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Growth and Differentation of Rat Mammary Epithelial Cells Cultured in Serum-free Medium

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A new serum-free defined medium was developed that supports the growth of normal rat mammary epithelial cells. Mammary organoids from the glands of female F344 rats were cultured in a serum-free medium. Monolayer culture colonies developed within a week and remained viable for months in culture. Upon subculture of one-week-old primary colonies, almost the same morphology of colonies was developed. The scrape loading/dye transfer technique showed that most of colonies that developed in a serum-free medium containing EGF, human transferrin, insulin, and hydrocortisone (basal serum-free medium, BSFM) failed to show cell-cell communication. However, colonies cultured in BSFM supplemented with prolactin, E_2, and progesterone (complete hormone serum-free medium, CHSFM) showed cell-cell communication at 14 days of primary culture or of subculture. By flow cytometry with FITC-PNA and PE-anti-Thy-1.1 monoclonal antibody, we distinguished four RMEC subpopulations in cultures in both media: Thy-1.1+ cells, PNA+ cells, cells negative to both reagents and cells positive to both reagents. It is likely that combined prolactin, cortisol, and insulin in CHSFM stimulate terminal differentiation of clonogenic cells.

Key words : Mammary epithelial cell, Differentiation, Flow cytometry, Cell-cell communication

INTRODUCTION

The glands of young adult (7-8 week-old) female rats contain more than one functional type of differentiated epithelial cells including ductal epithelia, alveolar epithelia and myoepithelia; they have also been postulated to contain pluripotent self-renewing stem cells (Dulbecco *et al.*, 1986; Rudland and Barraclough, 1988; Kim and Paik, 1995). They are also likely to be transitional cell types.

Culture of the heterogenous mammary epithelial cells in serum-containing medium also gives rise to several morphologically different cell types *in vitro*. Concentration of the clonogenic cells have thus been difficult. Recently, immunocytochemical stains have been used to distinguish the ductal epithelial, myoepithelial, and alveolar epithelial cell types in the rat *in vivo*. Peanut lectin (PNA) binds to normal human breast epithelial cells as well as to many human carcinoma cells; it has been described a marker for breast epithelial cell differentiation (Newman *et al.*, 1979a,b; Klein *et al.*, 1979). PNA also stains the luminal alveolar cells of rats (Rudland and Barraclough, 1988; Rudland, 1991). Thy-1 is a differentiation marker of potential rat mammary myoepithelial cells *in vitro* (Morris and Ritter, 1980). We have distinguished four different RMEC subpopulations from the cultured cells in serum medium by flow cytometry with fluorescein isothiocyanate (FITC)-PNA and phycoerythrin (PE)-anti-Thy-1.1 monoclonal antibody (Kim and Clifton, 1993; Kim *et al.*, 1993).

As the cultured mammary epithelial cells in a medium supplemented with 10% fetal bovine serum, insulin, hydrocortisone, prolactin, 17β-estradiol, and progesterone have higher clonal growth potentials in graft sites than cells from intact glands, we intended to improve the culture conditions to enrich the clonogenic cell population (Kamiya *et al.*, 1991a; Kim and Clifton, 1993). The culture system has allowed assessment of the ability of hormones such as prolactin, estradiol, insulin, progesterone, and hydrocortisone, known to stimulate growth *in vivo* and in organ and primary culture, to directly stimulate the growth of mammary epithelial cells. However, the use of medium containing serum which may mask, inhibit, or modulate the growth-

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promoting effects of exogenous hormones limited the evaluations of its effects.

The phenotypic characteristics of stem cells are a) their ability for self-renewal and b) their ability to differentiate into the specific cells of the tissue of origin. Trosko and Chang (1989) have suggested that stem cells also lack of cell-to-cell communication. They speculate that early stem cells do not have functional gap junctions but are controlled or suppressed by negative growth regulators produced by their differentiated progeny. The differentiation of at least one of the stem cell daughter cells would then be accompanied by, and dependent on, the expression of gap junctions.

This report describes the development of a serum-free culture system in which rat mammary epithelial cells undergo sustained growth, the determination of intercellular communication in culture colonies, and the identification and separation of four different RMEC subpopulations.

