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Presence of laminin and 67KDa laminin-receptor on endothelial surface of lung capillaries. An immunocytochemical study

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Summary. The existence of cell surface-associated molecules has been claimed to play a major role in cellular recognition and interaction. In this respect, different tumor cell lines express laminin and its receptor, and this expression has been correlated with metastatic potential. In the present work, we have studied, by electron microscopic immunolabeling methods, the presence of laminin and 67KDa laminin-receptor on the surface of endothelial cells of lung blood capillaries. To label these molecules, we have developed an easy method in which the labeling is carried out "in situ", in previously excised lungs. The presence of both molecules was observed on the luminal surface of endothelial capillaries and, in many cases, gold particles were associated to small open vesicles of the endothelial cells. The results suggest that these molecules, traditionally associated to extracellular matrix, are also expressed in cellular surface of the lung vascular bed.

Key words: Laminin, Laminin-receptor, Lung, Endothelium, Immunocytochemistry

Introduction

The blood-stream is the ultimate disseminating route for most metastatic cells. The interaction of cancer cells with blood-vessels plays a pivotal role in the metastatic cascade (Weiss et al., 1989). Neoplastic cells are subject to specific recognition and adhesion mechanisms (Rizzino et al., 1980; Baron-Van Euercooren et al., 1982; Rao et al., 1982). The existence of cell surface-associated molecules in the tumor cells has been claimed to play a major role in cancer cell interaction with other cancer cells, host cells and/or extracellular matrix. In this sense, different tumor cell lines express laminin and its receptor (Aliño et al., 1989; Bouzon et al., 1989), and this expression has been correlated with metastatic potential (Terranova et al., 1982; Situ et al., 1984).

Both in animal models and "in vitro", tumor cells have been shown to establish close contact to endothelial cells, which subsequently expose the underlining extracellular matrix (Kramer and Nicolson, 1979; Nicolson, 1982; Crissman et al., 1985, 1988; Lapis et al., 1988). Extracellular matrix molecules have been described to be correlated with metastatic potential (Kramer et al., 1980).

The presence of laminin and/or laminin-receptors on the luminal surface of the pulmonary endothelial cells, which may interact with the corresponding one of the metastatic cells, could contribute to the formation of metastatic foci. The aim of the present work is the immunocytochemical location of laminin and 67KDa laminin-receptor on the luminal surface of lung endothelial cells, which could corroborate this hypothesis.

Materials and methods

Chemicals

Antilaminin rabbit antibody (Chemicon International Inc., El Segundo, California, USA), anti 67KDa laminin-receptor rabbit antibody, generously donated by Dr. G.R. Martin (Institute of Dental Research, Bethesda, Maryland, USA) and anti-rabbit IgG-gold 10 nm antibody (Sigma Chemical Co., St. Louis, Mo., USA) were dissolved 1:10 in PBS-10 BSA.

Mice

Syngeneic C57BL/6 mice (9 to 10 weeks old) were purchased from Iffa Credo Laboratories (France). Water and food were given ad libitum. The experimental protocol met all regulations for animal research (EC Directive 86/609) and was approved by Institutional Research Committee.

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Experimental Procedure

For binding test of specific antibodies on endothelial cells lining the lung blood capillaries, we have developed an easy lung perfusion method. Mice were anaesthetized with Nembutal (Merck, Germany), 1.2 mg/mouse i.p. in phosphate-buffered saline (PBS). Afterwards, mice were injected i.p. with 0.2 ml of EDTA 2mM dissolved in PBS, and exanguinated. Then, the thoracic cavity was carefully dissected, and the heart cross sectioned through both ventricular cavities. A tiny polietilene tubes (Intramedic Adams, 0.027 mm and 0.06 mm, inner and outer diameters, respectively) was inserted into the pulmonary artery and another polietilene tube was placed into the left atrium. The former tube followed the perfusion medium and the latter collected the pulmonary venous effluent. The lungs were placed in a humidified environment at room temperature and afferent tube connected to a peristaltic pump (Gilson, monopuls 2).

Electron Microscopic Immunolabeling

To determine the presence of laminin and laminin-receptors on the endothelial cell surface of the lung vascular bed of mice, we have used a modification of the method of binding assay described by Schlepper-Schäfer et al. (1986). Initially, to remove blood, the lungs were perfused with PBS at a flow rate of 0.3 ml/min in a non-recirculating system at room temperature. Lungs were then very slightly fixed with 0.1% glutaraldehyde in 0.1M sodium cacodylate/HCl buffer (pH 7.4) for 2 min, and perfused with 0.5% bovine serum albumin (BSA) in PBS to saturate free aldehyde groups on the endothelial cells. After 2 min, the first antibody (antilaminin or anti 67KDa laminin-receptor) was injected through the perfusion tube. When perfusion medium containing the antibody began to come out of the lungs, the perfusion flow was stopped. After 20 min of incubation, the lungs were perfused to wash out unbound ligand with PBS-BSA for 5 min. Subsequently, the lungs were perfused with anti-rabbit IgG gold conjugate 10 nm, and incubated during 30 min. Then, the organs were primarily perfused to remove the unbound second antibody for 5 min with PBS-BSA and secondary with 0.5% glutaraldehyde in cacodylate buffer for 10 min. Controls for nonspecific labeling were performed with normal rabbit serum as first antibody. Each experiment was performed in three animals, except the control experiments in which five animals were used.

