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Short Contribution

Expression and immunohistochemical localization of laminin and type IV collagen in developing human fetal tracheal glands

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ABSTRACT The expression and distribution of laminin and type IV collagen, two major components of the basement membrane, were investigated at the epithelio-mesenchymal interface of the human developing tracheal glands from 10 to 37 weeks of gestation. The localization of these molecules was assessed by indirect immunoperoxidase and indirect immunofluorescence staining and correlated to morphogenesis and epithelial cell differentiation. Laminin and type IV collagen were detected as early as 10 weeks of gestation in a continuous, linear pattern in the basement membrane surrounding the epithelial tracheal tube. By 12 weeks of gestation the basement membrane developed large openings at the tips of the budding glands beneath poorly differentiated cells, concomitant with the onset of morphogenetic movements. The remodeling of the basement membrane led to branching epithelial morphogenesis. The maturation and the functional differentiation of the secretory cells appeared later in the epithelium, when the basement membrane was strongly labeled with both anti-laminin and anti-type IV collagen antibodies, after 24 weeks of gestation. At this time the basement membrane became regular and thick and the maturation of serous cells increased progressively. These results suggest that the remodeling of the basement membrane takes place very early during gestation and that the morphogenesis and the maturation of the tracheal glands are rapidly achieved in humans.

KEY WORDS: *laminin, type IV collagen, immunohistochemistry, human fetus, tracheal glands*

Cell-matrix interactions are major events during development and morphogenesis. Laminin (LN) and type IV collagen are two of the key substances involved in such interactions as they are important components of the basement membranes (BM) (Leblond and Inoue, 1989). LN is a large multidomain glycoprotein arranged in a cruciform shape composed of three chains (A, B1, B2) (Von der Mark and Kuhl, 1985; Beck *et al.*, 1990; Mecham, 1991). The type IV collagen molecule is composed of a triple helix formed by three a chains (Timpl and Martin, 1982). LN, the first extracellular matrix protein to appear during development, was reported as early as the 2-cell stage (Wu *et al.*, 1983; Richoux *et al.*, 1989), while type IV collagen takes place later, in the blastocyst (Leivo *et al.*, 1980). This suggests their important role during morphogenesis through the BM.

The alterations of the BM during the branching morphogenesis and the epithelial maturation of the kidney (Ekblom *et al.*, 1986), the salivary gland (Spooner and Faubion, 1980; Bernfield and Banerjee, 1982; Bernfield *et al.*, 1984) and the mammary gland (Sakakura, 1983) have been described in different experimental models. There are no data directly related to the development of human tracheal glands.

A few studies have described the morphological development of human fetal tracheal glands (Thurlbeck *et al.*, 1961; Tos, 1966; De Haller, 1969). They take into account their number during gestation or their histological and functional differentiation (Jeffery *et al.*, 1992). Bucher and Reid (1961) described the first bronchial glands at 14 weeks of gestation as primitive foci of cellular multiplication inside the endodermal epithelium which grow and ramify into the surrounding mesenchyme, canalize and then divide dichotomously. This way of development implies cell-matrix interactions between the epithelium and the underlying connective tissue.

The present report describes both the morphological changes of the epithelium and the alterations of the BM *in vivo*, in developing human fetal tracheal glands. In order to follow the chronological events and to determine the pattern of growth of these glands in connection with extracellular modifications, we have focused our study on the alterations of the BM surrounding the tracheal glands at different stages of gestation. We have approached this question

Abbreviations used in this paper: LN, laminin; BM, basement membrane.

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by studying the deposition of LN and type IV collagen, two major components of the BM, using immunohistochemistry.

Four different stages were identified, according to the morphological changes of the tracheal glands during their growth and maturation.

Before 12 weeks of gestation

Before 12 weeks of gestation the trachea appeared as a single epithelial tube lined by undifferentiated columnar cells with multilayered nuclei and clear cytoplasm. At this stage, there was no glandular structure.

