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Differentiation of the Fetal Rat Pulmonary Epithelial Cells in Organotypic Culture

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In order to study the differentiation of the epithelial cells during the development of fetal rat lung tissue, histological changes in organotypic culture and in vivo were examined. Light microscopy and scanning electron microscopy were used to analyze the histological change in rat lung from the 15th day of gestation to the 10th day after birth. In organotypic culture system, the pulmonary epithelial cell differentiation was studied by scanning electron microscopy.

The results obtained from this study were as follows.

1. During development of lung, the glandular stage lasted from the 15th day to the 16th day of gestation; the canalicular stage from the 17th day to the 19th day of gestation; the saccular stage from 20th day to the birth. Alveolar stage was observed at the 3rd day of postnatal rat lung.

2. In organotypic culture of fetal rat lung cells organized alveolar-like structures resembling those of *in vivo* state were observed on the gelatin matrix. In contrast with in vivo state, fetal lung cells formed group of type II pneumocytes predominently along the contours of the matrix. These cells have large apical surface, short microvilli and secreted materials which may be surfactant. These results suggested that an organotypic culture retaining epithelial--mesenchymal relationships is appropriate culture model to study the pulmonary epithelial cell (especially type II pneumocyte) differentation.

KEY WORDS: Organotypic culture, Pulmonary epithelial cell, Differentiation

Pulmonary organogenesis depends upon sequential branching of the primitive lung bud, initially into the two bronchi with subsequent subdivisions. At this stage, the future air passages are lined by tall, undifferentiated columnar cells surrounded by a loosely arranged band of mesenchyme. The branching process will continue in explant culture. However, if the epithelial component is cultured alone, branching will cease, but can be reinitiated if the epithelium is reassociated with mesenchyme (Wessells, 1970). The mesenchyme requirement is stringently organ specific, but mesenchyme derived from the embryonic lung of one species will support development of epithelium from another species. Regional differences in inductive capacity of mesenchyme within the lung have also been shown (Wessells, 1970).

Dameron (1968), using organ culture techniques, demonstrated that, pulmonary mesenchyme is necessary for the normal development of the bronchial tube and the bronchial epithelium. A later study by Marin and Dameron (1972) showed that the mesenchyme of the lung bud can influence the rate of differentiation of the secreting alveolar epithelial cells.

Developing lung is a good material for such a study since it consists of two easily distinguished parts, one bronchial, the other alveolar. In each

fixed specimens were processed routinely and embedded in paraffin.

Scanning electron microscopy

The excised lung tissues were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4) for 3 hours, washed in the same buffer for 30 minutes, and postfixed in 1% osmium tetroxide for 1 hour. After dehydration in graded concentration of alcohols, specimens were then critical point-dried in a chamber (Ladd Research Industries, Burlington, Vt.) according to standard procedure using liquid carbon dioxide. Dried samples were mounted on aluminum SEM stubs, coated with gold palladium alloy with Ladd Sputter Coater, and examined with a Hitachi scanning electron microscope operated at 25 KV.

Organotypic cultures of fetal rat lung

Female Sprague-Dawley rats were mated with male rats and killed following timed gestation of 19 days and the fetuses were surgically removed. The average litter consisted of 12 fetuses, and these were immdiately decapitated to prevent respiration. The lungs were excised, dissected free of major airways and minced with scissors into 1 to 2 mm^3 fragments. These fragments were washed three times with PBS, and stirred in PBS containing 0.05% trypsin, 0.02% DETA until the tissue was dispersed. The trypsin was then neutralized by the addition of fetal bovine serum (FBS), and the cells suspension was filtered through Cellector tissue screen (100 mesh, Pelco), followed by centrifugation to recover the cell pellet.

This was suspended in Eagle's minimum essential medium (MEM) and the suspension was applied to the surface of a 5 mm^3 gelatin sponge (Gelfoam^R, Upjohn, Kala- mazoo, MI). The gelatin sponges were transferred to plastic culture dishes (Corning Glassworks, Corning, NY) containing culture medium with 10% FBS or without FBS. These dishes were incubated at 37°C under an atmosphere of 95% air-5% CO_2. Medium changes were made 24 hours after plating, and then 48 hours intervals during the incubation period.

For the observation of cultured lung on the gelatin matrix, samples were processed by scanning electron microscopy as described previously.

Results

Light microscopic observations

Light microscopic study of pulmonary tissue from rats at the 15th day of gestation showed large, branching glands composed of pseudostratified columnar epithelium and surrounded by a mesenchymal tissue (Fig. 1). At the 16th and the 17th day of gestation, the glands were small and consisted of a single layer of columnar epithelial cells (glandular stage). The basal surface of gland is surrounded by elongated or round connective tissue cells with relatively small nuclei (Fig. 2). In comparison with the finding at the 16th to the 17th days, the lumen of the glands were mildly dilated and the epithelial lining was thinner at the 19th day of gestation (canalicular stage) (Fig. 3), especially in the proximal areas of the primitive alveolar zones. Many capillaries were present in the stroma surrounding the glands (Fig. 4).

At the 20th day of gestational age, the glands were round and consisted of cuboidal epithelium, while the primitive alveolar zones were lined by flattened or rounded epithelial cells (Fig. 5). The primordia of alveolar septa showed minimal protrusions into the lumen. Capillaries are prominent in stroma. At the 21st day of gestation, the lumen of the primitive alveolar zones were more dilated and the epithelium was thinner than in previous day. Some primordia of alveolar septa had grown and already protruded into the alveolar lumen, forming alveolar septa.

