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Reconstruction of Alveolus-Like Structure from Alveolar Type II Epithelial Cells in Three-Dimensional Collagen Gel Matrix Culture

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The purpose of this study is to reconstruct an alveolus-like structure from alveolar type II epithelial cells in a culture condition. Isolated alveolar type II epithelial cells of the rat were cultured in a three-dimensional collagen gel matrix. Single type II cells formed cellular aggregates that had a lumen after cell division in this culture condition. Through proliferation of the component cells, these aggregates grew to assume a globular or branching structure, part of which in turn developed into a large, cystic alveolus-like structure. This structure consisted of flattened epithelial cells intermingled with cuboidal epithelial cells. In these structures, the surfactant production was confirmed by immunohistochemistry and electron microscopy. To our knowledge, this is the first report of a reconstruction of an alveolus-like structure in a three-dimensional collagen gel matrix culture. This culture system seems to provide an appropriate physiological environment in which to study the differentiation and disorders of pulmonary alveoli. (Am J Pathol 1993, 142:783–792)

The extracellular matrix is thought to affect critically the differentiation of epithelial cells not only in the developmental stage of fetuses and newborns, but also in the adult stage. The induction of cellular differentiation has been well documented in culture experiments *in vitro* using an extracellular matrix. For example, reconstruction of ductal structure from the epithelial cells of breast,[1] pancreas,[2] and salivary glands,[3] maturation of preadipocytes to unilocular fat cells,[4] reconstruction of thyroid follicles from dissociated cells,[5] and differentiation of keratinocytes[6] have been reported in a collagen gel matrix culture system.

In this study, pulmonary epithelial cells, especially alveolar type II epithelial cells, were examined in terms of differentiation in three-dimensional collagen gel culture conditions. Type II epithelial cells are not only well-differentiated cells that synthesize and secrete pulmonary surface-active materials, but they are precursor cells for type I epithelial cells[7–10] as well. It has been reported that although type II cells actively proliferate *in vitro*, they rapidly lose their differentiative property in a culture condition at the same time, and that type II cells form small alveolar-like structures and produce surfactant proteins only on an extracellular matrix in a culture condition.[11–13]

The alveolus is a luminal structure in tissues *in vivo*. We expect that type II cells will reconstruct an alveolus-like lumen and produce the surfactant in the condition of a three-dimensional collagen gel. If true, such an experiment will help to elucidate the mechanism of differentiation of epithelial cells in connection with an extracellular matrix. The effects that some growth factors might have on epithelial cells were also studied in this culture condition.

Materials and Methods

Isolation of Alveolar Type II Epithelial Cells

Pulmonary tissue samples from 21- to 23-day-old rats of Wistar strain were used for the isolation of alveolar type II epithelial cells by the method of Dobbs et al and other investigators.[14–16] The pulmonary circulation was first perfused with a balanced salt solution and the trachea was cannulated. The lung was repeatedly lavaged through the trachea to remove macrophages. Elastase (4 U/ml, porcine pancreas, Sigma Chemical Co., St. Louis, MO) was instilled into the lung for 20 minutes at 37 C. After

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resection of the trachea and large bronchi, the lung was minced in the presence of fetal calf serum and shaken. After filtration and centrifugation, dissociated cells were obtained and incubated in bacteriological dishes coated with rat immunoglobulin G (Sigma) for 60 minutes. Nonadherent type II cells were thus collected. The purity of freshly isolated type II cell preparations was 85% ± 5% as judged by a modified Papanicolaou stain.[7] The contaminating cells contained fibroblasts and a small number of macrophages and lymphocytes. Fibroblasts were carried into the culture system of type II cells. However, fibroblasts did not proliferate very extensively in the collagen gel matrix; they did not disturb the proliferation of type II cells. These freshly isolated type II cells were used for collagen gel culture (direct embedding), and a part of these cells was incubated in the usual culture dishes with Ham F-12 supplemented with Ultrosera G (IBF Biotechnics, Villeneuvela-Garenne, France) and antibiotics. This complete medium is serum-free. The use of this type of medium is of vital importance in this study in view of the report that serum accelerated the loss of type II cell differentiation.[17] These cells were allowed to proliferate for 4 to 5 days to form monolayer sheets; they were then detached from the dish with treatment of 0.15% trypsin. We could completely dissociate these cells by gentle pipetting. The presence of lamellar bodies in these cells was confirmed by electron microscopy. These cells were also used for collagen gel culture (indirect embedding). This procedure was done to avoid any criticism that might be raised that incompletely digested alveoli were embedded and cultured in collagen gel.

Three-Dimensional Collagen Gel Culture

This culture method was initiated by Elsdale and Bard[18]; for convenience, however, we followed the procedure of Enami.[19] Briefly, eight volumes of acid-soluble type I collagen solution (Nitta Gelatin Co., Ltd., Osaka, Japan) were mixed with one volume of 10-fold concentrated Ham F-12 (without $NaHCO_3$) and one volume of reconstruction buffer (2.2 g of $NaHCO_3$ and 4.77 g of HEPES in 100 ml of 0.05 N NaOH). This mixture, kept in ice, was mixed with isolated alveolar type II epithelial cells obtained as above, ie, the freshly isolated cells (directly embedded) and their subcultured cells (indirectly embedded). A volume of 1.5 ml of the mixture containing 10×10^4 cells was placed in a 12-well dish and immediately warmed to 37 C to form a gel. Alveolar type II epithelial cells were thus embedded in the collagen gel. After 30 minutes, when it was strong enough, the gel was overlayered with 2 ml/dish of Ham F-12 supplemented with Ultrasera G, gentamicin, and amphotericin B. This complete medium was composed of 5 nmol/L and 0.02 ng/ml of vitamin A and epidermal growth factor, respectively. We also found it feasible to culture with Ham F-12 supplemented with 10% fetal calf serum.

