Retinoic Acid Induces Neuroblastoma Cell Death by Inhibiting Proteasomal Degradation of Retinoic Acid Receptor α

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ABSTRACT

To seek a novel therapeutic approach to neuroblastoma (NBL), we used three NBL cell lines (SK-N-DZ, NH12, and SK-N-SH) to examine the underlying molecular mechanisms of cellular reactions and sensitivity to all-trans-retinoic acid (ATRA). SK-N-DZ cells expressed relatively high levels of retinoic acid receptor α (RAR-α) and underwent ATRA-induced cell death that was blocked by an RAR-α antagonist. By contrast, RAR-α expression gradually decreased in NH12 and SK-N-SH cells, which did not experience increased cell death in response to ATRA. We report here the ubiquitin-dependent down-regulation of RAR-α expression during ATRA treatment. Our data suggest that SK-N-DZ cells have a defect in RAR-α down-regulation, resulting in sustained high expression of RAR-α that confers high sensitivity to ATRA. Accordingly, treatment with a proteasome inhibitor dramatically increased ATRA-induced cell death in NH12 and SK-N-SH cell lines. Our results reveal the crucial involvement of the RAR-α signaling pathway in NBL cell death and show that three NBL cell lines are differentially sensitive to ATRA. These data suggest a potential novel therapy for NBL involving retinoic acid treatment combined with the inhibition of RAR-α degradation.

INTRODUCTION

The survival rate of patients with widely metastatic neuroblastoma (NBL) generally is low, despite extensive multimodal therapy (1). In recent clinical trials, 13-cis-retinoic acid has been administered in an attempt to eradicate small numbers of residual tumor cells that survive after intensive induction and consolidation therapies (2). However, the retinoic acid signaling pathways involved in the growth and differentiation of normal and malignant neuronal cells remain largely unknown.

Two classes of retinoic acid receptors (RARs), α, β, and γ, and retinoid X receptors (RXRs), α, β, and γ, are structurally related members of the steroid hormone receptor family of ligand-dependent transcription factors (3, 4). Human NBL cells express all three RAR isoforms in response to retinoic acid treatment; however, the expression levels vary among the different isoforms. For example, in several NBL cell lines, RAR-α expression is increased and that of RAR-β is dramatically increased; however, no such increase in RAR-γ has been reported (5, 6).

It has been reported that high-level RAR-β expression is a favorable prognostic feature of primary NBL tumors (7) and that low RAR-γ expression contributes to the malignant phenotype of NBL (8). As for RAR-α, its expression in NBL is directly controlled by the RAR/retinoic acid signaling pathway (5).

In 1985, Nelson and Nelson (9) reported that the cell line SK-N-DZ was the only line in which 13-cis-retinoic acid caused cell death. In the present study, we treated SK-N-DZ and two other NBL cell lines, NH12 and SK-N-SH, with retinoic acid to investigate the mechanisms responsible for differential reactivity and sensitivity of cells to this compound. In particular, we examined the relationship between the level of RAR isofrom expression and cell death and/or cell proliferation. We confirmed that SK-N-DZ overexpresses RAR-α in response to all-trans-retinoic acid (ATRA), resulting in cell death. We investigated ATRA-induced cell death in SK-N-DZ using the RAR-α antagonist Ro 41–5253 and compared the findings with those for RAR-α-transfected NBL cell lines. We further examined the mechanisms of RAR-α degradation in NBL cell lines using a proteasome inhibitor and expression vectors encoding hemagglutinin (HA)-tagged ubiquitin. We show that the ATRA–RAR-α signaling pathway is involved in growth inhibition of NBL cells and that the RAR-α proteins are catabolized by the ubiquitin-proteasome pathway. Our data suggest that ATRA-induced cell death in SK-N-DZ may result from abnormal degradation of RAR-α. Our results may constitute the basis for a novel NBL therapy involving the inhibition of RAR-α catalysis in combination with retinoic acid treatment.

MATERIALS AND METHODS

Cells Lines and Transfections. The three human NBL cell lines used in this study were SK-N-DZ (9), SK-N-SH (10), and NH12 (11). These cell lines were maintained in RPMI 1640 with 10% heat-inactivated FCS containing penicillin (100 units/mL), streptomycin (100 μg/mL), and l-glutamine (2 mmol/L). The cells were grown at 37°C in a humidified atmosphere containing 5% CO2.

