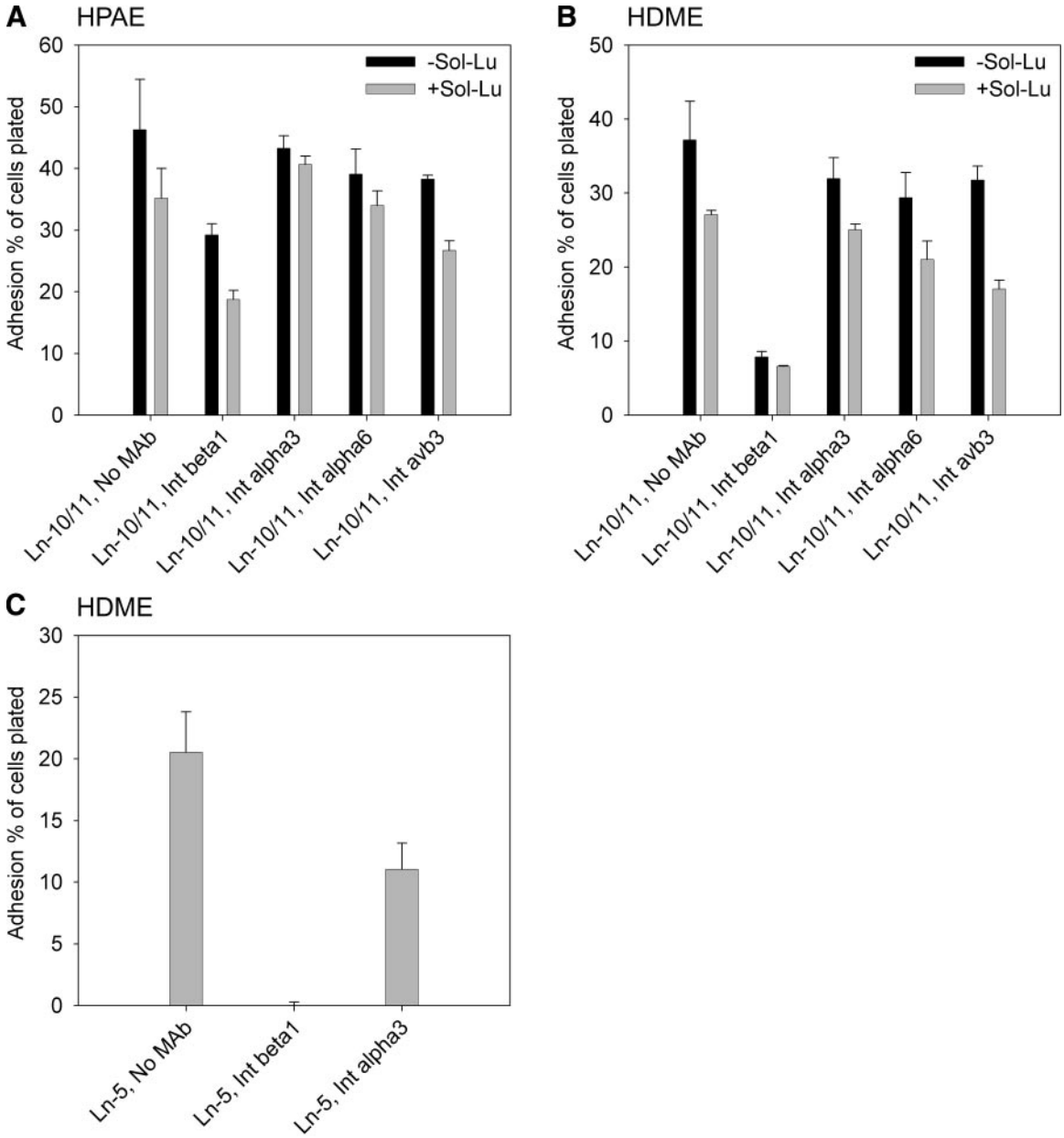

Fig. 8. Effects of Sol-Lu and MAbs to integrins on the adhesion of the endothelial cells. *A*: in the adhesion of HPAE cells to Ln-10/11, 45% of the plated cells adhered to this laminin. With Sol-Lu, the adhesion was diminished to 35%, and with MAb to integrin β_1, the adhesion was 30%. With the combination of MAb to integrin β_1 and Sol-Lu, the adhesion was diminished to <20%. *B*: in the adhesion of HDME cells to Ln-10/11, 35% of the cells adhered to this laminin. Adhesion was diminished to 25% with Sol-Lu. With MAb to integrin β_1, the adhesion was 25%, and the combination of MAb to β_1 integrin and Sol-Lu had a similar inhibitory effect. MAbs to integrins α_3, α_6, and $\alpha_v\beta_3$ had weak inhibitory effects on the adhesion of both cell types. *C*: in the adhesion of HDME cells to Ln-5, 20% of the plated cells adhered to this laminin. MAb to integrin β_1 prevented the adhesion nearly totally, whereas with the MAb PIB5 to integrin α_3, the adhesion diminished to 10%.

is encoded by the smaller, 2.5-kb transcript, was predominantly expressed by the cells. Immunofluorescence microscopy showed that Lu presented a punctate distribution on the endothelial cells, which did not resemble the distribution of any of the known adhesion structures.