MATERIALS AND METHODS

Cell culture medium

Serum-free medium (SFM) was comprised of a 1:1 (v/v) mixture of Eagle's modified essential medium (EMEM) and modified MCDB 153 medium (as Keratinocyte Basal Medium, Clonetics, San Diego, CA, USA). Basal serum-free medium (BSFM) was supplemented of SFM with EGF (10 ng/ml), human transferrin (10 μg/ml), gentamicin sulfate (50 μg/ml), insulin (5 μg/ml), and hydrocortisone (0.5 μg/ml). Complete hormone serum-free medium (CHSFM) was supplemented of SFM with insulin (5 μg/ml), hydrocortisone (0, 0.05, 0.5, or 5.0 μg/ml), progesterone (0.5 μg/ml), 17β-estradiol (0.005 μg/ml, Sigma, St. Louis, MO, USA), and 5 μg/ml bovine prolactin (Hormone Distribution Office, National Institute of Arthritis, Digestive Disorders and Kidney Diseases, Bethesda, MD, USA).

Mammary epithelial organoids isolation and culture

Virgin female F344 rats, 50-55 days old, were killed and their inguinal mammary fat pads were removed, and digested with collagenase solution (Type III, 2 mg/ml, Worthington Biochemical, Freehold, NJ, USA) in SFM with shaking at 37°C for approximately 3 hours. After digestion, the suspension was washed in serum medium (SM, consisted of SFM with 10% fetal bovine serum, FBS, HyClone, Logan, UT, USA) and centrifuged, and the pellet which contained cells, cell clumps, and mammary organoids was collected. The mammary digest was distributed to 10 cm polystyrene culture dishes (Lux, Naperville, IL, USA) in SM and incubated at 37°C for 2 hr to allow the rapidly adhering cells which are predominantly fibroblasts and other cells of mesenchymal origin to attach to the dish. The supernate, which contained the free epithelial cells, cell clumps, and organoids (ductal and endbud fragments) were collected, pooled and washed with centrifugation. The organoids and cells were resuspended in SM and the organoids were collected on a 40 m pore filter (Tetko, Briarcliff Manor, NY, USA) which allowed the dispersed cells and small cell clumps to pass. The organoids were resuspended by backwashing the filter, were distributed in culture dishes in an appropriate SFM with 5% FBS at 37°C for 1 day in a humidified 5% CO_2/air atmosphere. Next day, SFM with 5% FBS was removed and either BSFM or CHSFM was added. Each 10 cm petri dish contained one inguinal fat pad-equivalent of mammary organoids. The medium was changed 3 times weekly.

For subculture, the cultured cells and organoids for 7 days were dispersed by exposure to 0.2% trypsin-EDTA (GIBCO) with chick serum at 37°C for 6 min. The cells and organoids were collected, pooled and washed with centrifugation at ~350 g for 6 min. The organoids and cells were resuspended in an appropriate SFM with 5% FBS for 1 day and distributed in culture dishes with an appropriate numbers. Next day, SFM with 5% FBS was removed and either BSFM or CHSFM was added.

To make single cell suspension, the cultured cells were collected with 0.2% trypsin-EDTA with chick serum for 6 min, washed with SFM and resuspended in 0.05% trypsin-EDTA and incubated at 37°C for 9 min with shaking. The resultant cells were washed and resuspended in SFM. Three ml 0.05% DNase (Worthington Biochemical) was added per 10 ml suspension, and the mixture was broken up by pipetting and filtered in sequence through 40, 20 and 10 m pore size Nytex filters. The concentration of morphologically intact cells was determined by mixing 1 vol of cell suspension with 1 vol of 0.5% trypan blue in 0.85% saline and counting by phase microscopy in a hemacytometer.

Labeling cells with BrdU and PI

Bromodeoxyuridine (BrdU, 10 μg/ml) was added to organoid cultures which were then incubated at 37°C for 1 hr. The cells were then harvested, washed in PBS and fixed in 3 ml cold 70% ethanol with 0.5% Tween-20. The fixed cells (2×10^6) were treated with 0.04% pepsin (Sigma) in 0.1 N HCl at 37°C for 30 min. They were then washed once in PBS and resuspended in 1.5 ml of 2 N HCl with 0.5% Triton X-100 (Sigma) for 30 min to partially denature the DNA. After incubation, 3 ml borax (sodium tetraborate, 0.1 M $Na_2B_4O_7$, pH 8.5, Sigma) was added to neutralize any excess acid. The cells were then washed once with 3 ml PBS-TB (PBS with 1% BSA and 0.5% Tween-20, Sigma). The pellets consisting of permeabilized cells

