Differentiation of Tracheal Basal Cells to Ciliated Cells and Tissue Reconstruction on the Synthesized Basement Membrane Substratum \textit{In Vitro}

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\textbf{INTRODUCTION}

Although lung epithelial cells directly attach to the basement membrane underneath \textit{in vivo}, harvested epithelial cells are typically cultured on type I collagen gel (Col I-gel) \textit{in vitro}. Recently we developed new culture substratum, designated as \textit{“synthesized Basement Membrane”} (sBM), that has bared lamina densa on fibrillar collagen. To validate the usefulness of sBM substratum in airway tissue reconstitution \textit{in vitro}, we cultured rat tracheal epithelial cells on sBM substratum and Col I-gel. When starting the air-liquid interface culture, most of the epithelial cells were squamous and positive for the basal cell marker cytokeratin 14 (CK14). After 14 days on sBM substratum, CK14-positive cells differentiated not only to Clara and mucous cells, but also to ciliated cells. Those differentiated cells formed pseudostratified-like epithelium and the remaining CK14-positive cells were polarized to the basal side. However, on Col I-gel, the CK14-positive cells were still squamous and not polarized, and ciliated cells did not appear. In conclusion, we established a new culture model on sBM substratum in which basal cells could differentiate to ciliated cells. The application of sBM substratum is useful in the study of the airway epithelial cell differentiation \textit{in vitro}.

\textbf{Keywords}  
Air-Liquid Interface, Airway Epithelial Cells, Basal Cell, Basement Membrane, Cell Differentiation, Extracellular Matrix, Lamina Densa, Laminin, Type IV Collagen

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Col-I gel has been used in respiratory cell cultures as a good substratum for promoting cell proliferation and differentiation. However, epithelial tissues \textit{in vivo} normally stand on the basement membrane but not on type I collagen. The basement membrane has a highly integrated architecture composed of specific extracellular matrices such as laminin, type IV collagen, perlecan of heparan sulfate proteoglycan, and entactin
(nidogen); these macromolecular constituents regulate epithelial differentiation and morphogenesis as well as cell attachment and motility. In addition, the basement membrane works as a reservoir of growth factors and cytokines as well as a mechanical support [10, 11]. Although the deposition of laminin is observed beneath the basal epithelial layer on Col I-gel culture by immunofluorescent microscopy, it is not a thin and continuous line as the lamina densa in vivo [12]. Instead of Col I-gel, Goto and colleagues [13] have proposed using human amnion as a basement membrane substratum and reconstructing tracheal epithelial tissue using primary epithelial cells prepared from guinea pigs. However, human amnion is not always an available substratum for cell culture.

In previous studies, we have reported that rat immortalized alveolar type II epithelial cells (SV40-T2 cells) cultured on a fibrillar collagen substratum could form a continuous lamina densa in the presence of Matrigel® as an exogenous source of laminin and entactin [14]. The major basement membrane components described above were integrated into the lamina densa [14, 15]. By removing only SV40-T2 cells from the epithelial tissue without harming the remaining extracellular matrix structure, fibrillar collagen matrix with lamina densa on the surface could be easily prepared. We name this substratum “synthesized basement membrane” (sBM). In this study, we report on this new substratum for reconstructing tracheal epithelialium in vitro and evaluate the usefulness of sBM substratum in establishing in vitro reconstitution of airway epithelial tissue. Even though we seeded the rat tracheal epithelial (RTE) cells that were proliferated and undifferentiated by subculturing the freshly harvested primary cells, they could differentiate to ciliated cells on the sBM substratum, but not on Col I-gel.