Chemicals Added to Culture Cells

1) Epidermal growth factor (Collaborative Research, Lexington, MA) was administered into the medium of cultured type II cells at a concentration of 10 ng/ml for 14 consecutive days from day 1 of culture. 2) Cholera toxin (Seikagaku Kogyo Co., Tokyo, Japan) was administered into the medium of cultured type II cells at a concentration of 100 ng/ml for 14 days from day 1 of culture. Cholera toxin is a stimulating agent that induces a prolonged increase of intracellular cyclic adenosine monophosphate. 3) Transforming growth factor-β (Biomedical Technologies, Stougton, MA) was administered into the medium of cultured type II cells at a concentration of 20 pmol/L for 14 days from day 1 of culture.

Examination of Culture Cells

Culture cells were examined by the following procedures: 1) Phase contrast microscopy. 2) Histological observation: The cell layer gel was fixed with 10% formalin, routinely processed, and embedded in paraffin. Deparaffinized sections were stained with hematoxylin-eosin staining, periodic acid Schiff staining, and silver staining. 3) Immunohistochemistry: Deparaffinized sections were immunostained with anti-rat surfactant proteins and SP-A antibody (rabbit immunoglobulin G) (provided by Prof. T. Akino and Dr. Y. Kuroki, Department of Biochemistry, Sapporo Medical College, Sapporo, Japan[20–22]). The sections were also immunostained with mouse monoclonal antibody to cytokeratin Ks 18.04 (No.18) (Progen Biotechnik GMBH, Heidelberg, Germany) and cytokeratin PKK3 (No.18) (Labsystems Oy, Helsinki, Finland).[23,24] The indirect immunoperoxidase method was used for these procedures, and negative control experiments were done. 4) Transmission electron microscopy: The cell layer gel was fixed with 2.5% glutaraldehyde and processed by the standard method. 5) Examination of proliferation: labeling reagent (bromodeoxyuridine (BrdU) 2 µl, 30 mg/ml)[25,26] was added to 2 ml of culture medium and cultured for 3 to 8 hours. Deparaffinized sections

