Induction of Differentiation of the Cultured Rat Mammary Epithelial Cells by Triterpene Acids

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We investigated the effects of triterpene acids (TAs), ursolic acid (UA) and oleanolic acid (OA), on the induction of proliferation and differentiation of normal rat mammary epithelial cells (RMEC) or organoids cultured in Matrigel or primary culture system. To elucidate the effects, we tested their differentiation inducing activities with intercellular communication ability, cell cycle patterns, induction of apoptosis, and morphological differentiation in the three dimensional extracellular culture system. To study the changes of RMEC subpopulation in culture, the cultured cells were isolated, immunostained with peanut lectin (PNA) and anti-Thy-1.1 antibody and then analyzed with flow cytometry. Four different subpopulations, such as PNA and Thy-1.1 negative cells (B-), PNA positive cells (PNA+), Thy-1.1 positive cells (Thy-1.1+), PNA and Thy-1.1 positive cells (B+), were obtained and the size of each subpopulation was changed in culture with time in the presence of TAs. Intercellular communication was observed in culture for 7 days in TAs-treated cells, but not in culture for 4 days with scrape-loading dye transfer technique. G_2/M phase cells and the number of apoptotic population were increased in TAs-treated groups in cell cycle analyses. S phase fractions were reduced and the change of G_1 phase cells was not observed. The colonies with distinct multicellular structures, such as stellate, ductal, webbed, squamous, lobulo-ductal colonies, were observed in Matrigel culture and the frequencies of each colony were changed in the presence of TAs. These results suggest that UA and OA have differentiation inducing effects on rat mammary epithelial cells in primary or in Matrigel culture.

Key words : Triterpene acid, Ursolic acid, Oleanolic acid, Mammary epithelial cell, Differentiation

INTRODUCTION

Ursolic acid (UA) and oleanolic acid (OA) are triterpenoid compounds that exist widely in food, medicinal herbs and other plants, such as *Eriobotrya japonica, Rosmarinus officinalis*, and *Glechoma hederaceae* (Ohigashi *et al.*, 1986; Tokuda *et al.*, 1986; Huang *et al.*, 1994; Young *et al.*, 1995). Recently UA and OA were reported to have an antitumor action (Tokuda *et al.*, 1986; Ohigashi *et al.*, 1986). OA showed remarkable induction of differentiation of M1 cells into macrophage like cells, induction of tumor cell differentiation (Lee *et al.*, 1994), anti-angiogenic effect in chick chorioallantoic membrane (Sohn *et al.*, 1995), and anti-invasive activity in the HT1080 human fibrosarcoma cell by reducing the expression of matrix metalloproteinase-9 (MMP-9) (Cha *et al.*, 1996). In addition, it was reported that UA-induced down-regulation of MMP-9 gene is mediated through nuclear translocation of the glucocorticoid receptor in HT1080 human fribrosarcoma cells (Cha *et al.*, 1998).

To elucidate the effects of TAs compounds on the proliferation and differentiation on rat mammary epithelial cells (RMEC), we examined 1) the effects of the compounds on the proliferation and differentiation of normal RMEC or organoids grown in serum free medium, 2) the effects of the compounds on the cell cycle patterns in the same system, 3) the effects of the compounds on the cell-cell communication, 4) the effects of the compounds on apoptotic population of the cultured cells, and 5) the effects of the compounds on morphological development cultured in three dimensional extracellular matrix culture systems.

MATERIALS AND METHODS

Rat mammary epithelial organoids preparation

Mammary epithelial organoids were prepared as described previously (Kim *et al.*, 1997). Briefly, virgin female F344 rats, 50~55 day old, were killed with ex-

posure to overdosed CO_2 gas, and their inguinal mammary fat pads were removed, scissors-minced, and then digested with collagenase solution (Type III, 2 mg/ml, Worthington Biochemical, Freehold, NJ, USA) in mammary epithelial basal medium (MEBM, Clonetics, San Diego, CA, USA) 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 pellets which contained cells, cell clumps, and mammary organoids was collected. The pellet was washed, resuspended, and passed onto a 40 µm pore nylon mesh filter (Tetko, Brearcliff Manor, NY, USA) which allowed only the dispersed cells and small cell clumps to pass. The trapped organoids on the filter surface were collected and washed with MEBM. The numbers of these organoids were then calculated under microscope. These organoids composed of mammary epithelial cells and ducts (Kim and Clifton, 1993).

