Effect of Retinoids on Xenotransplanted Human Mammary Carcinoma Cells in Athymic Mice

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ABSTRACT

Previous studies have shown dose-dependent growth inhibition of the human mammary carcinoma cell line MDA-MB-231 xenotransplanted in athymic mice using retinol. In this study, the growth inhibitory effect of retinoic acid (RA) and 13-cis-retinoic acid (13-cis-RA) was examined in vitro and in vivo. With both agents there was dose-related growth inhibition in monolayer culture. The MDA-MB-231 cell line was more sensitive in monolayer culture to 13-cis-RA than to RA. Anchorage-independent growth of the MDA-MB-231 cell line was also inhibited by both of these agents but only in a dose-dependent manner with 13-cis-RA. Athymic mice inoculated with MDA-MB-231 human mammary carcinoma cells were treated with various doses of RA and 13-cis-RA for 30 days. RA doses greater than 90 µg were clinically toxic to the animals. There was a decrease in tumor size with all doses of RA tested but not in a dose-related fashion. Response at the higher doses of RA may be related to subclinical toxicity. Doses of 13-cis-RA above 300 µg were clinically toxic. Unlike RA, there was no statistically significant decrease in tumor size with treatment with 13-cis-RA. These findings show that there is significant reduction in growth in vivo of the human mammary carcinoma cell line MDA-MB-231 after treatment with RA. However, in vivo response to the retinoids is not always predicted by in vitro methods.

INTRODUCTION

Vitamin A alcohol or ROL3 is an essential nutrient for the maintenance of normal epithelial differentiation and proliferation (1, 2). This role has stimulated interest in using ROL and its derivatives, the retinoids, in the prevention and treatment of cancer. Studies in animal systems (3-6) have shown that the retinoids have also been shown to inhibit the growth of tumor cells in vitro and in vivo. Many animal and human malignant cell lines are sensitive to the growth inhibitory effects of the retinoids in vitro (7-17). There has been less evidence of a therapeutic effect in vivo. Early studies showed little sensitivity in vivo (18, 19), but in 1975, a transplantable rat chondrosarcoma was successfully treated (20). Since then, several studies have shown the inhibition of growth of other animal tumors in vivo by the retinoids (21-25). Although clinical studies have been published (18, 26-30), less work comparing the effects of these agents in vitro and in vivo on human tumor cells has been reported (2, 3, 5). One of the reasons for this has been the lack of a suitable animal model.

We have developed a model to study the in vivo effects of the retinoids on human carcinoma cells. We have reported previously on the therapeutic effects of ROL on tumor cells inoculated into athymic rats (31) and on human carcinoma cells inoculated into nude mice using the human mammary carcinoma cell line, MDA-MB-231, xenotransplanted into congenitally athymic mice (32, 33). The MDA-MB-231 cell line is sensitive to the retinoids in vitro and grows well in vivo without hormonal supplementation, thus, providing a useful system for studying the treatment of human breast carcinoma with these agents.

We now report the effects of treatment with RA and 13-cis-RA on human mammary carcinoma cells inoculated into athymic mice.

MATERIALS AND METHODS

Source of Cell Lines. MDA-MB-231 (ATCC-HTB-26) was obtained from the American Type Culture Collection (Rockville, MD). This human mammary carcinoma cell line was isolated from a pleural effusion (34).

Cell Line Maintenance. The cells were maintained in monolayer culture on Costar plasticware (Cambridge, MA) with Eagle's MEM supplemented with 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 1.5% MEM vitamin solution (100x, v/v), 2 mM L-glutamine, insulin (10 µg/ml), and 10% FBS (v/v) containing ROL in a final concentration of 0.01 µg/ml, pH 7.4 (Grand Island Biological Co., Grand Island, NY). Cultures were incubated in a humidified atmosphere of 5% CO2:95% air at 37°C. The cells were checked (Flow Laboratories, Ingelwood, CA) periodically for mycoplasma and were consistently found to be negative.

Growth Inhibition Determination In Vitro. Growth inhibition in monolayer culture was determined using all-trans-RA (Sigma Chemical Co., St. Louis, MO) and 13-cis-RA (Hoffmann-LaRoche, Inc., Nutley, NJ). Stock solutions of 10-10-10-10 were prepared using γ-irradiated 95% ethanol and diluted 1:1000 with growth media. Cells were allowed to settle in monolayer culture wells (Costar) and 24 h later 1 ml of a single concentration of each retinoid from 10-1010-10(M was added per well (11). Control cultures were given 0.1% ethanol. The media with or without the retinoids were replaced every 72 h. All procedures were carried out in subdued light. Exponentially growing cells were harvested 7 days later with EDTA:trypsin. Grossly viable cells were stained with 0.1% trypan blue and counted using a hemocytometer. The percentage of growth inhibition was calculated as:

\[ 100 - \left( \frac{R}{C} \times 100 \right) \]

where R and C are the number of cells from the retinoid-treated and control samples, respectively.

