Growth Inhibition by Retinol of a Human Breast Carcinoma Cell Line in Vitro and in Athymic Mice

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ABSTRACT

Although the anticarcinogenic and antiproliferative effects of vitamin A (retinol) have been extensively studied in vitro, there are few data regarding the response of human tumors to this agent in vivo. We have studied the effects of retinol on the human breast carcinoma cell line, MDA-MB-231. Initial in vitro studies on monolayer cultures demonstrated a retinol-induced growth inhibition that was reversible as well as time and dose dependent. A similar dose-dependent decrease in tumor cell growth was shown in vivo when BALB/c-nu/nu (athymic) mice were inoculated s.c. with MDA-MB-231 cells and given graded nontoxic doses of retinol intragastrically for 3 weeks. Tumor cells were also inhibited from lung colonization as "artificial" metastatic lesions when injected i.v. into athymic mice following retinol treatment. Spleen cells from these mice were assayed for natural killer cells as determined by their cytotoxic activity on 51Cr-labeled target cells. There was no change in natural killer activity with any dose of retinol. We conclude that retinol has a dose-dependent antiproliferative effect on human breast carcinoma in vivo as well as in vitro. Further, the retinol-induced tumor inhibition seen in T-cell-deficient mice does not appear to be due to enhancement of host immunity and thus may be solely a direct effect of retinol on the tumor.

INTRODUCTION

The role of retinol (vitamin A) in the differentiation and maintenance of normal epithelial tissues was recognized almost 60 years ago (52). In recent studies, retinol and its derivatives, the retinoids, appear to prevent the induction of tumors by carcinogens and to have antiproliferative effects on established neoplastic tissues. These antineoplastic effects have been noted in vitro as well as in vivo (for reviews, see Refs. 23, 34, and 43).

The therapeutic and prophylactic effects of retinol on human cancer have been examined. Retrospective human studies have suggested a prophylactic effect of dietary retinol or carotenoids against subsequent development of certain tumors, including those of the bladder (27), gastrointestinal tract (30), larynx (14), and lung (2, 28, 41). Prospective studies in humans have shown low serum retinol to be correlated with increased risk for carcinoma of the lung (51), prostate, skin, leukemias, and Hodgkin's disease (18), as well as tumors of the female reproductive tract and the gastrointestinal system (19). Human therapeutic trials have yielded poor results; basal cell carcinoma (4, 5), squamous cell carcinoma (47), bronchogenic carcinoma (29), cervical carcinoma (46), and melanoma (22) have not responded to retinol treatment. However, most treatment protocols have been limited to patients who have failed other treatment regimens.

The mechanism through which retinol exerts antineoplastic activity is unknown. Retinol and retinoic acid have been demonstrated to enhance several components of the immune system, including T-cell- and NK cell-mediated cytotoxicity (8, 9, 12, 13, 24, 26, 35, 42). It is thus unclear whether in vivo tumor inhibition by retinol is the result of a direct effect on tumor cells, a consequence of an altered immune response, or a combination of both. Further evaluation of the effects of retinol on human tumors would be aided by a system that allows both in vitro and in vivo manipulation. Results presented here show that retinol inhibits growth in vitro and in vivo of MDA-MB-231, a human breast carcinoma cell line. Tumor cells are inhibited from growing in local as well as in "artificial" metastatic sites established in congenitally athymic mice, and this inhibition appears to be a direct rather than an immune-mediated effect of retinol.

MATERIALS AND METHODS

Cells

MDA-MB-231 (ATCC-HTB-26), a human breast adenocarcinoma derived from a pleural effusion (6), was obtained from the American Tissue Type Culture Collection (Rockville, MD).

