

Human Neuroblastoma Cell Lines as Models for the *In Vitro* Study of Neoplastic and Neuronal Cell Differentiation

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Neuroblastoma is a childhood solid tumor composed of primitive cells derived from precursors of the autonomic nervous system. This neoplasm has the highest rate of spontaneous regression of all cancer types and has been noted to undergo spontaneous and chemically induced differentiation into elements resembling mature nervous tissue. As such, neuroblastoma has been a prime model system for the study of neuronal differentiation and the process of cancer cell maturation. In this paper we review those agents that have been described to induce the differentiation of neuroblastoma, with an emphasis on the effects and possible mechanisms of action of a group of related compounds, the retinoids. With this model system and the availability of subclones that are both responsive and resistant to chemically induced differentiation, fundamental questions regarding the mechanisms and processes underlying cell maturation have become more amenable to *in vitro* study.

Introduction

Neuroblastoma (NB) is a common childhood solid tumor of the autonomic nervous system with a yearly incidence of 8.7/1 million children. Stated differently, the disease represents 7% of the total cases of childhood cancer diagnosed annually with the tumor developing in approximately one in 7000 children before the age of 5 years and an average of 525 newly diagnosed cases in the U.S. in any given year (1). The most widely used clinical staging system was devised on the basis of the size of the primary tumor and the degree and sites of dissemination. Using this scheme, it has been noted that the tumor has spread beyond the organ of origin by the time it is first diagnosed in up to 55% of patients, thereby seriously worsening their prognosis (1). The histology of most NB includes a small primitive cell component with a variable number of more mature neural or ganglion cells (2). Although Beckwith and Martin (3) reported that more differentiated neoplasms were less aggressive, other investigators (4,5)

have not found that a simple relationship exists between the degree of cell differentiation and clinical prognosis. Given the degree of widespread disease in NB and the poor response to conventional therapies, more novel approaches to treatment have been sought.

In 1927, Cushing and Wolbach (6) first reported the singular phenomenon of spontaneous differentiation of NB to a benign ganglioneuroma. More detailed analysis of cases exhibiting spontaneous tumor regression shows that this phenomenon occurs with minimal or no therapy in a subgroup of infants designated as having stage II or IVS disease (7). Surprisingly, disease regression may be seen with or without maturation of tumor elements and progression may occur despite the presence of large areas of histological maturation in the specimen (8). Of interest is the fact that stage IVS patients, with metastases in the liver, skin, and bone marrow, have almost a 70% survival rate with minimal therapy (2,8). These observations, that a small proportion of tumors cease proliferating and even undergo spontaneous maturation, prompted interest in the study of NB as a model for the general process of tumor cell differentiation, as well as neuronal development.

This paper will review some of the current data available on the *in vitro* differentiation of human neuroblastoma cell lines. The usefulness of these cell lines for the general study of questions related to differentiation will be examined. In addition, we will propose several novel mechanisms of action for various agents used to in-

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duce *in vitro* differentiation, in particular, new developments from the authors' laboratory concerning retinoid compounds will be given special emphasis. Where the mechanism(s) of action in neuroblastoma has not been investigated, work from other cultured cell systems will be presented. No attempt will be made to review the entire field of neuronal differentiation nor of the differentiation of cell lines derived from nonhuman species, as this has recently been discussed (9,10). For an in-depth compilation of well-characterized human NB cell lines, see the review by Bottenstein (9). More recently characterized cell lines will be discussed under the pertinent sections dealing with agent-induced differentiation (*vide infra*).

Development and Differentiation of Human Neuroblastoma Cell Lines

Addressing fundamental questions concerning the cellular and molecular events controlling neural differentiation have been hampered by the lack of normal diploid, continuously dividing neuroblasts or their precursors. This factor has led to the use of cell lines derived from spontaneously arising tumors as sources of neuronal-like cells in various stages of development. Sundry cell lines have been developed that exhibit numerous characteristics of primitive and differentiated neurons and, despite their aneuploidy and obscure pedigree, have proven useful in the study of neural development. Whereas most murine neuroblastoma cell lines were derived from a single aneuploid tumor (9), many human neuroblastoma cell lines have been generated from a variety of peripheral nervous system neoplasms. Current evidence suggests that these tumors arise from cells containing characteristics of autonomic, adrenergic, or cholinergic neuroblasts (2). That these tumors are composed of a heterogeneous cell population is demonstrable by studying cell-surface antigens (11), catecholamine metabolites (12), and neurotransmitter-synthesizing enzymes (13). This heterogeneity has been further appreciated by studying subclones within established cell lines (2,13). Such dissimilarity within seemingly uniform tumors has major implications for therapeutic interventions as well as basic *in vitro* studies.

In investigating the process of differentiation, the first question that needs to be addressed is how does one best assess the process. The most commonly measured characteristic of differentiation has been neurite extension. A neurite is defined as a process whose length equals or exceeds the cell body diameter (9). These processes are akin to the axons and dendrites of fully differentiated neurons and can appear hours to days after the induction of the putative differentiated state (Fig. 1). Unfortunately, unlike the situation in some other cell systems, suitable criteria for determining very early stages of cell differentiation are lacking in NB. For example, differentiation of the HL-60 promyelocytic leukemic cell line is most easily appraised by monitoring the cells' ability to reduce nitroblue tetrazolium, a property that is acquired prior to any obvious morphologic alterations; this permits rapid monitoring of the differentiated state (14). In NB, such

convenient early markers have not been defined, thus hampering detection of the initial stages of commitment to a differentiated phenotype and forcing investigators to rely either on morphologic changes alone or on biochemical modulations (e.g., neurotransmitter synthesis) that accompany rather than control differentiation. However, recent developments in the detection of various oncogenes in human NB cells now present the possibility of studying the regulation of genes that are directly involved in early stages of the differentiation process (see "Oncogenes").