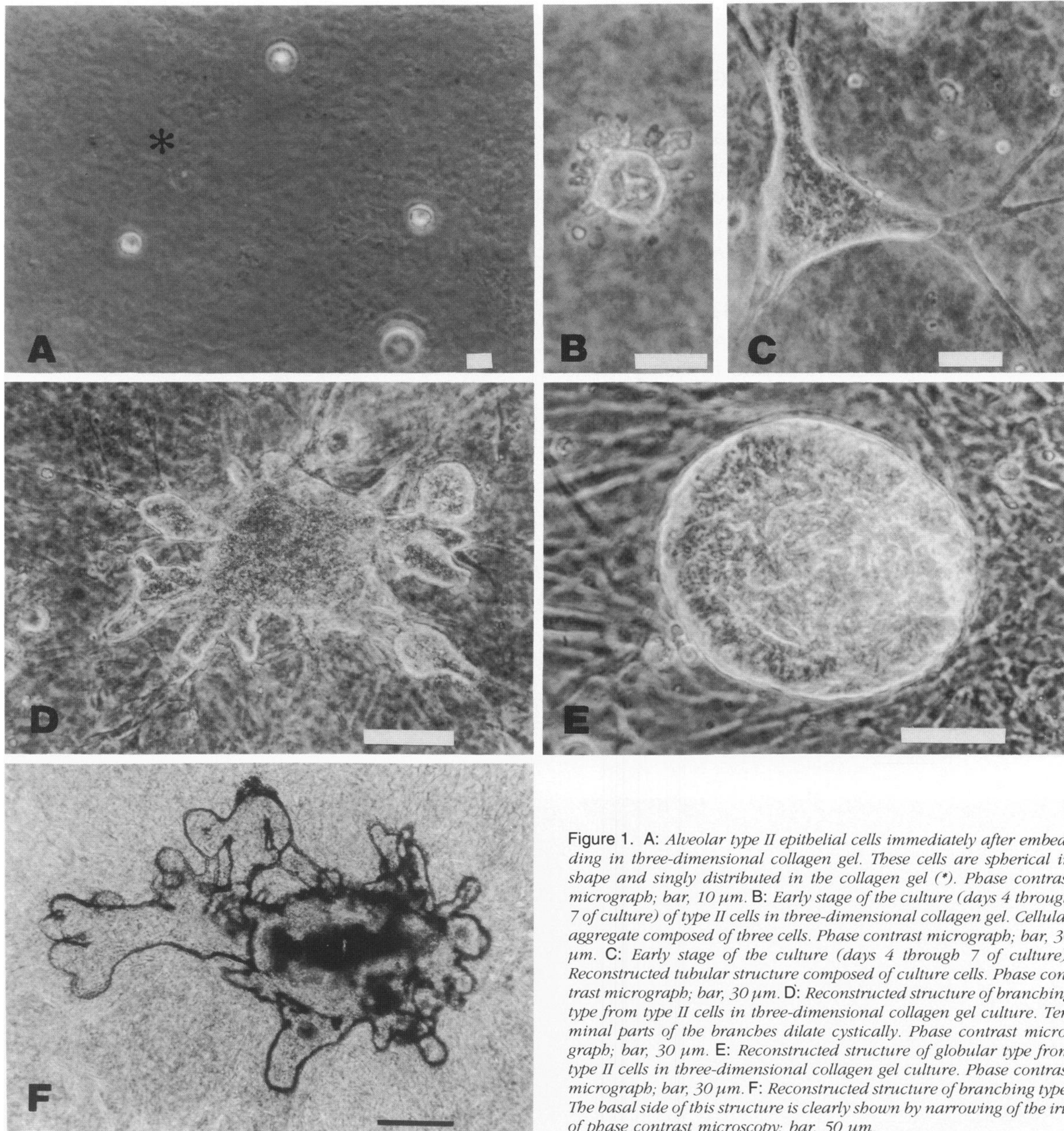

Figure 1. A: *Alveolar type II epithelial cells immediately after embedding in three-dimensional collagen gel. These cells are spherical in shape and singly distributed in the collagen gel (*). Phase contrast micrograph; bar, 10 μm.* B: *Early stage of the culture (days 4 through 7 of culture) of type II cells in three-dimensional collagen gel. Cellular aggregate composed of three cells. Phase contrast micrograph; bar, 30 μm.* C: *Early stage of the culture (days 4 through 7 of culture). Reconstructed tubular structure composed of culture cells. Phase contrast micrograph; bar, 30 μm.* D: *Reconstructed structure of branching type from type II cells in three-dimensional collagen gel culture. Terminal parts of the branches dilate cystically. Phase contrast micrograph; bar, 30 μm.* E: *Reconstructed structure of globular type from type II cells in three-dimensional collagen gel culture. Phase contrast micrograph; bar, 30 μm.* F: *Reconstructed structure of branching type. The basal side of this structure is clearly shown by narrowing of the iris of phase contrast microscopy; bar, 50 μm.*

from the gel fixed with 30% acetate ethanol were immunostained with anti-BrdU antibody by the procedures of BrdU kit (Amersham, Arlington Heights, IL). Five hundred cells were counted to estimate the labeling index.

Results

Morphological Observation

Freshly isolated alveolar type II epithelial cells were embedded into collagen gel as described in Materials and Methods. Embedded in collagen gel, type II cells were spherical in shape and distributed singly (Figure 1A). The cultured cells began to shoot out foot processes into collagen gel after 14 to 24 hours. Within 3 to 5 days, these cells proliferated and formed small cellular aggregates, each consisting of two to four cells (Figure 1B). These structures were enclosed by one layer of cells surrounding a lumen, gradually growing larger, and forming globular or tubular structures that consisted of seven to 10 cuboidal cells at the cross-section after 10 days (Figure 1C). These structures had distinct basal lamina at the basal side as shown later on electron micrograph. After 2 to 3 weeks, the reconstructed

structures were divided into three shape types, ie, globular (G) (Figure 1E), branching (B) (Figure 1, D and F), and intermediate between G and B (I). The ratios of the reconstructed structures of G, B, and I types in the medium condition of Ham F-12 supplemented only with Ultrasera G were about 50%, 30%, and 20%, respectively, on day 14 of culture, when only the structures over 100 μm in diameter were counted. As the cultures progressed, these structures became larger and the branching in type B increased in number (Figures 2 and 3). The globular structure regularly became cystic, and terminal parts of the branching structure dilated cystically in the late stage, ie, after 6 to 8 weeks (Figure 3). These cystic structures consisted mainly of flattened epithelium intermingled with cuboidal cells (Figure 4). This structure is compatible with the alveolus *in vivo*. From this point on, the reconstructive morphogenesis of type G, B, or I more or less converged into the G type at the rate of about 80%. These structures enlarged gradually to 1 mm in diameter after 8 to 12 weeks. Culturing in this condition was possible for 3 to 5 months.

After administration of epidermal growth factor for 14 days from day 1 of culture, the number of these structures increased at the rate of 70%, and the ratios of types G, B, and I were about 30%, 40%, and 30%, respectively. On the other hand, cholera toxin stimulated globular morphogenesis. Transforming growth factor-β had no constant effect on the reconstructive morphogenesis, but this requires further study.

Alveolar epithelial cells that had been cultured in monolayer in a usual plastic dish were also embedded three-dimensionally in collagen gel (indirect embedding). These cells also showed a reconstructive morphogenesis, but the better result was obtained in the directly embedded cultures.

Type II cells embedded in the surface zone of a collagen gel layer often migrated to the surface and proliferated. Epithelial sheets of type II cells were scattered on the surface of collagen gel, and these cells sporadically formed small aggregates.

Proliferation of Alveolar Type II Epithelial Cells

Growth of type II cells in the collagen gel culture has been clearly established through observation of the reconstructive morphogenesis in this culture

