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Short Contribution

Laminin fragment E4 inhibition studies: basement membrane assembly and embryonic lung epithelial cell polarization requires laminin polymerization

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ABSTRACT Laminins (LMs), the main constituents of basement membranes (BMs), are heterotrimeric glycoproteins composed of α, β, and γ chains held together by disulfide bonds. In the presence of Ca^{2+}, some laminins, such as laminin-1 self-assemble into a polymer through the interaction of their three NH_2 termini. Here we exposed lung organotypic cultures to a proteolytic fragment of laminin-1 that blocks laminin polymerization. This fragment, referred as E4, comprises the outer globular region of laminin β_1 chain. Inhibition of laminin polymerization in lung organotypic cultures resulted in impaired basement membrane assembly and failure of epithelial cells to polarize. In addition, we found that in control organotypic cultures, the bronchial smooth muscle cells were arranged in concentric layers around the newly formed epithelium. However, in E4-treated cultures, the smooth muscle cells were in disarray. Exposure of organotypic cultures to laminin-1 proteolytic fragment P1', that comprises part of α_1, β_1, and γ_1 chains, but does not overlap with fragment E4, had no effect in basement membrane assembly. Exposure to fragment E4 also caused an increased release of laminin-1 into the culture medium, suggesting a failure to retain laminin at the epithelial-mesenchymal interface. These studies provide the first direct evidence linking epithelial cell polarization to laminin polymerization at the epithelial-mesenchymal interface and assign a key role to the outer globular region of laminin β_1 chain.

KEY WORDS: *laminin, embryogenesis, basement membrane, extracellular matrix, smooth muscle*

Laminins (LMs) are main components of basement membranes (BMs) and play important roles in cell adhesion, proliferation and differentiation (Engel, 1993). More than eleven LM chains and eleven LM isoforms have already been identified (Timpl, 1996). Most LMs have the ability to interact with themselves, with other BM macromolecules, and with cell surface-associated molecules (Engel, 1992). We and others have shown that LMs are critical for normal organogenesis (for review, Ekblom, 1996, Schuger, 1997). In the developing lung, mRNA for LM chains α1, β1, and γ1 is detected in both epithelial and mesenchymal cells (Schuger *et al.*, 1992, Thomas and Dziadek, 1994, Lallemand *et al.*, 1995). LM-1 shares β1 and γ1 chains with LM-2 and β1 chain is also found in LM-6, LM-8, and LM-10 (Timpl, 1996).

In previous studies we found that a monoclonal antibody (mAb) against the globular regions of LM chains β1 and γ1, referred to as mAb AL-5 (Skubitz *et al.*, 1987,1988), impaired lung branching morphogenesis (Schuger *et al.*, 1991), inhibited epithelial cell polarization and caused severe BM structural alterations (Schuger *et al.*, 1995). Yurchenco and collaborators have proposed that, in the presence of Ca^{2+}, LM self-assembles into a polymer by an interaction between the outer globular regions of its short arms (Yurchenco and Cheng, 1993). We, therefore, reasoned that mAb AL-5 inhibited epithelial cell polarization by preventing LM self-assembly into a polymer at the epithelial-mesenchymal interface. However, we did not have direct evidence indicating that indeed mAb AL-5 blocks LM polymerization. To test this hypothesis, here we exposed organotypic epithelial-mesenchymal cocultures to a LM-1 proteolytic fragment that specifically blocks LM polymerization (Yurchenco *et al.*, 1992). This fragment, referred to as E4, comprises the outer globular region of LM β1 chain and the segment between this and the inner globule (domains V and VI). For comparison, additional cocultures were exposed to another LM-1 proteolytic fragment that does not block LM polymerization (fragment P1') (Yurchenco *et al.*, 1992). LM fragments E4 and P1' were not toxic for lung cells as indicated by a ^{51}Cr cytotoxicity assay (Schuger et al, 1989) which showed similar release of ^{51}Cr in cocultures exposed to 100 μg/ml of fragments P1', E4 or control untreated cocultures (less than 5% of total ^{51}Cr incorporated, data not shown).

