Retinoic Acid Confers Resistance to p53-dependent Apoptosis in SH-SY5Y Neuroblastoma Cells by Modulating Nuclear Import of p53*

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Many cell lines derived from neuroblastoma (NB) carry the wild-type p53 gene with a p53-dependent apoptotic pathway that is responsive to DNA damaging agents. A recent study has demonstrated that retinoic acid (RA) pretreatment of NB cells promotes chemoresistance to apoptosis induced by chemotherapeutic agents. We examine here the possible contribution of the p53 pathway to the chemoresistance response associated with the RA treatment in NB cells. Upon treatment with RA (1–10 μM) for 4 days, the human NB cells, SH-SY5Y, developed resistance selectively to p53-dependent apoptotic stimuli including γ-irradiation, etoposide, and 1-(5-isoquinolyl sulfonil)-2-methylpiperazine (H-7). Interestingly, RA affected the ability of p53 protein without altering its effect on elevating the steady-state level of p53, suggesting that drug-induced up-regulation and nuclear accumulation of the wild-type p53 protein are separable processes. The modulation of nuclear import of p53 protein by RA may thus represent a potential mechanism by which certain tumor cells with the wild-type p53 gene develop resistance to chemotherapeutic agents.

It is well established that tumor cells are able to acquire resistance to chemotherapeutic agents through a variety of mechanisms including enhanced drug metabolism, altered drug accumulation, drug-target amplification, and repair of damaged drug target (1). More recently, induction of apoptosis has been discovered as a novel mechanism by which many anti-neoplastic therapies achieve their therapeutic effects (2–4). Therefore, the inability of a tumor cell to activate the apoptotic response has been proposed as a pathway of resistance to antineoplastic therapy (5).

Neuroblastoma (NB) is one of the most common malignancies in childhood. Unlike other tumor types, nearly all human NBs were found to carry the wild-type p53 gene (6–9). Wild-type p53 protein is expressed in many of the cell lines derived from NB (10). However, the p53 protein is found to be restricted primarily to the cytoplasmic compartment of the NB cells (11).

This finding has led to the proposal that the p53-mediated response in NB cells is impaired because of an unknown cytoplasmic sequestration mechanism that prevents its translocation into the nucleus (11, 12). Our laboratory has recently reported a protein kinase inhibitor, 1-(5-isoquinolylsulfonyl)-2-methyl piperazine (H-7) that possesses novel activities of inducing nuclear accumulation of the p53 protein and triggering p53-dependent apoptosis in several NB cell lines that carry the wild-type p53 gene (13). H-7, similar to DNA damaging agents, increased the steady-state level of p53 in NB cells, which at least, in part accounted for the dramatic nuclear accumulation of p53 in these cells (12, 13).

All-trans-retinoic acid (RA) and many of its natural and synthetic derivatives, collectively known as retinoids, have been investigated extensively as potential therapeutics for human cancer because of their effects in inhibiting cell proliferation, inducing cell differentiation, and promoting apoptosis in many types of cancer, including NBs (14–17). The activities of retinoids are mediated by a network of RA receptors (RARs) and retinoid X receptors (RXRs) (18–21). RA at low concentration activates RARs in vivo (18–21). RA can also be metabolized to several active metabolites in vivo, including 9-cis-RA, the high-affinity ligand for RXR (22). In addition, RXRs are also known to heterodimerize with RARs and a subset of other nuclear receptors and co-regulator molecules to participate in a complex array of signaling events (18–21). Besides having a role in differentiation, RA has also been implicated in mediating apoptosis in NB cells (23). Paradoxically, a recent study has shown that treatment of NB cells with RA resulted in the development of resistance to apoptosis induced by chemotherapeutic agents (24).

In this report, we examine the possible contribution of the p53 pathway in the chemoresistance response rendered by RA treatment in the NB cell line, SH-SY5Y. RA treatment was found to confer resistance selectively to p53-dependent apoptotic stimuli in these cells. The effect was associated with the suppression of H-7-induced nuclear accumulation rather than the enhancement of protein stability of wild-type p53 protein in SH-SY5Y cells, suggesting that drug-induced up-regulation and nuclear accumulation of the p53 protein are separable processes.

