Articles

Immunohistochemical localization of extracellular matrix proteins in developing lung tissues

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ABSTRACT

Objectives: To examine the laminin specific receptor, known as β_1 integrin, and fibronectin distributions in 13, 15, 17, and 20 days prenatally old and 2 and 6 days postnatally old murine lung tissue by immunohistochemical methods at light microscope level.

Methods: Male and female swiss albino mice were used for experimental procedures from July 2001 to March 2002 at Gazi University Faculty of Medicine. The study was conducted on samples of 13, 15, 17, and 20 prenatally old and 2 and 6 days postnatally old lung tissues. Indirect immunohistochemical methods and fibronectin and laminin β_1 integrin antibodies were used; tissues were examined with a light microscope.

Results: In the prenatal group, fibronectin immunoreactivity was determined to be irregular in bronchiole epithelium cells, while it was strongly evident in mesenchymal cells and vascular endothelial cells. Laminin β_1 integrin immunoreactivity was also observed irregularly. Development period of lungs, immunoreactivity of fibronectin was clearly identified in the bronchiolus, ductus alveolaris and alveolar epithelial cells, mesenchymal cells, and vascular endothelial cells, and laminin immunoreactivity was strongly involved both in the apical and basal membranes of all the epithelial cells and within the basal lamina. The decreased immunoreactivity of fibronectin and laminin β_1 integrin was established after birth on the fetal period.

Conclusion: In this study we concluded: 1. During the development of the lung, fibronectin is necessary for the shaping of lung parenchyma and stroma. 2. β_1 integrin as the receptor of laminin is important in the process of lung maturation and the modelling of basal lamina.

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The extracellular matrix has supportive and structural functions in the development of lungs. Glycoproteins such as fibronectin and laminin that exist within the extracellular matrix mediate the interactions between cells.[1] Fibronectin is a dimeric glycoprotein with several disulfide bonds and is composed of 2 polypeptides transcribed from a single gene.[2,3] Besides acting in events such as cell migration, embryogenesis, wound healing, hemostasis, and thrombosis, it has important functions in the achievement of cell-cell and cell-adhesion interactions.[4] Fibronectin receptors are glycoproteins that interact with the structures of the cellular skeleton via special intracellular pathways, and therefore, induce cellular movement and differentiation.[5]

Laminin was first extracted from an Engelbreth Holm Swarm (EHS) tumor matrix.[6] Its synthesis is thought to be unique to cells and tissues.[7] It serves in the cell-matrix interactions that are important in hemostasis and the restructuring of tissues during embryogenesis.[8] Laminin also interacts with other important components of the basal membrane such as entactin, type IV collagen, and heparan sulfate. Cells adhere to laminin via several cellular receptors. There are at least 6 different laminin receptors that can adhere to different laminins. For instance, $\alpha_6\beta_1$ is the receptor of integrin laminin 1.[2] The integrins interact with proteins of the cytoskeleton such as actin and talin in the cytoplasm and with the important components of the extracellular matrix such as fibronectin and laminin.[3] Integrins such as $\alpha_3\beta_1$ and $\alpha_6\beta_1$ bind strongly to laminins, whereas integrin $\alpha_5\beta_1$ is bound strongly by fibronectin.[4,9]

The aim of this study is to determine the distributions of β_1 integrin, which are a laminin receptor, and fibronectin within the connective tissue and basal lamina in lung development. With this purpose, the distributions of β_1 integrin and fibronectin in mice were examined immunohistochemically in the pulmonary tissue obtained from fetuses on the 13th, 15th, 17th, and

20th prenatal days and from neonates on the second and sixth postnatal days.

Methods. Male and female swiss albino mice were used in this study. The experimental protocol was approved by the local Ethical Committee for animal studies and conducted at Gazi University Faculty of Medicine from December 2001 to January 2002. The mice were bred in 12 hour light/12 hour dark cycles, and fed with standard laboratory chow and tap water ad libitum. To achieve pregnancy, 5 female mice were placed overnight with a male. The mice in which vaginal plaques were observed; were considered to be on the first day of pregnancy and were separated into different cages. Either these pregnant females were sacrificed by cervical dislocation on the 13th, 15th, 17th, or 20th days of pregnancy for removal of the lungs of their fetuses, or the neonatal mice born from unsacrificed females were sacrificed for removal of their lungs on the second or sixth postnatal days.

