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In vitro Behavior of Human Fetal Lung Maintained in Organ Culture

Light and Electron Microscopic Studies

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Summary. Human fetal lung obtained from 9–26= weeks= old embryos were maintained in organ culture for 2–5 weeks. The in vitro survival and changes are clearly age dependent. The best survival was obtained with lung tissue from the early glandular period. With these young embryos tubular dilation was frequent during the 1st week. The relatively short duration of culture permitted only a fragmentary study of differentiation of the human lung in vitro but, with the exception of tubular dilations, most of the in vitro changes were also found during lung differentiation in vivo = monostratification of epithelium, bronchiolar development, decrease in glycogen, appearance of myelinlike figures, fibroblastic and myoblastic transformation of mesenchymal cells.

Key words: Human fetal lung — Organ culture — Light and Electron Microscopy — Differentiation — Degeneration.

The in vitro behavior of embryonic lung has been studied in the chick, the mouse, the rat, and the guinea pig (Alescio, 1961; Dameron, 1961; Sorokin, 1961; Taderera, 1967; Adamson and Bowden, 1975; Noack et al., 1970; Resnick et al., 1974). From these quickly maturing embryos, various processes of differentiation have been studied in vitro by organ culture (morphologic development, mesenchymal-epithelial interactions, etc.), but in vitro embryonic lung does not reach its full size and the alveolar period is rarely obtained, possibly due to deficiencies in hormonal regulation, vascularization, and mechanical expansion.

There are comparatively few observations on human embryonic lung culture. The duration of culture is very short compared to the duration of gestation and data on in vitro differentiation are therefore fragmentary (Chesterman and Franks, 1960; Glucksmann, 1964), or are concerned with toxicity studies in the neonatal period (Boat et al., 1973).

We have attempted to overcome these difficulties by maintaining in organ culture for 2–5 weeks human fetal lung obtained from embryos of 9–26 weeks gestation, thus covering the three periods of human fetal lung development.

Material and Methods

Lung tissue was obtained from 31 human embryos 9–26 weeks old (Table 1) procured by therapeutic abortion. The gestational age was evaluated by comparing the crown-rump length with Patten's table (1968).

The culture technique was as described previously (Rousseau et al., 1974), using "Falcon" plastic organ culture dishes and disks of "Millipore" filter supports. Cultures were grown at 37° C in Eagle's medium (Inst. Pasteur, Paris) supplemented with 10% decomplemented calf serum and antibiotics (penicillin 200 μ/ml, streptomycin 0.1 mg/ml), and in the presence of air containing 5% CO_2 inside Lwoff boxes. The culture was maintained for 2–5 weeks.

Table 1. Crown-rump length and gestational age

No.	Crown-rump length (mm)	Gestational age (week)	Electron micro-scopy	In vitro survival
1	40	9		Average
2	45	9–10	+	Good
3–4–5	50	10	+	Good
6	55	10	+	Good
7	58	10–11		Necrotic
8–9	65	11		Good
10	70	11–12		Good
11	80	12		Necrotic
12	85	12–13		Average
13–14	90	13	+	Good
15	95	13		Average
16	100	14		Good
17–18	110	14		Average
19	130	16		Average
20–21	150	17		Average
22–23	155	17–18		Average
24	160	18		Average
25	170	19		Average
26	200	22		Good
27–28	210	22		Average
29	214	23		Necrotic
30–31	250	26		Good

Samples were examined weekly by light microscopy. Explants were fixed for 3 h in alcoholic Bouin's fluid and embedded in paraffin; 5 μm sections were stained with hematoxylin eosin or with periodic acid schiff (PAS).

Electron microscopy was performed on three embryos aged $9^1/_2$–13 weeks of gestation (the early glandular period). Samples were examined weekly until the 3rd week in vitro.

The 2 h fixation in 1.7% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3 was followed by at least three rinses in buffer and by a 30′ postfixation in 1% Osmium tetroxide; all processing being carried out at 4° C. Specimens were dehydrated and embedded in Epon.

The 1 μ thick sections were stained by toluidine blue. Thin sections were contrasted by uranyl acetate in alcoholic solution and lead citrate. Glycogen was demonstrated by the Patag reaction (Thiery, 1967). Sections were examined in a Siemens 101 Elmiskop.

Results

Light Microscopy

The three periods (glandular, canalicular, alveolar) described by Loosli and Potter (1951) as covering human embryonic lung development were present in our material.

1. Glandular Period (20–130 mm). (a) In the early glandular period (20–70 mm) mesenchyma was predominant surrounding scattered pseudostratified epithelial tubules sometimes limited by a thin basal membrane. A few capillaries were also present. The mesenchyma was slightly PAS-positive while epithelial structures showed a very strong reaction.

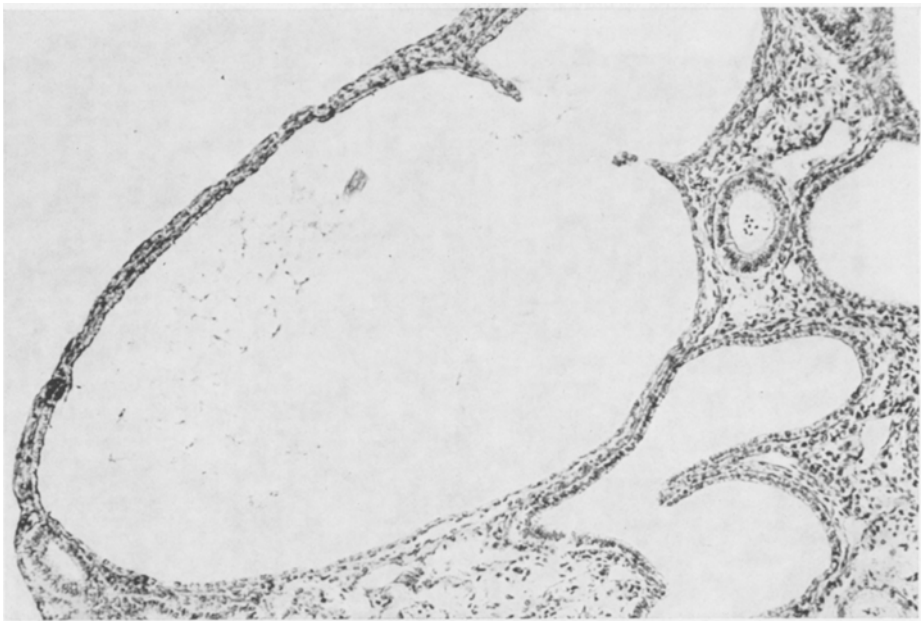

Fig. 1. Early glandular period (50 mm). Lung explant after 8 days in vitro. Tubular dilation is important but not all tubules are affected (×100)

From the 4th day of culture some tubules appeared dilated (Fig. 1) and the epithelial pseudostratification became simpler. In particular, the thickness of cells lining the dilated tubules was reduced to the size of the nucleus. The amount of interstitial tissue decreased. Mitoses could be seen in both interstitium and epithelium.

