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Isolation and Characterization of Mammary Epithelial Stem Cells in Culture

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Abstract

The mammary gland contains a subpopulation of epithelial cells with large proliferative potentials which are the likely targets for carcinogens. These clonogenic cells can proliferate and differentiate into functional glandular structures. Rat mammary epithelial cells (RMEC) were isolated and characterized *in vitro*. By flow cytometry of RMEC stained with fluorescein isothiocyanate-peanut agglutinin(PNA) and phycoerythrin anti-Thy-1.1 monoclonal antibody, it was possible to four cell subpopulations from 7-8 week old F344 female rat mammary glands: cells negative to both reagents (B-), PNA-positive cells (PNA+), Thy-1.1-positive cells (Thy-1.1+), and cells positive to both reagents (B+). When single PNA+ cells were isolated and cultured in Matrigel with irradiated (~50 Gray) 3T3 fibroblast feeder layer, they gave rise to multicellular clonal structures of three types: alveolar, foamy alveolar, and squamous colonies. The developed structures were similar to the mammary glands *in vivo*. These results suggest that some of PNA+ cells possesses many of the characteristics of multipotent clonogenic stem-like cells.

Key words – RMEC(rat mammary epithelial cell), clonogen, stem-like cells

Introduction

The mammary gland contains a subpopulation of stem-like clonogenic epithelial cells with a large potential for self-renewal and for proliferation and differentiation [1,2]. When grafted and hormonally stimulated, these cells, termed clonogens, give rise to clonal multicellular epithelial structures from which mammary cancer can arise. Identification of these putative stem-like cells is an important matter not only in developmental biology, but also because these cells appear to be prime targets for the action of carcinogens. Several mammary cell preparation methods have tested in an effort to concentrate and characterize the stem-like clonogenic epithelial cells mammary glands [1-3]. Immunocytochemical stains have been used to distinguish the ductal epithelial, myoepithelial, and alveolar epithelial cell types in the rat.

Peanut agglutinin (PNA) from *Arachis hypogaea* (peanut) binds to oligosaccharides containing the terminal sequence β-D-galactosyl-(1-3)-N-acetyl- D-galacto-samine [4]. The PNA has been described as a binding protein to normal human breast epithelium as well as many human carcinomas and as a marker for breast epithelial cell differentiation. PNA also stains most alveolar epithelial cells, as well as the luminal alveolar cells of rat [5,6].

Thy-1 was originally described as a cell surface allo-

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antigen of mouse thymocytes [7]. It is a small glycoprotein (MW: 17,500-25,000 D) found at the surface of cells [8]. In the mouse, it is encoded by a gene that maps on chromosome 9 [9] and the antigen has two allelic forms, Thy-1.1 and Thy-1.2. However, the rat has only Thy-1.1 [10]. In the rat, it was also described as a differentiation marker of potential rat mammary myoepithelial cells *in vitro* [11]. It is present on and immediately adjacent to the myoepithelial cells of ducts and alveoli [12].

To study the putative clonal origin of alveolar structure in mammary gland and the hierarchy of rat mammary epithelial cells evolution, the fluorescence-activated cell sorting technique has been used to sort cells which differentially express membrane antigens associated with either alveolar epithelial cells or myoepithelium.

Kim *et al.* [13] demonstrated that by flow cytometry of RMEC stained with PNA-FITC and anti-thy-1.1 monoclonal antibody, the PNA+ cells possess many of the characteristics of multipotent clonogen stem-like cells. Thy-1.1+ cells appears to be terminally differentiated; they may be primarily myoepitherial cells. B+ cells may be a transitional from one type of cell to another. No differences in morphology between alveolar unit (AU) that originated form mixed cells of organoid cultures and AU from sorted rat mammary epithelial cell (RMEC) subpopurations were detected.

These studies were designed 1) to study the growth kinetics of epithelial clonogens *in vitro*, 2) to determine the effects of feeder layer on the growth of sorted PNA+ cell subpopulations in Matrigel.