and nuclei were resuspended in 1 ml RNase (50 µg/ml in PBS) and incubated at 37°C for 20 min. Forty l anti-BrdU antibody (Becton Dickinson, Mountain View, CA, USA) was added to these cells and nuclei and they were incubated at room temperature for 30 min. Afterward, the cells and nuclei were washed once with 3 ml PBS-TB and resuspended in 200 µl PBS-TG (PBS with 0.5% goat serum and 0.5% Tween-20) containing 2.8 µg FITC conjugated F(ab')2 goat-anti-mouse IgG (Tago, Burlingame, CA, USA) and incubated at room temperature for 30 min in the dark. After incubation, the cells and nuclei were washed with 3 ml PBS-TB and resuspended in 1 ml PBS-TB containing 5 µg/ml PI. The dual-labeled cells were analyzed by Becton Dickinson FACScan flow cytometer. Laser excitation at 488 nm and standard FITC/PI emission filters were used. Ten thousand cells were analyzed for each sample. Analysis of the DNA distribution was done with Lysis II version 1.0 software (Becton Dickinson).

Immunostaining of monodispersed cells

The concentration of monodispersed cells in suspension was adjusted in PBS with 1.0% bovine serum albumin (BSA, Sigma) to 2×10^7 cells/ml. Fifty µl aliquots of the cell suspension (1×10^6 cells) were distributed in plastic tubes. Staining with 50 µl FITC-peanut lectin (PNA-FITC, 1.25 µg/ml, Vector Laboratories, Burlingame, CA, USA) and/or 8 µl Phycoerythrin-conjugated anti-Thy-1.1 (Thy-1.1-PE) monoclonal antibody (Bioproducts For Science, Indianapolis, IN, USA) was carried out at 4°C for 30 min. Some cell samples were single-labeled with either PNA-FITC or Thy-1.1-PE and others with both. A negative control for the lectin staining was prepared by pre-incubation with 0.2 M galactose (Sigma) for PNA-FITC before staining the cells as above. A negative control for anti-Thy-1.1 antibody was aliquots of cell suspension incubated with PE-conjugated IgG_1 isotype (Becton Dickinson). The stained cells were then washed and the final concentrations were adjusted to 1×10^6 cells/ml in PBS with 1% BSA.

Flow cytometric analyses

Fluorescence activated flow cytometric analyses of the monodispersed stained cells from 4, 7, and 14 days as primary and subcultures were performed with a Becton Dickinson FACScan. Cells were excited at 488 nm with 15 mW for analysis. Green FITC fluorescence was measured with a 530/30 band pass filter, and orange PE fluorescence with either a 585/42 or 575/26 band pass filter. Forward light scatter, side scatter, and fluorescence signals were collected in list mode files. For the analysis, dead cells were excluded on the basis of propidium iodide (PI) uptake (1.0 µg/ml, Sigma). Data were analyzed with Lysis II version 1.0 software (Becton Dickinson). Paint-A-Gate software (Becton Dickinson) was used for the analysis of double-positive cells.

Immunostaining of cultured cells *in situ*

About ~100 organoids were distributed into each well of 24-multiwell Primaria culture plates (Becton Dickinson), and were cultured in serum-free medium from 1 to 14 days as described as above. On days 1, 2, 4, 7, and 14 of culture, the selected culture plates were washed with PBS with 1% BSA and stained with PNA-FITC and Thy-1.1-PE as above. The cells were viewed under a Zeiss Axiovert 35 fluorescence phase microscope.

Determination of intercellular communication

The ability of rat mammary epithelial cells to perform gap junctional intercellular communication (GJIC) was determined by the scrape loading and dye transfer (SLDT) method (Chang *et al.*, 1987). The cells on plates cultured for 4, 7, and 14 days as primary and subcultures were rinsed with PBS before the addition of a mixture of fluorescent dyes (0.05% Lucifer yellow, MW 457.2, and rhodamine dextran, MW 10,000 in PBS, Sigma). The dye solution was added into plates and cut cultured colonies with a surgical blade. After 4 min at room temperature the dye solution was decanted and the plates were then rinsed several times with PBS. The colonies were examined for Lucifer yellow transfer across membrane gap junctions in culture medium under a Zeiss Axiovert 35 fluorescence phase microscope.