Further, multiple lung pieces (1 mm^3) were immersed in 2.5% glutaraldehyde in 0.1M sodium cacodylate/HCl buffer (pH 7.4) at 4 ºC for 6 hours. After washing overnight in cacodylate buffer containing 5% sucrose, the tissue blocks were postfixed in 1% osmium tetroxide in 0.1M cacodylate buffer (pH 7.4) for 2h. Subsequently, the tissues were rinsed in cacodylate buffer, dehydrated in ethanol series and embedded in Epon 812 (Fluka, Switzerland) by standard technique. Ultrathin sections (80-90 nm) were stained with uranyl acetate and lead citrate and examined with a Philips EM 300 electron microscope.

Quantitation of electron microscopic immunolabeling

From 3 to 8 lung blocks (1 mm^3) of each mouse were sectioned, and a minimum of 5 non-serial sections (400 nm apart) of each block were collected on the same grid. Four grids were collected from each block.

The presence, number, and the arrangement of gold particles were studied in the transversally sectioned capillaries. Tangential and longitudinal sections were discharged. A minimum of 100 capillaries of each lung were randomly counted.

Statistical Analysis

The percentage of lung capillaries with gold particles was expressed as the mean ± SD in every experimental group. Groups were compared by Student's t test for unpaired samples, and differences were statistically significant to $p<0.05$.

Results

The percentage of lung capillaries with gold labeling is shown in Table 1. Control experiments showed that the labeling was laminin or laminin receptor specific.

The presence of laminin and 67KDa laminin-receptor on the luminal surface of the lung capillary endothelium was observed. In both laminin and 67KDa laminin-receptor groups, the number of labeled capillaries was statistically significant in relation to control group ($p<0.001$, and $p<0.01$, respectively). Gold particles were exclusively restricted to the luminal surface of endothelial cells of the capillaries (Fig. 1). Gold particles were in many cases associated to small open-vesicles of the endothelial cell (Fig. 1B, C. Moreover, a small number of particles was located intracellulary inside a vesicle (Fig. 1C). Usually, the labels consisted of a single gold particle, but occasionally a small number of clustered gold particles were detected.

Presence of gold label on the remaining erythrocytes was not observed. Occasionally, isolated gold particles were observed on the surface of lymphocytes. No labeling was detected either on epithelial cells of the

Table 1. Percentage of lung capillaries with gold particles is expressed as means ± SD in every experimental group. AL: antilaminin rabbit antibody. ALR: anti 67KDa laminin-receptor rabbit antibody.

CONTROL	AL	ALR
1.11	38.66	21.00
2.00	56.66	27.82
8.18	62.40	31.33
10.43		
20.70		
8.46±7.92	52.58±12.39**	26.71±5.25*

*: $p<0.01$; **: $p<0.001$.

alveolar surface or in the interstitial connective tissue.

Discussion

In the present work, we have shown the presence of laminin and its 67KDa receptor on the luminal surface of endothelial cells of lung blood capillaries. Moreover, the experimental procedure used in this work allows one to carry out immunocytochemical studies with a good ultrastructural preservation.

Blood vessels are lined by endothelial cells, which show a multifunctional role, being involved in several recognition and cellular adhesion mechanisms. In this sense, they produce molecules such as ICAM, integrins and so on (for review see Simionescu et al., 1982; Cotran 1987). In this respect, the existence of a laminin-receptor for $\alpha_2\beta_1$ integrin in the human umbilical vein endothelial cells has been described (Languino et al., 1989), as well as a 67KDa laminin-receptor in cellular cytoplasm, which is indistinguishable from the transmembrane one (Hunt and Barker, 1988; Makrides et al., 1988).

Endothelium of capillaries exhibits particular biological properties that, in many cases, are different from those exhibited by the endothelium of large vessels (Folkman et al., 1979; Keegan et al., 1982; Madri and Williams, 1983), and these differences can be organ specific (Auerbach et al., 1985). Our results in the lung capillaries, together with those described in relation to umbilical vein (Hunt and Barker, 1988; Makrides et al., 1988; Languino et al., 1989), seem to suggest that these molecules could present a generalized distribution in the vascular endothelia. However, we cannot exclude regional differences, as Languino et al. (1989) previously described in the basal lamina with laminin and other laminin-related molecules.