MATERIALS AND METHODS

Culture Media and Supplements

The basal medium formula was an equal volume mixture of Dulbecco’s modified Eagle’s medium (DMEM) (#05915, Nissui Pharmaceutical, Tokyo, Japan) and Ham’s F12 medium (#N6760, Sigma, St. Louis, MO, USA) containing 10 mM Hepes (Wako Pure Chemicals, Tokyo, Japan), 38 mM sodium bicarbonate, 100 U/ml penicillin G potassium (Meiji Seika Kaisha, Tokyo, Japan), and 100 μg/ml streptomycin sulfate (#S6501, Sigma). The complete medium formula was the basal medium supplemented with 10 μg/ml insulin (#I6634, Sigma), 0.1 μg/ml hydrocortisone (#H0396, Sigma), 0.1 μg/ml cholera toxin (#100, List Biological Laboratories, Campbell, CA, USA), 5 μg/ml transferrin (#T1283, Sigma), 50 μM phosphoethanolamine (#P0503, Sigma), 80 μM ethanolamine (#E0135, Sigma), 25 ng/ml epidermal growth factor (#EGF-201, Toyobo, Osaka, Japan), 30 μg/ml bovine pituitary extract (#P1476, Sigma), 0.5 mg/ml bovine serum albumin (#A8806, Sigma), and fresh 50 nM retinoic acid (#R2625, Sigma).

Antibodies

Mouse monoclonal antibodies for MUC5AC (Clone 45M1, #MS-145-P), β-Tubulin IV (Clone ONS1A6, #MU-178-UC), and cytokeratin 14 (CK14) (#MAB3232) were purchased from NeoMarkers (Fremont, CA, USA), BioGenex (San Ramon, CA, USA), and Chemicon (Temecula, CA, USA), respectively. Rabbit polyclonal antibodies raised against Clara cell-specific 26-kD protein (CCSP) (#AB3700) and laminin (#10765) were obtained from Chemicon and Progen Biotechnik (Heidelberg, Germany), respectively. Affinity-purified anti-collagen IV antibody (#23709) was purchased from Polysciences (Warrington, PA, USA). Fluorescein-5-isothiocyanate (FITC)-conjugated goat affinity-purified antibodies to mouse (#55518) and to rabbit (#55664) were obtained from MP Biomedicals (Irvine, CA, USA). Normal mouse IgG (#sc-2025) and normal rabbit IgG (#sc-2027) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Isolation of Rat Tracheal Epithelial Cells

All animals were treated in accordance with the National Institutes of Health guidelines (Guide for the Care and Use of Laboratory Animals). RTE cells were isolated by enzymatic dissociation as previously described [3, 6]. Briefly, after 8- to 14-week-old male Sprague-Dawley rats (Japan Clea, Tokyo, Japan) were put under anesthesia with an intraperitoneal injection of 50 mg/kg sodium pentobarbital and then killed by exsanguination, the tracheas were removed and filled with 1% Pronase E for more than 8 hr. The RTE cells obtained from 2 rats were then treated overnight at 4°C. The dissociated cells were flushed out with Ham’s F12 medium containing 5% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, USA), and collected at 4°C by centrifugation at 500 × g for 10 min. The recovered cells were suspended in Ham’s F12 medium containing 0.5 mg/ml DNase I (#104159, Roche Diagnostics, Indianapolis, IN, USA) and 10 mg/ml bovine serum albumin to dissociate cell aggregates. After a 5-min treatment in an ice bath, the cells were spun down for 5 min and suspended in the complete medium containing 3 mg/ml bovine serum albumin.

Subculture of Primary RTE Cells

The primary RTE cells were subcultured on collagen coat plates in the complete medium. Six-well nontissue culture treated plates (#1146, Becton Dickinson Labware, Franklin Lakes, NJ, USA) were treated with 2.5 ml of 0.1 mg/ml acid-extracted type I collagen from bovine dermis (#IAC-13, Koken, Tokyo, Japan) in 1 mM HCl solution for 15 min. After the solution was removed, the culture plates were air-dried for more than 8 hr. The RTE cells obtained from 2 rats were combined, seeded on a collagen-coated well, and cultured at 37°C in a humidified atmosphere with 5% CO2. On day 1, the nonadherent cells were removed and complete medium was supplied every 2 to 3 days. When the RTE cells reached 80% confluence, they were subcultured, undergoing treatment first.
with Dulbecco’s phosphate-buffered saline without calcium and magnesium [D-PBS(−)] at 37°C for 5 min and next with 0.25% trypsin-1 mM EDTA (#25200-056, Gibco Laboratories, Grand Island, NY, USA) for 6 min. The reaction of trypsin was stopped with Ham’s F12 medium containing 10% FBS, and the cells were collected by centrifugation at 500 × g for 5 min. They were suspended with the complete medium and seeded on collagen coated plates. In this study, we use two types of RTE cells: one was freshly harvested primary RTE cells; a the other was subcultured RTE cells.

