Antiproliferative Effect of Vitamin A on Xenotransplanted CaMa-15 Cells

Neal T. Wetherall¹, William M. Mitchell, and Susan A. Halter

Department of Pathology, Vanderbilt University Medical Center [N. T. W., W. M. M., S. A. H.], and the Veterans Administration Medical Center [S. A. H.], Nashville, Tennessee 37232, and Ludwig Institute for Cancer Research, Bern Branch, Inselspital, University of Bern, 3010 Bern, Switzerland [N. T. W.]

ABSTRACT

In vitro and in vivo investigations have shown that nontoxic treatment with vitamin A (retinol) has an inhibitory effect on the growth of malignant cells. The tumorigenic CaMa-15 cell line responds to both retinol and retinoic acid under both anchorage-dependent and anchorage-independent conditions, reducing growth or colony formation by at least 50%. To date, there have been few studies on the effects of vitamin A on xenotransplanted neoplastic cells. Twenty-five adult female nude rats (nu/nu) were inoculated in the inguinal fat pad with 10⁶ CaMa-15 cells, a tumorigenic epithelial cell line. The rats were divided into three groups: ten high dose (3 mg retinol/day i.p.); five low dose (30 μg retinol/day i.p.); and ten controls (corn oil i.p.). All animals were housed in specific-pathogen-free conditions and permitted access to sterile laboratory chow (5.4 μg retinol/g chow) and water ad libitum. Rats were sacrificed at 21 days after inoculation. Onset of tumor development occurred between Days 9 and 13 in all groups. Tumors grew progressively and were reduced in mean diameter by 26% (p < 0.05) with high-dose retinol and 44% (p < 0.02) by low-dose treatment. No clinical signs of vitamin A toxicity were apparent. Necropsy and radiological examination revealed no evidence of toxic effects or metastases. These results indicate that vitamin A can reduce the growth of xenotransplanted tumor cells at nontoxic levels in T-cell-deficient hosts. The nude rat offers a potential model to study the inhibitory effects of retinoids on xenotransplanted cancers.

INTRODUCTION

The vitamin A alcohol, retinol, and the carboxylated derivative, retinoic acid, are essential for the maintenance of normal epithelial differentiation. It is this function which has led to increased interest in examining the possible therapeutic and prophylactic uses of these vitamins and their synthetic analogues in the treatment and prevention of cancer (40). In addition, recent studies have indicated that the retinoids have a direct effect upon the growth of malignant cells in vitro (22), in both anchorage-dependent and anchorage-independent culture systems (18, 24, 27).

The earliest attempts to alter the progression of neoplastic growth in vivo by retinoids centered around established transplantable rodent tumors (3). Retinoic acid or a synthetic analogue was administered either i.p. or p.o. without demonstrable growth inhibition. More recent investigations have demonstrated inhibition of tumor growth by the retinoids. Studies using the S-91 (Cloudman) melanoma in BALB/c mice (9), the mouse mammary adenocarcinoma C3HBA (35), and the rat chondrosarcoma (17) have shown significant tumor size and incidence reductions using retinoids. Other studies (30, 42) have demonstrated actual tumor regression. More recent studies have indicated that C3HBA tumor excision and vitamin A therapy increases animal survival (34), and provitamin A, or β-carotene, can yield similar growth inhibitory results (36).

All of the previous work has utilized syngeneic systems, while the effects of retinoids upon xenotransplanted cells have limited investigation (19). Several suitable recipients for heterotransplantation are available. One animal currently under investigation is the Rowett "nude" rat (nu/nu), established at the MRC Laboratory Animals Center (10). Like nude mice, these rats possess a rudimentary thymus almost completely devoid of lymphocytes (4). Although information regarding the nude rat is not as well documented (2, 13, 33) as for the nude mouse, natural killer cell activity appears to be present in the athymic rat (7, 14).

In this paper, we describe the influence of increased levels of retinoids on anchorage-dependent and anchorage-independent growth of tumorigenic epithelial cells and relate the effect of vitamin A, given at nontoxic doses, to tumor progression in T-cell-deficient hosts.

