In Vitro Activation of Distinct Molecular and Cellular Phenotypes after Induction of Differentiation in a Human Neuroblastoma Cell Line

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ABSTRACT

In this report we provide evidence for the activation of distinct differentiation pathways during treatment of the neuroblastoma cell line SMS-KCNR with 1 μm dibutyryl cyclic AMP (dbcAMP) and/or 5 μM retinoic acid (RA). Our results show that the adrenal gland specific gene pG2 is induced only during dbcAMP treatment, while RA induces a neuronal phenotype and expression of all neuronal related genes while decreasing the expression of many chromaffin related genes. Furthermore, dbcAMP does not affect the DNA content distribution of SMS-KCNR cells [G1 = 61.8 ± 4.1% (SD); S = 20.3 ± 6.3%; G2-M = 18 ± 5.4%] despite morphological and molecular signs of cellular differentiation. Conversely, RA arrests cell growth causing a decrease in cells in the growth fraction (S + G2 + M = 15.6 ± 6.1%) and an increase in cells in G1 (G1 = 84.3 ± 5%). Using cyclic AMP and RA in combination, we found that RA inhibited expression of adrenal gland specific gene pG2 and induced a neuronal phenotype. Since dbcAMP does not cause a significant G1 block in SMS-KCNR cells we propose that this agent may be able to induce SMS-KCNR only to an intermediate stage of chromaffin differentiation in which cells retain their proliferative potential.

INTRODUCTION

Cellular differentiation depends on specific environmental signals that alter intracellular gene expression affecting cell metabolism and growth. Recent studies have identified genes capable of directing cells along tissue specific differentiation pathways. The discovery of one of these genes, MYOD, that induces muscle gene expression in undifferentiated myoblasts and fibroblasts (1) suggests that a mature phenotype may be dependent on the function of lineage specific genes in other tissues as well. Conversely, tumor cells evolve toward an undifferentiated, highly proliferative, and invasive cell phenotype regardless of environmental regulatory signals. Numerous cancer causing genes, oncogenes, have been identified that are altered forms of genes involved in normal signal transduction pathways (2) that regulate cell growth and differentiation. Despite genetic alterations, treatment of some tumor cell lines and tumors with biological response modifiers reverts the malignant phenotype and induces cell maturation suggesting that alternative growth control and differentiation signal transduction pathways may be functional (3–6).

NB3 presumably arises from NC precursors that are altered during the course of normal development. Multipotent NC cells give rise principally to cells of the peripheral nervous system, chromaffin and nonchromaffin adrenal medullary cells, and melanocytes (7). Recent studies indicate that many NB cell lines and tumors retain some histopathological features of their normal NC progenitors (8, 9). In particular the expression of a panel of markers in neuroblastoma cells seems to recapitulate normal adrenal medulla histogenesis (9). Cooper et al. (10, 11) have identified, in fact, that the expression of markers such as tyrosine hydroxylase (12), CGA (13), β2-microglobulin (14), and a gene with an unknown function, pG2 (15), are developmentally regulated in the human fetal adrenal gland. Studies on the distribution of these markers in NB enabled NB cell lines and tumors to be classified into groups (I, II, and III) according to their apparent degree of chromaffin maturation (10, 11).

In the present study we investigated the changes in lineage related gene expression induced in a NB cell line that has an immature chromaffin phenotype (group I) (10) by treating it with dibutyryl cAMP, RA, or the combination of these agents. We assessed the effects of these agents on cell proliferation, morphology, and expression of lineage related genes. While most of the molecular markers utilized are expressed in both chromaffin and neuronal tissues, CGA and NPY (16) are typically more highly expressed in chromaffin cells while GAP43 (17) SYN (18), and NF (19) are more highly expressed in neuronal cells. Only the gene pG2 specifically marks adrenal chromaffin cells. By both Northern blot analysis and in situ analysis, pG2 expression has been found predominantly in chromaffin cells of the adrenal gland and specifically is not expressed in nonchromaffin adrenal neuroblasts (9–11) or brain (15). Markers clearly delineating chromaffin and neuronal lineages have not been well described even in animal studies (7). Our study indicates that arrest of cell growth, induction of a neuronal morphology, and increased expression of all neuronal markers were achieved only in RA treated cells. In contrast, only treatment with dbcAMP increased expression of the chromaffin related gene pG2. Concomitant increases in all chromaffin related genes as well as 2 of 3 neuronal markers were detected in cAMP treated cells. However, dbcAMP did not induce a G1 block in cell cycle progression. RA induced a neuronal morphology and inhibited expression of pG2 even in cells treated with cAMP. This study indicates that despite the malignant transformation of the cells, cAMP induces a more mature chromaffin phenotype while RA induces a more neuronal phenotype in SMS-KCNR NB cells.