Organoid culture and monodispersed cell preparation

RMEC organoids were resuspended and distributed in cultured dishes in an appropriate mammary epithelial growth medium [MEGM, supplemented MEBM 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)] with 5% FBS in the presence or absence of TAs at 37°C in a humidified 5% CO_2/air atmosphere for 1 day. Next day, MEGM with 5% FBS was removed and MEGM with or without TAs was added in appropriate concentrations. Each 100 mm or 60 mm petri dish contained one or a third inguinal fat pad equivalent of mammary organoids, respectively. TAs were prepared as stock solution in ethanol, and aliquots were stored at -20°C. Each TA (10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M) was added to the media immediately before each feeding and was present continuously thereafter. The culture medium was changed three times weekly.

After culture with TA, groups of dishes were selected, and the cultured cells and organoids were dispersed by exposure to 0.05% trypsin-EDTA (GIBCO, Grand Island, NY, USA) at 37°C for 9 min and then added 10 ml MEBM with 10% FBS for stopping the action of trypsin. The dispersed cells and organoids were collected to sample tube and centrifuged twice at ~350 g for 6 min. The pelleted organoids were then resuspended in 10 ml 0.05% trypsin-EDTA and incubated at 37°C for 9 min with shaking. The dispersed cells were washed and resuspended in MEBM. Three ml 0.05% DNase (Worthington Biochemical) 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. From this method,

monodispersed cell fraction was obtained with 95% of percentage. 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 by counting dye-free cells in a hemacytometer by phase contrast microscope.

Determination of intercellular communication

The capability of rat mammary epithelial cells for gap junctional intercellular communication (GJIC) was determined by the scrape-loading dye transfer method as previously described (Chang et al., 1987; Kim et al., 1997). The cells on plates cultured for 4 and 7 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, St. Louis, MO, USA). The dye solution was added to plates and cultured colonies cut 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 100 fluorescence phase microscope with a 485/20 band pass filter for green FITC fluorescence and 546/12 band pass filter, FT 580 chromatic beam splitter, and LP 590 barrier filter for red TRITC fluorescence.

Immunostaining of cultured cells in situ

About 100 mammary organoids were distributed into each well of 24-multiwell Primaria culture plates (Becton Dickinson, Mountain View, CA, USA), and were cultured in serum-free medium from 1 to 7 days as described as above. On days 1, 4 and 7 of culture, the selected culture plates were washed with PBS with 1.0% BSA and stained with PNA-FITC and Thy-1.1-PE as above. The cells were viewed under a Zeiss Axiovert 100 fluorescence phase microscope.

Immunostaining of monodispersed cells

The density 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 fluorescein isothiocyanate-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 anti-Thy-1.1-PE and others with both. A negative control for the lectin staining was prepared by pre-incuba-

tion 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). 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 and sorting of the monodispersed stained cells from 4 and 7 days as primary and subcultures were performed with FACScan and FACStar^{PLUS} (Becton Dickinson), respectively. Cells were exited at 488 nm with 15 mW for analysis and 50 mW for sorting. 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. For cell sorting, dead cells and debris were excluded by forward and side scatter, and sort windows were set on the appropriate positive or negative fluorescence signals. Cells were suspended in PBS with 1% BSA, and sorted fractions were collected into the same solution. Sorted cells were recovered by centrifugation (~350 g, 6 min), resuspended in MEBM, and counted in a hemacytometer.

Mammary epithelial organoid culture in Matrigel

Aliquots of 400 μl Matrigel were mixed with 100 μl MEGM containing ~100 organoids and immediately distributed into 24 multi-well Primaria tissue culture plates (Falcon, Lincoln Park, NJ, USA). The plates were then incubated at 37°C for 30 min to allow gel formation. The sample groups which treated one ml medium containing TAs and a control group which was not treated with TAs, were cultured for 4 weeks. The medium was changed every 3 days. Multicellular structures were observed every 2 or 3 days by phase contrast microscope, and made an observation to proliferation of the cells and took a picture when it be need.

Examination of multicellular structures

After 4 weeks culture, the Matrigel obtained from the culture plate and the postfixed in 10% neutral formalin solution for 1 hr. After fixation, the specimens were embedded in paraffin, and 5~6 μm thick sections were made. Those were stained with hematoxylin and eosin and then observed with optic microscope (Kim et al., 1993).

