Fibroblast growth factor-2 during postnatal development of the tracheal basement membrane zone

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Evans, Michael J., Michelle V. Fanucchi, Laura S. Van Winkle, Gregory L. Baker, April E. Murphy, Susan J. Nishio, Philip L. Sannes, and Charles G. Plopper. Fibroblast growth factor-2 during postnatal development of the tracheal basement membrane zone. Am J Physiol Lung Cell Mol Physiol 283:L1263–L1270, 2002. First published August 9, 2002; 10.1152/ajplung.00180.2002.—Thickening of the basement membrane zone (BMZ) is a characteristic of several airway diseases; however, very little is known about how this process occurs. The purpose of this study was to define development of the BMZ in the trachea of growing rhesus monkeys at 1, 2, 3, and 6 mo of age. We measured immunoreactivity of collagen types I, III, and V to detect structural changes in the developing BMZ. To detect more dynamic, functional components of the epithelial-mesenchymal trophic unit, we evaluated the distribution of perlecan, fibroblast growth factor-2 (FGF-2), and fibroblast growth factor receptor-1 (FGFR-1). One-month-old monkeys had a mean collagen BMZ width of 1.5 ± 0.7 μm that increased to 4.4 ± 0.4 μm in 6-mo-old monkeys. Perlecan was localized in the BMZ of the epithelium at all ages. FGF-2 was strongly expressed in basal cells at 1–3 mo. At 6 mo, FGF-2 was expressed throughout the BMZ and weakly in basal cells. FGFR-1 immunoreactivity was expressed by basal cells and cilia and weakly in the nuclei of columnar cells at all time points. These data indicate that development of the BMZ is a postnatal event in the rhesus monkey that involves FGF-2. basal cell; perlecan; fibroblast growth factor receptor-1; rhesus monkey

THE BASEMENT MEMBRANE ZONE (BMZ) of large airways occupies the central position in the epithelial-mesenchymal trophic unit (5, 6, 15). This anatomic and functional unit consists of opposing layers of epithelial and mesenchymal cells separated by the BMZ. The primary trophic unit consists of a layer of basal cells, the BMZ, and the attenuated fibroblast sheath. Recognition of the attenuated fibroblast sheath as a distinct layer of resident fibroblasts is key to the concept of an epithelial-mesenchymal trophic unit. The fixed position of the attenuated fibroblast sheath beneath the epithelium indicates that it responds in a local manner to bacterial products, tissue injury, or other environmental factors as they impinge on the epithelium and mediate the response of other tissues to these stimuli. The exchange of information between the epithelium and fibroblasts in the epithelial-mesenchymal trophic unit occurs via the BMZ.

The BMZ is specialized for attachment of epithelium with the extracellular matrix (ECM; see Refs. 1, 24, 33, and 35). With transmission electron microscopy, the BMZ appears as the following three component layers: the lamina lucida, the lamina densa, and the lamina reticularis (LR). Together they make up the basal lamina. The third component of the BMZ, the LR, is variable in its distribution, thickness, and composition. The LR is especially pronounced under the respiratory epithelium of large conducting airways, where it may be several micrometers thick. Immunohistochemical studies have shown that the collagen fibrils in the LR consist of types I, III, V, VI, and VII collagen. Collagen types I, III, and V form heterogeneous fibers that account for the thickness of the LR. Scanning electron microscopy revealed that the collagen fibers are arranged as a mat of large fibers oriented along the longitudinal axis of the airway. Smaller fibers are cross-linked with the larger fibers to complete this structure (10). Attenuated fibroblasts beneath the BMZ are thought to synthesize the collagen types I, III, and V component of the LR (5, 6, 15). Within and around the collagen fibrils are proteoglycans. BMZ proteoglycans store growth factors, hormones, and ions and are involved with cellular adhesion, electrical charge tissue hydration, and cell-cell communication. The predominant pulmonary BMZ proteoglycan is perlecan, and the predominant stored growth factor is basic fibroblast growth factor (FGF-2; see Refs. 31 and 32). FGF-2 is a ubiquitous multifunctional growth factor that influences development and is a regulator of growth and differentiation in adult tissues (2, 14, 34). FGF-2 is stored in the...
BMZ by binding to perlecan (11). Perlecan is a heparan sulfate proteoglycan that is an intrinsic constituent of the BMZ (17). FGF-2 can be released from perlecan in response to various conditions such as degradation of the BMZ by glycosaminoglycan-degrading enzymes or proteases (17). Thus perlecan functions as a regulator of FGF-2 transport, allowing for rapid responses to local environmental conditions (4). When released from the BMZ, FGF-2 is an important signaling molecule in the epithelial-mesenchymal trophic unit by binding to its primary receptor. In the lung, FGF-2 is stored in the BMZ of airway epithelium, alveolar epithelium, and endothelium of developing and adult rats (31, 32). In this central position, the BMZ and its components are well situated to regulate epithelial-mesenchymal trophic unit responses both during lung development and in response to injury.