Necrosis of capillaries was observed around the 7th day and red blood cells were no longer clearly seen. In rare cases an eosinophilic substance was noted in the lumen of slightly dilated tubules around the 14th day. Sometimes the epithelium showed folds, possibly due to cell proliferation of denoting bronchial differentiation (Fig. 2).

Limited additional changes were noted up to the 26th day, but tubular dilation sometimes progressed to rupture of tubules. The tubular epithelium was generally simple (nonstratified) and an encapsulation of the explant was often noted.

The PAS reaction was always positive but became less intense with time, except for the basal membranes, where it was stronger.

(b) Later, in the glandular period (70–130 mm), an increased number of capillaries was noted. The mesenchymal cell population was denser close to the tubules and frequent contacts were observed between capillaries and epithelium.

In culture the tubules were clearly delineated and the process of tubular dilation was still evident. The tubular epithelium was of the simple nonstratified variety (Fig. 3). During the 1st week of culture, slight fibrosis was noted and in the 3rd week some tubules were filled with an eosinophilic substance mixed with debris.

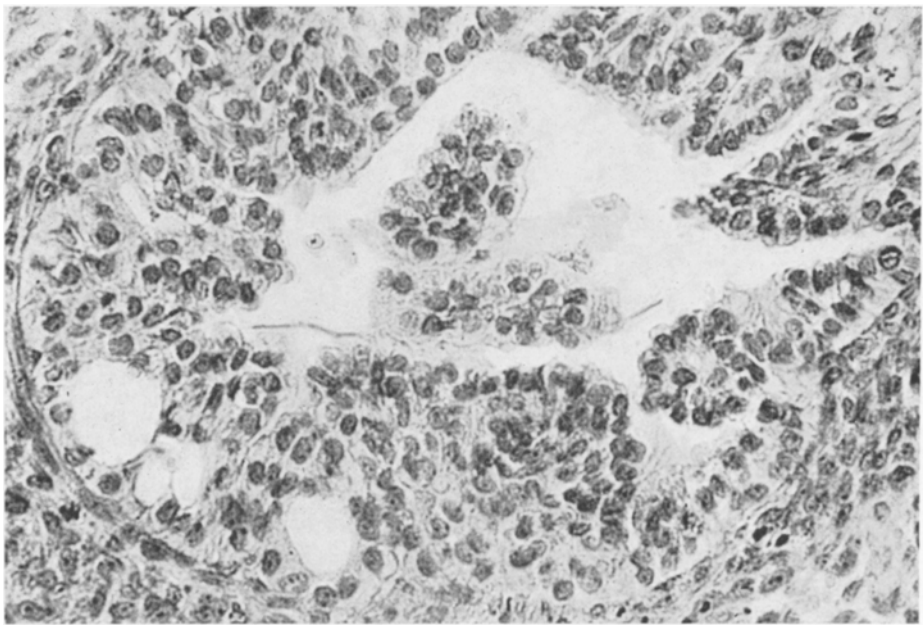

Fig. 2. Early glandular period (50 mm). Lung explant after 18 days in vitro. Folds are seen in some tubular epithelium (×400)

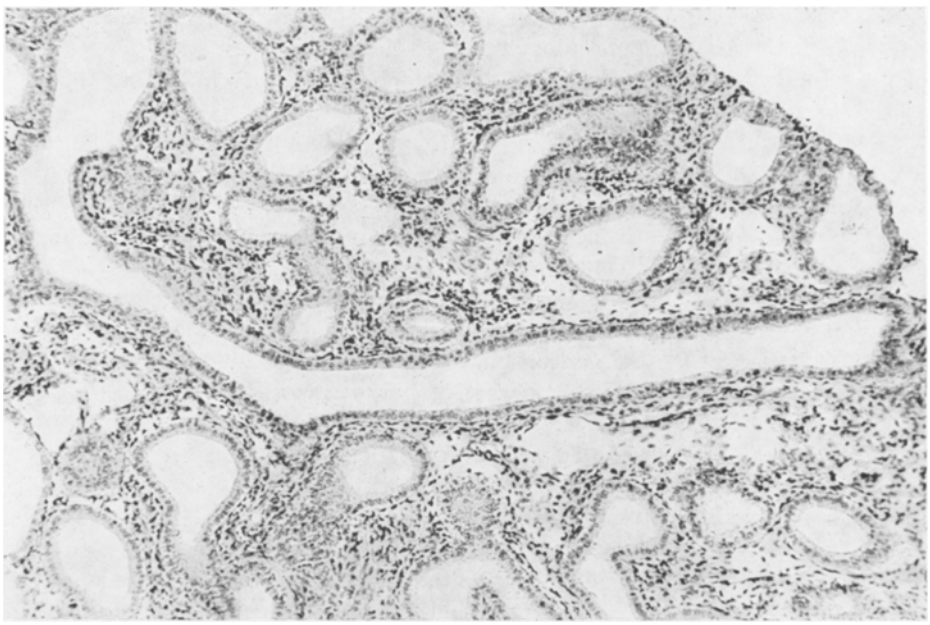

Fig. 3. Glandular period (90 mm). Lung explant after 4 days in vitro. Epithelium is of the simple and stratified variety (×100)

