Functional Overexpression of Wild-Type p53 Correlates With Alveolar Cell Differentiation in the Developing Human Lung

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ABSTRACT
At 15 weeks after conception (a.c.), the human pulmonary acinus is lined by distal low-columnar and more proximal cuboidal cells that are successive stages in alveolar type II cell differentiation (pseudoglandular period of lung development). From 16 weeks a.c. onward, there are also 'flatter' cells that are intermediate stages in the differentiation of cuboidal type II cells into squamous type I cells (canalicular period). We investigated the role of wild-type p53 protein and the proliferation marker Ki-67 in the differentiation of type II and type I cells in these two periods. Serial sections from fetal lungs (n = 30) were immunoincubated with antibodies against p53 and Ki-67. The presence of prospective type II and type I cells was confirmed using immunohistochemistry for surfactant protein SP-A as a differentiation marker and light and electron microscopy. The p53 and Ki-67 positive nuclei were quantified per alveolar cell phenotype (i.e., low-columnar; cuboidal; flatter). The occurrence of cell apoptosis was studied using propidium iodide (PI) and 4',6'-diamino-2-phenylindol dihydrochloride (DAPI) staining. The combined increase in p53 expression and decrease in Ki-67 expression during alveolar epithelial cell differentiation suggests that wild-type p53 protein plays a role in the differentiation of alveolar type II and type I cells in the human lung, and that this function is mediated through cell cycle arrest. The rare incidence of apoptotic nuclei in alveolar type II cells, together with their absence in alveolar type I cells, supports the view that p53 is involved in the differentiation, rather than the death, of alveolar epithelial cells. Anat Rec 263:25–34, 2001.

Key words: p53; Ki-67; alveolar type I cells; alveolar type II cells; cell differentiation; cell cycle arrest

The primitive pulmonary acinus is detectable in human fetal lungs from 10–12 weeks after conception (a.c.) onward. At that time, it is composed of acinar tubules lined by prospective alveolar type II cells (Otto-Verberne et al., 1988). These early prenatal type II cells show a large and round nucleus, large apical and basal glycogen fields, a rough and smooth endoplasmic reticulum, a well-developed Golgi apparatus with many associated vesicles and various types of inclusion bodies (cytoplasmic, granular/ flocculent, osmiophilic, multivesicular and dense), claimed to be precursory stages of multilamellar bodies (Ten Have-Opbroek, 1991; Ten Have-Opbroek et al., 1988, 1990b, 1991). On the basis of these ultrastructural features, alveolar type II cells can be easily recognized from bronchiolar Clara cells, the other major secretory cell type in the peripheral lung (Plopper et al., 1980; Ten Have-Opbroek and De Vries, 1993; Plopper and Ten Have-Opbroek, 1994). In this so-called pseudoglandular period of lung

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development, two stages in the differentiation of prospective type II cells can be distinguished: a low-columnar cell, located in the end-pieces of the acinus and having a high glycogen content, and a cuboidal cell situated more proximally and with a low glycogen content. Around 16 weeks a.c., there is also a third stage in the alveolar epithelial cell differentiation, mainly in those areas of the acinus that are close to capillaries. This is a ‘flatter’ cell type, ranging in shape from cuboidal to squamous, that represents an intermediate stage in the differentiation of prospective cuboidal type II cells into prospective squamous type I cells (Ten Have-Otbroek, 1979, 1991; Otto-Verberne and Ten Have-Otbroek, 1987; Otto-Verberne et al., 1988; Ten Have-Otbroek and Plopper, 1992; Brandsma et al., 1993, 1994). As a consequence of this cell ‘flattening,’ the acinar tubules start to transform into derivative structures, namely prospective alveolar ducts and sacs (cantalicular period of lung development). Our group has been the first to demonstrate that, in human fetal lungs, prospective type II and type I cells display specific protein expression, notably surfactant protein SP-A, as soon as they appear in the pseudoglandular period of lung development (Otto-Verberne et al., 1988, 1990). This has also been shown for the mouse (Ten Have-Otbroek, 1975, 1979), the rat (Otto-Verberne and Ten Have-Otbroek, 1987) and the Rhesus monkey (Ten Have-Otbroek and Plopper, 1992). These results have been confirmed by other groups (reviewed in Ten Have-Otbroek and Plopper, 1992).