The LR is the region of the BMZ in large airways that accumulates collagen and leads to BMZ thickening associated with asthma and other airway diseases (3, 30). Very little is known about the formation of the LR. The purpose of this research was to study postnatal development of the LR in the trachea of growing rhesus monkeys at 1, 2, 3, and 6 mo of age. To detect structural changes in the developing BMZ, we measured collagen types I, III, and V immunoreactivity. To detect indicators of functional change in the epithelial-mesenchymal trophic unit, we evaluated the distribution of perlecan, FGF-2, and FGFR-1 immunoreactivity.

Methods

Experimental animals. All monkeys selected for these studies were California Regional Primate Research Center colony-born rhesus macaques (Macaca mulatta). Care and housing of animals complied with the provisions of the Institute of Laboratory Animal Resources and conforms to practices established by the American Association for Accreditation of Laboratory Animal Care.

Preparation of animals. Groups of three male monkeys 1, 2, 3, and 6 mo of age were killed with an overdose of pentobarbital sodium after being sedated with Telazol (8 mg/kg IM) and anesthetized with Diprivan (0.1–0.2 mg·kg⁻¹·min⁻¹ IV). The attending veterinarian adjusted the dose as necessary. The monkeys were then necropsied after exsanguination through the abdominal aorta.

Immunohistochemistry. The trachea was sliced perpendicular to the long axis of the airway. Tracheal slices were fixed in 10% paraformaldehyde for 1 h and embedded in paraffin. For routine histology, 5 μm were stained with hematoxylin and eosin (H&E). For immunohistochemistry, 5-μm sections were deparaffinized in xylene, hydrated in ethanol, and washed in PBS. Four collagen sections were treated with pepsin (1.0 mg pepsin/ml 3.0% acetic acid) at 37°C for 2 h, blocked with BSA, and treated with antibody to either collagen I (250), collagen V (1500); rabbit, anti-human polyclonal antibody; Biogenex, Kingston, NH), or collagen III (1:10,000; mouse, anti-human monoclonal antibody; BioGenex, San Ramon, CA) overnight at 4°C. For perlecan, the sections were treated with 0.1% pronase in PBS for 30 min, rinsed in nanopure water followed by PBS, blocked with BSA for 30 min, and incubated with an antibody to perlecan (1:2,000; mouse, anti-human monoclonal antibody, clone 7B5; Zymed, San Francisco, CA) overnight at 4°C. For FGF-2, the sections were treated with 0.1% H₂O₂ in methanol for 60 min followed by 50 mg/ml bovine testicular hyaluronidase in 0.05 M Tris buffer (pH = 7.6) for 30 min. The sections are blocked with 5.0% horse serum for 30 min and incubated with an antibody to FGF-2 (1:750; mouse, anti-human monoclonal antibody, clone bFM-2; Upstate Biotechnology, Lake Placid, NY) overnight at 4°C. For FGFR-1 (FGFR-1), the sections were treated with 0.02% trypsin in PBS at room temperature for 30 min, washed, blocked with 25 μg purified goat IgG in PBS for 60 min, and incubated with an antibody to FGFR-1 (1500 rabbit, anti-chicken polyclonal antibody; Upstate Biotechnology) overnight at 4°C. After immunohistochemistry, the sections were washed in PBS, treated with the secondary antibody (1:100; Alexa Fluor 568; Molecular Probes, Eugene, OR) for 30 min, washed in PBS, and covered with a coverslip in enzyme-linked fluorescence-safe media (Molecular Probes). Fluorescence was visualized on an Olympus BH-2 fluorescence microscope.