LN and type IV collagen were present in the BM surrounding the trachea. Both molecules appeared as thin and regular lines along the epithelial tube beneath the surrounding mesenchyme. The labeling for LN was weak in the BM and was not detected in the mesenchyme. In contrast, the labeling for type IV collagen was strong in the BM and slighter throughout the mesenchyme around the tracheal epithelium .

Between 12 and 16 weeks of gestation

Between 12 and 16 weeks of gestation the tracheal glands appeared. When the surface epithelium began to differentiate with a few ciliated and secretory cells, the first glandular buds could be detected. They appeared first as small round nests of undifferentiated cells budding from the tracheal epithelium (Fig. 1). They grew and bulged downward, into the mesenchyme to form tubular structures with a small central lumen. Then, these ducts divided at their free end. They were lined by regularly well ranged but poorly differentiated cells which exhibited scarce mucous secretions. At the free end of the growing glandular tubes the cells remained undifferentiated, disorganized and piled up, exhibiting many mitoses.

At this stage the BM was dramatically changed and had an irregular labeling. In the proximal portion of the growing glandular tubule, the BM remained regular with a thin and delicate labeling with anti-LN antibody. At the tips of the epithelial tubules, the labeling became weak with focal disappearance along the disorganized epithelial cells. With the anti-type IV collagen antibody, the BM was clearly labeled with a strengthened positivity at the sites of division of the glandular tubules. At the free end of the tracheal budding glands the labeling of the BM disappeared.

In the mesenchyme the BM surrounding the vessels was also clearly labeled with both anti- type IV collagen and anti-LN antibodies.

Between 16 and 24 weeks of gestation

Between 16 and 24 weeks of gestation the ramifications of the glands carried out their growth caudally and in the deepness of the tracheal wall. The maturation of the glandular epithelium became clear. In almost all the tubes, the epithelial cells were perfectly aligned along the BM and exhibited abundant mucous secretions. However, some of the glands remained lined by poorly differentiated and disorganized cells (Fig. 2).

The mature glands were surrounded by a regular BM strongly stained with both anti-LN and anti-type IV collagen antibodies. This labeling was clear in the distal area of the glands as well as in their proximal portion.

The less differentiated glands were surrounded by an irregular, vanishing labeling with anti-LN and anti-type IV collagen antibodies.

At this stage, the BM surrounding the vessels and the muscle fibers in the mesenchyme were strongly labeled, whereas the extracellular matrix was unstained.

From 24 to 37 weeks of gestation

From 24 to 37 weeks of gestation the tracheal glands appeared mature, with a tubulo-acinar architecture. The cells were well differentiated with abundant mucous secretions and the number of serous cells increased progressively during the third trimester.

In these mature glands, the cells were always aligned along a regular BM. There was no more budding at the free end of the epithelial tubes. The acini were outlined by a strong positivity with anti-LN and anti-type IV collagen antibodies without any disruption (Fig. 3).

The most active period of growth of the tracheal glands has been reported between 13 and 27 or 30 weeks of gestation (Thurlbeck *et al.*, 1961; De Haller, 1969). Thurlbeck observed that about 92 percent of glands are present at 30 weeks of gestation. In our experiment we find a rather earlier development and a shorter period of active multiplication of the tracheal glands, reflected by the disruptions of the BM between 12 and 24 weeks of gestation. During this period and later, the visualization of the remodeling of the BM dramatically decreases. The precocity of the maturation in human trachea, in contrast with experimental models, has already been demonstrated for the surface epithelium which is fully mature and functional as early as the 24th week of gestation, thus less than 2/3 of gestation (Gaillard *et al.*, 1989). Our present findings show that the growth and maturation of the tracheal glands also appear earlier in humans than in experimental models usually studied (Leigh *et al.*, 1985a,b, 1986) where the number of glands increases after birth.