To determine whether the forced overexpression of exogenous RAR-α in NH12 and SK-N-SH generated ATRA-induced cell death, the RAR-α cDNA-containing expression vectors were transfected into these cell lines. The inserts of RAR-α expression vectors were constructed using monoclonal cell RNA from normal adult peripheral blood and primer sets specific for the 5’- and 3’-untranslated regions of RAR-α (forward primer, 5’-CTGCTTTCCCTTCTCAGTGTGCCCGTCTTGGCATG-3’; reverse primer, 5’-CAAGTCTGGGTTTCGAGAACAGT-3’). The products were cloned using the pBluescript cloning vector (Novagen, Madison, WI) and sequenced with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Purified RAR-α cDNA then was inserted into a pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA). The expression vectors were transfected into NH12 and SK-N-SH using the lipofection method (Qiagen, Valencia, CA). These procedures were conducted according to the manufacturer’s instructions. Stable transfectants were selected after 5 to 8 weeks. The expression of recombinant RAR-α was confirmed by immunoblot analysis.

Culture Reagents. Experimental culture reagents were as follows. ATRA was purchased from Sigma (St. Louis, MO). The RAR-α-specific antagonist Ro 41–5253 (12) was a gift from Dr. Peter Mohr (Hoffman-LaRoche, Basel, Switzerland). The proteasome inhibitor MG-132 (Z-Leu-Leu-Leu-H aldehyde; ref. 13) was purchased from EMD Chemicals, Inc. (San Diego, CA). ATRA, Ro 41–5253, and MG-132 were solubilized in DMSO as 10-mmol/L stock solutions kept at −80°C. EST was solubilized in ethanol as 10-mmol/L stock solutions kept at −20°C. Further dilutions were made in the appropriate culture medium.

Design of Experimental Cultures. The culture cells were treated continuously for up to 15 days with doses of ATRA ranging from 5 to 20 μmol/L. Culture medium was replaced every day during the experimental period.
Floating cells in the supernatants were collected by centrifugation at 1000 rpm for 5 minutes, and the cell pellets then were resuspended in fresh culture medium and returned to culture flasks for measurement of cellular proliferation and viability. The cultured cells were harvested every other day, and the cells were counted with an STKS hematology analyzer (Coulter, Hialeah, FL) to calculate cellular proliferation. Dead cells were determined by positive trypan blue staining, from which the dead cell fraction was calculated.

We tested the effect of Ro 41–5253 to determine whether the function of RAR-α in SK-N-DZ is related to ATRA-mediated cell death. Our preliminary experiments revealed the optimal concentration of ATRA to be 5 μmol/L and that of Ro 41–5253 to be 600 nmol/L, which is 10-fold higher than the retinoic acid concentration required to inhibit 50% of specific retinoic acid binding (IC50). These cultures were incubated in the presence of 0.15% DMSO alone, 600 nmol/L Ro 41–5253 alone, 5 μmol/L ATRA alone, or 5 μmol/L ATRA plus 600 nmol/L Ro 41–5253. The culture procedure and the determination of cell number and viability of the cultures have been described previously.

To ascertain whether the decreasing RAR-α expression in RAR-α-transfected NH12 and SK-N-SH cells is caused by proteasome- and/or lysosome-dependent degradation, we tested the effects of the MG-132 and EST. Our preliminary experiments revealed that the optimal concentration of MG-132 and EST was 10 μmol/L. The cultures containing MG-132 were incubated for 12 hours, whereas cultures containing EST were incubated for up to 3 days. The level of RAR-α in these cultured cells was analyzed by immunoblot analysis.

To assess whether the inhibition of RAR-α catalysis in SK-N-SH and NH12 generates ATRA-mediated cell death, we tested the effect of MG-132. Our preliminary experiments revealed that the optimal concentration of ATRA was 10 μmol/L for both cell lines, whereas that of MG-132 was 0.7 μmol/L for SK-N-SH and 1.0 μmol/L for NH12. The NH12 and SK-N-SH cells were grown to 50% confluence. These cultures then were incubated in the presence of 10 μmol/L ATRA plus 0.01% or 0.007% DMSO, 1.0 or 7.0 μmol/L MG-132 plus 0.1% DMSO, or 10 μmol/L ATRA plus 1.0 or 0.7 μmol/L MG-132. The culture medium was replaced every fourth day during the experimental period. The cultured cells were harvested every fourth day to determine viability of cultures as described previously. RAR-α levels in these cultured cells were assessed by immunoblot analysis.

