Increased lung expansion alters the proportions of type I and type II alveolar epithelial cells in fetal sheep

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Flecknoe, S., R. Harding, G. Maritz, and S. B. Hooper. Increased lung expansion alters the proportions of type I and type II alveolar epithelial cells in fetal sheep. Am J Physiol Lung Cell Mol Physiol 278: L1180–L1185, 2000.—Type I and type II alveolar epithelial cells (AECs) are derived from the same progenitor cell, but little is known about the factors that regulate their differentiation into separate phenotypes. An alteration in lung expansion alters the proportion type II AECs in the fetal lung, indicating that this may be a regulatory factor. Our aim was to quantify the changes in the proportion of type I and type II AECs caused by increased fetal lung expansion and to provide evidence for transdifferentiation of type II into type I cells. Lung tissue samples were collected from ovine fetuses exposed to increased lung expansion induced by 2, 4, or 10 days of tracheal obstruction (TO). The identities and proportions of AEC types were determined with electron microscopy. The proportion of type II cells was reduced from 28.5 ± 2.2% in control fetuses to 9.4 ± 2.3% at 2 days of TO and then to 1.9 ± 0.8% at 10 days. The proportion of type I AECs was not altered at 2 days of TO (63.1 ± 2.3%) compared with that of control cells (64.8 ± 0.5%) but was markedly elevated (to 89.4 ± 0.9%) at 10 days of TO. The proportion of an intermediate AEC type, which displayed characteristics of both type I and type II cells, increased from 5.7 ± 1.3% in control fetuses to 23.8 ± 5.1% by 2 days of TO and was similar to control values at 10 days of TO (7.7 ± 0.9%). Our data show that increases in fetal lung expansion cause time-dependent changes in the proportion of AEC types, including a transient increase in an intermediate cell type. These data provide the first evidence to support the hypothesis that increases in fetal lung expansion induce differentiation of type II into type I AECs via an intermediate cell type.

THE ALVEOLAR EPITHELIUM is composed of two cell types that are morphologically and functionally distinct. Type I alveolar epithelial cells (AECs) are large elongated cells that make up ~95% of the surface area of the lung and, therefore, comprise the vast majority of the epithelial component of the air-blood barrier (19). Type II cells are cuboidal in shape with rounded nuclei and have numerous cytoplasmic organelles, including lamellar bodies, which are the cytoplasmic storage sites for pulmonary surfactant (13). Because both AEC types play a crucial role in the respiratory function of the lung, it is important that the appropriate number of both AEC types exist within the gas-exchange regions of the lung. However, little is known about the factors that control proliferation and differentiation of these cells in vivo. This study is directed toward understanding the mechanisms that regulate AEC differentiation in vivo.

The type II AEC is considered to be the progenitor cell type that gives rise to both type I cells by differentiation and type II cells by division (22). Although numerous factors have been implicated in regulating the differentiation of type II into type I AECs, the precise mechanisms are largely unknown, particularly in vivo. Cell shape markedly influences both the morphology and phenotypic expression of AECs grown in culture (22), whereas cell shape is largely determined by the exertion of intracellular tensile forces onto a substrate through focal adhesion sites (21). This has led to the concept that physical forces, particularly those that alter cell shape, may regulate AEC differentiation. This concept is supported by in vivo studies showing that alterations in the degree of fetal lung expansion alter the density of type II AECs and greatly influence the mRNA levels for at least three of the surfactant proteins (SPs) (12).

During development, the fetal lungs are expanded by a liquid that is secreted across the pulmonary epithelium into the lung lumen (10) and leaves the lungs via the trachea (7). The degree to which the fetal lungs are expanded by this liquid is critical for normal lung growth and has been shown to influence the density of epithelial cell types in both rabbits (5) and sheep (3); epithelial cell differentiation is essentially complete by the end of gestation in these species. Prolonged increases in fetal lung expansion, which can be induced by obstructing the fetal trachea, increase the density of type II cells (3, 17), reduce the number of SP-B (6)- and SP-C (17)-positive cells, and markedly reduce the mRNA levels encoding SP-A, SP-B, and SP-C (12). On the other hand, prolonged periods of fetal lung deflation, caused by draining the lung liquid, increase the density of type I AECs (3) and increase mRNA levels for SP-C (12). However, the fate of the type II AECs that are lost by prolonged increases in fetal lung expansion and the effect of this treatment on the type I AECs are unknown.