EXPERIMENTAL PROCEDURES

Materials—Isoquinoline (1-(5-isooquinolinesulfonyl)-2-methylpiperazine; H-7) and staurosporine were purchased from RBI. N-Acetyl-L-erythro-sphingosine was from Calbiochem. All other chemicals were purchased from Sigma unless otherwise indicated.

Cells—The human neuroblastoma cell lines SH-SY5Y, LA-N-5, and IMR-32 have been described previously (13). RPMI 1640 medium was supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.) and antibiotics (100 μg/ml streptomycin and 100 μg/ml penicillin, Life Technologies, Inc.). H-7 was dissolved in water; all the other compounds were dissolved in Me2SO-ethanol (1:1). The cells were grown to subconfluence and then treated with RA for different durations and with different concentrations of RA. The medium was re...
The supernatant was discarded, and the pellet was resuspended in 400 μl of 1× PBS, just adjusted according to the protein concentration, were resolved by 10% SDS-PAGE, and the proteins were transferred to 0.2-μm pore size nitrocellulose membrane (Amersham Pharmacia Biotech). Filters were blocked with TBS containing 3% nonfat dry milk and 0.1% Tween 20 (TBST) for 3 h at room temperature and probed with primary antibodies diluted according to the supplier’s instructions for 1 h in the above buffer. The filters were then washed three times with TBST containing 0.1% Tween 20, probed with a 1:10,000 dilution of horseradish peroxidase-conjugated anti-mouse IgG (Amersham Pharmacia Biotech), washed again in TBST, and then washed using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech). To ensure identical amounts of protein extracts from different conditions were loaded, a duplicate set of 10% SDS-PAGE gels were routinely prepared and the gel was subjected to Coomasie staining to allow a visual inspection of the overall protein staining patterns.

**Immunofluorescence Analysis—** Cells were seeded onto glass coverslips and treated with various reagents after which monolayers were washed twice with ice-cold PBS, pH 7.4. The immunofluorescence staining procedure has been described previously (26). Briefly, cells were fixed for 3 min at 4 °C with absolute methanol (−20 °C). The washing step with PBS was then repeated once. The cells were blocked at 37 °C for 30 min with 2% bovine serum albumin, 5% fetal bovine serum, 5% normal goat serum in PBS. The cells were incubated at room temperature for 45 min with the monoclonal anti-p53 (Ab-2) antibody in blocking buffer and washed with PBS. The cells were then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Molecular Probes Inc., Eugene, OR). After washing, the coverslips were mounted in 80% glycerol in PBS containing 1 mg/ml 4,6-diamidino-phenylindole (DAPI) and examined with a Zeiss Axioplan microscope.

**Pulse-Chase Experiments—** Cells were grown in 90-mm plates, treated with solvent or RA for 4 days and then subjected to 50 μM H-7. The cells were labeled by incubation for 3 h in methionine-free medium (Life Technologies, Inc.) supplemented with diazylated fetal bovine serum and 25 μCi/ml [35S]methionine (NEN Life Science Products). After the labeling, the cells were washed three times with PBS before refeeding with fresh medium and then harvested 2, 4, 6, and 8 h later. The cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.6, 20 mM EDTA, pH 8.0, 0.5% v/v Triton X-100) in a microcentrifuge tube, and the samples were incubated at room temperature for 15 min. After centrifugation at 16,000 × g for 5 min, the supernatant was transferred to another microcentrifuge tube and subjected to further manipulations. The samples were extracted once with equal volume of phenol, once with phenol-chloroform (1:1), and once with chloroform-isoamyl alcohol (24:1). DNA was precipitated with 0.1 volume of 3M sodium acetate, pH 4.8, and 2.5 volumes of ethanol at −20 °C overnight. The DNA was then pelleted at 16,000 × g for 30 min the next day. After washing once with 70% ethanol, the samples were digested with 0.1 μg of RNase A at 37 °C for 30 min before loading onto 1.5% agarose gels and the DNA was visualized by ethidium bromide staining.