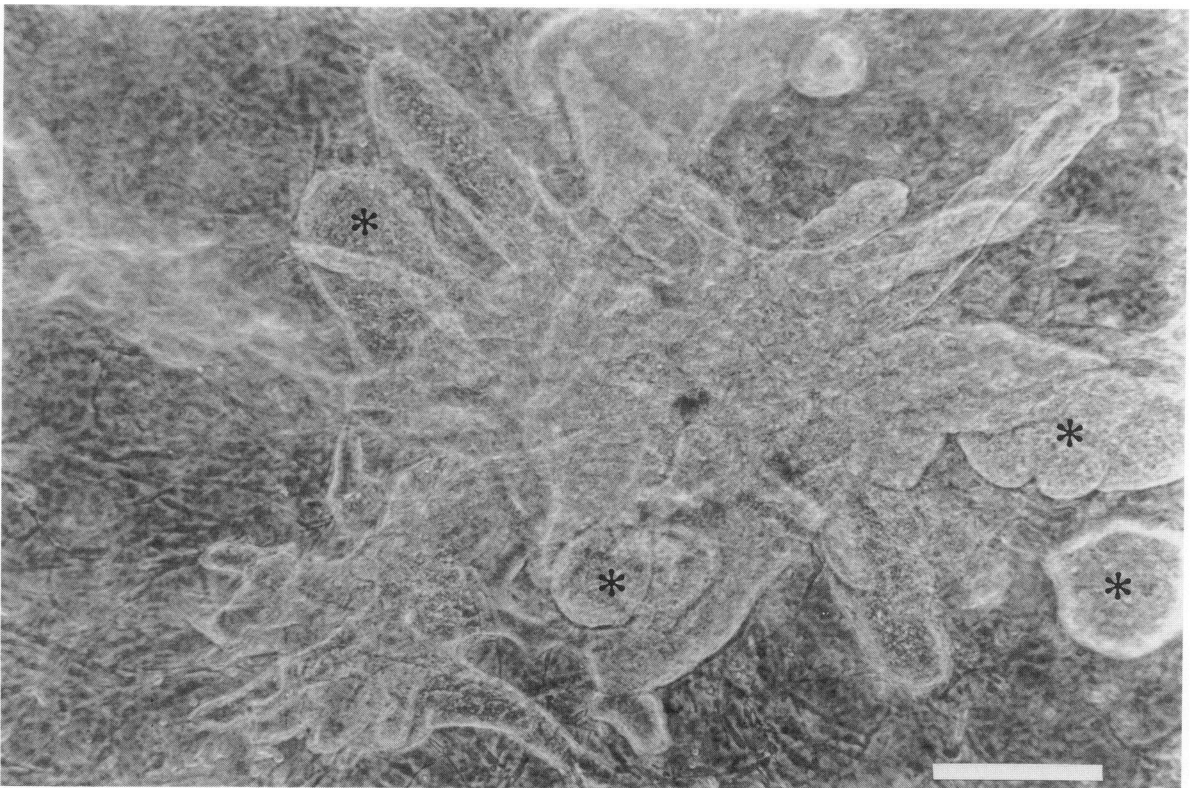

Figure 2. *Reconstructed structure of branching type has grown in size in the progress of culture in three-dimensional collagen gel. Terminal parts (*) of the branches dilate cystically. Phase contrast micrograph; bar, 100 μm.*

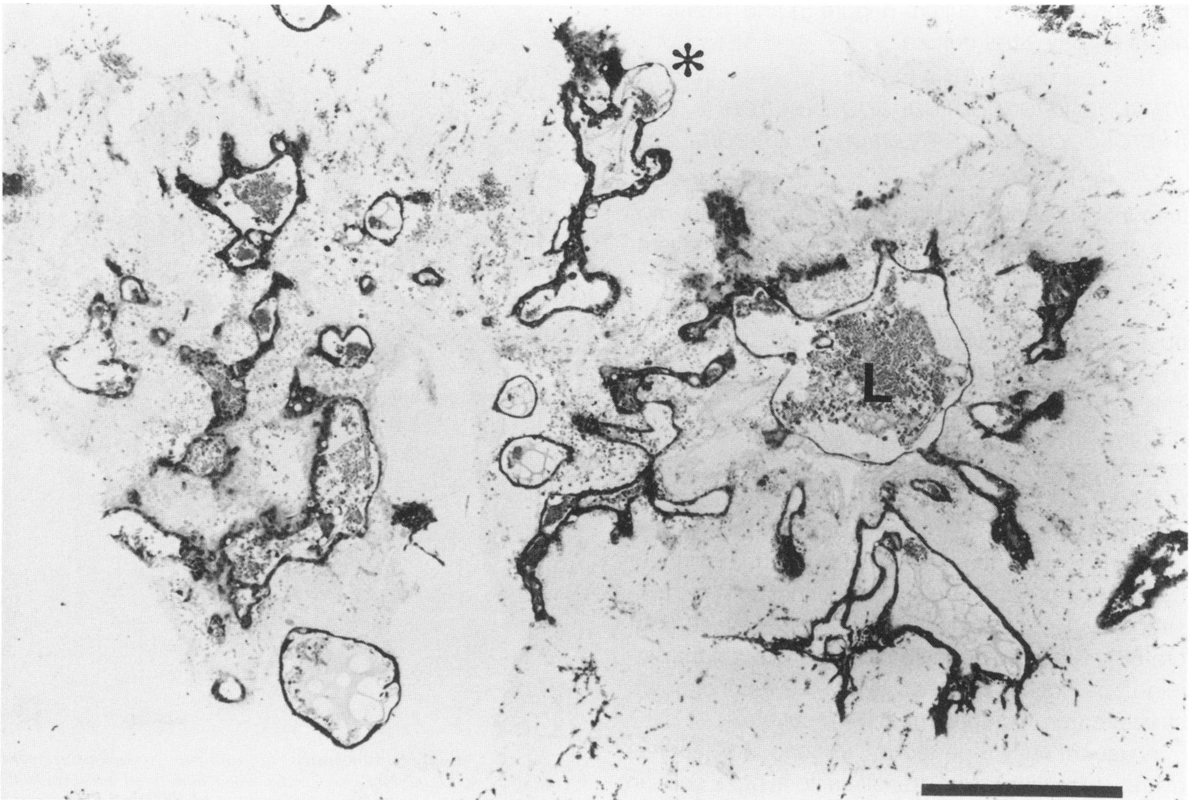

Figure 3. *Histological finding of large reconstructed structure in three-dimensional collagen gel culture. Large cystic lumen (L) exists in the center and multidirectional branches have grown out from it. Terminal parts of the branches dilate showing alveolus-like structure. One of them (*) is shown under higher power in Figure 5. Hematoxylin-eosin stain; bar, 500 μm.*