Both *in vivo* and *in vitro* NB cells, as derivatives of neural crest origin, are known to have the capacity to express multiple phenotypes, including neuronal, neurilemmal (Schwann), and melanocytic characteristics (15-24). This heterogeneity is observed despite the fact that the cells are presumably derived from common neuroblast progenitor cells (23). A compelling body of evidence has been presented by Biedler and her associates (15-17,20,22) that assists in clarifying the nature of this tumor cell mixture with the potential for coordinate biochemical and morphologic interconversion. This work has been extended in a recent study (23) where the *in vitro* differentiation of five human NB cell lines treated with cAMP and RA was examined. Three overall morphologic phenotypes were observed: neuronal, characterized by cell processes and neurosecretory granules; flat, nonpigmented cells displaying the characteristics of Schwann cells; and flat, pigmented cells displaying the characteristics of melanocytes. Similarly, it has been shown that two neuroblastoma variants derived from a common precursor cell can have dramatically different responses to a single differentiating agent (24), with one undergoing neuronal-like differentiation and the other transformed into large, flattened Schwannlike cells. Whereas it is currently unknown which genetic and epigenetic controls determine the particular path and characteristics chosen on assuming the differentiated state, these investigations emphasize the fact that neuroblastoma cells represent an early stage in neuronal development where the cells are pluripotent, and retain the capabilities for expressing multiple neural crest-derived phenotypes. This knowledge makes clear the potential risks in evaluating agents that induce differentiation if one only assesses neuronal maturation and ignores the possibility that the cell may be committed to a Schwann cell or melanocytic pathway.

Oncogenes

Recent progress in the understanding of cancer has focused on the role of cellular genes, termed "protooncogenes" or oncogenes, that can induce tumor growth in humans. The principal ways by which oncogenic activation takes place are point mutations, chromosomal activation and oncogene amplification (25-27). Sporadic amplifications of the oncogene *c-myc* were first noted in the promyelocytic cell line HL-60 (28) and in colon carcinoma cells containing double minute chromatin bodies (DMs) and long, nonbanding homogeneously staining regions of chromosomes (HSRs) (29). These findings prompted a

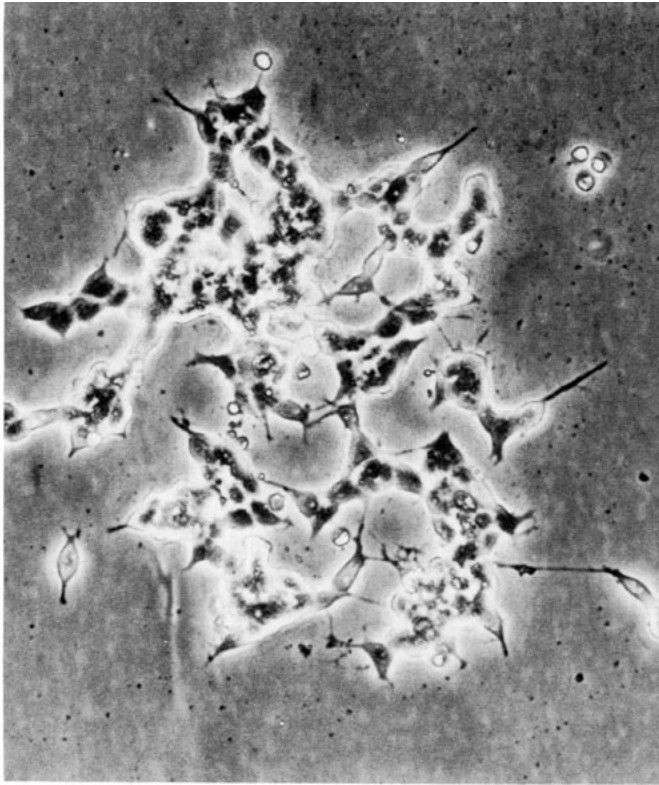
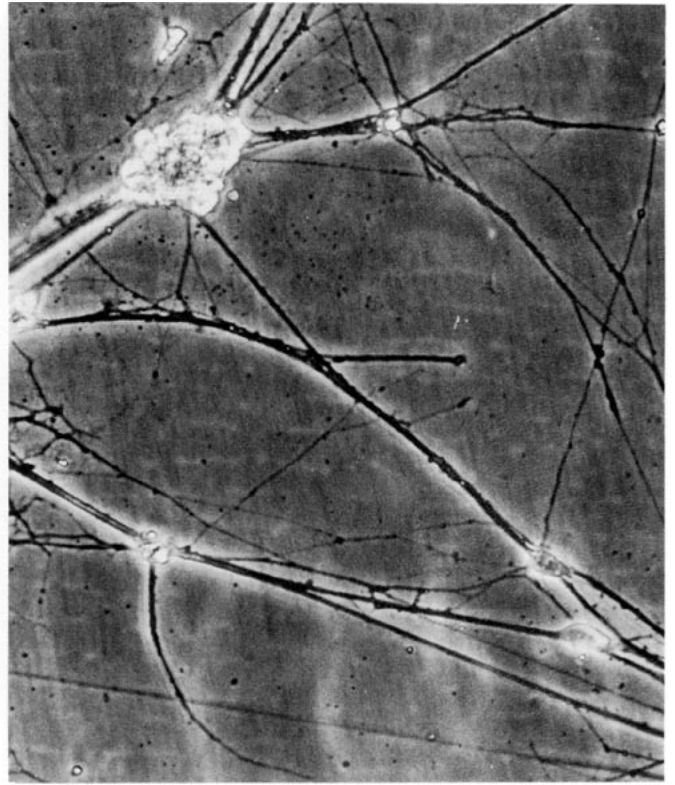
**A****B****C**

FIGURE 1. Differentiation of human neuroblastoma cells by retinoic acid. (A) Solvent-treated control culture of LA-N-5 human neuroblastoma cells. Very few neurites have extended from these loose aggregates of cells. (B) Scanning electron micrograph of 16-day retinoic acid-treated LA-N-5 cells showing the bundling patterns of individual nerve fibers, $\times 8000$. (C) Cells cultured in the presence of 3×10^{-6} M retinoic acid for 16 days. Dense cellular aggregates have formed and many neurites have grouped to form thick fascicles, $\times 250$.

search for nonsporadic amplifications in NB, as DMs and HSRs have both been described in primary NB tumors and cultured cell lines (30,31). This exploration resulted in the discovery of multiple copies of a gene with partial homology to the *c-myc* oncogene localized by *in situ* hybridization techniques to the DMs and HSRs of NB cells (27). The expression of this gene, called *N-myc*, has been detected in only a small number of cancer cell types including NB, retinoblastoma (32), small-cell lung carcinomas (33), and embryonal carcinoma cell lines (34). In addition, appreciable *N-myc* mRNA elevation has been shown in fetal neural cells with a reduced expression occurring during cellular differentiation (35).