**Preparation of Cell Extracts—** Cells were grown in 90-mm dishes, cultured, and treated as described. To prepare the whole cell lysates, the medium was removed and cells were washed twice with ice-cold Tris buffered saline (TBS) (150 mM NaCl, 10 mM Tris, pH 7.6) and lysed with 500 μl of lysis buffer (10 mM Tris-HCl, pH 7.6, 30 mM EDTA, pH 8.0, 0.5% v/v Triton X-100) in a microcentrifuge tube, and the samples were incubated at room temperature for 15 min. After centrifugation at 16,000 × g for 5 min, the supernatant was transferred to another microcentrifuge tube and sequential extractions were carried out. The samples were extracted once with equal volume of phenol, once with phenol-chloroform (1:1), and once with chloroform-isoamyl alcohol (24:1). DNA was precipitated with 0.1 volume of 3M sodium acetate, pH 4.8, and 2.5 volumes of ethanol at −20 °C overnight. The DNA was then pelleted at 16,000 × g for 30 min the next day. After washing once with 70% ethanol, the samples were digested with 0.1 μg of RNase A at 37 °C for 30 min before loading onto 1.5% agarose gels and the DNA was visualized by ethidium bromide staining.

**RESULTS**

**RA Pretreatment Inhibits H-7- and Etoposide-induced Nuclear Accumulation of p53 Protein in the Human Neuroblastoma Cell Line, SH-SY5Y—** To facilitate our investigation into the mechanism underlying the chemoresistance response in the neuroblastoma cells, SH-SY5Y, associated with RA treatment (24), we evaluated the effect of RA on cellular levels of a panel of proteins known to have a role in various apoptosis pathways, with and without H-7 treatment. Many of these proteins are known activators or suppressors (e.g. Bcl-xL, Bcl-2, Bax, c-Myc, RB) of apoptosis, and their expressions in SH-SY5Y cells have been documented (13, 27, 28). Protein levels were evaluated by Western analyses. With the exception of Bcl-2 and p53, no changes were noted in levels of all the proteins tested in mock- or RA-treated cells, with or without H7 (Fig. 1A). Similar to the results obtained in the earlier study, the level of Bcl-2 protein was found to be up-regulated in RA-treated SH-SY5Y cells (24) (Fig. 1A). However, H-7 treatment did not affect the Bcl-2 level in either mock- or RA-treated cells (Fig. 1A).

Interestingly, while RA treatment did not seem to affect the level of nuclear p53 in these cells, it suppressed the H-7-mediated nuclear accumulation of p53 that was observed in solvent-treated (Mock) SH-SY5Y cells (Fig. 1A).

The effect of RA in preventing nuclear accumulation of p53
mediated by H-7 in SH-SY5Y cells was further documented by immunofluorescence staining of endogenous p53 protein in these cells subjected to various treatments. Diffuse staining of p53 in the cytosol of SH-SY5Y cells was apparent in untreated cells (Fig. 1B). Upon stimulation by H-7, nuclear p53 signal was dramatically enhanced in the cells treated with solvent (Mock). However, in RA-treated cells, the strong nuclear staining was no longer observed upon H-7 treatment (Fig. 1B), supporting the conclusion drawn from Western analysis that RA treatment blocked H-7-mediated accumulation of nuclear p53 protein.

The ability of RA to block nuclear p53 induction in SH-SY5Y cells was not restricted to the H-7 paradigm, as essentially identical results were obtained with etoposide treatment (Fig. 1C), which is also an established paradigm for the induction of nuclear p53 (29).