MATERIALS AND METHODS

Mammary single epithelial cells preparation

Mammary organoids were prepared as described previously [13]. Briefly, virgin female F344 rats, 7-8 weeks, were killed with CO_2 and inguinal mammary fat pads were removed, minced and digested in collagenase solution (Type III, 2 mg/ml) in SFM (serum-free Dulbecco's Modifed Eagle Medium, 50 μg/ml gentamicin sulfate, and 0.33 mg/ml glutamin) with shaking at 37℃ for approximately 3 h. After digestion, the suspension was washed in serum medium [SM; SFM with 10% fetal bovine serum (FBS)] and centrifuged, and the pellet which contained cells, cell clumps and mammary organoids (ductal and endbud fragments) were collected. The pellet was washed, resuspended, and passed onto a 40 μm pore Nytex filter (Tetko, Briarcliff Manor, NY, USA) which allowed only the dispersed cells and small cell clumps to pass. The organoids trapped on the filter surface were collected by backwashing the filter with SM and were again washed and recentrifuged twice at ~20 g for 2 min. The organoids were then resuspended in 0.05% Trypsin-EDTA 10 ml and incubated at 37℃ for 9 min with shaking. The resultant dispersed cells were washed and resuspended in SFM. Three milliliters of 0.05% DNase was added per 10 ml suspension, and the mixture was triturated and filtered in sequence through 40-, 20-, and 10-μm 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 and in 0.85% saline and counting dye-free cells in a hemacytometer by phase microscopy. The monodispersed cells were immunostained with PNA-fluorescein isothiocyanate (FITC) and anti-Thy-1-phycoerythrin (PE) as described in the below.

Immunostaining of single cells

Immunostaining was performed as previously described [13]. Briefly, the concentraion of monodispersed cells in suspension was adjusted in PBS with 1.0% bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) to 2×10^7 cells/ml. Fifty μl aliquots of the cell suspension (1×10^6 cells) were stained with 50 μl of FITC-peanut lectin (PNA-FITC, 1.25 μg/ml, Vector Laboratories, Burlingame, CA, USA) and/or 8 μl of phycoerythrin-conjugated anti-Thy-1.1 (Thy-1.1-PE) monoclonal antibody

(Bioproducts For Science, Indianapolis, IN, USA) at 4℃ for 30 min. Some cell samples were single-labeled with either PNA-FITC or anti-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-PE antibody was an aliquot of cell suspension incubated with PE-conjugated IgG_1 isotype (Becton Dickinson, Mountain View, CA, USA). 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 and cell sorting

Fluorescence activated flow cytometric analyses and sorting of the monodispersed stained cells were performed with FACScan or FACStar^{PLUS} fluorometes (Becton Dickinson), respectively. Briefly, immunostained cells were excited at 488 nm with 15 mW for analysis and 50 mW for sorting. Green FITC fluorscence was measured with a 530/30 band pass filter, and orange PE fluorescence was measured 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 softwares (Becton Dickinson). Paint-A-Gate software (Becton Dickinson) was used for the analysis of double-positive cells. For cell sorting, dead cells and debris were excluded by forward and side scatter, and sort windows were set on the appropriate fluorescence signals and sorted four RMEC fraction. The sorted fractions were put into each well of 24-well cell culture plate with automatic cell disposition unit (ACDU, Becton Dickinson) attached to flow cytometry from one cell to 100 cells.

