Programmed Cell Death Contributes to Postnatal Lung Development

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The rat lung undergoes the phase of maturation of the alveolar septa and of the parenchymal microvascular network mainly during the third postnatal week. Speculating that programmed cell death may contribute to the thinning of the alveolar septa, we searched for the presence of DNA fragmentation in rat lungs between postnatal days 6 and 36 using the TUNEL procedure. The number of positive nuclei was compared at different days. We observed an 8-fold increase of programmed cell death toward the end of the third week as compared to the days before and after this time point. The precise timing of the appearance of the peak depended on the size of the litter. Double-labeling for DNA fragmentation (TUNEL) and for type I and type II epithelial cells (antibodies E11 and MNF-116), as well as morphologic studies at electron microscopic level, revealed that during the peak of programmed cell death mainly fibroblasts and type II epithelial cells were dying. While both dying cell types were TUNEL-positive, nuclear fragments and apoptotic bodies were exclusively observed in the dying fibroblasts. We conclude that programmed cell death is involved in the structural maturation of the lung by reducing the number of fibroblasts and type II epithelial cells in the third postnatal week. We observed that the dying fibroblasts are cleared by neighboring fibroblasts in a later stage of apoptosis, and we hypothesize that type II epithelial cells are cleared by alveolar macrophages in early stages of the programmed cell death process.


Mammals are born with a functioning but still immature lung. For example, the rat lung consists of smooth-walled, air-filled channels that end in saccules: alveoli, the future terminal gas exchange units, are not yet formed. The primary septa between the channels and saccules are thick and contain a double capillary network (primitive septa), which is required for the formation of the alveoli (1, 2). Each leaflet of this capillary layer is separated by a central sheet of connective tissue. In rats, alveolarization takes place after a short phase of expansion of the lung. The alveoli appear by the formation of new tissue ridges (secondary septa), which arise by lifting off from the existing primary septa (3, 4). Both types of septa still possess a double capillary network. As a result, the gas exchange surface increases dramatically. At about postnatal days 10 to 13, the bulk alveolar formation is completed, but additional alveoli may be formed later by a septation of secondary septa (5) and after a postulated branching in the subpleural region (6). Now the phase of microvascular maturation follows. The low and thick secondary septa are rapidly turned into high and slender interalveolar walls. Most of the double capillary layers inside the primary and secondary septa are transformed into a single central capillary layer with a high meshwork density. The central layer of connective tissue is reduced to a fibrous meshwork interwoven with the capillary network (7, 8). The process results not only in a reduction of the absolute mass of interstitial tissue, but also in a reduction of the absolute number of fibroblasts by 10–20%, a remarkable fact in view of an overall volume gain of the lung of 25% (9). In parallel, the type I epithelial cells are spreading and thinning out in order to contribute to the maturation of the air–blood barrier. Their total number is increasing slightly. The type II epithelial cells are viewed as putative stem cells for the type I epithelial cells (9, 10). In addition, they are biochemically very active and produce the surfactant found in the alveoli. Their total number decreases by more than 20% in this last step of lung maturation. The disappearance of the type II epithelial cells is not only due to a differentiation of type II to type I cells, because the total number of all epithelial cells decreases by more than 10% (4, 9, 11, 12). In rats most of the maturation of the mi-
crovasculature is completed by the end of the third postnatal week and normal growth of the lung ensues (5, 7, 8, 13; for review see References 4, 6, 12).

Programmed cell death represents an active form of physiologic cell death that was originally defined for developmental processes (14). Cell death through apoptosis is defined by a typical pattern of morphologic changes (15). These changes include shrinkage of the cell, reorganization and segmentation of the nucleus, active membrane blebbing, and ultimate fragmentation of the cell into membrane-enclosed vesicles (apoptotic bodies) (16). While many cells that undergo programmed cell death show all criteria of apoptosis, other cells die without the typical morphologic alterations (17), but all of them die without an inflammatory reaction. The latter distinguishes this process from necrosis, where cells disintegrate and inflammation is induced by cellular lysis (16).