MATERIALS AND METHODS

Cell Culture. The NB cell line SMS-KCNR (20) was cultured in RPMI 1640 supplemented with 10% fetal calf serum. Exponentially growing cells were seeded at 2 × 10⁶ cells/plate in 150-mm plastic sterile plates. Twenty-four h after being plated, cells were treated with all-trans-RA (5 μM) (Sigma, St. Louis, MO), dbcAMP (1 mM) (Sigma), and the combination of RA and dbcAMP or 1 mM VIP (Peninsula Lab, Belmont, CA). Controls were incubated with appropriate amounts of solvent. Medium was replaced every 48 h.

RNA Extraction and Northern Analysis. Total RNA extraction and Northern analysis were performed as previously described (21). In brief 5–10 × 10⁶ cells were pelleted and frozen at −70°C. Pellets were treated according to the hot phenol method for RNA preparation as previously described. Twenty-five μg of total RNA were run in a 1% formaldehyde-agarose gel at 20 V for 18 h. Ni trad (Schleicher and Schuell, Keene, NH) filters for Northern analysis were made by capillary transfer after

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3 The abbreviations used are: NB, neuroblastoma; NC, neural crest; RA, retinoic acid; cAMP, cyclic AMP; dibutyryl cAMP; CGA, chromogranin A; VIP, vasoactive intestinal peptide; NPY, neuropeptide Y; PKC, protein kinase C; PKA, protein kinase A; NF, neurofilament; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SYN, synaptophysin.
a pretreatment with 50 mM NaOH and 10 mM NaCl as previously reported (21). The following probes were labeled to a specific activity of at least $10^6$ cpm/µg by the nick translation procedure (Amersham, Arlington Heights, IL): NPY (16); SYN (18); M, 68,000; NF (19); GAP43 (17); PG2 (15); CGA (13); and GAPDH. Hybridizations were conducted at 42°C in the presence of 50% formamide for at least 18 h. Values from densitometric analysis of appropriately exposed autoradiograms were normalized to values determined for GAPDH expression utilizing a Bio-Rad 620 densitometer (Bio-Rad, Richmond, CA).

Cell Cycle Analysis. From each sample, $10^6$ cells were stained with propidium iodide according to standard protocols. In brief, cells were washed twice in phosphate buffered saline and resuspended directly in a solution of RNase (5 mg/ml) dissolved in phosphate buffered saline (0.5 ml); after 5 min of incubation on ice 0.5 ml of propidium iodide buffer (0.1 mg/ml propidium iodide-0.1% Triton X-100-1 mM EDTA) was added, and cells were incubated in the dark for 30 min at room temperature. After staining, cells were filtered through 35 µm nylon mesh (Potomac Scientific Supply, Inc., Bethesda, MD). In each sample, $10^6$ events were analyzed on a FACSscan cytometer (Becton Dickinson, San Jose, CA) using the automated computer program Cellfit (Becton Dickinson). DNA content and cell cycle distribution were determined manually.

In Vitro Transcription Assay. Nuclear RUN/ON procedure was performed as previously described (22). In brief, 2 µg of indicated plasmid DNA were spotted onto Nytran membrane (Schleicher and Schuell) using a Manifold II slot blot apparatus (Schleicher and Schuell). For the transcription reaction 2 x $10^6$ nuclei frozen at -70°C were thawed on ice and incubated for 60 min at 26°C with transcription buffer (5 X): containing 50 mM Tris (pH 8); 50 mM MgCl₂; 5 mM dithiothreitol; 175 mM KCl; 5 mM concentrations each of ATP, GTP, and CTP and 500 µCi of [³²P]UTP (specific activity, 3000 Ci/mmol; Dupont, Boston, MA). Radiolabeled RNA was isolated from nuclei and 10⁶ cpm/ml of hybridization buffer containing 50% formamide, 4 x SSPE (1 x SSPE = 0.15 M sodium chloride-0.05 M sodium phosphate-1 mM EDTA), 1 mg/ml salmon sperm, and 1 x Denhardt's solution, were incubated with filter for 48 h at 42°C. Filters were washed in 0.5 x standard saline-citrate (1 x SSC = 0.15 M sodium chloride-0.05 M sodium citrate, pH 7.2) at 55°C. Densitometric analysis was carried out using a Bio-Rad 620 densitometer.