RA Blocks p53-dependent Apoptosis in SH-SY5Y Cells—The proteins that we have examined in Western analysis represent only a fraction of all the possible proteins that might have a role in the apoptotic pathway affected by RA. In order to assess whether the up-regulation of Bcl-2 and the suppression of H-7-induced p53 nuclear accumulation by RA had any functional impact on the apoptotic response of SH-SY5Y cells, we compared the apoptotic responses of these cells upon induction by paradigms that are either p53-dependent or p53-independent. H-7, etoposide, and γ-irradiation have been regarded as p53-dependent apoptotic paradigms in many experimental systems (5, 11–13, 30), while staurosporine, N-acetyl-L-erythro-sphingosine, and menadione are believed to act through p53-independent pathways (31–33). Indeed, treatment of SH-SY5Y cells with staurosporine, N-acetyl-L-erythro-sphingosine, or menadione effectively resulted in apoptosis as demonstrated by DNA ladder assay (Fig. 2A), and yet none of these paradigms induced nuclear accumulation of p53 under identical conditions (Fig. 2B), confirming that their apoptotic effects are likely to involve p53-independent mechanisms. On the other hand, treatment of SH-SY5Y cells with H-7, etoposide, or γ-irradiation induced apoptosis as well as nuclear accumulation of p53 (Fig. 2, A and B).

We next assessed the effect of RA pretreatment on the sensitivity of SH-SY5Y cells to these apoptotic stimuli. The cells were treated with either solvent or 10 μM RA for 4 days before exposure to the different apoptosis-inducing paradigms, and the cell viability was measured by MTT assay (Fig. 3). RA treatment did not alter the apoptotic response of the cells to staurosporine, N-acetyl-L-erythro-sphingosine, and menadione...
Interestingly, RA-treated cells were considerably more resistant to apoptosis induced by H-7, etoposide, or γ-irradiation (Fig. 3, A–C), as demonstrated by a shift of the dose-response curves to the right. These results support the idea that pretreatment of these cells with RA led to the development of resistance selectively to the p53-dependent apoptotic stimuli.

TPA Pretreatment Up-regulates Bcl-2 Protein Level but Fails to Confer Resistance to H-7-induced Apoptosis in SH-SY5Y Cells—In agreement with a previous study, the protein level of Bcl-2 was found to be up-regulated by 3–4-fold in SH-SY5Y cells upon RA treatment (24). Since Bcl-2 has been reported to be an effective suppressor of apoptosis triggered by a variety of apoptotic stimuli (34–37), it is therefore possible that the chemoresistance response observed in SH-SY5Y cells was a consequence of the elevated Bcl-2 level. The observation that RA-treated cells develop resistance selectively to only p53-dependent apoptosis argues against the elevated Bcl-2 levels to have a dominant role in conferring the RA-mediated resistance response in these cells. To clarify the issue further, we subjected SH-SY5Y cells to 12-O-tetradecanoyl-13-acetate (TPA) treatment, which is known to up-regulate Bcl-2 in SH-SY5Y cells (27). Similar to RA, TPA treatment (16 nM × 5 days) resulted in 3–5-fold induction of the Bcl-2 protein (Fig. 4A). In contrast to RA-treated cells, TPA-treated cells remained sensitive to H-7 and exhibited nuclear accumulation of p53 (Fig. 4B). Moreover, TPA was unable to confer resistance to the p53-dependent apoptotic response mediated by H-7. Despite a rise in Bcl-2 level, SH-SY5Y cells pre-exposed to TPA remained as sensitive as the solvent-treated cells (Mock) to H-7-mediated apoptosis, as evidenced by the DNA ladder assay (Fig. 4C).

Kinetics of RA-induced Resistance to p53-dependent Apoptosis—The effect of RA in conferring resistance to p53-dependent apoptosis appeared to be time- and concentration-dependent. The effect was not detectable within the first 24 h of exposure (Fig. 5A). After 48 h of exposure to RA, resistance to p53-dependent apoptosis became apparent and the degree of resistance was enhanced in a time-dependent manner. The chemoresistance effect of RA was found to be dose-dependent as higher concentrations produced more pronounced effects (Fig. 5A). RA at concentration above 10 μM was toxic to the cells and therefore was not used.

In contrast to an earlier study, which showed that SH-SY5Y cells treated with RA at 10–100 nM for 4 days developed chemoresistance to adriamycin and cisplatin (24), the resistance effect studied here appeared to require a relatively higher concentration of RA (1 μM). This raises the possibility that a metabolite of RA, rather than RA itself, was responsible for this effect.