Currently, programmed cell death is seen as deliberate cell death, a suicide that is necessary to achieve the final morphology as a result of an endogenous developmental program (18–20). During embryogenesis, cell elimination by programmed cell death seems to be necessary for fashioning of the body, molding of tissues, canalization of ducts and tubes, formation of digits, fusion of palatal shelves, closure of the neural tube, and development of the nervous system (19, 20). Furthermore, programmed cell death is very important for the development and maintenance of the immune system (19–21).

Programmed cell death has been shown to be tightly controlled by a number of suppressing or inducing genes. However, regardless of how a cell enters the process of programmed cell death, endogenous endonucleases are activated in most cases. The DNA is cleaved into nucleosomal fragments (multiples of 180 base pairs), thus generating a characteristic DNA ladder during separation on electrophoretic gels (22–24). As a consequence, the large number of 3’ ends provides the basis for a histochemical staining of nuclei of cells dying by programmed cell death. In this assay, the DNA fragments are 3’ end-labeled by the enzyme terminal transferase, using labeled deoxyuridine triphosphate (dUTP) (25).

In the present study, we investigated whether the reduction of the total number of fibroblasts and lung epithelial cells during the third postnatal week may be a result of programmed cell death. We searched for the appearance of programmed cell death during early postnatal lung development, and have shown in rats that in the third postnatal week, during remodeling of the interalveolar walls, interstitial fibroblasts and type II epithelial cells are dying due to programmed cell death.

Materials and Methods

Animals and Tissues

All rat tissues were obtained from SIV-Z-50 or Wistar rats, respectively. Lungs were prepared according to Schittny and colleagues (26). Briefly, the blood vessels were perfused with phosphate-buffered saline (PBS, 10 mM sodium phosphate, containing 127 mM sodium chloride, pH 7.4), containing 5 units/ml (U/ml) heparin, 10 mg/ml procaine, and 10 mM ethylenediaminetetraacetic acid (EDTA) (Fluka Chemie A G, Buchs, Switzerland), and the air space filled with PBS, containing 4% freshly prepared paraformaldehyde (Merck, Darmstadt, Germany) at a constant pressure of 20 cm H₂O. At this water pressure, the lung reaches roughly its midrespiratory volume. In order to prevent a recoiling of the lung, the pressure was maintained during the fixation.

In order to assess clearly the morphology of dying type II epithelial cells, programmed cell death was induced in C57BL/6 mice by a programmed cell death-inducing anti-FAS antibody (27). Mice weighing approximately 20 g were anesthetized with methoxyflurane before positioning on a slope board apparatus for intratracheal instillation. Following visualization of the vocal cords with a dissecting microscope, 50 μg of the anti-FAS antibody in 50 μl of sterile PBS or an isotype control antibody were intratracheally injected (28). After 24 h, the mice were killed and the lungs were prepared as described previously.

The handling of the animals before and during the experiments, as well as the experiments themselves, were approved and supervised by the Swiss authorities.

Histochemistry

For paraffin embedding, lungs were fixed for 0.5 to 24 h with 4% paraformaldehyde freshly dissolved in PBS, briefly washed in PBS three times, and embedded in paraffin (Histology) as intermedium. Under all conditions no recoil of the lungs was observed. The 3.5-5 μm sections were cut, transferred onto silanized (aminopropyl-trimethoxysilane) or gelatinized micro slides, and air dried overnight at 37°C. All sections were dewaxed in three changes of Histoclear and a graded series of ethanol and a treatment with 5 μg/ml proteinase K in 20 mM tris-(hydroxymethyl)aminomethane (Tris), pH 8.0, containing 5 mM EDTA (Fluka Chemie AG) at 21°C for 10 min. Proteinase K was removed by four changes of distilled water or Tris-buffered saline (TBS), respectively.