**Type I Collagen Gel Substratum**

An acid-extracted type I collagen solution at the concentration of 3 mg/ml neutralized in DMEM, pH 7.2 was placed on the polyethylene terephthalate (PET) membrane of a cell culture insert (#3102, Becton Dickinson Labware) and allowed to polymerize at 37°C in CO2 incubator for 1 hr. After polymerization, the Col I-gel substratum was used for the RTE cell culture.

**Synthesized Basement Membrane Substratum**

sBM was prepared as previously described [14] (Figure 1). An acid-extracted type I collagen solution at the concentration of 0.45 mg/ml neutralized in DMEM, pH 7.2 was placed on the PET membrane of a cell culture insert, polymerized in CO2 incubator for more than 20 hr, air-dried, and rinsed with D-PBS(−) before use. This substratum is stiffer than the Col I-gel substratum to mimic the dense collagen matrix. We designated this hard substratum as fibrillar collagen substratum. Matrigel® (#354234, Becton Dickinson Labware) was placed on the bottom of a tissue culture plate (#3502, Becton Dickinson Labware) and allowed to polymerize in a CO2 incubator for 1 hr. Immortalized rat alveolar type II epithelial cells transfected with the SV40-large T antigen gene (SV40-T2 cells, a gift from Dr. A. Clement [16]) (8.0 × 10^5) were seeded on the fibrillar collagen substratum and cultured in DMEM containing 1% fetal bovine serum and 0.2 mM ascorbic acid 2-phosphate. After 14 days of culture, the cells were lysed and removed with D-PBS(−) containing 50 mM NH4OH, 0.1% Triton X-100, and protease inhibitor cocktail (5 µg/ml leupeptin [#L2884, Sigma], 5 µg/ml pepstatin A [#P5318], 1 µg/ml antipain [#A6191], 1 µg/ml chymostatin [#C7268], and 5 µg/ml phosphoramidon [#P7385]). The fibrillar collagen substratum holding synthesized lamina densa on the bared surface was termed sBM substratum.

**Preparation of Other Substratum**

For laminin-1 coating, PET membrane of cell culture insert was coated with laminin-1 (#354232, Becton Dickinson Labware) solution at the concentration of 10 µg/cm² in DMEM and placed at 37°C in CO2 incubator overnight. For type I collagen coating, PET membrane of cell culture insert was coated with acid-extracted type I collagen at the concentration of 10 µg/cm² in 1 mM HCl solution for 15 min. After the solution was removed, the culture inserts were air-dried for more than 8 hr. Fibrillar collagen substratum is the same substratum used for preparation of sBM substratum. In this study, we used 5 substrata: sBM substratum, Coll I-gel substratum, fibrillar collagen substratum, type I collagen coating, and laminin-1 coating.

**Cell Culture**

The primary and subcultured RTE cells were seeded on each substratum at the density of 2.0 × 10^5 cells/cm². Nonadherent RTE cells were removed after 24 hr. The culture medium was changed every other day with 2.5 ml of the complete medium in both compartments. The RTE cells submerged in the medium came to be confluent for 3–7 days and were lifted to the ALI. In the ALI culture, the RTE cells also were placed in a humidified atmosphere with 5% CO2. The apical surface of the cells was
rinsed every other day with Ham’s F12 medium and the bottom compartment was filled with only 1.6 ml of the medium.

Electron Microscopy

All fixatives and dyes for electron microscopy were obtained from TAAB (Berkshire, UK), except for Quetol™ 812 resin (Nissin EM, Tokyo, Japan). Cultured tissues of RTE cells were fixed at 4°C with 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, supplemented with 0.2 M sucrose and 0.1% tannic acid, and postfixed with 1% osmium tetroxide. The tissues were dehydrated through a series of graded ethanol. For scanning electron microscopy, the solvent was replaced with t-butyl alcohol. After the tissues were frozen and lyophilized, they were sputter-coated with a gold/palladium mixture and observed with a JEOL JSM-840 scanning electron microscope. For transmission electron microscopy, the solvent was replaced with n-butyl glycidyl ether and the tissues were embedded in Quetol-812 resin. The embedded tissues were processed to ultrathin sections, stained with lead citrate and uranyl acetate, and examined with a JEOL JEM-2010 microscope.

