Antiproliferative Effect of Vitamin A on Xenotransplanted CaMa-15 Cells

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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 tumorogenic 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 (nmu/nmu) were inoculated in the inguinal fat pad with 10⁶ CaMa-15 cells, a tumorogenic 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 tumorogenic 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 (nmu/nmu), 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 increase of increased levels of retinoids on anchorage-dependent and anchorage-independent growth of tumorogenic epithelial cells and relate the effect of vitamin A, given at nontoxic doses, on 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 Cal515 cell line maintains epithelial characteristics when transplanted into athymic rodents. The cell line was routinely cultured in IMDM 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 subbed 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 Company.
Co. or provided as a gift from Dr. W. E. Scott of Hoffmann-La Roche Inc., Nutley, N.J. Retinoid diluted with ethanol in IMDM was added to the wells to yield final concentrations of 0.1 mm to 1 nm. Control cultures received 0.1% ethanol (v/v) in IMDM alone. After 7 days of treatment, the ethanol or the retinoid concentration was 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.

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

where $f_i$ is the number of retinoid-treated cells, and $C$ is the number of cells in control cultures (28).

Anchorage-independent Growth Assay. Cells were cultured in triplicate 35-mm Petri dishes using 2 layers of media, as described by Hamburger and Salmon (13). 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 (Difco Laboratories, Inc., Detroit, MI). Conditioned media were not used. CaMa-15 cell suspensions were washed in Hanks’ 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. (26) was used with modifications. Twenty thousand well-separated cells were suspended in Connaught Medical Research Laboratories medium with 15% horse serum, additional nutrients (16), 0.6% agarose (SeaPlaque; FMC Corp., Marine Colloids Division, Rockland, ME), and either 0.1% ethanol only (control) or a concentration of retinoid (10 µM, 0.1 µM, or 1 nm). One-mi aliquots were seeded in 35-mm dishes to which 1 ml of underlayer, as described previously, was added. The cultures were fed every 72 hr with 1 ml of fresh medium containing 0.1% ethanol or the retinoid concentration in liquid Connaught Medical Research Laboratories medium. The cells were incubated at 37° in 7.5% CO₂ in a humidified atmosphere. Cultures were examined on Day 1 and periodically thereafter, using an inverted microscope. Aggregates containing more than 30 cells were considered to be colonies. Colonies were counted after 14 days at ×10.

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/nu males with rnu/+ 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 athymic rats were housed at 27 ± 1°, without antibiotic coverage, in a specific-pathogen-free room under laminar-flow HEPA-filtered air. All bedding, cages, water, and other material coming in direct contact with the animals were autoclaved before use. Animals were permitted access to food and water ad libitum. The rats were fed a diet of powdered chow (Wayne Sterilizable Lab-Blox; Allied Mills, Inc., Chicago, IL), which contains elevated levels of heat-sensitive nutrients. Autoclaved chow yields approximately 5.4 µg retinol/g.

Xenotransplantation and Vitamin A Administration. Twenty-five female nu/nu rats were chosen at random from the breeding stock and distributed into 3 groups on the basis of animal weight. No significant difference in weight was demonstrated by Student’s t test. All animals were approximately 3 months old and were maintained under standard housing conditions described previously. The athymic rats were permitted access to sterile laboratory chow and water ad libitum throughout the experiment.

For xenotransplantation, the cells were prepared as follows. Routine culture conditions were observed by growing cells in 75 sq cm flasks (Costar). Harvesting was followed by washing the cells in IMDM and counting the viable cells in a hemocytometer. Cells (1 × 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, dermatosis, 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 × ab², 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%
considered moderately sensitive, and 75 to 100% considered extremely sensitive to the retinoids.

The CaMa-15 cell line responded to the retinoids with at least a 50% reduction in cell growth at all levels of retinoic acid and low doses of retinol. Higher doses of retinol did not increase response. The dose-response curves showed that the degree of growth inhibition varied with the concentration of retinoid tested, but not in a linear fashion. Levels of retinoids less than $10^{-4}$ M were not considered cytotoxic, since the viability of the cells as determined by trypan blue exclusion was always greater than 90%.

**Effect of Retinol and Retinoic Acid on Anchorage-independent Growth.** CaMa-15 cells grow readily in semisolid media, at the concentration of cells plated, with a cloning efficiency of 1.3%. The ability of the retinoids to inhibit the anchorage-independent growth of CaMa-15 cells is presented in Chart 2. Both retinol and retinoic acid can reduce the ability of CaMa-15 cells to form colonies in 0.6% agarose, with retinol being slightly more effective than retinoic acid at all doses tested, 1 nM being somewhat less inhibitory than 0.1 or 10 $\mu$M.

**In Vivo Effects of Vitamin A.** 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 painful or difficult motion was observed in the surviving animals.

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 tumorigenic 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 tumorigenic 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-
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 CaMa-15 cells were more sensitive to retinoic acid in monolayer culture, they differed by retinol and retinóte acid. Although CaMa-15 cells tumor size with the low-dose retinol treatment in vivo. Although both doses showed approximately 40%

The in vivo study on the growth-inhibitory properties of retinol indicated that a relatively low dose, 30 μg/day, can retard a progressively growing tumor. The high i.p. dose, 3 mg/day, may be marginally toxic to the host, without detection by standard criteria, thereby rendering the antiproliferative function of vitamin A less effective. A dose of 300 μg was not included in the experimental design, however, the data suggest that a daily dose of retinol in a range between the doses examined may prove to be optimal.

Other than the usefulness of the nude rat in xenotransplantation, the animal also offers another means to study the role of vitamin A in vivo. It has been suggested that vitamin A has a direct action on the cell-mediated immune system. Thymopoiesis (39) and lymphopoiesis (38) are enhanced, and antigen-dependent T-cell-mediated immunity is stimulated (1, 8, 32). Lacking a functional thymus, the nude animal is still capable of demonstrating the antiproliferative function of vitamin A less effective. A dose of 300 μg was not included in the experimental design, however, the data suggest that a daily dose of retinol in a range between the doses examined may prove to be optimal.

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REFERENCES


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