MATERIALS AND METHODS

Cells. CaMa-15, originally described as a human mammary cell line derived from the primary tumor of a patient with infiltrating ductal carcinoma of the breast (31), was obtained from Dr. Luciano Ozzello (Institut d'Anatomie Pathologique, Lausanne, Switzerland). Subsequently, other investigators have suggested Syrian hamster (Mesocricetus auratus) interspecies contamination (29). Isoenzyme patterns determined by Dr. W. D. Peterson, Jr., at the Child Research Center of Michigan, Detroit, MI, have also indicated cross-contamination with our cell population. Transmission electron microscopy (not shown) demonstrated that the CaMa-15 cell line maintains epithelial characteristics when transplanted into athymic rodents. The cell line was routinely cultured in IMDM² supplemented with insulin (10 μg/ml), gentamicin (50 μg/ml, Sigma Chemical Co., St. Louis, MO), and 10% calf serum at pH 7.2 to 7.4 in a humidified atmosphere with 7.5% CO₂ at 37°. The IMDM and serum were obtained from Grand Island Biological Co., Grand Island, NY. Fluorochrome Hoechst 33258 was used for routine screening of cells for Mycoplasma (5).

Preparation of Retinoids and Anchorage-dependent Growth Assay. Dose-response curves with retinol and retinoic acid were determined, using a modification of the method of Lacroix and Lippman (20). Triplicate cultures for each treatment were seeded in six 8-sq cm well dishes (Costar, Cambridge, MA) with IMDM. Density of inocula was adjusted to achieve confluency of controls after 8 to 10 days of incubation. After allowing overnight attachment of the cells to the substrate, the media were supplemented with appropriate retinoid concentrations. All procedures involving retinoids were carried out in subdued light. Subsequent media changes were performed every 72 hr until at least 90% confluent growth in the control cultures was reached. All-trans-retinol and all-trans-retinoic acid were purchased from Sigma Chemical

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² To whom requests for reprints should be addressed. Received October 11, 1983; accepted March 6, 1984.
³ W. D. Peterson, Jr., personal communication.
⁴ The abbreviation used is: IMDM, Iscove's modified Dulbecco's medium.
Co. or provided as a gift from Dr. W. E. Scott of Hoffmann-La Roche Inc., Nutley, NJ. Retinoid diluted with ethanol in IMDM was added to the wells to yield final concentrations of 0.1 mM to 1 mM. Control cultures received 0.1% ethanol (v/v) in IMDM alone. After 7 days of treatment, the ethanol or the retinoid not used after brief trypsinization, and cell numbers were determined using a hemocytometer. Trypan blue exclusion was used to determine gross cell viability. Dose-response curves were calculated as percentage of inhibition of cell proliferation from the following equation.

$$100 - \left( \frac{F}{C} \right) \times 100$$

where \( F \) is the number of retinoid-treated cells, and \( C \) is the number of cells in control cultures (26).

**Anchorage-independent Growth Assay.** Cells were cultured in triplicate 35-mm Petri dishes using 2 layers of media, as described by Hamburger and Salmon (15). The underlayer consisted of 1.0 ml of McCoy's medium with 10% fetal calf serum (Grand Island Biological Co.), various nutrients (16), and 0.5% agarose (Seaplaque; FMC Corp., Marine Colloids Division, Rockland, ME). Conditioned media were not used. CaMa-15 cell suspensions were washed in Hank's balanced salt solution, and the total number of cells was counted with a hemocytometer. Gross viability was determined by trypan blue exclusion.

To assess the effects of retinoids on the growth of cells, the method of Lotan et al.

