Differential Effects of Laminin, Intact Type IV Collagen, and Specific Domains of Type IV Collagen on Endothelial Cell Adhesion and Migration

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Abstract. Laminin and type IV collagen were compared for the ability to promote aortic endothelial cell adhesion and directed migration in vitro. Substratum-adsorbed IV promoted aortic endothelial cell adhesion in a concentration dependent fashion attaining a maximum level 141-fold greater than controls within 30 min. Aortic endothelial cell adhesion to type IV collagen was not inhibited by high levels (10^{-3} M) of arginyl-glycyl-aspartyl-serine. In contrast, adhesion of aortic endothelial cells on laminin was slower, attaining only 53% of the adhesion observed on type IV collagen by 90 min. Type IV collagen when added to the lower well of a Boyden chamber stimulated the directional migration of aortic endothelial cells in a concentration dependent manner with a maximal response 6.9-fold over control levels, whereas aortic endothelial cells did not migrate in response to laminin at any concentration (.01–2.0 × 10^{-7} M). Triple helix–rich fragments of type IV collagen were nearly as active as intact type IV collagen in stimulating both adhesion and migration whereas the carboxy terminal globular domain was less active at promoting adhesion (36% of the adhesion promoted by intact type IV collagen) or migration. Importantly, aortic endothelial cells also migrate to substratum adsorbed gradients of type IV collagen suggesting that the mechanism of migration is haptotactic in nature. These results demonstrate that the aortic endothelial cell adhesion and migration is preferentially promoted by type IV collagen compared with laminin, and has a complex molecular basis which may be important in angiogenesis and large vessel repair.

Endothelial cells are derived from primitive mesoderm (47) which in the adult forms a simple squamous epithelium that lines the cardiovascular and lymphatic systems (8, 38). In vivo endothelial cells are attached to basement membrane and under normal conditions are mostly nonmotile (13, 45). There are several collagenous and noncollagenous extracellular matrix (ECM)[1] glycoproteins which have been identified in or are closely associated with basement membrane. Laminin (LMN) and type IV collagen (IV) comprise the majority of protein found in basement membrane being present in equimolar concentrations in many locations (12), with nidogen/entactin, fibronectin, heparan sulfate proteoglycan, and bullous pemphigoid antigen being detected in certain basement membranes (4, 15, 19, 32, 48). This structural heterogeneity suggests that the function(s) of basement membranes may vary in a tissue specific fashion.

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1. *Abbreviations used in this paper*: AEC, aortic endothelial cells; ECM, extracellular matrix; IV, type IV collagen; LMN, laminin; NC1, noncollagenous domain 1; PIV, pepsin-generated helical fragments of type IV collagen.

IV is localized almost exclusively to basement membranes. The molecule exists as a flexible 420-nm rod composed of two α1(IV) and one α2(IV) chains (43). There are three primary regions within the IV molecule. The first 60 nm at the amino terminus represents the "7S" region whereby the molecule is able to form lateral associations with four other IV molecules (18). The central 330 nm of the molecule represents the major triple helical portion. This helical region has numerous areas where the normal gly-Xaa-Yaa amino acid sequence is interrupted with nonhelical segments. The third primary region of IV is the collagenase-resistant carboxyl terminal globular domain termed NC1. This domain has sites for both end-to-end and lateral associations (52) important for the complex macromolecular organization of IV in vivo. Until recently, IV was considered as largely a structural component of basement membrane where it forms a scaffold with which other basement membrane components such as LMN, fibronectin, or heparan sulfate proteoglycan could associate (21). It is now becoming increasingly apparent that can function directly in mediating various cell functions (1, 30, 41).

LMN is a large complex glycoprotein (1,000 kD) containing binding sites for many epithelial and mesenchymal cells

and is capable of binding to several ECM constituents (20). LMN promotes cell attachment, neurite extension, has a role in orchestrating early embryonic development and may function in mediating the extravasation of cells during blood bourne metastasis (3, 16, 31, 32, 36, 40, 51).

If a blood vessel wall is damaged and endothelial continuity is disrupted then the marginal endothelial cells will initiate sheet migration (35) and rapidly move over the exposed subendothelium to restore cellular continuity (13). If the damaged area is limited, continuity may be restored within hours. If the damaged area is more extensive, then individual endothelial cells will eventually migrate away from the margins of the defect out over the exposed ECM. The migration of individual endothelial cells is also required for angiogenesis. For example, it has been shown that the initial reaction of capillary endothelial cells to stimulating factors in a corneal angiogenesis assay is one of cell migration before mitosis (44, 45). Furthermore, the migration rate in vitro of confluent endothelial cells in an experimental wound has been shown to be influenced by various ECM proteins preadsorbed to the culture plates (56). Several studies have shown that certain components of basement membranes may be important modulators of endothelial cell functions important to wound healing, vessel growth, and development (5, 9, 11, 23, 24, 25, 29, 37).

The present studies were designed to contrast the ability of LMN, intact and purified domains of IV, to stimulate the adhesion and directed migration of AEC. IV was extremely active at promoting AEC adhesion and migration. LMN, in contrast, was less effective on a molar basis at promoting AEC adhesion and was ineffective at promoting AEC migration. Analysis of AEC adhesion and migration on purified proteolytic fragments of IV demonstrated that adhesion to IV has a complex molecular basis involving multiple domains on the molecule.

Materials and Methods

Cell Culture

AEC were isolated after the protocol described by Schwartz (42) with a few modifications. Briefly, aortas were obtained from 6-m-old bovine calves at a local slaughterhouse, washed in cold PBS, pH 7.2, and processed within 2 h from the time of death. The vessel was clamped at the distal end, filled with the collagenase solution (crude collagenase CLS III, 125-145 U per mg dry weight; Cooper Biomedical, Inc., Malvern, PA) used at 2 mg/ml in DME, (Gibco, Grand Island, NY) and digestion was carried out for 10 min. The lumenal contents were harvested, followed by addition of fresh collagenase, for two more 10-min periods. The enzyme-cell suspension was added to an equal volume of DME containing 10% FBS (Sigma Chemical Co., St. Louis, MO) and spun at 400 *g*. The resulting cell pellet was resuspended in DME containing 10% FBS, 100 units/ml of penicillin G, 100 μg/ml of streptomycin. Cells were cultured in 75 cm^2 flasks (Falcon Plastics, Oxnard, CA) in a 5% CO_2/95% humidified air atmosphere at 37°C. Cultures were fed twice a week with the same media and cells were used in assays when 75-95% confluent. Cells were identified as endothelial in nature by characteristic cobblestone morphology, contact inhibition of growth upon reaching confluency, and positive immunofluorescence staining for factor VIII:RAg (Miles Laboratories, Naperville, IL).