The p53 tumor suppressor gene encodes a 53 kD protein that is an important key in the regulation of the normal cell cycle. The wild-type p53 protein is active as a transcription factor and able to initiate cell cycle arrest at the G1/S checkpoint (Dulic et al., 1994; Mercer et al., 1990; Yiu et al., 1992). Recent in vitro and in vivo studies indicate that the process for cells to undergo differentiation involves up-regulation of the p53 expression (Rotter et al., 1992; Aloni-Gurstein et al., 1995; Eizenberg et al., 1996; Almog and Rotter, 1997). These data, together with the finding that p53 mRNA and protein are expressed during mouse embryogenesis, with high levels of expression in specific differentiating tissues (Louis et al., 1988; Schmid et al., 1991), suggest that p53 up-regulation plays a role in organogenesis.

Therefore, the aim of this study in the early fetal human lung (15–20 weeks a.c.) was to investigate the expression of wild-type p53 protein in relation to the expression of a cell proliferation marker (Ki-67) during alveolar cell differentiation. For this, we used immunohistochemistry in serial sections and morphometric analysis. Prospective alveolar cells were identified using an antibody against SP-A and light and electron microscopy. In addition, two apoptosis stainings were used to examine the occurrence of cell death. Our results are consistent with the possibility that wild-type p53 regulates alveolar type II and type I cell differentiation during human lung development through cell cycle arrest.

### MATERIALS AND METHODS

Permission to use human fetal tissue specimens was given by the Committee for Medical Ethics of the Medical School of the University of Leiden.

### TABLE 1. Developmental ages of human fetuses included in Group I (pseudoglandular period of lung development) and Group II (canalicular period of lung development) in the present study

<table>
<thead>
<tr>
<th>Developmental agea</th>
<th>Group I</th>
<th>Group II</th>
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<tbody>
<tr>
<td>15</td>
<td>15 ± 0  (n = 6)</td>
<td>16 ± 4 (n = 1)</td>
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<tr>
<td>16</td>
<td>16 ± 2 (n = 1)</td>
<td>16 ± 5 (n = 1)</td>
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<td>17</td>
<td>17 ± 0 (n = 1)</td>
<td>17 ± 2 (n = 1)</td>
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<tr>
<td>18</td>
<td>18 ± 0 (n = 4)</td>
<td>17 ± 5 (n = 1)</td>
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<tr>
<td>19</td>
<td>19 ± 0 (n = 1)</td>
<td>18 ± 3 (n = 1)</td>
</tr>
<tr>
<td>20</td>
<td>20 ± 0 (n = 1)</td>
<td>19 ± 2 (n = 1)</td>
</tr>
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aExpressed in weeks and additional days after conception.

The developmental age was estimated by measuring the dis-tantia biparietalis (head diameter) using ultrasound. When the fetus was younger than 18 weeks a.c., the foot length was included.

### Tissue Preparation

The study was carried out on lungs from 30 human fetuses (aged 15–20 weeks a.c.), obtained from the “Medical Center for Birth Control" in Leiden. Curettage material was placed in ice-cold phosphate buffered saline (PBS, pH 7.3) immediately after abortion. Macroscopically identifiable lung lobes were transferred to 4% buffered formaldehyde (pH 7.3) and fixed by immersion for 16 hr at room temperature (rt). After dehydration through a graded ethanol series (70–100%, each step lasting 30 min), the tissue was embedded in paraffin and serially sectioned at 5 μm. The sections were mounted on precoated slides (Knittel Gläser, Germany) and stored at rt.

### Light Microscopy

Staining with hematoxylin and eosin (H&E) was performed on serial sections of each specimen to select blocks for immunohistochemistry and for general orientation. Based upon the presence of only prospective type II cells or both prospective type II and type I cells in the lining of the pulmonary acinus, specimens were divided in Group I (pseudoglandular period) and Group II (canalicular period), respectively (Table 1).