During our initial observations of fetal lung tissue exposed to periods of increased lung expansion, we observed numerous AECs that displayed characteristics of both type I and type II AECs. This “intermediate”

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were identified and counted. The AECs were categorized into one of four groups (stem cells, type I AECs, type II AECs, and intermediate AECs) based on their morphological appearance following strict criteria. To categorize a cell as an AEC, the basement membrane had to be visible and the cell had to lie on the luminal surface of the basement membrane. The following criteria were used to categorize AECs. Alveolar epithelial stem cells were cuboidal in shape and contained abundant cytoplasmic glycogen deposits. Type I AECs had long flattened cytoplasmic extensions that could extend over several adjacent alveoli, flattened and elongated nuclei, and very little perinuclear cytoplasm and few cytoplasmic organelles (Fig. 1B). Type II cells were rounded with a rounded nucleus and had apical microvilli and abundant cytoplasmic organelles including lamellar bodies (Fig. 1A). The intermediate cells were a heterogeneous group that displayed characteristics of both type I and type II AECs (Fig. 1C). Their classification depended on the presence of a flattened elongated nucleus with marked cytoplasmic extensions, but they must also have contained lamellar bodies and usually also had apical surface microvilli.

Statistical analysis. All data are presented as means ± SE. All data were tested for normality, and differences in the proportion of each cell type were analyzed between treatment groups with a one-way ANOVA; differences between individual groups were determined by the least squares difference test. The accepted level of significance for all statistical analysis was \( P < 0.05 \).

RESULTS

In control fetuses at 128 days of gestation, most (64.8 ± 0.5%) of the AECs counted were classified as type I cells. Type II AECs represented 28.5 ± 2.2% of the total number of AECs counted, whereas the remainder (5.7 ± 1.3%) were of the intermediate cell type. At this stage of gestation, only a few undifferentiated stem cells (~1%) were observed.

Type II AECs. The proportion of type II AECs was markedly reduced from 28.5 ± 2.2% in control fetuses to 9.4 ± 2.4% by 2 days of tracheal obstruction. Although the proportion of type II AECs was not reduced further at 4 days of tracheal obstruction (6.1 ± 1.6%), it was markedly reduced to 1.9 ± 0.3% at 10 days of tracheal obstruction (Fig. 2).

Type I AECs. The proportion of type I AECs, expressed as a percentage of the total number of AECs counted, was similar after 2 days of tracheal obstruction (63.1 ± 2.3%) to that in control fetuses (64.8 ± 0.5%). At 4 days of tracheal obstruction, the proportion of type I AECs was elevated to 75.2 ± 4.1%, whereas at 10 days of tracheal obstruction, the proportion of type I AECs was significantly increased further to 89.4 ± 0.9% (Fig. 2).

Intermediate AECs. The proportion of intermediate cell types observed in control fetuses was 5.7 ± 1.3% of all AECs and was markedly increased to 23.8 ± 2.3% by 2 days of tracheal obstruction. By day 4 of tracheal obstruction, the proportion of intermediate cells had decreased to 14.2 ± 2.0% and had returned to control values by 10 days of tracheal obstruction (Fig. 3). The morphological appearance of the intermediate cells varied widely in that many displayed type I and type II cell characteristics to differing degrees. In particular,
Fig. 1. Electron micrographs of a type II alveolar epithelial cell (AEC; A), nuclear region of a type I AEC (B), and nuclear region of an intermediate AEC (C). Type II AECs are rounded in shape, with rounded nuclei, and contain lamellar bodies and apical surface microvilli (A). Type I cells have flattened nuclei, little perinuclear cytoplasm, and long cytoplasmic extensions (B, arrows) that extend beyond edges of photograph. Cells identified as intermediate AECs displayed characteristics of both type I and type II cells. They had flattened nuclei and long cytoplasmic extensions (C, arrows) as well as lamellar bodies and apical surface microvilli. Bars, 1 μm.
the cytoplasmic location of the lamellar bodies as well as the amount of perinuclear cytoplasm varied widely. In most intermediate cells, the lamellar bodies primarily resided within the cytoplasmic region between the nucleus and alveolar space (Fig. 1C). However, in cells with a reduced amount of perinuclear cytoplasm, they were more commonly found adjacent to the nucleus.