**Antibodies.** The antibodies used in this study were as follows: mouse monoclonal antibodies against p53 (clone DO-7; Novocastra Laboratories, Newcastle, United Kingdom) and p27kip1 (clone 57; Transduction Laboratories, Lexington, KY), and rabbit polyclonal antibodies against RAR-α, RAR-β, and RAR-γ (Santa Cruz Biotechnology, Santa Cruz, CA). All of the antibodies were used for immunoblot analysis at appropriate dilutions as follows: anti-p53 (1:1000), anti-p27kip1 (1:1000), and anti–RAR-α, -β, and -γ (1:3000). The anti-HA monoclonal antibody used for immunoprecipitation was a gift from Dr. Keiji Wada (National Institute of Neuroscience, Tokyo, Japan).

**Immunoblot Analysis.** Cultured cells were obtained before each experiment and were either used as controls or subjected to various treatments. The cultured cells were harvested by SDG followed by 15°C. The electrophoresed samples were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) that were incubated with appropriate antibodies. Antibody binding then was visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). Anti–β-actin (Sigma) was used to detect β-actin as the internal control.

**Ubiquitylation Assay.** We used an ubiquitylation assay (16, 17) to ascertain whether RAR-α ubiquitylation could be detected in RAR-α transfecteds. Stable transfectants of RAR-α expression vectors were preincubated with 10 μmol/L MG-132 for 12 hours, and the expression vectors encoding HA-tagged ubiquitin (provided by Dr. Shigetsugu Hatakeyama, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan) then were transiently cotransfected into RAR-α transfecteds using the lipofection method (Qiagen). These procedures were conducted according to the manufacturer’s instructions. After transient transfection, cultured cells were obtained for immunoblot analysis with anti–RAR-α or immunoprecipitation with anti-HA monoclonal antibody to detect RAR-α.

To examine whether SK-N-DZ fails to mult ubiquitylate RAR-α, RAR-α ubiquitylation was compared with that of the other cell lines by immunoprecipitation. Expression vectors encoding HA-tagged ubiquitin or RAR-α were transiently cotransfected into the three intact NBL cell lines, after which the cells were cultured for 48 hours. MG-132 (5 μmol/L) was added 12 hours before harvesting. Whole-cell lysates were subjected to immunoprecipitation with the anti-HA monoclonal antibody, followed by immunoblot analysis with anti–RAR-α.

**Immunoprecipitation.** Immunoprecipitation assays were performed to detect ubiquitylated RAR-α in intact NBL cell lines and/or stable transfecteds of RAR-α. The cells were subjected to various ubiquitylation conditions as described previously and then were harvested. Cells (5 × 10⁶) were lysed in 200 μL lysis buffer (20 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L EDTA, 10 μg leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 1.0% (w/v) Triton X-100, 0.5% deoxycholate, and 0.1% SDS), and the lysates were centrifuged at 14,000 rpm for 30 seconds to collect supernatants. The supernatants were preincubated with 5 μL of protein G-Sepharose (Amersham) treated by Tris-buffered saline containing 0.05% (w/v) Tween 20 and 0.1% BSA for 1 hour at 4°C to eliminate nonspecific binding proteins, and the supernatants then were collected by centrifugation at 10,000 rpm in a table-top centrifuge for 10 seconds. The supernatants then were incubated with the anti-HA monoclonal antibody–conjugated protein G-Sepharose (5 μL) for 2 hours at 4°C, and target proteins were precipitated. Immunoprecipitated proteins were analyzed by immunoblot analysis to detect RAR-α and derivatives thereof.

**Southern Blot Analysis.** To ascertain whether the expression of RAR-α in SK-N-DZ is caused by amplification of copy number and gross structural changes in the RAR-α gene, genomic DNA containing the RAR-α gene in SK-N-DZ was compared with that of other cell lines by Southern blot analysis.

Probes of RAR-α for Southern blot analysis were generated from normal human blood cell DNA by PCR using the primers: forward, 5′-TCCGCCG-CAGCATCCGAGAACAT-3′; and reverse, 5′-CAGCCGGCCAGCT-CCGAAAAGGT-3′. The genomic DNA (50 ng) including RAR-α primers were incubated at 95°C for 120 seconds, followed by 30 cycles at 95°C for 60 seconds, 61.7°C for 20 seconds, and 72°C for 60 seconds using a DNA thermal cycler and a PCR kit (TAKARA, Kyoto, Japan). The PCR products were electrophoresed through 1% agarose/Tris-borate-EDTA gels, and the shifted bands were excised and eluted into 1× Tris-borate-EDTA using an eluculator (Bio-Rad, Hercules, CA). The PCR products then were labeled with digoxigenin (Boehringer Ingelheim, Ingelheim am Rhein, Germany). High molecular mass DNA was extracted from cultured cells for Southern blot analysis. BamHI-digested DNA (10 μmol/L) was separated on 1% agarose/Tris-borate-EDTA gels and capillary transferred onto hybridization transfer membranes (NEN Life Science, Boston, MA). Southern hybridization was performed with digoxigenin-labeled DNA fragments in a hybridization mixture according to the manufacturer’s instructions. The hybridized probe was detected using the DIG luminescent detection kit (Boehringer Ingelheim), and the signals were visualized with scientific imaging film (Kodak, Rochester, NY).