RESULTS

Morphological Differentiation and DNA Content Analysis. To evaluate in vitro morphological differentiation, SMS-KCNR cells were cultured for 2 to 8 days in the presence of 5 µM RA, 1 mM dbcAMP, or both drugs in combination. Concentrations of drugs were chosen which minimized toxic effects to cells. Fig. 1 shows the effect of these treatments on cellular morphology after 2 and 8 days of treatment. Neurite spreading is visible with either dbcAMP (Fig. 1B1) or RA (Fig. 1C) at 2 days. However, by 8 days neurite extension and formation of neuritic bundles are evident only in RA treated culture and not in cAMP treated cultures (Fig. 1C2 and 1B2, respectively). To determine the effects of these agents on the cell cycle distribution we analyzed the DNA content by FACSscan after 8 days of treatment. Cells cultured in the presence of RA demonstrated a G1 block greater than 80% (Fig. 1C3). Conversely, cells incubated with cAMP alone (Fig. 1B3) did not show any significant change in their cell cycle distribution. RA and dbcAMP in combination produced a remarkable morphological differentiation in SMS-KCNR cells apparently increasing the intercellular neurite network. However, G1 enrichment (76.4%) (Fig. 1D3) was reproducibly lower than the G1 arrest induced by RA alone. Results of 3 independent experiments (Table 1) indicated that a significant reduction in S phase was detected only in cultures treated with RA. Sodium butyrate may be a contaminant in dbcAMP preparation and may induce differentiation in some cell lines. However, sodium butyrate is toxic to SMS-KCNR cells.4 To ascertain whether effects noted with dbcAMP would occur with another agent which increased cAMP levels, we treated cells with VIP which is known to raise intracellular cyclic AMP levels (35). After 4 days, 1 µM VIP treated SMS-KCNR cells had a morphology similar to that of dbcAMP treated cells. Cell cycle analysis reveals G1 = 61%, S = 21%, and G2 + M = 18% in VIP treated SMS-KCNR; G1 = 64%, S = 20%, and G2 + M = 16% in dbcAMP treated cells; G1 = 62%, S = 22%, and G2 + M = 16% in control; while G1 = 85%, S = 8%, and G2 + M = 7% in RA treated cells. Conclusively, neither dbcAMP nor VIP was able to arrest cell cycle progression, a hallmark of terminally differentiated cells.

Gene Expression Analysis. The effects of RA and dbcAMP are thought to be mediated by different cellular signaling pathways (2). While both RA and cAMP induce aspects of neuronal morphology in NB cells, the inability of dbcAMP to arrest cells in G1 suggests that a different degree of maturation or lineage may be stimulated in NB cells. The combination of RA and dbcAMP was utilized as previous studies evaluating morphological and growth of NB cells suggest additive or synergistic effects when these agents are used in combination (23). To date, studies have not evaluated changes at a molecular genetic level. For these reasons we evaluated the expression of genes related to neuronal or chromaffin differentiation pathways in RA and dbcAMP treated cells.

Fig. 2 shows that RA was effective in increasing the neural related gene expression but not chromaffin genes (Fig. 2A and B). According to densitometric analysis in which values were normalized to GAPDH levels (Fig. 3), GAP43 mRNA levels increased approximately 2-fold after 8 days of treatment with RA or the combination of RA and dbcAMP but were unaffected by dbcAMP treatment. The GAP43 gene is expressed predominantly on developing neuronal growth cones and may reflect the acquisition of neurosecretory properties (17). NF mRNA was induced by all treatments (RA, 1.5-fold; dbcAMP, 4.5-fold; and RA + dbcAMP, 5.5-fold), and expression of the SYN mRNA was increased 3-fold by RA, 6-fold by dbcAMP treatment, and 4-fold by both. Increased levels of mRNA encoding neuronal markers are detected in cells treated with either RA, cAMP, or the combination of RA and cAMP.

In contrast, when a series of chromaffin related genes were evaluated, RA failed to significantly increase expression of these genes (Fig. 2B). The steady-state levels of CGA and NPY mRNA genes increased 1.6- and 7-fold, respectively, compared to their basal level of expression in dbcAMP treated cells. However, a 9-fold induction of expression of the adrenal gland specific gene pG2 was detected only in dbcAMP treated cells. Furthermore, Fig. 2C illustrates that VIP also stimulates pG2 mRNA expression. The product and function(s) of this gene are unknown but the very restricted pattern of pG2 mRNA expression in the adrenal cortex and medulla and its developmental regulation suggest that it may be an important marker to monitor the activation of specific adrenomedullary differentiation (10, 11, 15). Of interest is the observation that in the presence of RA and dbcAMP, the steady-state levels of pG2 did not increase (Fig. 2B). These observations confirm that SMS-KCNR expresses a relatively immature chromaffin phenotype (group I) (10, 11) and indicate that cAMP increases pG2 expression, thus inducing a transition to a more mature chromaffin phenotype (10, 11).