Statistics

Statistical significance was determined using Student's t test. $P<0.05$ was judged to be statistically significant.

RESULTS

Cell growth and immunostaining of cells *in situ*

The organoid cultures which had been plated with one inguinal fat pad-equivalent of mammary tissue each had received ~2×10^4 (20%) organoids per 10 cm dish. In BSFM, about 47% of the organoids were adherent after 1 day. In CHSFM, about 60% of the organoids were adherent after 1 day. With time in culture media, monolayer cells spreaded out from the organoids. *In situ* immunostaining of cultured organoids and cells with PNA-FITC and Thy-1.1-PE showed very distinctive cell growth patterns. There were three morphologically different organoids: elongated, spherical, and mixed types. The majority of these organoids proliferated into the different types of colonies. Most of cells in elongated organoids were positive to

Thy-1.1-PE antibodies. However, most of cells in spherical shape organoids were positive to PNA-FITC, and cells in mixed type were positive to both markers. Representative staining patterns are shown in Fig. 1.

As we early reported (Kim and Clifton, 1993), there were three morphologically major cell types were observed by phase-contrast microscopy: a) small cuboidal, cobble stone appearance epithelial cells growing in tightly packed colonies, b) larger epithelioid cells that grew in colonies and often surrounding small cuboidal epithelial cells and colonies, and c) elongated cells often found in areas between the epithelial colonies. Several other minor cell types were sometime observed. Cells with spindle shape were coming from tubular-shape organoids which contained red blood cells. At day 1, most of out-growth cells from spherical organoids were tightly packed cobble stone appearance. Most of out-growth cells cultured in BSFM were completely negative to either Thy-1.1-PE or PNA-FITC. However, out-growth cells cultured in CHSFM showed very different patterns. The cells in outer layer of out-growth were also completely negative to