Because function-blocking antibodies to Lu, suitable for our experimental setup, were unavailable, the role of Lu in endothelial cell adhesion was studied by inhibition of Lu function by saturation of the Lu-binding sites of laminin α5-chain with recombinant protein Sol-Lu as described previously (27). Although Sol-Lu clearly inhibited endothelial cell adhesion to Ln-10, it did not inhibit adhesion to fibronectin, EHS-Ln, or Ln-5, suggesting that the inhibitory effect of Sol-Lu in the adhesion assay was specific for the laminin α5-chain.

Compared with the adhesion without any function-blocking MAbs or Sol-Lu, Sol-Lu alone inhibited the endothelial cell adhesion to Ln-10 by 60–70%. MAb to integrin β_1 inhibited the adhesion by 65–85%, whereas with the combination of Sol-Lu and MAb to integrin β_1, the adhesion was nearly abolished. These findings suggest that endothelial cell adhesion to Ln-10 requires both Lu and integrin β_1. Interestingly, MAb to integrin $\alpha_v\beta_3$ alone had no inhibitory effect on the adhesion,

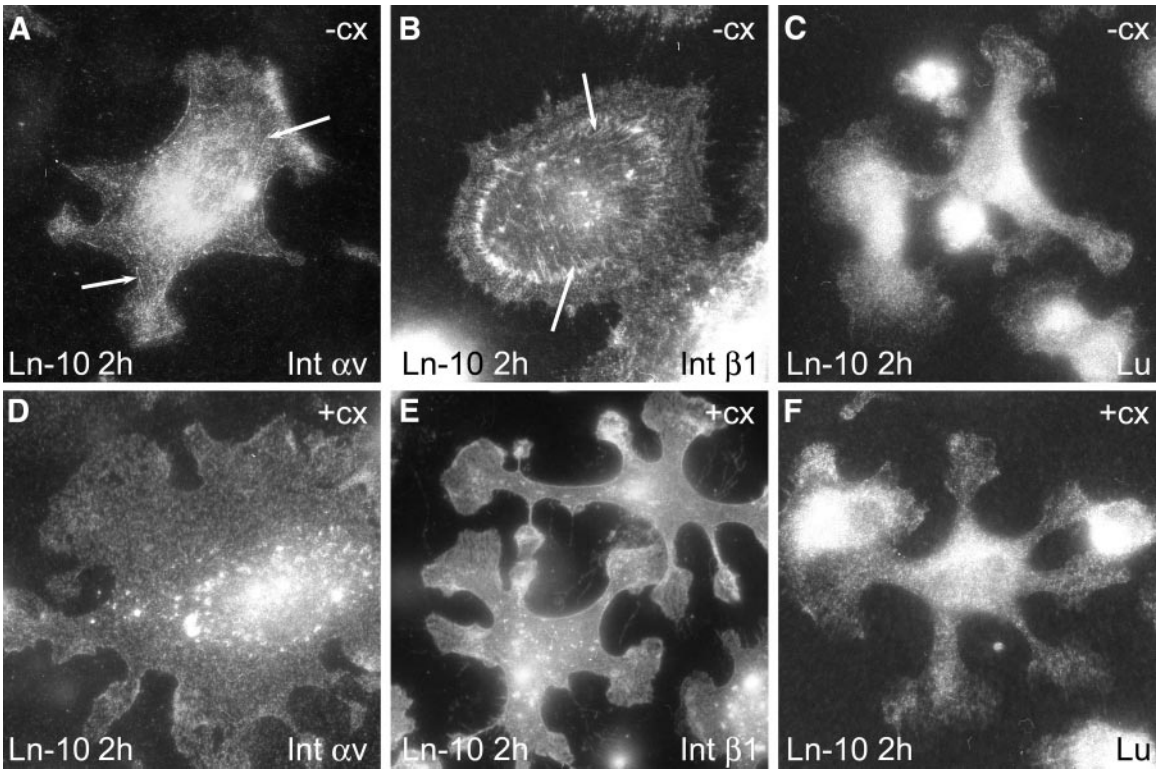

Fig. 9. Distribution of the adhesion receptors in endothelial cells adhering to Ln-10. *A*: in the absence of cx, immunoreactivity for integrin α_v had a diffuse distribution, but in some cells it was located in tiny fibrillar structures (HPAE, arrows). Immunoreactivity for integrin β_1 was located in fibrillar structures (*B*, HDME), which were often located circularly near the periphery of the cells (arrows). Immunoreactivity for Lu had a punctate distribution (*C*, HPAE) on the cells. In the presence of cx, immunoreactivities for both α_v (*D*, HPAE) and integrin β_1 (*E*, HDME) were diffusely distributed, and immunoreactivity for Lu (*F*, HPAE) had a punctate distribution on the cells.

but in combination with Sol-Lu, it prevented the cell adhesion to Ln-10 almost completely. The inability of MAb to integrin $\alpha_v\beta_3$ alone to inhibit the adhesion suggests that this integrin is not crucial for endothelial cell adhesion to Ln-10. It could, for example, partially replace the function of primary adhesion receptors Lu and integrin β_1, if the function of either one of them is prevented. Another explanation for this phenomenon could be the *trans*-dominant inhibition of other adhesion receptors, such as integrin β_1 via integrin $\alpha_v\beta_3$ as previously suggested by Hynes (20).