### Apoptosis Labeling

Using the technique of Coles et al. (1993), one series of sections was incubated with 4 μg/ml PI (propidium iodide; Serva Feinbiochemica, Heidelberg, Germany) and 100 μg/ml RNase (Sigma, St. Louis, MO) in PBS for 30 min at rt. A second series of sections was stained with 0.1% DAPI (4′,6′-diamino-2-phenylindol dihydrochloride; Boehringer Mannheim, Germany) in aqua dest for 15 min at rt (Salido et al., 2000). All sections were mounted with antifading mounting medium for fluorescence (500μg/ml p-phenylendiamine, Sigma, and 38% glycerol, Merck, Darmstadt, Germany, in PBS) and stored in darkness at −20°C until examination.
Antibodies

For type II cell detection, we used a polyclonal antibody against surfactant protein A (SP-A) called SALS-Hu (Otto-Verberne et al., 1988, 1990). SALS-Hu was prepared in our laboratory by injecting rabbits with the pellet fraction of bronchoalveolar lavage fluid from adult human lung. The resulting immune serum was absorbed with serum and cross-reacting organs (Otto-Verberne et al., 1988). The SP-A specificity of the final immune serum was assessed by western blot analysis, in vitro transcription assays of mRNA from human lungs, immunoprecipitation and immunohistochemistry. SALS-Hu recognizes recombinant human SP-A in western blots and can be depleted with this substance (Otto-Verberne et al., 1988, 1990). The antibody was used in our further studies of lung morphogenesis (Otto-Verberne et al., 1991; Ten Have-Opbroek et al., 1991; Ten Have-Opbroek and Plopper, 1992) and pathogenesis (Ten Have-Opbroek et al., 1990a, 1997).

In addition, we used a monoclonal antibody against the DNA replication control protein p53 (Clone DO-1, mouse anti-human; Santa Cruz Biotechnology, Santa Cruz, CA), and a monoclonal antibody against the cell proliferation marker Ki-67 (Clone MIB-1, mouse anti-human; Immunotech, Marseille, France). Ki-67 is a nuclear antigen present in all proliferating cells that are in active phases of the cell cycle, i.e., G₁, S, G₂, and mitosis, but which is absent in G₀ cells, and therefore it can be used as an proliferation marker (Gerdes, 1990).

Immunohistochemistry

Immunohistochemical staining was performed using the avidin-biotin complex (ABC) method with peroxidase labeling, and 3-3’-diaminobenzidine (DAB) as the chromogen (Vector; Burlingame, CA). Briefly, the procedure involves the following steps: 1) hydration of the paraffin sections through xylene and a graded ethanol series (100—70%, each step lasting 30 min) and quenching of the endogenous peroxidase activity with 100% methanol containing 0.4% hydrogen peroxide (H₂O₂) for 20 min at rt; 2) three times rinsing in Tris Maleate buffer (TMB, pH 7.6) for 1 min at rt and incubation with 10% normal horse serum for 1 hr at rt; 3) incubation with the primary antibody (SALS-Hu, 1:50; anti-p53, 1:2,000; anti-Ki-67, 1:100; all diluted in TMB, pH 7.6) overnight at 4°C and rinsing in TMB; 4) incubation with a 1:400 dilution of biotinylated swine anti-rabbit IgG (Dako, Denmark) (after SALS-Hu) or biotinylated horse anti-mouse IgG (after anti-p53 or anti-Ki-67) for 60 min at rt and rinsing in TMB; 5) incubation with ABC for 30 min at rt and rinsing in TMB; and 6) incubation with TMB containing 0.04% DAB and 0.008% H₂O₂ for 10 min at rt. Finally, the sections were washed in TMB for 1 min and in tap water for 10 min, then counterstained for 5 sec with hematoxylin that stained the various nuclei bluish, rinsed in tap water for 10 min, dehydrated through a graded ethanol series (70—100%) and xylene and mounted with xylene-soluble mounting medium Depex (H.D. Supplies, England).

For the detection of wild-type p53 protein, we used an enhancement procedure, i.e., deposition of biotinylated tyramide (Kerstens et al., 1995). After incubation with ABC (Step 5), the sections were incubated with 1:400 biotinylated tyramide in TMB containing 0.006% H₂O₂ for 10 min at rt, then rinsed in TMB, and again incubated with ABC before continuing with Step 6.

Immunohistochemical controls were performed on the serial human fetal lung sections using preimmunization serum or normal mouse serum as the primary antibody, or omission of one of the incubation steps.