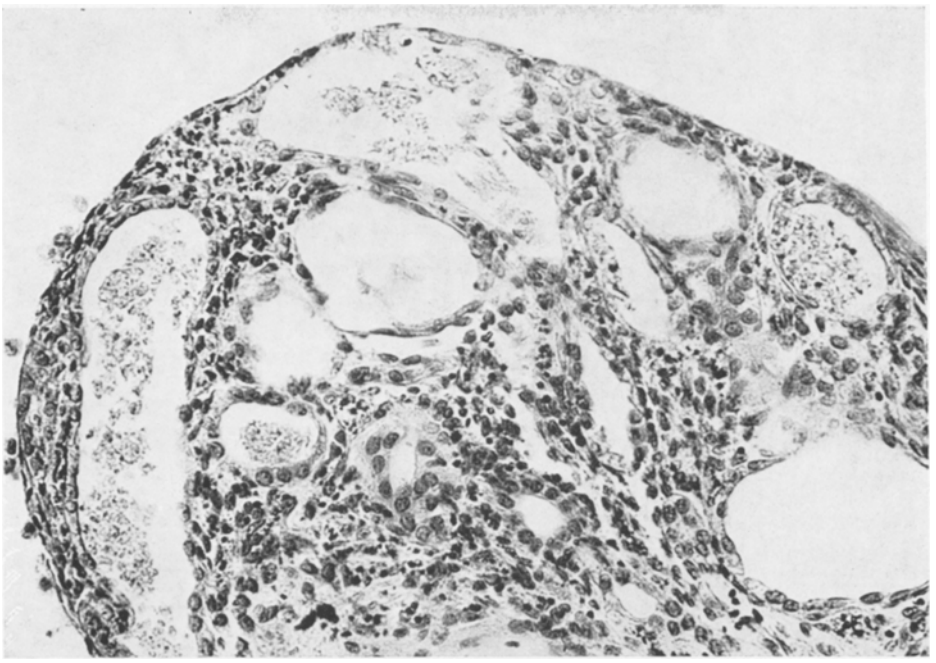

Fig. 4. Canalicular period (210 mm). Lung explant after 8 days in vitro. Necrotic cell clusters are seen in some tubules. Mesenchyma becomes fibrous and necrotic (×250)

The PAS reaction was slightly less intense than before and the tubules were not equally well stained. As culture progressed, the reaction became more positive in epithelium of the dilated tubules.

2. Canalicular Period (150–210 mm). Before culture, capillaries were in close contact with the cuboidal epithelium. Two kinds of tubules could then be distinguished. Some had a ciliated columnar epithelium, others a defined cuboidal one with an irregularly shaped lumen.

The bronchiolar structures sometimes have a clearly ciliated epithelium. The interstitium was reduced. There was a slight PAS reaction localized only on tubular membranes.

As culture progressed, the mesenchyma became fibrous, especially around some capillaries. About the 7th day some tubules were filled with necrotic cell clusters (Fig. 4). In one case dilation was noted. The two kinds of tubules remained distinct throughout culture. Only the tubular epithelium was still proliferating and showed mitotic activity until the 35th day.

During culture, a strongly positive PAS reaction persisted on tubular basement membranes.

3. Alveolar Period (214–250 mm). Capillaries were very numerous and intimately associated to prealveolar structures. Ciliated bronchiolar tubules were clearly seen.

During culture fibrosis occurred around some capillaries. Necrotic blood cells, macrophages, and epithelial cells were found in the tubular lumens amidst PAS-positive material (Fig. 5).

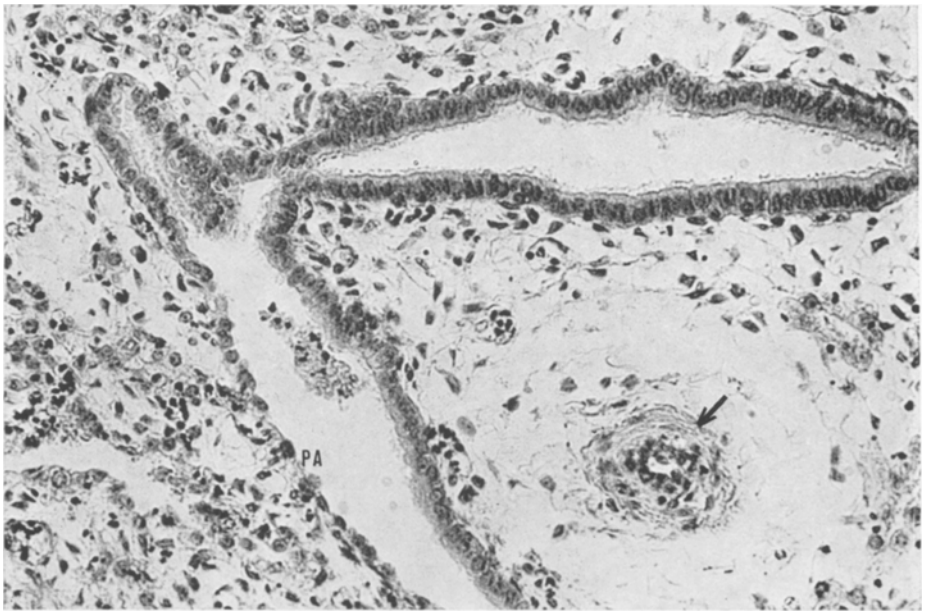

Fig. 5. Alveolar period (250 mm). Lung explant after 11 days in vitro. Ciliated bronchiolar tubules are clearly seen. That ciliated epithelium is continuated with prealveolar epithelium (PA). Note fibrosis of a capillary (*arrow:* →) (×250)

Sometimes a very slight tubular dilation was noted, but not comparable to that observed earlier. Only one case (250 mm) showed a good survival until the 4th week.

Electron Microscopy

Electron microscopic observations were limited to the early glandular period.

Day 0, from a $9^1/_2$ Week-Old-Fetus. In 1 μ= thick sections, stratified tubules with a narrow lumen were seen in a vascularized stroma. The interstitial tissue consisted of a mixed population of cells more numerous in the vicinity of the tubules.

The superficial epithelial cells of the tubules exhibiting short microvilli are separated from each other by large intercellular spaces. At the luminal pole, the cells were held by a junctional complex of a superficial tight junction and by series of gap junctions (Fig. 6). The cells had a large ovoid interphasic nucleus, with a large nucleolus in the shape of a coiled skein. Dispersed chromatin was regularly scattered in the nucleoplasm with a few clumps of condensed chromatin, the latter more concentrated along the nuclear envelope.