TUNEL assay. The terminal transferase-mediated dUTP nick end labeling (TUNEL) assay was adapted from Gavrieli and associates (25). Briefly, the sections were incubated with terminal transferase reaction solution, containing 9 mM dioxigenin-11-dUTP and 0.165 U/ml enzyme (Boehringer Mannheim, Mannheim, Germany) for 50 min at 37°C. Before the application of an FITC-labeled anti-dioxigenin antibody (Boehringer Mannheim; diluted 1:40 in blocking reagent for nucleic acid hybridization and detection from the same company), the sections were washed two times with 0.03 M sodium citrate, pH 7.4, containing 0.3 M sodium chloride; two times with TBS; and blocked with blocking reagents. Then the sections were washed three times with TBS, and either mounted using Mowiol (PBS, containing 15% of the substance Hoesch 4.88; Hoesch A.G., Frankfurt a.M., Germany) and 50 mg/ml 1,4-diazabicyclo(2.2.2)-octane (Merck, Darmstadt, Germany) or further processed for double-staining (see below).

Immunofluorescence staining. Immunostaining was performed with or without preceding TUNEL assay. Sections were blocked with TBS containing 1 mg/ml bovine serum albumine.
albumin (TBS/BSA) for 30 min and incubated with the first antibody for 1–15 h and the second one for 30–60 min. After both incubation steps, sections were washed three times with TBS. The rabbit antilaminin antiserum (a kind gift of Dr. Mats Paulsson, University of Köln, Germany) was diluted 1:100, and the monoclonal mouse antibody MNF-116 (Dakopatts, Gostrup, Denmark), which recognizes type II epithelial cells, 1:10 in TBS. The cell culture supernatant containing the monoclonal mouse antibody E11 (a kind gift of Dr. A. N. Wetterwald, University of Bern, Switzerland [29]), which recognizes type I epithelial cells, was used undiluted. A secondary antibody, we used rhodamine-labeled goat antirabbit IgG (Cappel Research Products, Organon Teknika Co., Durham, NC; 1 mg/ml diluted 1:60 in TBS/BSA), or rhodamine-labeled sheep antimouse IgG, F(ab')2 fragments (Boehringer Mannheim; diluted 1:40–1:60), respectively.

After having been single- or double-stained, the sections were mounted in Mowiol (see above). All steps were carried out in a moist chamber at room temperature. Tests on negative control animals were performed with nonspecific rabbit and mouse IgG. None or little nonspecific background was observed in all negative control animals. The shown samples were taken from all parts of the lung. The method used was not sensitive enough to show significant differences between central and peripheral regions of the lung. The stained sections were examined and imaged with filters appropriate for fluorescein isothiocyanate or rhodamine staining and interference contrast, respectively. Microscopic images were obtained on a Polyvar (Reichert-Jung, Leonberg, Germany) and ethanol.

Counting of Apoptotic Nuclei

A reas of 1.5 × 1.0 mm of lung parenchyma were systematically, randomly photographed in a meandering order on the Polyvar microscope using the ×10 objective. The micrographs covered 50% to 80% of the total area of the parenchyma. The images were enlarged in a slide projector and TUNEL-positive nuclei were counted. A apoptotic bodies, which were very close to each other, were counted as one dying cell. In lung tissue the time lag between the first sign of programmed cell death and the disappearance of the dying cells is not known. Furthermore, no information is available on how long the TUNEL assay stays positive. Therefore, it is not possible to determine the total number of dying cells. Using a single image as standardized reference space, we obtained only a relative number of dying cells, which we compared between samples of different postnatal days (days 6, 10, 13, 16, 19, 21, 24, and 36). Whenever data were compared, the sections were processed on the same day and in the same batch to minimize variations due to different enzyme activities. Every batch was repeated one to three times. In order to establish the time point of the highest rate of apoptosis during the third postnatal week, we performed the following series of experiments. At postnatal days 10, 13, 16, 19, and 21 and a litter size of 9–11 pups, we used three animals per postnatal day (Figure 1). The reproducibility of this first experiment was shown by repeating it two times for postnatal days 10–21 and two times for postnatal days 6–36 using one animal per postnatal day per repetition. The experiments leading to Figures 2a and 2c were performed two times for every litter size, using one animal per postnatal day. As a further control we performed the same experiment at postnatal days 6 to 36 using one Z jur:SD rat (Sprague-Dawley) per day. The same results were obtained in all repetitions. The data of postnatal days 6 and 36 are not shown because we obtained them in different batches under slightly different conditions (time of fixation) from those shown in Figures 1 and 2.