Unlike the *ras* oncogenes, no mutations are required for *N-myc* activation, thus focusing attention on quantitative differences in expression between tumor and normal cells (26,27). Several lines of evidence suggest that *N-myc* amplification and expression are related to the malignant potential of NB tumors and to cell growth *in vitro*. Amplification is correlated with advanced pathologic staging of the disease, a predominance of undifferentiated tumor histology, and an enhanced capacity for the cells to form continuously growing cell lines (36-38). There is also a positive correlation between genomic amplification and tumor progression that is independent of the extent of initial disease (36-38). The amplified *N-myc* copy number is consistent within a tumor, at different tumor sites, and at different times during the course of the patient's disease (38). In addition, the majority of NB cell lines contain amplified copies of *N-myc*, although a few, e.g., the SK-N-SH line, do not. The latter line contains a mutationally activated dominant gene, *N-ras*, which is not specific for NB and is found amplified in some other tumors (39,40). The role of *N-myc* as a protooncogene capable of inflicting cell transformation without the need to cooperate with other oncogenes has been demonstrated by Small et al. (41) who have shown that it can act alone to elicit neoplastic growth

of an established line of rat fibroblasts. Since the primary function of the *myc* protein itself is not known, it remains unclear as to how elevations in *N-myc* expression enhance tumor cell transformation and growth.

It has been shown in several NB cell lines that differentiation results in a reduction in cellular *N-myc* mRNA content with no change in genome amplification (42,43). In the NB cell line SMS-KCNR, decreased *N-myc* expression was detected within 6 hr of treatment with retinoic acid (RA) and preceded both cell-cycle changes and morphological differentiation (43). This down-regulation of *N-myc* expression could result from decreased transcription or from changes in the turnover of mRNA; a choice between these alternatives in NB is currently not possible. However, in teratocarcinoma cells, treatment with retinoic acid results in a 50% reduction in the level of *c-myc* mRNA within 3 hr after treatment (44) possibly as a result of posttranscriptional regulation mechanisms affecting the short half-life of the *c-myc* transcript (45). Recently, rabbit antisera has been produced that specifically recognizes a 62 to 64 kD fragment of the *N-myc* protein product (46). As seen in Figure 2, nuclei of untreated LA-N-5 neuroblastoma cells strongly react with the anti-*N-myc* sera while those of RA-treated LA-N-5 cells show little, if any, reactivity. Using this antisera, we have observed reduced staining of LA-N-5 cells after only 4 to 6 hr treatment with RA, which is consistent with similar rapid decreases in *N-myc* mRNA. These results demonstrate that expression of oncogenes in NB can be modified by differentiating agents and oncogene regulation can occur prior to morphological modifications.

The recent work by Thiele et al. (47), as well as the work in our laboratory (48) has permitted detection of transcripts of a variety of other well-characterized oncogenes. As seen in Table 1, the majority of those oncogenes detected show altered expression during RA-induced differentiation. Some of these (*N-myc*, *c-myb*, *H-ras*, *c-erb*)

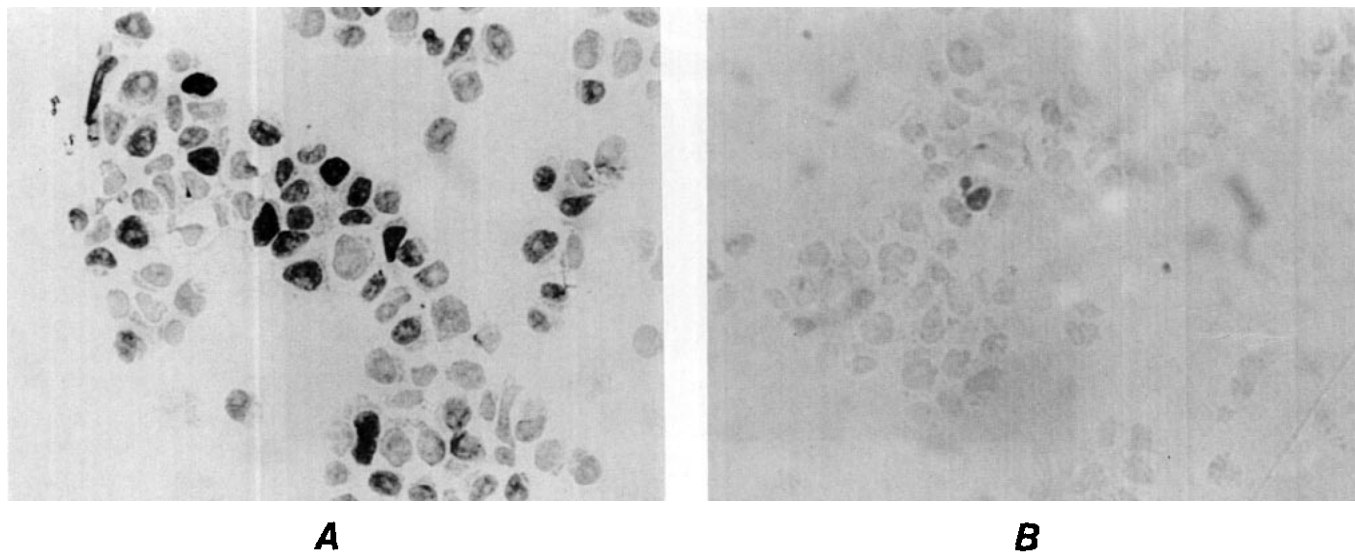


FIGURE 2. Immunoperoxidase staining of LA-N-5 neuroblastoma cells with antibody against the *N-myc* protein product. Cells were grown in the absence (A) or presence (B) of retinoic acid (2×10^{-6} M) for 3 days, then prepared and stained according to the method of Slamon et al. (46). Cells were not counterstained; therefore, all staining was due to the reaction between the primary antiserum and *N-myc* proteins.