4 Unpublished data.
Fig. 1. Morphological differentiation and DNA content analysis. Effect of treatment with RA, dbcAMP, or both in combination, on the cell line SMS-KCNR. A, B, C, and D, morphological results obtained with various treatments: A, control; B, dbcAMP; C, RA; D, RA+dbcAMP. A1, B1, C1, and D1, effect of treatment after 48 h of culture. A2, B2, C2, and D2, changes occurring after 8 days of culture. Right, results of the DNA content distribution and analysis determined at 8 days of in vitro culture with the various treatments are shown in the right column: A3, control; B3, dbcAMP; C3, RA; and D3, RA+dbcAMP.

In Vitro Transcription Assay. Studies indicate that during cell differentiation many developmental decisions depend on transcriptional events. The elevated steady-state level of pG2 in dbcAMP (9-fold) but not in RA treated NB cells prompted us to investigate the regulation of this gene at the transcriptional level. Our result (Fig. 4) showed a surprisingly high basal level of pG2 mRNA transcription in control as well as in dbcAMP treated cells. This finding suggests the possibility of a posttranscriptional level of regulation for pG2 mRNA steady-state levels that is activated by dbcAMP treatment. Neither RA nor cAMP induced significant transcriptional alteration in neural or chromaffin related genes in NB cells (data not shown).

DISCUSSION

Cellular differentiation is a process marked by the coordinated expression of regulatory molecules that depends on precise environmental signals and these signals are transduced to cells through distinct membrane, cytoplasmic, or nuclear receptor signaling pathways. *In vitro* agents such as RA, dbcAMP, and phorbol esters are capable of promoting differentiation and controlling cell growth even in NB tumor cells (23). RA functions are mediated by a family of nuclear receptors, with ligand dependent DNA binding and *trans*-activating properties (24). dbcAMP and phorbol esters mimic cytoplasmic membrane
receptor-ligand binding and are associated with the activation of a cascade of events that cause protein phosphorylation and transduction of intracellular regulatory signals mediated by PKA and PKC, respectively (2). Studies conducted principally on hematopoietic malignancies show that tumors may arise from cells blocked at intermediate steps along their normal differentiation pathway (25) and distinct differentiation lineages may be induced in vitro via activation of RA, PKA, or PKC signal transduction pathways (26, 27). Recent evidence based on an analysis of the expression and timing of chromaffin markers in human fetal adrenal glands permitted a classification of NB cell lines into at least three patterns of differentiation based on their expression of tyrosine hydroxylase, CGA, NPY, and pG2. 4. Our studies indicate that alterations in lineage associated gene expression occur in the SMS-KCNR cell line depending on treatment with dbcAMP or RA.

Treatment of a SMS-KCNR (10, 11) that has an immature chromaffin phenotype [type I NB cell (10, 11)] with agents that cause an increase in intracellular cAMP levels (dbcAMP or VIP) results in enhanced expression of the adrenal gland specific marker, pG2, which marks an intermediate chromaffin phenotype [type II NB cell (10, 11)]. Furthermore, CGA and NPY mRNA expression are dramatically increased in cells treated with dbcAMP. Although dbcAMP does induce increases in NF and SYN, GAP43 levels are unaltered and the majority of cells fail to morphologically differentiate to the same extent as RA treated cells. In contrast, RA does not significantly alter expression of chromaffin related genes yet does increase the steady-state mRNA levels of all the neuronal genes including GAP43, which encodes a phosphoprotein predominantly expressed on developing neural growth cones. RA also induces an extensive network of neuritic processes in cells. The combination of both agents results in a phenotype marked by coexpression of GAP43, NPY, NF and CGA, but not pG2, suggesting that RA blocks or inhibits the cAMP stimulated increases in pG2 expression. Consistent with these data is our finding in the cell line LAN-5 which expresses chromogranin A and pG2 (an intermediate chromaffin phenotype) that the steady-state levels of these genes are dramatically decreased when cells are treated with RA.