Extensive areas of the cytoplasm were occupied by particulate material, shown by the PATAG reaction to be glycogen, leaving little room for organelles (Fig. 7). Centriolar structures were seen in a very apical position. Supranuclear mitochondria, showed regular cristae. The Golgi apparatus was apical and well developed, the rough endoplasmic reticulum (RER) scanty and lysosomes few in number. Near the basal plasma membrane, the cytoplasm had a denser aspect

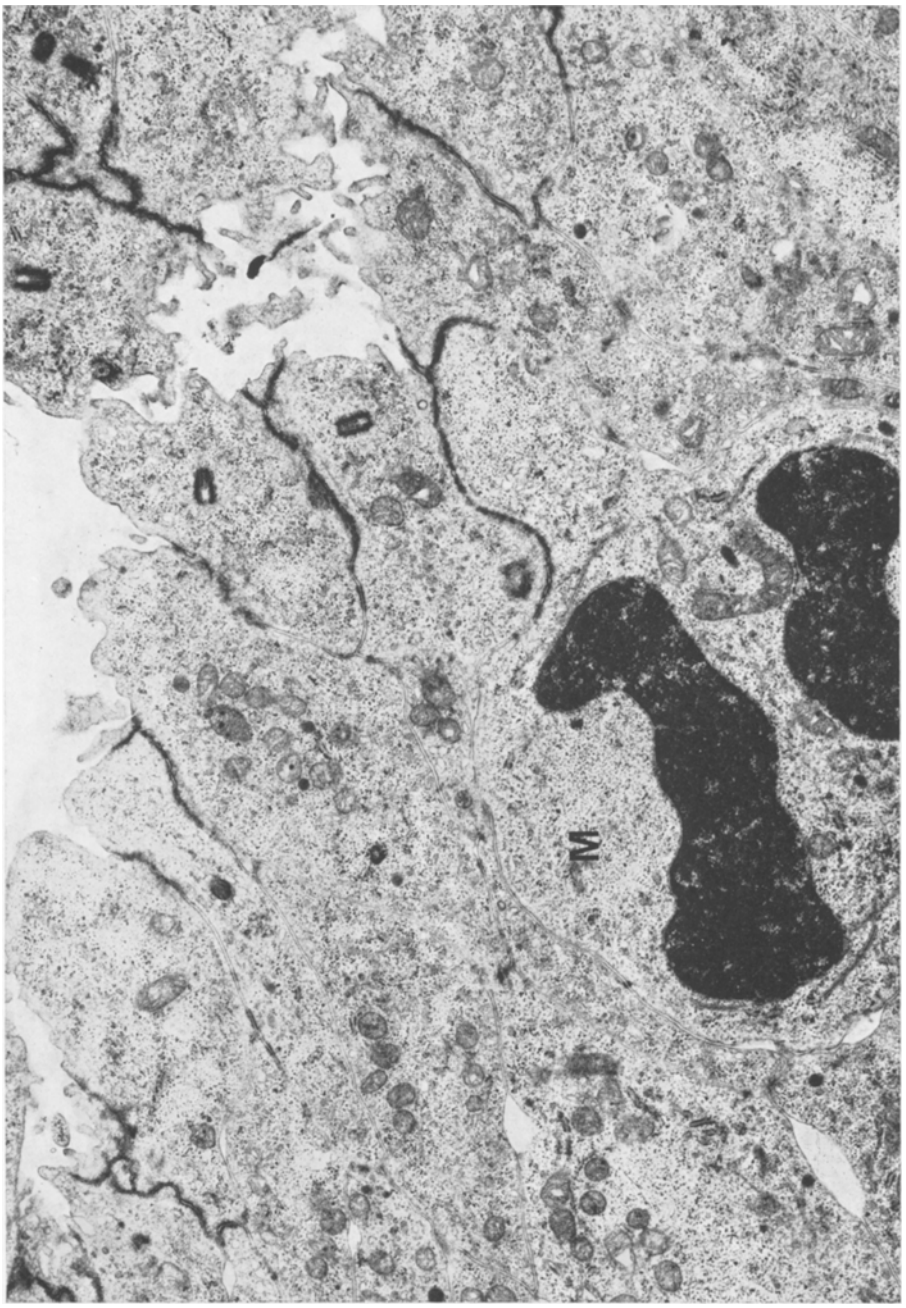

Fig. 6. Early glandular period (65 mm). Part of a pluristratified tubule. Cells are held by junctional complexes, the cytoplasm is filled with glycogen. Mitochondria are grouped together and centrioles are seen in apical position (M = mitosis) ($\times 7{,}600$)

due to an accumulation of fibrillar material arranged parallel to the basal plasma membrane. The latter exhibited thickenings opposite corresponding densities of the basal lamina to which were attached strands of fibrous material. Apart from fibroblasts and associated collagen near the basal lamina, interstitial cells were

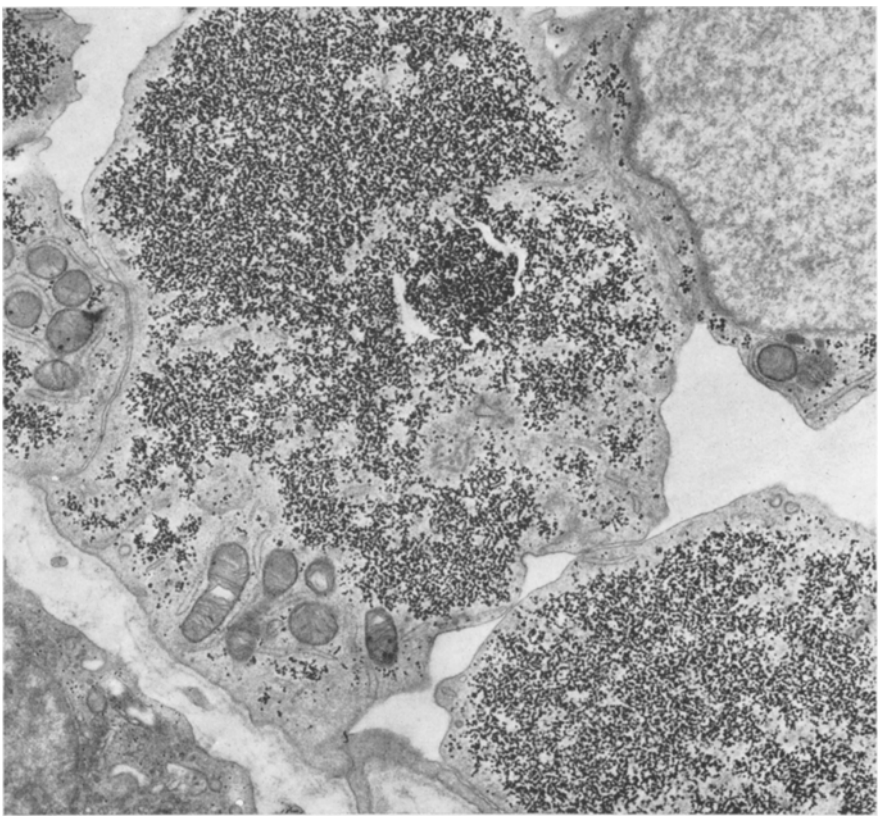

Fig. 7. Early glandular period (67 mm). Glycogen occupying much of the cytoplasm is shown by PATAG reaction (×11,500)

