Vitamin A Inhibition of Keratinization in Rat Urinary Bladder Cancer Cell Line Nara Bladder Tumor No. 2 in Meniscus Gradient Culture

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SUMMARY

The Nara Bladder Tumor No. 2 cell line, established from a urinary bladder carcinoma in the Wistar rat, formed keratinizing cells and multicellular pearls in meniscus gradient culture when fed with a medium of 15 or 30% fetal calf serum in Eagle’s minimal essential medium. Confluent monolayer cultures were first prepared with the tubes in conventional horizontal position. When cultures were then changed to a vertical position, stratification, piling up, and aggregation of cells were observed in a few days at the aerobic end of the gradient. Keratinization appeared 1 week after the tubes were placed in vertical position. Squamous differentiation proceeded to the formation of keratin pearls, a phenomenon never observed so distinctly and in such abundance in horizontal culture. Supplements of vitamin A, as low as 1 IU/ml, added to the medium did not inhibit piling up or aggregation but did prevent keratinization. Inhibition of keratinization by vitamin A was reversible. After vitamin A was removed from the medium, the cells in the aggregates progressed to keratinization.

INTRODUCTION

The initial studies of NBT-II in meniscus gradient culture were conducted at the University of Tokyo (2) using as the medium 15 or 30% calf serum in MEM. The most striking feature in those studies was the appearance of a zone of giant tumor cells about 3 or 4 mm below the gas-medium interface. Keratinization of single cells was noted immediately below the gas-medium interface. The present investigation began as a result of observations made when 30% calf serum in MEM was compared with 30% fetal calf serum in MEM in the meniscus gradient system (6).

Materials and Methods

Stock Cultures. The NBT-II cell line (5) was maintained in stoppered stationary bottles at 36° in MEM with Earle’s balanced salt solution (Microbiological Associates, Inc., Bethesda, Md.) and 20% calf serum with L-glutamine (2 mmoles/ml), kanamycin (50 mg/ml), penicillin (50 units/ml), streptomycin (50 mg/ml), and fungizone (1 mg/ml).

Vitamin A. Aquasol A, generously supplied by USV Pharmaceutical Corp. (Tuckahoe, N. Y.), contained 50,000 USP units of vitamin A (retinyl palmitate) per ml. The solution was diluted to the appropriate concentration with culture medium just before use. The control, solvent without vitamin A, was provided by the USV Pharmaceutical Corp. and was used diluted to the same concentration in the medium. The solvent was composed of 12% sorbitan oleate, 0.5% sodium hydroxide, and 0.5% chlorobutanol.

Meniscus Gradient Culture. In general, the same procedure was used as reported originally (2). Cell suspensions were made with EDTA-trypsin (Grand Island Biological Co., Grand Island, N. Y.) and sedimented by centrifugation at 100 x g for 5 min. The pellet was resuspended in a medium consisting of 30% fetal calf serum in MEM with all of the supplements itemized above for stock cultures, and then 1-ml aliquots of the cell suspension were distributed in small Leighton tubes each containing a glass coverslip (11 x 22 mm). The tubes were incubated at 36° in a horizontal position, and the medium was replaced as indicated by changes in the pH. As growth became nearly confluent, the tubes were fed with 2 ml of medium and transferred to a vertical position for subsequent cultivation. Medium was replaced with exactly the same volume 2 or 3 times each week. The pH ranged from 7.4 after feeding to as low as 6.6.

Numerous prominent typical keratin pearls formed after 2 weeks when the medium contained fetal calf serum; there was no pearl formation when calf serum was used. The appearance of giant tumor cells was prominent in cultures with calf serum and was less impressive with fetal calf serum.

Since vitamin A deficiency is associated with squamous metaplasia of the urothelium of rats (1, 7), we studied the effect of medium supplemented with vitamin A on the keratinization of NBT-II in meniscus gradient culture.
just before the medium was replaced.

Observation of the Cultures. A phase contrast-inverted microscope was utilized for the living cultures. When cultures were terminated, the coverslips were removed for fixation and staining. Fixatives were 10% alcoholic formalin for hematoxylin and eosin stain, and 95% alcohol for Papanicolaou's stain.

RESULTS

After a few days in vertical culture, there were great differences in appearance of the cells along the course of the entire gradient from the gas-medium interface to the bottom of the coverslip, a span of 12 to 15 mm. In the studies reported here, observations were limited to the zone at and just below the gas-medium interface to a depth of 3 mm, because greatest cell density and prominent keratinization were regularly found in this area.

The preliminary observation that gave rise to this study, that keratinization reached an advanced state in a medium containing fetal calf serum but not in one containing calf serum, was repeated several times with consistent results. See Table 1, Groups A and B.