In the 1st to the 3rd day of postnatal rat lung, light microscopic observations showed that many of the alveolar septa were well developed (alveolar stage) (Fig. 6). The septa of lung are rather thick and they mostly possess capillaries on either side (primary septa). The air spaces seem to be irregular in shape, short ridges appearing on the surface of septa.

On the 7th day after birth (Fig. 7) the lungs exhibit some changes, when compared to those of the Fig. 6. The previously straight and smooth septa have now become covered on either side with buds bulging into the air space. The saccules start to be transformed into alveolar sac. The

part epithelial cells are different and underlying connective tissue cells undergo different transformation. Since there is only one mesenchymal cell type, the septal cell, it is possible to observe the intermediate stages of mesenchymal cell transformations in the developing alveolar septa (Ryan, 1969; Yu and Sun, 1972).

One of the most important processes in pulmonary development is the formation of alveolus, which subdivides the terminal saccules and provides a large surface for gas exchange (Fukuda *et al.*, 1983). the normal mammalian alveolus is lined by two types of epithelium: type I pneumocytes form a complete but very thin layer lining all alveolar spaces, and type II pneumocytes occur singly or in small groups of two to three cells between the surface squamous cells with which they are globular in shape. Type II pneumocytes are considered to be responsible for the production of pulmonary surfactant (Askin and Kuhn, 1971; Van Golde, 1976).

The appearance of these type II pneumocytes during fetal life is a major event in the course of functional lung develoment, and a better understanding of mechanisms triggering or regulating their differentiation has been the aim of most experiments on fetal lung maturation. Moreover, the association between immature lung development, insufficient storages of pulmonary surfactant, and the manifestation of respiratory distress syndrome (RDS) has stimulated many investigations concerning factors which influence fetal lung maturation and the differentiation of type II pneumocytes (Avery *et al.*, 1981).

A well defined *in vitro* system in which the cellular organization and degree of fetal lung cell differentiation closely resemble the *in vivo* pattern would be an ideal model in which to study normal fetal lung cell differentiation and to examine agents that may influence the regulation of lung maturation. Thus several *in vitro* systems of fetal lung have been developed to study the regulation of the lung function during fetal development.

Torday (1980), using fetal rabbit lung in monolayer cell cultures, found that the ongoing epithelial maturation occurred in response to hormones normally present in the bovine fetal calf serum used as a mitogen in the culture medium. Tanswell and his colleagues (1983) confirmed this observation in studies of fetal rat lung in monolayer cell cultures Although this culture method was a good model for observing gestation-dependent effects of various agents, the lack of cellular organization in monolayer cell cultures limited the study of cellular interrelationships.

While cell-to-cell interactions are retained in organ cultures of fetal lung, these cultures cannot be maintained *in vitro* for adequate periods of time (Gross *et al.*, 1978). Dougls and Teel (1976) and coworkers (Engle *et al.*, 1980) have described an organotypic culture system in which fetal rat lung cells cultured on a gelatin sponge matrix. These culture models gave the advantage of offering a stable and defined environment in which to compare biochemical analyses with direct observations of morphogenesis.

Therefore, the purpose of the present work is to compare the morphological evolution of connective tissue cells with that of epithelial cells in perinatal rat lung, with emphasis on the early stages of formation of alveolar structures. We also studied the role for epithelio-mesenchymal interactions in the control of cell proliferation and morphogenesis. For this purpose, we have used the organotypic cell culture system which was designed to characterize the epithelial cell types by scanning electron microscopy.

Materials and Methods

Tissue preparation

Female Sprague-Dawley rats were placed overnight with males and examined in the following morning either for vaginal plugs or for the presence of sperm in vaginal smear. If positive findings were made, this day was considered day zero of gestation. Specimens were obtained from the 15th fetal age to the 10th postnatal day. For the tissue preparation of the lung, postnatal rats were anesthetized with ether. After laparotomy and perforation of the diaphragm, in order to create a pneumothorax, the lung were fixed by intratracheal instillation of buffered formalin. After fixation for 1 hour, blocks of tissue were taken from right middle part of the lung. In the case of fetuses, the whole lung was immersed in the same fixative solution. For ordinary light microscopy,

smoothness of the primary septa is altered by the outgrowth of many small ridges. These forming septa (secondary septa) subdivide the primitive air spaces and give rise to the alveoli.

Scanning electron microsopic observations

Examination of the 15th day fetal rat lung showed that large canal lining epithelial cells were open (Fig. 8A). Fig. 8B depicted scanning electron micrograph of cubodidal and flattened epithelial cells lining a canal. These epithelial cells were relatively same in size and shape. At the 19th day of gestation, in the canalicular stage of lung development, rapid changes in cell structure commence with the appearance of differentiating type II pneumocytes (Fig. 9). Differentiating type II pneumocytes, bearing small and scanty microvilli, and particularly observed in the transition zones from bronchioli to sacculi.

In lung tissue of newborn rat, developed large sacculi can be recognized, separated from each other by a primary septum (Fig. 10). Concomitant with the development and thining of the primary septa, pores of Kohn may sometimes be detected. The smooth walled primary septa was modified by the development of low secondary crests. A secondary crest was a ridge running along a primary saccule wall dividing the saccule into two or more parts.

At the 7th day of postnatal periods, primary septa give rise to secondary septa into the saccular lumen, thus forming alveoli. As the alveolization progresses, the septa often show a single central capillary system. In Fig. 11A, large tubule of lung at the 7th day after birth is shown. As seen in Fig. 11B, two types of tubular cells were recognized. Some tubular cells were ciliated and others were non-ciliated.