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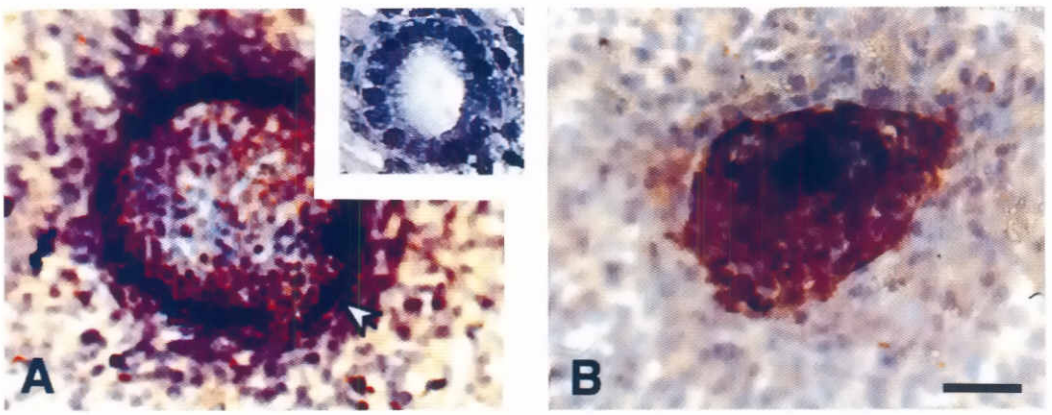

Fig. 1. Organotypic cocultures exposed to LM-1 proteolytic fragments P1' and E4. *The cocultures have been immunostained with antibodies to cytokeratins to identify the epithelial cells (dark brown cytoplasm, arrow) and counter-stained with hematoxylin. Cocultures exposed for 48 h to 100 μg/ml of fragment P1' show tri-dimensional epithelial cysts surrounded by mesenchymal cells* **(A)** *(notice that the cyst's lumen is covered by the hematoxylin-stained nuclei of the cells constituting its wall; compare with Figure 3A, in which the coculture was not counter-stained). The insert represents a section through a 24 hour-old cyst showing a central lumen and polarized epithelium. The later indicated by basally located nuclei and apical cytoplasm. Cocultures exposed for 48 h to 100 μg/ml of fragment E4 showed mainly clusters of unpolarized epithelial cells growing as a flat monolayer* **(B)**. *Bar, 40 μm.*

In untreated organotypic cocultures or in cocultures exposed to 100 μg/ml of LM fragment P1' or 100 μg/ml of heat-denatured fragment E4, the epithelial cells rearranged into a polarized epithelium leading to the formation of cysts surrounded by mesenchyme (Fig. 1A and insert). In the cocultures exposed to 100 μg/ml of fragment E4 most of the epithelial cells did not polarize into cysts but formed flat, irregular islets which grew as a monolayer. These islets were identified by immunohistochemistry with an antibody to cytokeratins, a marker for epithelial cells (Fig. 1B). Determination of cell number at the end of the experiment indicated no differences in epithelial cell number or epithelial/mesenchymal cell ratio in all the cultures regardless of the treatment received (data not shown). Dose-response studies showed that fragment E4 blocked epithelial cell polarization up to 75±10% in a concentration-dependent manner, whereas fragment P1' and heat-inactivated E4 had no effect (Fig. 2A). The number of unpolarized epithelial cell clusters was directly proportional to the concentration of fragment E4 (Fig. 2B). These results, therefore, confirmed the role of the outer LM β1/γ1 globular domains in epithelial cell polarization suggested by our previous studies using mAb AL-5 (Schuger *et al.*, 1995).

In organotypic epithelial-mesenchymal cocultures, a BM-like structure is formed at the interface between the two cell types. This *in vitro* BM has the ultrastructural and immunohistochemical characteristics of an *in vivo* BM (Schuger *et al.*, 1995). Therefore, the BM formed in organotypic cultures is immunodetected by antibodies to LM and type IV collagen as a continuous polymer at the epithelial-mesenchymal interface (Schuger *et al.*, 1995). In the present study, we used immunohistochemistry to identify the BM in cocultures exposed to fragment E4, P1' and denatured fragment E4. These studies revealed a linear pattern of LM-1 and type IV collagen deposition at the epithelial-mesenchymal interface in cocultures exposed to fragment P1' (Fig. 3A-C). This pattern was identical to that seen *in vivo* and in untreated cocultures (Schuger *et al.*, 1995). In contrast, cocultures exposed to fragment E4 showed no LM-1, or type IV collagen deposition at the epithelial-mesenchymal interface. These cocultures presented instead a diffuse granular distribution of the two BM components surrounding and within the areas of epithelial clustering (Fig. 3D-F). ELISA demonstrated that the absence of LM-1 at the epithelial-mesenchymal interface was not due to a decrease in its synthesis, since cocultures exposed to 100 μg/ml of fragment E4 released approximately 10-fold more LM-1 to the medium than cocultures exposed to fragment P1', heat-denatured E4, or unexposed cocultures (Table 1). We concluded from these studies that, in the presence of fragment E4, LM-1 was not retained at the epithelial-mesenchymal interface but it was rather released to the medium. Since fragment E4 specifically blocks LM self-assembly (Yurchenco *et al.*, 1992), these experiments demonstrated the essential role of LM polymerization at the epithelial-mesenchymal interface in the formation of the airway BM suggested by our previous study using mAb AL-5 (Schuger *et al.*, 1995).