Proteins and Antibodies

Intact IV and LMN were isolated from the EHS sarcoma grown in lathrytic mice (16). The carboxyl terminal noncollagenous domain 1 (NC1) was isolated from purified intact IV by extensive collagenase digestion (Cooper Biomedical) substrate/enzyme ratio 50:1 followed by chromatography on a Sephacryl S-300 column (Pharmacia Fine Chemicals, Piscataway, NJ) as described (52, 53). Helix-rich fragments of IV were prepared by a brief treatment with pepsin (s/e 100:1, for 1 h at 28°C) (Sigma Chemical Co.) which releases the globular NC1 domain but preserves the collagenous structure of the molecule. The reaction was stopped by raising the pH to 7.4 before the triple helical segments were purified by passing the digest over a Sephacryl S-1,000 column (Pharmacia Fine Chemicals) as described (50). The proteins and fragments thus generated were characterized by discontinuous SDS-PAGE, rotary shadowing electron microscopy (16, and references therein), and where applicable, tested for crossreactivity with panels of polyclonal antisera in solid-phase ELISA assays (28). Affinity purified polyclonal anti-LMN antibodies were generated as previously described (27).

SDS-PAGE

A 2-15%, 1.5-mm thick linear gradient of SDS-polyacrylamide (Boehringer Mannheim Biochemicals, Indianapolis, IN) was formed according to the method of Laemmli (19). The samples were mixed with an equal volume of sample buffer (3% SDS, 80 mM Tris-HCl, 15% glycerol, .01% bromphenol blue) reduced (.25% vol/vol 2-mercaptoethanol, 3 min at 100°C) and loaded (30 μg total protein) into each lane. The proteins were chromatographed by applying 45 mA of constant current (E.C. 500; E. C. Apparatus Corp., St. Petersburg, FL) for 2.5 h at 4°C. The gel was fixed and stained by incubation overnight in dH_2O-95% ethanol-glacial acetic acid (1:1:.1) containing .25% wt/vol Coomassie Brilliant Blue R stain (Sigma Chemical Co.). The gel was destained by soaking in four successive changes (2 h each) of 95% ethanol-acetic acid (2:3, 1.5:3.5, 1:4, .5:4.5) before being soaked in dH_2O containing 5% vol/vol glycerol (Columbus Chemical Industry, Columbus, WI) for 30 min, and then dried under vacuum.

Rotary Shadowing

IV, pepsin-generated helical fragments of IV (PIV), and NC1 were dialyzed overnight into PBS and then processed for rotary shadowing as follows. All samples were centrifuged at 38,000 rpm (~150,000 *g*) for 30 min to remove aggregates before being added to a solution of 50% glycerol (vol/vol) in .15 M NH_4HCO_3, pH 7.7, to a final concentration of 5-10 μg/mL. These final solutions were then sprayed onto freshly cleaved mica sheets and air dried. The mica sheets were then placed onto the stage of an evaporator (Balzers Union, Hudson, NH); the chamber was evacuated to less than 10^{-5} torr and while rotating, the mica sheet was shadowed by evaporating a filament of 95% platinum/5% carbon at an angle of 4°. The shadowed proteins were then stabilized by evaporating a rod of 100% carbon at a 90° angle. The carbon-stabilized protein replicas were floated off the mica sheet onto the surface of dH_2O and picked up on Copper 300 mesh naked grids (Ted Pella Inc., Tustin, CA). The replicas were viewed in a Phillips 300 transmission electron microscope (Mahwah, NJ) at an accelerating voltage of 60 kV with a 30-μm objective aperture.

Protein Binding and Cell Adhesion

Adhesion assays were performed in 96-well microtiter plates (Immulon-1; Dynatech Labs, Inc., Chantilly, VA) to which test proteins had been previously adsorbed (28). The amount of substratum adsorbed LMN, IV, NC1, or PIV was calculated by coating plates overnight at 37°C with radiolabeled proteins in Vollers buffer (15 mM Na_2CO_3 and 34.8 mM $NaHCO_3$) pH 9.6. Unbound protein was washed away and the bound material solubilized with .5 N NaOH in 1% SDS before counting bound radioactivity. IV and LMN were chemically radiolabeled using [^{3}H]formaldehyde (New England Nuclear, Boston, MA) with sodium cyanoborohydride (Sigma Chemical Co.) as the reducing agent (14, 39) yielding specific activities of 14 and 8.8 × 10^{-3} μCi/μg, respectively. Under these mild reducing conditions, the α-amino terminus and ε-amino groups of lysine are converted to stable, N,N-dimethyl derivatives by reductive methylation. The conditions used also minimize intra and intermolecular crosslinking and shows no disulfide bond reduction. NC1 was radiolabeled using lactoperoxidase (Sigma Chemical Co.) immunobilized on enzymobeads (Biorad Labs, Richmond, CA) and carrier free [^{125}I]Na by the method of LeBien et al. (22). Radiolabeled NC1 was mixed with nonlabeled NC1 and serial dilutions were made into Vollers buffer before plating. TCA precipitation determined that 91% of ^{125}I- was bound to NC1. The specific activity of [^{125}I]NC1 was 1.56 × 10^{-3} μCi/μg. PIV was iodinated with ^{125}I using Iodogen (Pierce Chemical Co., Rockford, IL) following the methods specified by Fraker and Speck (10). The specific activity of [^{125}I]PIV was 6.61 × 10^{-4} μCi/μg and TCA precipitation showed 56% of ^{125}I was bound to PIV.