**DISCUSSION**

Our results demonstrate that increased fetal lung expansion induced by tracheal obstruction causes a rapid (within 2 days) and sustained reduction in the proportion of type II AECs within the fetal lung. After 10 days of increased lung expansion, type II cells represented only ~2% of the total AEC population compared with ~30% in control fetuses. On the other hand, the proportion of type I AECs within the fetal lung was not altered until 4 days of tracheal obstruction, whereas at 10 days of tracheal obstruction, the proportion of type I AECs was markedly increased; type I AECs represented ~90% of all AECs at this time. The reduction in the proportion of type II cells without a corresponding increase in type I cell number at 2 days of tracheal obstruction may be explained by the transient increase in the proportion of the intermediate cell type at this time. We suggest that the time course for the changes in the proportions of all three AEC types provides strong evidence to support the hypothesis that increases in fetal lung expansion induce type II cells to differentiate into type I cells via an intermediate cell type. This cell type clearly has morphological characteristics of both type I and type II cells.

Previous studies have indicated that prolonged periods of increased fetal lung expansion induced by tracheal obstruction are “deleterious” for type II cells, resulting in a reduction in type II cell density (3, 17, 18) as well as a reduction in the number of SP-A (6)- and SP-C (17, 18)-positive cells. Our study has provided the first information on the time course for the changes in type II cell number, the possible fate of the type II cells that are lost, and the effect of this treatment on type I cell number. Furthermore, in view of our finding of a population of morphologically distinct cells that were of an intermediate AEC type (i.e., clearly had both type II and type I cell characteristics), we considered that the effect of increased lung expansion on type II cell number needed to be reexamined. In particular, it is unclear as to how these intermediate cells would have been classified in previous studies. Presumably, some would have been classified morphologically as type II cells due to the presence of lamellar bodies. However, based on the fact that SP mRNA levels are greatly reduced within 2 days of obstructing the fetal trachea (12), it is unlikely that these intermediate cells would be positively labeled for SP-C mRNA with in situ hybridization techniques.

Our observation of an intermediate AEC type that possesses characteristics of both type I and type II cells is not unique to this study. Studies (1, 2) in adult mice have provided compelling evidence that type II cells are the progenitor cells that give rise to both cell types via division and differentiation after damage to the epithelium. In addition to the evidence provided by [3H]thymidine labeling in the presence and absence of mitotic arrest, AEC types that possessed both type II and type I cell characteristics were also observed (2). In view of their other findings, these authors (2) suggested that these cells represented cells in the process of differentiating from type II into type I AECs. Similarly, cells possessing characteristics of both type I and type II cells have been observed in the developing lung of fetal cats (14). In describing the initial differentiation of type I cells, it was concluded that the only reliable criterion for identifying differentiating type I cells is the commencement of attenuated cytoplasmic extensions distal to the nucleus (14). In our study, all cells that were

**Fig. 2.** Percent change in type I and type II AECs in control fetuses (0 days) and in fetuses exposed to 2, 4, and 10 days of tracheal obstruction. All AECs were identified morphologically by electron microscopy. Values are means ± SE. Values indicated by a different letter are significantly different from each other.

**Fig. 3.** Percent change in number of AECs identified as having an intermediate morphology in control fetuses (0 days) and in fetuses exposed to 2, 4, and 10 days of tracheal obstruction. Intermediate AECs had morphological characteristics, as observed by electron microscopy, of both type I and type II cells. Values are means ± SE. Values indicated by a different letter are significantly different from each other.
classified as intermediate AECs had pronounced extension and attenuation of the distal cytoplasm as well as gap junctions with neighboring cells that were adjacent to the basement membrane (see Fig. 1C). They also possessed lamellar bodies and usually had microvilli on their apical surface, which are characteristics usually associated with type II cells.

We consider it likely that the intermediate cells are not only morphologically distinct but are also functionally distinct from type II cells. Tracheal obstruction causes a rapid and marked reduction in SP-A, SP-B, and SP-C mRNA levels whereby mRNA levels are reduced to 10–15% of control values within 2 days (12). Thus the time course for the changes in mRNA levels closely correlates with the time course for the changes in type II cell number based on our morphological criteria. If, however, the intermediate cells were classified as type II cells simply because they contained lamellar bodies, the time course for the changes in type II cell number would lag behind the changes in SP mRNA levels by at least 4 days (12). Thus it is highly likely that the AECs we classified as intermediate cells are functionally distinct from the true type II cell phenotype. Indeed, we suggest that the large reduction in SP-A, SP-B, and SP-C mRNA levels induced by 2 days of tracheal obstruction (12) is due to the rapid transformation of more than half of the type II cell population into the intermediate cell type, which does not express these genes. Thus in view of the time lag between the reduction in mRNA levels for SP-A and the protein levels (12), it is possible that some of the intermediate cells would not be positive for SP-A, SP-B, and SP-C mRNAs but may be positive for the mature proteins measured with immunocytochemical techniques.