scattered in a ground substance sparse in structured collagen fibers. The interstitial cells appeared rather undifferentiated with a large nucleus and dispersed chromatin, similar to the nuclei of epithelial cells. Capillaries did not show any unusual features.

Second Day, from 10-Week-Old-Fetus. Tubules lined by a simple or stratified epithelium had lumen and stood out in a loose interstitial tissue. Epithelial intercellular spaces were wider but focal gap junctions were still clearly noticeable. Microvilli appeared fewer. In the nucleus the dispersed chromatin showed a marked change in its pattern, becoming diffuse and more evenly distributed throughout the nuclear sap; while the condensed chromatin was no longer evident. The nucleolus retained its skeinlike structure. Glycogen was as abundant as at day 0 and mitochondria were grouped either at the base or at the apex of the cell.

Among the epithelial cells, one with secretory characteristics was noted (Fig. 8). It was situated on the basal lamina, sometimes protruding into the interstitial tissue below and did not extend to the lumen. The cytoplasm con-

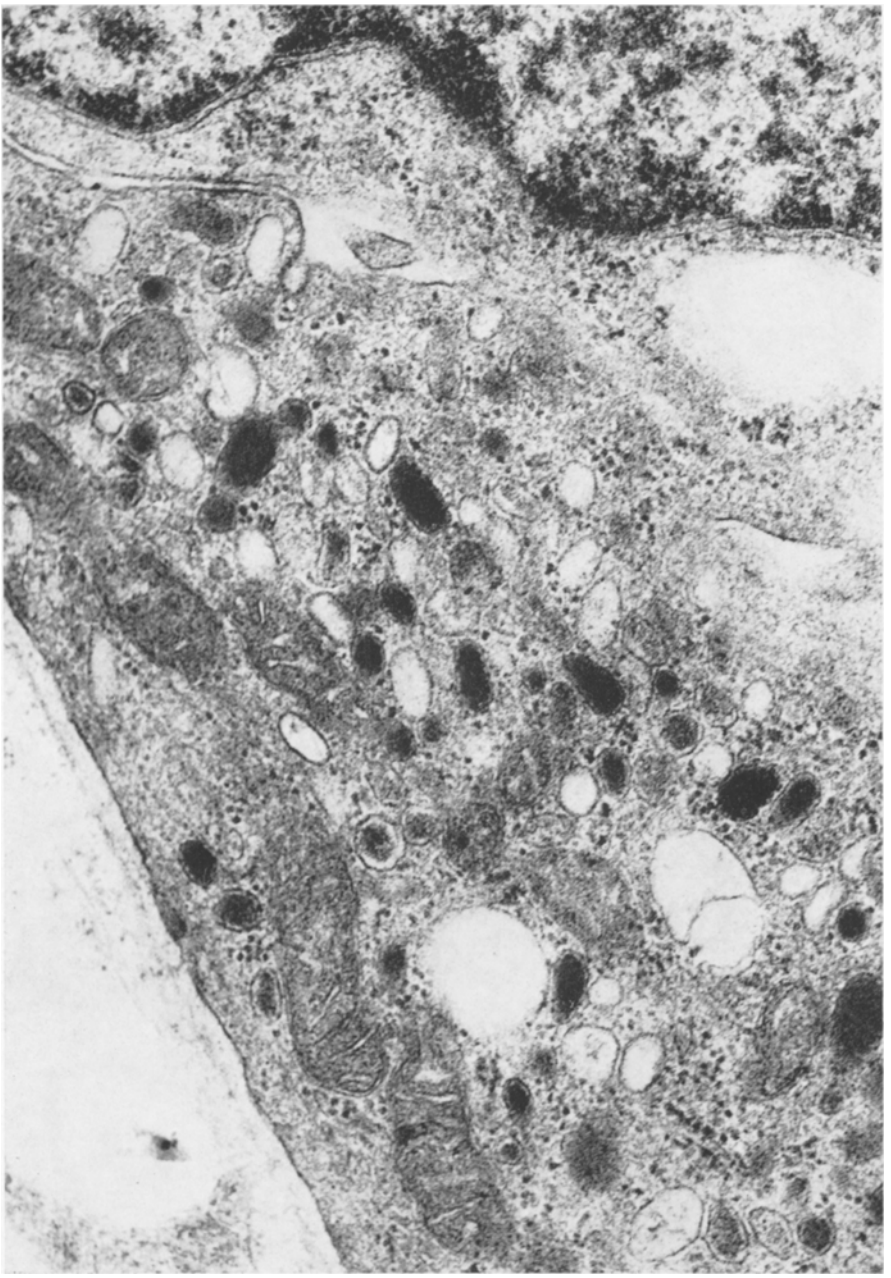

Fig. 8. Early glandular period. In culture 2 days. A portion of secretory cell, or Kultzchitsky type cell, resting on the basal lamina and exhibiting secretion granules of various sizes and densities (×38,000)

tained a large number of ribosomes and organelles among which were numerous small circular vesicles (120 nm in diameter) or oval ones (220 nm in length) surrounded by a single limiting membrane, and rather more concentrated in the basal part of the cell. They were sometimes empty or contained an electron opaque core of varying density.