Organotypic cultures of fetal rat lung

Organotypic cultures of fetal rat ung cells taken at day 19 of gestation exhibited tubular epithelial structure. Fig. 12 shows a piece of gelatin sponges containing a group of fetal rat lung cells (culture day 2) as viewed by the scanning electron microscope. Scanning electron microscopy showed several types of cellular regions in the organotypic lung cell cultures. Areas of loosely accociated cells were observed on the outer surface of the gelatin matrix. At higher magnification, these areas were found to consist of a variety of cell types ranging from very flat to cuboidal cells (Fig. 13).

In culture day 4, more differentiated regions showed a distinct tubule-like epithelial structures (Figs. 14 and 15), which were found to contain both cuboidal and flattened cells. Beneath the tubule-like structure, quite deep alveolar structures were also formed.

In culture day 5, closely associated fetal lung cells forming an organized cell layer along the contours of the culture matrix are shown in Fig. 16. Cell boundaries were well defined in this cellular layer, which had region of cells with large apical surface and short microvilli (Fig. 17). On the outer surface of these cells, secreted materials which may be surfactant were observed.

Discussion

In the course of the development of the mammalian lung, periods can be discerned which are characterized by distinct histological features. The distinguishing feature of the pseudograndular period is the presence of tubules lined by a columnar epithelium and surrounded by mesenchyme. The canalicular period is characterized by the occurrence of tubules lined by cuboidal epithelium and situated close to the capillary system. The specific feature of the terminal-sac period is the presence lumen and rather smooth walls and form clusters (Banks and Epling, 1971; Adamson and Bowsen, 1975). The alveolar period is marked by the occurrence of distinct pouches (called alveoli or alveolar pouches) in the hitherto relatively smooth walls of the respiratory structures.

In the present study, the glandular stage occurred between the 15th and the 16th day of gestation. During this period the major conducting airways developed by growth and ramification of primitive epithelial tubules. The canalicular stage of development, occurred between the 17th and the 18th day of gestation. During this period the appearance of the lung dramatically altered with the rapid expansion of peripheral airway units. These developed from the original tubules by growth and subdivision in an elongated manner,

effectively increasing the future air space volume of the lungs. The saccular stage of development, occurred from day 19 to birth.

During this period saccules were formed by compartmentalizing the larger canal structures. According to Burri and Weibel (1977), alveolar septa of adult lung can be classified into primary alveolar septa, which are the walls of the saccules, and secondary alveolar septa, which protrude from the saccular walls and form the tissue crests that subdivide the terminal saccules. It has been suggested that the time of formation of secondary alveolar septa differs from species to species; for example, in the rat lung the bulk of the alveoli are formed between the 4th day and the 13th day after birth (Burri, 1974).

At birth the rat lung is practically devoid of alveoli in the present investigation. The newborn parenchyma is made of smooth-walled channels and saccules delineated by relatively thick septa, called primary septa. The primitive alveoli were detected at postnatal developmental periods. The outgrowth of secondary septa from buds of smooth-walled terminal saccules agrees fairly closely with the general process of alveolarization observed in rats (Burri, 1974) and in mice (Amy *et al.*, 1977). Boyden and Tompsett (1971) and Emery (1969) have described two patterns of lung alveolarization. One involves outpouching of gas exchange units from the walls of terminal bronchioles, transforming these conducting airways into respiratory airways. The second mechanism of alveolar formation involves the budding of new septa from the saccule walls of the immature lung.

The alveoli of the mammalian lung are lined with a lipid-rich substance known as surfactant. Earlier studies of pulmonary epithelial cells have generally employed in vivo methods, or mixed lung cell cultures. Using these techniques a number of investigators have found that a variety of precursors can be used for pulmonary phospholipid synthesis. However, because the lung is composed of approximately 40 cell types (Sorokin, 1970), the precise mechanisms of surfactant synthesis in the type II pneumocytes cannot be obtained from such studies.

The organotypic cell culture system has several advantages over the other lung cell culture methods. The present work described that monodisperse fetal rat lung cells reaggregate to form alveolar-like structures when cultured on three-dimensional gelform matrices. Unlike mixed lung cell cultures or explants, the cells in organotypic culture are predominantly type II pneumocytes. Especially, type II pneumocytes in organotypic cultures retain the structural integrity, alveolar structure of the intact lung, and cell to cell contact.

The principal differences between monolayer and organotypic cell cultures lie in the organization of the fetal rat lung cells. The gelatin sponges used in organotypic cultures provide a matrix against which the cells can reaggregate to form spatial relationships close to those seen in vivo. The cellular arrangements observed in organotypic lung cell cultures may play a role for the mechanisms of epithelial cell differentiation. Since there is a strong temporal relationship according to the degree of direct contact between mesenchymal and epithelial cells during fetal lung development (Marin *et al.*, 1982), it is possible that epithelial cell differentiation may be under mesenchymal influence. As spatial relationships are retained in organotypic culture system, this organotypic cultures is appropriate culture system to study the pulmonary epithelial cell (especially type II pneumocytes) differentiation under mesenchymal influences.