DNA fragmentation, a hallmark of late stage apoptosis, induced by H-7, was dramatically reduced only when the SH-SY5Y cells were pretreated with RA for more than 24 h (Fig. 5B). These data provide evidence to support the hypothesis that the effect of RA on NB cells was to prevent death resulting from apoptosis. The loss of sensitivity of SH-SY5Y cells to p53-dependent apoptosis upon RA treatment may represent a gradual metabolic process, which may involve re-programming of complex signaling processes in the cells.

RA Confers Resistance to H-7-mediated p53-dependent Apoptosis in Only a Subset of NB Cell Lines—We further investigated the effect of RA in preventing the induction of functional
p53 and subsequent apoptosis mediated by H-7 in two additional NB cell lines. These two cell lines, LA-N-5 and IMR-32, have been previously demonstrated to be sensitive to the effect of H-7 in mediating nuclear accumulation of p53 and apoptosis (13). In our experiments, only LA-N-5 cells responded to RA in a similar manner as SH-SY5Y cells. LA-N-5 cells showed a reduction in sensitivity to H-7 in the induction of nuclear p53 as well as apoptosis upon treatment with 10 μM H-7 for 4 days (Fig. 6A). In contrast, RA pretreatment had no effect on H-7-induced nuclear p53 accumulation and also failed to confer resistance to apoptosis in IMR-32 cells (Fig. 6B).

RA Treatment Does Not Affect H-7-induced Enhancement of the Steady-state Level of the p53 Protein—H-7 has been shown to elevate the steady-state level of p53 in NB cells by increasing the half-life of the protein (13). The effect may, in part, account for the nuclear accumulation of p53 in the cells upon exposure to this drug. It is conceivable that RA treatment in SH-SY5Y cells may affect the protein stabilizing effect of H-7 on p53 in these cells.

We examined the amount of p53 protein in whole cell lysates and nuclear extracts of SH-SY5Y cells by immunoblotting before and after exposure to RA treatment. RA treatment did not significantly affect the low basal level of p53 detected in the whole cell lysate of SH-SY5Y cells (Fig. 7A, lanes 1 and 3, and B). Upon H-7 treatment, however, a marked increase in p53 level was detected in the whole cell extract of both mock and RA-treated cells (Fig. 7A, lanes 2 and 4, and B). Apparently, H-7 was still able to enhance the steady-state level of p53 in RA-treated cells to a similar extent as in the control cells (Fig. 7B).

The effect of H-7 on nuclear accumulation of p53, however, was essentially absent in RA-treated SH-SY5Y cells (Fig. 7A, lane 8, and B). Indeed, pulse-chase experiments revealed that pre-exposure to 10 μM RA for 4 days had minimal effect on the H-7-induced stabilization of p53 protein in SH-SY5Y cells (Fig. 7C), suggesting that the effect of RA on blocking the H-7-induced nuclear accumulation of p53 is independent from its effect on the stability of p53 in these cells.

Therefore, RA may modulate the p53-dependent response in SH-SY5Y cells by inhibiting the nuclear localization process from taking place despite the high level of p53 in the cytoplasm induced by drug treatments.

**DISCUSSION**

In this study, we investigated the role of the p53 pathway in the development of chemoresistance response to p53-dependent apoptosis in NB cells as a result of exposure to RA. The SH-SY5Y, LA-N-5, and IMR-32 NB cell lines are known to be sensitive to p53-dependent apoptosis pathway regulated by H-7 (13). RA treatment effectively conferred resistance in SH-SY5Y and LA-N-5 but not IMR-32 cells to H-7-mediated apoptosis.

The differential responses among NB cell lines to RA may be due to a variation of genetic backgrounds in NBs. RA-treated SH-SY5Y and LA-N-5 cells failed to accumulate nuclear p53 upon H-7 treatment, consistent with the suggestion that the p53 pathway might be involved in this effect. Interestingly, RA did not significantly affect the H-7-mediated up-regulation of the cytoplasmic pool of the wild-type p53 protein in SH-SY5Y...
cells, raising the possibility that the drug-induced nuclear accumulation of p53 is a separable process from enhanced protein stability.