Cell Adhesion

For adhesion assays, endothelial cells in log phase growth were metaboli-

cally radiolabeled by overnight incubation in 25 μCi/mL of ^{3}H-amino acids (sp act 235 mCi/mg; ICN Radiochemicals, Irvine, CA). The radiolabeled cells were washed twice with DME before being released from the substratum by treatment with 0.05% trypsin (Sigma Chemical Co.), containing 0.75 mM EDTA, pH 7.2, for 2 min. Trypsin was inhibited by the addition of an equal volume of DME containing 10% FBS. Cells were then pelleted at 400 *g* and washed twice in DME before being resuspended to a final concentration of 3–5 × 10^4 cells/ml in serum-free DME containing 2 mg/ml BSA. Care was taken to assure that a single cell suspension was attained. Cells were added in a volume of 100 μl per well and the plates were incubated at 37°C in a humid atmosphere for 15–90 min (standard assay time being 60 min). The wells were then vigorously washed three times with 200 μl warm PBS containing 2 mg/ml BSA to remove nonadherent cells. The adherent cells were solubilized in 200 μl of 0.5 N NaOH containing 1% SDS, quantitated by liquid scintillation counting (model No. 3801; Beckman Instruments, Inc., Irvine, CA).

The antibody inhibition experiments were performed by preincubating radiolabeled AEC for 15 min with 1–50 μg/mL of polyclonal affinity purified anti-LMN antibody. The AEC were seeded in the continued presence of antibody on substrata coated with an equimolar solution of LMN or IV (described above) and allowed to become attached and spread for 60 min before washing, solubilizing, and quantitating as described.

To test the dependence of cell adhesion on de novo protein synthesis, cycloheximide (Sigma Chemical Co.) was included in parallel adhesion assays. AEC were incubated with cycloheximide at 20 μg/ml for 2-h before harvesting and then maintained in cycloheximide for the duration of the assays.

Effects of RGDS on Cell Attachment

Attachment of AEC cells to substrata coated with the various proteins was also tested in the presence of the soluble synthetic peptide arginyl-glycyl-aspartyl-serine (RGDS) (Peninsula Laboratories, Inc., Belmont, CA). Peptides containing this sequence have previously been shown to modulate adhesion of a variety of normal and transformed cells to fibronectin (28, 33, 55) as well as certain other noncollagenous adhesive glycoproteins (46). For these studies, aliquots of the final cell suspension were incubated at 25°C for 15–20 min with 10^{-3} to 10^{-6} M RGDS or RGES (an inactive analog containing the conservative amino acid substitution, glutamic acid [E] for aspartic acid [D]) (34) before the adhesion assay. Adhesion assays were then performed on LMN or IV-coated substrata. In addition, the effects of RGDS on cell adhesion to fibronectin-coated substrata were included as a positive control.

Cell Migration

Migration assays were performed in Boyden microchemotaxis chambers (Neuroprobe, Bethesda, MD) as previously described (27, 28). Subconfluent (60–95%) AEC were released with trypsin, washed, and resuspended at a final concentration of 3–5 × 10^5 cells/ml in DME containing 20 mM N-2-hydroxy-ethylpiperazine-N′-2-ethane sulfonic acid (Hepes), pH 7.4. Putative attractants were added in solution to the lower wells at concentrations ranging from .01–13 × 10^{-7} M. In addition to testing the proteins in fluid phase, an insoluble solid-phase gradient was established by floating the filters overnight at 37°C on a solution of Vollers buffer containing 25 μg/ml of the protein being tested. The filters were washed by floating on DME + Hepes before being used in migration experiments in the absence of a fluid phase attractant. The lower wells were then overlaid with an 8-μm pore size polycarbonate filter and the upper half of the chamber was secured into place. The upper wells received 50 μl of the final cell suspension and the chambers were incubated at 37°C in a humid atmosphere for 6 h. At the end of the assay period, the filters were removed, fixed, stained (Diff-Quick fixative, American Scientific Products, McGaw Park, IL) and mounted on glass slides. The migrated cells were quantitated by viewing the stained cells with a Zeiss Universal microscope (Carl Zeiss, D-7082; Oberkochen, Federal Republic of Germany) integrated with an Optomax Image Analysis System (Optomax, Inc., Hollis, NH) equipped with an Apple IIe computer (Cupertino, CA).

Results

Cell Culture

After enzymatic dissociation from the bovine thoracic aorta a cell population is obtained that is morphologically homogeneous with a cobblestone like appearance, shows contact inhibition of growth, and stains positive for factor VIII:RAg (Fig. 1). The number of cells staining positive for factor VIII:RAg and the intensity of staining was found to be influenced by the degree of confluency and monolayer organization. The method gives high yield with little contamination (<5%) by smooth muscle cells, pericytes, or fibroblasts.

SDS–PAGE and Rotary Shadowing

The intact IV molecule is a heterotrimer consisting of two α1 (IV) and one α2 (IV) chains interwoven in a triple helical conformation 420-nm long from end to end. At the amino terminus is the 7S region involved in lateral association with other IV molecules and network formation. The central 330 nm represents the major triple helical portion of IV and contains several short interruptions in the usual gly-Xaa-Yaa sequence. Near the carboxy terminus is the globular NC1 domain which mediates both end-to-end and lateral associations of IV both vital to the architecture of IV in vivo.

Fig. 2, panel *A* shows intact IV and the purified domains of IV separated on a 2–15% linear gradient gel under nonreduced (*a*, *b*, and *c*) or reduced (*d*, *e*, and *f*) conditions. When run nonreduced, intact IV migrates primarily as large disulfide-bonded molecular aggregates (Fig. 2 *a*) but upon reduction it migrates as two bands at ~180 kD corresponding to the α1(IV) and α2(IV) chains (Fig. 2 *d*). NC1 migrates nonreduced in both dimeric (55 kD) and monomeric (~25 kD) forms (Fig. 2 *b*). Reduction results in dissociation of some of the 55-kD dimer to 25-kD monomeric NC1 (Fig. 2 *e*). Pepsin digested IV whether reduced or nonreduced migrates as a series of fragments with molecular weights ranging from 130–170 kD with some remaining intact and aggregated IV (Fig. 2, *c* and *f*).