**Quantitative Reverse Transcription-PCR Analysis and Sequencing of RAR-α mRNA.** To ascertain whether the expression of RAR-α mRNA changed in ATRA-treated transfecteds of NH12 and SK-N-SH cells, RAR-α mRNA expression was quantitated by reverse transcription-PCR. Total RNA (10 μg) was reverse transcribed using the guideline thioctate-phenol chloroform extraction method, and RNA (1 μg) was then converted to cDNA using AMV-reverse transcriptase and Oligo dT-Adaptor primers (TAKARA). PCR was conducted in polypropylene reaction tubes using a final concentration of 0.3 μmol/L each for forward (5′-CGTGTCTCCTCGAGACATGA-3′) and reverse (5′-CGGAGCAGCAGTTGATG-3′) primers, 0.05 units/μL Ex TaqDNA polymerase, 3 mmol/L Mg2⁺ solution, 0.3 mmol/L dNTP mixture (TAKARA), 10% of diluted (30,000×) SYBR Green I (Cambrex, Rockland, ME), and 8% of cDNA samples (0.1 μg total RNA). PCR was performed on a Smart Cycler (Cepheid, Sunnyvale, CA). The following PCR protocol was applied: 40 cycles at 95°C for 3 seconds (denaturation), 70°C for 20 seconds (annealing and extension), and 87°C for 6 seconds (monitoring of intercalation of SYBR Green I for specific products). Each DNA sample was quantified twice in each of two separate PCR reactions.

The sequence of SK-N-DZ RAR-α mRNA was analyzed using reverse transcription-PCR. RNA was extracted and converted to cDNA, and samples including RAR-α primers (described previously) then were incubated at 95°C for 120 seconds, followed by 30 cycles at 95°C for 60 seconds, 62°C for 60 seconds, and 72°C for 60 seconds using a DNA thermal cycler (TAKARA).

The PCR products of RAR-α cDNA were sequenced with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). These procedures were conducted according to the manufacturer’s instructions.
Statistical Analysis. Statistical significance was determined by the paired two-tailed t test using a commercially available computer program (StatView 4.0 software; Abacus Concepts Inc., Berkeley, CA). P < 0.05 was considered significant.

RESULTS

ATRA-Induced Cell Growth/Death. The three cell lines were treated continuously for up to 15 days with doses of ATRA ranging from 5 to 20 μmol/L. All three ATRA-treated lines showed reduced proliferation compared with control cultures (Fig. 1A, top). In particular, the number of SK-N-DZ cells decreased slightly by about day 7. The growth of NH12 cells was almost inhibited by about day 3 to day 7. The growth of SK-N-DZ and NH12 was significantly lower (P < 0.05) compared with the control cultures. With respect to cell death, on day 3 the ATRA-treated SK-N-DZ cells had a significantly higher (P < 0.05) percentage of trypan blue-stained cells (dead cells) compared with the control (Fig. 1A, middle), whereas there were no significant differences between the control and ATRA-treated NH12 or SK-N-SH cells. ATRA-treated NBL cell lines displayed altered morphology (Fig. 1A, bottom). ATRA treatment caused dramatic morphologic changes in SK-N-DZ cells, including the formation of long neurites. By comparison, there were minimal morphologic changes in the ATRA-treated NH12 and SK-N-SH cells.

ATRA-Induced Expression of RAR-α, -β, and -γ. ATRA-induced RAR expression was assessed over time by treating cells with 10 μmol/L ATRA for various periods ranging from 1 to 15 days. RAR isoforms were analyzed by immunoblot analysis. For SK-N-DZ, basal RAR-α expression was higher than that of the other cell lines, and RAR-α expression remained relatively high during the entire period. For NH12 and SK-N-SH, RAR-α expression peaked after 1 to 3 days and then returned to the basal level by 5 to 7 days. Although basal RAR-β expression was low in all of the cell lines, it was up-regulated 1 day after ATRA treatment. The induction of RAR-β was higher in SK-N-DZ than in the other cell lines. Basal RAR-γ expression was lower in SK-N-DZ than in the other cells. ATRA did not induce changes in RAR-γ expression in SK-N-DZ and NH12. SK-N-SH showed decreased RAR-γ expression over time to a level below that of the control (Fig. 1B).