Maintenance. The cells were cultured and maintained as monolayers 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 (1 mg/ml), sodium bicarbonate (2.2 g/100 ml), and 10% fetal bovine serum. Cultures were incubated in a humidified atmosphere of 5% CO2-95% air at 37°C. Cells were harvested by the addition of 0.25% trypsin:0.02% EDTA (K-C Biologicals, Kansas City, KS) for 2 to 5 min until the cells detached. Trypsin was inactivated by the addition of a 10- to 20-fold excess of fresh medium, followed by a low-speed centrifugation to remove the trypsin/medium mixture. Cells were periodically assayed for Mycoplasma by indirect immunofluorescence (Flow Laboratories, Inglewood, CA) and were consistently negative.

Retinol Treatment. For use in tissue culture experiments, all-trans-retinol (Sigma Chemical Co., St. Louis, MO) or the phenyl analogue of retinoic acid (Dr. David Ong) was prepared as a 10-5 M stock solution in 7-irradiated 95% ethanol. The stock solution was filtered through a 0.4 µm filter (Millipore, Bedford, MA) and stored in the dark for up to 1 week at -70°C. Immediately prior to use, the 10-7 M stock solution was serially diluted in ethanol to concentrations of 10-2 to 10-5 M. These solutions were used in all experiments.
were further diluted 1:1000 in growth medium, and 1 ml of each (10^{-5} M to 10^{-10} M) was added per well to 6-well tissue culture dishes (Costar) 24 hr after plating the cells. Control medium contained 0.1% ethanol. Medium with or without retinol was replaced every 72 hr, and all procedures were carried out in subdued light. Seven days after beginning retinol treatment (or daily when indicated), exponentially growing cells were harvested with trypsin:EDTA, and viable cells were counted by trypan blue exclusion. For each retinol concentration, the percentage of growth inhibition was calculated as 100 – (R/C × 100), where R and C are the number of cells from the retinol-treated and control wells, respectively.

Animals

Three- to 4-week-old, age-matched, congenitally athymic female BALB/c-nu/nu mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and maintained in a laminar flow room under sterile conditions. The mice were fed autoclaved chow (Wayne Sterillzable Lab-Blox; Allied Mills, Chicago, IL) from which they received approximately 20 µg retinyl palmitate per day. All manipulations (tumor injections, weights, retinol feedings, etc.) were done under sterile conditions.

Tumors. For tumor growth studies, mice were given injections s.c. in the interscapular region with various (1 to 20 x 10^5) concentrations of tumor cells in 0.1 ml volumes through a 22-gauge needle within 5 to 10 min after harvesting the cells. The mice were monitored daily for tumor development. Once visible, the tumors were measured in 3 dimensions (a, b, and c) with callipers. Mean tumor volume was calculated as a x b x c (cu mm). Body weight and retinol toxicity were also assessed on an alternate-day basis. For the establishment of artificial metastases, 1 x 10^6 viable tumor cells/0.2 ml (untreated or pretreated with retinol for 7 days) were injected via the lateral tail vein with a 27-gauge needle.

Retinol Feedings. Retinol feeding solutions for the mice were prepared fresh weekly and stored in the dark at -70°. The highest concentration of retinol was dissolved in 0.1 to 0.2 ml ethanol and diluted with γ-irradiated corn oil containing α-tocopherol (2 mg/ml). This stock solution was then serially diluted with corn oil:α-tocopherol to obtain the desired feeding concentrations in 0.1 ml. Control animals received corn oil:α-tocopherol only. These solutions were given to the mice i.g. through stainless steel feeding tubes (Popper and Sons, New Hyde Park, NY). Daily feedings were begun 4 days after s.c. injections or 5 days before i.v. injections of tumor cells.

Retinol Assay

Retinol was extracted from serum samples with hexane (high-pressure liquid chromatography grade; Fisher Scientific, Pittsburgh, PA), following initial solubilization in 100% ethanol according to the method of Thompson et al. (48). Extracted retinol was measured in a Perkin-Elmer 650-40 fluorescence spectrophotometer, taking readings with emission at 475 nm and excitation at 330 nm. These readings were converted to serum concentrations (µg/dl) by the following formula

\[
[\text{retinol}] = \frac{(\text{Samples} - \text{Blank}) \times 1.437}{(\text{Samples} - \text{Blank}) \times 1.027} \times 1.778
\]

Statistical Analysis

Statistical significance was determined for each set of experiments using Student’s t test analysis.