Intact IV, NC1, and the pepsin generated, major triple helical fragments were rotary shadowed before being viewed by transmission electron microscopy. In Fig. 2, panel *B* (1) intact IV is shown to be a long rod-like molecule joined end-to-end by the globular NC1 carboxyl terminal domain. Isolated molecules of the globular NC1 are shown in Fig. 2, panel *B* (2), this is entirely free from helical collagenous segments. The major triple helical fragments devoid of NC1 are shown in panel *B* (3) and are primarily rod-like collagenous segments somewhat shorter than intact IV.

Protein Adsorption

Purified preparations of LMN, IV, NC1, and PIV were radiolabeled as described in Materials and Methods. These proteins were serially diluted into Vollers buffer and allowed to adsorb to the plastic surface overnight at 37°C. The wells were washed free of unbound protein and adsorbed protein was solubilized and quantitated.

All proteins bind to the plastic surface in a concentration dependent saturable fashion. When added at an equimolar concentration (Table I) the number of moles of bound protein ranges from 0.26–0.52 pmol with IV binding being greatest and PIV binding being least. LMN and NC1 binding is intermediate giving .41 and .38 pmol bound, respectively.

Cell Adhesion

LMN or IV were adsorbed to microtiter plates at various concentrations to measure the ability to promote AEC adhe-

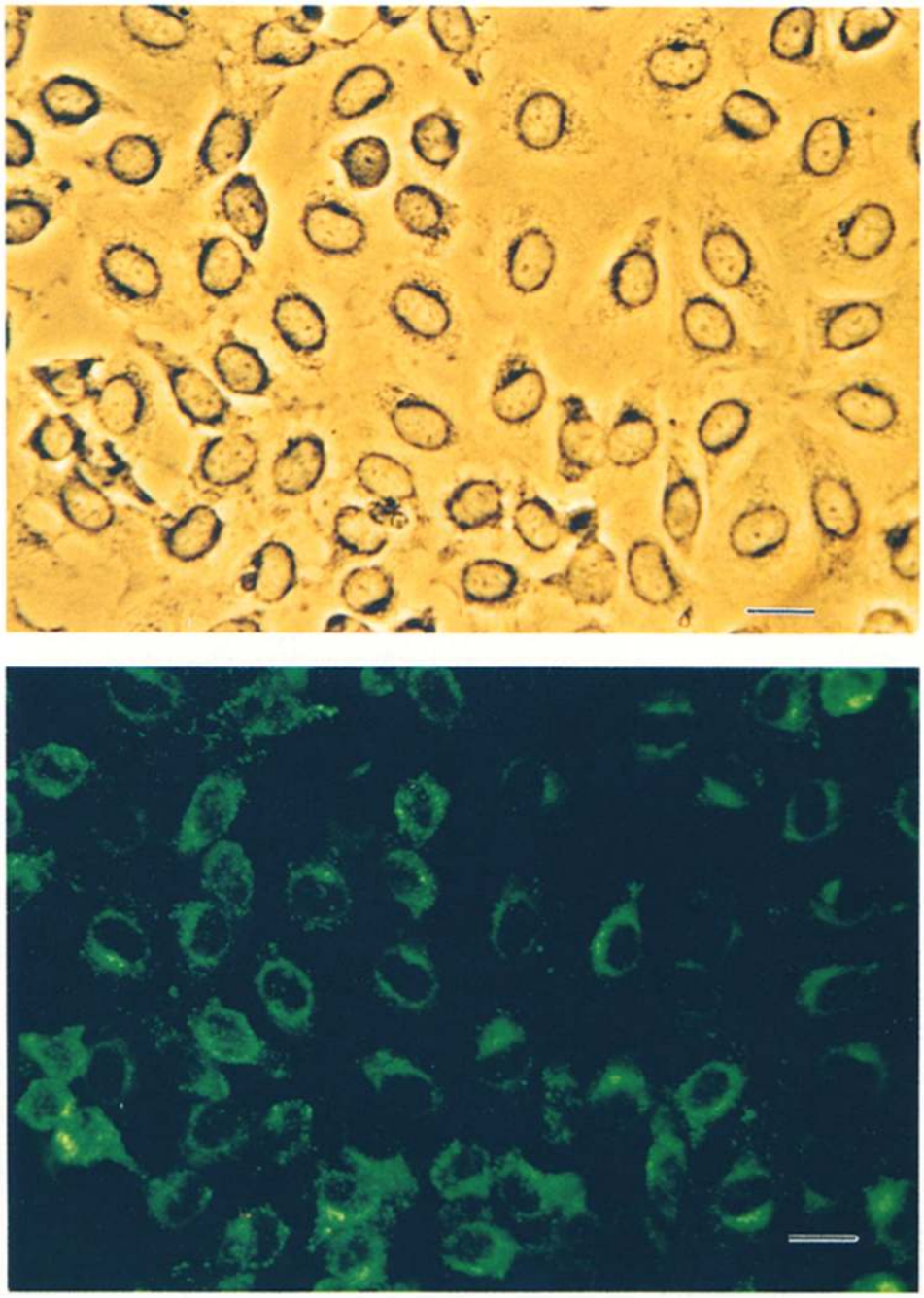

Figure 1. Endothelial cell characterization. (*a*) Phase contrast photomicrograph of confluent AEC. The cells form a contact inhibited monolayer with flat polygonal cells demonstrating the characteristic cobblestone morphology of endothelium. (*b*) Indirect immunofluorescence for factor VIII: RAg. This micrograph shows the positive intracellular granular fluorescence for VIII:RAg seen in a confluent monolayer of AEC. Bars, 5 μm.

sion and spreading in a concentration dependent fashion. At coating concentrations as low as 1 μg/ml (2×10^{-9} M) IV promotes significantly greater levels of cell adhesion than LMN (Fig. 3). The adhesion promoted by IV begins to saturate at coating concentrations of 5–10 μg/ml ($1–2 \times 10^{-8}$ M) at which point 93% of available cells have become attached. In contrast to the IV-mediated AEC adhesion, LMN promotes endothelial cell adhesion only when used at coating concentrations of 20 μg/ml (2×10^{-8} M) or greater. The AEC adhesion to LMN will attain but never exceed 30% absolute cell adhesion even at saturating coating concentrations of LMN, thus demonstrating that the molecule is biologically active but is incapable of promoting the levels of adhesion attained with IV.