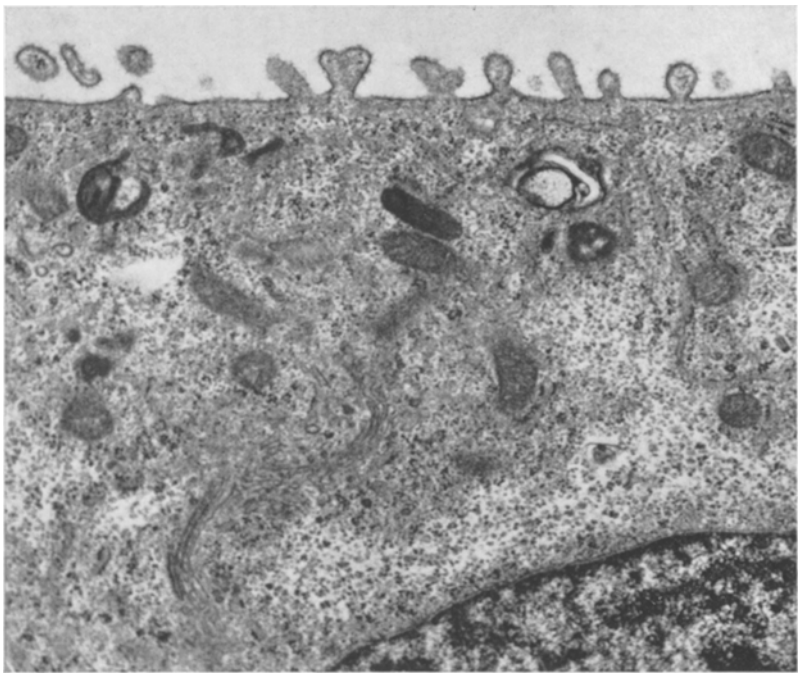

Fig. 9. Early glandular period. In culture 7 days. Part of an epithelial cell exhibiting short microvilli with its glycocalyx. A myelin figure and a well-developed golgi apparatus are seen in cytoplasm (×17,500)

The interstitial cells had a high nuclear-cytoplasmic ratio and presented a dendritic appearance. Some had a dilated RER of fibroblastic type, others had microfibrils 5.3 nm thick and pinocytotic vesicles 140 nm in diameter, reminiscent of those of myoblasts.

A diffuse and loose mesh of fine interstitial fibers was attached, sometimes slantwise to the basal lamina of the epithelial structures.

6th and 7th Days, from $9^1/_2$ to 13-Week-Old Fetuses. The tubular epithelium became mono or bilayered in a less vascularized stroma. Mitoses were observed in both epithelial and connective tissues.

The epithelial intercellular spaces were less obvious but gap junctions and desmosomes remained unchanges. In nondividing cells, the nucleoli underwent no change. Microvilli were perhaps longer, exhibiting a fine glycocalyx (Fig. 9).

Due to a decrease in glycogen reserves, mitochondria were more spread through the cytoplasm, RER was more prominent and microtubules and free ribosomes were visible. Membrane-bound glycogen pockets were sometimes capped by an electron-dense material. Myelin figurelike structures and autophagosomes were more numerous and larger than in the earlier stages.

Interstitial cells were more uniformily spread in the stroma. The fibroblastlike cells also had a better developed RER and their nuclei resembled those on day 0; their cytoplasm contained lipid droplets and lysosomes.

10th Day, from $9^1/_2$-Week-Old Fetus. Branching tubules with a very wide lumen were seen in a looser interstitial tissue where blood vessels were no longer evident.

There were two types of tubules: some had regularly arranged cuboidal cells with a high nuclear cytoplasmic ratio (Fig. 10). Their nuclei were basal, sometimes indented. Their lateral plasma membranes were straight and parallel to each other, except at both poles where they interdigitated. At the apical pole, the cells were held by a tight junction and a desmosome. On the straight part of the lateral membranes, only occasional thickenings of the membrane were seen.

The other type of tubule had a less orderly structure with marked swelling of the intercellular spaces (Fig. 11). The superficial cells were held by a tight junction and a desmosome near the lumen and by a few desmosomes on their lateral membranes which were more interdigitated. On the whole, these cells had a lower nuclear cytoplasmic ratio. In the nucleus the chromatin was adjacent to the nuclear envelope while the nucleolus retained its structure. The number and localization of organelles and cytoplasmic inclusions remained identical to those of 6th and 7th days. As was found in the earlier stages, a matting of irregularly thick microfibrils adhered to the basal plasma membrane.

Below the regular tubules with cuboidal cells were found interstitial cells with a cytoplasm rich in microfibrils and pinocytotic vesicles resembling typical leiomyoblasts.

15 and 17 Days, from a $9^1/_2$ and 13-Week-Old Fetus. Both kinds of tubules were seen in a quite cellular but avascular interstitial tissue.

The irregularly structured tubules had a similar architecture to that of the 10th day and showed no further changes. Glycogen was scanty and mostly basal.

Occasional lipid droplets occurred in the cytoplasm. Bodies resembling autophagosomes, more numerous at this late stage, were seen in both kinds of tubules. The regular tubules exhibited increased differentiation. But ciliated and secretory cells were present, the latter usually with apically positioned nuclei. The cells in the interstitium showed a definite fibroblastic and myoblastic appearance.

Discussion

The appearance of the lung in culture naturally varied in relation to the embryonic development period. The best survival was obtained with lung tissue from the early glandular period. During that period vascularization was not well developed. The increase in the number of capillaries which occurs during lung development appeared to be a nonfavorable factor for the in vitro survival. Red blood cells died and disappeared after the first few days in vitro. Endothelial cells persisted longer but the general aspect of the culture was modified.

Epithelial survival was always excellent in organ culture, as is the case with embryonic kidneys and nephroblastoma (Rousseau et al., 1974). Cellular cohesion by tight junctions perhaps preserved metabolic exchanges (Loewenstein, 1973)