Culture of normal mammary epithelial cells in Matrigel

First, appropriate numbers of mixed or sorted PNA+ cells (2×10^4 cells/well) were distributed into 24 multi-well plate in Matrigel without feeder layers and cultured for 4 weeks to see whether Matrigel itself could support the growth of sorted single RMEC *in vitro*. Second, several feeder layers were employed for the growth of sorted single RMEC in Matrigel. The feeder layers used in this study were 3T3 fibroblast cell line, F344, and WF (Wistar-Furth) rat mammary organoids. About 300~400 mammary organoid separated from F344 and WF rat put into 24 well plate and cultured for 3 days. After those feeder layers were cultured confluently in complete hormone medium (CHM: CHM consisting of SM with 0.5 μg/ml progesterone, 0.005 μg/ml 17β-estradiol, 0.5 μg/ml cortisol, 5 μg/ml insulin (Sigma), and 5 μg/ml bovine prolactin (Hormone Distribution Office, National Institute of Arthritis, Digestive Disorders and Kidney Diseases, Bethesda, MD, USA).

Confluent cells were irradiated with ~50 Gray ^{137}Cs γ-rays in 4 π radiation chamber and in case of Swiss 3T3 fibroblast cell, 25,000 cells put into 24 well plate and cultured for 2 days and confluent cells were irradiated as the same method in the above. And then 300 μl of diluted Matrigel (100 μl SFM in 400 μl stock Matrigel) was added on feeder layer, and then kept the cell culture plate at 4℃ until use (Fig. 1). The PNA+ cells separated by flow cytometry were directly sorted into each well of 24-well cell culture plate containing Matrigel and feeder layer using ACDU. The sorted cell

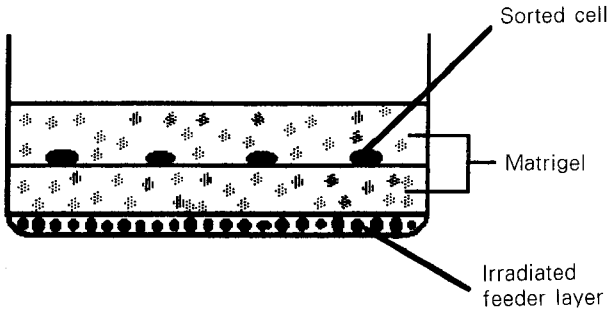

Fig. 1. Matrigel culture of irradiated feeder layer and sorted cells.

number in each well was one or 100 cells. After sorting, 250 μl of diluted Matrigel was added into each well, and let the gel formed at 37℃. After Matrigel formed gel, 1 ml of CHM was added in each well and cultured for 4 weeks.

Observation of multicellular structures and histological features

Multicellular structures and morphological features were observed every 2-3 days by phase contrast microscope. The numbers of colonies were determined in triplicate wells as described previously [14]. After 4 week-culture, the Matrigel obtained from the culture plate were fixed in Carnoy fixative (methanol : chloroform : acetic acid = 6 : 3 : 1) at 4℃ overnight.

By the standard method for tissue specimen, paraffin blocks containing the gel were made and cut with microtomb at 5-6 μm thickness. Those were stained with hematoxylin and eosin and observed with microscope.

Statistics

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

RESULT

Culture of normal mammary epithelial cells in Matrigel

When the mixed unsorted cells isolated from normal F344 rat mammary organoids and sorted PNA+ cells were cultured in Matrigel without feeder layer for 4 weeks, the only small number of multicellular colonies were formed and were not grown well. From the PNA+ cells, about 27 times more multicellular colonies were developed than from the mixed cells (Fig. 2).