The kinetics of endothelial cell adhesion promoted by substrata coated with a 2×10^{-8} M solution of either IV, LMN, or purified domains of IV was determined (Fig. 4). Within 15 min intact IV and the major triple helix of IV (PIV) promoted levels of cell adhesion from 49–110-fold greater than control values (wells coated with PBS/BSA only). This adhesion continued to increase up to 30 min, at which point the level of cell adhesion plateaued, 90–140-fold greater than the time matched controls. In contrast, adhesion to NC1 and LMN occurs more slowly and not to the same degree. Cell adhesion promoted by NC1 and LMN was only 2 and 28-fold greater than controls, respectively by 15 min into the assay. The amount of AEC adhesion on NC1 and LMN was only 33% and 53% of the observed cell adhesion on intact IV at 90 min.

The AEC adhesion to substrata coated with IV is not medi-

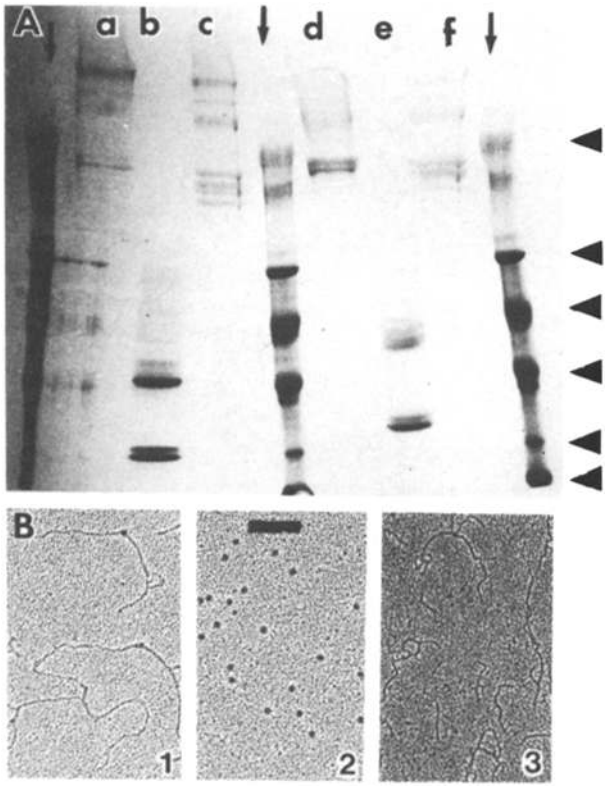

Figure 2. (*A*) Purity of intact type IV collagen and its fragments. 2–15% SDS–PAGE gradient gel of nonreduced (lanes *a*, *b*, and *c*) and reduced (lanes *d*, *e*, and *f*) samples (treatment with .25% mercaptoethanol and heated for 3 min at 100°C). Lanes *a* and *d*: intact type IV collagen: lanes *b* and *e*: NC1; and lanes *c* and *f*: pepsin-digested triple helix. Arrows indicate molecular weight standards, some of which have spilled over into lane (*a*) during electrophoresis: fibronectin (220 kD), phosphorylase A (93 kD), BSA (68 kD), ovalbumin (43 kD), soybean trypsin inhibitor (21 kD) and cytochrome C (12 kD). (*B*) Electron micrographs of rotary shadowed purified preparations of (*1*) intact type IV collagen, (*2*) globular NC1, and (*3*) pepsin-digested type IV collagen. Bar, .1 μm.

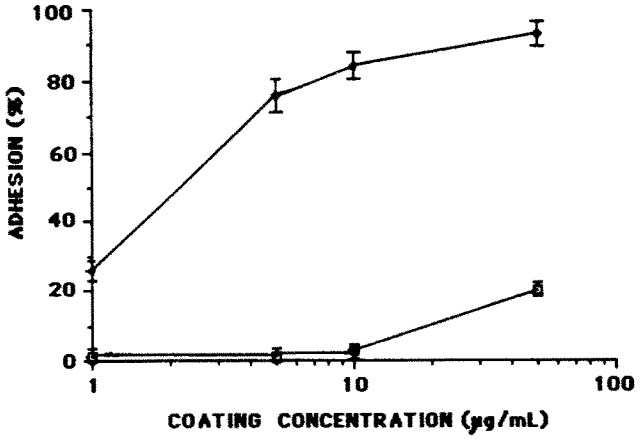

Figure 3. Concentration dependent adhesion of AEC to substratum-bound type IV collagen or laminin. Wells were coated overnight with soluble LMN or type IV collagen at concentrations from 1–100 μg/ml (.01–2 × 10^{-7} M). The next day the wells were coated with 2 mg/ml BSA in PBS to prevent nonspecific cell attachment. Radiolabeled cells were added to each well and allowed to attach and spread for 60 min. The wells were washed and the remaining cells solubilized. The percent adhesion was determined by the following equation and represents the mean of quadruplicate samples ± SEM: Percent adhesion = DPMS (cells) attached/DPMS (cells) added × 100. Type IV collagen (◆——◆); laminin (■——■).

Table I. Binding of ECM Proteins to Immunlon-1 Microtiter Plates

*Protein adsorbed	‡Coating concentration	§Coating concentration	‖Moles bound
	μg/ml	*×10⁻⁸M*	*×10⁻¹⁴*
Laminin	10	1.0	4.1
IV	5	1.0	5.3
NC1	1.5	1.0	3.8
PIV	4.9	1.0	2.7

* Proteins were radiolabeled with ^{3}H (LMN, IV) or ^{125}I (NC1, PIV) as described in Materials and Methods. Free radioactivity was removed by dialysis and the proteins were diluted in low ionic strength carbonate buffer.