Table 1. Effects of retinoic acid-induced different on oncogene mRNA levels in human neuroblastoma cell lines.^a

Oncogene	mRNA level	Time
<i>N-myc</i>	↓	3 hr ^b , 2-4 days ^c
<i>c-myb</i>	↓	3 hr, 2-4 days
<i>H-ras</i>	↓	3 hr, 2-4 days
<i>N-ras, K-ras</i>	NC ^d	
<i>c-fos</i>	↑	48 hr, 8 days
<i>cets-1</i>	↑	48 hr, 4 days
<i>c-erb</i>	↑↓	0.25 hr, 4 days ^e
p53	↓	48 hr, 6-7 days
<i>c-myc, c-cis</i>	ND ^f	

^aSMS-KCNR and LA-N-5 cell lines were used in these studies. Data for p53 are from Sidell and Koeffler (48). All other results are from Thiele and Israel (unpublished data).

^bTreatment time to detect initial changes in mRNA levels.

^cTreatment time for showing maximal changes in mRNA levels.

^dNC, no change.

^eInitial increase, then not detected at 4 days.

^fND, not detected.

show changes that temporally precede any detectable morphologic or biochemical changes and may therefore play a primary role in the differentiation process. In summary, it appears that oncogenes play an important role in the growth and malignant potential of NB cells, and some are regulated during differentiation. With the availability of antisera to oncogene-encoded proteins (46), the potential for monitoring early events associated with differentiation and for determining the role of the proteins in this process will become feasible.

Macara (49) has proposed that certain oncogenes may be regulated by alterations in cellular ionic signals (e.g., Na, H, Ca), which in turn induce further changes in oncogene expression and cell replication and differentiation. This coupling of changes in cellular ionic milieu with alterations in oncogenes and differentiation has been borne out in some cell systems using various agents. Morgan and Curran (50) described induction of the oncogene *c-fos* in the pheochromocytoma cell line PC12 with agents that affect voltage-dependent Ca channels. In the leukemic cell line HL-60, and in murine erythroleukemia cells (MEL), alterations in Na-H exchange, increases in voltage-activated K current, and changes in intracellular Ca concentrations have been observed accompanying chemically induced cell maturation (51-55). In murine NB cell lines, the K ionophore valinomycin (55) and high levels of extracellular Ca (56) have been shown to cause neurite extension. Hence, although highly speculative at present, it is plausible that induced differentiation in NB may in part be triggered by perturbations in the ionic environment. This possibility is more fully explored below in discussing the mechanism(s) of action of retinoids on NB.

Retinoic Acid and Derivatives

Retinoids are a class of natural and synthetic analogues of retinol that have long been known to have potent effects on cell proliferation and differentiation. In 1925, Wollbach and Howe (57) noted that vitamin A deficiency in the rat resulted in a failure of stem cells to properly differentiate into mature epithelial cells accompanied by an abnor-

mal differentiation of the preexisting epithelial cells. Since then, an extensive body of literature has grown documenting the *in vivo* and *in vitro* effects of retinoids in suppressing the development of the malignant phenotype (58-60). These effects are visible after short-term culture with nanomolar concentrations of retinoids. Since the physiological serum concentration of vitamin A metabolites are in the nanomolar range, and micromolar levels can be achieved pharmacologically, these effects are of biological significance.

As a group, human NB cell lines are extremely sensitive to retinoic acid (RA)-induced growth inhibition and phenotypic changes (24,61-63). However, despite an abundance of work describing the morphologic changes induced by RA in NB, there is a dearth of mechanistic detail. Although RA can inhibit the growth, reduce colony formation in soft agar, and induce morphologic differentiation of a host of human neuroblastoma cell lines, a neuronal-like differentiation effect is not universal. For example, RA treatment of a subline of SK-N-SH does not cause neurite extension, but instead yields large fibroblastic epithelial-like cells (24,62). This effect may in part reflect the fact that this cell line contains some cells capable of expressing a melanocytic phenotype (22,23,64). Indeed, the differentiation of neuroblastoma cells within a single line need not always result in neurite sprouting since many lines are composed of heterogeneous cell elements with different phenotypic potential (21,22,65).

In order to first assess the potential clinical applications and limitations of RA treatment of NB in humans, a nude mouse model system was established by the present authors. First, we tested the effects of *in vitro* treatment with RA on the tumorigenic potential of NB cells as judged by measurement of gross tumor formation. As shown in Figure 3, there was a marked reduction in the number of tumors in mice injected with LA-N-5 cells that had previously been treated *in vitro* with RA. In those tumors that did develop, no major differences were noted in the average size between the two groups nor in the

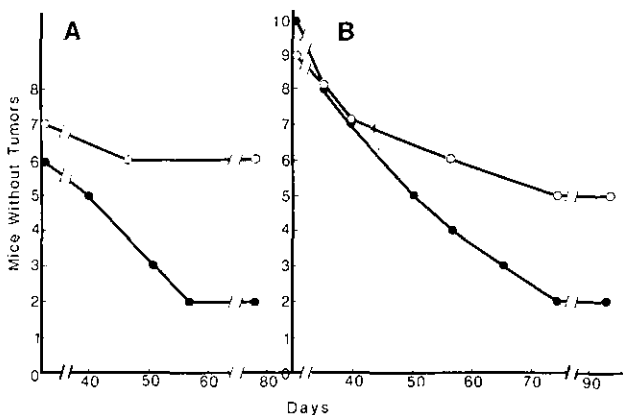


FIGURE 3. Tumor development in nude mice after injection of *in vitro*-treated LA-N-5 neuroblastoma cells in two separate experiments (A,B). Cells (10^7) were injected (SC) after being cultured in the absence (●) or continuous presence (○) of 4×10^{-6} M RA for 18 days. Tumor development was recorded on the day that a nodule could first be definitively detected (~ 3 -5 mm diameter).

histologic appearance of the tumors. The establishment of tumors in some mice receiving RA-treated cells may have resulted from the outgrowth of a subpopulation of cells resistant to RA, a phenomenon that has previously been described *in vitro* (65).