Fujiwara et al. (15) recently suggested that human microvascular endothelial cells form focal adhesions on Ln-10/11. The likely reason for the discrepancy with our results is the production of endogenous proteins during the adhesion assays, which they did not take into consideration. We showed that the effects of these endogenous proteins on the results of the adhesion assays could be prevented with the use of cycloheximide. However, this raises the question of whether cycloheximide prevents the formation of focal adhesions. Although some previous studies about the effects of cycloheximide on the focal adhesion formation have remained controversial (22, 57), our results show that in the presence of cycloheximide, the addition of fibronectin to Ln-10 coat induced typical focal adhesions, indicating that cycloheximide did not prevent the formation of these adhesion structures.

Furthermore, Fujiwara et al. (15) showed that the spreading of human microvascular endothelial cells on Ln-10/11 was completely inhibited with function-blocking MAb to integrin β_1. Of the function-blocking MAbs to α-subunits, MAb 3G8 to integrin α_3 had the strongest effect by inhibiting the cell spreading by 40%. Doi et al. (9) studied the adhesion of human saphenous vein endothelial cells and immortalized mouse brain capillary endothelial cells to recombinant human Ln-10. Of the function-blocking MAbs, MAb PIB5 to integrin α_3 and MAb to integrin β_1 had the best, but only partial, inhibitory effects on the adhesion. Although in neither of these studies did MAbs to integrin α_3 inhibit the adhesion completely, the investigators concluded that the adhesion of human endothelial cells to $\alpha5$ laminins was most probably mediated by integrin $\alpha_3\beta_1$.

Our results regarding the role of integrin β_1 are in agreement with the aforementioned studies (9, 15); however, although we used the same two MAbs to integrin α_3 (PIB5 and 3G8), our results did not support the suggested primary role for integrin α_3 in the adhesion of the endothelial cells to Ln-10. Although integrin $\alpha_3\beta_1$ appears to be the primary receptor for Ln-10 in some cell types (26, 56), the binding specificity of certain integrins in endothelial cells differs from their specificity in other cell types (29). The binding specificity of an integrin depends on the amount of its expression, activation state, and interaction with other proteins such as CD151 (19, 21, 41). Therefore, we studied whether the $\alpha_3\beta_1$-integrin is functional in our cells. Ln-5 is one of the high-affinity ligands for integrin $\alpha_3\beta_1$ (59), and some studies have proposed that this laminin is located to BMs of some capillaries (32, 63), suggesting that human microvascular endothelial cells could interact with Ln-5 via this receptor. The results show that the adhesion of HDME

cells to human Ln-5 is clearly reduced with MAb PIB5 to integrin α_3, indicating that these cells have a functional α_3-integrin.

Because the endothelial cells of capillaries live in a microenvironment different from that of the endothelial cells of larger vessels, we performed the adhesion experiments with both HDME and HPAE cells. The MAb to integrin β_1 clearly inhibited the adhesion of HDME cells better than that of HPAE cells, and the inhibitory effect of Sol-Lu was more pronounced with HPAE cells than with HDME cells, suggesting that even the microvascular and pulmonary artery endothelial cells differ to some extent in their adhesion characteristics.

The MAbs to both integrin β_1 and Sol-Lu had more pronounced effects on the cell adhesion to native Ln-10 than to the Ln-10/11 preparation. Furthermore, the combination of Sol-Lu and MAb to integrin $\alpha_v\beta_3$ did not inhibit the cell adhesion to Ln-10/11, as it inhibited the adhesion to native Ln-10, suggesting that experiments performed with the mixture of Ln-10/11 are not fully comparable to the experiments performed with native Ln-10. The commercially available Ln-10/11 is produced by pepsin digestion and contains partially degraded Ln-10 and -11 (14, 25, 53). The proteolysis can expose masked receptor binding sites and can therefore lead to erroneous results.

In conclusion, the findings show that Ln-10 is an adhesion substrate of endothelial cells but does not stimulate focal adhesion formation. The adhesion to Ln-10 is mediated by Lu together with integrins β_1 and $\alpha_v\beta_3$. Lu has previously been shown to mediate the adhesion of normal and sickle red blood cells, Lu-transfected human erythroleukemia cells, and Lu-transfected murine fibroblasts (10, 43, 60, 68). On the basis of a study of human mesangial cells, Kikkawa et al. (27) recently suggested that Lu alone would be unable to mediate the adhesion of adherent human cells. Our findings show that Sol-Lu alone markedly inhibited the adhesion of human endothelial cells to Ln-10, suggesting a role for Lu in the adhesion of adherent human cells. Moreover, the findings suggest interplay between Lu- and integrin-mediated adhesion processes.

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