condition. In the reconstructive structure, such as globules or branching, mitotic figures were not infrequently observed in the component cells. The mitotic index was 0.7% ± 0.2% in globular structures and 0.9%± 0.3% in branching structures on day 14 of culture. In the examination of BrdU intake, the nuclei of single epithelial cells that were identified by cellular and nuclear shape were positively immunostained with anti-BrdU antibody at the rate of 20% to 30% for 3 hours on day 3 of culture (Figure 5, inset), and the rates of positive staining of epithelial cells of the globular or branching structure were 20% for 8

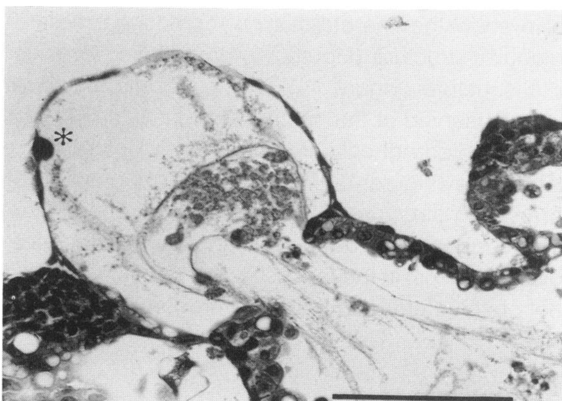

Figure 4. *A high power view of alveolus-like structure in collagen gel. This structure consists of mainly flattened epithelial cells intermingled with cuboidal epithelial cell (*). Hematoxylin-eosin stain; bar, 100 μm.*

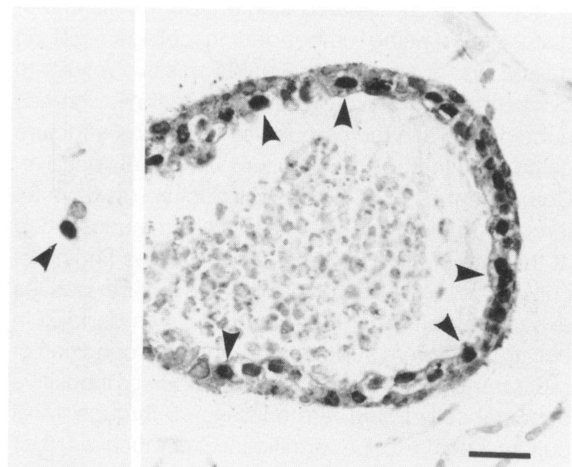

Figure 5. *Examination of proliferation of type II cells in three-dimensional collagen gel culture. Nuclei of cells comprising globular structure and a nucleus of single epithelial cells* (inset) *are positively immunostained with anti-BrdU antibody (arrowhead). Immunoperoxidase and hematoxylin stain; bar, 50 μm.*

hours on day 10 of culture (Figure 5) and 13% for 8 hours on day 24 of culture.

Immunohistochemical and Electron Microscopical Examination of Alveolar Type II Cells

Immunohistochemistry using anti-rat surfactant protein antibody was performed. In the previous examination of this antibody in the pulmonary alveolar tissue of rats, granular reaction products were observed in the cytoplasm of cuboidal cells, and linear products at the alveolar surface (Figure 6A). This immunostaining result is compatible with the distribution of the surfactant, as reported by the investigators of this antibody.[20–22] In culture cells, reaction products were observed in the center of cellular aggregates composed of several cells, and in the lumen of reconstructed globular, branching, or cystic structures. Scattered component cells in these structures were also positively immunostained (Figure 6B). These findings suggest that surfactant protein is produced and secreted in the reconstructed structure from component cells in culture.

It was recently reported by Woodcock-Mitchell et al that type II cells in transition to type I cells in injured lung and type II cells in culture were specifically positively immunostained for monoclonal antibody to cytokeratin No.18.[23,24] They concluded that type II cells in culture were supposedly in the condition of transition to type I cells. Before an application of this immunostaining to culture cells, we checked its specificity to pulmonary tissues of the rat. We established that scattered cuboidal cells of the alveolus were positively immumostained to this antibody, especially to antibody Ks 18.04 (Progen Biotechnik) (Figure 7, inset). Isolated single type II cells immediately after being embedded in collagen gel on days 2 and 3 of culture revealed positive results to cytokeratin No.18 at the rate of 30% to 50%, whereas cuboidal cells in the globular or branching structure on days 10 through 14 of culture were rarely positive. Component cells in the cystic structure on days 24 through 28 of culture were positively immunostained at the rate of 20% to 30% (Figure 7). The finding of culture cells on days 2 and 3 of culture was as expected. Some parts of type II cells embedded in collagen gel were supposed to be in the condition of rapid transition to type I cells. The finding of positive cuboidal cells being rare on days 10 through 14 of culture seems to be significant inasmuch as they mimick normal alveolar tissues *in vivo*. This finding is suggestive of the restoration of the phenotype of type II cells in a collagen gel culture using serum-free medium.