In a different series of experiments, long-term *in vivo* treatment of mice with RA at doses achieving serum levels of $1-2 \times 10^{-6}$ M resulted in a reduction in the percentage of mice that developed tumors as compared to solvent-treated controls (Fig. 4). In a similar vein, RA treatment of mice with established neoplasms substantially reduced the growth rate of the tumors (Fig. 5). Indeed, while the tumors continued to progressively grow over the entire treatment period in the control group, there was little growth in tumor size in the RA-treated group after the first 2 weeks of dosing. Histological examination of selected specimens by H & E and Bielschowsky staining revealed no major morphologic differences in tumors obtained from the two treatment groups although, as was expected from their slower growth rate, a reduced number of mitotic figures was apparent in tumors obtained from RA-treated animals. Thus, although systemic administration of RA resulted in a plateauing of tumor growth, there was no obvious morphologic differentiation as evidenced by neurite formation similar to that observed with *in vitro*-treated cells. This finding does not exclude the possibility that other, more subtle changes are occurring that are consistent with neuronal differentiation, such as a reduction in *N-myc* gene expression (Fig. 2). Taken together, these data indicate that RA has a significant role in diminishing the malignant phenotype of *in vitro*-treated NB tumors, or as a biological modifier once the tumor has been implanted.

Cellular Retinoid-Binding Proteins

Because of its lipophilic nature, the transport of retinol and RA requires specific binding proteins. Two of these proteins, cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP) are ubiquitous, not tissue specific, and are located in the cytosol of cells (60), although plasma membrane-bound CRABP has also been described (66). By analogy to steroid hormones, it has been proposed (67) that the retinoid binding proteins translocate retinoids to the cell nucleus where they exert an effect on chromatin and gene expression. Although current evidence supports the view that retinoids are capable of being transported to the nucleus via CRBP and CRABP (67), direct evidence for the role of these proteins in exclusively mediating the actions of retinoids is lacking. For example, in embryonal carcinoma cell lines, CRABP appears to be necessary but not sufficient for the induction of differentiation (68,69). Matthaie et al. (70) have reported that cells from several human embryonal carcinoma lines possess high levels of CRABP but fail to undergo differentiation in the presence of RA. Conversely, in the HL-60 cell line no binding activity has been detected, although the cells are very sensitive to differentiation by RA (71).

Haussler et al. (72) have shown that there is a lack of

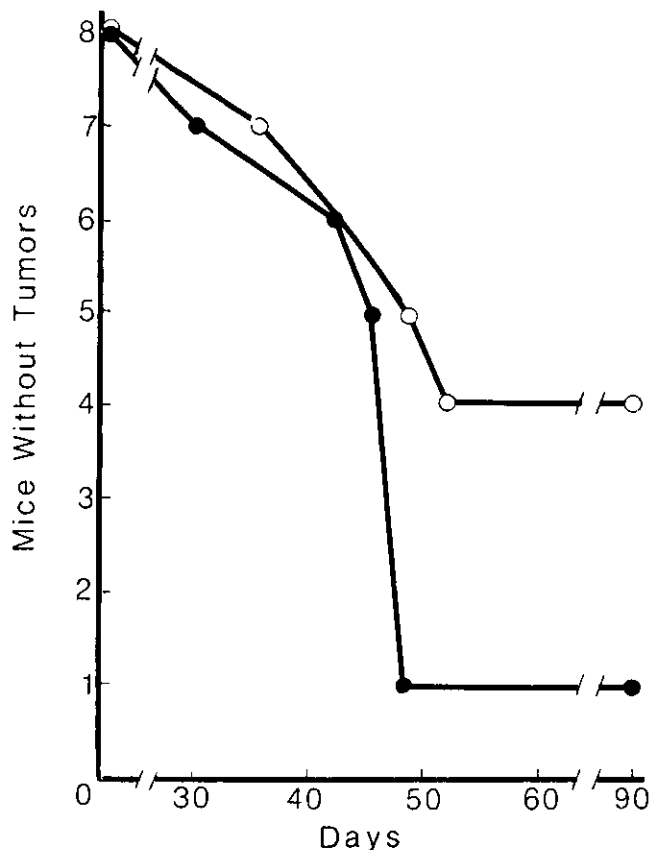


FIGURE 4. Development of LA-N-5 tumors in *in vivo*-treated nude mice. Four- to six-week-old nude mice were dosed daily with 0.1 mL of 4×10^{-3} M RA PO (in corn oil) (○) or solvent control (●) starting 5 days before injection (SC) of 10^7 LA-N-5 cells and continuing for 14 days after injection of the cells. The mice were examined twice weekly for tumor development, which was recorded on the day that a palpable nodule could first be detected ($\sim 3-5$ mm in diameter).

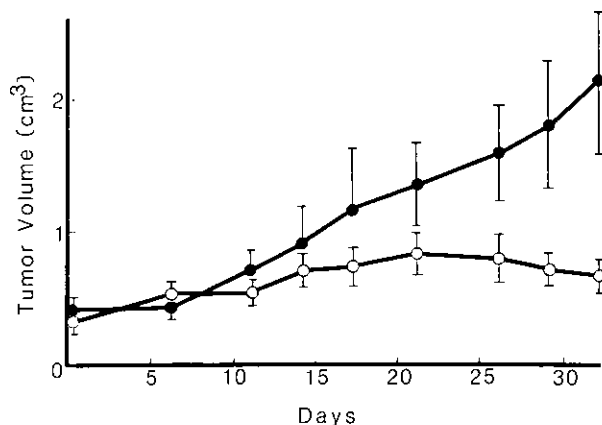


FIGURE 5. Growth of established LA-N-5 tumors in *in vivo*-treated nude mice. Mice were treated daily for 32 days with either 0.1 mL of 4×10^{-3} M RA PO (in corn oil) or solvent control after development of roughly 1-cm diameter nodules. The tumors were measured once or twice a week. The points represent the mean tumor volume (\pm SE) measured in the control (●) (5 mice) and RA-treated groups (○) (6 mice).