Immunohistochemistry. For immunohistochemical examination, all groups of tissue specimens were fixed in 10% neutral formalin solution for 72 hours, after which they were routinely observed by light microscopy and embedded in paraffin. Sections of 4 μm thickness were taken from paraffin blocks, transferred to polylysine glass slides, deparaffinize, and dehydrated by passing through xylol and alcohol series. Streptavidin-biotin indirect immunohistochemical methods were performed on the slides. For this purpose, the Zymed Histostain-Plus Broad Spectrum System (Cat # 85-9043, Lot # 00961827, USA) was used. The slides were first exposed to 3% hydrogen-peroxide to block the endogenous hydrogen peroxidase activity. After that non-immune protein blocking solution was applied to the glass slides. They were then divided into 2 groups, and the first group was subjected to Fibronectin (mouse monoclonal Ig, Lot#110501, Novacastra, USA) primary antibodies application, whereas the second was subjected to Laminin β_1 (goad polyclonal Ig, Cat # SC-6018, Lot#E030, Santa Cruz, USA). All slides were stored overnight at +4^{O}C. The tissue specimens washed with phosphate buffered saline (PBS) were treated with a biotinated secondary antibody, after which an avidin-biotin complex was applied to the slides. Finally, substrate 3,3'-Diaminobenzidine Tetrahydrochloride (Liquid DAB-Plus Substrate System, Lot #73139668, Zymed, USA) was applied to obtain a visible product. Mayer's hematoxylin was used as the counter stain. Negative control was made in the primary antibody section by using of normal goad serum. The dehydrated slides were mounted with Entellan.

Results. In the 13-day-old fetal murine lung tissues, the formation of bronchioles was noted, whereas that of alveoli was not. Irregular fibronectin involvement was observed in the bronchioles. It was also found that the apical membrane reactivity in the bronchiole epithelium cells was remarkably strong in some regions, whereas it was only moderate in others. In the cytoplasms, diffuse but weak staining was observed while occasional weak staining was noted in the basal regions of the cells. Smooth muscle cells were not completely formed, and therefore, there was no apparent fibronectin involvement. However, strong fibronectin immunoreactivity was observed in the stromal mesenchymal cells (data not shown). In the 15-day-old fetal lung tissues, the terminal bronchioles were found to have formed. Involvement within the epithelium was again not uniform. In the apical cell cytoplasms, both strong and weak involvement was observed in different locations. The vascular wall endothelial cells and the adventitial layer were shown strong and diffused immunoreactivity. The cells with strong involvement observed in the external layer of the vascular wall were proposed to be the mesenchymal cells that form the vascular smooth muscle layer. There was also diffuse and strong fibronectin immunoreactivity in the stromal mesenchymal cells **(Figure 1).** In the pulmonary tissue of 17-day-old fetal mice, the alveolar ducts were found to have formed. In this group, fibronectin involvement was distributed to the apical and basal cytoplasmic membranes of epithelial cells that form the bronchioles. Similar to the previous group, strong or weak involvement in the apical cell membrane was observed. The involvement in the epithelial cells forming the alveolar ducts was a variable. While there was moderate immunoreactivity in the membranes and diffuse moderate to strong immunoreactivity in the cytoplasms of some cells, prominent fibronectin involvement was observed in the basal cell membranes of some others. Likewise, there was strong involvement in the mesenchymal cells (data not shown). In the 20-day-old fetal pulmonary tissues, the alveoli were found to have developed, however, the alveolar septa were considerably thicker than early developmental stages. The involvement in the bronchioles was observed in the apical cell membranes and the apical and basal cytoplasms of epithelial cells. There was also prominent involvement in the smooth muscle cells of the bronchioles. In the alveoli, reactivity was observed in type I and type II alveolar cells. In these cells, fibronectin immunoreactivity was observed in the basal and apical regions and diffusely in the cytoplasm. While diffuse and strong involvement was observed in the endothelial cells of the vascular walls, the smooth muscle