Morphometry

In sections immunoincubated with anti-p53 or anti-Ki-67 antibodies, microscopic fields were chosen by random serial sampling (Gundersen and Jensen, 1987). We took 10 sections per specimen with an interval of 50 μm. The number of p53 and Ki-67 positive nuclei were counted per alveolar cell type (i.e., low-columnar, cuboidal and flatter cells). For each cell type, 100 or more nuclei were counted over at least 10 microscopic fields.

Statistical Analysis

The morphometric data are expressed as mean ± SEM. Quantitative data of the p53 and Ki-67 positive nuclei were analyzed statistically by using the one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls’ Multiple Range Test in two ways: a) per alveolar cell type, and b) per protein expression; a difference was considered to be statistically significant if P < 0.05.

Electron Microscopy

Lung lobes were fixed by immersion in 2% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 24 hr at 4°C and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 2 hr at rt. After dehydration through a graded ethanol series (70—100%), the tissue was processed for transmission (TEM) and scanning electron microscopy (SEM) as described previously (Ten Have-Opbroek et al., 1988).

RESULTS

Light Microscopy

In the pseudoglandular period of lung development, the epithelial lining displayed two stages in alveolar cell differentiation, which differed in morphology and location: the low-columnar cells were located in the end-pieces of the acini, whereas the cuboidal cells were situated more proximally. Large areas that did not stain with eosin could be recognized in the basal and apical cytoplasm. As reported (Otto-Verberne et al., 1988) and shown below through electron microscopy, these areas corresponded to glycogen fields. Such fields were especially visible in the distal low-columnar shaped cells. At this early fetal age, only some capillaries were present underneath the alveolar epithelial layer. Similar to the period before, the epithelial lining in the canalicular period of lung development exhibited distal low-columnar and proximal cuboidal cells with a similar distribution (see above). Additionally, there was a third stage in alveolar cell differentiation, namely cells ranging in shape from cuboidal to squamous, which have been indicated as ‘flatter’ cells (Ten Have-Opbroek, 1979, 1991). These flatter cells were also found more proximally in close relationship with capillaries that were more abundant in this period.

Electron Microscopy

Figure 1A is a representative scanning electron micrograph of the tubular system in the early fetal human lung. It shows the transition from the pseudostratified column-
The nar epithelium of the prospective bronchial portion to the approximately cuboidal epithelium of the prospective respiratory portion (pulmonary acinus) of the lung. Figures 1B–D are transmission electron micrographs of the epithelial cells lining the acinar tubules of fetuses in Group I (Fig. 1B) and in Group II (Fig. 1C,D). In the pseudoglandular period of lung development, there were low-columnar and approximately cuboidal cells with large and more or less round nuclei, showing blunt microvilli and cellular protrusions at the luminal side, a basal lamina, and interdigitations and junctional complexes with adjacent epithelial cells. Large glycogen fields located in the apical and basal cytoplasm. Mitochondria, endoplasmic reticulum and cytoplasmic inclusions are mostly present close to the apical cell border. BL: basal lamina (×4,370). C: As of 16 weeks a.c. (TEM), the epithelial lining also contains shorter cells (arrowhead) situated above capillaries (CA) (×4,370). D: Eighteen weeks a.c. (TEM). An attenuated cell (arrowhead) is present adjacent to a capillary (CA) (×3,200).
Fig. 2. Immunohistochemistry of the pulmonary acinus in human fetuses (incubated with SP-A antibody). A: Sixteen weeks a.c. The low-columnar and cuboidal epithelial cells located in the distal (asterisk) and in the more proximal (square) regions of the acini, respectively, show positive (yellow to red) cytoplasmic staining for SP-A, especially in the apical area (×132). B: Detail of A, exhibiting the cytoplasmic SP-A staining. Note the absence of staining in the glycogen fields around the large and roundish nuclei (bluish) (×330). C: At 19 weeks a.c., a strong apical staining for SP-A is seen in both cuboidal and flatter (FL) cells. Nuclear counterstaining: hematoxylin (×330).