Antibody specificity. The antibodies for collagen types I and V and FGFR-1 had negligible cross-reactivity with other collagens or noncollagen matrix proteins (per supplier). The antibody for collagen type III has no cross-reactivity with other collagens (13). The antibody for FGF-2 has no cross-reactivity with FGF-1 or heparin-binding growth factor (23). The antibody for perlecan may cross-react with the short arm of laminin A and B chains (25).

Quantitation. Collagen types I, III, and V form heterogeneous fibers that account for the width of the BMZ. The collagen width of the BMZ was measured morphometrically to quantitate the immunohistochemical results. Because of the normal variability in airway BMZ width, the average width was determined from 32 measurements/animal. To determine the width of the BMZ, eight micrographs were taken in sequence around the circumference of the trachea from each animal for each collagen type (I, III, and V) studied. The width of the BMZ was measured at four equidistant points, determined by random placement of a grid on each micrograph, and the mean was determined for each collagen. Measurements of tracheal diameters were made from the epithelial surface of the long and short diameters of the tracheal rings stained with H&E, and the average diameter was determined. The height of the epithelium was measured on these same sections. The data are presented as means ± SD.

Table 1. Changes in weight, tracheal diameter, and columnar height during postnatal development of the rhesus monkey

<table>
<thead>
<tr>
<th>Age, mo</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>6</th>
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<tbody>
<tr>
<td>Weight, kg</td>
<td>0.56 ± 0.01</td>
<td>0.97 ± 0.08*</td>
<td>1.00 ± 0.21*</td>
<td>1.48 ± 0.12</td>
</tr>
<tr>
<td>Tracheal diameter, mm</td>
<td>2.7 ± 0.3</td>
<td>2.8 ± 0.2</td>
<td>2.9 ± 0.1</td>
<td>3.6 ± 0.4†</td>
</tr>
<tr>
<td>Columnar cell height, μm</td>
<td>44.0 ± 4.2</td>
<td>48.4 ± 1.5</td>
<td>45.0 ± 2.7</td>
<td>49.3 ± 5.2</td>
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</tbody>
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Values are means ± SD. *P < 0.05 when compared with 1-mo-old animals. †P < 0.05 when compared with the 3-mo-old animals.

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SD. The differences between groups were compared with ANOVA using Fisher’s least protected significant difference, with significance set at $P < 0.05$.

RESULTS

Morphology. The mean weight of the monkeys, tracheal diameter, and columnar epithelial cell height are given in Table 1. At 1 mo, the mean weight of the monkeys was 0.56 ± 0.01 kg. By 6 mo, it had increased to 1.48 ± 0.12 kg. At 1 mo, the mean diameter of the trachea was 2.7 ± 0.3 mm and was unchanged at 2 and 3 mo. By 6 mo, the diameter had increased significantly to 3.6 ± 0.4 mm. The mean height of the columnar epithelium at 1 mo was 44.0 ± 4.2 μm and was not significantly different at 2, 3, and 6 mo. In each age group, the epithelium was morphologically similar. The columnar epithelium comprised mainly ciliated and goblet cells. The basal cells formed a layer covering most of the BMZ. Although the epithelium was morphologically developed at 1–6 mo, the BMZ developed postnatally. At 1 mo, there was very little evidence of the BMZ in H&E-stained sections (Fig. 1A). However, the BMZ was visible at 2 and 3 mo (Fig. 1B) and had developed into a distinctive and wide structure by 6 mo (Fig. 1C).

The attenuated fibroblast sheath surrounds the BMZ. It is thought to be associated with synthesis of the BMZ (5, 6, 15). At 1 mo, the attenuated fibroblast sheath was not present under the BMZ. However, by 6 mo, a layer of attenuated fibroblasts lined the mesenchymal surface of the BMZ. The attenuated fibroblast sheath was also present at 3 and 6 mo (Fig. 1, A–C).