Immunofluorescent Microscopy

For immunohistochemical analysis, the cultured tissues were fixed with cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, containing 0.2 M sucrose. For vertical observation of the cell layer on a permeable support, tissues were embedded and frozen in Tissue Tek® OTC compound (Miles, Elkhart, IN, USA) and 5-µm thick transverse sections were cut with a cryostat. The sections were rinsed with 0.1 M phosphate buffer-0.2 M sucrose solution and nonspecific bindings were blocked with normal goat serum (#CL1200, Cedarlane, Ontario, Canada) and bovine serum albumin (#A2058, Sigma). The sections were treated with the primary antibodies in a humidified chamber at room temperature for 30 min. The control sections, which were treated with normal IgG instead of primary antibodies, were run in parallel. FITC-conjugated secondary antibodies were then applied at room temperature for 30 min. The sections were rinsed with 0.1 M phosphate buffer and 0.2 M sucrose and 0.1% tannic acid, and postfixed with 1% osmium tetroxide. The tissues were dehydrated through a series of graded ethanol. For scanning electron microscopy, the solvent was replaced with t-butyl alcohol. After the tissues were frozen and lyophilized, they were sputter-coated with a gold/palladium mixture and observed with a JEOL JSM-840 scanning electron microscope. For transmission electron microscopy, the solvent was replaced with n-butyl glycidyl ether and the tissues were embedded in Quetol-812 resin. The embedded tissues were processed to ultrathin sections, stained with lead citrate and uranyl acetate, and examined with a JEOL JEM-2010 microscope.

Total and Ciliated Cell Counting

To determine the cell densities of RTE cells, the cultured tissues were fixed with cold 4% paraformaldehyde in phosphate buffer-0.2 M sucrose solution and stained with DAPI. To determine the total cell number, the cell nuclei were counted by immunofluorescent microscopy. The cell numbers in 5 fields of each sample were counted.

The numbers of ciliated cells were counted from the pictures of scanning electron microscopy. The numbers of ciliated cells in 10 fields per sample were counted. The appearance rate of ciliated cells was evaluated as the ratio of the number of ciliated cells to the total number of cells in each culture.

Western Blot Analysis

Laminin isoform secreted from SV40-T2 cells was detected by Western blot analysis. SV40-T2 cells were cultured on fibrillar collagen substratum in DMEM containing 1% fetal FBS and 0.2 mM ascorbic acid-2-phosphate. After 7 days culture the conditioned medium at the side of basal surface was collected for 3 days from 11th to 14th day and mixed with the protease inhibitors’ cocktail of 5 µg/ml leupeptin, 5 µg/ml pepstatin A, 1 µg/ml antipain, 1 µg/ml chymostatin, and 5 µg/ml phosphoramidon. The collected medium was further processed to more than 10-fold condensation with microcon centrifugal filter device (Amicon Bioseparations YM-10, Millipore, USA), dissolved in SDS-PAGE sample buffer under reducing condition, and then separated by 5% polyacrylamide gel electrophoresis. The separated proteins were electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore) and the laminin isoform was identified with polyclonal antibody for human laminin α5 chain (#sc-20145, Santa Cruz) and mouse laminin α1-chain (#sc-6017, Santa Cruz). The immunoreactive protein bands were detected by with ECL Plus (Amersham Biosciences, Buckinghamshire, UK) and Polaroid 667. Nonspecific staining was blocked with Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan). Mouse laminin-1 was purchased from Becton Dickinson (#354232). The conditioned medium of the 293 cells endowed with human laminin α5-, β1-, and γ1-chain genes was the gift from Dr. Karl Tryggvason [17].

Statistics

Data are expressed as means ± SE. The results were analyzed by one-way analysis of variance for comparison between the two groups. Differences of p < 0.05 were considered significant.