It is widely believed that restoring and/or enhancing wild-type p53 functions in tumor cells represent a promising treatment strategy for human cancers (38). Unlike other tumors, NBs have an extremely low rate of mutation in their p53 gene (6, 8). Irradiation and DNA damaging agents are known to induce p53 expression in NB cells harboring the wild-type gene (12). Recently, we reported that the protein kinase inhibitor, H-7, has the ability to elevate the p53 level in the nucleus of NB cells, raising the possibility that the drug-induced nuclear import of p53 is a separable process from enhanced protein stability (12) proposed that the cytoplasmic retention of p53 in NB cells is a saturable process and a sudden increase in p53 level can saturate the storage capacity of the cytoplasmic pool of p53 leading to nuclear accumulation of the protein. In this study, RA treatment did not substantially affect the H-7-induced enhancement of the p53 steady-state level but dramatically inhibited the nuclear accumulation of p53. This observation leads us to propose that the nuclear import of p53 is an independent and active process that can be selectively regulated by RA in a subset of NB cells that expresses wild-type p53 protein. While the loss of p53-dependent response has been associated with chemoresistance in certain tumor cells, the molecular basis of the resistance has primarily been associated with gene mutation and protein stability (5, 39). Our current findings represent the first documentation that the process of nuclear import of wild-type p53 protein can be a target for modulating the chemoresistance response in some tumor cells.

An earlier study has reported that RA treatment, at 10–100 nm, renders chemoresistance to Adriamycin and cisplatin in SH-SY5Y cells (24). Bcl-2 up-regulation upon RA treatment was proposed as a likely mechanism for the development of the chemoresistance (24). In agreement with this report, we observed a 3–4-fold up-regulation of total Bcl-2 protein in SH-SY5Y cells upon RA (10 μM) treatment (Fig. 4). However, Bcl-2 has been shown to prevent apoptosis resulting from treatments with various apoptotic stimuli including sphingosine, staurosporine, and chemotherapeutic agents (34–37). Since RA treatment did not confer resistance to apoptotic stimuli rendered by sphingosine or staurosporine in our system, it is therefore

**Fig. 6. Differential effects of RA on H-7-dependent apoptosis in human neuroblastoma cell lines.** Left panels, RA treatment selectively inhibited the H-7-dependent apoptotic response in only a subset of NB cells. NB cell lines were treated for 4 days with solvent (open squares) or with 10 μM RA (filled squares) prior to exposure to 50 μM H-7 for an additional 24 h, and the cell viability was assessed by MTT assay as described in Fig. 3. The 100% values of viable cells were defined by measurement obtained from mock- and RA-treated cells in the absence of H-7 treatment. Values shown are means ± S.D. of six samples. Each treatment paradigm was repeated at least twice and gave similar results. Right panels, immunoblot analysis of nuclear extracts from NB cell lines, with mock or RA (10 μM × 4 days) pretreatment, to determine the level of nuclear p53 protein upon treatment with 50 μM H-7 for 10 h.

**Fig. 7. RA does not affect the H-7-mediated increase in p53 stability in SH-SY5Y cells.** A, Western blot analysis of total and nuclear p53 protein upon H-7 (50 μM × 10 h) treatment in mock- or RA-treated (10 μM × 4 days) SH-SY5Y cells. Extracts from whole cells or nuclei were prepared and fractionated using SDS-PAGE. Equivalent amounts of lysates were analyzed by immunoblotting using the p53-specific mouse monoclonal antibody P21210. B, graphic representation of the data in A. Data from immunoblotting experiments were quantitated with a densitometer using Image^®^ program, and the -fold increase in p53 protein was plotted as the ratio of the amount of p53 from H-7 treated cells versus untreated cells. Results from mock- and RA-treated cells are represented by open and filled bars, respectively. Values shown are means ± S.D. of three separate experiments. C, pulse-chase analysis of p53 protein stability in SH-SY5Y cells. The cells were pretreated for 4 days with solvent or 10 μM RA, and the stability of the p53 protein upon H-7 treatment was determined as follows; cells were pulsed for 3 h in the presence of [3H]leucine and then chased with non-radioactive medium for the indicated times. H-7 (50 μM) was added to the media at the beginning of the pulse period and throughout the chase period. Whole cell extracts were then subjected to immunoprecipitation using the anti-p53 antibody DO-1. The immune complexes bound to protein A were then fractionated on a 10% SDS-polyacrylamide gel followed by autoradiography.
questionable whether the mild up-regulation of Bcl-2 protein could have a major role in conferring selective resistance to the p53-dependent apoptosis.