RESULTS AND DISCUSSION

Effects on growth and immunostaining of cells in situ by TAs

The cells cultured in the presence or absence of 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M TAs showed three morphologically different organoids: elongated, spherical, and mixed structures. Most of cells spreaded out from elongated organoids were positive to Thy-1.1-PE antibodies at day 1. However, most of cells from spherical organoids were positive to PNA-FITC and cells from mixed type were positive to both of them. The structure of organoids rapidly degenerates clumps of cells surrounded by out-growth cells that have migrated out of the organoids. These out-growth cells have grown as major types of cell colonies through
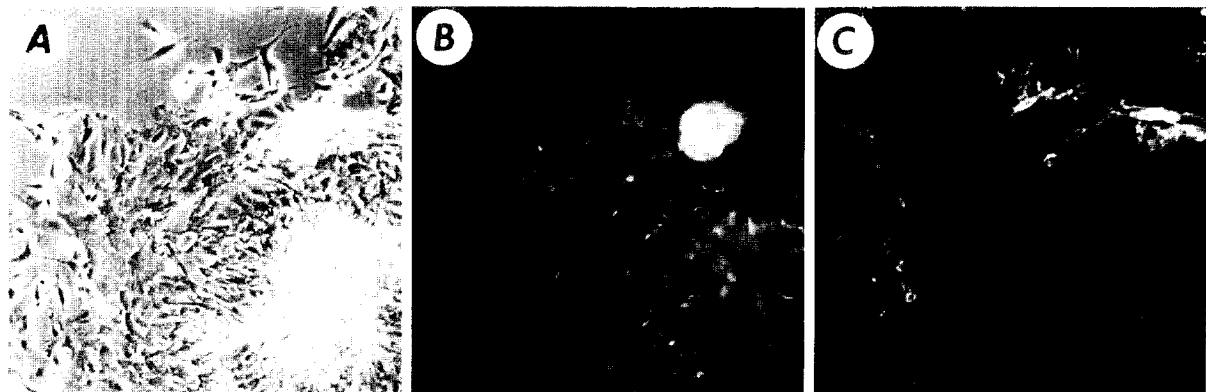

Fig. 1. Immunostaining of RMEC in primary cultures in MEGM for 4 days. Cultured cells were stained with PNA-FITC and Thy-1.1-PE. The cells were viewed under a Zeiss Axiovert 100 fluorescence phase microscope (A) with a filter for green FITC fluorescence (B) and with a filter for orange PE fluorescence (C). Phase contrast, ×100.

three or four days. The results of immunostaining at fourth day are shown in Fig. 1. Morphologically three different major types were observed by phase contrast microscopy: (A) Most of tightly packed small cuboidal epithelial cells located near the center of the colonies or edge of remaining organoids were positive to PNA-FITC. These small cuboidal cells were gradually reduced as culture time goes. (B) Large epithelioid cells which located at the colony boundaries of the small cuboidal cells elongated with the stick shape around the small cuboidal cells in one or many folds. These large epithelioid cells were positive to Thy-1.1-PE. These cells were gradually increased as culture time goes. (C) The last type of the cells were elongated cells between the epithelial colonies. These cells were stretched loosely and positive to Thy-1.1-PE. The structures formed in the plate culture system seems that of

the mammary gland, i.e., the growth of the structures may have the similarities with that of the end buds.