RESULTS

Preparation of sBM Substratum

The structure of sBM substratum was confirmed by immunofluorescent and electron microscopies as previously described [14]. SV40-T2 cells on fibrillar collagen substratum formed sharp and continuous lamina densa structure beneath the cells (Figure 2A). After SV40-T2 cells were removed from the epithelial tissue, the remaining lamina densa was exposed on the surface of the residual tissue (Figure 2B). The surface of the sBM substratum was a flat and continuous sheet of fine felt-like meshwork (Figure 2C). By immunofluorescent microscopy, a thin and continuous line of laminin and type IV collagen, major basement membrane components, was observed (Figures 2D and 2E). The sBM substratum had the common characteristics of basement membrane structure in vivo.
FIG. 2. Electron and immunofluorescent microscopy observations of the sBM substratum formed by SV40-T2 cells in vitro. (A) A continuous thin lamina densa (arrows) was formed beneath SV40-T2 cells. (B) SV40-T2 cells were removed and the remaining lamina densa (arrow) was exposed on the surface of the residual tissue. The fibrillar collagen substructure was observed connecting below the lamina densa (arrowhead). (C) The sBM substratum was a flat and continuous sheet and had a fine felt-like meshwork seen by scanning electron microscopy. Observed by immunofluorescent microscopy, laminin (D) and type IV collagen (E) were continuously integrated into a thin layer on the surface of the sBM substratum. Bars = 0.4 µm (A, B), 1.0 µm (C), 50 µm (D, E).

Differentiation of RTE Cells on Collagen Gel and sBM Substratum

The primary and subcultured RTE cells were seeded on Col I-gel. Some differentiated ciliated cells were observed among the primary RTE cells on day 14 (Figure 3G). Although no ciliated cells were seen among the subcultured RTE cells on Col I-gel (Figure 3H), many ciliated cells were seen among the same cells on the sBM substratum (Figure 3I). The total number of cells on the sBM substratum increased during culture and was significantly higher than that on the Col I-gel on day 14 (Figure 4A). The appearance ratio of ciliated cells to the total number on the sBM substratum on day 14 was significantly higher than that of on the Col I-gel (Figure 4B). By day 14, the cultured epithelium showed pseudostratified-like structure on the sBM substratum, whereas it was still flat on the Col I-gel (Figures 3J, 3K and 3L). The sBM substratum was partially degraded by RTE cells at day 14. These results suggest that the sBM substratum could facilitate the growth and differentiation of RTE cells.

Polarization of RTE Cells

The number of RTE cells expressing CK14 as a basal cell marker decreased and came to be localized on the basal side in a time-dependent manner on the sBM substratum. In contrast, CK14-positive cells remained dominant and randomly distributed on Col I-gel (Figure 5). The differentiation of subcultured RTE cells to ciliated cells also was confirmed on the sBM substratum, whereas the cells on Col I-gel were negative with immunofluorescent microscopy for β-tubulin IV (Figures 6A, 6B, and 6C). The CCSP-positive Clara cells (Figures 6D, 6E, and 6F) and the MUC5AC-positive mucous cells (Figures 6G, 6H, and 6I) also were observed on the sBM substratum as the primary and subcultured RTE cells on Col I-gel. These data suggest that the sBM substratum could induce the polarity and the differentiation of the tracheal epithelial tissue, but Col I-gel failed.

Differentiation of RTE Cells on Laminin-1

Laminin-1 is one of the components integrated into the sBM substratum [14]. To investigate the contribution of laminin-1 and type I collagen to the differentiation, the subcultured RTE cells were cultured on a laminin-1 coating, type I collagen coating, and fibrillar collagen substratum (Figure 7). The subcultured RTE cells were not differentiated to ciliated cells on any kind of substratum. These results suggest that laminin-1 or type I collagen alone could not replace sBM substratum.

Laminin Isoform of SV40-T2 Cells

To identify the laminin isoform of SV40-T2 cells cultured on fibrillar collagen substratum, the condition medium from 11–14 day culture was collected, more than 10-fold condensed, Western blotted, and detected by chemiluminescence with ECL Plus (Figure 8). The protein band of MW 350k was immunoreactive to the antilaminin α5-chain polyclonal antibody raised for human H-160 peptide. The positive control of human laminin-10 also was detected in the condition medium of laminin-10-endowed 293 cells. Mouse laminin-1 and FBS were negative. As laminin β1- and γ1-chains also were detected in the conditioned medium (data not shown), rat SV40-T2 cells secreted at least laminin-10 isoform. Comparing with laminin α5-chain, a trace of protein band was reactive to anti-α1-chain polyclonal antibody raised for mouse M-20 peptide only by ECL advance (data not shown).