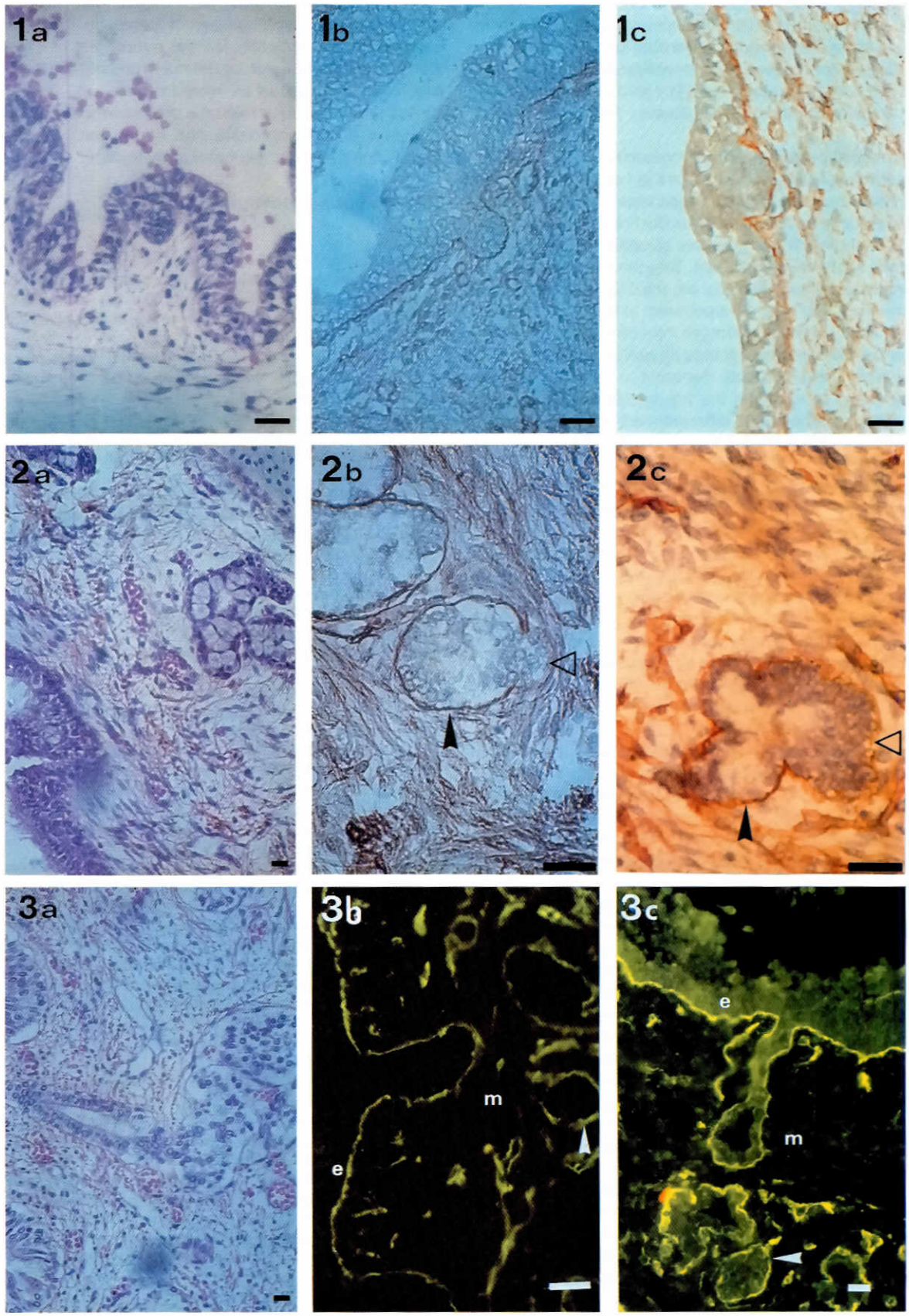
Budding and ramifying epithelial tubes into the underlying connective tissue imply modifications of the extracellular matrix. However, the molecular mechanisms of growth are poorly studied in the tracheal glands.