The effect of feeder layer on development and proliferation of clonogens

The sorted PNA+ cells were cultured in CHM with

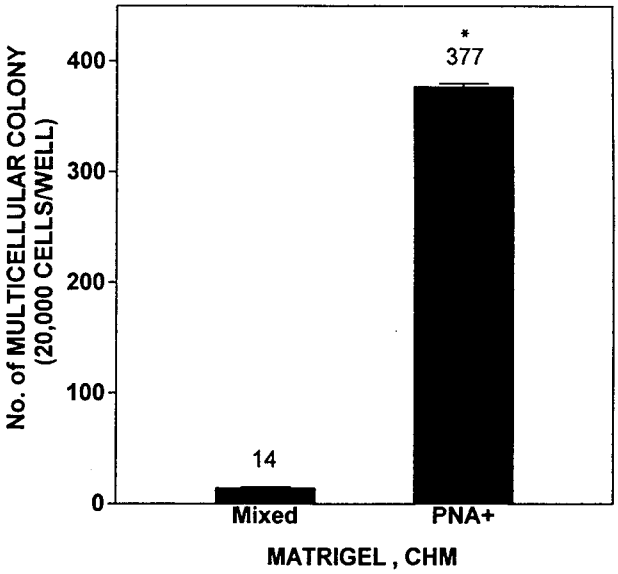

Fig. 2. Comparison of numbers of multicellular colonies which developed from mixed or PNA+ cells cultured in Matrigel. The numbers of cells (2×10^4/well) were distributed into 24 multiwell plate and cultured for 4 weeks.
Values were expressed as mean±S.D. Statistical significance: *<0.05.

F344 rat, WF rat, or 3T3 fibroblast feeder layer and multicellular structures developed from cells were observed. When cultured in CHM with 3T3 fibroblast feeder layer, the more multicellular colonies and especially foamy AU structures were formed than when cultured in CHM with F344 or WF rat feeder layers And then, when cultured in CHM with 3T3 fibroblast feeder layer, the fractions of foamy alveolar, alveolar, and squamous colonies were about 70%, 25%, and 4-5%, respectively. In contrast, when cultured in CHM with F344 or WF rat feeder layer, more than 90% of colonies were alveolar and very few were foamy alveolar and squamous colonies (Fig. 3 and Fig. 4).

Observation of multicellular structures and morphological features

When the sorted PNA+ cells were cultured in CHM with 3T3 fibroblast feeder layer, multicellular structures were formed from single cells. With time in culture, the

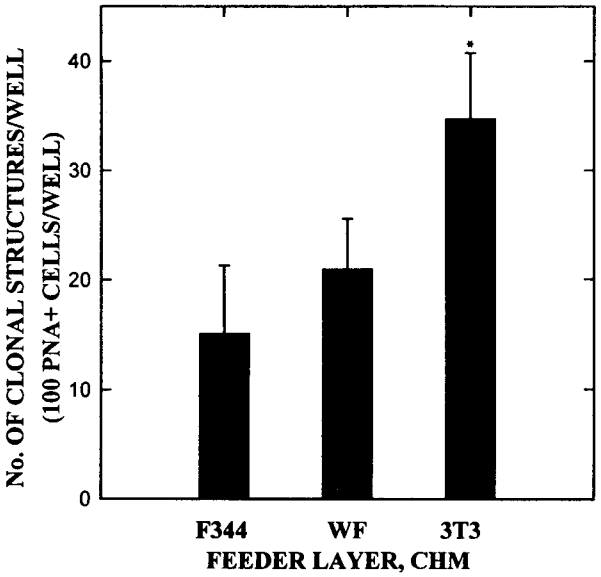

Fig. 3. Effects of feeder layers on the development of colonies. Organoids from F344 or WF female rats or Swiss 3T3 fibroblasts were used for the feeder layers.
One hundred PNA+ cells were cultured in CHM with each feeder layer for 4 weeks. Values were expressed as mean±S.D. Statistical significance: *<0.05.