RESULTS

In Vitro Retinol Sensitivity

Increasing concentrations of retinol caused a dose-dependent decrease in growth of MDA-MB-231 cells treated in vitro for 7 days (Chart 1). Retinol concentrations greater than 10^{-6} M corresponded to levels that were toxic to the nude mice and were also lethal to the tumor cells in vitro. At 10^{-5} M, retinol produced a 60% inhibition of cell growth without loss of cell viability as assessed by trypan blue exclusion.

In contrast, MDA-MB-231 showed minimal response to treatment with a phenyl analogue of retinoic acid (Chart 1). This analogue was less than 50% as effective at inducing growth inhibition than was retinol, causing no inhibition at 10^{-10} M and only slightly greater than 20% inhibition at 10^{-8} M concentrations.

Growth in culture with or without retinol was then examined as a function of time. As shown in Chart 2, inhibition of growth was apparent by Day 2 and increased linearly through the 7 days of the experiment (see Chart 2, inset).

An experiment to test the reversibility of growth inhibition by retinol led to several observations (Chart 3). Initially, it was found that cells maintained in growth medium without added retinol for 7 days and subsequently treated with retinol for 7 days were inhibited in an equal manner to cells treated immediately after plating. Further, cells treated with retinol for 7 days and then changed to growth medium without added retinol resumed the
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Chart 1. Dose-response relationship of growth inhibition of MDA-MB-231 cells in vitro. On Day 0, cells were plated in 6-well cluster dishes at 5 x 10^3 cells/35-mm well in growth medium. On Day 1, medium was removed from all wells and replaced with medium containing the indicated concentrations of retinol (•) or the phenyl analogue of retinoic acid (O) in 0.1% ethanol or medium with 0.1% ethanol only. Medium was changed on Days 3 and 6. Cells were detached by trypsinization on Day 7 and counted. Points, mean of 3 experiments, each done in triplicate; S.E. (not shown) is ≤ 1% for all points.

Chart 2. Time course of MDA-MB-231 growth inhibition by retinol in vitro. On Day 0, cells were plated in 6-well cluster dishes at 5 x 10^3 cells/35-mm well. All media was replaced on Day 1 with either medium containing 10^-5 M retinol (A) or control medium (O). Triplicate control and retinol-treated wells were trypsinized and counted each day. Points, mean of 3 experiments. Inset, kinetics of inhibition by retinol on MDA-MB-231 cells. S.E. for each point is < 1%, and p < 0.05 for all points by a Student t test.

Chart 3. Reversibility of retinol-induced growth inhibition in MDA-MB-231 cells. Cells were plated at 5 x 10^3 cells/35-mm well in 6-well cluster dishes and grown in medium with and without 10^-5 M retinol for 6 days. On Day 6, one-half of the control cells were continued in control medium (C) and the other half received medium containing 10^-5 M retinol ( Officers). Similarly, one-half of the retinol-treated cells were continued in retinol-containing medium (Δ), and the other half changed to growth medium (Δ). Triplicate wells of the 4 treatment groups were counted each day.

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normal growth curve within 2 days. Cells which continued to receive retinol for a total of 14 days, on the other hand, were still sensitive to retinol and exhibited the same degree of growth inhibition during the last 7 days as during the first 7 days of treatment. Sensitivity to retinol, therefore, was a reversible phenomenon that exhibited both dose and time dependence.

