All-trans-retinoic Acid Inhibits the Growth of Human Rhabdomyosarcoma Cell Lines

Gary D. Crouch and Lee J. Helman

Molecular Genetics Section, Pediatric Branch, National Cancer Institute, Bethesda, Maryland 20892

ABSTRACT

We have been evaluating the role of all-trans-retinoic acid (RA) in the differentiation and growth of human rhabdomyosarcoma (RMS) cell lines. Treatment of both embryonal (RD) and alveolar (RH30) human RMS cell lines with all-trans-RA resulted in a dose-dependent inhibition of cell growth with a maximal inhibition of 92 and 66%, respectively, at $5 \times 10^{-6} \, \text{M}$. When 13-cis-RA was used under identical experimental conditions, maximal growth inhibition was 41 and 37%, respectively. This stereo-specific growth inhibition was not associated with morphological or biochemical evidence of myogenic differentiation. Furthermore, all-trans-RA demonstrated no evidence of competition with binding of insulin-like growth factor II (IGF-II), an autocrine growth factor in RMS, to its membrane receptor as evaluated by an [125I]IGF-I receptor-binding assay. Attempts to rescue all-trans-RA growth-inhibited RMS cells with exogenous IGF-II resulted in no increase in growth compared to cells treated with all-trans-RA alone. We conclude that RA inhibits the growth of human RMS cell lines in a dose-dependent, stereo-specific manner, is not associated with differentiation, and does not appear to be directly related to IGF-II.

INTRODUCTION

Retinoids have been shown to have dramatic effects on development and differentiation. The mechanism by which retinoids exert these diverse biological effects is unclear. Specific cellular binding proteins and receptor molecules for various retinoids have been described (1, 2). RA, an active metabolite of vitamin A, influences epithelial cell growth and differentiation (3), suppresses malignant transformation in vitro and in vivo (4, 5), and influences the development of the regenerating amphibian limb (6).

All-trans and 13-cis-RA are isomers of RA that have been used in the treatment of acute APL as two of many agents that can induce differentiation and terminal cell division of leukemic cells in vitro (7). All-trans-RA has been demonstrated to have striking effects on the developing chick limb bud (8, 9), and the exposure of a rat RMS cell line to all-trans-RA resulted in terminal differentiation and growth inhibition (10).

RMS is a tumor thought to arise from skeletal muscle cells that are presumably arrested along the normal myogenic pathway to maturation (11). It is the most common soft tissue sarcoma in persons younger than age 21 years and accounts for 5–8% of all cases of childhood cancer (12). Since RA has been demonstrated to cause differentiation in leukemic cell lines, which are presumed to be disorders of cellular maturation, as well as affecting muscle development, we have been evaluating the effects of these compounds on the growth and differentiation of human RMS cell lines.

MATERIALS AND METHODS

Cells

RD is a human embryonal RMS cell line obtained from the American Type Culture Collection (13). RH30 is a human alveolar RMS cell line obtained from Peter Houghton (St. Jude Children's Research Hospital) (14). These two cell lines are capable of growing in serum-free (N2E) conditions. Cell cultures were maintained in N2E media as previously described at 37°C and 6% CO₂ (15).

Chemicals

All-trans and 13-cis-RA (Sigma, St. Louis, MO) were resuspended in 95% filter-sterilized ethanol at a stock concentration of $5 \times 10^{-5} \, \text{M}$. Final dilutions were made in N2E media and 1% ethanol. All-trans- or 13-cis-RA was added to the media when the cells were plated and was not replenished for the duration of the experiment. Recombinant human IGF-II (Bachem, Inc., Torrance, CA) was added to N2E media to final concentrations and added to each assay when the cells were plated and was not replenished for the duration of the experiment.