At present, the factors that control differentiation of type I and type II AECs into their separate phenotypes are not well understood (22). The type II cell is considered to be the progenitor cell type that gives rise to both type I and type II cells (2). After an injury to the alveolar epithelium in adults, replacement of type I cells is thought to result from division of a type II cell followed by differentiation of one of the daughter cells into a type I cell (22). However, there is also evidence to indicate that type II cells can directly differentiate into type I cells without having to divide (22). If the intermediate cells we observed in this study represent cells in the process of differentiating into type I cells, it is unclear as to whether or not these cells had divided first or were in the process of dividing before entering the pathway leading to type I cell differentiation.

The process leading to differentiation into the type I and type II cell phenotypes must involve activation and repression of specific genes. A number of factors have been implicated in the regulation of AEC phenotypes, including growth factors and cell-cell and cell-matrix interactions as well as changes in cell shape, at least in vitro. With regard to the role of cell shape, the flattening and elongation of AECs in culture is associated with the loss of type II cell characteristics and the development of some type I cell characteristics (20). This change in cell shape is primarily due to the exertion of intracellular generated tensile forces onto the extracellular matrix, causing the cells to flatten (21). If, however, the matrix is unattached, these cellular generated forces cause the edges of the matrix to curl, allowing the cells to retain their cuboidal shape and type II cell characteristics (20). Combined with our findings that increased lung expansion appears to stimulate differentiation of type II cells into type I cells, these data provide strong evidence that the mechanical load experienced by an AEC may play an important role in determining its phenotype.

It is possible that the reduction in type II cell number after an increase in lung expansion is not due to differentiation into type I cells but to apoptosis. This is unlikely because increased fetal lung expansion is a potent stimulus for type II cell division (16), not programmed cell death. Although it was expected, it is interesting that type I AECs did not divide in response to an increase in fetal lung expansion (16), which is consistent with previous findings that these cells do not divide (1). Similarly, the proportional increases in lung tissue and luminal volumes (16) that are associated with the lung growth induced by tracheal obstruction could not account for the changes in the proportions of AEC types we measured. Indeed, measurement of AEC proportions, by counting a fixed number of epithelial cells, is not influenced by changes in lung structure that might alter the numerical density of the different epithelial cell types. Thus the only plausible explanation for the changes in AEC number that we observed is the result of increased differentiation from the type II into the type I cell phenotype.

Tracheal obstruction is a potent and rapid stimulus for fetal lung growth (9, 15), and, therefore, it has been suggested that this procedure may be used therapeutically to reverse lung growth deficits in human fetuses with lung hypoplasia. However, the finding that tracheal obstruction rapidly decreases the number of type II cells and mRNA levels for SP-A, SP-B, and SP-C (12) indicates that this procedure may impair lung function postnatally. However, hypoplastic fetal lungs are considered to have a much greater proportion of type II AECs than control lungs (3), which is probably because lung hypoplasia primarily results from underexpansion of the fetal lung over prolonged periods of time (10). Thus in the absence of a mechanical distending influence, the majority of epithelial cells may differentiate into type I cells. It is conceivable that obstructing the trachea in fetuses with severe lung hypoplasia may not only stimulate lung growth but may also restore the normal balance in AEC types. Although the number of type II cells is often used as an index of lung maturity, it is important to note that, in normal fetuses, the number of type II cells gradually decreases in late gestation, whereas the number of type I cells increases (4). It is interesting that lung luminal volume in relation to body weight increases markedly over this same time period (10) and may be involved in this changing pattern of AEC numbers.
Our study demonstrates that increases in fetal lung expansion cause a rapid and large reduction in the proportion of type II AECs within the fetal lung that closely coincides with a transient increase in the proportion of an intermediate cell type. This cell type, which displays characteristics of both type II and type I cells, has previously been described as a cell in the process of differentiating into a type I cell. This concept is supported by our findings that the increased proportion of this cell type is transient and that gradually the proportion of type I cells increases and eventually (after 10 days) represents ~90% of the AECs. These data demonstrate that, at least in the fetus, sustained alterations in lung expansion can have profound effects on the AEC population and indicates that mechanical forces may be an important determinant of AEC phenotype in vivo.

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