strict correlation between RA-induced anchorage-dependent growth and CRABP levels in human NB cell lines. Correspondingly, there is a variable relationship between retinol-induced cell differentiation and levels of assayable CRBP activity (73). In a further effort to ascertain the role of these binding proteins in the mechanism of action of retinoids, Jetten et al. (74) compared the biological activity of several newly synthesized benzoic acid derivatives with their binding capacity to CRABP. Whereas some of the compounds exhibited high biological activity, they did not bind to CRABP. From these and other data, it appears unlikely that retinoid-induced cell differentiation is brought about solely by the interactions of the binding protein-retinoid complex with nuclear chromatin.

RA and Protein Kinase Activity

The role of protein phosphorylation in modulating cell growth and differentiation has been actively investigated, particularly with reference to changes in the cAMP-dependent kinase system, the Ca-dependent protein kinase C (PKC), and the oncogene-derived protein kinases (75-77). Consequently, interest in the actions of RA on these systems has grown.

Plet et al. (78) have shown that RA treatment of differentiation-responsive embryonal carcinoma cells caused an increase in the cytosolic and plasma membrane-bound cAMP-dependent kinases with a preferential increase in the RII regulatory subunit associated with the membrane fraction. It should be noted, however, that some investigators were unable to demonstrate changes in cAMP-dependent protein kinase activity upon treating embryonal carcinoma cells with RA (69).

Retinoid treatment has also been reported to enhance protein kinase activity in HL-60 and melanoma cells (79,80). In HL-60 cells and in the myeloblast cell line RFD, there is also a direct correlation between the degree of cell differentiation and elevation in cAMP-dependent protein kinase activity, as well as an increase in the phosphorylation of various specific substrates of this enzyme (81). To our knowledge, studies directly assaying changes in cAMP-dependent protein kinase in RA-treated NB cell lines have not yet been reported. Given the responsiveness of these lines to RA and cAMP and the availability of subclones that are relatively resistant to these agents (65), it would be of interest to study the role that this kinase system plays in the differentiation phenomenon. From studies in other cell systems, it would be fair to conclude that retinoids may exert some of their effects in NB by modulation of cAMP-dependent protein kinase activity or enzyme levels.

In addition to the cAMP-dependent kinase, the Ca-dependent PKC has been shown to be important in the differentiation process. This membrane-bound kinase, originally identified in brain (82) is widely distributed in cells, appears to be the target for the tumor promoting phorbol esters, and is important in the control of signal transduction and tumor differentiation (83). In myeloid precursors, there is a stimulation of PKC enzyme activ-

ity and phosphorylation of its various substrates correlating with RA-induced differentiation (81) occurring prior to the commitment of the cells to the myeloid pathway (84). In contrast, embryonal carcinoma cells exhibit an increase in PKC activity that accompanies but does not precede differentiation (85), suggesting that in the latter system, these effects may be a consequence rather than a cause of differentiation. In other tissues, depending on the assay conditions, retinoids have been reported to have a stimulatory and an inhibitory effect (86), solely an inhibitory effect (87), or no effect at all (88) on the Ca-dependent kinase system. These disparate observations preclude any facile inferences as to how RA may act on PKC in NB and whether this has any primary effect on NB differentiation.

RA Effects on Ion Channels

Since changes in cation transport across the plasma membrane have been implicated as controlling factors in the regulation of cell growth and differentiation (*vide supra*), we have been interested in the prospect that RA-induced differentiation of NB cells is partly triggered by alteration of ion channel activity in the plasma membrane. It was first demonstrated that RA can alter membrane ion-channel activity in mammalian cells by showing that it blocks K channels in human lymphocytes and that this activity can account for some of its immunomodulating effects (89,90). Because the K channels in these cells are unusual in that they are sensitive to both K channel blockers and to some classical Ca channel blockers (91), we have further investigated the specificity of RA blockage in other cell types. As seen in Figure 6, RA blocks T-type Ca channels in a mouse hybridoma cell line (92,93) but does not affect the K channels in human neuroblastoma cells (which are insensitive to Ca-channel blockers). Thus, the effects of RA on human lymphocyte K channels may in part be the result of an interaction with components that are shared with Ca channels, as reflected in the sensitivity of these K channels to blockage by Ca channel blockers. In the mouse hybridoma cells, the dose-dependent effects of RA on Ca channels correlate with the ability of this compound to inhibit cell proliferation,

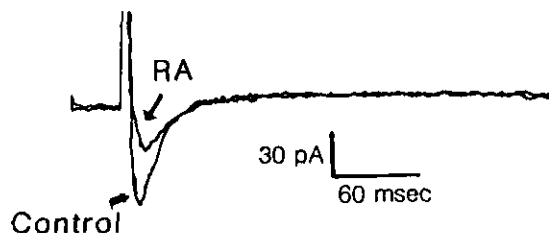


FIGURE 6. Retinoic acid reduces the inward Ca current in MHY206 murine hybridoma cells. Using the whole-cell patch clamp technique (146), currents were measured during a voltage pulse from a holding potential of -80mV to -20mV . The figure shows the Ca current through T-type Ca channels (147) recorded before (control) or 5 min after $5 \times 10^{-5}\text{ M}$ RA had been added to the bath solution. The channel block by RA was reversible such that after washout, the currents were very close to control values (not shown).

Table 2. Blockage of ion channels and biological action of retinoic acid on different cell types.