Fig. 3. Immunohistochemistry of the pulmonary acinus in human fetuses (incubated with p53 antibody). A: Sixteen weeks a.c. The low-columnar cells present in the distal regions (asterisk) of the acini show less p53 positive (brown) nuclear staining than the cuboidal (CU) cells present in more proximal regions of the acini (×132). B: Nineteen weeks a.c. The cuboidal cells display the same p53 staining pattern as seen at 16 weeks a.c. (see A). In addition, almost all the flatter (FL) cells are positive for p53 (×132). C: Detail of B, showing the intense p53 immunostaining in flatter cells. Nuclear counterstaining: hematoxylin (bluish) (×330).
development, the epithelial cells lining the acinar tubules exhibited the same ultrastructural characteristics as described above. Capillaries were seen located in the proximity of mildly (Fig. 1C) and strongly (Fig. 1D) attenuated cuboidal cells.

**Immunohistochemistry**

After incubation with the anti-SP-A antibody (SALS-Hu), the sections of fetal lungs in Group I showed positive (= yellow to red) staining in the epithelial lining composed of distal low-columnar and proximal cuboidal cells (Fig. 2A). The SP-A reactivity was present in the cytoplasm, especially in the apical areas and along the cell borders. The large glycogen fields around the nucleus (= bluish) often remained unstained (Fig. 2B). In the sections of fetal lungs in Group II, the distal low-columnar and proximal cuboidal cells showed a staining pattern for SP-A similar to the one described above. Additionally, SP-A reactivity was also found in the proximal flatter cells (Fig. 2C).

**Fig. 4.** Immunohistochemistry of the pulmonary acinus in human fetuses (incubated with Ki-67 antibody). A: Sixteen weeks a.c. Strong Ki-67 positive (= brown) nuclear staining locates to the low-columnar cells present in the distal regions (asterisk) of the acini; the cuboidal cells present in more proximal regions (square) of the acini show less Ki-67 immunostaining (×132). B: Nineteen weeks a.c. The cuboidal (CU) cells display the same Ki-67 staining pattern as found at 16 weeks a.c. (see A), whereas the nuclei of the flatter (FL) cells are Ki-67 negative (×132). C: detail of B. Nuclear counterstaining: hematoxylin (= bluish) (×330).

**Fig. 7.** Apoptosis staining of the pulmonary acinus in human fetuses (incubated with DAPI). Representative image of acinar tubules in a 19 weeks a.c. specimen. Two nuclei of mesenchymal cells near the epithelium appear brighter and smaller or fragmented, showing two different stages in the apoptotic process (arrows). The nuclei of alveolar cells lining the acinar tubules do not show any sign of apoptosis. A: Acinar tubule. B: Bronchiole (×565).
After incubation with the anti-p53 antibody, the sections of fetal lungs in both Group I and Group II demonstrated positive (5 brown) nuclear staining in the distal low-columnar and the proximal cuboidal cells (Fig. 3A). In Group II, p53 positive nuclei were more abundant in the proximal flatter cells than in the other alveolar cell types (Fig. 3B,C).

The sections of fetal lungs in Group I immunoincubated with the anti-Ki-67 antibody exhibited positive (5 brown) nuclear staining in the two alveolar cell types present, i.e., distal low-columnar and proximal cuboidal cells, but particularly in the former one (Fig. 4A). In the sections of fetal lungs in Group II, the nuclear reactivity for Ki-67 protein was positive and comparable in both distal low-columnar and proximal cuboidal cells, whereas the proximal flatter cells were almost negative for Ki-67 expression (Fig. 4B,C).

**Quantification of p53 and Ki-67 Expression**

In sections of fetal lungs in Group I, the counting of the p53 positive nuclei showed no significant difference between the distal low-columnar and the proximal cuboidal cells. The percentage of the Ki-67 positive nuclei was significantly smaller, however, in the proximal cuboidal cells compared to the distal low-columnar cells (P < 0.05) (Fig. 5). Remarkably, when comparing the expression of both proteins, there were more p53 than Ki-67 positive nuclei in the proximal cuboidal cells (P < 0.05).

The quantification of the p53 and Ki-67 expression data in Group II indicated that there was no significant difference between the distal low-columnar and the proximal cuboidal cells for the expression of both proteins. By contrast, the percentage of p53 positive nuclei was significantly higher in the proximal flatter cells compared to the other two alveolar cell types (P < 0.001) (Fig. 6). Likewise, the percentage of Ki-67 positive nuclei was significantly lower in the proximal flatter cells when compared with the distal low-columnar cells (P < 0.001) and the proximal cuboidal cells (P < 0.01) (Fig. 6). Finally, when comparing the expression of p53 and Ki-67, we found an inverse relationship between them during the progression of alveolar cell differentiation, being most prominent in the proximal flatter cells (P < 0.001).