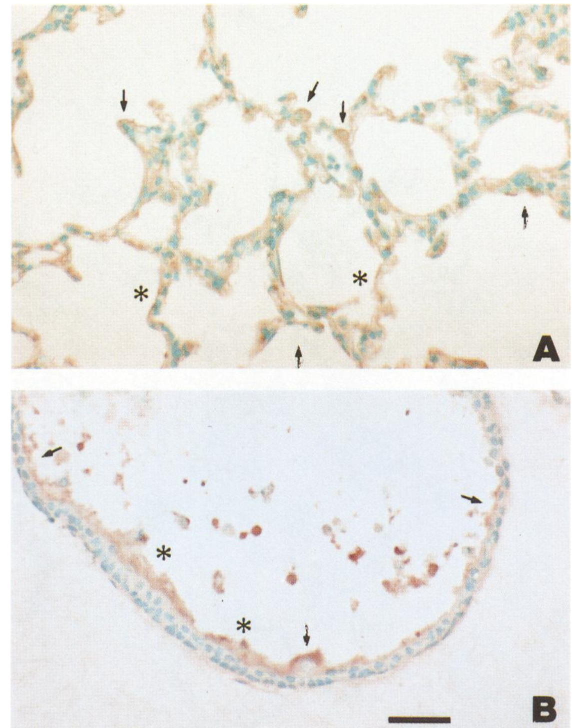

Figure 6. *Immunohistochemistry of anti-rat surfactant protein antibody.* **A:** *Pulmonary alveolar tissue of 1-month-old male rat. Scattered cuboidal cells are granularly positive (arrow) and alveolar surface is also linearly positive (*). Original magnification, ×400.* **B:** *A part of reconstructed alveolus-like structure in collagen gel culture. Some cells (arrow) and fragments stored in lumen (*) are positively immunostained. Immunoperoxidase and methyl green stain; bar, 50 μm.*

Electron microscopical examination revealed inclusion bodies possessing a multilamellar structure of stored surfactant, ie, lamellar bodies in single cells on days 2 and 3 of culture, in aggregated cells on days 7 through 10 of culture (Figure 8), and in some cells of alveolus-like structure in the late stage (Figure 9). Electron microscopical examination detected more differentiated type II cells than did immunohistochemistry of surfactant protein or cytokeratin. We also show the fine structure of component cells of globular structure (Figure 10) and cystic, alveolus-like structure (Figure 11). In these cells, microvilli were observed at the apical side. Basal lamina was clearly noted not only around cystic structure in the late stage (Figure 12), but also around globular or branching structure in the early stage. These findings suggest that normal cellular polarity of the alveolus does exist in the reconstructed structure. We did not observe any cilia in the cultured cells.

Discussion

In pulmonary alveolar tissues, two main types of epithelial cells, ie, alveolar type II and type I epithelial

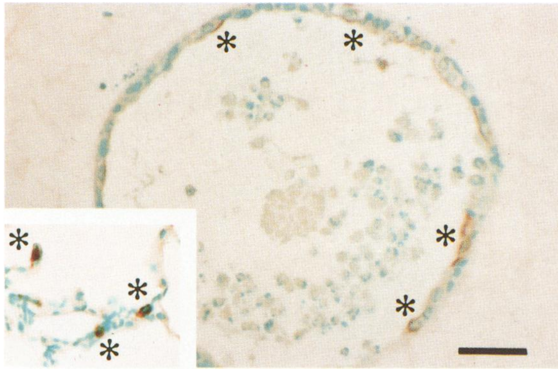

Figure 7. *Immunohistochemistry of cytokeratin No.18 that is specific to alveolar type II cells in transition to type I cells. Reconstructed alveolus-like structure in collagen gel culture. Some cells comprising this structure are positively immunostained (*); bar, 50 μm.* Inset: *Pulmonary alveolar tissue of 1-month-old male rat. Some cuboidal cells in the alveolus are positively immunostained (*). Immunoperoxidase and methyl green stain. Original magnification, ×200.*

cells, line the walls of alveoli. Type II cells proliferate in the wall, and those cuboidal cells differentiate into flat type I cells. The alveolus is a three-dimensional structure that has lumen. We attempted to make type II cells proliferate and reconstruct an alveolus-like structure in a three-dimensional collagen gel matrix.

Dissociated type II cells proliferated in collagen gel and reconstructed alveolus-like luminal structures as described in Results. In these structures, cultured type II cells had a morphological polarity specific for the alveolar epithelial cells *in vivo*: the apical side facing the lumen with microvilli and the basal side confronting collagen gel matrix with basal lamina. To the best of our knowledge, this is the first report that isolated type II cells reconstructed a large alveolus-like structure in a three-dimensional collagen gel matrix culture. Relevant in this respect are reports by Shannon et al,[11] Yoshida et al,[12] and Blau et al[13] that type II cells formed alveolar-like structures that had a lumen on an extracellular matrix *in vitro*. However, the small structures mentioned by these investigators seem incomplete in comparison to those we have obtained.