Immunostaining pattern of RMEC cultured with TAs by PNA-FITC and anti-Thy-1.1-PE

The cells cultured in the presence or absence of TAs from the first day were isolated from culture plates at 4 or 7 day. These monodispersed cells were immunostained with PNA-FITC and anti-Thy-1.1-PE and performed flow cytometric analyses with a Becton Dickinson FACScan. Multiparameter flow cytometric analysis of cells stained with both PNA-FITC and Thy-1.1-PE showed four different populations of cells: cells positive to PNA-FITC (PNA+), cells positive to Thy-1.1-PE (Thy-1.1+), cells negative to both reagents (B-), and cells positive to both reagents (B+).
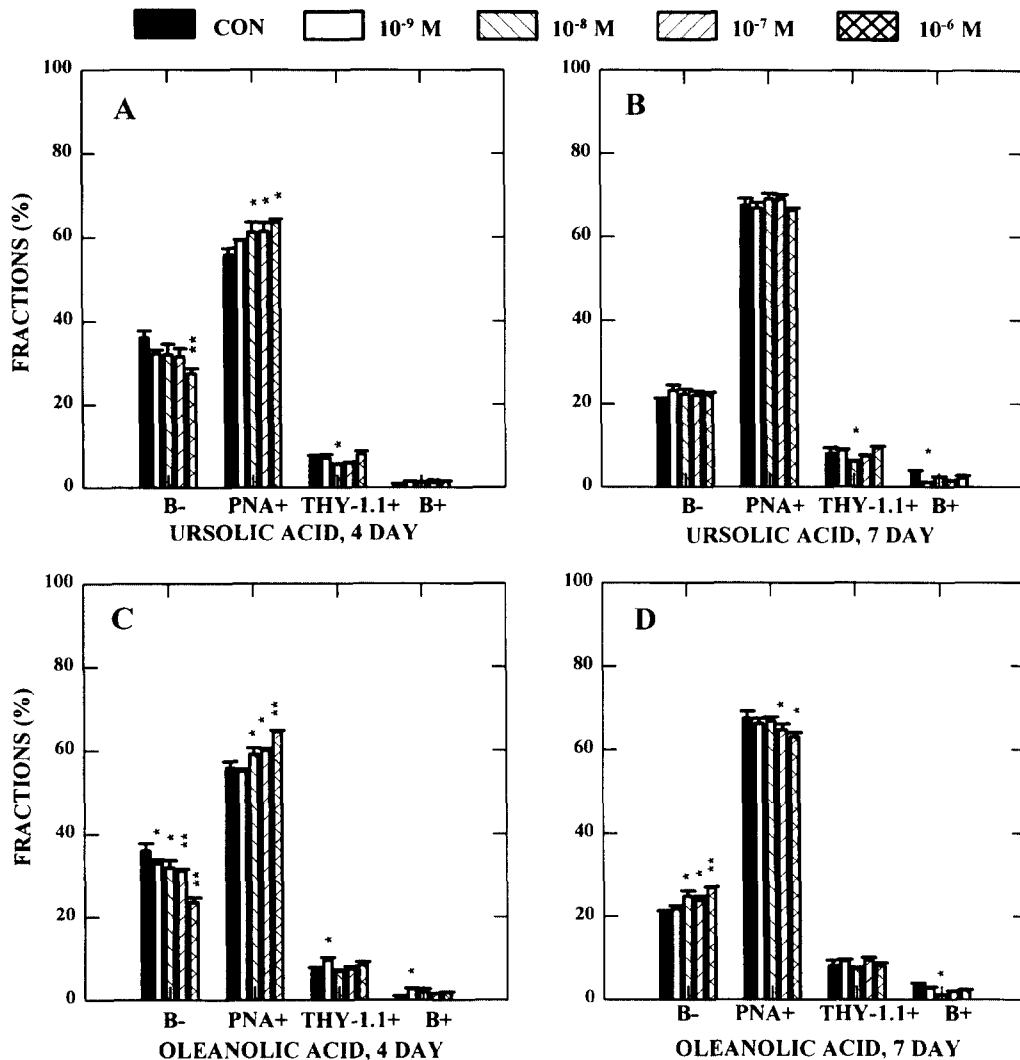
Fig. 2. Fractions in the various RMEC subpopulations in organoid cultures in MEGM with ursolic acid for 4 days (A) or for 7 days (B) or with oleanolic acid for 4 days (C) or for 7 days (D). The organoids were cultured in 10 cm petri dishes which contained one inguinal fat pad equivalent of mammary tissue each. Statistical significance: *$p<0.05$ vs. control group. Con, control.

According to transplantation assay *in vivo* in our laboratory (Kim and Clifton, 1993; Kim and Clifton, 1997), PNA+ cell subpopulation contains most of clonogenic cells (stem-like cells) and Thy-1.1+ cell subpopulation mainly contains finally differentiated cells, myoepithelial cells. On the basis of these, we have examined the effects of TAs on each cellular subpopulations. When the sample groups treated with UA or OA and control group which was not treated with TAs were cultured by 4 days (Fig. 2A, 2C), the B-cells were reduced dose-dependently in sample groups. In the case of PNA+ cells, they represents the tendencies of gradual increase as the increase of dose of TAs. However, Thy-1.1 and B+ cells did not affected much by TAs.

