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Comparison of the Inhibitory Effects of Retinoic Acid Derivatives on Development of Squamous Metaplasia from Rat Mammary Epithelial Organoids Cultured in Matrigel

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To compare the inhibitory effects of retinoic acids derivatives among all-trans, 13-cis and 9-cis retinoic acids on the development of squamous metaplasia (SQM) from rat mammary epithelial cells (RMEC), female F344 rat mammary organoids were cultured in reconstituted basement membrane, Matrigel, under either a complete hormone medium (CHM) or serum-free mammary epithelium growth medium (MEGM). From the culture, five different types of multicellular colonies were developed: stellate, ductal, webbed, squamous, and lobulo-ductal colonies. Organoids cultured in CHM without retinoids gave rise to fewer such SQM (~5%) than those in MEGM without retinoids (~16%). Formation of SQM was completely suppressed when three different retinoic acids derivatives [10^{-9} M], all-trans, 13-cis and 9-cis RAs, were added to CHM. However, a few SQM were still observed in cultures in MEGM with the added retinoids [10^{-6} M]. In the presence of retinoids in culture medium, lobulo-ductal colonies, one of well-differentiated structures, were increased with the augmentation of retinoids concentrations. However, there were no difference in the differentiation inducing activities among three different retinoic acid derivatives.

Key Words: Squamous metaplasia, all-trans retinoic acid, 13-cis retinoic acid, 9-cis retinoic acid

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INTRODUCTION

The histogenesis of squamous metaplasia (SQM) of mammary epithelial cells is very rare event in vivo. However, there were several reports about the development of SQM from rat mammary epithelial cell (RMEC) subpopulations or organoids in a reconstituted basement membrane, Matrigel, overlaid with media.[1,4] In addition to SQM, four different types of multicellular colonies were also developed: stellate, ductal, webbed, and lobulo-ductal colonies.[1] Generally, SQM is known as a preneoplastic change in the formation of bronchogenic squamous cell carcinoma. However, little is known about the role of SQM in formation of mammary tumor. Injury, whether caused by chemical, mechanical, or nutritional (such as vitamin A deficiency) is the leading stimulus for the development of squamous metaplasia.[5,10] Recently, all trans retinoic acid (ATRA) added in culture medium completely inhibited the development of SQM from rat mammary epithelial cells (RMEC) cultured in reconstituted basement Membrane (RBM), Matrigel.[11] Retinoids have also been found to be the most potent vitamin A analogues for the reversal of SQM in organ cultures of hamster trachea or rat tracheal cells.[12,13]

In the current study, we have compared the effects of three different retinoic acid derivatives, all-trans retinoic acid (ATRA), 13-cis retinoic acid (13-cis RA) and 9-cis retinoic acid (9-cis RA), on the suppression of SQM development from mammary organoids cultured in Matrigel, and on the development of lobulo-ductal structures formation by retinoids.

MATERIALS AND METHODS

1) Organoid preparation and culture in Matrigel

Mammary organoids were prepared as we described previously [6]. Briefly, virgin female F344 rats, 50~55 days old, were killed with ether, and their inguinal mammary fat pads were removed, and scissors-minced, and then digested with collagenase solution (Type III, 2 mg/ml, Worthington Biochemical, Freehold, NJ) in SFM (Serum free Dulbecco's Modified Eagle Medium). After digestion, the suspension was washed in serum medium (SM: SFM with 10% fetal bovine serum, FBS, HyClone, Logan, UT), centrifuged, and the pellet which contained cells, cell clumps, and mammary organoids (multicellular duct and end bud fragments) was collected. The pellt was washed, resuspended, and passed onto a 40 μm pore nylon mesh filter (Tetko, Brearcliff Manor, NY) which allowed only the disperesd cells and small cell clumps to pass. The trapped organoids on the filter surface were collected. These organoids were then placed in culture plate.