The process of the reconstruction of the alveolus-like structure may be explained as follows. When dissociated alveolar type II epithelial cells are embedded into a three-dimensional collagen gel matrix, the entire surface of the cells comes into contact with the collagen matrix. After cell division, cellular aggregates composed of several type II cells are formed in the gel, and a small intercellular lumen develops in the cellular aggregate. This process is the first step of the reconstruction. The cells comprising this small lumen undergo further cell division, and the lumen gradually grows in size to eventually become a dilated cystic lumen. When those cells compose a lumen of fair size, the cuboidal cells turn into flattened cells, resulting in an alveolus-like structure consisting of flattened epithelial cells intermingled with cuboidal epithelial cells. To our knowledge, the occurrence of flat epithelia in a collagen gel culture was specifically observed for the first time in this study using alveolar epithelium. Component cells of a luminal structure in a culture of mammary, thyroid, or some other glands remain cuboidal in the reconstructive state.[1,5] Despite the fact that the cytoplasm of flattened epithelia in this study is very thin in the cystic structure as shown in Figure 4, these cells are not, of course, mature alveolar type I cells that facilitate gas change. Identification of alveolar type I cells is difficult in culture cells *in vitro*. A monoclonal antibody specific for rat alveolar type I cells has been developed by Dobbs et al.[27] Further studies of the degree of differentiation of flattened epithelia in this study should be performed by using such a specific marker.

It has been reported that alveolar type II cells placed *in vitro* rapidly lose their differentiation property, ie, surfactant protein, but they are able to keep their surfactant protein in the culture condition on extracellular matrix.[7–13] The same mechanism may explain the differentiation of type II cells in this study in which cells are cultured within collagen gel matrix. As described in the Introduction, extracellular matrix, especially collagen, induces morphological and

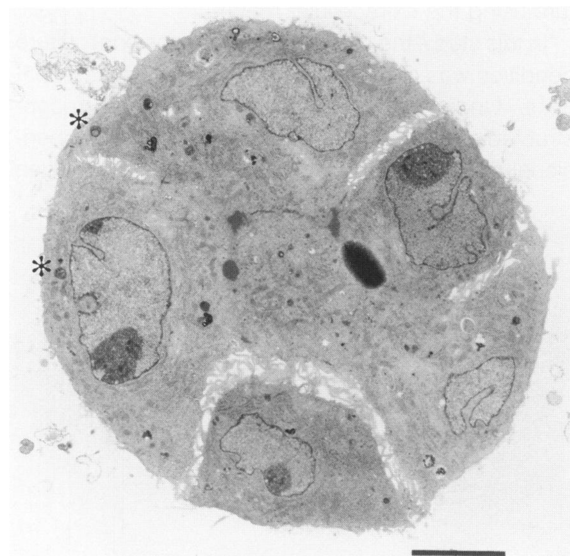

Figure 8. *Electron micrograph of a cellular aggregate in collagen gel culture. These cells reconstruct a small globular structure of the early stage and nuclei are situated at the basal side. Lamellar bodies (*) and amorphous, electron-dense bodies are observed in the cytoplasm. Higher power view of lamellar bodies are shown in Figure 9. Uranyl acetate and lead citrate stain. Following electron micrographs (Figures 9–12) are of the same stain; bar, 5 μm.*