In Vivo Retinol Sensitivity

Serum and Liver Retinol Concentrations. Serum retinol concentrations were assayed in tumor-bearing athymic mice fed corn oil or graded doses of retinol in corn oil for 28 days. Samples were taken 24 hr after the last retinol feeding. None of the treated mice had serum retinol levels significantly higher than the control level of 18 μg/dl (data not shown). For this reason, the kinetics of retinol clearance from the serum of tumor-bearing mice following i.g. feedings was investigated. Chart 4 shows that animals maintained on the 2 highest doses of retinol for the standard treatment period and then bled at intervals following a bolus ingestion of the same amount of retinol exhibited a sharp peak in serum retinol concentration at 6 hr, followed by rapid clearance. Serum retinol reached control levels in each case by 8 hr, a finding which explained the similar levels seen in all groups at 24 hr.

Retinol concentrations in the livers of these animals were also determined after the 28-day treatment period and were increased as much as 8-fold with the highest retinol dose compared to controls (Chart 6A). While serum levels reflected only transient increases in retinol concentration, hepatic levels demonstrated increased retinol storage with the daily ingestion of 300 and 600 μg retinol in corn oil.

Inhibition of Tumor Growth. Initial experiments established the reproducibility of tumor induction and latent period by MDA-MB-231 cells in athymic mice. A dose of 1 x 10^7 cells injected s.c. was sufficient to obtain 100% tumor incidence by 10 days (Chart 5). In subsequent experiments using this inoculum, tumors in control animals attained mean volumes of 159 cu mm by 28 days. Animals treated daily with graded doses of retinol demonstrated a dose-dependent decrease in tumor size, with maxi-
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Chart 4. Kinetics of clearance of a bolus retinol dose from the serum of mice treated daily with retinol. Mice given injections of tumor cells and fed retinol doses of 300 and 600 µg/day for the 28-day treatment period were given an i.g. bolus of retinol 24 hr after their last dose and bled retroorbitally at various time intervals. The serum was assayed for retinol as described in "Materials and Methods." Points, average of serum levels from 4 animals. S.E. was ≤ 12 µg/dl.

Chart 5. Tumor development in athymic mice after injection of MDA-MB-231 cells. Mice were given injections s.c. in the interscapular region with 1 x 10^6 to 2 x 10^7 cells/mouse and examined daily for local tumor development. The number of days from tumor injection to the presence of a palpable lesion was denoted "tumor onset." Points, mean of 6 animals from 2 experiments. S.E. was ≤ 0.01%.

Chart 6. Effect of i.g. retinol on tumor inhibition and liver retinol stores in nude mice. In A, livers were removed from the same animals on Day 28 and assayed for retinol as described in "Materials and Methods." In B, mice were given injections of 1 x 10^6 MDA-MB-231 cells on Day 1 and were fed i.g. doses of retinol in corn oil 5 days/week for 24 days starting on Day 4. Once palpable, tumors were measured, and tumor volumes were calculated as described in "Materials and Methods." Each treatment group consisted of 4 animals, with the exception of the highest dose group, in which data from only one animal were available. Bars, S.E.; *, statistical significance (p < 0.05) as determined by the Student t test. The values for the 2000-µg dose were not included in the statistical analysis but are included in the graph to demonstrate the consistent trends seen.

Chart 7. Effect of i.g. retinol on tumor retinol concentrations. As presented above, these mice were given injections of 1 x 10^6 MDA-MB-231 cells on Day 1 and were fed i.g. doses of retinol in corn oil 5 days/week for 24 days starting on Day 4. At the end of the treatment period, the tumors were removed and assayed for retinol levels as described in "Materials and Methods." Bars, S.E.; *, p < 0.05.

Maximum inhibition of 88% at the highest concentration, 600 µg/day. The next highest doses of 300 and 30 µg/day also significantly inhibited tumor growth. Inhibition was not seen, however, at the smallest dose of 3 µg/day, which is only a 15% increase of the amount ingested in the chow as retinyl palmitate (Chart 5B).