Matrigel (without phenol red, Collaborative Research) was prepared according to the manufacturer's recommended protocol. Aliquots of 400 μl of Matrigel were mixed with 100 μl of SFM containing ~100 organoids or ~10^5 monodispersed cells and immediately distributed into 24 multi-well Primaria tissue culture plates (Falcon). The plate were then incubated at 37°C for 30 min to allow gel formation. One ml of medium containing an appropriate concentration of ATRA, 13-cis RA, or 9-cis RA (Sigma) (structures in Fig. 1) was then added to which well and the cultures were incubated at 37°C. The retinoids were prepared as stock solution in ethanol, and aliquots were stored at -20°C. Each RA was added to the media immediately before each feeding and was present continuously thereaffer. Two basic culture media were used in this study. The first was complete hormone medium (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 ovine prolactin (Hormone Distribution Office, National institution of Arthritis, Digestive disorders and Kidney Diseases). The second was serum-free mammary epithelial growth

medium (MEGM) based on MCDB 170 (Mammary Epithelial Basal Medium, MEBM, without phenol red, Clonetics). The MEGM consisted of MEBM supplemented with EGF (10 μg/ml), insulin (5.0 μg/ml), hydrocortisone (0.5 μg/ml), human transferrin (10 μg/ml), and gentamycin (50 μg/ml). The media were changed three times per week. Photomicrographs were taken with a Zeiss Axiovert 100 phase microscope.

2) Morphological analysis and histology

Morphology of the colonies from mammary organoids were quantitated by light microscopic observation. Numbers of colonies were quantitated in triplicate wells as we described previously.[1,11] For histology, cultures grown in RBM were washed with PBS, fixed in methanol/chloroform/acetic acid (6 : 3 : 1) at 4°C overnight, routinely processed, and embedded in paraffin. Sections 4 μm thick were mounted on poly L lysine-coated slides. Deparaffinized and rehydrated sections were rinsed and stained with hematoxylin and eosin. Statistical significance was determined by Student's t-test. $P < 0.05$ was judged to be statistically significant.

RESULTS

1) Multicellular structures and squamous metaplasia

Organoids from donor rats initially retained their multicellular lobulo-ductal structure. During the first three days in cultures, organoids appeared to gather matrix around themselves, to increase in size and to develop cellular outgrowths. By the third day, the various types of structures were observed and representative structures of squamous and lobulo-ductal colonies were shown in Fig. 2A. Squamous structures commonly had developed from tiny multicellular spheres by ten days of culture. These could be identified in undisturbed cultures by their concentric swirl appearance and rust coloration.[1,11] In section, SQM were seen to contain keratin pearls surrounded by multi layered basophilic cuboidal to squamous epithelial cells and were similar to SQM that occur in many epithelial tissues in vivo in situation of stress (Fig. 2B). The flattened squamous layers of SQM were positively stained with anti-pan cytokeratin antibody (data not shown). The presence of SQM suggests a) that the culture conditions, particulary serum-free conditions, were less than optimal and b) that there were significant numbers of cells capable of such differentiation in each od the cell subpopulations we have observed in RMEC cultures. Lobulo-ductal structures with secretory cells were composed of a mondayer of well-polarized cuboidal cells with large secretory vesicles in their cytoplasm (Fig. 2B).

2) Effects of media on morphological differentiation

The type of structure that developed from mammary organoids generally depeneded on the type of culture media, CHM or MEGM. About 75% of the colonies that developed from organoids cultured in RBM in CHM were either ductal or webbed, and very few were squamous (Fig. 3). When cultured in RBM in MEGM, organoids gave rise to a pattern of colony types similar to those in CHM cultures except that somewhat fewer webbed and two to three times more squamous colonies were present.

3) Effects of retinoids

The most striking changes in response to retinoids were the decrease in squamous colonies and the increase in the lobulo-ductal colonies. Squamous colony formation from organoids in CHM decreased with increasing concentrations of each RA; development of SQM was completely suppressed at 10^{-9} M ATRA, 13-cis RA, and 9 cis RA (Fig. 4). However, squamous colony formation from organoids cultured in MEGM decreased but was not totally suppressed even at 10^{-6} M retinoids (Fig. 5). Moreover, 9-cis RA showed slightly lesser inhibitory effects on the squamous formation than the other two RAs. The numbers of lobulo-ductal

colonies from organoids increased with increasing retinoids concentrations in both media (Fig. 6); however, the frequencies of stellate and webbed colonies were not significantly changed (data not shown). The total numbers of colonies were little changed by treatment of the retinoids tested.