In the last set of experiments we studied the behavior of bronchial

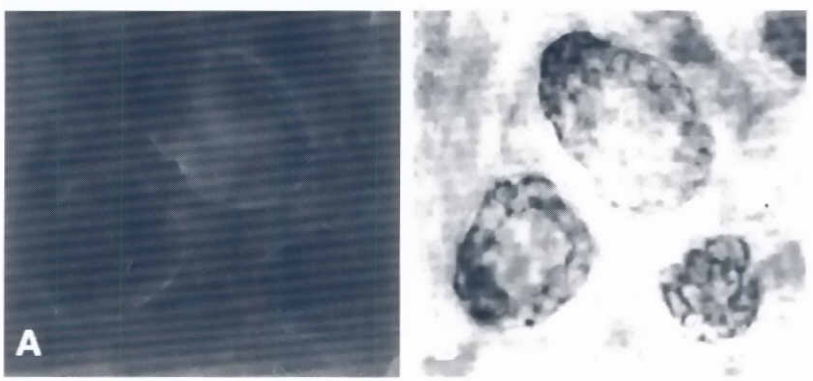

Fig. 2. Organotypic cocultures were established in the presence of various concentrations of LM-1 fragments P1', E4 and heat-denatured E4, or were left untreated. *After 24 h in culture the number of epithelial cysts and unpolarized epithelial clusters formed per well was determined. Only fragment E4 inhibited cyst formation* **(A)**. *This inhibition was concentration-dependent and was statistical significant for concentrations of 50 and 100 μg/ml respectively (P =< 0.003 and 0.001, respectively). The number of unpolarized clusters* **(B)** *was directly proportional to the concentration of fragment E4 and was statistical significant for concentrations of 50 and 100 μg/ml respectively (P =< 0.005 and 0.001, respectively). The bars represent standard deviations (SD). The means and SD are based on four samples in a single experiment. The experiment was repeated four times with similar results.*