In the respiratory tract, the alterations of the BM have been studied during the maturation of the pulmonary alveolar ducts (Grant *et al.*, 1983; Riso, 1983; Jaskoll and Slavkin, 1984;

Fig. 1. Development of the first tracheal glandular bud. (a) *Tracheal glands at 12 weeks of gestation appear in the epithelium as small nests of undifferentiated cells. (Hematoxylin-Phloxine-Safran: HPS).* **(b)** *Immunolocalization of LN.* **(c)** *Immunolocalization of type IV collagen. At this stage a thin and regular BM is observed around the epithelial nests. There is no disruption in front of the budding areas.*

Fig. 2. Ramification and growth of the glands. (a) *Tracheal glands at 22 weeks of gestation are made of mucous cells, and some undifferentiated cells are observed at their free end. (HPS).* **(b)** *Immunohistochemical localization of LN.* **(c)** *Immunostaining with anti- type IV collagen antibody. The staining with both antibodies is intense and regular in the areas where the cells are mature. Conspicuous disruptions of the BM are observed in front of undifferentiated and piled up cells at the free end of the growing glands.*

Fig. 3. Maturation of the glands. (a) *Tracheal glands at 24 weeks of gestation are mature and contain mucous cells; serous cells begin to differentiate. (HPS).* **(b)** *Immunofluorescent localization of LN.* **(c)** *Immunofluorescent localization of type IV collagen. Both immunostainings show a continuous BM around the tracheal glands. The arrows (▲) indicate the areas where the BM is regular; the arrows (Δ) point to disruptions of the BM. e, surface epithelium; m, mesenchyme. Bars, 20 μm.*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Adamson and King, 1985; Takaro *et al.*, 1985). Most of these experimental results correlated the disappearance of the BM to the late maturation of the pneumocytes in the fetal lung. The alterations of the BM during earlier stages of lung development have only been described in chicken (Koch *et al.*, 1991) by immunohistochemistry and in the embryonic murine lung (Csato, 1989) by electron microscopy.

Our results also show very important remodeling of the BM during the morphogenesis of the tracheal glands in human fetuses. The disruptions, visualized through the disappearance of LN and type IV collagen, take place very early, as soon as the first epithelial tubules grow into mesenchyme. These gaps are conspicuous, as they are visualized at the light microscopy level. They are located at the tips of the growing tubes where the cells are piled up undifferentiated and exhibit mitoses. The poor differentiation of these cells may be related to their ability to divide and move into the mesenchyme. The disruptions of the BM in tracheal glands as well as in other ramified tissues allow early process of cell migration in order to form new epithelial ducts and to maintain the lobular morphology. On the other hand, the secreting well differentiated cells in the fetal tracheal glands are observed in areas where the BM is thick and regular.

The epithelio-mesenchymal interactions are thus implicated in early morphogenesis and not only in late maturation process and functional differentiation of the epithelium.

Different hypotheses may be proposed to explain the remodeling of the BM during the branching morphogenesis of the tracheal glands. The disappearance of macromolecules such as LN and type IV collagen may be correlated to their degradation by metalloproteinases. These enzymes have been reported to be expressed very early during embryonic development (Brenner *et al.*, 1989). Their tissular inhibitor (TIMP) is implicated in morphogenesis of ramified organs as it stimulates cleft formations of mouse salivary gland *in vitro* (Nakanishi *et al.*, 1989).

This enzymatic system may be also involved in degradation of BM during development of the tracheal glands. Besides, the plasmin system can degrade type IV collagen and activate collagenases (Barret, 1981; Mackay *et al.*, 1990). Enzymatic proteolysis seems to be a very important process during the fetal development.

However, numerous studies have demonstrated that the loss of BM implies disorganization, regression and abnormalities in the development of epithelial cells *in vitro* (Lwebuga-Mulkasa *et al.*, 1984; Shuger *et al.*, 1990). In our study, in spite of the disappearance of the BM components at the light microscopy level, budding and branching of the glandular ducts are not affected: the cell migration and the epithelial morphogenesis remain normal.