cells were also noted to have formed and to display diffuse involvement. Strong fibronectin involvement was observed in the connective tissue. Also, large fibroblast-like cells within the connective tissue, which we have suggested to be myofibroblasts, were found to have developed and to display strong involvement (data not shown). The 2-day-old neonatal murine lung tissue was found to display the characteristics of the adult tissue. The alveolar septa were thinned. The fibronectin immunoreactivity was reduced as compared with those observed in the fetal tissue groups. In the alveolar cells, moderate involvement was observed compared to the other groups. In the vascular endothelium, moderate or strong fibronectin immunoreactivity was observed. No involvement was observed in the vascular smooth muscle cells (data not shown). The 6-day-old neonatal lung tissue displayed adult tissue characteristics with prominent respiratory bronchioles and alveolar sacs. While strong staining was observed in the apical cell membranes of the epithelial cells within these structures, there was moderate involvement basally. There was also diffuse moderate involvement in the cytoplasms in the alveolar epithelium. In the vascular endothelium, strong involvement was found in the apical cell membrane and the basal region, whereas diffuse and moderate involvement was seen in the cytoplasm **(Figure 2)**.

In the 13-day-old fetal lung tissues, it was found that the β_1 integrin staining was not uniform in the bronchiole epithelium. In the apical cell membrane, there was strong involvement in some regions and no involvement in the others. The involvement in the basal membrane was considerably weak. In the vascular endothelium, strong and weak involvement was found apically and basally. The involvement also varied from weak to strong within the mesenchymal cells (data not shown). In the 15-day-old fetal lung tissues, the involvement of laminin β_1 integrins was distributed unevenly in the bronchiolar epithelium both apically and basally with strong and weak involvement. However, strong immunoreactivity was observed in the vascular wall. Mesenchymal cells with strong immunoreactivity that was suggested to be the predecessors of smooth muscle cells were also found in this region **(Figure 3).** In the 17-day-old fetal pulmonary tissues, the laminin β_1 integrin involvement was found to have descended to basal lamina in the bronchioles as development proceeded. In the basal lamina, strong to moderate reactivity was seen. The involvement in the bronchiole epithelial cells were irregular. While the involvement was strong in the apical region and diffuse in the cytoplasm in some cells, it was indefinite in some others. In the predecessors of bronchiole smooth muscle cells, strong involvement was observed. Diffuse endothelial and smooth muscle cell

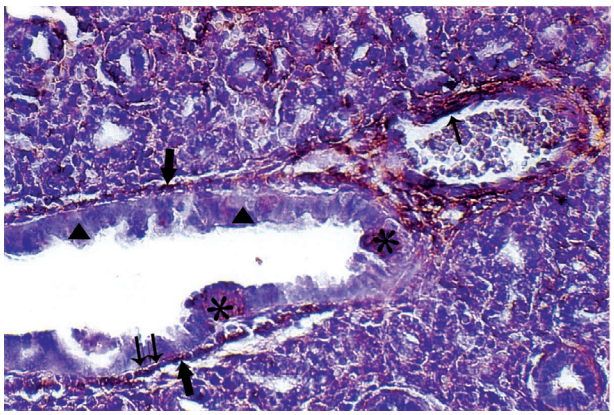

Figure 1 - On the fifteenth prenatal day, the distinguishing feature is that the terminal bronchioles (TB) have formed in the fetal lung tissue. In the bronchiolar epithelium, the fibronectin involvement is not uniform. Within the mesenchymal cells, diffuse and strong fibronectin staining is prominent (*). In the vascular endothelial cells (↑↑) and the external vascular layer (↑), diffuse and strong immunoreactivity is observed.

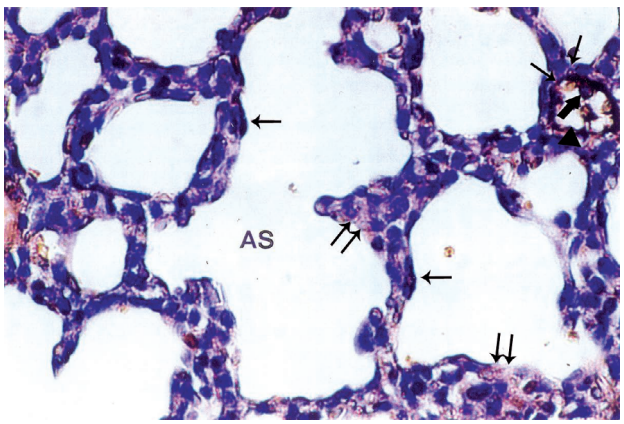

Figure 2 - On the sixth postnatal day, the alveolar sacs (AS) are noted to have formed. Strong immunoreactivity is observed in the apical cell membrane of the alveolar epithelium (↑). Moderate cytoplasmic involvement is noted in some alveolar cells (↑↑). In the vascular endothelial cells, there is prominent involvement in the basal region and the apical cell membrane. Moderate immunoreactivity (▲) is noted in the cytoplasm of some endothelial cells.