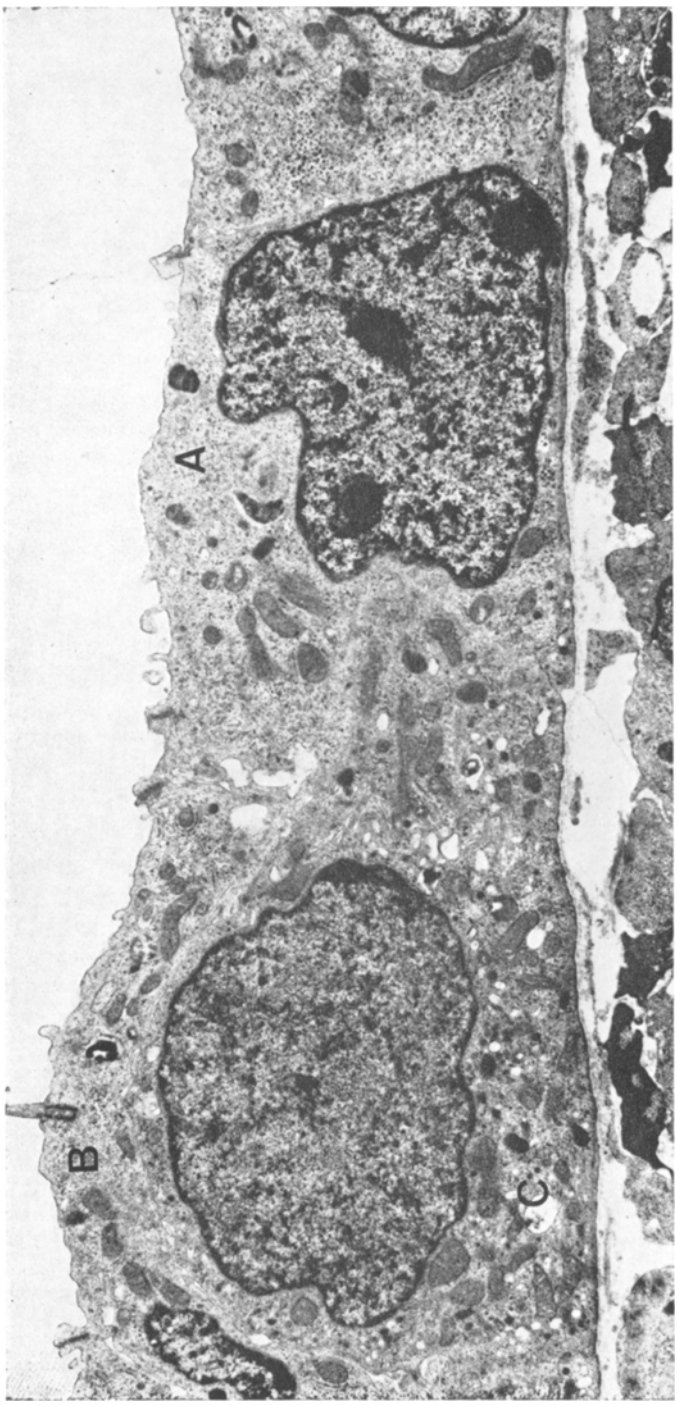

Fig. 10. Early glandular period. In culture 10 days. A regular tubule showing three types of cells: (A) an ordinary cuboidal cell, (B) a ciliated cell, and (C) a secretory one. In the interstitium, myoblastlike cells are seen among less differentiated ones ($\times 10{,}500$)

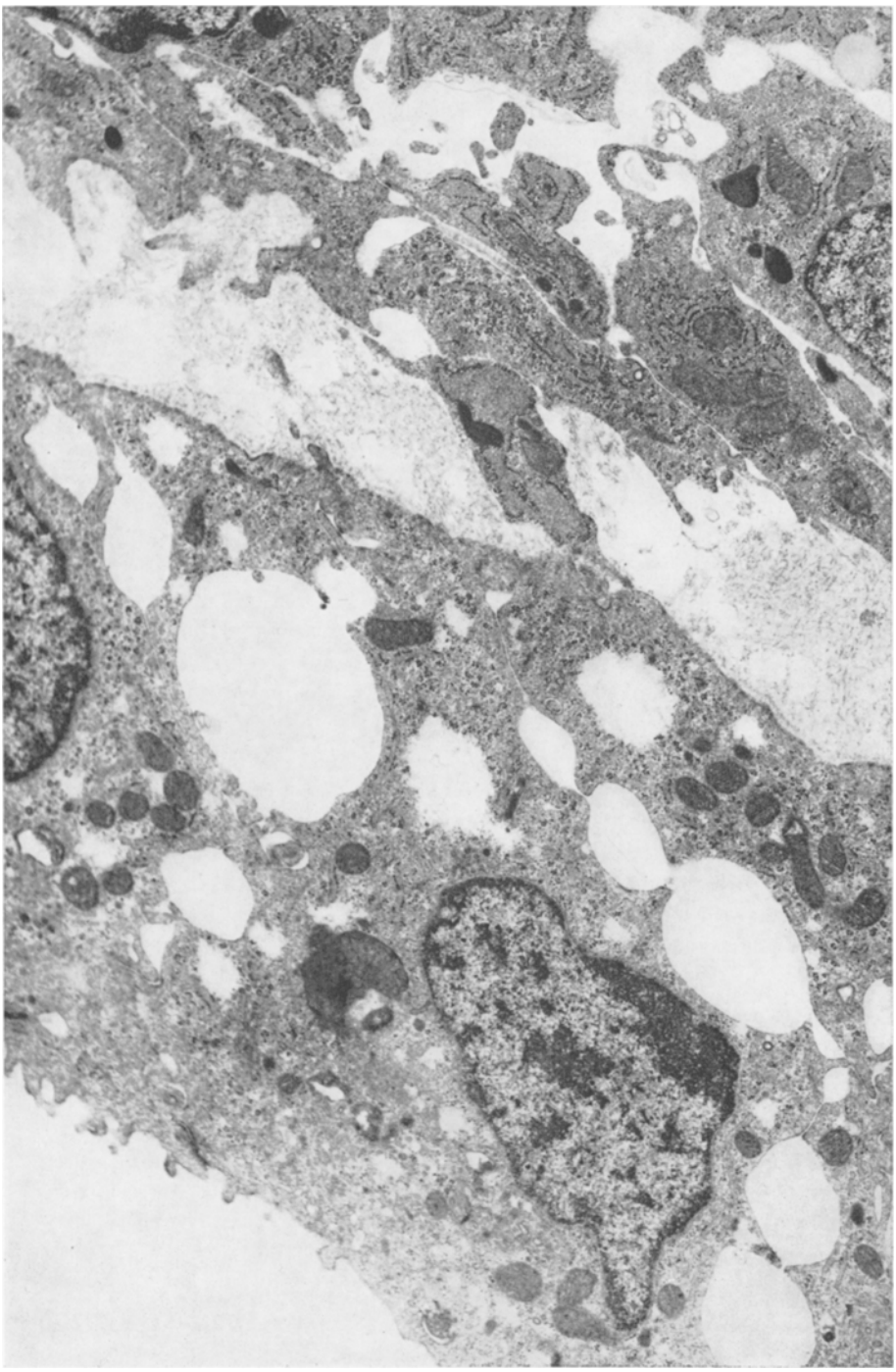

Fig. 11. Early glandular period. In culture 10 days. This micrograph shows marked swelling of intercellular spaces in irregular tubule. There are fibroblastlike cells in interstitium and one can see a loose mesh of fibrils attached to basal lamina. ($\times$10,000)

and ensured a mechanical protection against the unusual nutritive and gaseous circumstances. During culture, mitoses were numerous and the cellular features remained unchanged except for folds or dilatation.