Cells	Channel type studied	Dose (M) for 50% maximum effect	
		Channel block	Biological response ^a
T-lymphocytes (human)	K	2.5×10^{-5}	3×10^{-5}
Natural killer (human)	K	2.5×10^{-5}	5×10^{-5}
206 (mouse B-cell hybridoma)	Ca (T-type)	5×10^{-5}	3×10^{-5}
GH ₃ (rat pituitary cells)	Ca (T-type)	No ^b	No
	Ca (L-type)	No	
LA-N-5 (human neuroblastoma)	K	No	
	Ca (??) ^c	$<10^{-6}$	10^{-7}

^aEffect of RA on T-lymphocytes (89) and 206 cells (92) is antiproliferative at the doses shown; its effect on natural killer cells is to inhibit killing (89); on LA-N-5 cells RA induces differentiation (62).

^b 10^{-4} M RA was the highest concentration tested and showed no effects.

^cThe exact ion selectivity and dose range for channel block have not yet been characterized.

suggesting that blockage of the channels contributes to its antiproliferative activity (92). In contrast to the notion that RA's effects on Ca channels are nonspecific is our unpublished observation that RA has no effect on Ca channels in the rat pituitary cell line GH₃ (which contains both T-type Ca channels like those in the hybridoma cells and L-type Ca channels) (94) and does not inhibit the proliferation of these cells. Thus, the ability of RA to modulate ion-channel activity, and perhaps to ultimately induce a biological response, may in part be dependent not only upon channel type but also on the presence of specific RA-sensitive receptors or components that can influence channel function. As seen in Table 2, preliminary experiments suggest that RA can also block Ca currents in neuroblastoma cells, and at lower concentrations ($<10^{-6}$ M) than seen in the other cells ($1-5 \times 10^{-5}$ M). This parallels the substantially greater sensitivity of neuroblastoma cells to the biological action of RA.

Although it is difficult at this point to hypothesize how blocking Ca channels by RA might contribute to the differentiation of neuroblastoma cells, recently acquired knowledge of the second messenger functions of Ca could serve as a point of departure for future studies. For example, PKC activity is regulated both by the amount of activated C-kinase bound to the plasma membrane and by the rate of Ca cycling across this membrane (77). Thus, an RA-induced block of Ca influx through Ca channels could theoretically lead to an inhibition of C-kinase activity with subsequent modification of a variety of cellular process dependent on this phosphorylating enzyme. This hypothesis is consistent with results in some other systems where RA has been shown to decrease C-kinase activity (87) albeit not universally (86,88). Another possible consequence of blocking Ca channels with RA that could involve the phosphatidylinositol-mediated second messenger system is inhibiting a membrane depolarization that is necessary during cell growth (95). Clarification of a general role for ion-channel modulation in the biological action of RA will require future characterization and correlation of these activities in a variety of cell systems.

parts of sympathetic neuroblasts, then nerve growth factor (NGF) would be regarded as a likely triggering agent for differentiation (96,97). With little experimental data, this rationale prompted an early clinical trial in children with metastatic neuroblastoma where NGF was given to promote tumor cell maturation and retard malignant growth; disappointingly, the clinical course was not affected (98).

In brief, NGF is a basic polypeptide dimer that binds to cell surface receptors in a specific and saturable fashion. It can be isolated from a variety of endocrine and exocrine sources, and the presence of NGF receptors has been demonstrated in several, but not all, human NB lines (96,97,99,100). Within hours following NGF treatment of some sensitive NB cells there is an increase in amino acid uptake, protein synthesis, and neurite extension (99,101). For example, after 5 days of treating a cloned subline of SK-N-SH designated by SY5Y, there is a 5-fold increase in the rate of protein synthesis and extensive neurite outgrowth (101,102). These neurites are capable of firing action potentials as judged by electrophysiologic and pharmacologic criteria, whereas the undifferentiated counterparts are not (103,104). In addition, the intracellular stores of monoamines and levels of neuron-specific enolase increase, with associated increases in plasma membrane resting potentials (105). Some human NB cell lines have also been reported to respond to NGF with an accelerated rate of attachment to culture dishes (106). Despite these observations, responses to the effects of NGF in many NB lines can only be considered to be feeble at best (i.e., minimal neurite outgrowth with sparse changes in growth rate) (10,100).

From studies in other cell systems, it appears that NGF's biological actions are not triggered exclusively by interactions with the cell surface receptor. Indeed, it has been shown that NGF or NGF and its internalized receptor complex can interact with target cells at the level of the cell membrane, cytoplasm, and nucleus, suggesting that more than one type of receptor is involved in mediating its actions (96). Consistent with this concept is the observation that in the cultured pheochromocytoma cell line PC12, which undergoes neuronlike differentiation, NGF-stimulated neurite outgrowth requires both transcription-dependent and independent pathways (107). As suggested by Green and Shooter (96), it is possible that transcription-dependent actions require internalization

Other Differentiating Agents

Nerve Growth Factor

If NB cells are considered to be the neoplastic counter-

and interaction with nuclear receptors, whereas transcription independent actions are triggered via cell-surface receptors and second messengers.

Cyclic AMP (cAMP) is a putative second messenger for a host of hormones and has been implicated as mediating some of the actions of NGF. However, the evidence for cAMP acting as the sole second messenger is conflicting (96). From studies on PC12 lines using the adenylate cyclase activator forskolin and chemically modified cAMP analogues, it appears that NGF can act through cAMP-independent pathways and that cAMP activation of protein kinases is neither necessary nor sufficient for NGF-induced neurite formation (108). It has also been hypothesized that NGF might exert its effects by primary regulation of ion fluxes. In this connection, Boonstra et al. (109) have shown that within minutes of treating PC12 cells with NGF, there is stimulation of an amiloride-sensitive electroneutral Na influx system. However, prolonged incubation of cells with amiloride failed to block NGF-induced cellular differentiation. More recently it has been shown that NGF induces electrical excitability in PC12 cells by augmentation of the membrane density of Na channels, presumably by increasing the transcription of Na channel genes (110,111). Although it could be speculated that this action is a secondary effect of the differentiation process, it has recently been shown that electrical excitability can, by itself, influence neurite outgrowth (112). As such, the causal relationship between NGF-induced changes in ion fluxes and neuronal differentiation remains to be elucidated.