TPA is as effective as RA in up-regulating Bcl-2 in SH-SY5Y cells. However, only RA is able to confer resistance to p53-dependent apoptosis in SH-SY5Y cells. These data offer further evidence arguing against the up-regulation of Bcl-2 alone being responsible for the RA-mediated chemoresistance response studied here. However, it remains formally possible that Bcl-2 might have a role in collaborating with p53 or some other molecule in the apoptotic pathway to confer the resistance response seen in these cells.

The network of signaling pathways influenced by RA is extremely complex and diverse (40). Indeed, some synthetic retinoids, which have selective affinity to a particular RAR or RXR subtype, have been shown to promote different effects in NB cells in vitro (41, 42). It is possible that the pleiotropic effect of RA on NB cells is mediated by a complex interplay of multiple retinoid signaling pathways. Additional studies involving receptor subtype-specific retinoid ligands are, therefore, necessary to further our understanding of the contribution of individual retinoid signaling pathways to the processes of differentiation, proliferation, apoptosis, and chemoresistance in NB cells.

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REFERENCES

1. Biedler, J. L. (1994) Cancer Res. 54, 666–678
2. Dive, C., and Hickman, J. A. (1991) Biochem. Pharmacol. 40, 2353–2362
3. Barry, M. A., Behnke, C. A., and Eastman, A. (1990) Biochem. Pharmacol. 40, 2353–2362
4. Sen, S., and D’Incalci, M. (1992) FEBS Lett. 307, 122–127
5. Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. (1993) Cell 74, 957–967
6. Vogan, K., Bernstein, M., Leclerc, J. M., Brisson, L., Brossard, J., Brodeur, G. M., Pelletier, J., and Gros, P. (1993) Cancer Res. 53, 5269–5273, 1993
7. Komuro, H., Hayashi, Y., Kawamura, M., Hayashi, K., Kaneko, Y., Kamoshita, S., Hanada, R., Yamamoto, K., Hongo, T., and Yamada, M. (1993) Cancer Res. 53, 5269–5273
8. Imamura, J., Bartram, C. R., Berthold, F., Harms, D., Nakamura, H., and Koeffler, H. P. (1993) Cancer Res. 53, 4053–4058
9. Hess, G., Hara, J., Okamura, T., Osagi, Y., Ishihara, S., Fukuzawa, M., Okada, A., Okada, S., and Tawa, A. (1994) Cancer Res. 53, 1087–1093
10. Davidoff, A. M., Pence, J. C., Shorter, N. A., Iglehart, J. D., and Marks, J. R. (1992) Oncogene 7, 127–133
11. Moll, U. M., LaQuaglia, M., Benard, J., and Ruig, G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4407–4411
12. Moll, U. M., Ostermeyer, A. G., Haladay, R., Winkler, B., Frazier, M., and Zambetti, G. (1996) Mol. Cell. Biol. 16, 1126–1137
13. Ronca, F., Chan, S. L., and Yu, V. C. (1997) J. Biol. Chem. 272, 4252–4260
14. Abosirf, A. M., Dahiyat, R., Narayana, P., and Cunha, G. R. (1997) Prostate 31, 261–267
15. Kaba, S. E., Kyritis, A. P., Conrad, C., Gleason, M. J., Newman, R., Levin, V. A., and Yung, W. K. (1997) J. Neurooncol. 34, 145–151
16. Szondy, Z., Reichert, U., Bernardon, J. M., Michel, S., Toth, R., Ancian, P., Ajzner, E., and Feus, L. (1997) Mol. Pharmacol. 51, 972–982