Tubular dilation was more frequent during the 1st week of culture and mostly in embryos less than 150 mm (glandular period). With embryonic rat lungs in culture, Noack et al. (1970) observed the same process of dilation but chiefly in the portion of the explants in contact with air. For these authors this tubular expansion was related to a necrotic process which does not correspond to our observations. Resnick et al. (1974) observed dilation phenomena with cultures of embryonic rat lung in the presence of an excess of oxygen. Some of their control cultures in air showed, after 48 h, some "dilated bulbous, terminal bronchiolar buds" besides deficient bronchiolar development. They explained these changes as a reaction against oxygen toxicity of embryonic lung accustomed to a relative hypoxia *in utero*. These authors also demonstrated an identical toxic action of oxygen on embryonic rat kidney cultures. The same dilation process has been observed with embryonic human kidney (3–4 months old) and nephroblastoma culture in the presence of air (Rousseau et al., 1974). With embryonic rat kidney in organ culture, Monie and Morgan (1975) showed that a tendency to cyst formation could be correlated with a deficiency in kidney maturation or to a secretory action of the tubules. The dilations obtained in our culture of human lung from the early embryonic period were more marked than in the above observations. The process of dilation was perhaps related to the slight overpressure of the gas mixture which occurs when the cultures are enclosed in a Lwoff-box at 20° C and incubated at 37° C ($\Delta P = 5\%$). The fact that earlier embryonic periods were more affected than later ones may be explained by the greater plasticity of immature tissues.

At the ultrastructural level, the most significant changes observed during culture also concerned the epithelial structures. Besides the dilation of tubules noted above, electron microscopy showed the appearance of two different types of tubules during the second week of culture from $9^1/_2$- and 13-week-old embryos: irregular tubules with loosely attached cells and regular ones with cuboidal cells.

In the regularly arranged tubules, the presence of secretory and ciliated cells, the cuboidal shape of the majority of cells with basal nuclei, and the presence of smooth muscle below the tubules, all indicated the beginning of bronchiolar structures, previously described by Campiche et al. (1963) and Cutz et al. (1972, 1974). The latter found Kultzchitsky type cells with secretory vesicles as early as the 8th week. Hage (1971) mentioned groups of several types of endocrine cells in 10–11-week-old fetuses, but reported an isolated endocrine cell only in the smallest bronchi of the youngest fetuses (Hage, 1973). Our present findings of secretory cells in bronchiolar structures correspond to all these descriptions. According to Reid (1967), cilia appear between the 10th and the 13th week, which is the age span of our material.

As is well known, the developing lung consists of two easily recognizable parts: the bronchial and the alveolar. Having assumed that regular tubules were bronchiolar, we deduced that the irregular ones, were the precursors of alveolar structures.

Among the intracellular changes of the epithelial cells was the marked decrease in glycogen content. In vivo, glycogen is abundant, increasing until the 11th week and then slowly decreasing during further maturation (Hage, 1973). The necessity of glycogen for the development of bronchial branching has been established in vitro by Sorokin (1961), while Mc Dougall and Smith (1975) have suggested that glycogen is used by the cell for anaerobic glycolysis before mitochondria initiate aerobic metabolism. But it must be remembered that our material coming from relatively anaerobic conditions in utero was exposed to air in vitro.

Myelin figures became more numerous with time in vitro. These bodies are well known in organ culture (Rousseau et al., 1974) and are a sign of metabolic difficulty in cells. They might be interpreted, in the present tissue, as a step toward differentiation into the lamellar inclusions of granular pneumocytes. To support this would require testing the culture medium for traces of surfactant. Gluck (1972) has shown that surfactant is not detectable before the 22nd week and although true maturation has been obtained by Adamson et al. (1975) in culture explants of fetal mouse lung exposed to prednisolone at the 18th day, it is likely that in our material the myelin figures represent degeneration phenomena.

The presence of necrotic cells (blood elements, macrophages, epithelial cells), inside the tubular lumens in the oldest embryonic stages, might be related to the fragility of the numerous capillaries noted during these experiments and which is enhanced by oxygen (Resnick et al., 1974).

Eosinophilic or PAS-positive material was seen in vitro in the lumen of the tubules of oldest embryonic stage; the nature of this substance is unknown. A previous report has shown abnormal type II pneumocyte secretion in organ culture of mature lung (Basset et al., 1974).

In the glandular period the interstitial cells increased slightly in number, and acquired quite early during culture the more differentiated characters of fibroblasts as seen in electron microscopy. After 10 days in culture, cells with the characteristics of leiomyoblasts were also seen, particularly about the regular tubules. Collet et al. (1975) attribute both cells to a single origin, the primitive mesenchymal cell, which, according to its environment, would differentiate into fibroblasts near would-be alveolar territories and into myoblasts under bronchial epithelium. Our findings support this hypothesis.

The in vitro transformation of mesenchymal cells into fibroblastic cells has been noted by others (Alescio, 1961; Resnick et al., 1974). In vivo excessive fibroblastic proliferation in the alveolar septa occurs with various harmful agents, in particular oxygen (Anderson et al., 1973; Resnick et al., 1974).

In conclusion, an excellent in vitro survival, confirmed by electron microscopy, has been observed, particularly in the epithelial component of developing embryonic lung. The relatively short duration of culture permits only a fragmentary study of differentiation of the human lung in vitro but, with the exception of tubular dilations, most of the in vitro changes are also found during lung differentiation in vivo = monostratification of epithelium, bronchiolar development, decrease in glycogen, appearance of myelinlike figures, fibroblastic and myoblastic transformation of mesenchymal cells. The in vitro survival and changes are clearly age dependent.

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