Mitogenic Assays

Colorimetric MTT Assay. Cell number was assayed using MTT (Sigma) and a protocol modified from Mossmann (16). For growth assays, cells were plated in 96-well microtiter plates at a density of $5 \times 10^4$ cells/well for RD cells and $5 \times 10^3$ cells/well for RH30 cells. MTT was dissolved in phosphate-buffered saline at $5 \, \text{mg/ml}$, filter sterilized, and diluted to 10% (v/v) in RPMI media without phenol red. Media were removed from all wells of an assay, replaced with 10% MTT (100 µl) and incubated at 37°C for 3 h. Individual cell viability was assessed by visualization of intracellular blue crystal formation after MTT incubation by light microscopy. Isopropanol (100 µl) was added to each well and mixed thoroughly to dissolve the dark blue crystals. Absorbances were then obtained on each test well on a Titertek Multiskan MCC/340 MKII enzyme-linked immunosorbent assay reader (FLOW Labs, Montgomery, AL) using a test wavelength of 570 nm and a reference wavelength of 690 nm. Cell numbers were obtained from standard curves constructed for each experiment and each cell type from the test absorbance. Each data point was obtained in triplicate, and cell growth assays were repeated at least once.

[3H]Thymidine Incorporation. Exponentially growing cells were plated in 96-well microtiter plates at a density of $5 \times 10^4$ cells/well for RD cells and $5 \times 10^3$ cells/well for RH30 cells and treated with various concentrations of RA and/or N2E media for 24, 48, and 72 h and 6 days. Twenty-four h prior to harvest $[3H]$thymidine (1 µCi) (New England Nuclear, Boston, MA) was added to each well and the incubation continued for an additional 24 h. Cells were collected using a semiautomatic cell harvester and lysed hypotonically, and the macro-molecules were collected on a glass fiber filter. Cellular uptake of $[3H]$ thymidine was determined by liquid scintillation counting. Each data point was obtained in triplicate and repeated several times.

Ligand-Binding Assays for IGF Cell Surface Receptors. Confluent RD cells were suspended in Puck’s saline containing 1 mm EDTA, pelleted, and resuspended in 4 (2-hydroxyethyl)-1-piperazineethanesulfonic acid binding buffer (pH 8.0) (17). In order to determine whether all-trans-RA competes with IGF-I at displacing $[125I]$IGF-I from its membrane receptor, each assay tube received $[125I]$-labeled human IGF-I.
(20,000 cpm; Amersham, Arlington Heights, IL). 1 x 10⁶ cells, and the indicated concentration of either recombinant IGF-II alone or in the presence of 5 x 10⁻¹⁰ m all-trans-RA in a final volume of 0.5 ml. Cell-associated radioactivity was determined after incubation for 18 h at 4°C as previously described (17). Binding studies were also performed as described above on cells pretreated with 5 x 10⁻¹³ m all-trans-RA for 72 h prior to harvest to determine whether treatment of RD cells with all-trans-RA affected the number or the affinity of the IGF-I membrane receptors. Binding data were analyzed by LIGAND (18).