‡ Wells were coated with 100 μl of the indicated concentrations of each protein as described in Materials and Methods. Adsorption was carried out overnight at 37°C.

§ Conversion of concentrations in (b) to molar values was calculated by using the following molecular weights: laminin:1000 kD; Type IV collagen:500 kD; NC1:150 kD; PIV:490 kD.

‖ Based on the calculated specific activities of each radiolabeled protein, the amount of bound radioactivity was quantitated and used to first calculate the number of μg's of protein bound per well and then converted to moles using the molecular weights above. The SD in all cases is less than 7% of the given value.

ated by LMN. Fig. 5 shows that affinity purified polyclonal anti-laminin antibody has no effect on AEC adhesion to IV even when used at antibody levels up to 50 μg/mL. In comparison, the inclusion of even small concentrations of the same polyclonal anti-laminin antibody (1 μg/mL) in AEC adhesion assays on LMN-coated substrates causes a marked decrease in cell adhesion which plateaus at ~77% inhibition of cell adhesion with 25 μg/mL or greater anti-LMN antibody.

The adhesion of endothelial cells to all of the substratum bound proteins was partially inhibited by pretreatment of the cells with cycloheximide (Fig. 6). The cell adhesion to NC1 was most affected (80% inhibition of adhesion) while adhesion to IV was least affected (52% inhibition) with the inhibition on PIV or LMN-coated substrata being 57 and 70%, respectively.

Effects of RGDS

Since previous work had demonstrated that the amino acid sequence RGDS is an important adhesion promoting domain present in several collagenous and noncollagenous molecules we tested the ability of soluble RGDS to disrupt the cell adhesion promoted by substratum bound intact IV and LMN. When trypsin released AEC were pretreated with soluble RGDS at concentrations ranging from 10^{-6}–10^{-3} M and then plated on either IV or LMN-coated substrata in the continued presence of RGDS there was no inhibitory effect on cell adhesion (Fig. 7). In contrast, when included as a positive control, fibronectin-mediated endothelial cell adhesion was markedly inhibited by soluble RGDS in a dose-dependent fashion reaching 90% inhibition at 10^{-3} M RGDS (Fig. 7). An analog of RGDS containing a conservative substitution at the third amino acid position (RGES) also had no inhibitory activity for AEC adhesion on either LMN

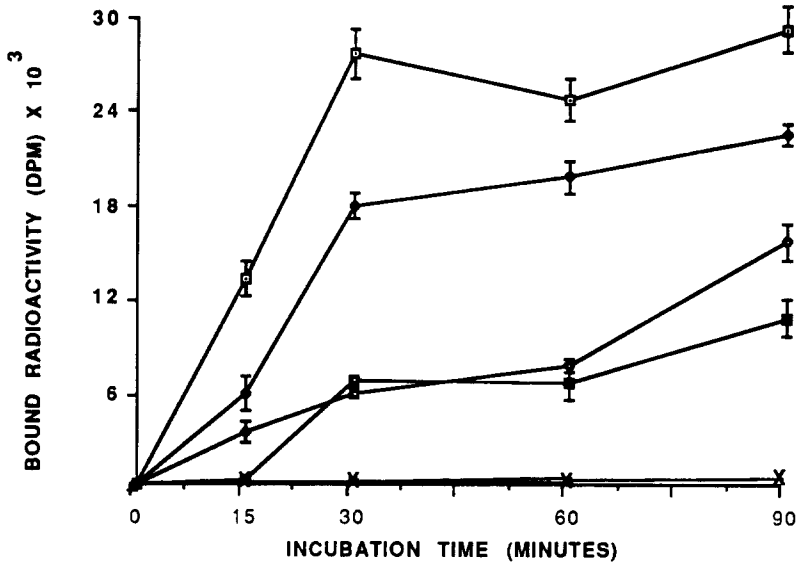

Figure 4. The kinetics of endothelial cell adhesion after plating on substrata coated with a 2×10^{-8} M solution of LMN, IV, or purified domains of IV. Radiolabeled cells were allowed to attach for 15–90 min after which the unattached cells were washed away, and the remaining radiolabeled cells were solubilized and quantitated by liquid scintillation counting. The experimental values (expressed as bound radioactivity in DPMS) represent the mean of quadruplicate samples ±SEM. Type IV collagen, □. PIV fragments, ♦. LMN, ◇. NCI, ■. Control, X.

or IV, and importantly, fibronectin-mediated cell adhesion was also unaffected by RGES (data not shown) thereby confirming the specificity of the RGDS sequence as a cell binding signal.

Cell Migration

The ability of LMN intact IV, the PIV and NC1 domains of IV to stimulate the directional migration of AEC was measured in Boyden microchemotaxis chambers. The proteins were added to the lower wells at equimolar concentrations. When used at 2×10^{-8} M concentration IV and the helix-rich PIV fragments promote the directional migration of AEC 4.6- and 2.3-fold above controls (Fig. 8). While the NC1 domain and LMN do not stimulate migration. LMN was inactive at promoting AEC migration even when added at 40-fold greater soluble concentrations.

Previous work had demonstrated that cell migration in this system may be haptotactic in nature, meaning that the cell migratory response was due to the generation of a substratum bound density gradient of protein adsorbed onto the filter surface during the assay (27 and references therein). To more clearly define the mechanism of AEC migration the polycarbonate filters were precoated on the distal surface with the test proteins and then used in the absence of any fluid phase attractant. As shown in Fig. 9, IV and the PIV fragments of IV promote haptotactic migration of AEC at levels 6.4- and 7.4-fold over controls (filters coated with BSA). NC1 was

