Morphological Differentiation of Human Neuroblastoma Cell Lines by a New Synthetic Polyprenolic Acid (E5166)

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ABSTRACT

The prognosis of patients with advanced neuroblastoma remains poor despite recent progress in chemo/radiotherapy. Therapeutic trials on the induction of differentiation of neuroblastoma by chemical and biological agents have been attempted to improve patients' prognosis. Recently a new synthetic polyprenolic acid, E5166, having retinoic acid properties, has been described. In this study two human neuroblastoma cell lines, KP-N-RT(LN) and SK-N-DZ, were treated in vitro by E5166. Morphological differentiation of KP-N-RT(LN) and SK-N-DZ cells could be induced by E5166 in the presence of 1.7 x 10^{-4} M E5166 for 10 days in culture. Levels of catecholamines (dopamine, adrenaline, and noradrenaline) were not elevated in the E5166-differentiated cells. E5166-induced differentiation may not be cyclic AMP dependent, since levels of cyclic AMP did not increase after exposure of cells to this agent. No significant increase in neuron-specific enolase levels could be demonstrated in E5166-treated neuroblastoma as compared to control untreated cells. E5166 treatment of KP-N-RT(LN) and SK-N-DZ cells was found to inhibit colony formation in soft agar in a dose-dependent manner. Colonies of KP-N-RT(LN) cells in the presence of E5166 showed morphological differentiation as defined by the expression of long neurite processes.

E5166 is a less toxic reagent than the retinoic acids used for the induction of differentiation, it can be administrated to patients p.o., and the concentration of E5166 which induces the morphological differentiation in vitro can be achievable in vivo. Therefore our study suggests that E5166 could be a useful therapeutic agent in advanced neuroblastoma to differentiate residual anaplastic tumor cells to a benign form (ganglionenuroma) after surgery and chemotherapy.

INTRODUCTION

NB, one of the most common malignant solid tumors in childhood, arises from primitive sympathetic neuroblasts. The prognosis of patients with advanced NB is still poor, despite recent progress in chemotherapy. Induction of differentiation of NB in vitro by chemical and biological agents, such as dbc AMP, prostaglandin E2, papaverine, sodium butyrate, phorbol ester, serum-free medium, RA, and nerve growth factor, has been reported (1-8). In addition therapeutic trials for the induction of differentiation in vivo by prostaglandin E2, papaverine, and nerve growth factor have been attempted (9-11). These trials, however, have not proved successful. Recently a new synthetic polyprenolic acid, E5166, which has properties similar to RA and is less toxic than synthetic RA, has been described by Muto et al. (12, 13).

In this study two human NB cell lines, KP-N-RT(LN) and SK-N-DZ established from bone marrow of patients with advanced NB, were differentiated in vitro by E5166. The results of these studies suggest a possible therapeutic role for E5166 in vivo. Hopefully the differentiation of induction of NB may lead to the total eradication of residual NB cells after extensive chemotherapy and surgery.

MATERIALS AND METHODS

Human NB Cell Lines. Two human NB cell lines, KP-N-RT(LN) (14) and SK-N-DZ (15, 16), were cultured in RPMI 1640 medium containing penicillin (100 units/ml), streptomycin (100 ìg/ml), and 10% heat-inactivated fetal calf serum at 37°C in a 5% CO2 incubator. The identification of these cell lines as NB was determined by conventional morphological as well as membrane phenotypic study (14, 16).

Morphological Differentiation Induction of NB Cell Lines Induced by dbc AMP and E5166 in Liquid Culture. Adenosine dbc AMP (P-L Biochemical, Inc., Milwaukee, WI) was dissolved in distilled water, filtered through a 0.45-mm Millipore filter, and frozen at -20°C. An optimal concentration of dbc AMP, for the in vitro induction of morphological differentiation, was determined by adding this to cultures at concentrations ranging from 0.5 to 3.0 x 10^{-3} M. E5166 (M, 302.46) (Eisai Co., Ltd., Tokyo, Japan) (12, 13), the chemical structure of which is illustrated in Fig. 1, was dissolved in dimethyl sulfoxide to a concentration of 0.17 M and frozen as stock solution directly into complete medium, the final solvent dilution being 0.1% by volume. An optimal concentration of E5166, for the in vitro induction of morphological differentiation, was determined by adding this to cultures at concentrations ranging from 0.8 to 3.3 x 10^{-3} M. Control cultures were established containing 0.1% solvent alone. Medium in the cultures was replaced every 4 days, and cells were maintained for a total of 12 days.

Assessment of Morphological Differentiation in Liquid Culture. Cells were plated into 60-mm Petri dishes containing 10- x 10-mm-sized coverslips. On Day 0 of either dbc AMP or E5166 treatment and on Days 2, 4, 6, 8, and 10, coverslips were stained with May-Giemsa. Two hundred cells from at least 3 different regions were examined by light microscopy and scored as morphologically differentiated, if they possessed one or more processes at least twice as long as the soma diameter (1).