However, when the sample and control groups were cultured by 7 days, they represents some different tendencies from those of 4 days. Especially in the case of PNA+ cells, the changes were somewhat stagnant (UA, Fig. 2B), or gradually decreased (OA, Fig. 2D). Therefore, the proliferation of these PNA+ cells containing the clonogenic cells were induced when they were cultured for short periods (4 days) and then the proliferated cells were started their differentiation and it makes the proliferation rate relatively reduced at longer times (7 days).

The effects of TAs on intercellular communication

In order to measure the effect of TAs on intercellular junctional communication, the scrape-loading dye-transfer (SLDT) technique was used on rat mammary epithelial cells grown in serum-free medium *in vitro* (Kim *et al.*, 1997). This technique introduces macromolecules into cells by a transient perturbation of the cell membrane that does not affect cell viability or colony-forming ability (McNeil *et al.*, 1984). Intercellular communication was not observed at short cultural time, four days. But when it cultured for seven days, cell to cell communication was observed in triterpene acid treated cells at the highest concentration (10^{-6} M) (Table I).

Intercellular communication mediated via gap junctions has been postulated to be an important mechanism by which communicating cells could exchange regulatory ions and molecules to control tissue home-

ostasis, cell growth and differentiation, and synchronization of tissue regeneration (Chang *et al.*, 1987). Disrupted gap junctional communication has been implicated in abnormal growth, such as tumor promotion during tumorigenesis (Madhukar *et al.*, 1989; Kao *et al.*, 1997).

Tumor-promoting chemicals, such as phorbol esters, have been reported to inhibit gap-junction-mediated intercellular communication *in vitro* and *in vivo* (Madhukar *et al.*, 1989). Inhibition of gap junctional communication by various chemicals, including tumor promotors, is thought to be a factor in carcinogenesis, teratogenesis, and other disorders. Indeed, neoplastic transformation mediated by oncogenes, such as v-*ras*, v-*src*, and the gene coding for polyoma virus middle-sized tumor antigen can decrease intercellular junctional communication in certain cell systems although not all cells show the same degree of junctional uncoupling as those in transformed epithelial cells (Nicolson *et al.*, 1988).

Less is known concerning the possible role that junctional communication plays in the neoplastic progression of tumor cells to the highly malignant phenotype. Evidence indicates that intercellular junctional communication exists in benign epithelial tumors and there is some evidence that invasive epithelial tumor cells show reduced abilities to communicate via intercellular junctions (Nicolson *et al.*, 1988).

The analyses of cell cycle and apoptosis population with flow cytometry

The cells which have been treated or untreated with TAs for 7 days were obtained from culture plate and fixed. After fixations, staining DNA of the cells with PI were performed and then flow cytometric analyses were performed.

We compared the PI distributions of the cells cultured in the presence or absence of TAs for 7 days. The size of each cell cycle fraction was changed with the concentrations of TAs in culture (Fig. 3). When we compared the groups which have been treated with UA or OA with controls in G_0/G_1-phase, we could not find any differences between these groups. However, the sizes of S-phase fraction was decreased in the UA- or OA-treated groups and the sizes of G_2/M-phase fraction in the sample groups were increased dose-dependently (Fig. 3A, 3B). These results suggest that TAs have anti-proliferative and G_2/M-phase arrest effects on RMEC. However, more experiments are needed further.

In addition to this, our results revealed that UA or OA affected on the size of apoptotic population (Fig. 4). As the concentration of UA and OA were increased, the number of apoptotic population was increased. Especially in the concentration above 10^{-7} M, the sample groups significantly showed the diff-

Table I. Results of scrape loading and dye transfer tests of colonies cultured for 4 days (4-D) or 7 days (7-D) in the presence or absence of ursolic or oleanolic acids cultured in MEGM