The relative importance of the expression of pG2, in our study, is related to the possibility to study the transition of NB from an immature chromaffin cell to a more mature chromaffin phenotype (10, 11) and develop a model to study regulation of chromaffin cell differentiation. Although the function of pG2 is unknown, the product of this gene seems to be a protein with an estimated size ranging from 30 to 45 Kda based on in vitro translation (28) and translation of nucleic acid sequence (29). The peculiar pattern of pG2 mRNA expression, restricted to the adrenal gland, both medulla and cortex, and not adrenal neuroblasts, suggests that this protein may be an organ specific protein (15). In our system it appears that the mRNA for the pG2 gene becomes detectable only after treatment with dbcAMP. However, the presence of an actively transcribed pG2 mRNA in control cells suggests that dbcAMP alters posttranscriptional regulation of this gene. In sympathetic neurons, evidence suggests that the cholinergic or adrenergic phenotype of a neuron can be altered by posttranscriptional regulation of gene expression (30, 31).
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Fig. 3. Densitometric scanning. Quantitative, densitometric analysis of appropriately exposed autoradiograms from the same data shown in Fig. 2. A and B, that have been normalized to the relative level of GAPDH mRNA expression •, RA; △, dbcAMP; and □, RA+dbcAMP.

Fig. 4. Nuclear transcription assay. Representative nuclear transcription experiment using nuclei that were isolated from cells in control condition or treated with dbcAMP for 8 days (D) (see “Materials and Methods”).

C

db-cAMP

pG2

pGEM

GAPDH

Fig. 4. Nuclear transcription assay. Representative nuclear transcription experiment using nuclei that were isolated from cells in control condition or treated with dbcAMP for 8 days (D) (see “Materials and Methods”).

Level of expression of differentiated neuronal molecular markers and a dramatic alteration in cell morphology. We have shown that RA markedly decreases the cellular content of the cell cycle gene product p34cdc2 (22). That study provided a molecular rationale for the growth inhibition induced by RA since p34cdc2 is a protein kinase that functions as a central regulator of the eukaryotic cell cycle progression (32). In contrast to RA, our data indicate that treatment with dbcAMP failed to alter the cell cycle distribution of NB and also fails to significantly alter p34cdc2 levels. The failure of dbcAMP to achieve a G1 block, typical of terminally differentiated cells, may be due to alterations in the cAMP dependent pathway of signal transduction in SMS-KCNR cells. Amplification of the MYCN gene has been associated recently with a direct alteration in the expression and function of PKC subunits (33). Thus, it is possible that amplified MYCN may alter transduction of PKA signaling pathways as well.

The ability of dbcAMP to up-regulate the adrenal specific gene pG2 permits the reallocation of SMS-KCNR cells from group I to group II of the adrenal chromaffin differentiation schema described by Cooper et al. (10, 11). The most mature phenotype of chromaffin cells described by Cooper et al. (10) is marked by the expression of β2-microglobulin protein. Although we detected small increases in β2-microglobulin mRNA expression in SMS-KCNR treated with either dbcAMP, or RA,4 previous studies have shown that neither RA nor dbcAMP increases membrane bound β2-microglobulin expression in SMS-KCNR cells (34), thus supporting the concept that cAMP stimulates an intermediate stage of differentiation. Furthermore, these observations are consistent with the activation by dbcAMP of a cellular differentiation program apparently similar to the original adrenal differentiation program interrupted when the transformation event took place. That dbcAMP also increases expression of some neuronal associated genes implies at this stage of differentiation lineage plasticity is retained. Conversely, many neural associated genes are expressed in normal developing adrenal medullary cells. The cell cycle studies also indicate that this is a nonterminal differentiation state representing an intermediate differentiated phenotype. In contrast RA induces the arrest of cell growth, differentiation...
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Fig. 5. Model of NB cell differentiation. A model of neuroblastoma cell differentiation based on the data presented in this work. According to the differential expression of neuronal and chromaffin markers, dbcAMP induces cells to express a more mature chromaffin phenotype thus indicating a transition of the SMS-KCNNR cell line from the group I (immature chromaffin cell) to the group II (more differentiated) based on the data presented in this work. Furthermore, when RA is used in combination with dbcAMP, it inhibits or prevents expression of the chromaffin related gene pg2, arrests cell growth, and induces a neuronal morphology.

The ability of dbcAMP and VIP, agents that raise intracellular cAMP levels, to increase expression of chromaffin-related genes suggests that signals utilizing PKA may be important in stimulating a more mature chromaffin phenotype in NB cells. The inability of these agents to induce terminal differentiation as marked by arrest of all growth and expression of membrane β2-microglobulin indicates that other or complementing signal transduction paths may be needed to induce terminal chromaffin differentiation. However, it remains possible that SMS-KCNNR cells are prevented from proceeding along a terminal chromaffin differentiation pathway by specific genetic alterations associated with tumorigenesis.

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