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기관형 배양에서 흰쥐 태자 폐상피세포의 분화

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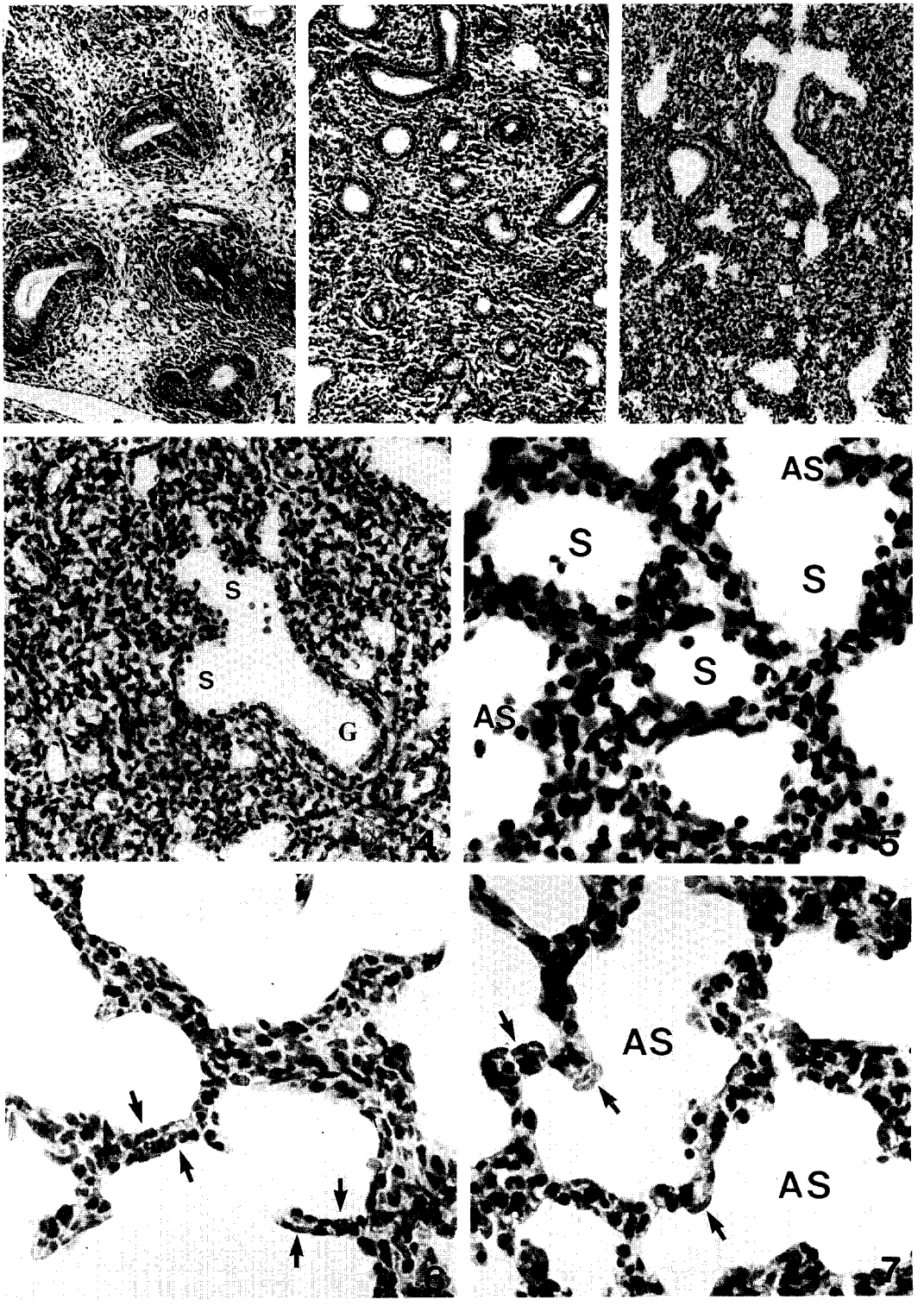
폐조직의 발달과정 동안 상피세포의 분화과정을 관찰하기 위하여 기관형 배양계에서와 생체에서의 조직학적 변화과정을 조사하였다. 출생전후의 흰쥐를 대상으로 태생 15일부터 10일까지 조직학적 변화양상을 광학현미경과 주사전자현미경으로 관찰하였고, 태자 폐조직의 기관형 배양을 시행하여 상피세포와 간엽세포간의 관계가 폐상피세포 분화에 미치는 영향을 주사전자현미경으로 관찰하여 다음과 같은 결론을 얻었다.

1. 폐조직발달은 태생 15일 및 16일의 선기, 태생 17일부터 19일의 세관기 그리고 태생 20일 이후부터 생후 1일까지의 폐포낭기를 관찰하였다. 생후 1일의 폐조직에서는 폐포를 관찰할 수 없었고 대신 일차격막을 가진 saccule이 관찰되었으며 생후 3일 이후부터 이차 격막으로 나누어진 폐포를 관찰할 수 있었다. 따라서 흰쥐에서 폐포는 생후에 발달되는 것으로 생각된다.

2. 기관형 배양계에서 흰쥐 태자 폐조직세포는 젤라틴 기질 위에서 다시 재집합하여 생체에서와 같은 폐포구조를 나타내었는데 주로 type II 폐포상피세포가 군집을 이루어 나타나는 것이 생체계와 다른점이었다. 이 세포는 type II 폐상피세포의 특징인 미세융모와 surfactant로 추정되는 분비물질을 갖고 있어, 상피세포와 간엽세포간의 관계가 유지되는 기관형 배양이 폐상피세포의 분화과정에 미치는 간엽세포의 영향을 알아보는데 적합한 배양모델임을 알 수 있었다.