Butyric Acid

The natural four-carbon fatty acid, butyric acid, has been studied as an inducer of differentiation in several systems. For example, butyric acid treatment has been reported to increase the production of human gonadotropin in HeLa and human lung carcinoma cells (113,114) and human myelocytic leukemia cells cultured with this agent have been shown to differentiate along the granulocytic pathway (115). Prasad and co-workers (116,117) have reported that butyrate at a concentration of 0.5 mM induced both cell death and some neurite production in human NB cell lines, whereas it reversibly inhibited proliferation without causing differentiation in murine NB cells. In a neural crest-derived human retinoblastoma cell line, butyrate treatment has been reported to result in growth inhibition as well as a flatter-appearing morphology (118). The exact mechanism of action responsible for the effects of Na butyrate are unknown, although it is thought to involve a direct action on the cell genome. In HeLa cells, butyrate treatment results in an increase in the acetylation of histones H3 and H4 through an inhibition of the deacetylating enzyme. The consequence of an increase in acetylated histones includes alterations in chromatin structure and function (119). In murine NB cells, butyrates affect the synthesis and turnover of histone H1 subtypes with an enrichment of subfraction H1^o (120). The significance of this observation is underscored by reports that the state of histone acetylation can be cor-

related with the transcriptional activity of certain genes (121).

Polar-Planar Compounds

Low molecular weight polar-planar compounds such as dimethyl sulfoxide (DMSO) and hexamethylene bisacetamide (HMBA) constitute a class of agents that has been reported to induce differentiation of various cell lines *in vitro*, including NB (122). HMBA induces differentiation in several leukemic lines, solid tumor lines, and some NB cell lines (122-126). The addition of 2% DMSO to cultures of the human NB cell line LAN-1 and the murine cell line C-1300 resulted in morphologic differentiation within 36 hr following addition of the agent and was accompanied by a marked inhibition of cell multiplication and colony growth (127). Interestingly, prior *in vitro* treatment of the murine cells with DMSO delayed but did not prevent tumor formation in nude mice and did not significantly change animal survival. Similarly, there was no significant effect on tumor growth or animal survival if DMSO was given *in vivo* following the induction of tumors. How these agents act in inducing differentiation in NB is subject to speculation. However, from work in other cell systems there is compelling evidence to suggest that DMSO and HMBA cause a significant decrease in the levels of inositol triphosphate and diacylglycerol (DAG) within a short time of addition to culture (128). Since DAG activates PKC (129), this implies that the effects of DMSO and HMBA may be brought about by perturbations in cell membrane structure which in turn can inhibit PKC. Indeed, in leukemic cell lines, DAG inhibits DMSO-induced differentiation (130). However, inhibition of PKC alone cannot be the sole initiator of differentiation since the phorbol diester 12-O-tetradecanoyl-phorbol-13-acetate, which activates PKC (131), can also induce morphologic and biochemical differentiation in some cells, including NB (132-135). Further explorations in the actions of these compounds on PKC activity in NB would be of great interest.

Cyclic AMP System

The rapid modulation of cAMP levels makes this second messenger a prime candidate for influencing cellular processes involving maturation and differentiation. That cAMP was involved in cell growth and differentiation became clear beginning in 1971 when two simultaneous reports of studies with cultured mammalian cells demonstrated an effect of this substance on inducing phenotypic maturation (136,137). Since then, substantial experimental evidence has been accumulated implicating alterations in cyclic nucleotide regulatory mechanisms as being important in the process of differentiation (76). With some human neuroblastoma cell lines, an increase in neurite extension and reduced growth rate can be demonstrated when cAMP derivatives, agents that activate adenylate cyclase or inhibit phosphodiesterase, are administered singly or in combination (138,139). These observations, along with dramatic phenotypic changes seen with murine

neuroblastoma cells (138), have prompted limited application of cAMP-elevating agents for the treatment of this disease (140,141). In some reports, apparent beneficial results were claimed as a result of promoting *in vivo* maturation of cancer cells (140,141). However, these cases lacked objective standards of evaluation and to date no rigorous trials have been published.

Cyclic AMP is thought to affect changes in mammalian cells through the activation of one or both cAMP-dependent protein kinases (142). Two forms of the kinases, referred to as type I and II, have been identified and are composed of the same catalytic subunit with two different regulatory subunits (RI and RII) (143). Activation of the kinase system occurs when cAMP binds to the RI or RII subunit with release of the catalytic subunit, resulting in the phosphorylation of various proteins (143). Omitted from this highly simplified depiction are a host of interactions between the cAMP system, other kinases, and Ca (77). Although the role of cAMP in regulating cell cytosolic pathways such as glycogenolysis have been well described (77), the mechanisms underlying its effects on cell differentiation remain obscure. Recent investigations in cell lines other than NB have focused on interactions between cAMP and oncogenes. Elevations in cAMP levels in HL-60 result in rapid (3 hr) decreases in *c-myc* RNA levels preceding detectable changes in proliferation and differentiation (144,145). In the NB cell line SMS-KAN, a significant decrease in *N-myc* expression has been observed following maximal morphologic differentiation induced by dibutyryl cAMP (43). Hence, cAMP may induce differentiation through a kinase-mediated modulation of oncogene expression.

Conclusion

As a malignant tumor of childhood, neuroblastoma has proven to be fascinating from a biological as well as clinical viewpoint. Several agents have been reported to induce *in vitro* differentiation of NB into a variety of cells sharing a neural-crest lineage. Treatment with retinoids has been shown to inhibit *in vitro* cell proliferation and retard tumor growth in nude mice, suggesting that these agents may prove useful in the treatment of metastatic, refractory disease.

The biochemical and ionic changes necessary for the induction of differentiation in NB cells remains subject to investigation. Aside from relying on morphologic alterations, early markers of differentiation are being sought; monitoring changes in oncogene products may serve such a purpose. In so doing, the initial events triggering cell maturation may be more amenable to study. In this connection, modulation of ion channel function by a variety of agents, which in turn can modulate gene expression, promises to be a fruitful area to explore in NB. The study of NB promises to open new vistas in the analysis of tumor growth and differentiation and may provide clinicians with potent chemotherapeutic agents capable of altering the basic biology of neoplasms.

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