Effect of Ro 41–5253 on ATRA-Induced Cell Death of SK-N-DZ. To determine whether the continuously high RAR-α expression in SK-N-DZ is related to ATRA-induced cell death, we tested the effect of Ro 41–5253, an RAR-α–specific antagonist. Ro 41–5253 (600 nmol/L) or DMSO alone did not significantly affect SK-N-DZ cell proliferation (Fig. 1C, left). In contrast, 5 μmol/L ATRA significantly decreased the number of cells compared with DMSO alone (P = 0.03). The growth of SK-N-DZ cells treated with 5 μmol/L ATRA plus 600 nmol/L Ro 41–5253 was not significantly different compared with DMSO alone. Moreover, cell death did not increase in the presence of Ro 41–5253 or DMSO alone, whereas ATRA alone caused substantial cell death (Fig. 1C, right). However, Ro 41–5253 significantly (P = 0.005) reduced ATRA-induced cell death. These data suggest that ATRA controls cell proliferation/death in SK-N-DZ cells via the RAR-α signaling pathway.

ATRA-Induced Cell Growth and Cell Death in RAR-α–Transfected Cell Lines. Expression vectors encoding RAR-α were transfected into NH12 and SK-N-SH cells to effect RAR-α expression comparable with the basal expression in SK-N-DZ cells. Transfected cells were treated continuously with 10 μmol/L ATRA for up to 15 days. During days 3 to 15, the ATRA-treated RAR-α–transfected NH12 cells showed significantly reduced proliferation (P = 0.0028) compared with the empty vector transfectant (Fig. 2A, top). Similarly, during days 3 to 15, the RAR-α–transfected SK-N-SH cells treated with ATRA showed significantly reduced proliferation (P = 0.0004) compared with the empty vector transfectant.

We also measured the frequency of cell death in RAR-α–transfected NH12 and SK-N-SH cells. Surprisingly, cell death in these ATRA-treated lines did not differ from that of the empty vector transfectants (Fig. 2A, bottom).

Expression of RAR-α mRNA and Protein in ATRA-Treated RAR-α–Transfected Cell Lines. To clarify that the lack of ATRA-induced cell death observed was not a consequence of the forced expression of exogenous RAR-α in the NH12 and SK-N-SH transfectants, we monitored changes in RAR-α mRNA and protein expression in transfectants treated continuously with 10 μmol/L of ATRA for up to 8 days. Basal RAR-α protein expression was nearly the same as in SK-N-DZ cells (Fig. 2B). However, the protein levels in transfected NH12 and SK-N-SH cells gradually declined over time. The mean copy number of RAR-α mRNA (per 0.1 μg of total RNA) of NH12 and SK-N-SH cells in the control culture was 38,260 and 203,680, respectively (Fig. 2C), likewise, the copy numbers on ATRA treatment day 2 were 90,660 and 207,500; on ATRA treatment day 4, 106,300 and 482,570; on ATRA treatment day 6, 124,660 and 531,890; and on ATRA treatment day 8, 86,250 and 605,900. These data suggest that the level of RAR-α is regulated post-translationally.

Proteasome-Dependent Degradation and Ubiquitylation of RAR-α in RAR-α Transfectants. To determine whether the decreasing expression of RAR-α protein over time in RAR-α–transfected NH12 and SK-N-SH cells was caused by post-translational regulation, we first tested the effect of the proteasome inhibitor MG-132. RAR-α expression in transfected NH12 and SK-N-SH cells drastically increased after treatment with 10 μmol/L MG-132 for 12 hours (Fig. 3A), indicating that RAR-α is degraded by ubiquitin-dependent proteolysis. RAR-α ubiquitylation subsequently was assayed using transient transfection with a vector encoding HA-ubiquitin. Ubiquitylated RAR-α was detected as smeared protein bands from the transfected NH12 and SK-N-SH cells (Fig. 3B). We also tested whether RAR-α protein could be detected in anti-HA immunoprecipitates from RAR-α stable transfectants that were transiently transfected with HA-ubiquitin. HA-ubiquitin expression significantly and specifically increased the level of ubiquitylated RAR-α immunoprecipitated from cells cultured with MG-132 (Fig. 3C). We also used the cytotoxic protease inhibitor EST to determine whether RAR-α is degraded by lysosomes. EST did not accumulate RAR-α in the RAR-α transfectants (Fig. 3D). These results indicate that RAR-α in the NBL cell lines is catabolized by the ubiquitin-proteasome pathway.