To determine the inhibition of anchorage-independent growth of the cells in soft agar, the method previously described (31) was used. Exponentially growing MDA-MB-231 cells were trypsinized, washed in Hanks' balanced salt solution, and single cell suspensions prepared. Samples were plated in 35-mm Petri dishes using the method of Hamburger and Salmon (35). The underlayer was prepared without conditioned media using 1.0 ml of McCoy's medium with 10% fetal bovine serum and 5% horse serum (Grand Island Biological Co., Grand Island, NY), various nutrients (36), and 0.5% agar (Difco Laboratories, Inc., Detroit, MI). Triplicate samples of 5 x 10-6 cells were suspended in 1 ml of Connaught Medical Research Laboratories media with 15% horse serum, additional nutrients (36), and 0.3% agar (Difco), and various doses of retinoid. Control cultures received 0.1% ethanol instead of the retinoid in the culture media. One-mL aliquots were plated on top of 1

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3The abbreviations used are: ROL, retinol; RA, retinoic acid; 13-cis-RA, 13-cis-retinoic acid; MEM, minimal essential media; NK, natural killer.
ml of underlayer. Fresh media containing the retinoid was added to the treated cultures every 72 h. Control cultures received fresh media containing 0.1% ethanol every 72 h. All procedures were carried out in subdued light. The cultures were incubated in 5% CO2:95% air in a humidified incubator. Colonies were defined as aggregates of more than 40 cells (37). The colonies were counted using an inverted microscope at 10x after 7 days incubation. An average of 30 or more colonies were considered significant.

Animals. Three- to 4-week-old, age-matched, congenitally athymic female mice with a BALB/c-nu/nu background were obtained from Harlan Sprague-Dawley (Indianapolis, IN). They were housed in a laminar flow room under sterile conditions. The mice were fed autoclaved mouse chow (Wayne Sterilizable Lab-Blox; Allied Mills, Chicago, IL) which supplied approximately 5 μg retinyl palmitate per gram after sterilization.

Tumors. The method for producing the tumors in athymic mice has been described previously (32). The cells were harvested using EDTA:trypsin and 1 × 10^7 cells in a 0.1-ml volume of media were injected s.c. within 5 to 10 min in the intrascapular region with a 22-gauge needle. The mice were examined every other day for tumor development. When the tumors became visible and palpable, they were measured in three dimensions (a, b, and c) with calipers, 3 days per week on alternate days. Retinoid toxicity was evaluated every other day by assessing general habitus, skin quality, presence of bone fractures, and body weight. At the time of sacrifice, the tumors were dissected free from the muscle and connective tissue, weighed, and measured in three dimensions (a, b, and c) in mm. Mean tumor volume was calculated as

\[ V = \frac{a \times b \times c}{2} \text{ mm}^3 \]

Retinoid Treatment. Sterile RA and 13-cis-RA feeding solutions were prepared fresh weekly and stored in the dark at −70°C. The retinoids were diluted in 0.1 ml of γ-irradiated corn oil containing α-tocopherol (2 mg/ml). Control animals received 0.1-ml corn oil and α-tocopherol only. The mice were fed these solutions intragastrically through stainless steel feeding tubes. Feedings began 3 days after s.c. injection of the tumor cells and continued for 5 days/week for 30 days. On Day 30, the mice were sacrificed.

Statistical Analysis. Differences between treated and control samples were analyzed using one-way analysis of variance. Values with p < 0.05 were considered significant.

RESULTS

In Vitro Retinoid Sensitivity. The effect of in vitro treatment of the MDA-MB-231 cells in monolayer culture with various concentrations of RA and 13-cis-RA is illustrated in Fig. 1. With both agents, there was increasing growth inhibition with increasing dose of the retinoid. The cells were extremely sensitive to both retinoids at the highest dose tested. Sensitivity to RA and 13-cis-RA is similar at most points examined except at 10^-5 M where the MDA-MB-231 cells are much more growth inhibited after treatment with 13-cis-RA.

The anchorage-independent growth of MDA-MB-231 cells with retinoid treatment is shown in Fig. 2. There was a dose-dependent effect on cellular proliferation with increasing concentrations of 13-cis-RA. Similar to monolayer culture, there was maximum inhibition of anchorage-independent growth with treatment with 10^-3 M of this retinoid. In contrast, sensitivity to RA was not dose dependent in soft agar culture. Maximum anchorage-independent growth inhibition occurred at 10^-4 M RA and the degree of growth inhibition decreased with 10^-3 M RA.

In Vivo Retinoid Sensitivity. Fig. 3 shows the results of treatment with 13-cis-RA on the growth of MDA-MB-231 cells in athymic mice. Doses of 13-cis-RA above 300 μg daily were toxic to the mice producing a severe exfoliative dermatitis, extreme lethargy, weight loss, and eventual death. Although treatment with 3 μg resulted in a mean tumor size larger than the control, this was not statistically significant. There was a decrease in tumor size after treatment with 30 and 300 μg 13-cis-RA, but this was not statistically significant.