Electron Microscopy

For electron microscopy tissues were fixed with 2.5% glutaraldehyde in 0.03 M potassium phosphate buffer (pH 7.4, 370 milliosmole [mOsm]) instead of 4% paraformaldehyde in PBS (7). Samples were postfixed in 0.1 M sodium cacodylate (pH 7.4, 340 mOsm), containing 1% OsO4, dehydrated in a graded series of ethanol and embedded in Epon 812. Sections of approximately 80-nanometer (nm) thickness were cut, picked up on Formvar-coated copper grids, double-stained with lead citrate (30) and uranyl acetate (31), and viewed in a Philips 400 electron microscope. The material of the ant-FAS-treated animals was first paraffin-embedded. Embedding in Epon 812 was done without any postfixation after the tissue was dewaxed by three changes of both Histoclear (Life Sciences International, Frankfurt, Germany) and ethanol.

Results

Detection of DNA Fragmentation In Situ

DNA fragmentation is one marker for cells dying by programmed cell death. We adapted the TUNEL assay (25) for double-labeling and laser-scanning microscopy and used it for the staining of nuclei containing fragmented DNA. In rat lung we observed a peak of programmed cell death during the third postnatal week. The method was not sensitive enough to show specific rabbit and mouse IgG. None or little nonspecific background was observed in all negative control animals. The shown samples were taken from all parts of the lung. The method used was not sensitive enough to show significant differences between central and peripheral regions of the lung. The stained sections were examined and imaged with filters appropriate for fluorescein isothiocyanate or rhodamine staining and interference contrast, respectively. Microscopic images were obtained on a Polyvar (Reichert-Jung, Leonberg, Germany) and ethanol.

Figure 1. Relative number of cells dying by programmed cell death in alveolar septa. Lung sections obtained from rats between postnatal days 10 and 24 were stained for DNA fragmentation (TUNEL assay). The number of apoptotic nuclei was counted and normalized to an area of 1.5 × 1.0 mm (one micrograph). Bars include the standard error, N = 3 animals per data point.
death toward the end of the third postnatal week (Figure 1). As compared to baseline labeling on postnatal days 10, 13, and 24 (Figure 1), the number of TUNEL-positive nuclei increased about 8-fold on postnatal days 19 and 21. Furthermore, at postnatal days 6 and 36 we observed only baseline labeling (data not shown). We could express the number of cells undergoing programmed cell death only as relative numbers, because we do not know how long the process of programmed cell death takes in lung and during which interval we are able to detect residual apoptotic cells. Therefore, it is not possible to calculate the total number of dying cells in the lung.

The exact day of the appearance of the peak of programmed cell death was dependent on the litter size. In animals obtained from litters containing 9 to 11 pups, the peak was observed on postnatal day 19 (Figures 1 and 2b). In smaller litters (< 9 pups) development seemed to be faster and the peak appeared earlier, between days 16 and 19 (Figure 2a). A larger litter size (> 14 pups) resulted in a later appearance of the peak on postnatal day 21 (Figure 2c).

The distribution of cells dying by programmed cell death was relatively homogeneous. Whole lung lobes were cut every 3-mm (9 blocks) and the first section of every block was stained for DNA fragmentation. Comparing the sections of one lung lobe, we determined a standard error of 5% and did not see any significant differences between the first, a middle, and the last section, or within single sections. Due to the small number of dying cells, we cannot exclude regional differences, but none were apparent.

**Light Microscopic Identification of the Dying Cells**

On postnatal days 19 and 21 a double-labeling for DNA fragmentation using the TUNEL assay, and of basement membranes using anti-laminin, was performed. It revealed that epithelial cells as well as nonepithelial cells undergo DNA fragmentation and programmed cell death. Figure 3a shows an example of a dying epithelial cell located on the alveolar side of the basement membrane. Based on its morphology and its typical location in a niche, this cell represents most likely a type II epithelial cell. In order to verify this result, we performed a double-labeling experiment using the TUNEL assay and the antibody MNF-116. The antibody MNF-116 recognizes a wide range of cytokeratins (32). In lung parenchyma it binds specifically to the apical surface of type II epithelial cells (33). A cell that is positive for both labels is shown in Figure 3b, and its interference contrast image in Figure 3c. From these results, we conclude that type II epithelial cells undergo programmed cell death between postnatal days 19 and 21.