Control Studies

Thirty % Fetal Calf Serum in MEM without Vitamin A Supplement. Within 1 week after confluent cultures were turned to the vertical position, piling up and aggregation of cells were seen just below the gas-medium interface. Single keratinized cells occurred, occasionally in the center of aggregates of cells. At the end of the 2nd week, fully formed typical keratin pearls were frequent. These became more numerous by the end of the 3rd week. In addition, abundant multicellular groups of cells were arranged as intersecting ridges with a distinctive architecture. The centers consisted of cords of keratinized cells, and these were covered by stratified cells with prominent nuclei and without keratinization (Figs. 1, 2, 5, and 6). Mitoses were numerous during the entire period of cultivation, even after keratinization appeared in the cultures. Control cultures, with or without the addition of the solvent for vitamin A, were indistinguishable.

The concentration of cells in suspension in the original inoculum determined the interval of time required for confluent growth to be attained. Once confluent cultures were placed in a vertical position, whether initiated with a very light or heavy seeding, the 1st keratinization was seen in about 1 week.

Meniscus Gradient Cultures in 0, 5, 15, 30, and 50% Fetal Calf Serum in MEM. Experiments using the different concentrations of fetal calf serum revealed that results with 15 and 30% fetal calf serum were identical (Table 2, Groups C and D). The highest concentration, 50% fetal calf serum, did not permit maturation of keratinizing cells in spite of early aggregation, as seen in Table 2, Group E. In the case of the 5% concentration (Table 2, Group B), both aggregation and keratinization were delayed as compared with Table 2, Groups C and D, 15 and 30% serum, respectively, but cell viability was maintained well enough so that keratinizing cells could mature if the culture was continued longer than the 2-week test period. The cells cultured

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Meniscus gradient cultures with medium containing 30% CS*, or 30% FCS with and without vitamin A</th>
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<td>Group</td>
<td>Culture medium</td>
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<tr>
<td>A.</td>
<td>30% CS; 12 cultures</td>
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<tr>
<td>B.</td>
<td>30% FCS; 12 cultures</td>
</tr>
<tr>
<td>C.</td>
<td>30% FCS and 1 unit vitamin A; 12 cultures</td>
</tr>
<tr>
<td>D.</td>
<td>30% FCS and 5 units vitamin A; 9 cultures</td>
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* CS, calf serum; FCS, fetal calf serum.
* Mit, mitotic figures as observed with light microscope (× 200); ±, very rare or abnormal figure; +, sporadic mitoses.
* Agg, cell aggregations stained blue-green or sometimes blue-violet, but never clear orange by Papanicolaou’s stain; -, no aggregation; ±, very few areas of minimal piling up; +, focal piling up and diffuse stratification; ++, many distinct aggregates and extreme stratification.
* Ker, cell keratinization, i.e., brilliant copper color with Papanicolaou’s stain; -, no keratinization; ±, very rare single, partially, or fully keratinized cells; +, many fully keratinized individual cells with small nuclei staining densely without detail. Scattered distinctive “cancer pearls”; + +, many cancer pearls.
without serum (Table 2, Group A) could not be maintained and became detached after a week.

Effect of Vitamin A on Keratinization

Supplements of vitamin A, as low as 1 IU/ml, added to the medium throughout the time of cultivation in a vertical position, prevented the formation of keratin pearls and reduced markedly the presence of single keratinized cells. However, it had no inhibitory effect on the aggregation of cells. The contrast between the 30% fetal calf serum-MEM control and the same medium with vitamin A supplements is seen in Table 1, Groups B and C. Figs. 3, 4, 7, and 8 are from cultures treated with vitamin A and should be compared with Figs. 1, 2, 5, and 6, the controls. In cultures receiving 5 IU of vitamin A per ml, after 2 and 3 weeks, large signet-ring cells were seen frequently in the anaerobic areas. None of these cells stained positively with either mucicarmine or periodic acid-Schiff.

In order to see the effects on keratinization of periodic exposure to vitamin A, 6 groups were prepared, as designated alphabetically in Chart 1. Two media were used, a control medium of 30% fetal calf serum in MEM, and the same medium to which a supplement of 5 units of vitamin A per ml had been added. The experimental period of 3 weeks was divided into 3 segments of 1 week each. Results are shown in Chart 1.

In Groups A and B, keratinization was marked after 3 weeks in control medium and was completely absent in supplemented medium. This is a confirmation of the observations shown in Table 1, Groups B and C. In Groups C and D, pretreatment with vitamin A for 1 or 2 weeks suppressed keratinization and also delayed its reappearance.

When vitamin A was removed after the 1st week, the initiation of keratinization could be seen only 2 weeks later, whereas it occurred in 1 week in the control group. When vitamin A was added after 1 week, the immature keratinization present at that time was prevented from further development in the subsequent 2 weeks (Chart 1, Group E). When vitamin A was added after 2 weeks of cultivation on
control medium, the keratinization that developed during the 2nd week was not inhibited but continued to progress so that, by the end of the 3rd week, it was comparable to the control (compare Groups A and F). This experiment shows that vitamin A could inhibit the initiation and early stages of maturation of keratinization but was obviously not effective once maturation had reached a certain point.