Explanation of Figures

Fig. 1. The immature lung of a fetus at the 15th day, showing large glands composed of pseudostratified columnar epithelium and surrounded by a mesenchymal cells. × 100.
Fig. 2. The glandular stage of the 17th day fetal rat lung. The glands are small and consisted of a single layer of columnar epithelial cells. × 100.
Fig. 3. The canalicular stage of the 19th day fetal rat lung. In comparison with figure 2, the lumen of the glands are mildy dilated, and the epithelial lining become thinner. × 100.
Fig. 4. Fetal rat lung, day 19. A gland (G) divides into two primary saccules (S). Many capillaries are present in the stroma surrounding the glands. × 200.
Fig. 5. Fetal rat lung, day 20. Primary saccules (S) are formed and the primordia of alveolar septa (AS) protrude into the lumen of the saccules. × 400.
Fig. 6. Rat lung of the 3rd day after birth. The septa of lung possess capillaries on either side (Primary septa, arrows). × 400.
Fig. 7. Rat lung of the 7th day after birth. The saccules start to be transformed into alveolar sac (AS). The smoothness of the primary septa is altered by the outgrowth of many small ridges. These ridges possess capillaries on one side (secondary speta, arrows), and subdivide the alveolar sac into the alveoli. × 400.
Fig. 8A. A low power scanning electron micrograph of fetal rat lung at the 15th day of gestation. The large canal lining epithelial cells are open. × 300.
Fig. 8B. High magnification of epithelial cells lining a canal show regularity in shape. × 2,000.
Fig. 9. At the 19th day of gestation, the fetal rat lung commence with the appearance of differentiating type II pneumocytes. Differentiating type II peneumocytes bearing small and scanty microvilli are observed. × 6,000.
Fig. 10. Newborn rat lung. The saccules are smoothly contoured by straight and thick septa. These septa present at birth are called primary septa. × 600.
Fig. 11A. At the 7th day, postnatal rat lung show a large tubule. × 400.
Fig. 11B. Magnification field of tubular cells illustrated in figure 11A. Two types of tubular cells consisting of ciliated and non-ciliated cells are observed. × 3,000.
Fig. 12. In 2 days cultured fetal rat lung cells taken at the 19th day of gestation exhibit a distinct saccule-like epithelial structure. Trabeculae of gelatin matrix (G) are also seen. × 300.
Fig. 13. Similar structure of saccules containing cuboidal and flat cells are found at high magnification. × 2,000.
Fig. 14. In culture day 4, highly orgainzed saccular and tubule-like structures are seen. Beneath the tubule-like structures, quite deep alveolar structures (arrow) are recognized. × 2,000.
Fig. 15. Alveolar-like epithelial structures which are composed of cubolidal and flat cells are observed. × 4,000.
Fig. 16. Closely associated fetal lung cells (arrows) cultured for 5 days forming an orgnized cell layer along the contours of the culture matrix are seen. × 300.
Fig. 17. High magnification of organized cell depicted in figure 16. These cells have large apical surface and short microvilli. On the outer surface of these cells, secreted materials which may be surfactant, are observed. × 8,000.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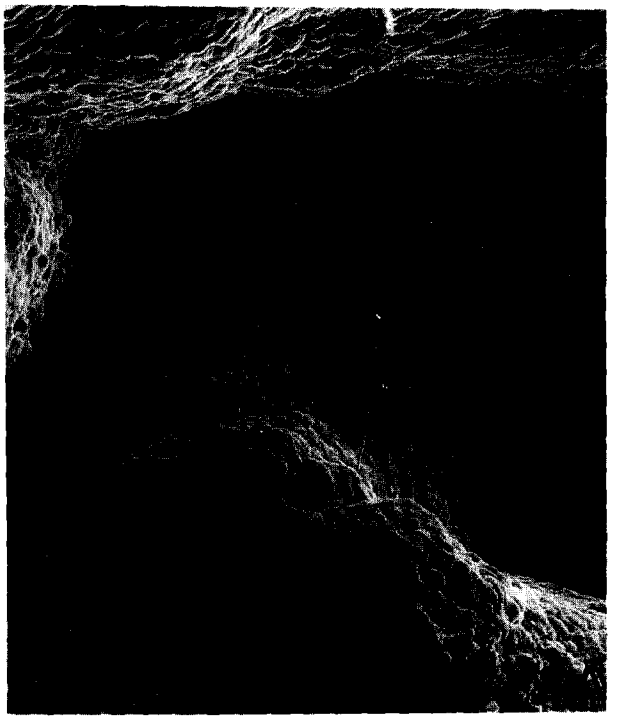