In order to detect programmed cell death in epithelial type I cells, we performed a labeling on successive serial sections using the TUNEL assay and anti-laminin on a first section and the type I epithelial cell marker antibody E11 on the second section. This monoclonal antibody recognizes a protein homologous to the murine OTS-8/gp38 sequence at the apical surface of type I epithelial cells and at the cell surface of osteoblasts and osteocytes (29). We did not find any evidence that type I epithelial cells undergo programmed cell death. We can, however, not exclude that a small number of dying type I epithelial cells may have remained undetected (data not shown).

In Figure 4, examples of dying nonepithelial cells are shown. A gain, based on their location and morphology it is very likely that many of these cells are fibroblasts (Figure 4a), but at this level of resolution we were not able to clearly distinguish between fibroblasts and other interstitial cells. Therefore, we investigated the same material by electron microscopy (see below). Many of these cells were not only TUNEL-positive, but also contained labeled apoptotic bodies (Figure 4b). By laser scanning microscopy we
verified that the apoptotic bodies were not part of a larger nucleus, but were separated particles.

Electron Microscopic Identification of the Dying Cells

In order to identify clearly the dying nonepithelial cells, we searched for apoptotic bodies in lungs on postnatal days 19 and 21 using electron microscopy. Most of the apoptotic bodies were found in fibroblasts within the alveolar septa (Figure 5). Some of these fibroblasts appeared to be in the stage of nuclear segmentation (Figure 5a). A later stage, where fragments of the nucleus are phagocytosed by neighboring cells (fibroblasts), was also found (Figure 5b). In addition, few nonparenchymal cells, such as leukocytes and macrophages, containing apoptotic bodies were also observed (data not shown). In contrast to the observations made in dying fibroblasts, we did not find any morphologic signs of apoptosis such as nuclear fragmentation or apoptotic bodies in epithelial cells, neither at the light microscopic (TUNEL assay) nor electron microscopic level.
Discussion

As described in more detail previously, the total numbers of type II epithelial cells and of fibroblasts decrease in rat lung during the third postnatal week (9, 11). Knowing that cell elimination by programmed cell death seems to be important during several developmental processes (19, 20), we speculated that programmed cell death may be involved in postnatal lung development. Therefore, we searched for apoptosis during the phase of microvascular maturation and applied the TUNEL assay to rat lung tissue between postnatal days 6 and 36. We found a peak of programmed cell death in the lung parenchyma on postnatal day 19 (Figure 1). The time point of appearance depended on the size of the litter. Apparently development is faster in smaller litters than in larger ones (Figure 2). This result is in good agreement with earlier findings of Kauffman and colleagues (9). If the present data are normalized to the litter size (Kauffman and colleagues [9] used eight pups per litter), the peak of programmed cell death appears just in the middle of the third postnatal week. Furthermore, as predicted from their work (9), we found mainly type II epithelial cells (Figure 3) and fibroblasts (Figures 4 and 5) undergoing programmed cell death and no dying epithelial type I cells or endothelial cells. As explained in RESULTS, it was not possible to determine the total number of dying cells, but we can state that at a given time point only a small number of dying cells was detected. Because programmed cell death may be a fast process, this small number may be large enough to remove a significant number of cells. The type II epithelial cells are viewed as putative stem cells for the type I epithelial cells (9, 10). Therefore, it is likely that, in contrast to fibroblasts, the total number of type II epithelial cells is not only reduced by programmed cell death but also by the differentiation of these cells into type I epithelial cells.