Electron Microscopy. For scanning electron microscopy, adherent NB cells on coverslips were fixed in 2.0% glutaraldehyde, dehydrated in a graded concentration of ethanol, and dried by the critical point method. These were coated with platinum and observed with a Hitachi S520-LB scanning electron microscope. For transmission electron microscopy, cells attached to culture flasks were fixed to 2% glutaraldehyde in 0.1 M cacodylate buffer. These were subsequently harvested by scraping from the surface of the dishes using a rubber policeman. Cells were refixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated through a series of graded ethers, and embedded in epoxy resin. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined in a Hitachi H-500H electron microscope.

Catecholamine, Cyclic AMP, and Neuron-specific Enolase Estimations. Cells were harvested from culture flasks and washed with phosphate-buffered saline. For quantitative assays of catecholamine, harvested cells were homogenized, and dopamine, adrenaline, and noradrenaline levels were determined by high-performance liquid chromatography (1, 14). The level of cyclic AMP in treated cells was determined by the method of Honma et al. (17). The levels of neuron-specific enolase in treated cells were determined by radioimmunoassay using a Pharmacia kit (18). Results are expressed as neuron-specific enolase levels proportional to total cellular protein levels.
NEUROBLASTOMA DIFFERENTIATION BY POLYPRENOIC ACID

RESULTS

Induction of Morphological Differentiation by dbc AMP and E5166 in Liquid Culture. The optimal concentration of dbc AMP that induced differentiation of KP-N-RT(LN) cells after 10 days in culture was determined as $1.0 \times 10^{-3} \text{ m}$. At this point 58% of cells showed morphological differentiation. In contrast more cells showed a differentiated morphology treated with doses of E5166 ranging from 0.8 to $3.3 \times 10^{-5} \text{ m}$. Ten days after beginning the experiment between 77 and 92% of the NB cells appeared morphologically differentiated. The dose of E5166 where optimal differentiation occurred was $1.7 \times 10^{-5} \text{ m}$. In the absence of E5166 a maximum of 35% of the cells appeared differentiated (Fig. 2). With regard to SK-N-DZ cells, the maximum number of cells showing a differentiated morphology (45%) was obtained by treated cells with $1.7 \times 10^{-5} \text{ m}$ E5166 for Day 10. Control cells treated in an identical manner apart from the omission of E5166 from the medium showed 7% of the cells with a differentiated phenotype. Only 26% of SK-N-DZ cells could be induced to differentiate upon incubation with $1.0 \times 10^{-3} \text{ m}$ dbc AMP. Scanning electron microscopy showed that dbc AMP- and E5166-treated KP-N-RT(LN) cells formed long neurites after 4 days of exposure to the agent inducing differentiation. Following 12-day exposure to E5166, cells aggregated and formed cellular clusters that were connected with long-bundled neural networks. In contrast, nontreated cells were spindle-shaped soma with short neurite processes (Fig. 3, A to C). After the treatment of E5166 for 12 days, SK-N-DZ cells also showed long neurite processes. However, the long-bundled neural networks, observed in KP-N-RT(LN) cells, were not observed (Fig. 3, D and E). Transmission electron microscopy showed the development of Golgi apparatus, mitochondria, and neural tubules in both E5166-treated KP-N-RT(LN) (Fig. 4) and SK-N-DZ cells (data not presented).

Catecholamine, Cyclic AMP, and Neuron-specific Enolase Levels in Morphologically Differentiated Cells. KP-N-RT and SK-N-DZ cells were treated for 10 days with either dbc AMP or E5166. Dopamine levels in dbc AMP-treated KP-N-RT(LN) and SK-N-DZ cells and the noradrenaline level in dbc AMP-treated SK-N-DZ were significantly higher than in control nontreated cells ($P < 0.05$). However, no increase in dopamine, adrenaline, and noradrenaline levels was noted in cells exposed to E5166 for 10 days. Similarly while cyclic AMP levels in dbc AMP-treated KP-N-RT(LN) and SK-N-DZ cells were significantly elevated as compared with nontreated cells ($P < 0.05$), exposure to E5166 did not increase cyclic AMP levels over those of control cells. Neuron-specific enolase activity did not increase significantly upon E5166 treatment (Table 1).

Dose-dependent Growth Inhibition and Morphological Differentiation in Soft Agar by E5166. The inhibitory effect of E5166 on the ability of KP-N-RT(LN) cells to form colonies in soft agar was determined. In control dishes not exposed to E5166, 179 ± 12 (mean ± SE in triplicate cultures) colonies were formed, with a colony-plating efficiency estimated to be 1.7%. A 76% reduction in colony numbers as compared to controls (not exposed to E5166) was found on exposure of cells to $6.7 \times 10^{-5} \text{ m}$ E5166. This reduction was dependent on the concentration of E5166 with 50% inhibition occurring at $2.0 \times 10^{-5} \text{ m}$ E5166. In addition, colonies that developed in the presence of E5166 were smaller than nontreated cells and showed morphological differentiation by the criteria of expressing long-bundled neurite processes (Fig. 6). Under similar conditions control culture of SK-N-DZ cells gave 181 ± 15 colonies with a colony-plating efficiency of 1.8%. As with SK-N-DZ cells a dose-dependent growth inhibition was observed following incubation of cells with E5166. Fifty % growth inhibition occurred at $0.67 \times 10^{-5} \text{ m}$ E5166 (Fig. 5). However, under these conditions SK-N-DZ cells forming colonies were not morphologically differentiated.