Immunohistochemistry of collagen and perlecan. At 1 mo, collagen types I, III, and V immunoreactivity in the region of the BMZ was present as a thin discontinuous line (Fig. 2A). A high intensity for collagen type V immunoreactivity was present in the walls of blood vessels. Immunoreactivity for perlecan was present as a distinct labeling pattern within the BMZ. Perlecan immunoreactivity was also present in the walls of blood vessels (Fig. 2B). These observations indicate that the BMZ was beginning to form at 1 mo, but it could not be defined as a discrete structure in H&E-stained sections. At 2 and 3 mo, collagen types I, III, and V immunoreactivity in the BMZ was distinct with many areas of high intensity compared with the ECM (Fig. 2C). Immunoreactivity for perlecan was present as a distinct labeling pattern within the BMZ similar to that at 1 mo. Perlecan immunoreactivity was also present in the walls of blood vessels (Fig. 2D). Collagen types I, III, and V immunoreactivity was similar at 6 mo (Fig. 2E). There was a distinct pattern of immunoreactivity with focal areas of high-intensity label compared with label in the ECM. The collagen type III mesenchymal surface was irregular and continuous with the ECM in many areas. Also, the intensity of collagen type V immunoreactivity at 6 mo was now less than that in the walls of blood vessels. At all time points, the width of the BMZ was variable. Immunoreactivity for perlecan in the BMZ was less than that in the walls of blood vessels (Fig. 2F).

**Fig. 1.** Hematoxylin and eosin-stained sections of tracheal rings from 1- to 6-mo-old monkeys. Bar = 20 μm. A: at 1 mo the basement membrane zone (BMZ) is not apparent (arrowheads), and the layer of attenuated fibroblasts beneath the epithelium is not present. B: at 2 mo the BMZ is present (arrowheads), and the attenuated fibroblast sheath beneath the epithelium is organized (arrows). C: at 6 mo the tissue has the characteristics of the adult. Arrowheads, BMZ; arrow, attenuated fibroblast sheath.
Collagens types I, III, and V form heterogeneous fibers that account for the width of the BMZ. The width of the BMZ measured for these collagen types was similar at each time point (Table 2). ANOVA indicated there was not a statistical difference between measurements of these three collagens. We determined the average collagen BMZ by determining the mean of types I, III, and V collagen at each time point. The mean BMZ width increased significantly from 1.5 ± 0.7 μm at 1 mo to 3.4 ± 0.9 μm at 2 mo and 3.5 ± 0.2 μm at 3 mo. At 6 mo, the mean collagen width again increased significantly to 4.4 ± 0.4 μm.

**Immunohistochemistry of FGF-2 and FGFR-1.** At 1 mo, FGF-2 immunoreactivity was not evident in the BMZ. However, it was strongly expressed in basal cells and the lateral intercellular space (LIS; Fig. 3A). Immunoreactivity for the FGFR-1 was present on the surface and in the cytoplasm of basal cells. It was also associated with the surface of ciliated cells and the nuclei of subepithelial mesenchymal cells. There was
weak immunoreactivity for FGFR-1 in the nuclei of both ciliated and goblet cells (Fig. 3B). At 2 and 3 mo, FGF-2 immunoreactivity was expressed strongly in basal cells and the LIS but not the BMZ (Fig. 3C). FGFR-1 immunoreactivity was expressed in the nuclei of some basal cells and the cell surface and cytoplasm. In ciliated and goblet cells, it was again present in the nuclei and now also the cilia (Fig. 3D). At 6 mo, the opposite distribution of FGF-2 immunoreactivity was observed. Strong immunoreactivity was present within the BMZ, and weak immunoreactivity was present in basal cells and the LIS (Fig. 3E). FGFR-1 immunoreactivity was expressed on the surface and cytoplasm of basal cells. It was also expressed in ciliated and goblet cell nuclei, cilia, and in some basal cell nuclei (Fig. 3F). Weak immunoreactivity was present in attenuated fibroblasts at 2, 3, and 6 mo but not at 1 mo. Variable FGF-2 immunoreactivity was present in the walls of blood vessels at each time point studied.