Northern Analysis. Cells were plated at 2 x 10⁶/150-mm² plate and cultured for 6 days. Total RNA was extracted from untreated cells and cells treated with various concentrations of all-trans- and 13-cis-RA using the guanidine thiocyanate/CsCl method (19) with the following modifications. Cells were lysed by guanidine thiocyanate solution, CsCl was added to a final concentration of 1.2 M, and the mixture was layered into ultracentrifuge tubes which were one-quarter filled with 5.7 M CsCl solution (19). Tubes were placed in a Beckman SW40 rotor and centrifuged for 15 h at 31,500 rpm and 15°C. Poly(A⁺) RNA was extracted using the Fast Track kit (Invitrogen, San Diego, CA). Twenty μg of total RNA or 3-5 μg of poly(A⁺) RNA was separated on a 1% agarose/2.2 M formaldehyde-denaturing gel by electrophoresis, transferred to Nytran filters, hybridized to 32P-labeled probes, and exposed by autoradiography as previously described (15). Plasmid probes used for Northern analysis included cDNAs for hamster desmin (20), human muscle CKM (21), human MHC (supplied by Spiapiro (21)), human RAR-α (23), human IGF-II (24), and human type 1 IGF receptor (25). Antisense oligodeoxynucleotide probes were synthesized on an ABI 381 DNA synthesizer (Applied Biosystems, Foster City, CA) using cyanoethylphosphoramidite chemistry for the following sequences: human α-actin according to the sequence 3'-CGACGTTAGCATTTGACATCGTCAAAATA-5' corresponding to the reverse complement of bases 2663-2692 of the 3'-untranslated region of the sequence for human α-actin described by Taylor et al. (26); human RAR-β according to the sequences 3'-GGGGTTCCTGAGT-GACTGTTCATTGATGT-5' and 3'-ATTTGTCAATACCTT-GTATCTTCTGCCTCAAACA-5' corresponding to the reverse complement of bases 331-361 of the 5'-translated region and bases 2895-2925 of the 3'-untranslated region of the sequence for human RAR-β described by de The et al. (27), respectively; and human RAR-γ according to the sequences 3'-GGGTTTTCACGGAGGAACTACCACCGGTA-5' and 3'-CAGACCCCTCTACAGTCAGAGCCGGACCT-5' corresponding to the reverse complement of bases 415-444 of the 5'-translated region and bases 1816-1846 of the 3'-untranslated region of the sequence for human RAR-γ described by Krust et al. (28), respectively. Plasmid probes were labeled in vitro using a nick translation kit according to the instructions of the manufacturer (Amersham). Antisense oligonucleotide probes were end labeled in vitro using terminal deoxynucleotidyl transferase methods as previously described (15). Filters were prehybridized in 50% formamide, 5 x Denhardt's solution (0.1% Ficoll [Type 400], 0.1% bovine serum albumin [Fraction V], 0.1% polyvinylpyrrolidone), 5 x SSPE (1 x SSPE = 0.15 M sodium chloride-0.01 M sodium phosphate-0.001 M EDTA-Na₂, pH 7.4), 0.1% SDS, and 200 μg/ml salmon sperm DNA at 42°C for 1 h. The same conditions were used in hybridization except 5 x SSC (1 x SSC = 0.15 M sodium chloride-0.015 M sodium citrate) was used instead of 5 x SSPE. Filters were hybridized at 42°C overnight for plasmid probes and 37°C overnight for oligonucleotide probes. Filters were washed as previously described (29), with the final wash being 0.1x SSC and 1% SDS at 65°C for 1 h for plasmid probes and 1x SSC and 0.5% SDS at 37°C for 30 min for oligonucleotide probes. Films were exposed using Kodak XAR5 film and intensifying screens at -70°C. Normalization for variation in the amount of RNA loaded on the gel was done using densitometry of the exposed film compared to the intensity of ethidium bromide staining of the 28S ribosomal band in a photograph of the original gel. Rehybridization of filters was performed as described above after treating the filters for 1 h in 50% formamide-1x SSC, at 75°C.

Immunohistochemistry. Cytosplains of each cell line were prepared and stained by an immunoperoxidase method using a Vector avidin-biotin-complex anti-mouse strep-avidin kit (Vector Laboratories, Burlingame, CA) with 3',3'-diaminobenzidine (Sigma) as the chromogen. Monoclonal antibodies were obtained for human muscle actin and human desmin (Accurate, Westbury, NY) and human skeletal muscle MHC (Sigma). Counterstaining was with Mayer's hematoxylin and eosin-phloxine (American Histo Labs, Gaithersburg, MD). Positive controls were paraffin sections of human RMS tissue. Negative controls were cyto-

RESULTS

Retinoic Acid Inhibits Growth of RMS Cell Lines in a Stereo-specific Manner. Exposure of RD and RH30 to various concentrations of all-trans-RA and 13-cis-RA resulted in a dose-dependent decrease in cell growth. As demonstrated in Fig. 1 (A and B), treatment of RD cells with all-trans-RA resulted in a maximal inhibition of 92% at 5 x 10⁻⁶ M versus a 41% maximal inhibition with 13-cis-RA as assayed by [³H]thyminidine assay. Treatment of RH30 demonstrated similar results with a maximal growth inhibition of 66% at 5 x 10⁻⁶ M all-trans-RA compared with a 37% maximal growth inhibition with 13-cis-RA demonstrated in Fig. 1C and D.
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A.