DISCUSSION

A primary culture model of airway epithelial cells from a variety of species including rat has been developed for studies of airway physiology in health and disease [3, 6–8, 13, 18–21]. The airway epithelial tissue and surrounding interstitium are complicated in their structure and function,
and the epithelial cells constituting the airway surface are associated with each other in the lineage of cell differentiation [22], in which basement membrane beneath the epithelial cells plays crucial roles. Because it has been difficult to mimic the complicated airway epithelial tissues in vitro, two important technical clues have been proposed to simplify the culture model: serum-free medium with growth and differentiation supplements including retinoic acid [2, 3] and ALI culture as an inducer of differentiation [1, 9]. Collagen matrix typically has been used for an airway epithelial cell culture model as a solid growth stimulant [4, 23, 24], ignoring that these cells stand on the basement membrane rather than on the type I collagen substructure in vivo.

One of the remaining problems is a source of airway epithelial cells; immortalized cell lines such as BEAS-2B [25] and SPC-1 [26] are not adequate for reconstructing airway tissue in vitro because of their high proliferation as well as less normal differentiation. On the other hand, the primary epithelial cells isolated by protease treatment of dissected trachea or bronchi have other disadvantages: low yield, reduced cell adherence, and heterogeneous cell population. The heterogeneity is especially disadvantageous for investigating cell differentiation.

Primary RTE cells are a mixture of heterogeneous cell population: mainly ciliated and secretory cells and a few basal cells. Each cell type appears to have a different adherence activity on culture substratum and the cell adherence changes depending on the impairment degree by the protease treatment in preparation, so that more than half the population of primary cells fails to adhere [27]. These characteristics of the primary cells have made it complicated to analyze the differentiation of basal cells to secretory and ciliated cells in vitro. To solve the problem of the heterogeneous population, we subcultured the primary RTE cells on a type I collagen coating up to the second passage and allowed the cells to proliferate, undifferentiating to a homogeneous population of basal cells. However, the immature basal cells alone failed to differentiate to ciliated cells not only on soft (Col I-gel) and stiff (fibrillar collagen substratum) matrices of type I collagen, but also on a laminin-1 coating even in the ALI culture. The basal cells squamously proliferated and multilayered on them. Once
FIG. 4. The primary rat tracheal epithelial cells were cultured on Col I-gel (□). The subcultured rat tracheal epithelial cells were cultured on Col I-gel ( Continent ). The subcultured rat tracheal epithelial cells were cultured on the sBM substratum (■). The total cell number in each culture is seen in upper graph; the appearance rate of ciliated cells in the lower. [Values are expressed as means ± SE; ∗ = significant at $p < .05$.]

the heterogeneous primary cells became an undifferentiated cell population in subculture, those cells appeared to lose the potential of differentiation as long as they were cultured on the traditional substratum.

Recently, we proposed the use of a sBM substratum for epithelial tissue reconstruction [14]. The basement membrane beneath epithelial cells has a highly integrated architecture composed of extracellular matrices including laminin, type IV collagen, heparan sulfate proteoglycan, and entactin (nidogen) [10]. Depending on the biological nature of each component and the relationship between molecular events such as masking and subsequent unmasking by limited proteolysis with matrix metalloproteinases, the basement membrane controls various kinds of cellular behaviors including differentiation [11]. Previously, we reported that alveolar type II epithelial cells immortalized by SV40-large T antigen (SV40-T2 cells) cultured on fibrillar collagen substratum could form the basement membrane beneath the cells with a large amount of exogenous laminin and entactin from Matrigel® [14, 15, 28, 29]. By removing only SV40-T2 cells from the culture tissue without impairing the extracellular matrices, the lamina densa could be prepared. The major basement membrane components were located in the lamina densa. We named this unique substratum sBM. Because not only type I collagen, but also laminin-I substratum were impotent to realize the potential for differentiation of the immature basal cells, we have shifted to using, sBM substratum for the differentiation of subcultured RTE cells.