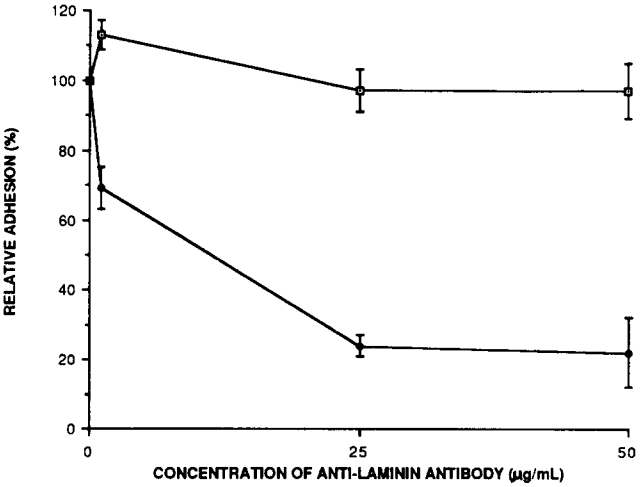

Figure 5. AEC adhesion to substrata coated with either LMN (♦———♦) (40 μg/mL) or type IV collagen (□———□) (20 μg/mL) in the presence of various amounts of anti-LMN polyclonal antibodies (1–50 μg/mL). Cells were released, washed, and resuspended (as described) in media containing from 1 to 50 μg/mL polyclonal anti-LMN antibody. After ~15 min the cells were plated in the continued presence of antibody on the respective substrata and allowed to attach and spread for 60 min. The wells were washed and attached cells quantitated as described above. The values represent the mean of quadruplicate determinations ± SEM. Normal rabbit IgG included as controls had no inhibitory effect on adhesion to either LMN or IV.

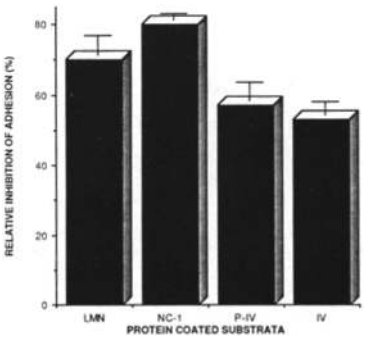

Figure 6. The effects of cycloheximide treatment of endothelial cell adhesion to LMN ($.5 \times 10^{-7}$ M), IV, NC1, or PIV-coated substrata (1×10^{-8} M). Radiolabeled cells were pretreated with cycloheximide (20 μg/ml) for 2 h before being released with trypsin, washed and resuspended in DME (containing cycloheximide) for adhesion assays as described in Materials and Methods. Parallel assays were performed with endothelial cells not exposed to cycloheximide. The results compare adhesion of AEC to each individual protein in the presence of cycloheximide relative to the adhesion seen in the absence of cycloheximide (this value is set to be 100% and is expressed as the percent inhibition of adhesion). Values are the mean of quadruplicate samples ± SEM.

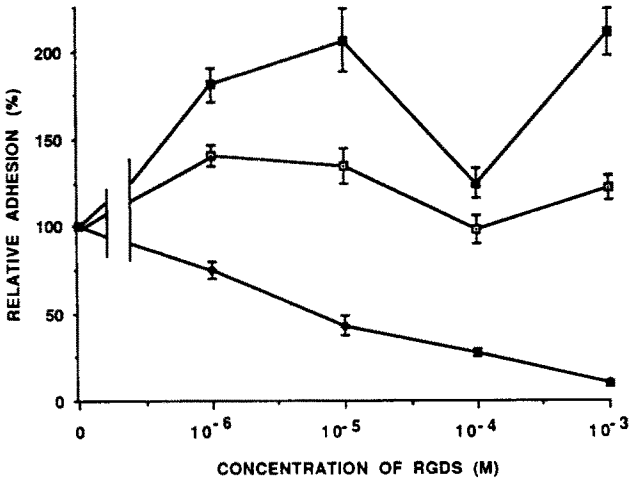

Figure 7. Effect of varying concentrations of the synthetic peptide RGDS on endothelial cell adhesion. Plates were precoated with a 2.5×10^{-8} M solution of laminin, a 5×10^{-8} M solution of intact type IV collagen or a 0.4×10^{-8} M solution of plasma fibronectin. Radiolabeled cells were incubated for 30 min in DME containing from 10^{-3}–10^{-6} M soluble RGDS or in DME alone. The cells were then added to wells precoated with one of the matrix proteins and allowed to become attached and spread for 60 min. The wells were then washed and the remaining cells were solubilized. The degree of cell adhesion in the presence of RGDS was compared with that in the absence of RGDS (standardized to equal 100%) and expressed as relative percent adhesion (absolute adhesion values would be ~60% for IV and 30% for LMN in the absence of RGDS). Values are the mean of quadruplicate determinations ± SEM. LMN (■——■) type IV (□——□), fibronectin (◆——◆).

moderately active at promoting haptotactic migration at this coating concentration and laminin was inactive at this, and all coating concentrations subsequently tested (up to 2×10^{-7} M).

Discussion

We have shown that at equivalent concentrations, substratum-bound IV is substantially more effective than substratum-bound LMN at promoting endothelial cell adhesion. The mechanism(s) by which AEC using IV or LMN as an adhesive substratum is independent of an RGDS adhesion sequence and is partially dependent on de novo protein synthesis. This requirement of protein synthesis for attachment is possibly due to the fact that when trypsin released cells are used in adhesion assays they may require resynthesis and surface expression of a trypsin sensitive molecule to fully use the signals provided by a specific ECM component. At the concentrations of cycloheximide used the decrease in cell attachment does not appear to be due to cytotoxicity as judged by trypan blue exclusion. In addition, when similar experiments were performed including cycloheximide and using human plasma fibronectin as the adhesive substratum, the percent inhibition is only 20–25% (not shown). Therefore we feel that the inhibitory effects of cycloheximide on AEC attachment were a function of the requirement for the resynthesis of receptors or other cell surface associated molecules disrupted during trypsin release.

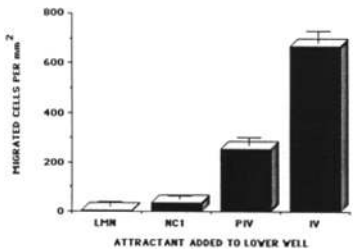

Figure 8. Migration of AEC to extracellular matrix proteins. Laminin, NC1, P-IV, or intact type IV were added in solution (2×10^{-8} M) to the lower well of Boyden chambers as described in Materials and Methods. Cells were added to the upper well and the chambers incubated at 37°C for 6 h. The number of AEC having migrated to the lower filter surface (*solid bars*) by the end of 6 h are quantitated and expressed as cells/mm². Values are the mean of triplicate determinations ± SEM. Control values representing the level of random cell migration when DME alone is added to the lower well have been subtracted out of the displayed values.