Fig. 2. Dose-response growth curves of RH30 cells grown in N2E media/1% ethanol (●) and N2E media with 5 × 10^{-7} M (▲), 5 × 10^{-8} M (●), 5 × 10^{-9} M (■), and 5 × 10^{-10} M (△) all-trans-RA or 13-cis-RA (●) for 6 days.

B.

by directly interfering with IGF-II. RA demonstrated no evidence of competition with binding of IGF-II to the type I membrane receptor on RD as evaluated by an [^{125}I]IGF-I receptor assay (Fig. 4A). To determine whether the growth inhibition noted in all-trans-RA-treated RD cells reflected a change in the affinity or the number of type I IGF membrane receptors, Scatchard analyses were carried out (Fig. 4B). After 72 h pretreatment of RD cells with 5 × 10^{-7} M all-trans-RA, Scatchard analysis demonstrated 7 × 10^{5} binding sites/cell which was unchanged from that of untreated RD cells. The affinity of the type I IGF membrane receptor was essentially the same for both treated and untreated RD cells [0.12 ± 0.013 nm (mean ± SE, n = 4) versus 0.10 ± 0.011 nm, respectively]. No change in

All-trans-RA Does Not Induce Differentiation. Because RA is a known differentiating agent in some cell lines, we investigated whether growth inhibition was related to differentiation. A dose of 5 × 10^{-7} M all-trans-RA was chosen to study the effects on differentiation since this dose led to moderate, but not complete, growth inhibition without being cytotoxic. Examination by light microscopy of RD and RH30 exposed to 5 × 10^{-7} M all-trans-RA showed no evidence of terminal differentiation, defined as formation of myotubes, compared to untreated cells (data not shown). Fig. 3A demonstrates Northern analysis of the expected 2.2-kilobase MyoD1 message in normal adult skeletal muscle (lane 1) and a slight decrease (2-fold determined by densitometry) of expression of MyoD1 mRNA in all-trans-RA-treated cells at 48 h (lane 4 versus lane 5) and 6 days (lane 6 versus lane 7) compared to control cells. Similarly, Fig. 3B demonstrates expression of the 1.7-kilobase skeletal muscle-specific CKM in normal adult muscle (lane 1) but no detectable expression in treated and untreated RD cells (lanes 2–7). No expression for α-actin or MHC was noted by Northern analysis for untreated cells or cells treated with 5 × 10^{-7} M all-trans-RA (data not shown).

By immunohistochemistry, RD cells show a low level of staining for actin which was unchanged in cells treated with 5 × 10^{-7} M all-trans-RA. Desmin was strongly positive in untreated and treated cells. No staining for MHC was seen in treated or untreated RD cells (data not shown).

All-trans-RA Does Not Interfere Directly with the IGF-II Autocrine Growth Pathway. Since IGF-II has been demonstrated to act as an autocrine growth factor in RMS cell lines (30), we explored the possibility that RA may inhibit growth under identical conditions (Fig. 2). Corresponding results were obtained using the MTT assay for cell number (Fig. 1, C and D). The growth inhibition induced by RA is dependent upon the continued exposure to RA. If RA is removed from the growth media, cells recover log-phase growth (data not shown).

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expression by Northern analysis was noted for the type I IGF receptor in RD cells treated with $5 \times 10^{-7}$ M all-trans-RA compared with untreated cells at 6 days (data not shown). Attempts to rescue RA growth-inhibited RD cells with exogenous IGF-II at doses up to 50 ng/ml resulted in no increase in growth compared to RD cells treated with RA alone (Fig. 5). Although exogenously added IGF-II does not increase the growth of exponentially growing RD or RH30 cells, it has previously been shown to increase the growth of these cells prior to their achieving log-phase growth (30). IGF-II has also been demonstrated to rescue the growth inhibition of RD cells treated with the nonspecific agent suramin.\(^3\) Northern analysis of RD cells treated with $5 \times 10^{-7}$ M all-trans- and 13-cis-RA showed no difference in expression of IGF-II compared to untreated RD cells (Fig. 6).