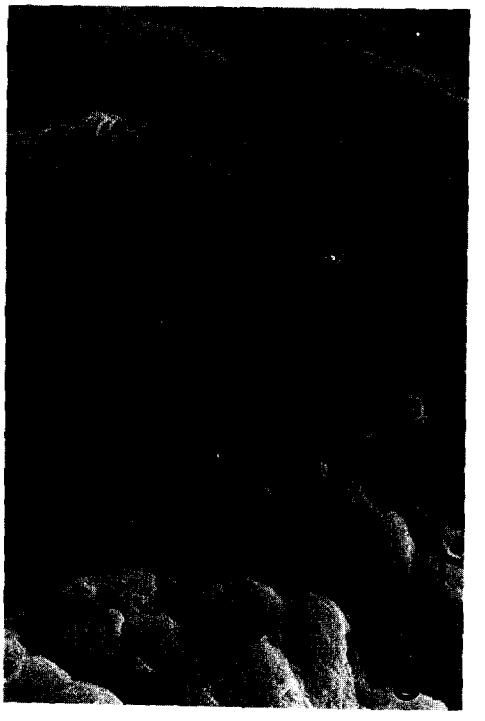

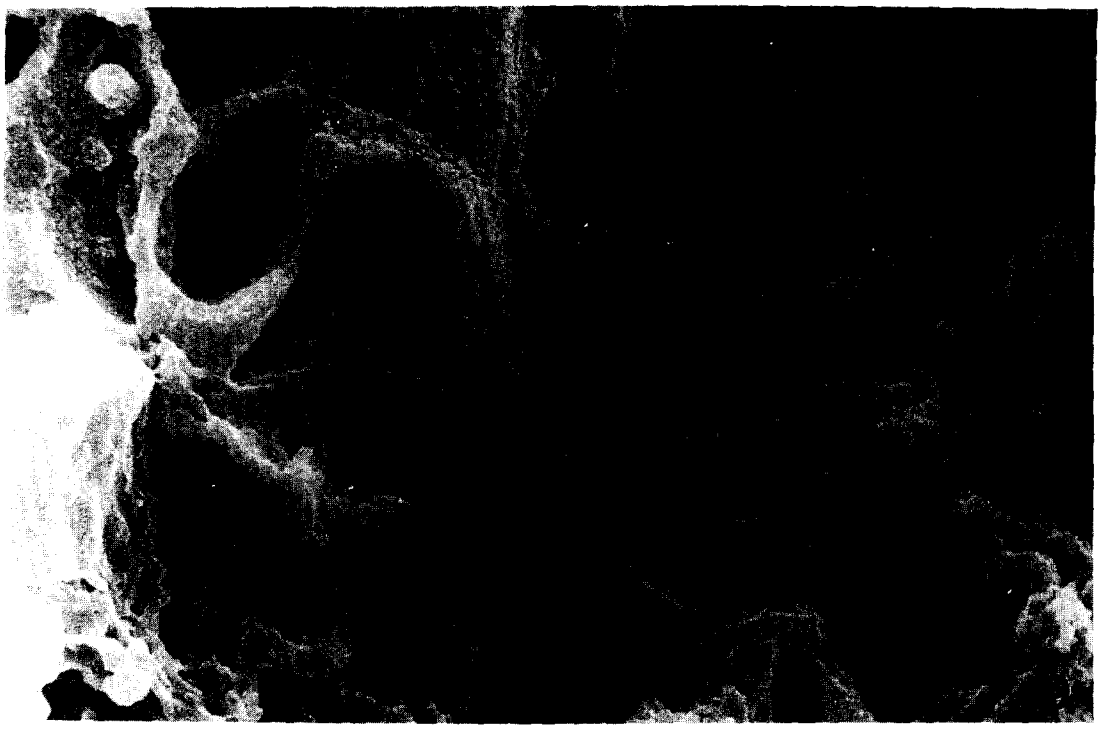

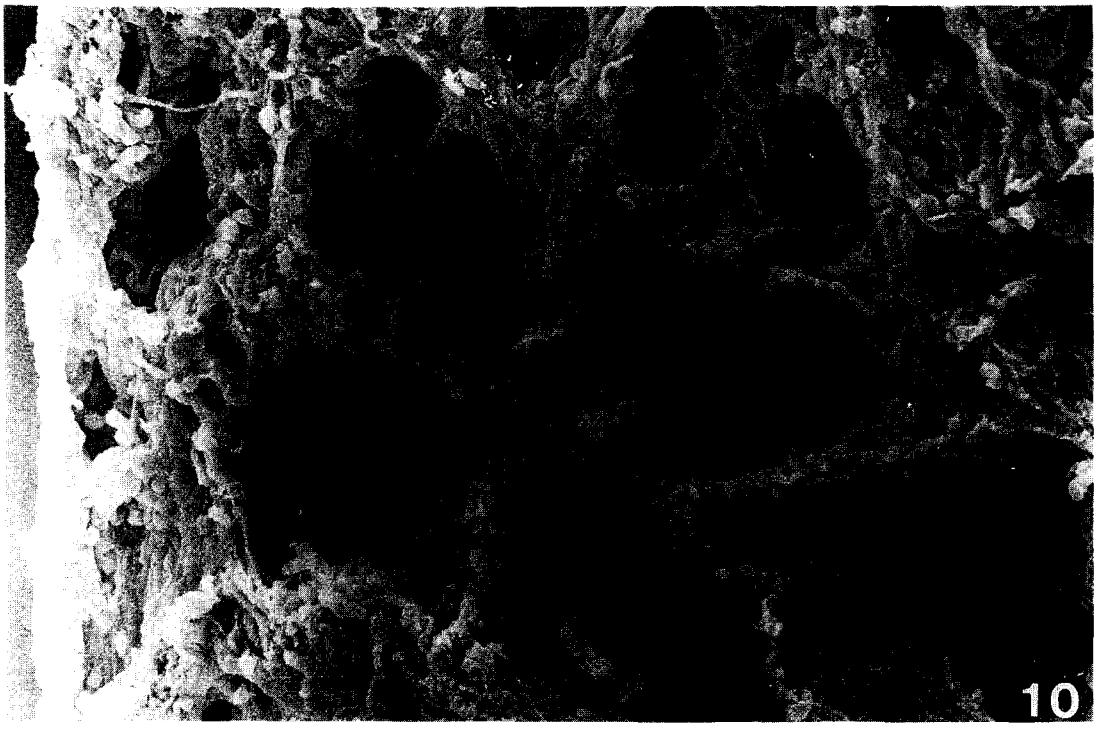
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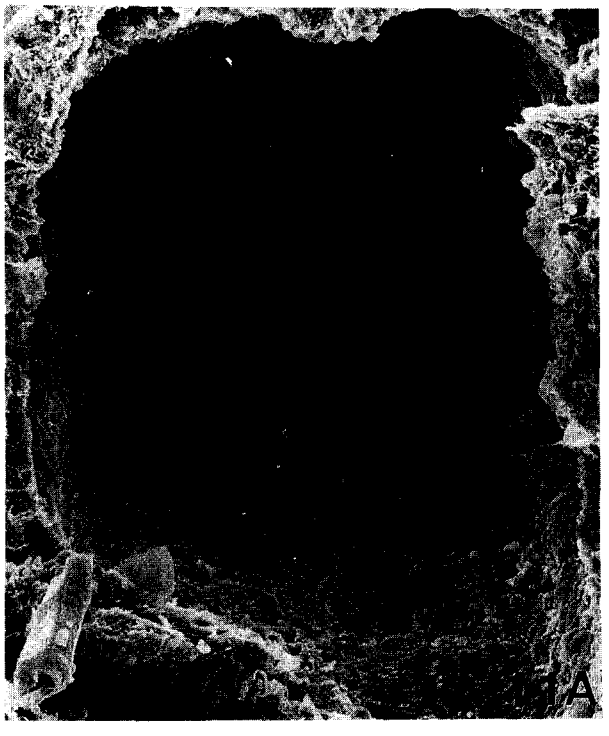