Tumors taken from retinol-treated mice showed a 2- to 4-fold increase of retinol at the 2 highest doses (Chart 7), but this accumulation was small relative to corresponding liver concentrations of retinol. Retinol doses of 3000 µg/day and higher were toxic to the animals, producing a significant dermatitis and weight loss. No toxicity was seen using doses equal to or less than 600 µg/day.

Tumor cells were injected i.v. to investigate the effects of retinol on the establishment of "artificial" lung metastases. The lungs pictured in Fig. 1 demonstrate the dramatic difference seen in the number of metastatic lesions in control animals receiving injections of untreated tumor cells, when compared to the retinol-treated animals receiving injections of retinol-treated tumor cells. Lungs from 5 control animals had an average of 80 histologically confirmed tumor foci, while only one similar lesion was found in the lungs of 5 retinol-treated mice.

Effects on NK Cell Activity. In order to determine whether alterations of tumor growth in vivo were direct results of retinol activity or were indirect effects of immune intervention, NK cell-mediated cytotoxicity was assayed in these T-cell-deficient animals at the end of the retinol treatment period. As shown in Table 1, the percentage of cytotoxicity exhibited by spleen cells did not change significantly with any dose of retinol. These data suggest that enhancement of this activity does not play an

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Fig. 1. Effect of retinol on the ability of MDA-MB-231 to colonize lung tissue. On Day 1, control animals began receiving daily feedings of corn oil, and treated animals received 300 µg of retinol in corn oil. On Day 6, mice in both groups were given injections i.v. of 1 x 10⁶ cells; control animals received untreated cells, and retinol-treated animals received cells pretreated with retinol (10⁻⁸ M) for 7 days. Daily feedings (5 days/week) were continued for a total of 5 weeks. Each group consisted of 5 mice; representative lungs from a control (C) and a treated (R) animal are shown.

Table 1
Effect of retinol on NK cell-mediated cytotoxicity
NK cell activity was measured in the spleen cells of tumor-bearing nu/nu mice treated with corn oil with or without retinol in the concentrations indicated.

<table>
<thead>
<tr>
<th>Retinol dose (µg/day)</th>
<th>No. of animals</th>
<th>% of cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3²</td>
<td>19.9 ± 5.0²</td>
</tr>
<tr>
<td>3</td>
<td>3⁶</td>
<td>15.6 ± 1.0 (NS)⁶</td>
</tr>
<tr>
<td>30</td>
<td>9³</td>
<td>17.6 ± 0.2 (NS)³</td>
</tr>
<tr>
<td>300</td>
<td>9³</td>
<td>24.8 ± 7.7 (NS)³</td>
</tr>
<tr>
<td>600</td>
<td>4⁴</td>
<td>24.8 ± 7.3 (NS)⁴</td>
</tr>
</tbody>
</table>

¹ Calculated as described in "Materials and Methods."
² Three independent experiments.
³ Mean ± S.E.
⁴ Two independent experiments.
⁵ NS, not statistically significant.
⁶ One experiment.

important role in the in vivo effects of retinol on the growth of MDA-MB-231 in nude mice.

DISCUSSION

In this study, congenitally athymic mice served as in vivo hosts for human tumor cells grown in tissue culture. Use of this system allows us to present the first report of a human tumor cell line whose response to retinol has been examined in vitro and in vivo. Both environments provide unique information regarding the effects of retinol.

In vitro analysis of growth inhibition by retinol suggests that altered proliferation was a specific retinol effect rather than a nonspecific one. A phenyl analogue of retinoic acid caused only minimal growth inhibition of the MDA-MB-231 cells, although its chemical structure is very similar to that of retinol. This suggests that retinol-induced growth inhibition did not result from a nonspecific interaction of an amphipathic molecule with the tumor cell membranes. Rather, the inhibition is likely to have occurred through the presumed physiological pathway of specific binding protein interactions, since the analogue shows minimal binding to cellular retinoic acid-binding protein (33) and fails to promote growth and reverse vitamin A deficiency in functional assays (44).