Effect of MG-132 on p53, p27, RAR-α, and Ubiquitylation of RAR-α in Intact NBL Cell Lines. We used MG-132 to assess whether proteasome inhibition impairs the catabolism of p53, p27, and RAR-α proteins in the empty vector–transfected SK-N-SH, NH12, and intact SK-N-DZ cells. RAR-α expression increased in MG-132–treated SK-N-SH and NH12 cells but not in treated SK-N-DZ cells (Fig. 4A). The intensity of the p53 and p27 bands increased slightly in all three cell lines.

To determine whether SK-N-DZ cells fail to mult ubiquitylate RAR-α, we performed a ubiquitylation assay on all three intact NBL cell lines. Although the presence of expression vectors encoding HA-ubiquitin or RAR-α in intact NH12 and SK-N-SH cells led to increased ubiquitylated RAR-α, the smeared ubiquitylated RAR-α band in SK-N-DZ cells was less intense (Fig. 4B), indicating that SK-N-DZ is impaired for RAR-α degradation via the ubiquitin-dependent proteasomal pathway.

Southern Blot of RAR-α Genomic DNA and Sequence Analysis of SK-N-DZ RAR-α mRNA. Southern blot analysis was performed to compare the amplification of copy number and gross structural changes in the RAR-α gene in the three NBL cell lines. RAR-α
Fig. 1. Effects of ATRA on NBL cell lines. A, dose-dependent effects of ATRA on the time course of cellular proliferation and viability of cultures of three NBL cell lines: NH12 (left), SK-N-DZ (middle), and SK-N-SH (right). Top, The total cell number was counted with a hematology analyzer. All of the cell lines showed reduced cellular proliferation compared with control cultures. Middle, Cell viability was assessed by trypan blue dye exclusion, and the results are expressed as the percentage of dead cells. A significant increase in dead cells was observed in SK-N-DZ but not in the other cell lines. Data points of control and 10 μmol/L ATRA represent the mean and SE of at least three replicate experiments. Bottom, phase contrast light microscopy showing the morphology of the NBL cell lines after 11 days of treatment with ATRA (10 μmol/L final concentration). The SK-N-DZ cells showed numerous neurite formations after treatment with ATRA (middle). However, there was little morphologic differentiation in NH12 and SK-N-SH cells. B, Western blot analysis of RAR contents in the three NBL cell lines treated with 10 μmol/L ATRA for different periods. In SK-N-DZ, the basal level of RAR-α expression was continuously elevated and was higher than that of the other cell lines. RAR-β was markedly up-regulated on day 1 in all of the cell lines after ATRA treatment. β-Actin was used as a control. C, effects of the RAR-α antagonist Ro 41–5253 on ATRA-induced cell death and reduced cellular proliferation of SK-N-DZ. Left, time course of cellular proliferation. Ro 41–5253 rescued ATRA-mediated inhibition of cellular proliferation in SK-N-DZ. Right, viability of SK-N-DZ cultures. Ro 41–5253 nearly blocked ATRA-mediated cell death in SK-N-DZ. The cell number and viability of the cultures were measured as described in Materials and Methods. All of the values represent the mean and SE of at least three replicate experiments.
genomic DNA of SK-N-DZ was not substantially different from that in the and other two cell lines (Fig. 4C), nor was there any nucleotide sequence abnormality in the SK-N-DZ RAR-α/H9251 cDNA (data not shown).

Cell Death in NH12 and SK-N-SH Lines Treated with ATRA and MG-132. NH12 and SK-N-SH cells were treated with ATRA only, proteasome inhibitor MG-132 only, or ATRA plus MG-132. Both cell lines had increased trypan blue–stained cells around 7 days after treatment with ATRA plus MG-132 (Fig. 5A, top). The cell death rate was significantly different (P < 0.05) between the ATRA-only and MG-132-only groups. We assessed morphologic changes in NBL cell lines after treatment with ATRA plus MG-132 on day 11 (Fig. 5A, bottom). The NH12 cells had numerous long neurites (left) similar to that seen for SK-N-DZ cells treated with ATRA (Fig. 1A, bottom), and SK-N-SH cells showed increased cell volume and neurite formation (right).