staining was noted in the vascular walls (data not shown). In the 20-day-old fetal lung tissues, the alveoli, alveolar canals, and sacs were found to have differentiated. There was prominent laminin β_1 integrin involvement in the apical and basal membranes of all epithelial cells. In the vascular endothelium, strong membranous, and diffuse cytoplasmic involvement was noticed. Strong involvement was also observed in the basal lamina (data not shown). Following birth, the laminin β_1 integrin immunoreactivity was particularly at basal lamina. On the second postnatal day, there was moderate and irregular involvement in the apical cell membranes of

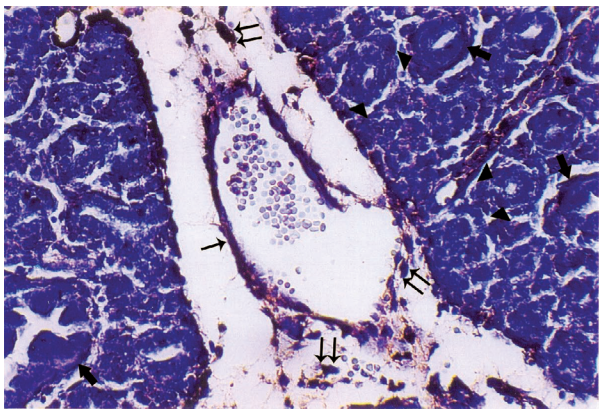

Figure 3 - In the fetal lung tissue on the fifteenth prenatal day, the bronchiolar basal membrane observed strong (➔) and weak (▲) involvement, whereas strong immunoreactivity is observed in the vascular wall (↑). In this region, strongly stained mesenchymal cells that probable are the progenitors of vascular smooth muscle cells have been noted (↑↑).

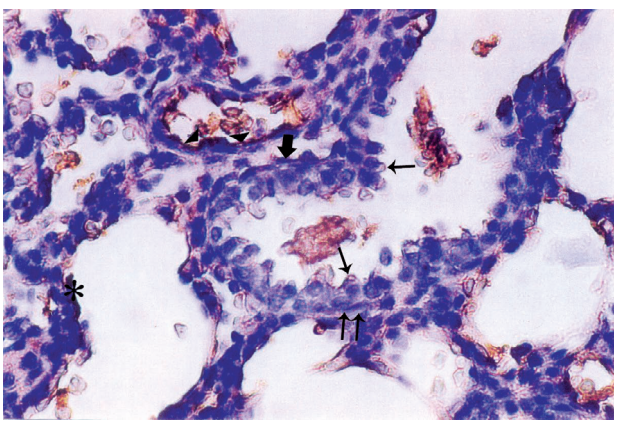

Figure 4 - On the 2nd postnatal day, the lung tissue displays diffuse involvement at the level of basal lamina. There is irregular moderate involvement (↑) in the apical membranes of bronchiolar epithelial cells. In the basal lamina, the immunoreactivity varies from weak (↑↑) to strong (➔). In the vascular wall, strong involvement is observed in the endothelial cell cytoplasm and the basal lamina (▲). Within the alveoli, there is prominent (*) membranous involvement, particularly in the type I cells.

the bronchiole epithelial cells, whereas the involvement observed within the basal lamina ranged from weak to strong. In the alveoli, strong involvement was observed particularly in the membranes and cytoplasms of type I alveolar cells. In the vascular walls, there was strong involvement particularly in the cytoplasm and basal lamina of endothelial cells **(Figure 4).** On the sixth postpartum day, the lungs had adult tissue structures. The alveolar septa were found to have thinned and to contain cells with strong immunoreactivity. It was determined that the large cells in the alveolar walls, which were possibly septal cells, displayed moderate cytoplasmic but strong membranous involvement. In the vascular endothelium, strong involvement was observed. Also, strong involvement was noticed in the type I alveolar cells (data not shown).