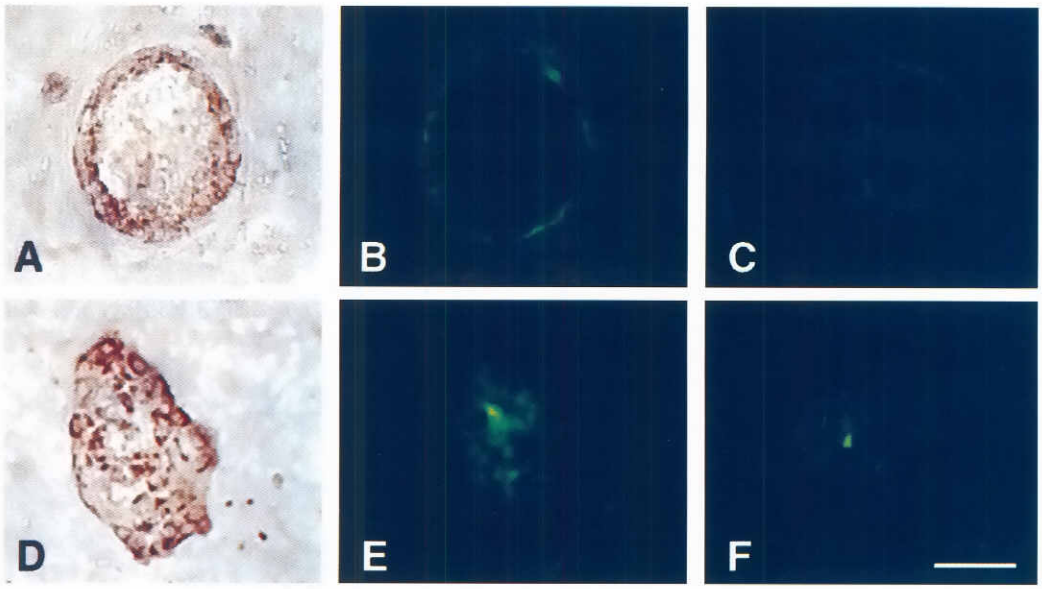

Fig. 3. Deposition of LM-1 and type IV collagen in cocultures exposed to 100 g/ml of LM fragments P1' and E4 for 24 h. (A-C) *Cocultures exposed to fragment P1'. Photomicrographs A and B show the same epithelial cyst double-stained with antibodies to cytokeratins by immunoperoxidase (A) and to LM-1 (anti-LM_1 chain mAb) by immunofluorescence (B). Photomicrograph C represents an epithelial cyst single-stained with antibodies to type IV collagen. A linear pattern of LM-1 (B) and type IV collagen (C) are seen at the epithelial-mesenchymal interface, surrounding epithelial cysts.* **(D-F)** *Cocultures exposed to fragment E4. Photomicrographs D and E show the same epithelial cluster double-stained with antibodies to cytokeratins by immunoperoxidase (D) and to LM-1 by immunofluorescence (E). Photomicrograph F represents an epithelial cyst single-stained with antibodies to type IV collagen. No LM-1 (E) or type IV collagen (F) are seen at the epithelial-mesenchymal interface, but some granular deposits of both BM components are observed within the unpolarized epithelial cell clusters. Bar, 80 μm.*

smooth muscle cells in organotypic cocultures and whether it is affected by LM polymerization. In these experiments the cocultures were exposed to 100 μg/ml of fragments P1', E4 and denatured E4 and after 24 h culture were immunostained with antibodies to smooth muscle α-actin, a marker for smooth muscle cells. As seen in the developing lung, the cocultures exposed to fragment P1' or heat-inactivated E4 showed several concentric layers of elongated smooth muscle cells surrounding the epithelial cysts (Fig. 4A). However, the cocultures exposed to fragment E4 showed smooth muscle cells running in haphazard directions with no particular alignment around epithelial clusters (Fig. 4B). Thus, our studies suggested that normal bronchial smooth muscle architecture requires polymerization of LM at the epithelial-mesenchymal interface. Interestingly, we recently found a correlation between LM α1 chain deposition in the airway BM and peribronchial mesenchymal cell elongation and differentiation into bronchial smooth muscle (Schuger *et al.*, 1997). Based on the above, we propose that LM polymerization through its short arm globular domains supplies an organized meshwork to which mesenchymal cells can attach and subsequently spread by binding to the LM α1 chain.

In this study we show for the first time that LM polymerization at the epithelial-mesenchymal interface induces BM assembly and normal arrangement of bronchial epithelium and smooth muscle layers. Further studies are required to elucidate whether alterations in the LM domains involved in self-assembly may affect lung morphogenesis and tissue repair.

Experimental Procedures

LM fragments and antibodies

LM isolated from lathyric EHS tumors was purified as described (Schittny and Yurchenco, 1990). Fragment E4 was generated by digestion with elastase and purified by Sepharose CL-6B gel filtration and DEAE-5PW ion-exchange HPLC (Schittny and Yurchenco, 1990). Fragment P1' was pre-