To assess changes in RAR-α expression in the presence of ATRA and MG-132 over time, the RAR-α levels were analyzed by immunoblot analysis (Fig. 5B). In NH12 and SK-N-SH cells, RAR-α expression did not decrease during ATRA/MG-132 treatment, in contrast to that observed with ATRA only.

DISCUSSION

Retinoic acids have antiproliferative effects in a variety of malignant cells (18, 19). Similarly, we observed that the three NBL cell lines treated with ATRA show reduced cellular proliferation compared with untreated cells. Moreover, ATRA treatment further reduces the proliferation of NH12 and SK-N-SH cells expressing exogenous RAR-α. These results indicate that RAR-α directly inhibits the proliferation of ATRA-treated NBL cells.

RARs bind to ATRA and 9-cis-retinoic acid, but RXRs do not bind to ATRA (20, 21). Therefore, we examined the expression level of the three RAR isoforms in the NBL cell lines. It has been reported that in the NBL cell line, SK-N-DZ is lysed by 13-cis-retinoic acid, whereas other cell lines are refractory to this compound (9). We observed that
the basal level of RAR- expression in SK-N-DZ is significantly higher than that in the other two cell lines and that RAR- is constitutively expressed in SK-N-DZ even after long-term treatment with ATRA. The other two NBL cell lines, NH12 and SK-N-SH, showed gradual decreases in RAR- at later periods during culture and reduced cell proliferation but did not show increased cell death. In fact, the RAR- antagonist Ro 41–5253 (600 nmol/L final concentration) significantly blocked ATRA-mediated cell death in SK-N-DZ. It has been reported that the IC50 of Ro 41–5253 for RAR- is 60 nmol/L for RAR-, 2400 nmol/L for RAR-, and 3300 nmol/L for RAR-, respectively (12). Therefore, it is reasonable to assume that 600 nmol/L Ro 41–5253 would not influence the RAR- signaling pathways to inhibit cell death, whereas RAR- function would be almost entirely blocked. Hence, we conclude that the retinoid signal-

Fig. 3. Ubiquitin-proteasome–dependent degradation of RAR- in NBL cells. A. Effects of the proteasome inhibitor MG-132 were investigated by immunoblot analysis using anti-RAR-. RAR- transfected NH12 and SK-N-SH cells were treated with or without MG-132 for 12 hours. MG-132 significantly blocked proteasome-dependent degradation of RAR-. Band intensities of transfectants treated with or without MG-132 showed no significant differences with those of cultures treated together with ATRA. B, the ubiquitination assay for RAR- proteins in the RAR- transfectants. Cultured cells were preincubated with MG-132 for 12 hours and then transiently transfected with an expression vector encoding HA-tagged ubiquitin. Smear ubiquitinated bands were detected in the RAR- transfected NH12 and SK-N-SH cells by immunoblot analysis using anti-RAR-. C, immunoprecipitation assay to detect ubiquitin-conjugated RAR-. The stable transfectants of RAR- treated with MG-132 (5 μmol/L final concentration) were transiently cotransfected with an HA-tagged ubiquitin expression vector. Ubiquitlated RAR- was immunoprecipitated from RAR- transfected NH12 (left) and SK-N-SH (right) cells with anti-HA. Immuno-precipitation with the anti-HA monoclonal antibody from nontreated cultured cells served as a control (CTR). D. The RAR- transfectants of NH12 (top) and SK-N-SH (bottom) cells were incubated with cysteine protease inhibitor EST on the indicated day to examine whether RAR- is degraded by the endosome-lysosomal pathway. The EST did not block RAR- catabolism. β-Actin was used as a control.

Fig. 4. A. The effect of MG-132 was tested to assess whether this proteasome inhibitor impairs the catabolism of p53, p27, and RAR- in empty vector-transfected SK-N-SH, NH12, and intact SK-N-DZ cells. β-Actin was used as a control. B. To examine whether SK-N-DZ fails to mult ubiquitinate RAR-, ubiquitination of RAR- in SK-N-DZ was compared with that of other cell lines using immunoprecipitation. The expression vectors encoding HA-tagged ubiquitin or RAR- were transiently cotransfected into the three intact NBL cell lines, and whole-cell lysates were subjected to immunoprecipitation with the anti-HA monoclonal antibody and then immunoblotted using anti-RAR-. The smears band of ubiquitinated RAR- was detected in SK-N-SH and NH12, but this band was obviously less intense for SK-N-DZ. C. RAR- genomic DNA was analyzed by Southern blot analysis. The three cell lines SK-N-DZ (left), NH12 (middle), and SK-N-SH (right) showed almost the same level of RAR-.