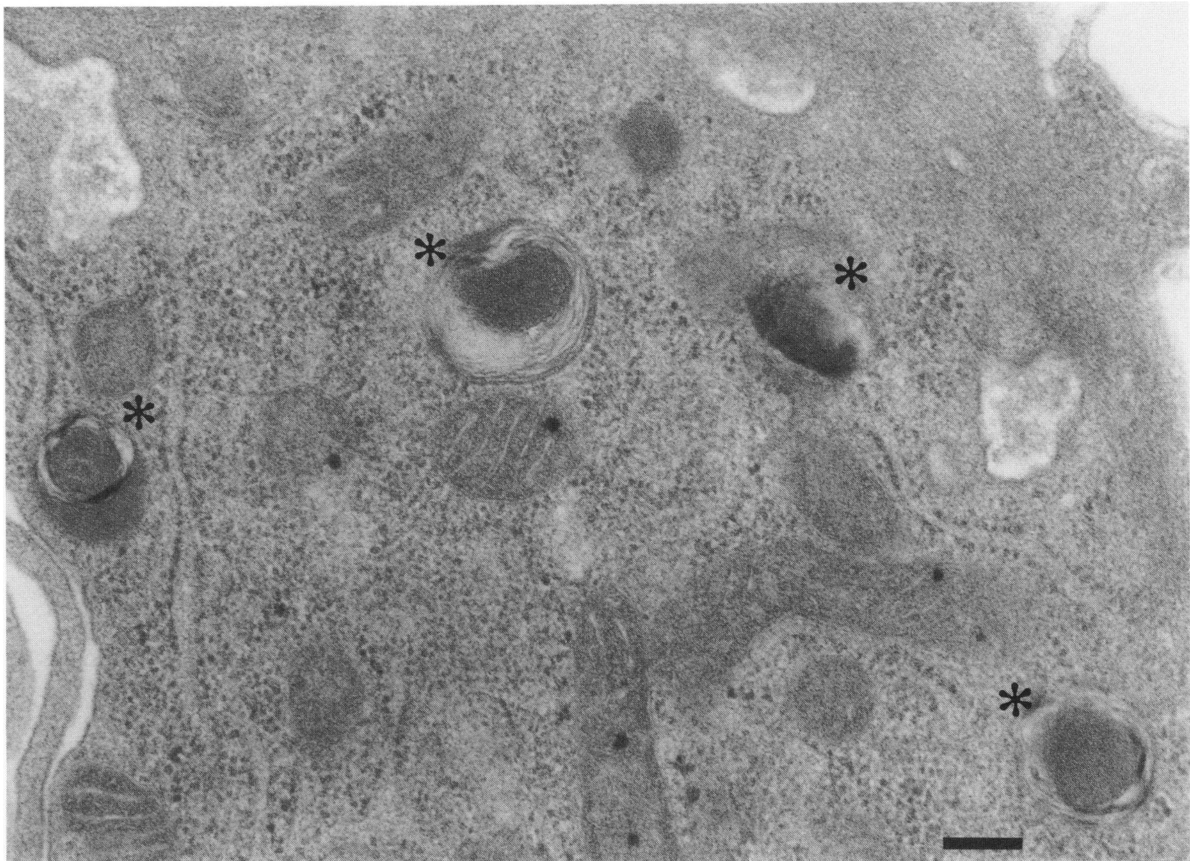

Figure 9. *Electron micrograph of one cuboidal epithelial cell in alveolus-like structure in collagen gel culture. Typical lamellar bodies (*) are seen in the cytoplasm; bar, 0.2 µm.*

functional differentiation of many tissue cells in culture using the extracellular matrix.[1–6]

In this study, the use of a three-dimensional culture condition was chosen specifically for alveolar type II cells. Under this condition, they indeed reconstructed an alveolus-like structure, ie, a three-dimensional structure. Thus, a three-dimensional collagen gel matrix culture seems to be suitable for the study of type II cells in terms of structural differentiation. Recent investigations have demonstrated that cellular surface receptors to extracellular matrix, such as collagen and fibronectin, exist in many kinds of cells.[28,29] These receptors of the integrin family help the cells communicate with the extracellular matrix

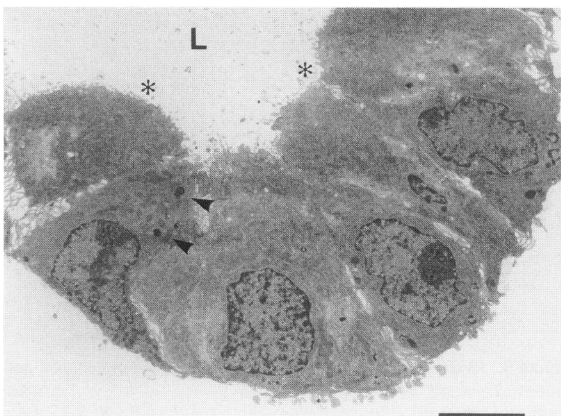

Figure 10. *Electron micrograph of reconstructed structure of globular type. These cells have numerous microvilli (*) at the apical surface of the lumen (L); arrowhead, lamellar bodies; bar, 5 µm.*

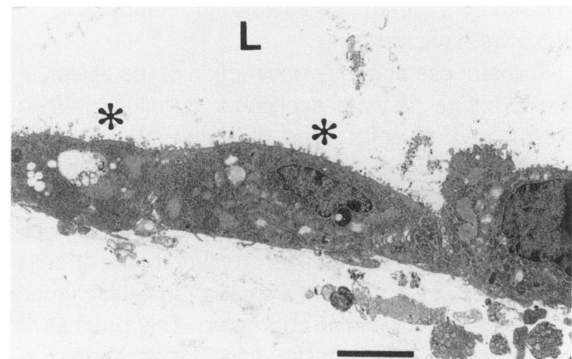

Figure 11. *Electron micrograph of the wall of cystic, alveolus-like structure composed of flattened epithelial cells and cuboidal epithelial cell (right-hand side). These cells have numerous microvilli (*) at the apical surface of the lumen (L); bar, 5 µm.*

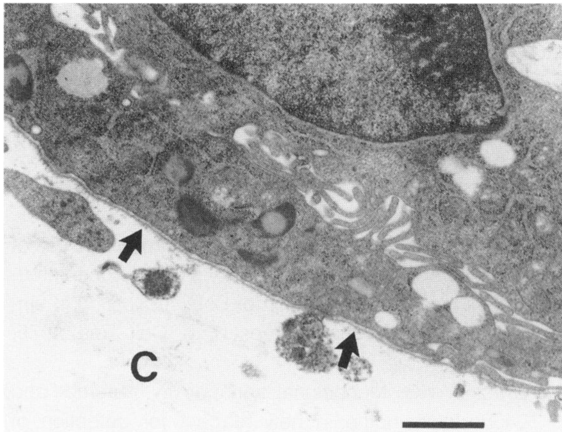

Figure 12. *High power view of an epithelial cell in alveolus-like structure. Basal lamina (arrow) are distinctly observed at the basal side in contact with collagen matrix (C); bar, 1 μm.*

and regulate cellular structure and function. In this manner, these receptors play an essential role in cellular differentiation.

Alveolar epithelial cells in vivo are exposed to air. This condition must be sought for culture cells in vitro. We could have exposed keratinocytes to air in reconstructed skin in vitro with our novel method.[6] The maximum diameter of reconstructed cystic lumen was 1 mm in this culture. Artificial infusion of air into the lumina seems to be a possibility, and might promote cellular differentiation, eg, production of surfactant in the culture cells.

Epidermal growth factor promoted the growth of the alveolus-like structure, and cholera toxin especially made the structure relatively globular. However, even in the culture condition of serum-free medium containing very small amounts of growth factors, alveolar type II cells proliferated and reconstructed the alveolus-like structure. This finding suggests that alveolar type II cells are self-differentiating cells,[8,9] and that a collagen matrix supports the expression of differentiation.

Finally, we hope that this culture system of alveolar type II cells will provide a physiological environment to study the differentiation and disorders of pulmonary alveoli in more detail.

Acknowledgments

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