DISCUSSION

The present study was conducted to determine: (a) whether retinoids, all-trans RA, 13-cis RA, and 9-cis RA, can suppress the development of SQM from mammary organoids and (b) whether there were difference in the efficacy of these inhibitory effects on the suppression of SQM development and induction of well-differentiated lobulo-ductal formation by retinoids. The study demonstrated a) that each retinoid inhibited the development of SQM and induce the differentitation of multicellular structures, such as lobulo ductal colonies, b) that there were differences between CHM and MEGM on the development of multicellular structures, and c) that three retinoids showed almost no difference to suppress the development of SQM and induce the lobulo-ductal formation.

Commercial FBS contains ~190 ng retinol per ml or about 10^{-6} M. As the retinol and several unknown growth factor(s) are present in FBS, it is likely to have contributed to the difference of between CHM and MEGM in the current studies. Ip et al[14] have observed squamous colonies at very low frequent in rat mammary cell cultures in growth factors-optimized serum-free medium; squamous colony frequencies were dramatically greater in suboptimal conditions. These investigators suggested that the concentration of EGF, the presence of TNF-α, and the condition (thickness) of RBM affected SQM formation.[14] Wada et al.[15] observed that phorbol 12 myristate 13-acetate completely suppressed the development of the squamous-like colonies in rat mammary organoid cultures; SQM were frequently observed under conditions of limiting EGF concentrations. Morever, a retinobenzoic acid derivative RF80 also suppress the development of SQM.[4] Schaefer et al.[16,17] observed a similar suppressive effect on SQM formation in mouse mammary organ cultures by phorbol ester or retinoids. Thus to completely suppress SQM formation, mammary organoids require factors other than RA which are not present in RBM in MEGM.

The development of the mammary glands and the differentiation of mammary epithelial cells are regulated by a variety of hormones and growth factors. The complex interactions among these regulatory components are not completely understood. Moreover, the effect of these regulators on the development and functional differentiation of individual cells as well as the intercellular interactions within the mammary gland are unclear. The current model system may allow further systemic investigations of the effects of these regulators on in vitro morphogenesis of mammary epithelial cells and on mechanisms of aberrant differntiation such as SQM formation.

In summary, ATRA, 13-cis RA, and 9-cis RA completely inhibit development of SQM from rat mammry organoids cultured in RBM in CHM and partially inhibits SQM in cultures in RBM in serum-free MEGM. Moreover, there were no difference in the efficacy of inhibition of SQM development. This system may serve as a useful model for investigation of the mechanism of SQM formation, differentiation of epithelial cells, development of multicellular structures, and chemoprevention of aberrant structure formation in vitro.

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Fig. 1. Structures of retinoids. (A) All-*trans* retinoic acid, (B) 13-*cis* retinoic acid, (C) 9-*cis* retinoic acid.

Fig. 2. Morophological appearance of multicellular squamous metaplasia (SQM) and lobolo-ductal colonies (LD) cultured in Matrigel in the absence or presence of RAs for 28 days (A). Light micrographs of cross sections of colonics in Matrigel for 4 weeks, stained with hematoxylin and eosin (B). Squamous structures were composed of a multilayer of cells with a keratin pearl (KP) at center. Lobulo-ductal structures were composed of highly polarized epithelial cells with well developed secretory vesicles in their apices. Bars: 50 μm.

Fig. 3. The effect of medium on morphological differentiation of normal rat mammary organoids grown in CHM or in MEGM. Colnies were quantitaed on day 28 of culture. Each bar represents the mean±SEM of triplicated wells. $*P<0.05$, statistically from CHM.

Fig. 4. The effect of various concentration of retinoids on the development of squamous metaplasia from organoids cultured in CHM. Each bar represents the mean numbers of squamous colonies per well of triplicate wells determinations. Vertical lines indicate SEMs. Morphology was quantitated on day 28 of culture.

Fig. 5. The effect of varients concentration of retinoids on the development of squamous metaplasia from organoids cultured in MEGM. Each bar represents the mean numbers of squamous colonies per well of triplicate wells determinations. Vertical lines indicate SEMs. Morphology was quantitated on day 28 of culture. $*P<0.05$, statistically different from ATRA and 13-cis RA.

Fig. 6. The effect of retinoids on the development of lobulo-ductal structures from organoids cultured in CHM. Each bar represents the mean of squamous colonies per well of triplicate wells. Vertical lines indicate SEMs. Morphology was quantitated on day 28 of culture.