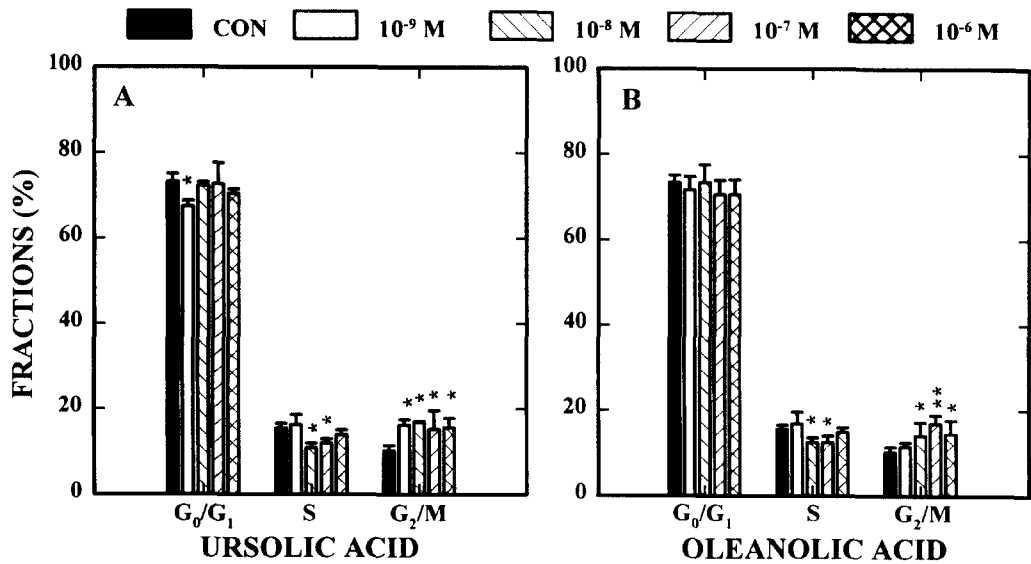|	Ursolic acid [M]					Oleanolic acid [M]				
	0	10^{-9}	10^{-8}	10^{-7}	10^{-6}	0	10^{-9}	10^{-8}	10^{-7}	10^{-6}
4-D	-	-	-	-	-	-	-	-	-	-
7-D	-	-	-	-	+	-	-	-	-	+

Fig. 3. Fractions of G_1, S, and G_2/M phase cell populations in 7-day cultures as shown by incorporation of propidium iodide. Ursolic acid (A); Oleanolic acid (B). Statistical significance: *p<0.05, **p<0.01 vs. control group. Con, control.
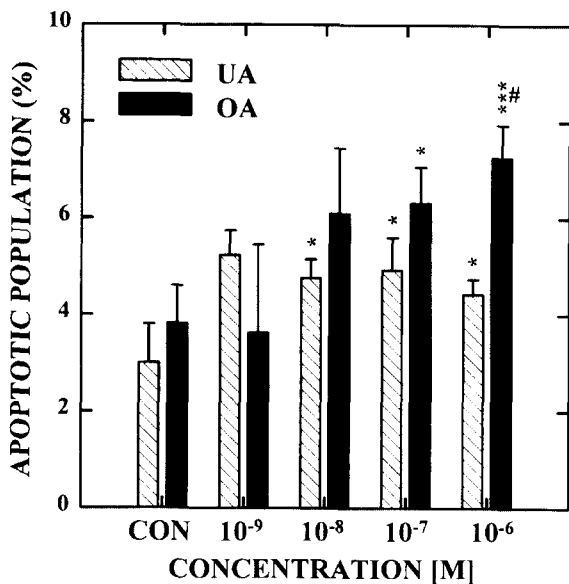

Fig. 4. Apoptotic populations from cultured cells treated with ursolic acid or oleanolic acid. Cells were cultured for 7 days and fixed, stained with propidium iodide, and analyzed with flow cytometry. Bars represent the mean±SEM of triplicated cell preparations. Statistical significance: *p<0.05, ***p<0.001 vs. control group (CON) of each triterpene acid. #p<0.001 between 10^{-6} M of ursolic acid and oleanolic acid.

erences (p<0.05). However, to clarify the differences between UA and OA, more experiments are needed.

Effects of TAs on multicellular structures developed from mammary organoids cultured in Matrigel