At the light and electron microscopic levels we sought apoptotic bodies and nuclear fragments, the classic signs for apoptosis (15). As expected, we detected fragmented nuclei in fibroblasts (Figures 4b and 5). Late stages of apoptosis (Figure 5b) suggest that the fragmented fibroblasts are phagocytosed by neighboring cells. In contrast to this finding, we did not find any nuclear fragments in type II epithelial cells. To clarify this discrepancy we studied a mouse cell death model. Programmed cell death of type II epithelial cells was induced by the intra-alveolar application of a programmed cell death-activating anti-FAS antibody. This antibody induces cell death of 10–20% of the type II epithelial cells. The identification of the dying cells was done by double-labeling of DNA fragmentation and in situ hybridization for surfactant protein C (28). We studied the same material with light and electron microscopy and could not find any evidence that type II epithelial cells produce nuclear fragments and/or apoptotic bodies while they are undergoing anti-FAS–induced programmed cell death (data not shown). From the results of both experimental systems we conclude that type II epithelial cells undergo programmed cell death without detectable apoptotic bodies—at least as long as they are part of the alveolar epithelium.

While searching for apoptotic bodies in electron microscopic sections, we found, besides many normal type II epithelial cells (Figure 6a), a few cells that appeared normal by morphologic criteria but which exhibited a macrophage adhering to them (Figure 6b). In addition, we found phagosomes of macrophages containing both apoptotic nuclear fragments and material of lamellar bodies (Figure 6c). Furthermore, labeled Maclura pomifera lectin histochemically stains the apical surface of rat type II epithelial cells and cyttoplasmic vacuoles of rat macrophages (34, 35, and Schittny and associates, unpublished results). A II three observations indicate that macrophages may phagocytose type II epithelial cells or material that is derived from these cells. Based on these observations, we would like to propose the following hypothesis: Type II epithelial cells undergoing programmed cell death are cleared by macrophages in early stages of the death process before nuclear fragments and apoptotic bodies are detectable. Fragmentation of the type II cell may not be necessary, because macrophages appear to be large enough to handle whole type II epithelial cells. This hypothesis is supported by the findings that, following an airway inflammation of newborn babies, intact neutrophile granulocytes are phagocytosed by bronchoalveolar macrophages (36). The situation may be different for fibroblasts, because the dying fibroblasts are cleared by neigh-
boring fibroblasts (Figure 5b), which are not specialized phagocytic cells and have the same size as the dying cells themselves.

Type II epithelial cells are not the only cells dying by programmed cell death without nuclear fragmentation. In many instances of cell death in embryos, phagocytosis appears to occur before fragmentation of the nucleus; in fact, phagocytosis can occur at any stage of programmed cell death (19, 37).

Until now, programmed cell death of noncancerous lung cells was only described for epithelial cells after lung injury. Bardales and coworkers (38) described that in early stages of acute lung injury type II epithelial cells are proliferating and that during the resolution phase of the regenerative process the disappearance of these cells was largely due to programmed cell death. Results of in vitro experiments suggest that lung fibroblasts release soluble factor(s) that induce programmed cell death of alveolar epithelial cells during repair of acute lung injury (39), and that bronchoalveolar lavage fluid obtained from patients during lung repair induces programmed cell death of both fibroblasts and endothelial cells (40). A similar function of programmed cell death was described for the transition between granulation tissues and scars of skin wounds (41).

In summary, we propose the following model for the role of programmed cell death during postnatal lung development. Normal alveolarization requires a double capillary network in the pulmonary parenchyma with a sheet of connective tissue between both layers. Dying type II epithelial cells can be removed during the repair process without nuclear fragmentation. The excess cells are removed by programmed cell death with all signs of apoptosis. Apparently, in rats more type II epithelial cells are produced during the first two postnatal weeks than needed at the end of the third week. The larger number of type II epithelial cells may serve two different functions. First, as the putative stem cells for the type I epithelial cells (9, 10), an excess of these cells ensures that type I cells can be formed in a sufficient number during alveolarization, when the alveolar surface area increases steeply. Second, the cellular excess may be beneficial for the increasing production of surfactant. A gain, the excess of type II epithelial cells is removed by programmed cell death, but here the typical morphologic signs of apoptosis do not appear, most likely because the dying cells may be removed by macrophages in an early stage of the death process.

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