**DISCUSSION**

The retinoids constitute a class of pharmacological agents made up of retinol, retinol derivatives, and closely related compounds called the aratinoinds. More than 1500 different synthetic analogues of retinol have been developed and biologically tested (31). Although their functions and biological activities frequently overlap, they are not identical. They play a critical role in growth, vision, reproduction, epithelial cell differentiation, and immune function (32). When animals are fed a retinol-deficient diet to which all-trans-RA has been added, they grow normally but have impaired reproductive capability and vision (32). This limited ability of all-trans-RA to substitute for retinol suggests that it cannot be reduced in vivo to supply the necessary retinal for vision. Results of nutritional experiments also indicate that the metabolic functions of RA and retinol may differ. These results indicate a striking difference in the pharmacokinetics of RA and retinol.

Intracellular binding proteins for either retinol or RA are widely present in tissues throughout the body (1, 2). The direct effects of retinoids appear to be mediated by several recently

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\(^3\) Data from C. P. Minniti, M. Maggi, and L. J. Helman. Suramin inhibits the growth of human rhabdomyosarcoma by interrupting the IGF-II autocrine growth loop, submitted for publication.
described nuclear RARs, which act as ligand-inducible transcriptional enhancer factors and belong to the nuclear receptor superfamily (33). RARs are variously distributed in different tissues. These RARs include RAR-α, which exhibits widespread expression in numerous different tissues and cell lines; RAR-β, which is expressed in a wide variety of epithelial cell types; and RAR-γ, whose expression appears to be confined primarily to skin (28, 34). The identification of these receptors suggests that RA may exert its biological effects in a manner similar to that of glucocorticoids, i.e., the interaction of RA with its specific receptor allows binding of the receptor to specific DNA sequences leading to certain target gene transcription. All-trans-RA was more potent in inhibiting the growth of both embryonal (RD) and alveolar (RH30) human RMS cell lines compared to 13-cis-RA, as demonstrated by [3H]thymidine uptake and MTT assay. However, unlike the ability of all-trans-RA to cause terminal differentiation and growth inhibition in a rat RMS cell line (10), we saw no evidence of differentiation in the two human RMS cell lines tested by the above methods. Since it is likely that the diverse biological effects of RA are mediated through a number of specific RARs, we investigated whether the stereo-specific growth inhibition of RA on human RMS cell lines was due to a change in expression of these RARs in cells treated with all-trans or 13-cis-RA. By Northern analysis, RD and RH30 express RAR-α and RAR-γ which does not change with exposure to 5 x 10⁻⁷ M all-trans or 13-cis-RA. No expression of RAR-β mRNA was seen in treated or untreated RD or RH30 cells (data not shown).

When administered in vivo, all-trans-RA is isomerized to the 13-cis-derivative, and, conversely, when 13-cis-RA is administered, it is recovered as all-trans-RA (32). The physiological significance of isomerization of RA is undetermined. Both isomers have been used clinically to treat patients with various dermatological conditions and hematological malignancies (35). All-trans-RA has been demonstrated to be a more potent differentiating agent than 13-cis-RA in promyelocytic cells of patients with acute promyelocytic leukemia (7). All-trans-RA is capable of inducing remission in patients with APL (36). Recent reports describe the discovery of a genomic rearrangement in RAR-α in patients with APL who have the t(15;17) abnormality, which may play a role in the pathogenesis of this disease. Pharmacological doses of all-trans-RA may be able to overcome this abnormality and lead to maturation of promyelocytic cells (37, 38).

We conclude that RA treatment of human RMS cell lines causes a significant inhibition of growth in a stereo-specific manner, with all-trans-RA having more potent activity. The mechanism of this growth inhibition is not known, but determining the molecular basis for this may lead to new insights into the mechanisms of RA specificity. Soft agar cloning experiments are currently ongoing to determine whether the tumorigenic capacity of the RD cells is altered with all-trans-RA treatment. Finally, since the doses of all-trans-RA used in this study to cause growth inhibition of RMS cells in vitro are clinically achievable in vivo, our data suggest a potential role for these agents in the treatment of rhabdomyosarcoma tumors. Currently, all-trans-RA is being used in pediatric phase I studies that have included patients with refractory RMS to determine its antitumor effects in this tumor.

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