Discussion. Cell-cell interactions have significance in the development of several systems. Large glycoproteins in the extracellular matrix, such as laminin and fibronectin mediate such interactions.[10] Fibronectin is involved in the adhesion and migration in important physiological events such as embryogenesis, hemostasis, and thrombosis.[11]

In recent studies, it has been proposed that fibronectin and fibronectin-rich complexes facilitate binding of cells to the extracellular matrix and therefore accelerate cell migration.[12,13] In their immunohistochemical studies, Rosenkrans et al[14] examined fibronectin distribution in the pseudoglandular stage and newborn lung tissues. In the pseudoglandular stage of lung development, the primary septa are formed and tight connective tissue is synthesized on the fifteenth prenatal day. During this stage, fibronectin involvement in the lungs was found to be strong on the apical cell membranes. Fibronectin involvement was also observed in the septal buds and basal lamina.

In the present study, antifibronectin polyclonal antibodies and the peroxidase anti-peroxidase (PAP) immunohistochemical method were utilized to demonstrate the presence of fibronectin in pulmonary tissues. There was diffuse and strong fibronectin involvement in the mesenchymal cells beginning from the early stages of development (the thirteenth prenatal day). Between the fifteenth and twentieth prenatal days, strong immunoreactivity was found in the progenitors of smooth muscle cells and the developing vascular endothelium. During the stages in which the bronchioles begin to differentiate, strong involvement was found particularly in the apical cytoplasms and cell membranes of epithelial cells. During the alveolar stage (second to sixth postnatal days), it was noted that the involvement was in the apical and basal cell membranes of alveolar epithelium, and there was immunoreactivity in type II alveolar cells. In the later stages of development, diffuse fibronectin involvement was found in the connective tissue. As the vascular development was complete, no immunoreactivity was found in the smooth muscle cells. However, there was diffuse and strong fibronectin involvement in the endothelial cells in all stages of development.

In all studies conducted, the presence of fibronectin has been shown in all basal membranes in murine lungs. Besides, laminin has been shown to mediate the interactions between the epithelial and mesenchymal

cells in murine lungs.[15] It functions by binding integrins located on the cell surface, which is transmembrane proteins composed of α and β subunits.[16] Among these, the $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins are specific laminin receptors.[17] Several studies have been conducted in order to determine the locations of laminin-integrin complexes in fetal and mature pulmonary tissues.[13] To illustrate, Virtanen et al[18] has demonstrated the integrins that act as laminin receptors in the developing and mature lungs. They have identified the α_1, α_3, and β_3 subunits of laminin in every stage of development in the bronchi. While α_2 was found only in the epithelial buds, β_2 was observed in both the epithelial buds and basal membrane. β_1 is also found in the basal membrane of mature lungs, but is not identified in the early stages of development. In the canalicular stage, the α_2 and α_6 subgroups vanish and only the α_3 subgroup is found in the alveolar wall. The subgroup α_6 is identified in the capillaries.

In order to demonstrate the distribution of laminin in the developing lung, we have utilized antibodies for β_1 integrin, the specific laminin receptor. Unlike Virtanen et al,[18] we found increased involvement in the developing fetal lung tissue by the thirteenth prenatal day. In these early stages of development, the laminin involvement was not uniform, however, it varied from weak to strong in the mesenchymal cells, the bronchiolar epithelium and the endothelial cells of developing vessels. In this stage, there was rather weak involvement in the basal membrane. The involvement was found to have increased on the fifteenth prenatal day, being particularly strong in the mesenchyme where cell differentiation and migration are higher. By the seventeenth prenatal day, it was found that the β_1 integrin immunoreactivity was restricted to the bronchiolar basal membrane, and the involvement in the basal membrane had increased in parallel with the development. As the alveoli had formed, the β_1 integrin involvement in the alveolar epithelium had increased. The involvement in the epithelium was strong at the levels of the apical cell membrane and the basal membrane. At every stage of development, strong β_1 integrin involvement was observed in the vascular endothelial cells and their basal membranes.

As a result, fibronectin may be the important marker, which indicates the development of lung stromal and parenchymatic tissue and β_1 integrin, the receptor of laminin, may have a role in the complete development of mature lung structure and the figuring of basal laminae.

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