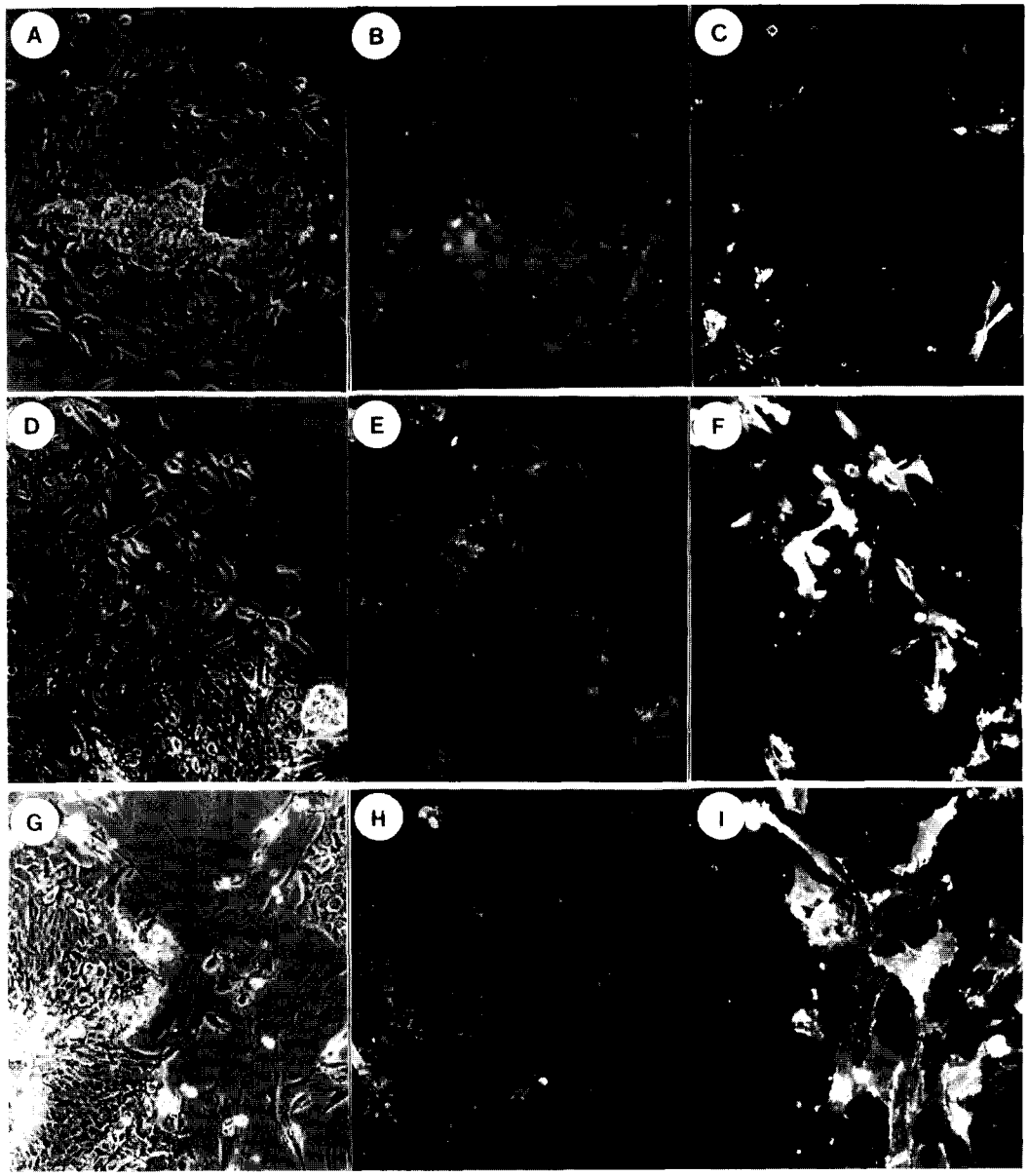

Fig. 1. Immunostaining of RMEC in primary cultures in BSFM for 2 days (A,B,C), 7 days (D,E,F), and 14 days (G,H,I) with PNA-FITC and Thy-1.1-PE. The cells were viewed under a Zeiss Axiovert 35 fluorescence phase microscope with a 485/17 band pass filter for green FITC fluorescence (B,E,H) and a 546/12 band pass filter for orange PE fluorescence (C,F,I). After two days, small cuboidal, cobble stone like cells in out-growing portion are positive PNA-FITC (B,E,H) and epithelioid cells at boundary of colonies are staining to Thy-1.1-PE (C,F,I). Phase contrast ×100.

either markers and the cells in inner layer of out-growth were positive to PNA-FITC and negative to Thy-1.1-PE (data not shown). Most of out-growth cells from elongated organoids were positive to Thy-1.1-PE in both media. By day 2, the cells which were negative to PNA-FITC became positive cells (Fig. 1A, 1B, 1C). The Thy-1.1+ cells remained as it were. Most of mono-layer cell colonies were established by day 7 (Fig. 1C, 1D, 1E). PNA+ cells had a cuboidal, cobble stone appearance and Thy-1.1+ cells were spindle shaped. Most of cells located near the center of the colonies were positive to PNA-FITC and cells located at the colony boundaries were positive to Thy-1.1-PE. By 14 days in culture, most of remained organoids were degenerated and finally disappeared (Fig. 1G). Most of Thy-1.1+ cells were elongated or flat shapes and had come to predominate (Fig. 1I); only a small area of PNA+ cells remained in organoids (Fig. 1H). Some of colonies positive to PNA-FITC remaining at 14 days had very weak strength of FITC fluorescence (Fig. 1H).

Gap junctional intercellular communication

The cell colonies cultured in BSFM were found to be not able to transfer Lucifer yellow as revealed by the SLDT till 14 days in primary and subcultures (Fig. 2A and 2B). When a second fluorescent dye of larger molecular weight, the rhodamine dextran, was concurrently used, it remained and thus labeled the primary loaded cells which have been already damaged by the scrape (data not shown). However, the cell colonies cultured in CHSFM were found to be able to transfer Lucifer yellow at 14 days in primary and subcultures (Fig. 2C and 2D). The summarized data are in Table 1.