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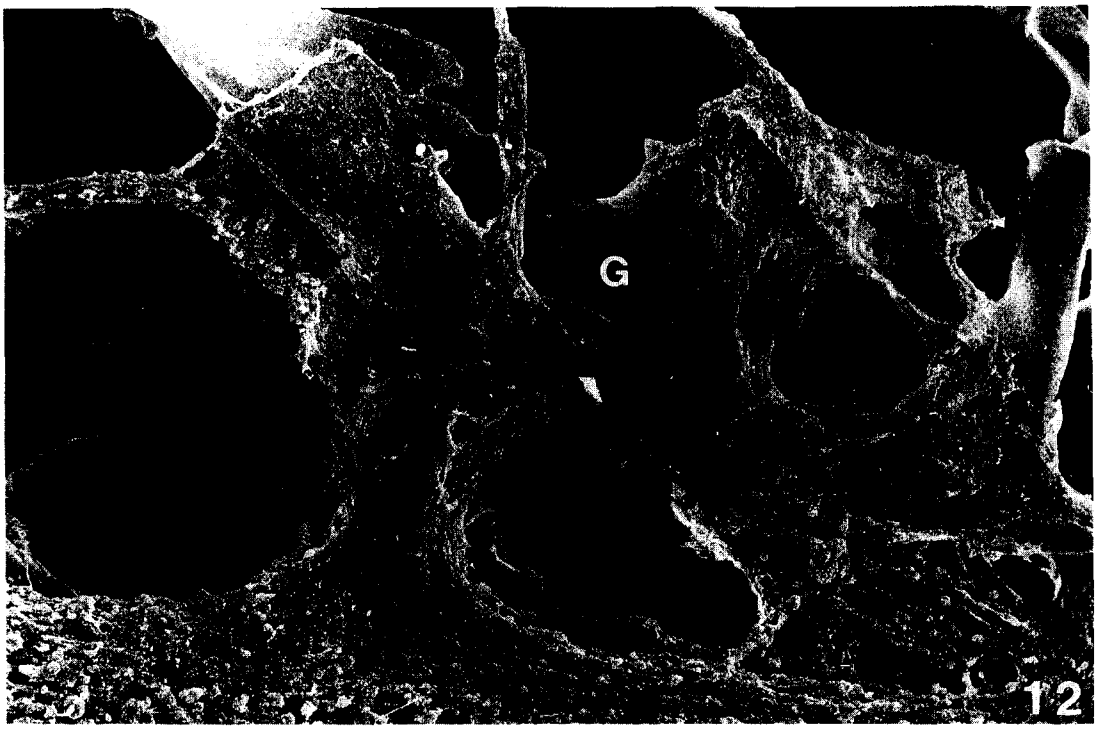
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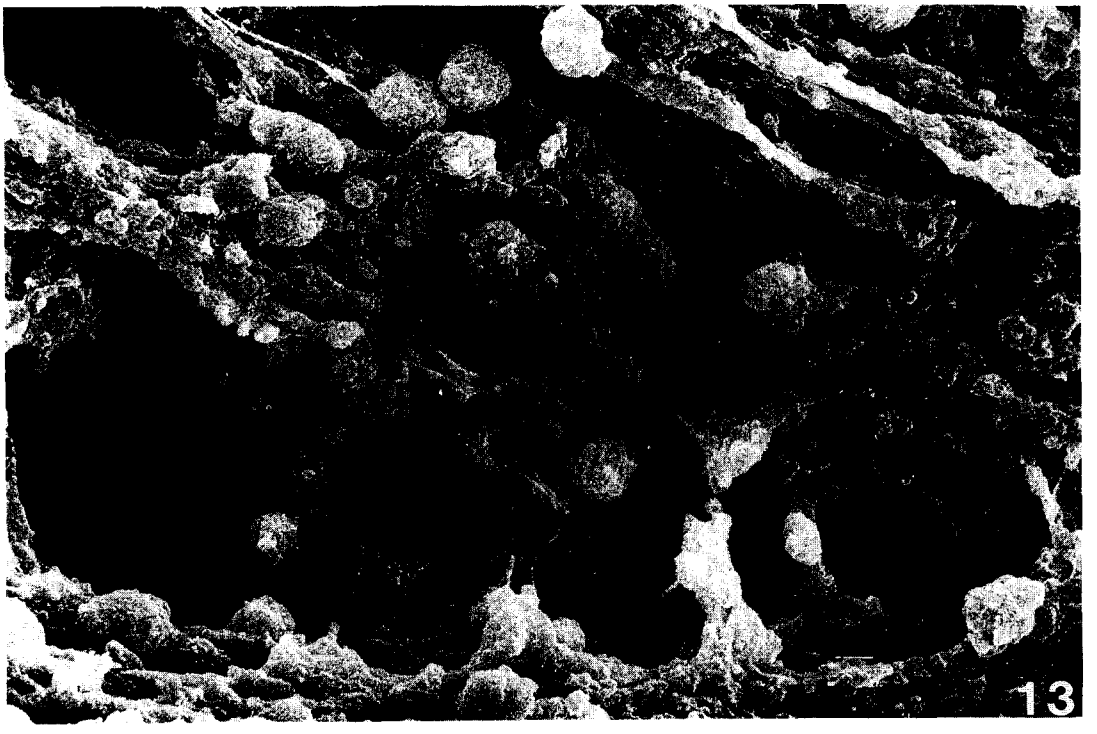