**Animals, Diets, and Environments.** A colony of noninbred hooded Rowett nude rats (nu/nu) had been maintained in our breeding facility for over 4 years. Homozygous and heterozygous animals are produced by mating run/mu males with run/+ females. Heterozygotes were removed when identified. Weaning occurred at approximately 21 days, and the animals were distributed to no more than 4 rats/cage. The animals were autodosed before use. Animals were permitted access to sterile laboratory chow and water ad libitum throughout the experiment. The animals were housed at 27 ± 1°, without antibiotic coverage, in a housing conditions described previously. The athymic rats were permitted access to sterile laboratory chow and water ad libitum throughout the experiment.

**For transfection, the cells were prepared as follows.** Routine culture conditions were observed by growing cells in 75 cm² flasks (Costar). Harvesting was followed by washing the cells in IMDM and counting the viable cells in a hemocytometer. Cells (1 x 10⁶) were resuspended in 0.1 ml of IMDM and injected into the right inguinal mammary fat pad.

For retinol supplementation, 6 mg all-trans-retinol/ml (Hoffmann-La Roche) were dissolved in 95% ethanol. To this dilution, the antioxidant α-tocopherol was added (2 mg/ml), and the final volume was completed with corn oil (Sigma Chemical Co.). The final concentration of ethanol in the injection milieu was 10%. After gentle but thorough mixing to avoid aeration, the preparation was sterilized by γ-irradiation and stored in the dark at 2° for no more than 48 hr. Immediately after the implantation of the CaMa-15 cells, 10 animals received an i.p. injection of 0.5 ml of this high-dose retinol supplement. This provided an additional 3 mg of vitamin A/day over the 5.4 μg/g of laboratory chow. An i.p. injection of retinol supplement was given mid-morning on a daily basis for 21 days.

Five animals were provided with a low-dose (30 μg/day i.p.) supplementation by reducing the amount of all-trans-retinol in the formula to 60 μg/ml. The control group consisted of 10 rats which received identical i.p. injections as the treated groups, without the addition of retinol. Each animal was weighed daily and examined for lethargy, kinesalgia, dermatitis, and infections. The inoculation site was gently palpated to determine the date of gross tumor onset. When the tumor appeared, its size was measured daily by 2 independent observers. The tumor diameter was determined by averaging the major and minor diameters of the oval neoplasm, and the tumor volume was calculated from the formula \(4/3 \pi \times a \times b^2\), where \( a \) is one-half of the long axis, and \( b \) is one-half of the short axis (41). Significance of differences between groups was evaluated by the unpaired Student t test. Probability values are specified in 2 tails.

After 21 days of treatment, all animals were sacrificed by ether overdose, and a necropsy was performed within 12 hr of the final tumor measurement and vitamin A treatment. The primary tumor was excised, and relative necrosis was estimated. Internal viscera were examined for the appearance of gross metastasis. Representative sections of the lung, liver, spleen, kidney, heart, diaphragm, and primary tumor were fixed in 10% buffered formaldehyde solution and submitted for histological examination. All sections were stained by hematoxylin-eosin and alizarin red for the detection of calcium deposition. Three carcasses of the survivors from each group were subjected to X-ray examination of the pelvic girdle and caudal appendages.

**RESULTS**

Effect of Retinol and Retinoic Acid on Anchorage-dependent Growth. Dose-response curves for the CaMa-15 cell line tested with the various concentrations of retinol and retinoic acid are illustrated in Chart 1. Growth was assessed by the criteria of Lotan and Nicolson (26), with 10% inhibition considered unresponsive, 25 to 50% inhibition considered sensitive, 50 to 75%
difficult motion was observed in the surviving animals. During the experimentation, no lethargy, anorexia, or painful or pathological symptoms could be determined. Except for the rats which died of peritonitis or septicemia, all the animals survived the experiment. Only 5 of the initial 25
nu/nu rats introduced into the study, 5 died during the course of the experiment; 3 high-dose (3 mg retinol/day i.p.) animals and 2 controls. None of these animals developed tumors before death, nor was any neoplastic growth observed at necropsy. All 5 animals exhibited a purulent peritonitis, which was the apparent cause of death. No peritonitis was observed in any of the survivors.