The sBM substratum has proved to be powerful for inducing differentiation even after RTE cells were subcultured and undifferentiated to immature cells. The immature RTE cells homogenously expressed basal cell marker CK14 and could differentiate to ciliated cells on the sBM substratum. The differentiation of the subcultured basal cells on the sBM substratum was superior to that of the same cells on type I collagen substratum in the population of ciliated cells, and comparable with those of MUC5AC-positive and CCSP-positive cells. In addition, the CK14-positive cells on the sBM substratum were well polarized in the pseudostratified-like tissue developed from the homogenous subcultured basal cells, while the cells scarcely polarized in the squamously developed tissue on Col I-gel. This polarization of CK14-positive cells is similar to the observation

FIG. 5. Immunofluorescent microscopy observations of rat tracheal epithelial cells with anti-CK14 antibody as a basal cell marker. The primary (A, D, G) and subcultured (B, E, H) rat tracheal epithelial cells were cultured on Col I-gel. The subcultured rat tracheal epithelial cells were cultured on sBM substratum (C, F, I). The upper (A, B, C), the middle (D, E, F), and the lower (G, H, I) rows are the cultured epithelial tissues at days 0, 7, and 14, respectively. The basal cell marker CK14 was stained in green (arrows) with FITC and the nuclei were stained blue with DAPI. (Bars = 10 µm.)
of epithelial renewal induced by the secretory cell ablation with naphthalene in vivo reported by Hong et al. [30].

The differentiation of the basal cells on the sBM substratum appears to resemble the repair process in airway injury in vivo, in which the basement membrane does not appear to be damaged. Previously, Goto et al. [13] reported the use of human amnion for the construction of differentiated airway tissue in vitro. After the epithelial layer of amnion was removed, primary tracheal epithelial cells of guinea pig were seeded on the epithelial side and cultured along with fibroblasts. However, human amnion is scarcely available because of ethical and transport problems and medical safety. On the other hand, our sBM substratum can be easily produced. Once frozen, the substratum can be stored over 1 year in a deep freezer, transported while still frozen, and thawed before use. Easy handling also is of great advantage. Thus, the sBM substratum in this study is superior for epithelial tissue construction.

The subcultured RTE cells could realize the potential for normal differentiation on sBM substratum but not on Col-I gel. Although we cannot identify the reasons why sBM substratum induced differentiation of the tracheal epithelial cells, we can speculate about two possibilities: extracellular matrices and growth factors. Lammin is the most abundant glycoprotein in the basement membrane and plays an important role in the differentiation of epithelial cells [11]. In the sBM substrate, lammin-1 derived from Matrigel® is incorporated [14]. Alveolar epithelial cells synthesize lammin-α5 chain (lammin-10/11) rather than α1 (lammin-1) and α3 chains (lammin-5) [31]. SV40-T2 cells, immortalized rat alveolar type II epithelial cells, normally synthesize lammin-α5 [32], and we confirmed the secretion of lammin-α5-chain rather than α1-chain as well as β1- and γ1-chains in our culture models.

Therefore, lammin-10 at least may be integrated into the lamina densa of sBM substratum. Because the subcultured RTE cells at the second passage could not differentiate on the lammin-1 coating, lammin-10/11 may be critical in the differentiation. Extracellular matrices other than lammin, such as type IV collagen, perlecan (heparan sulfate proteoglycan),
and entactin (nidogen) have been proven to be integrated in the sBM substratum [14]. The association of laminin with other components of the sBM substratum also may be important to cooperatively facilitate cell differentiation.

Second, Matrigel® contains several kinds of growth factors: insulin-like growth factor 1, platelet-derived growth factor, transforming growth factor beta, fibroblast growth factor, and epidermal growth factor [33]. Because growth factors such as vascular endothelial growth factor and hepatocyte growth factor bind to basement membrane components [34], the growth factors from Matrigel® and conditioned medium of SV40-T2 cells, and fetal bovine serum in the preparation of sBM may have been adsorbed. According to transmission electron microscopy, the sBM substratum was partially degraded by the subcultured RTE cells. Therefore, the adsorbed growth factors may have been freed from the partially degraded sBM substratum and have contributed to the differentiation.

In conclusion, we have established a means to induce the differentiation of basal cells to ciliated cells on sBM substratum and to reconstruct airway epithelial tissue in vitro. In this study, we have clarified that the epithelial-basement membrane interaction plays a crucial role in the differentiation and morphogenesis of tracheal epithelium.

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