12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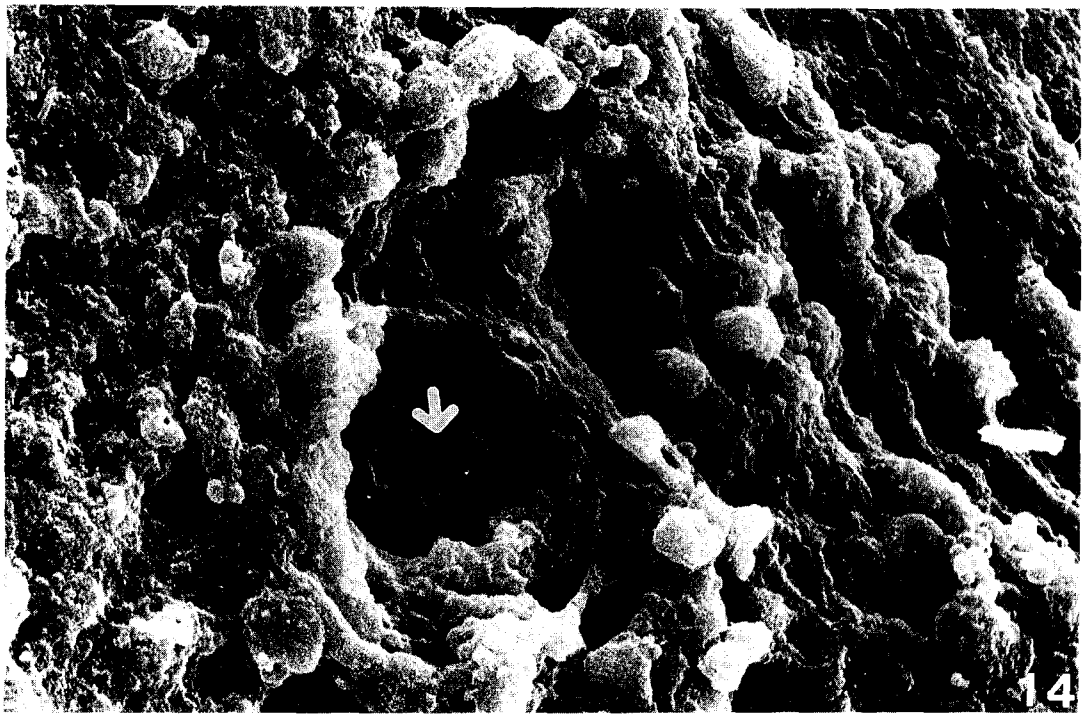
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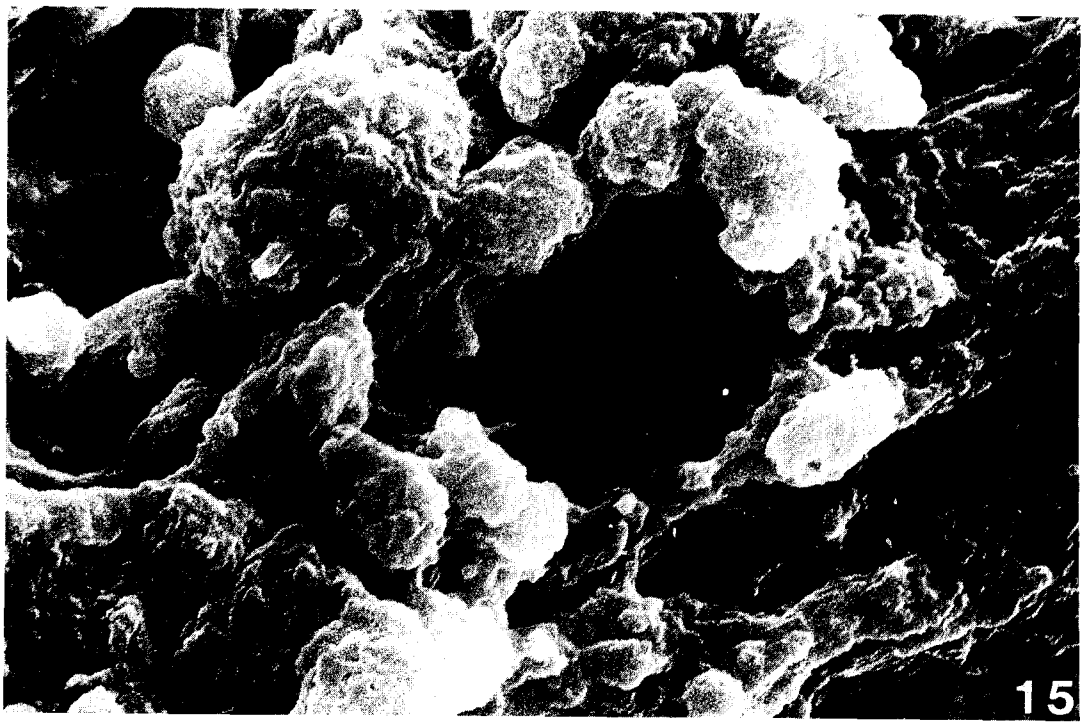
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