The 3 groups of animals were initially well matched for weight and continued to gain weight steadily over the entire experimental period. No statistically significant differences between groups could be determined. Except for the rats which died of peritonitis during the experimentation, no lethargy, anorexia, or painless or pathological symptoms were observed in any of the survivors.

Onset of tumor development occurred between Days 9 and 13 in all 3 groups. Once the lesions appeared, they always grew progressively, as shown in Chart 3, which applies regression analysis to the mean tumor volume. At the midpoint of tumor progression (Day 15), the high-dose treatment reduced tumor progression by 43\% (p = <0.05), and the low-dose treatment reduced tumor size by 62\% (p = <0.02). At the end of the experiment (Day 21), the tumors in the high-dose group were 26\% (p = <0.05) smaller, and tumors in the low-dose group were 44\% (p = <0.02) smaller than in the control population. Postmortem examination revealed rats free of dermatological lesions and infections. Gross and microscopic examination of the viscera did not reveal any metastases. All tumors were well encapsulated without microinvasion of surrounding tissues.

In most cases, cross-sections of the primary growth revealed a highly necrotic tumor. Greater than 50\% of the tumor mass in all of the control and high-dose animals was necrotic. Sixty \% of the low-dose animals, having much smaller tumors, were not necrotic. Histologically, the tumors consisted of highly undifferentiated cells that were frequently spindle shaped.

Osseous lesions were not observed radiographically in any of the sampled rats in the 2 experimental or control groups. No osteoporosis was detected in the scapula or long bones. No calcium deposits were seen in the soft tissues histologically.

**DISCUSSION**

Lotan et al. (23) and Lotan and Nicolson (26) provided the first evidence that retinoids have a direct effect upon the proliferation of cancer cells in vitro. Our results, based upon use of a similar anchorage-dependent culture method, agree with Lotan’s findings (21), that the growth of tumorogenic cells is substantially affected by the retinoids. CaMa-15 cells did not demonstrate any dose responsiveness in monolayer culture to retinol; all doses (1 nM to 10 \(\mu M\)) inhibited growth approximately 40 to 50\%. However, retinoic acid reduced the proliferation of CaMa-15 cells to a greater extent over the same concentration range. This demonstrates that retinol and retinoic acid may not affect cells to the same degree.

The correlation between anchorage-independent growth of cells in semisolid medium and their ability to form tumor xenografts has long been known (12). This culture system has been used to test the susceptibility of several neoplasms against a variety of retinoids, achieving mixed results (6), but in general, there is agreement that dose response may not be observed in all cases (28). Using the criterion accepted for primary cultures, which may not apply to cells grown in continuous culture, the assay did not predict susceptibility of the tumorogenic cells to the retinoids, since there was not a 70\% reduction in clone formation. In addition, this assay system was not able to discriminate between the different response in vivo to the high-dose and low-

Chart 2. Effect of retinol (\(\bullet\)) and retinoic acid (x) on the anchorage-independent growth of CaMa-15 cells. The cultures were grown and incubated as described in "Materials and Methods." Points, mean of triplicate cultures; bars, S.D. The area below the dashed line (\(<30\%\)) indicates the suggested colony reduction which equates with in vivo retinoid susceptibility.
dose retinol. Although both doses showed approximately 40% reduction in colony formation, there was a greater reduction in tumor size with the low-dose retinol treatment in vivo.

Anchorage-independent cell growth in soft agar is also affected differently by retinol and retinoic acid. Although CalMa-15 cells were more sensitive to retinoic acid in monolayer culture, they showed greater suppression of colony formation when treated with retinol in soft agar. It has been suggested that growth in soft agar is selective for stem cells (37), and since the cell population may be more heterogeneous in monolayer culture, this difference may reflect a variance in cell population sensitivity to retinoic acid.

No experimental system has been established which can assess the in vivo effects of retinoids on cells of human origin, with the possible exception of clinical trials. The athymic rodent tumor size with the low-dose retinol treatment in vivo.

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