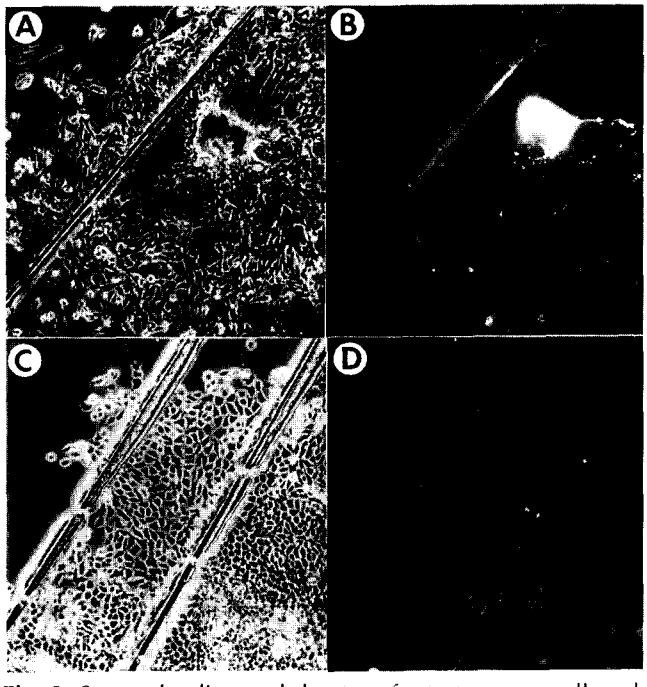

Fig. 2. Scrape loading and dye transfer test among cells cultured in BSFM (A,B) or CHSFM (C,D) for 14 days (×100). Photomicrograph B is negative and D is positive for intercellular exchange of Lucifer yellow dye.

Table 1. Results of scrape loading and dye transfer tests of colonies cultured for 4, 7, or 14 days as primary or subcultures in BSFM or CHSFM

	Primary Culture			Subculture		
	4-D	7-D	14-D	4-D	7-D	14-D
BSFM	-	-	-	-	-	-
CHSFM	-	-	+	-	-	+

Flow cytometric analysis of immunostained cultured cells

Multiparameter flow cytometric analysis of cells stained with both PNA-FITC and Thy-1.1-PE showed four different populations of cells: PNA-positive cells (PNA+), Thy-1.1-positive cells (Thy-1.1+), cells negative to both reagents (B-), and cells positive to both reagents (B+). Cells cultured in BSFM and CHSFM showed very different distribution patterns of RMEC subpopulations. Generally, the fraction of PNA+ cells in BSFM was almost constant or slightly changed with time in culture (Fig. 3A). However, the fraction of PNA+ cells in CHSFM significantly decreased with time in primary (Fig. 3B). The fractions of B- cells in BSFM generally decreased with time (Fig. 3A) However, B- cells in CHSFM showed opposite response to the variation of concentration and culture period (Fig. 3B). Fraction of Thy-1.1+ cells were almost constant in BSFM and their relative sizes were also small (Fig. 3A). However, in CHSFM, these cells were signifi-

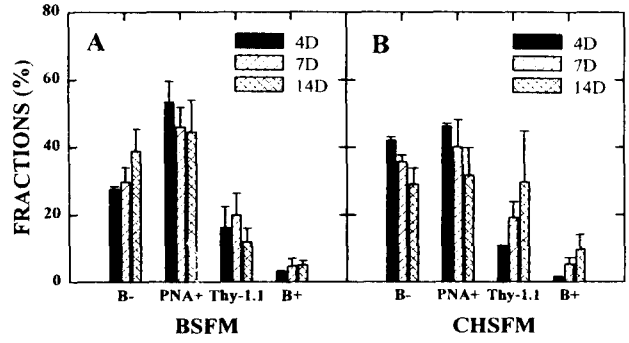

Fig. 3. The effect of culturing time on the fractions in the various RMEC subpopulations in organoid cultures in basal serum-free medium (BSFM) (A) or in complete hormone serum-free medium (CHSFM) (B) after 4 (4D), 7 (7D), and 14 (14D) days in culture. The organoids were cultured in 10 cm petri dishes which contained one inguinal fat pad-equivalent of mammary tissue each. Bars represent the mean SEM.

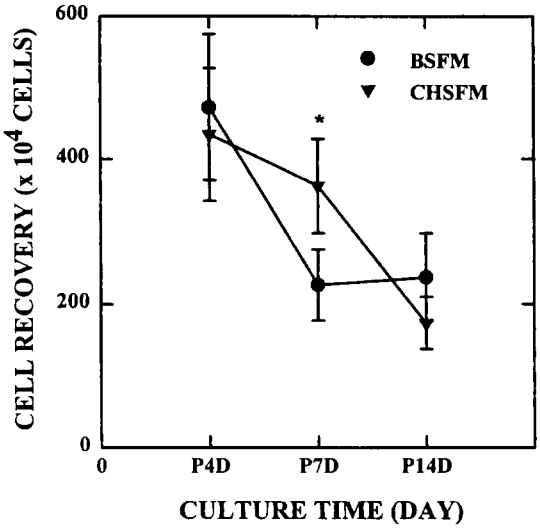

Fig. 4. Cell recovery rates from organoid cultures in BSFM or in CHSFM with different culturing times. Cell numbers were determined with trypan blue exclusion method. Each point represents the recovered cell numbers from three petri dishes. Each point represents the mean SEM. Statistical significance: *P<0.05. P4D, P7D, and P14D: 4, 7, and 14 days after primary culture, respectively.