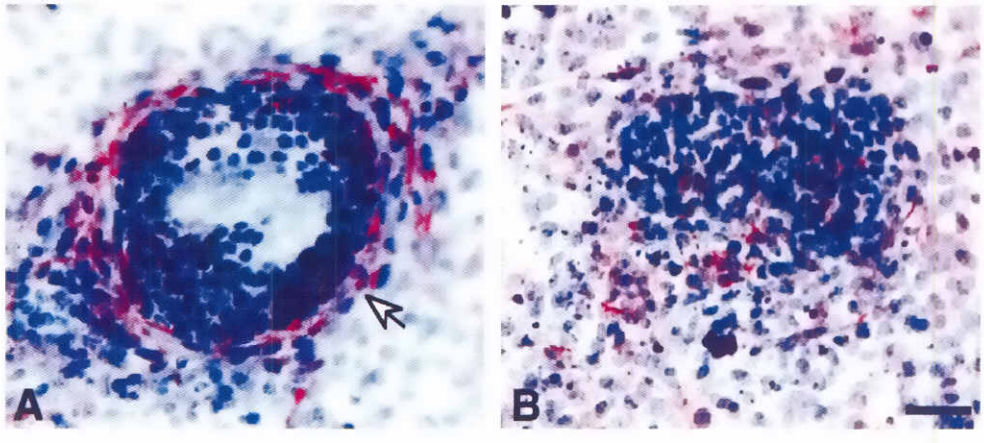

Fig. 4. Immunohistochemical detection of bronchial smooth muscle rearrangement in organotypic cocultures exposed to LM-1 fragments P1' and E4. *The cocultures have been immunostained with antibodies to smooth muscle α-actin to identify the smooth muscle cells (reddish cytoplasm). Hematoxylin has been used for counter-staining. Cocultures exposed to 100 μg/ml of fragment P1' showed epithelial cysts surrounded by smooth muscle cells aligned concentrically to the cyst (arrow)* **(A)**. *Cocultures exposed to 100 μg/ml of LM fragment E4 showed smooth muscle cells scattered through the mesenchyme. A cluster of unpolarized epithelial cells is seen in the center of the photomicrograph* **(B)**. *It should be stressed that mesenchymal cells do not form clusters. Bar, 40 μm.*

TABLE 1

RELEASE OF LM-1 BY COCULTURES EXPOSED AND UNEXPOSED TO LM FRAGMENTS P1', E4 AND HEAT-INACTIVATED E4

Treatment (100 μg/ml)[a]	mean ± SD x 10^6 cells (pg)[b]
None	45±0.08
P1'	30±0.08
E4	420±0.15
D-E4	14±0.22

[a] Organotypic cocultures were treated for 24 hours.
[b] The means and SD are based on four samples in a single experiment. The experiment was repeated three times with similar results.

pared from a pepsin digest of EHS LM by agarose gel filtration (Schittny and Yurchenco, 1990). Rabbit polyclonal antibody to EHS-derived LM and type IV collagen were purchased from Collaborative Biomedical (Boston, MA). A rat site-specific mAb to the G domain of LM -1 chain (globular region of LM-1 long arm), not found in fragments P1' or E4, was a gift from Dr. Amy Skubitz (Skubitz *et al.,* 1987,1988). A rabbit polyclonal antibody to low and high molecular weight cytokeratins was purchased from Dako (Carpinteria, CA) and a mouse mAb to smooth muscle α-actin was obtained from Sigma (St. Louis, MO).

Blocking experiments

Organotypic cocultures were generated in 96-well plates as described previously (Schuger *et al.,* 1993,1995) and various concentrations of LM fragments E4 and P1' (ranging from 5 to 100 μg/ml) and heat-denatured fragment E4 (100 μg/ml, boiled for 5 min) were added to quadruplicate wells at time zero. Additional quadruplicate wells received no treatment. The cocultures were maintained for 24 and/or 48 h, then washed, fixed with ethanol for 5 min, immunostained with anti-keratin antibodies as described below and counterstained with hematoxylin. The number of polarized and unpolarized epithelial cell clusters per well was then determined. Epithelial cells were considered polarized when they formed small cysts with a central lumen. The culture media was used for determination of LM-1 by ELISA as described (Varani *et al.,* 1985) using a mAb to LM-1 G domain. The experiments were repeated four times and the results were evaluated with the Student's t-test for statistical significance.

In additional studies, P1' and E4-treated and untreated cocultures were trypsinized at the end of the experiment, washed and replated. After three hours, the attached cells were stained with antibodies against cytokeratins to identify epithelial cells (cytokeratin positive). Cytokeratin-negative cells were considered mesenchymal. The number of epithelial and mesenchymal cells and the epithelial/mesenchymal cell ratio was then determined in a field outlined on a projection screen.

Immunohistochemistry

Double immunostaining (immunofluorescence followed by immunoperoxidase) was done for LM-1 (using the mAb against 1 chain G domain) and cytokeratins combined, and single immunostaining was done for type IV collagen. Cocultures were fixed for 5 min in absolute alcohol, exposed to 5% normal goat serum followed by treatment with a 1:50 dilution of antibodies to LM-1, type IV collagen, or control IgG for 45 min at room temperature. The sections were then washed in PBS and exposed to a 1:50 dilution of the secondary antibody (FITC-conjugated goat anti-rat or anti-rabbit IgG for LM-1 and type IV collagen, respectively; Cappel, Malvern, PA) for 30 min at room temperature. To identify epithelial cells, the LM-1-stained cocultures were then immunostained with anti-cytokeratin antibodies using a commercial peroxidase-anti-peroxidase kit (Dako) and following the manufacturer's instructions. In the case of type IV collagen, duplicate cocultures were stained with anti-type IV collagen and anti-cytokeratin antibodies respectively. To identify smooth muscle cells, the cocultures were stained with 1:50 dilution of anti-smooth muscle α-actin using a commercial peroxidase-anti-peroxidase kit (Dako) and following the manufacturer's instructions.

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