Tumor response to treatment with RA in vivo is illustrated in Fig. 4. The mean size of the control and treated tumors is larger than those seen in the experiments performed with 13-cis-RA. Although it is not entirely clear why the tumors are larger, improvement in technique and a lower average passage
of the MDA-MB-231 cell line could account for these differences. RA doses greater than 90 µg were clinically toxic to the animals, similar to the toxicity seen in the 13-cis-RA treated animals. Although there was a decrease in tumor size with all doses of RA, the decrease was statistically significant only at 30 µg of RA per day. Reduction in tumor size was not dose related with this retinoid with maximal tumor growth inhibition occurring at 30 µg of RA.

Table 1 lists the weight of the mice in both treatment groups at the start of the study and after sacrifice. There was no statistically significant difference between the weights of the animals before treatment in any study group. In the group receiving RA there was a statistically significant lower weight gain at the time of sacrifice in the group receiving 90 µg of RA when compared with the control mice. Weight gain in all mice receiving 13-cis-RA was not statistically different from the control mice.

**DISCUSSION**

The sensitivity of cells to the *in vitro* inhibition of the retinoids has been well documented in monolayer and soft agar culture (7, 8, 11, 39, 40). The growth of the MDA-MB-231 cell line is inhibited by ROL (32) in monolayer culture in a dose-related fashion. When these results are compared with the results of treatment with RA and 13-cis-RA, the cells are more sensitive to RA and to 13-cis-RA except at the lower concentrations where sensitivity to all three retinoids is similar. Sensitivity to 13-cis-RA in monolayer culture is comparable to sensitivity to RA at most of the dosages examined.

When anchorage-independent growth of the MDA-MB-231 cell line is evaluated after retinoid treatment, there is a difference between treatment with RA and 13-cis-RA. There is no dose response to RA in soft agar culture as there is with 13-cis-RA. Lack of dose-response to a variety of chemotherapeutic agents has been well described in many human malignancies (39, 40) grown in soft agar.

Treatment with RA *in vivo* results in significant growth inhibition of the human MDA-MB-231 carcinoma cell line in athymic mice. Treatment with 30 µg of RA causes a 60% reduction in tumor size after 30 days of treatment. An earlier study with 30 µg of ROL (32) resulted in a 77% reduction in tumor size after 28 days. In contrast, tumor growth is not diminished to a statistically significant degree after treatment with 13-cis-RA. The reasons for the difference between RA and 13-cis-RA are not clear.

The clonogenic assay using soft agar (35, 36) was proposed as an *in vitro* method to predict patient response to chemotherapy. Greater than 70% growth inhibition is considered significant for predicting *in vivo* sensitivity (40). In the present study, treatment with both RA and 13-cis-RA resulted in significant growth inhibition *in vitro* in soft agar culture. Although it is not clear what concentration RA or 13-cis-RA reached in the blood of the treated mice, treatment *in vivo* only at 30 µg of RA resulted in significant tumor inhibition. The clonogenic assay did not predict the *in vivo* response accurately for either retinoid at any other dose which showed significant anchorage-independent growth inhibition.

The mechanism of action of the retinoids is unknown. It has been suggested that these agents act *in vivo* through enhancement of T-cell function. Since the athymic mouse is deficient in T-cells, this is unlikely in this host. However, the athymic mice do have intact NK cell function which is stimulated by ROL treatment when the mice have tumors (33). Reports on the effects of RA on NK cell activity have been contradictory (41–43). It is not clear in this study whether RA is acting through immune mechanisms or in a direct way on the tumor cells. The effects of 13-cis-RA on the immune system are less well characterized. Although 13-cis-RA has been shown to enhance cell mediated immunity (44), its effects on NK cell activity have not been reported to our knowledge. However, treatment with 13-cis-RA may require intact T-cell function to be effective *in vivo*. Since the athymic mouse is deficient in T-cells, treatment may be ineffective in this host.

The use of ROL for the treatment of malignancies is limited by its storage in the liver with possible toxic overload. Although RA is excreted more rapidly (45), our findings demonstrated an increased sensitivity to RA in nude mice compared with
ROL or 13-cis-RA. In a previous study (32), athymic mice were able to tolerate doses of 600 μg ROL per day while in this study only 90 μg of RA could be given daily without clinical evidence of toxic symptoms. Up to 300 μg of 13-cis-RA could be given without toxicity.

This study suggests that the use of low dose RA may be more efficacious in the treatment of human tumors. Although treatment with ROL resulted in a dose-related decrease in tumor size in athymic mice inoculated with human tumor cells (32), this was not the case with RA. 30 μg of RA exerted more growth inhibition in vivo than higher doses. Although the reasons for this are not clear, weight gain was not as great in the mice treated with 60 or 90 μg of RA as it was in the control mice. This may represent early toxicity since several studies of sub-chronic and chronic toxicity with RA and other retinoids have shown weight loss to be a feature of retinoid toxicity (46, 47). The mice in the present study may not have been treated long enough to see weight loss or other signs of toxicity. Low weight gain may have been the only sign of toxicity. A similar difference between low and high dose treatment with ROL was seen in athymic rats xenotransplanted with tumor cells (31). The lack of significant response seen with the higher doses of RA may also explain why earlier studies using high doses of this retinoid did not result in significant growth inhibition (18, 19).

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