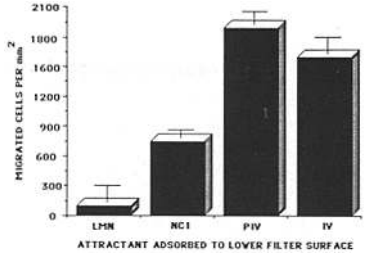

Figure 9. Migration of AEC on filters precoated with laminin, NC1, PIV, or IV. The polycarbonate filters were precoated by floating them overnight on the surface of a 25 μg/mL solution of the respective matrix protein as described in Materials and Methods. The filters were then washed and used in migration experiments with DME added to the lower well. Control filters were floated overnight on buffer only and these numbers have been subtracted out from the experimental values shown. Values were determined in triplicate and represent the mean ± SEM.

The adhesion studies performed in the presence of RGDS and the differences in AEC adhesion kinetics on IV, PIV, NC1, and LMN suggest that these cells may have a class of preexisting receptors for IV that are distinct from receptors for either fibronectin (17) or LMN (26, 54). The gradual rate of cell attachment, the lack of an adhesion plateau within 90 min, and the cycloheximide sensitivity, suggests that when plated on LMN or NC1-coated substrata AEC may be stimulated to synthesize new receptors or they may be generating molecules for export which are able to crosslink the cell surface to substratum-bound LMN or NC1 thus further facilitating cell adhesion and spreading. In this regard, it is well established that cell surface-associated LMN is able to bind certain cell types to substratum adsorbed IV promoting cell adhesion (49). To address this possibility we have performed adhesion experiments with AEC attaching to either substratum bound IV or LMN in the presence of affinity purified polyclonal anti-LMN antibodies. When included in the assay the anti-LMN antibodies have no inhibitory effect on AEC adhesion to IV, while they strongly inhibit adhesion to LMN. Therefore we feel that the cell adhesion promoted by IV is not mediated via LMN, but rather it involves distinct cell surface receptors for IV.

The triple helical region of IV (PIV) contains much of the molecular information necessary for mediating the cell

adhesion promoted by intact IV. Within the pepsin-generated helical segments are numerous amino acid sequences which are divergent from the standard gly-Xaa-Yaa sequences characteristic of collagenous proteins. These discontinuities in the type IV triple helix are highly conserved and may contribute to the ability of PIV fragments to promote AEC attachment and spreading. The diminished activity seen on the purified NC1 domain suggests that this globular carboxy terminal section of the IV molecule is somewhat lacking in the molecular architecture necessary to mediate the adhesion and spreading response of AEC observed on either PIV or intact IV.

The AEC adhesion studies on IV and purified domains of IV are in partial agreement with recent work by Aumailley and Timpl (1). They have shown that a variety of normal and transformed cell lines are able to use IV for attachment. In addition, they show that a human fibrosarcoma cell line is also effectively able to use the major triple helical domain of IV for cell attachment but that the globular NC1 domain of IV was relatively ineffective. However, in contrast to their results, the cell adhesion and spreading in our assays was sensitive to pretreatment with cycloheximide, and, AEC were very capable of using IV directly as an adhesive substratum. These differences in results are possibly due to the fact we were using bovine aortic (large vessel) endothelial cells compared with the murine microvessel (capillary) endothelial cells.

In our studies LMN was much less effective than IV or PIV at promoting the adhesion of AEC. Other investigators have also observed that LMN is less potent than other serum or ECM proteins at mediating the adhesion of various epithelial cell types. For example, Donaldson et al. have demonstrated differences in the ability of purified components of the basement membrane to support reepithelialization of wounded amphibian epidermis (6, 7). These studies reported that the basement membrane components fibronectin and IV, as well as nonbasement membrane proteins fibrinogen and type I collagen were far more potent than LMN at promoting wound closure. Additionally, immunolocalization studies of reepithelializing corneal wounds have indicated that LMN appears at the basal surface of the epithelial cells, only after closure has been completed (11). In contrast, several studies have clearly demonstrated an important role for LMN in promoting the adhesion and migration of normal and transformed cells, including neurons (3, 40), hepatocytes (51), and tumors of epithelial (49, 51), mesenchymal (26), and neural crest origin (27, 31). In light of these results it is clear that LMN mediates its biological effect in a cell specific manner and that it may not be as important an adhesive substratum as IV for AEC during the early events of wound healing or vessel growth.

The migration of endothelial cells has been shown to be an important early response to large vessel injury and to also play a dominant early role in neovascularization. In comparing LMN with IV and purified domains of IV, we have shown that under the conditions outlined herein, LMN is completely ineffective at stimulating the directed migration of AEC. On the other hand, IV and the triple helical fragments of IV are quite active at promoting the directed migration of AEC, and on a molar basis NC1 is much less active than IV or PIV. It still promoted AEC migration. Since previous work (Basara et al.) had shown that LMN does bind to the surface of the polycarbonate filters used in these experiments we feel that the lack of any measurable migration by AEC to LMN does not represent a failure of the protein to bind to the filter surface.

The fact that the directed migration elicited by soluble proteins could also be accomplished by an insoluble substratum adsorbed gradient in the absence of any fluid phase attractant suggests that the mechanism of AEC migration in this system is haptotactic in nature. This type of directed cell movement has been demonstrated with malignant cells on a variety of proteins (2, 27) but has not been previously demonstrated with endothelial cells on IV or any purified domains of IV.

In summary, we have shown that basement membrane IV is able to directly promote the adhesion and directed migration of AEC. The IV molecule has at least two bioactive domains able to mediate differential effects on endothelial cell adhesion and migration via a partially cycloheximide sensitive, RGDS independent mechanism. This information suggests that like other adhesive glycoproteins, IV contains multiple distinct sites able to promote specific aortic endothelial cell functions which may be important to in vivo mechanisms of vessel growth and repair.

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