DISCUSSION

The history of gradient methods in vitro has been reviewed in recent publications from this laboratory (3, 4). A few additional points should be raised. When a culture is transferred from the horizontal to the vertical position, we believe that the initial gradient is one of $p_{CO_2}$, with the highest concentration of oxygen in the medium at the air-medium interface, and lowest concentration in the depths of the tube. Other gradients must appear promptly. As 1 example, the lowest $p_{CO_2}$ will be at the gas-medium interface, and the highest concentration must be at a deeper level. As days and weeks pass and cells pile up in 3-dimensional groups just below the gas-medium interface, additional gradients must become operative. The cells in the center of an aggregate and those in the periphery of the aggregate are in a gradient association to one another with relation to the metabolites in the medium. In view of the passage of time in culture, a gradient of maturation or aging may be present, with the keratinized cells in the center of the pearl being the oldest cells of the aggregate.

Urothelium is second to none in its potential for metaplastic response as either a glandular epithelium, a keratinizing squamous epithelium, or a combination of the two. Carcinomas arising in urothelium in humans may show the same spectrum of metaplastic changes. The relevance of such changes to the evolution and malignant behavior of individual tumors is obscure, but may be extremely important. The NBT-II tumor used in these studies provides a laboratory model for studying metaplastic changes of a malignant urothelium in vitro.

The NBT-II tumor in the rat of origin was observed originally to be a transitional cell carcinoma with areas of squamous metaplasia. On serial s.c. passage in rats, and on tissue culture passage, the histopathological picture rapidly became one in which squamous cell carcinoma with keratinization was predominant. In vitro, the morphology of NBT-II is extremely responsive to conditions of cultivation. In the aerobic part of the culture the cells pile up on one another; in the deeper anaerobic part of the culture, they survive and proliferate only as a monolayer. This suggests that there exists in the same culture both noncontact-inhibited and contact-inhibited behavior as a response to the gradient, i.e., to the different microenvironments of the cells. Furthermore, the histogenesis in the aerobic zone follows different paths, depending on whether the medium contains fetal calf serum, calf serum, or fetal calf serum supplemented with vitamin A.

A matter of importance to determine is whether NBT-II on fetal calf serum and vitamin A is, on ultrastructural examination, squamous cell carcinoma the maturation of which is blocked by vitamin A, or whether by the criteria of fusiform vesicles and asymmetrical membranes it is recognizable as a urothelial derivative. Similar studies should also be conducted on bladder carcinoma cell lines derived from human tumors, as well as on the primary growth of newly resected surgical specimens of transitional cell carcinomas.

As data become available on a number of bladder cancers relating to their metaplastic and nonmetaplastic forms, we may be able to ask new kinds of questions concerning the regulation of histotypic expression and malignant behavior of urothelial cancer.

ACKNOWLEDGMENTS

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REFERENCES

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Figs. 1 to 4. All × 20. All cultures were stained by the Papanicolaou method in which keratin becomes a metallic orange color and the remaining structures, blue of varying intensity. A Wratten no. 11 yellow-green filter was used in the photography. Figs. 1 and 2 are controls. Figs. 3 and 4 are from cultures exposed to 5 IU of vitamin A per ml medium. The gas-medium interface is on the left.

Fig. 1. A 2-week-old culture. A narrow zone of piling up is seen in which cells have coalesced to form a coarse branching pattern and in which the central part of the intersecting cords stains very well for keratin, although it is not evident in a black-and-white photograph.

Fig. 2. A 3-week-old culture. The piling up and keratinization increases very much between the 2nd and 3rd weeks, both in density and in the width of the zone.

Fig. 3. The effect of vitamin A from a 2-week-old culture is seen.

Fig. 4. The effect of vitamin A from a 3-week-old culture is shown. Keratinization is not seen in either Fig. 3 or 4. Instead, the pattern is one of a more diffuse piling up of cells in a dense band with scattered aggregates of cells just below the gas phase.

Figs. 5 to 8. Higher magnifications of Figs. 1 to 4, stained by the Papanicolaou method.

Figs. 5 and 6. Zone of branching keratinized cells after 3 weeks in control medium. Fig. 5 (× 170) shows a thin terminal cord of cells superimposed on a monolayer of cells. Occasional mitotic figures are seen in the monolayer. The cord of cells contains several discrete aggregates. In the upper right is 1 large aggregate with densely packed nuclei and without obvious keratinization. In the lower left, at the distal end of the cord, is a discrete keratin pearl. In Fig. 6 (× 670), a keratin pearl is seen in greater detail with its onion-skin arrangement of keratinizing cells.

Figs. 7 and 8. Zones of piling up in 3-week-old cultures that received medium with an addition of 5 IU vitamin A. In Fig. 7 (× 170), 3 small aggregates are seen superimposed on the monolayer. In Fig. 8 (× 670), a typical aggregate is seen. No keratinization is found with the light microscope.
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