The restoration of the injured tracheal epithelium after bronchitis involves the reappearance of fetal morphological characteristics concerning ciliogenesis as well as secretory products (Reid, 1960; MacDowell *et al.*, 1987; Gaillard *et al.*, 1989). Important changes in the rate of synthesis of extracellular matrix components have been reported after a chemical injury of the lung (Turino, 1985) and the same pattern of response has been observed in different experimental models such as regenerating limbs (Toole and Gross, 1971) and salivary glands (Thompson and Spooner, 1983). The modifications of the extracellular matrix during reparation of an injury have also been studied in lung parenchyma (Riso, 1983). They are described as a cascade of events: disappearance of the BM, reparation of the injured epithelium and then, restoration of the BM.

Taken together, all these latter experiments suggest that knowledge of normal morphogenesis in human, including cellular maturation and differentiation, as the remodeling of the extracellular matrix components, may be helpful to understand the mechanisms implied in the repair of an injured tissue.

Our present results reinforce the concept of the BM as a major regulator of organogenesis and of the early development and maturation of the human respiratory tract.

Experimental Procedures

Material

Twenty normal non-infected fetuses ranging in gestational age between 10 and 37 weeks were studied. They were the result of spontaneous miscarriages, medical inductions or voluntarily interrupted pregnancies. This study was carried out in accordance with the Regional Ethics Committee on development and reproduction.

The gestational age was determined according to the menstrual age and to usual morphological criteria: weight, crown-rump length, foot-length, organ weight (Streeter, 1920; Trolle, 1948; Shepard *et al.*, 1988). The respiratory tract was dissected under a dissecting microscope and the trachea was transversally cut to collect the two rings under the thyroid gland. The tissues were then fixed in 10% formalin or immediately frozen.

Immunohistochemical methods

The immunohistochemical staining was carried out either by an indirect method using a peroxidase conjugate second antibody or by an indirect immunofluorescence technique.

Immunoperoxidase method

For this method the tracheal specimens were fixed in 10% formalin and embedded in paraffin. The 6 μm thick transverse sections were mounted on glass slides coated with gelatin. Deparaffinized and rehydrated sections were treated with 0.4% pepsin in 0.01N HCl for 90 min at 37°C. Hydrogen peroxide was used to remove endogenous peroxidase in tissue. Sections were reacted with an immunoperoxidase based method (Universal Immunoperoxidase Staining Kit CRL-Ortho Diagnostic system Inc.). The sections were incubated with normal sheep serum for 20 min and were rinsed in phosphate buffered saline (PBS) (pH 7.4). The primary antibody was a polyclonal rabbit immunoglobulin directed against human LN (Biolyon) used at a dilution 1:200 in PBS or a monoclonal mouse immunoglobulin directed against human type IV collagen (Dako) used at a dilution 1:100. The sections reacted overnight at 4°C in a humidified chamber. Thereafter the sections were rinsed with PBS and incubated with the secondary antibody used as a linking reagent. The peroxidase anti-peroxidase immunoenzyme complex was used to mark the antigen's localization. The localization of LN and type IV collagen was visualized by addition of a complex peroxidase anti-peroxidase revealed by a chromogenic substrate (3 amino-9 ethyl carbazole: AEC). The BM was labeled by a brownish-red precipitate. The slides were counterstained by hematein. Negative control slides were stained using the same procedure and either omitting the primary antibody or adding a non-immune serum.

Immunofluorescence method

Frozen sections of 4 μm thick were spread on glass slides and allowed to dry at room temperature for one hour. Thereafter they were fixed in acetone for 10 min. The staining procedure was the same as for immunoperoxidase but without hydrogen peroxide step. The primary antibody was a rabbit serum directed against mouse-LN (Institut Pasteur), used at a dilution of 1:40. It was incubated for 30 min at room temperature. Then the slides were washed in PBS and incubated for 30 min with a fluorescent rabbit antibody (Institut Pasteur) used at a dilution of 1:20 at room temperature. The sections were washed again in PBS and mounted in 90% glycerol. Specimens were observed and photographed on an Aristoplan (Leitz) immunofluorescence microscope.

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