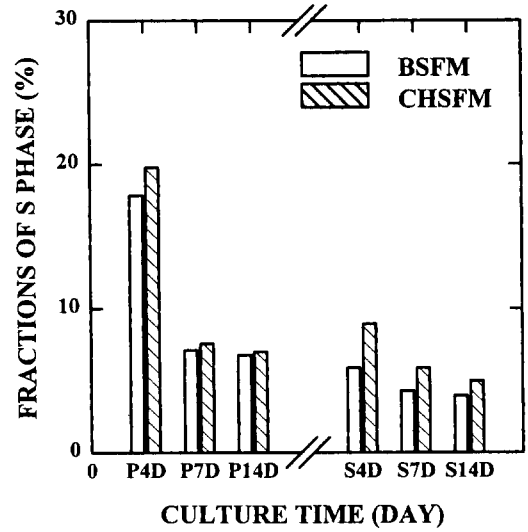

Fig. 5. Variations in the S phase populations in primary cultures of organoids cultures in BSFM or in CHSFM with time. The organoids were cultured in 10 cm petri dishes which each contained one inguinal fat pad-equivalent of mammary tissue. Cultures were pulsed with BrdU (10 μg/ml) for 1 hour immediately before dispersion for analysis. P4D, P 7D, and P14D: 4, 7, and 14 days after primary culture, respectively. S4D, S7D, and S14D: 4, 7, and 14 days after subculture, respectively.

cantly increased with time and its fraction was greater than fraction in BSFM (Fig. 3B). There were small amount of B+ cells throughout in both media (Fig. 3A and 3B). However, unlikely to cell preparation from culture in serum medium (Kim and Clifton, 1993), very prominent B+ cell populations were detected at dot plots of FACS analysis data (data not shown). Although the numbers of B+ cells are small, the presence of these cells supports the conversion of epithelial cells into a mesenchyme-like phenotype (Barraclough *et al.*, 1984).

After each cell culture period, the cell recovery rates were calculated after cell counting (Fig. 4). The cell numbers recovered from three petri dishs were counted. There were not significantly difference between cells cultured in BSFM and in CHM for 4 or 14 days. However, cells cultured for 7 days in each medium showed statistically significance.

Growth kinetics of epithelial cells

We compared the BrdU-PI bivariate distributions of organoid cultures in BSFM and CHSFM with 0.5 μg/ml hydrocortisone after different culture times. The percentages of cells in th S phase of the cell cycle changed with time in culture (Fig. 5). Generally, cells cultured in CHSFM contained more S phase cells than cells cultured in BSFM.

DISCUSSION

The present study has clearly shown that a) serum-free defined media (BSFM or CHSFM) was able to support the growth of primary normal rat mammary epithelial cells; b) the rat mammary epithelial cells developed in these media contain two types of cultured colonies in response to the supplemented hormones: one is deficient in GJIC and the other is capable of GJIC; c) four different subpopulations of RMEC were discerned; d) these mixed cells or sorted cells with PNA-FITC and Thy-1.1-PE induced AU in grafts; and e) the manipulation of hormones in culture media showed the change of morphological and clonal growth potential of cultured cells. Proliferation of RMEC was shown to be sensitive to a number of hormones and growth factors as well as as to the tissue culture medium.

Primary cultures of virgin rat mammary glands in serum-free medium gave three morphologically distinct major cell types: small cuboidal, cobble stone appearing epithelial cells, larger epithelioid cells, and elongated cells. The small cuboidal cells were positive to peanut lectin which binds only to alveolar epithelial cells or binds to ductal lining epithelial cells in the virgin rat mammary gland *in vivo* after their treatment with neuraminidase (Warburton *et al.*, 1985). Larger epithelioid cells and elongated cells were all positive to Thy-1.1+.