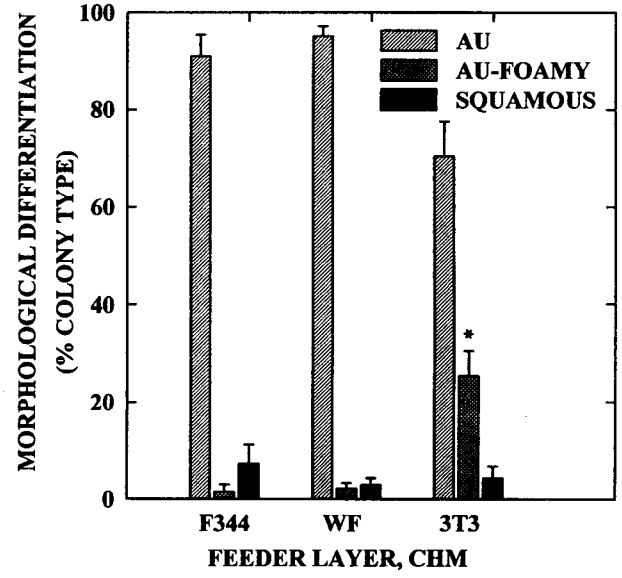

Fig. 4. Effects of feeder layers on the development of colony types. Organoids from F344 or WF female rats or Swiss 3T3 fibroblasts were used for the feeder layers.
Values were expressed as mean±S.D. Statistical significance: *<0.05.

immature single cells were grown and very distinctive patterns of multicellular structures were noticed within 7-day cultures. The results were same whether a single cell or 100 cells were sorted into well (Fig. 5).

After 4 week-culture, the resulting colonies were classified into three types: foamy alveolar (Fig. 6A),

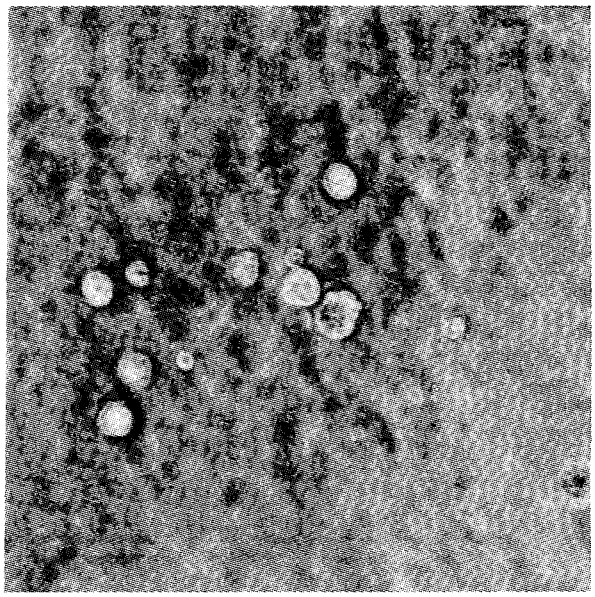

Fig. 5. Morphological appearance of sorted PNA+ cells and multicellular structures cultured in CHM within 7 days.

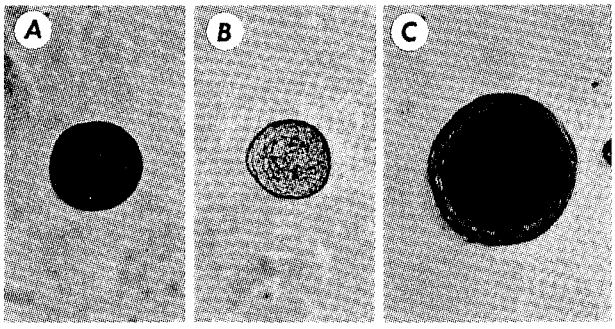

Fig. 6. With time in culture, the immature cells were observed to undergo dramatic morphological differentiation.
The resulting colonies were classified into one of three colony types: foamy alveolar (A), alveolar (B), or squamous (C).

alveolar (Fig. 6B), or squamous colonies (Fig. 6C). The colonies containing foamy vesicle in structure were described as foamy alveolar, those with glossy appearance as alveolar, and those containing gray and multilayer structure as squamous colonies.

DISCUSSION

When PNA+ cells were sorted and cultured in Matrigel with feeder layer, it gave more clonogenic growth into multicellular structures than when cultured in Matrigel without feeder layer. Especially, PNA+ cells very efficiently developed into foamy alveolar unit which are very likely mammary structures, alveolar unit which are histologically and ultrastructurally similar to alveolar structure found in intact gland.

