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# Viability of Cultured Lewis Lung Cell Populations Exposed to β-Retinoic Acid (40753)[1]

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Retinoids have a prophylactic and therapeutic effect on certain premalignant and malignant epithelial lesions (1). Retinoids can retard the appearance, reduce the incidence, or inhibit the *in vivo* growth of chemically induced tumors of the skin (2–4), lungs (5, 6), intestines (7), mammary glands (8, 9), and urinary bladder (10), and also virally induced tumors (11, 12). In addition, the growth of transplantable chondrosarcoma in rats fed retinoic acid (13–15) and of mammary adenocarcinomas in mice fed retinyl palmitate (16) is reduced, and the development of tumors by S91 melanoma cells injected into mice pretreated with retinyl palmitate is inhibited (17). However, Bollag (18) has reported that the development of transplantable tumors is *not* inhibited by orally or i.p.-administered retinoic acid.

Studies *in vitro* with organ cultures of trachea (19) and prostate (20–22) show that the hyperplastic and metaplastic changes induced in culture by chemical carcinogens can be inhibited and reversed by retinoids. Also, studies with organ cultures of trachea (1) or chick skin (23, 24) demonstrate that retinoids can modulate epithelial differentiation *in vitro*. Recent reports indicate that retinoids may inhibit proliferation, without evidence of cell death or lysis, of certain cultured cells derived from tumors (25–28).

The demonstration of intracellular retinoid-binding proteins (29–31) has led to the postulation that retinoids modulate the process of cell proliferation and differentiation by a mechanism similar to that of steroid hormones; complexes of retinoids and specific intracellular-binding proteins may be transported to the nucleus and modify gene transcription. Since retinoic acid-binding protein has been demonstrated in the Lewis lung tumor (32), we used cultured Lewis lung cells to determine if β-retinoic acid can have a direct effect on the proliferation and viability of Lewis lung cells.

*Materials and methods.* Cultured Lewis lung cell populations were derived from the tumor propagated in C57BL/6 mice. The growth medium used was a modification of Eagle's minimal essential medium (MEM) (33) supplemented with 20% fetal bovine serum. Cells were seeded into either 32-oz Brockway tissue culture bottles or 75-cm$^2$ tissue culture flasks at a cell density of 4.0–6.6 × 10$^4$ cells/cm$^2$ and exponentially proliferated to a cell density of approximately 4.0–6.6 × 10$^5$ cells/cm$^2$. At this cell density there was a confluent layer of cells on the growth surface. Under these conditions the population doubling time was 21 ± 2.9 hr (mean ± standard deviation).

Following 160, 400, and 640 population doublings of the cultured cells, the cells were monitored for tumorigenicity. Cultured cells (4–6 × 10$^7$ cells/mouse) were implanted s.c. into C57BL/6 mice and tumors of approximately 1 g appeared 20–33 days postimplant. These tumors were histopathologically compatible with Lewis lung carcinoma.

After the Lewis lung cells were established in culture, stock cultures were propagated in Eagle's MEM supplemented with 10% fetal bovine serum. Periodic monitoring of the stock cell populations for mycoplasma indicated that the cells were free of mycoplasma contamination.

Stock cultures of Lewis lung cells were harvested using 0.05% trypsin, and the resulting cell suspension was diluted in medium and counted in a hemacytometer. Cultures for experiments were initiated with 3.6 × 10$^4$ cells/cm$^2$ in plastic tissue

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culture flasks (Corning, Corning, N.Y.) in a total volume of 6 ml medium per flask. In the experimental flasks, cells were inoculated into Eagle's MEM supplemented with 10% fetal bovine serum and β-retinoic acid (0.5 to 50.0 μg/ml medium); and as a control, cells were inoculated into flasks containing Eagle's MEM supplemented with 10% fetal bovine serum. A single flask was seeded for each concentration-time interval and control cultures were prepared for each time interval.

β-Retinoic acid (all-*trans*-β-retinoic acid) was supplied through the courtesy of the Lung Cancer Segment of the National Cancer Institute. Stock solutions of β-retinoic acid, made immediately before use, were prepared by dissolving β-retinoic acid in a minimum volume of dimethylsulfoxide (DMSO) and diluting to the desired concentrations with medium. The final DMSO concentration never exceeded 0.3% in the culture medium. DMSO at this concentration was also added to the control cultures. Experimental work with β-retinoic acid was performed in subdued light.

Cultures were incubated at 37°. At 48 hr, the medium containing β-retinoic acid was decanted from the experimental cultures and 6 ml of fresh medium supplemented with β-retinoic acid added to the cultures. The control cultures received 6 ml of fresh medium without β-retinoic acid at 48 hr.

At the end of 72 hr the medium from the cell populations exposed to β-retinoic acid and the control cell populations was decanted into 15-ml conical centrifuge tubes. Two milliliters of 0.25% trypsin solution was added to each flask and the cells were allowed to detach at 37°. The trypsin cell suspensions were pipetted into the centrifuge tubes. The tissue culture flasks were rinsed with medium and scraped with a rubber policeman to remove any remaining cells. The medium and cells were added to the centrifuge tubes. The cells were sedimented at 250 *g* and the trypsin-medium supernatant was discarded. The cells were resuspended in 3 to 5 ml of medium and counted in a hemacytometer.

Growth inhibition was calculated according to the formula:

$$100 - \left[ \frac{Nr}{Nc} \times 100 \right],$$

where $N_r$ was the cell density of the cultures exposed to β-retinoic acid for 72 hr and $N_c$ was the cell density of the control cultures at 72 hr.