A

B

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ing pathway mediates cell death in SK-N-DZ through RAR-α and that the high sensitivity of SK-N-DZ to retinoic acid is related to the RAR-α signaling pathway. In contrast, ATRA treatment did not induce cell death in RAR-α-transfected NH12 and SK-N-SH lines. These transfectants exhibited a gradual increase in the expression of RAR-α mRNA during the ATRA treatments, whereas the expression of RAR-α protein decreased gradually. These findings suggest that RAR-α protein expression is regulated post-translationally. Therefore, we further examined the mechanism of RAR-α catabolism in NBL cells, concluding that the RAR-α degradation in the transfectants is mediated by the ubiquitin-proteasome pathway based on the following arguments. First, the addition of the proteasome inhibitor MG-132 to RAR-α transfectants resulted in a drastic increase in the RAR-α level, indicating that the proteasome inhibitor impairs RAR-α degradation. The smeary band representing ubiquitylated RAR-α was detected in the transfectants transiently transfected with an HA-ubiquitin expression vector. Furthermore, RAR-α was detected in anti-HA immunoprecipitates from RAR-α transfectants in NH12 and SK-N-SH cells transiently transfected with the HA-ubiquitin vector. The cysteine protease inhibitor EST did not block RAR-α degradation in the RAR-α transfectants. Consequently, we speculate that ATRA does not induce cell death in the RAR-α-transfected NH12 and SK-N-SH lines because stimulation by ATRA is attenuated by the ubiquitin-dependent degradation of RAR-α by the proteasome. Regarding RAR-α catabolism, it has been reported that RAR-α itself, like RAR-α fusion proteins, is catabolized by the proteasome after exposure to retinoic acid in acute promyelocytic leukemia cells and non-

acute promyelocytic leukemia cells (16). In NBL cell lines, it seems that RAR-α catabolism ultimately generates an increase in RAR-α proteins because the band intensities of RAR-α protein in the transfectants treated with or without MG-132 showed no significant differences between those of cultures treated together with ATRA.

As for the cause of the continuous expression of RAR-α in the SK-N-DZ line, it is known that RAR-α catabolism is impaired by mutations of some regions within the RAR-α gene, such as those that abolish heterodimerization or affect the DNA binding domain and/or the AF-2 transcriptional activation region (16). However, the RAR-α gene is not amplified or mutated in SK-N-DZ. The proteins p27 and p53 are reportedly catabolized by the ubiquitin-proteasome system (22, 23). We have shown that p27, p53, and RAR-α accumulate in empty vector–transfected NH12 and SK-N-SH cells treated with the proteasome inhibitor MG-132, whereas RAR-α does not accumulate in SK-N-DZ, except for p27 and p53. We also investigated RAR-α ubiquitylation using anti-HA immunoprecipitation of lysates of the intact NBL cell lines transiently cotransfected with HA-ubiquitin and RAR-α expression vectors. The intensity of the smeared ubiquitylated RAR-α band in SK-N-DZ was clearly lower than that in the other NBL cell lines. It is known that polyubiquitylated proteins are targeted to the 26S proteasome for degradation, and at least three classes of enzymes are engaged in ubiquitylation processes, namely, E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligase) (24). Although the molecular elucidation of the defect in the ubiquitin-dependent degradation of RAR-α in SK-N-DZ cells is
unsolved, our results indicate that RAR-α polyubiquitylation is impaired in SK-N-DZ.

On the basis of these findings, we hypothesize that the inhibition of RAR-α catabolism in NBL cells promotes maximal efficacy of retinoic acid treatment. Incubation of SK-N-SH and NH12 cells with MG-132 and ATRA resulted in increased cell death, similar to that observed with SK-N-DZ after treatment with ATRA. Although MG-132 blocks the degradation of many types of ubiquitylated proteins in addition to RAR-α, the inhibition of proteasome function by specific inhibitors has been reported to amplify RAR-α-mediated transcription (25). We speculate that the effects of the proteasome inhibitor, such as the inhibition of RAR-α catabolism and amplification of RAR-α transcription, increase cellular sensitivity to retinoic acid and that ATRA treatment may induce cell death in NBL cells that are normally insensitive to retinoic acid.

In conclusion, our current results indicate that the inhibition of RAR-α degradation by the proteasome may form the basis of a novel therapy that facilitates maximal therapeutic efficacy of retinoic acid treatment.

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Retinoic Acid Induces Neuroblastoma Cell Death by Inhibiting Proteasomal Degradation of Retinoic Acid Receptor α


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