On the other hand, the specificity of adhesion between tumor cells and capillary endothelium is an important event in the organ preference in metastatic localization (Nicolson, 1982; Auerbach et al., 1985, 1987). Tumor cells express laminin and its receptor (Terranova et al., 1982; Rao et al., 1983; Malinoff et al., 1984; Aliño et al., 1989; Bouzon et al., 1989), and this expression has been correlated with its metastatic potential (Terranova et al., 1982, 1984; Barsky et al., 1984; Situ et al., 1984; Aliño et al., 1990). The involvement of laminin and its receptor in the metastatic process could be related to a specific interaction between tumor and endothelial cells of target organ. In a subsequent step, these molecules would participate in the attachment and migration of tumor cells in the subendothelial matrix (Crissman et

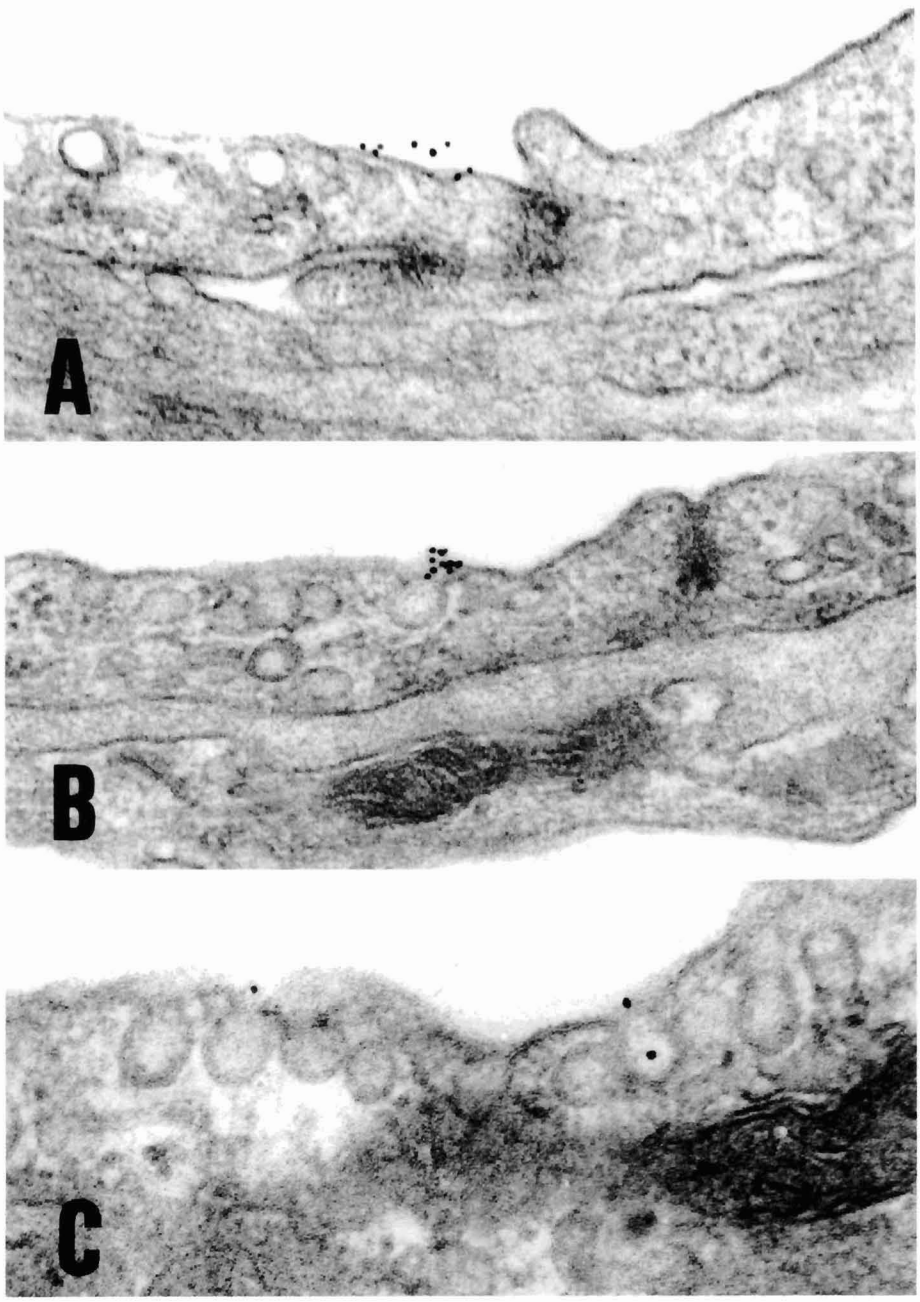

Fig. 1. A. Immunogold labeling of laminin on luminal surface of endothelial cells. In **B** and **C** the gold particles are associated to open cytoplasmatic vesicles. A, x 84,000; B, x 70,000; C, x 113,000

al., 1988).

Further studies will be needed to clarify the role that laminin and/or 67KDa laminin-receptor play in the interaction between tumor and endothelial cells in the lung, contributing to the tumor progression. Moreover, the function that these molecules carry out in physiological conditions in the pulmonary vascular bed, remains to be clarified.

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