**Detection of Apoptosis**

Under the fluorescence microscope, normal alveolar cell nuclei in human fetal lungs in groups I and II displayed a

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**Fig. 8.** Diagram showing the spatial distribution of the various nuclear markers used in the present study in a representative fetal acinus in Group II. Ki-67 positive nuclei predominate in the end-pieces of the acinus, lined by low-columnar (i.e., immature) alveolar type II cells. By contrast, p53 positive nuclei prevail in more proximal regions of the acinus, lined by cuboidal (i.e., more mature) alveolar type II cells and flatter (i.e., immature) alveolar type I cells. Occasionally, immature alveolar type II cells show an apoptotic nucleus. For further explanation, see Results.
The present study detects positive reactivity for p53 protein in the pulmonary acinus of the human fetal lung from 15–20 weeks a.c. The p53 nuclear staining is present in all three stages of alveolar epithelial cell differentiation (i.e., distal low-columnar, proximal cuboidal, and proximal flatter cells). Expression of wild-type p53 protein in the pulmonary acinus [called acinar tubules (Ten Have-Opbroek, 1979)] was often located in close proximity to capillaries. As presently shown, these cells were always SP-A positive, irrespective of their varying shapes (cuboidal to almost squamous). These so-called ‘flatter’ cells (Ten Have-Opbroek, 1979, 1991) are considered to be intermediate stages in the differentiation of cuboidal type II into squamous type I cells. Fully differentiated squamous alveolar type I cells as seen in the adult lung are always SP-A negative (Ten Have-Opbroek et al., 1991). All these observations agree with, and also extend, previous reports on the presence of prospective alveolar type II and type I cells in developing lungs in the human as well as in other species (reviewed in Ten Have-Opbroek, 1979, 1991) are considered to be intermediate stages in the differentiation of cuboidal type II into squamous type I cells. Fully differentiated squamous alveolar type I cells as seen in the adult lung are always SP-A negative (Ten Have-Opbroek et al., 1991). All these observations agree with, and also extend, previous reports on the presence of prospective alveolar type II and type I cells in developing lungs in the human as well as in other species (reviewed in Ten Have-Opbroek and Plopper, 1979).

The aim of the present work was to investigate the role of p53 and Ki-67 in the differentiation of prospective alveolar type II and type I cells in the late pseudoglandular and early canalicular periods of lung development. Our present data are consistent with the possibility that, at this specific time of lung development, one alveolar cell lineage with different morphologic phenotypes and locations lines the components of the primitive pulmonary acinus [called acinar tubules (Ten Have-Opbroek, 1979)]. The distal low-columnar and the proximal cuboidal alveolar cells in the epithelial lining exhibited the distinctive ultrastructural features of prospective alveolar type II cells (see Introduction) and showed positive cytoplasmic staining for SP-A. The third, also proximal, alveolar cell type that appeared in the early canalicular period of lung development, was often located in close proximity to capillaries. As presently shown, these cells were always SP-A positive, irrespective of their varying shapes (cuboidal to almost squamous). These so-called ‘flatter’ cells (Ten Have-Opbroek, 1979, 1991) are considered to be intermediate stages in the differentiation of cuboidal type II into squamous type I cells. Fully differentiated squamous alveolar type I cells as seen in the adult lung are always SP-A negative (Ten Have-Opbroek et al., 1991). All these observations agree with, and also extend, previous reports on the presence of prospective alveolar type II and type I cells in developing lungs in the human as well as in other species (reviewed in Ten Have-Opbroek and Plopper, 1979).

The present study detects positive reactivity for p53 protein in the pulmonary acinus of the human fetal lung from 15–20 weeks a.c. The p53 nuclear staining is present in all three stages of alveolar epithelial cell differentiation (i.e., distal low-columnar, proximal cuboidal, and proximal flatter cells). Expression of wild-type p53 protein in the cell nuclei is a normal physiological response to slow down the cell cycle at the G1 phase. This protein is not usually detectable by immunohistochemistry because it is short-lived in comparison with its mutated counterpart and present at low levels. It may become detectable, however, by immunohistochemical means in normal tissues if accumulated due to functional overexpression (Battifora,
Correlation of p53 Functional Overexpression to Alveolar Cell Differentiation