The cells harvested from control and β-retinoic acid-treated cultures were also assayed for viability by growth in semisolid medium. The semisolid medium was composed of modified Eagle's MEM supplemented with 20% fetal bovine serum and a final concentration of 0.15% agarose (Type II, Sigma Chemical Co., St. Louis, Mo.). Cells from the β-retinoic acid-treated cultures were cloned at $1.0 \times 10^4$, $1.0 \times 10^3$, and $1.0 \times 10^2$ cells/tube in quadruplicate, and cells from the control cultures were cloned at $1.0 \times 10^2$ cells/tube in a series of eight tubes. Tubes were incubated 14 days at 37° in 5% $CO_2$. Only clones with 50 or more cells were counted (34). Cloning efficiencies of control cultures were about 60%.

*Results and discussion.* In five separate experiments, a concentration of 5.0 μg/ml β-retinoic acid, which is effective in modulating epithelial differentiation of chick embryo skin explants (23, 24) and inhibiting or reversing carcinogen-induced hyperplasia of mouse prostate explants (21, 22), did not affect the proliferation or viability of cultured Lewis lung cells. Although this concentration may cause membrane destabilization and presumably a degree of cytotoxicity in certain experimental systems (35–38), it had no significant effect on the proliferation or viability of the Lewis lung cells.

In another series of experiments, we exposed cultured Lewis lung cells to a series of concentration of β-retinoic acid between 0.5 and 50 μg/ml (Table I). Inhibition of proliferation was concentration dependent. There was, however, no reduction in the number of viable cells in the cell populations at any of the concentrations. The $ED_{50}$ (the concentration in μg/ml which inhibits cell growth to 50% of control) for Lewis lung cells was about 25 μg/ml. We had previously reported $ED_{50}$ concentrations for

TABLE I. EFFECT OF DIFFERENT CONCENTRATIONS OF β-RETINOIC ACID ON THE PROLIFERATION AND VIABILITY OF CULTURED LEWIS LUNG CELLS[a]

| μg/ml | Percentage growth inhibition[b] (mean ± SD) | $S_f$[c] |
|---|---|---|
| 0 | 0 | $1.0 \times 10^0$ |
| 0.5 | 0 | $1.0 \times 10^0$ |
| 5.0 | 10 ± 1.1 | $1.0 \times 10^0$ |
| 10.0 | 22 ± 2.3 | $1.0 \times 10^0$ |
| 25.0 | 50 ± 5.9 | $1.0 \times 10^0$ |
| 50.0 | 69 ± 7.1 | $1.0 \times 10^0$ |

[a] Proliferating cultured Lewis lung cells were exposed to 0.5 to 50.0 μg/ml all-*trans*-β-retinoic acid for 72 hr harvested by trypsinization and sedimentation, and counted in a hemacytometer. After determining cell densities, the number of viable cells in the cell populations was determined by cloning in semisolid medium as described under Materials and Methods.

[b] Mean and standard deviation of three experiments.

[c] $S_f$ is the surviving fraction as determined by assay in semisolid medium.

β-retinoic acid of 24 μg/ml for cultured KB cells and 5.6 μg/ml for cultured fibroblast L-929 cells (39).

Similar observations have been reported in other studies. β-Retinoic acid and retinyl acetate inhibited the proliferation of a number of transformed and tumor cell lines in culture (27) and proliferation of cultured murine melanomas S91 and B16 (28). In both of these studies, inhibition of cell growth was not a result of direct cytotoxicity (based on dye exclusion and the absence of detached cells in adherent cell cultures). Excess retinol inhibited the proliferation of cultured murine fibroblast 3T6 cells with no evidence of cell death or lysis (25). β-Retinoic acid also had an inhibitory effect on the proliferation of cultured murine fibroblast L-929 cells (26). Based on results from experiments involving dye exclusion, uptake of tritiated uridine, and vesicular stomatitis virus plaque formation, cytotoxicity was excluded as a cause of decreased proliferation. Inhibition of proliferation of Chinese hamster ovary cells by retinol occurred in a concentration-dependent manner and was not the result of direct cytotoxicity (40). The reported inhibition of proliferation of cultured mastocytoma P815Y cells by a series of retinoids was considered to result from toxicity associated with the presence of aldehyde groups (41).

Although we observed a direct effect of β-retinoic acid on the proliferation of cultured Lewis lung cells, preliminary *in vivo* experiments did not show an inhibitory effect on the *in vivo*-passaged tumor (personal communication, Dr. W. R. Laster, Jr., Southern Research Institute). Thus, 40 mg/kg/dose β-retinoic acid administered i.p. q.d. 1–5, 8–12, and 15–19 did not produce an increase in life span in mice implanted i.v. with $1.0 \times 10^6$ Lewis lung tumor cells. The dose used was the highest nontoxic dose possible on this schedule. Whether this lack of therapeutic effect of β-retinoic acid in mice bearing the Lewis lung tumor was due to a lack of effective concentration-time parameters at the target cell site remains to be determined.

β-Retinoic acid evidently has a differential effect on cultured Lewis lung cells in that it will inhibit proliferation without decreasing the viability of the cell population. This implies that growth inhibition of these cells by β-retinoic acid is reversible. Indeed, such inhibition of cultured melanoma S91 or B16 cells by β-retinoic acid is reversible (28). Retinol arrested Chinese hamster ovary cells in the $G_1$ phase of the cell cycle, and this effect was also reversible (40). Perhaps β-retinoic acid also modulates the transit of Lewis lung cells through the replicative cycle. However, the mechanism of inhibition of proliferation of Lewis lung cell populations by β-retinoic acid is not known at present.

*Summary.* β-Retinoic acid inhibited the proliferation of cultured Lewis lung cells without decreasing the viability (as determined by cloning in semisolid medium) of the cell populations.

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