Human tumors appear to be monoclonal, suggesting that they arise from a single transformed cell [15]. Many believe the cell of origin is a stem cell. The concept has gained support from one of evidence that common clonal markers can be found in all the cells of some types of tumors. It is thought that the carcinogenic insult causes defects in control of the normal stem-cell function of self-renewal and differentiation.

Stem cells can be defined as those cell which have the capacity to repopulate the mammary fat pads with parenchyma that undergoes the entire range of morphological and differentiative changes exhibited in the normal developmental cycle of the mammary gland [16]. Some of the progeny produce milk proteins, carbohydrates, and lipids, undergo involution and start the complete cycle again upon the renewal stimulus of pregnancy and lactation. Investigators in several laboratories have attempted to identify mammary stem cells with animal and culture models. Stem-like mammary cells which have been described include the RAMA 25 cell line [17], cap cells [18], basal cells [19], and pale-staining cells [20]. The detection and characterization of mammary gland stem cells, if present, is of more than academic interest. Because of properties of stem cells are relevant to the behavior of mammary cancers, elucidation of the nature of stem cells has implications regarding the origins and behavior of preneoplasms and neoplasms of the mammary gland. Although the number of stem cells in the various differentiative stages of the mammary gland have been difficult to quantify, there is little doubt that such cells exist. Phenotypic markers specific for stem cells would be valuable in following their fate and their cellular progeny.

Several studies in our laboratories have been done to identify the clonogen of RMEC and postulated that by flow cytometry of RMEC stained with PNA-FITC and anti-Thy-1.1-PE monoclonal antibody, PNA+ cells contain the most stem-like clonogenic mammary cells and Thy-1.1+ cell were the least [13].

It has been known that casein protein is secreted from the internal of the well differentiated structures among multicellular structures of mammary epithelial cells [14]. To investigate where the developed structures are originated, multicellular structures were stained with anti-casein antiserum. This process identified the presence of casein protein and it also confirmed that the developed structures forming brown precipitates were originated from mammary epithelial cells. The developed structures are similar to the mammary gland *in vivo* including the presence of myoepithelial cells around the cultured mammary organoids and secretion of casein by secretory cells.

In summary, these results demonstrated that when sorted PNA+ cells were cultured with irradiated feeder layers, it gave rise into multicellular clonal structures. The developed structures were similar to the mammary glands *in vivo* including secretion of casein protein by secretory cells. These result are consistent with the conclusion that some of PNA+ cells possesses many of the characteristics of multipotent clonogenic stem-like cells.

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초록 : 유선상피 간세포의 분리 및 특성연구

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흰쥐의 유선조직에는 증식과 분화능력을 가지는 일련의 세포군, 즉 clonogen 이라 불리는 세포군들이 존재하는 것으로 알려져 있으며 이들은 발암물질의 표적이 되기도 한다. 흰쥐의 유선상피세포를 분리, 단일세포로 만든 뒤 세포의 표면표식자들 (PNA-FITC와 anti-Thy-1.1-PE) 을 이용하여 immunostaining하고 flow cytometry로 분석하면 PNA+, Thy-1.1+, 두 세포표면표식자에 음성인 B-, 두 세포표면표식자에 양성인 B+의 4개의 세포군으로 분리된다. PNA+ 세포군만을 따로 분리하여 feeder layer를 사용한 Matrigel에서 배양하였을 때 생기는 다세포 구조물의 형태학적 특징을 관찰하였다. 4주간 CHM으로 배양하였을 때 alveolar, foamy, squamous구조을 가지는 다세포 구조물이 생성되는 것을 관찰하였다. 생성된 이들 구조물은 *in vivo* 상태의 유선조직과 유사하게 casein protein을 분비하는 것을 확인하였다. 이상의 실험결과로부터 PNA+ 세포군이 가장 stem cell과 유사한 기능을 가지는 세포군(clonogenic stem-like cells)으로 확인되었다.