The quantification of p53 and Ki-67 expression in prospective alveolar type II cells (i.e., distal low-columnar and proximal cuboidal cells) in the pseudoglandular period of lung development showed that the percentage of wild-type p53 positive nuclei was higher in proximal cuboidal cells in comparison with distal low-columnar cells, although the difference was not statistically significant. At the same time, proximal cuboidal cells exhibited a lower percentage of Ki-67 positive nuclei compared to distal low-columnar cells. Because the wild-type p53 protein has been shown to have an antiproliferative effect by blocking cell cycle progression (Mercer et al., 1990) and to selectively down-regulate the expression of the proliferating cell nuclear antigen (Mercer et al., 1991), we assume that the decrease in the proliferation rate (as measured by Ki-67 expression) of proximal cuboidal cells in relation to distal low-columnar cells is the result of a p53-induced growth arrest. A similar mechanism seems to be involved in type I cell differentiation. For in the canalicular period of lung development, we observed the same events as described above together with a remarkable inverse correlation between p53 and Ki-67 expression in the proximal flatter cells. In summary, the finding of rising p53 expression levels synchronically with declining Ki-67 expression levels during alveolar epithelial cell attenuation (from low-columnar to cuboidal and from this to flatter) strongly suggests that the wild-type p53 protein participates in alveolar type II and type I cell differentiation in the developing human lung, probably through cell cycle arrest.

CONCLUSIONS

Our conclusion that p53 plays a role in alveolar cell differentiation is in line with other reports in the literature. Lutzker and Levine (1996) have shown that induction of cell differentiation in undifferentiated teratocarcinoma cell lines enhances p53 half-life and p53 target genes transcriptional activity. Embryonic studies indicate that the wild-type p53 protein may play an important role as a positive regulator of cell differentiation (Halevy, 1993; Woodworth et al., 1993; Keren-Tal et al., 1995; Eisenberg et al., 1996; Almog and Rotter, 1997). Likewise, other tumor suppressor genes have been found to be essential for embryonic development in the mouse (for review, see Jacks, 1996). Throughout mouse embryogenesis, a strong p53 signal is expressed during specific differentiation stages of certain tissues like brain, lung, thymus, intestine, salivary gland and kidney (Schmid et al., 1991). This participation of the wild-type p53 protein in mouse embryogenesis seems to be in contradiction with the first studies (Donehower et al., 1992) carried out on p53 knockout mice because the latter describe an apparent normal prenatal and postnatal development. One possible explanation for this discrepancy might be that the function of p53 in cell differentiation is masked by other compensating genes or alternative pathways in mammalian species as has been proposed by Almog and Rotter (1997) and Choi and Donehower (1999). Later findings have reported that p53 null mice show, at certain frequencies, developmental alterations (Armstrong et al., 1995; Sah et al., 1995). Furthermore, the fact that transgenic mice overexpressing mutant alleles of the p53 protein show a high incidence of lung adenocarcinomas (Lavigueur et al., 1989), confirms the direct role of this protein as a negative regulator of cell proliferation in the epithelial compartment of the lung.

Because wild-type p53 is also implicated in the pathway inducing apoptosis (Yonish-Rouach et al., 1991, 1993; Oren, 1994), it could be that the p53 overexpression in the fetal lung reported in the present experiments is related to the induction of cell death rather than cell maturation. The present study did not detect any distinct sign of nuclear fragmentation in differentiating alveolar type I cells, i.e., proximal flatter cells, and only sporadic apoptotic nuclei in distal low-columnar and proximal cuboidal alveolar type II cells. In agreement with our observations, it has been reported that all apoptotic cells found in human fetal lungs aged 15–24 weeks a.c. were located in the interstitium, whereas in epithelial cells no apoptotic nuclei were detected (Scavo et al., 1998). This finding further supports our present concept of p53 acting exclusively as a regulator of cell differentiation in alveolar epithelial cells of the early fetal human lung.

In conclusion, our results indicate that the wild-type p53 protein may play a role in alveolar epithelial cell differentiation in the developing human lung by interfering with the normal cell cycle progression. More research is needed to achieve further insight into the mechanism through which wild-type p53 regulates normal cell proliferation and differentiation, as well as its role in developmental biology, carcinogenesis and wound repair.

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