17. Melino, G., Thiele, C. J., Knight, R. A., and Piacentini, M. (1997) J. Neurooncol. 31, 65–83
18. Yu, V. C., Deletet, C., Andersen, B., Holloway, J. M., Devary, G. V., Naar, A. M., Kim, S. Y., Boutin, J. M., Glass, C. K., and Rosenfeld, M. G. (1991) Cell 67, 1251–1266
19. Yu, V. C., Naar, A. M., and Rosenfeld, M. G. (1992) Curr. Opin. Biotechnol. 3, 597–602
20. Kastner, P., Mark, M., and Champon, P. (1995) Cell 83, 859–869
21. Mangelers, D. J., and Evans, R. M. (1995) Cell 83, 841–850
22. Heyman, R. A., Mangelers, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M., and Thaller, C. (1992) Cell 68, 397–406
23. Piacentini, M., Annichiarico-Petruzzelli, M., Oliviero, S., Piredda, L., Biedler, J. L., and Melino, G. (1992) Int. J. Cancer 52, 271–278
24. Lasorella, A., Iavarone, A., and Israel, M. A. (1995) Cancer Res. 55, 4711–4716
25. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
26. Yee, K. S., and Yu, V. C. (1998) J. Biol. Chem. 273, 5366–5374
27. Hanada, M., Krajewski, S., Tanaka, S., Cazals-Hatem, D., Spengler, B. A., Roelofs, A. J., Biedler, J. L., and Reed, J. C. (1993) Cancer Res. 53, 4972–4986
28. Hammerling, U., Rijffman, C., and Pahlman, S. (1987) Oncogene 2, 73–77
29. Fritsche, M., Haessler, C., and Brandner, G. (1993) Oncogene 8, 307–318
30. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. (1993) Nature 362, 847–849
31. Bertrand, R., Solary, E., O’Connor, P., Kohn, K. W., and Pommier, Y. (1994) Exp. Cell Res. 211, 314–321
32. Santana, P., Pena, L. A., Haimovitz-Friedman, A., Martin, S., Green, D., Moulignon, M., Cordon-Cardo, C., Schuchman, E. H., Fukuji, Z., and Kolesnick, R. (1996) Cell 86, 189–199
33. Ninikawa, Y., Curry, B. I., Wang, M., Kar, S., Finn, F., Dowd, P., Zheng, Z. B., Kerno, J., and Naganathan, S. (1995) J. Biol. Chem. 270, 28304–28310
34. Huang, D. C., Cory, S., and Strasser, A. (1997) Oncogene 14, 405–414
35. Cai, J., and Jones, D. P. (1998) J. Biol. Chem. 273, 11401–11404
36. Yoshimura, S., Banno, Y., Nakashima, S., Takenaka, K., Sakai, H., Nishimura, Y., Sakai, N., Shimizu, S., Eguchi, Y., Tsujimoto, Y., and Nozawa, Y. (1998) J. Biol. Chem. 273, 6921–6927
37. Green, D. R., and Reed, J. C. (1998) Science 281, 1309–1312
38. Lowe, S. W. (1995) Curr. Opin. Oncol. 7, 547–553
39. Shaulian, E., Resnitzky, D., Shifman, O., Blandino, G., Amsterdam, A., Yayon, A., and Oren, M. (1997) Oncogene 15, 2717–2725
40. Gudas, L. J., Sporn, M. B., and Roberts, A. B. (1994) in The Retinoids: Biology, Chemistry, and Medicine (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) 2nd Ed., pp. 443–520, Raven Press, New York
41. Ponzoni, M., Buca, P., Chiesa, V., Deecensi, A., Pistoni, V., Raffaghello, L., Rozzi, C., and Montaldo, P. G. (1995) Cancer Res. 55, 853–861
42. Hsu, C. A., Rishi, A. K., Su-La, X., Gerald, T. M., Dawson, M. I., Schiffer, C., Reichert, U., Shroot, B., Poirer, G. C., and Fontana, J. A. (1997) Blood 89, 4470–4479