All-trans Retinoic Acid Induced Differentiation of Rat Mammary Epithelial Cells Cultured in Serum-free Medium

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Retinoids are applied to not only cancer prevention but also cancer chemotherapy by stimulating differentiation of cells. We studied differentiation inducing effect of all-trans retinoic acid (ATRA) by studying proportion of high dense fractions of stem-like cells and the size of S phase fraction in cell cycle. From mammary organoids obtained from 7- to 8-week old F344 female rat mammary gland, we cultured rat mammary epithelial cells (RMEC) and treated physiological doses of 10^{-6}, 10^{-7}, and 10^{-8} M ATRA from the first day and then cultured for 4, 7, and 14 days. After that, immunostaining was performed using peanut agglutinin (PNA) and anti-Thy-1.1 monoclonal antibody (Thy-1.1) that can be used as markers of differentiation. We separated four different cell subpopulations by flow cytometry: 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+). We observed continuous decreases of high dense fractions of stem-like cells (PNA+ subpopulations) for 14 days and as much decreases as high doses of ATRA, which were thought to be proportional to doses of ATRA. We labeled RMEC with bromodeoxyuridine and investigated cell cycle fractions that went through S phase. We observed a tendency of decrease of S phase fraction with time in culture, which is thought to be related to continuous decreases of PNA+ subpopulations and inhibitory role of ATRA on cell cycle. These results suggest that physiological doses of ATRA could stimulate differentiation of RMEC and convert stem-like RMEC to differentiated cells in SFM for a relatively long period of 14 days.

Key words: All-trans retinoic acid, Mammary epithelial cell, Differentiation, Flow cytometry

INTRODUCTION

Retinoic acid (RA) and related compounds play important regulatory roles in growth and differentiation of a wide variety of cell types. Moreover, retinoids are required to maintain normal differentiation and proliferation of epithelial tissues in general (Dembinski and Shiu, 1987). RA showed inhibitory effects on the growth of squamous metaplasia formation from rat mammary epithelial cells cultured in reconstituted basement membrane, Matrigel (Kim et al., 1996).

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 (Clifton, 1990; Clifton and Gould, 1985; Dubbecco et al., 1986; Kim and Clifton, 1993; Kim and Paik, 1995; Rudland and Barraclough, 1988). In recent years, stem-like cells from rat mammary glands were isolated with flow cytometry using peanut lectin and anti-Thy-1.1 antibody and demonstrated the clonal growth potential in the transplanted sites (Kim and Clifton, 1993, 1996). Peanut lectin (PNA) has been used to identify luminal mammary epithelial cells that line the mammary ducts in vivo (Newman et al., 1979a, b). It also binds to the small cuboidal cells in primary cultures only after treatment with neuraminidase (Warburton et al., 1985).

Kim and Clifton (1993) sorted four different RMEC with PNA and anti-Thy-1.1 antibody, such as PNA-positive cells (PNA+), Thy-1.1-positive cells, cells negative to both reagent, and cells positive to both reagents, from intact rat mammary glands with flow cytometry and transplanted in hyperprolactinemic syngenic recipient rats to study the clonogenicity of these cells. We found that PNA+ cells contained the highest numbers of clonogenic cells among subpopulations of rat mammary epithelial cells (RMEC). Moreover, single
sorted PNA+ cells gave rise to multicellular colonies in a reconstituted basement membrane, Matrigel (Kim and Clifton, 1996; Kim et al., 1993). Culture of the heterogeneous mammary epithelial cells in serum-free medium also gives rise to several morphologically different cell types in vitro (Kim et al., 1997).

To study the effects of all-trans retinoic acid (ATRA) on the proliferation and differentiation of mammary stem-like cells cultured in serum-free medium, mammary organoids were collected, cultured in the presence of various concentration of ATRA, and examined the patterns of in situ immunocytochemical staining patterns, and analyzed S phase fractions with bromodeoxyuridine labeling technique.

MATERIALS AND METHODS

Cell culture

Serum-free medium (SFM) was MEGM (Mammary Epithelial Growth Medium, Clonetics, San Diego, CA, USA). MEGM was supplements-added MEBM (Mammary Epithelial Basal Medium) with epidermal growth factor (EGF) (10 ng/ml), human transferrin (10 µg/ml), gentamicin sulfate (50 µg/ml), insulin (5 µg/ml), and hydrocortisone (0.5 µg/ml). ATRA was prepared as stock solution in ethanol, and aliquots were stored at -20°C. Each ATRA (10⁻⁶, 10⁻⁷, and 10⁻⁸ M) was added to the medium immediately before each feeding and was present continuously thereafter.

Mammary epithelial organoids isolation and culture

Rat mammary epithelial organoids were prepared as described previously (Kim and Clifton, 1993). In brief, 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 MEBM supplemented with gentamicin sulfate (50 µg/ml) with shaking at 37°C for approximately 3 hr. After digestion, the suspension was washed in MEBM 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 MEBM with 10% FBS 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 by centrifugation. The organoids and cells were resuspended in MEBM with 10% FBS and the organoids were collected on a 40 µm 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 MEGM with 5% FBS in the presence or absence of ATRA at 37°C in a humidified 5% CO₂/air atmosphere for 1 day. Next day, MEGM with 5% FBS was removed and MEGM with or without ATRA was added. Each 10 cm petri dish contained one inguinal fat pad-equivalent of mammary organoids. The medium was changed 3 times weekly.

To make single cell suspension and count cell numbers, the cultured cells were collected with 0.2% trypsin-EDTA with chick serum for 6 min, washed with MEBM 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 MEBM. 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 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 in 0.85% saline and counting by phase microscopy in a hemacytometer.

Labeling cells with BrdU

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 (2×10⁶) 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 Na₂B₄O₇, 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 µl 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. The labeled cells were analyzed by Becton Dickinson FACScan flow cytometer. Laser excitation at 488 nm and standard FITC em-