Further analysis of growth inhibition by retinol in the MDA-MB-231 cells suggests that the breast carcinoma cell line studied consists of a population of cells that is heterogeneous with respect to its retinol sensitivity. The progressive increase in growth inhibition with time produced a nonlinear growth curve that is not compatible with either a homogeneously sensitive cell population or a 2-component population of resistant and sensitive cells. If all cells in the population were equally sensitive to retinol, treatment with retinol would have caused a uniform growth inhibition and a pattern of cell growth that progressed at a linear rate slower than the control. Similarly, retinol treatment of a 2-component population of resistant and sensitive cells would have resulted in a linear growth curve composed of an average of the doubling times of the slower, inhibited subpopulation and the faster, uninhibited subpopulation. Rather, the data suggest that the MDA-MB-231 line is composed of a spectrum of cell subpopulations whose retinol sensitivities are variably expressed, producing a nonlinear growth curve for the total population. Heterogeneity within the cell line is an attractive hypothesis in view of the evidence that many primary tumors are heterogeneous with respect to a number of characteristics, including metastatic potential (15, 32) and retinoid responsiveness (25). Other explanations for the observed pattern of growth inhibition are possible, however, and include the retinol-induced production of a growth-inhibitory factor, such as the one described for fibroblasts (45).

In the in vivo studies, retinol concentrations in the serum and livers of mice treated with retinol confirmed the ability to reproducibly administer retinol via the i.g. route. Previous animal studies have used either ad libitum feedings of retinol-chow pellets (10, 39, 50) or i.p. injections of retinol solutions (3, 21, 49) as a means of providing retinol. Variability in retinol ingestion and absorption is a potential problem with both methods, and neither serum nor tissue retinol concentrations have been reported in those studies.

Retinol was shown to inhibit the growth of MDA-MB-231 cells in a solid tumor in a dose-dependent manner similar to their inhibition in vitro. The peak serum concentration reached with 600 µg ingested retinol is roughly equivalent to 10⁻⁸ M in vitro, although alkaline hydrolysis analysis of retinol measures all forms of retinol including retinyl esters. Thus, similar concentrations of retinol and/or retinyl esters have similar effects on growth both in vitro and in vivo. The retinol-induced effects on growth of tumor cells in vitro must occur, in the absence of other cellular elements, through a direct effect. By analogy, the similar response seen in vivo also suggests a direct action. Accumulation of retinol in the tumors confirmed the direct exposure of the tumor cells to the vitamin in vivo.

There is considerable evidence, however, to suggest that the in vivo inhibition of tumors by retinol is not a direct effect but is mediated through immune mechanisms. Retinol and retinoic acid appear to enhance T-cell-mediated cytotoxicity against specific tumor antigens in allogeneic and syngeneic hosts (9, 26, 35). Retinoic acid has also been demonstrated to increase the NK cell-mediated (nonspecific) cytotoxicity of murine and human lymphocytes in vitro (1, 13). The absence of T-cells in athymic mice and the inability of retinol to increase NK cell activity in this host make it improbable that antitumor immunity is enhanced by retinol in this in vivo system. Our data thus support a direct role.
for vitamin A in tumor growth inhibition. Our results also strongly refute the suggestion that retinol is an effective antitumor agent only in the presence of an immune-competent host and/or the presence of a highly immunogenic tumor (35).

Growth of MDA-MB-231 cells as experimental metastases was also inhibited by retinol in vivo. Nicolson (31) has suggested that establishment of metastases is more clinically relevant than growth of the primary tumor, since metastases are often the cause of patient death. The data presented here indicate that treatment of the host with retinol prevents either the implantation of the circulating tumor cells into lung tissue or their subsequent clonal expansion. The observation that the population of cells capable of completing the metastatic process is inhibited from establishing metastases more clinically relevant than the presence of a highly immunogenic tumor (35).

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