The mammary organoids were developed into several morphologically different multicellular structures with time. The multicellular colonies were classified
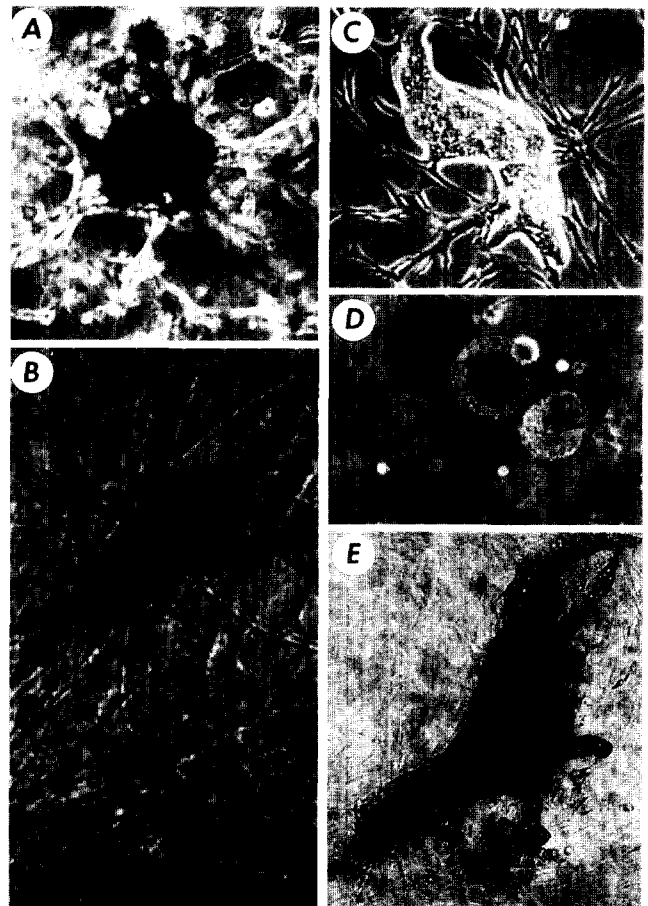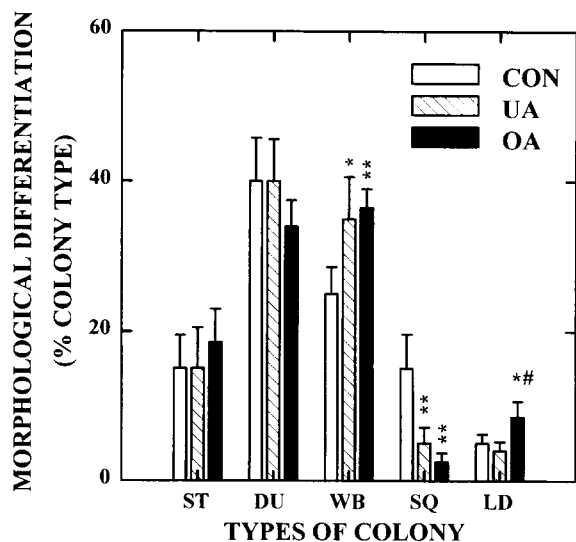

Fig. 5. Morphological appearance of multicellular colonies developed from mammary organoids cultured in Matrigel under serum-free MEGM for 28 days. The resulting colonies were classified into one of five types: stellate (A), webbed (B), ductal (C), squamous (D), or lobulo-ductal colonies (E).

Fig. 6. The effects of triterpene acids (UA or OA) on morphological differentiation of multicellular colonies developed from normal rat mammary epithelial organoids grown in MEGM. Morphology was quantitated during days 28 of cultures. Stellate (ST), ductal (DU), webbed (WB), squamous (SQ), or lobulo-ductal (LD) colonies. Bars represent the mean±SD of colonies per well of triplicate wells. Statistical significance: *p<0.05, **p<0.01 vs. control group. #p<0.05 between 10^{-6} M of ursolic acid and oleanolic acid. Con, control.

into five types, based on their general appearance at the light microscopic level: stellate, webbed, ductal, squamous and lobulo-ductal colonies (Fig. 5) (Kim and Clifton, 1993).

In this current experiments, we demonstrated that RMEC organoids treated with 10^{-6} M UA or 10^{-6} M OA have influenced to their multicellular structures (Fig. 6). Especially, webbed colonies which are thought to be deduced from myoepithelial cell in UA- or OA-treated sample groups were more developed than those of control groups. And the squamous metaplasia, the results of dedifferentiation of mammary epithelial stem cell (Kim et al., 1993), were markedly reduced in UA- or OA-treated sample groups. Moreover, the lobulo-ductal colonies, which is known as a structure formed in well-differentiated mammary epithelial cells, were more developed in OA treated groups. However, more experiments are needed about more detailed effects in the developments of multicellular colonies.

In summary, in an effort to find substances from natural products that may be utilized as differentiation inducing agents, we have identified two compounds of pentacyclic TAs, UA and OA, having the ability to induce the differentiation of rat mammary epithelial clonogenic cells in serum-free culture.

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