Peanut lectin has been used to identify luminal mammary epithelial cells that line the mammary ducts in vivo (Newman *et al.*, 1979a,b) and it also

binds to the small cuboidal cells in primary cultures only after treatment with neuraminidase (Warburton *et al.*, 1985). Anti-Thy-1.1 antibodies binds to the cell surface Thy-1.1 antigen on myoepithelial cells and myoepithelial-like cell line (Barraclough *et al.*, 1987). The cells cultured in serum medium were discriminated into four cell subpopulations with the immunocytochemical cell surface markers peanut agglutinin (PNA) labeled with FITC and phycoerythrin (PE)-conjugated anti-Thy-1.1 monoclonal antibody and flow cytometry (Kim and Clifton, 1993).

One of the determination for stem-like cells cultured in several different culture conditions was their lack of gap junction-mediated intercellular communication. Chang *et al.* (1987) demonstrated that small subpopulation of normal human fetal kidney epithelial cells were deficient in gap junction-mediated intercellular communication and therefore contact-insensitive. These cells were able to proliferate on the fibroblast cell mat. They also had greater proliferative potential than the parental cell population. Most of our cultured colonies as primary or subcultures in BSFM till 14 days or in CHSFM for 7 days showed no cell-cell communication.

The purpose of subculture after seven day of primary culture is to separate and induce the clonal growth of some heterogeneously present subpopulations in primary cultures. However, in this study, we could not get any colonies after the culture of monodispersed cells from primary culture. Almost all of colonies in subculture were coming from the remaining organoids in primary culture.

An effect of glucocorticoids on mammary epithelial cell proliferation in primary culture may be species specific or depend on the substratum on which the cells are grown or both. Kidwell *et al.* (1984) demonstrated a requirement for glucocorticoids for proliferation of RMEC grown on plastic or type I collagen; however, this requirement was significantly reduced in RMEC growing on type IV collagen. No requirement for glucocorticoid was reported for proliferation of RMECs growing on type IV collagen (McGrath *et al.*, 1985); it was required, however, for mouse mammary epithelial cells grown under the same conditions (Imagawa *et al.*, 1985). Glucocorticoids were also shown to be required for human mammary epithelial cells growing on plastic (Hammond *et al.*, 1984) or within collagen gels (Yang *et al.*, 1982). The RMEC grown within a reconstituted basement membrane under serum-free medium without hydrocortisone slightly enhanced cell proliferation (Hahm and Ip, 1990). Furthermore the morphology of colonies was considerable altered, with an increase in ductal formation resulting in colonies with an extensive branching network, and a decreased number of the colonies with lobular-like budding clusters. They also found that casein production was decreased when RMECs were grown in the absence of hydrocortisone.

The cells cultured in serum-free media without hydrocortisone gave rise to sizable colonies with time. Although their sizes were relatively smaller than colonies cultured in serum-free media with various concentration of hydrocortisone. The cell-cell communication ability in colonies were not affected the presence or absence of hydrocortisone in both media. There may be other factors to induce protein(s) which control the ability of intercellular cellular junctional complex system. The fractions of individual subpopulations from both media showed very different changing patterns with time and concentrations of hydrocortisone. Generally, the fractions of PNA+ cells in BSFM with various concentration of hydrocortisone were greater than PNA+ cells in CHSFM. Moreover, the fractions of Thy-1.1+ cells, the least clonogenic fraction, from CHSFM were larger than cells from BSFM. The transplantation studies of cells cultured in media showed that cells cultured in BSFM contained more clonogenic fractions than cells in CHSFM. Fraction of cells in S phase of cell cycle were greater in cell population from CHSFM than in cells from BSFM. The study of cell recovery rate after culture in either BSFM or CHSFM suggest that cells cultured in CHSFM were more persistent in colonies than cells in BSFM. From these finding, we speculate that cells in CHSFM are in conversion to terminally differentiated cells, *i.e.*, Thy-1.1+ cells, with time in culture.

In summary, the serum-free defined media were able to support the growth of primary normal rat mammary epithelial cells, the cells cultured in these media contain two types of cells with different ability cell-cell communication, four different subpopulations of RMEC were discerned with PNA-FITC and Thy-1.1-PE and the manipulation of hormones in culture media showed the change of morphological and clonal growth potential of cultured cells.

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