DISCUSSION

These findings demonstrate that the tracheal epithelial cell populations are established at birth in the rhesus monkey (27); however, development of the tracheal LR occurs postnatally. Structural development of the LR requires synthesis of a collagen framework and the incorporation of proteoglycans in this framework. Collagen types I, III, and V form heterogenous fibers that make up the structural framework that accounts for the width of the BMZ. Collagen types I and III make up most of the fiber volume, and collagen type V makes up only a small amount. Collagen type V determines the diameter of collagen fibrils (20, 22). High levels of collagen type V are associated with thin fibrils and low concentrations with large fibrils. With the use of immunohistochemistry, collagen types I, III, and V were visible at 1 mo as a thin layer. This layer doubled in width at 2 and 3 mo and increased more at 6 mo. Immunoreactivity was intense for collagen types I, III, and V at 1–3 mo; however, the intensity for collagen V was lower at 6 mo. Initial fibril formation is associated with high levels of collagen type V. As the fibril continues to grow, the collagen type V levels in the fibrils decrease. These findings suggest that between 1 and 2 mo may be a critical time in the formation of the collagen framework of the BMZ. They also demonstrate that the collagen framework of the LR is present and beginning to form at 1 mo of age. By 2 mo, it has formed and continues to develop throughout the 6 mo studied. At 6 mo, the BMZ width of 4.4 ± 0.4 µm is similar to the width measured in adult rhesus monkeys (4.2–6.3 µm; see Ref. 8).

Structurally and functionally, development of the BMZ requires incorporation of proteoglycans into the collagen framework. Proteoglycans are an intrinsic component of the BMZ that is associated with several of its functions, such as storage of growth factors, hormones, ions, cellular adhesion, electrical charge, and cell-cell communication. The predominant heparan sulfate proteoglycan in the LR is perlecan. In this study perlecan was colocalized with the collagen fibers at each time point studied, suggesting that the structural and functional aspects of the BMZ develop together. A major function of perlecan is storage of FGF-2 to be used for rapid cellular/tissue responses to changes in local conditions (2, 17, 20, 26, 33, 34). FGF-2 can be released from perlecan in response to these conditions and become an important cytokine within the microenvironment of the epithelial-mesenchymal trophic unit (2, 4). Thus perlecan functions, in part, as a regulator of FGF-2 function. In the lungs of developing and adult rats, FGF-2 is stored in the BMZ of airway and alveolar epithelium, endothelium, and smooth muscle cells (28, 31). Although perlecan was present at each time point of this study, FGF-2 was only stored in the LR at 6 mo. This is in contrast to the rat where FGF-2 is found stored in the BMZ during postnatal development of the airway (28, 31). In the present study, FGF-2 immunoreactivity was associated with basal cells and the LIS and to a lesser degree attenuated fibroblasts at 1, 2, and 3 mo of age. Presumably, the basal cell-associated FGF-2 was being synthesized or used in an autocrine or paracrine manner by the basal cells.

A major receptor for FGF-2 is FGFR-1. The presence of FGFR-1 in the airway during postnatal development is in agreement with the results of Powell et al. (28). In quiescent airways of adult humans, only the basal cells express FGFR-1 (16). In the present study basal cells expressed most of the immunoreactivity, indicating they are the primary target cell for FGF-2 in the epithelium. We also describe FGFR-1 immunoreactivity in the nuclei of basal and columnar cells and some attenuated fibroblast. Translocation of FGFR-1 to the nuclei has been observed by others and is associated with activated cells (1). Maher (21) showed in Swiss 3T3 fibroblasts treated with FGF-2 that there is a time- and dose-dependent increase in the association of FGFR-1 with the nucleus. Later Reilly and Maher (29) showed that translocation of FGFR-1 to the nucleus is
dependent on importin-β, a component of multiple nuclear import pathways. They conclude that this is a novel signal transduction pathway for transmembrane growth factor receptors from the cell surface to the nucleus.

There appears to be a significant relationship between basal cells and FGF-2. Other investigators have also reported that basal cells in the skin (12, 16) and prostate (36) express FGF-2 protein. Basal cells occupy a central position in the epithelial-mesenchymal trophic unit, existing as a separate layer of cells covering most of the basement membrane. In this central position, they interact with columnar epithelium, neurons, the basement membrane, and underlying mesenchymal cells. In addition, they interact with trafficking immune cells, neutrophils, and eosinophils. These interactions take place in the LIS between basal and columnar cells. Basal cells express various molecules associated with these processes, such as neutral endopeptidase, CD44 adhesion molecule, intercellular ad-
expression of FGF-2 immunoreactivity in basal the BMZ increased 4.2 times between 1 and 6 mo of animal. In this study, the total cross-sectional area of the tracheal BMZ in infant rhesus monkeys (Abstract). Am J Respir Crit Care Med 161: A276, 2000.