DISCUSSION

A new synthetic polyprenoic acid, E5166, was originally discovered by Muto et al. E5166 is a derivative of RA since this chemical reagent has binding affinity to CRABP (12, 13). In the present study, two human NB cell lines, KP-N-RT(LN) and SK-N-DZ established from 14-mo and 12-mo-old infants with Stage IV NB, respectively, were induced to differentiate in liquid culture. The highest percentage of differentiated cells (92%) was found after 10 days of exposure to $1.7 \times 10^{-5} \text{ m}$ E5166 (Fig. 2). A similar differentiation effect of E5166 was observed on SK-N-DZ cells. Scanning electron microscopy showed that E5166-treated KP-N-RT(LN) cells aggregated, forming cellular clusters that were connected with long-bundled neurite processes, all indications of a mature "differentiated" state. With regard to SK-N-DZ, E5166 induced long neurite processes, but long-bundled neural fibers, observed in KP-N-RT(LN) cells, were not observed (Fig. 3). Transmission electron microscopy showed the development of Golgi apparatus, mitochondria, and neural tubules, analogous to those found in differentiated cells. However, no significant increase in dense core granules (catecholamine granules) was observed in E5166-treated cells (Fig. 4). Quantitative assays of catecholamine...
Fig. 3. A, untreated KP-N-RT(LN) grown in culture for 12 days. NB cells with short neurite processes were seen. × 500. B and C, KP-N-RT(LN) cells after culture for 12 days in the presence of $1.7 \times 10^{-5}$ M E5166. NB cell clusters were connected with long-bundled neural networks. × 500 (B) and × 2000 (C), respectively. D, untreated SK-N-DZ grown in culture for 12 days. × 500. E, SK-N-DZ cells after culture for 12 days in the presence of $1.7 \times 10^{-5}$ M E5166. × 500.
Fig. 4. Transmission electron microscopy of KP-N-RT(LN) cells after culture for 12 days in the presence of $1.7 \times 10^{-5}$ M ES 166. Left, untreated cells, $\times 10,000$. Right, ES 166-treated cells, $\times 10,000$. The development of Golgi apparatus, mitochondria, and neural tubules was observed.

Table 1  Levels of catecholamine, cyclic AMP, and neuron-specific enolase in dbc AMP- and E5166-treated KP-N-RT(LN) and SK-N-DZ cell lines

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Catecholamine (ng/mg protein)</th>
<th>Cyclic AMP (pmol/mg protein)</th>
<th>Neuron-specific Enolase (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dopamine</td>
<td>Adrenaline</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>KP-N-RT(LN)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nontreated</td>
<td>1.05 ± 0.46*</td>
<td>ND</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>dbc AMP</td>
<td>2.35 ± 0.56</td>
<td>ND</td>
<td>0.74 ± 0.24</td>
</tr>
<tr>
<td>E5166</td>
<td>0.68 ± 0.20</td>
<td>ND</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>SK-N-DZ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nontreated</td>
<td>7.08 ± 2.69*</td>
<td>ND</td>
<td>17.93 ± 3.47*</td>
</tr>
<tr>
<td>dbc AMP</td>
<td>27.68 ± 5.95*</td>
<td>ND</td>
<td>85.56 ± 12.40*</td>
</tr>
<tr>
<td>E5166</td>
<td>2.81 ± 0.84</td>
<td>ND</td>
<td>12.02 ± 0.54</td>
</tr>
</tbody>
</table>

* Means ± SE from 5 to 6 separate experiments.
* Significant differences from dopamine, noradrenaline, and cyclic AMP levels in dbc AMP-treated cells, and nontreated cells ($P < 0.05$).
* ND, not detectable.

The formation of KP-N-RT(LN) and SK-N-DZ colonies in soft agar was inhibited by increasing the concentration of E5166. Following treatment of these with E5166, neurite cells with processes forming differentiated colonies were observed in the KP-N-RT(LN) cell line (Figs. 5 and 6).

Morphological differentiation of NB by RA (all-trans-retinoic acid) was first reported by Sidell et al. (5). Their subsequent study demonstrated biochemical differentiation, based on the increased acetylcholinesterase activity, and electrophysiological differentiation of NB by RA (6–8).

Recently two independent reports have demonstrated de-
increased levels of N-myc oncogene mRNA following RA-induced differentiation of NB in vitro. This suggests the regulation of N-myc mRNA could occur through the direct action of RA, leading to a decrease in cellular proliferation and neural maturation (21, 22). E5166 has a similar effect on KP-N-RT(LN) cells which show decreased expression of N-myc and morphological differentiation after exposure to the reagent. Further studies need to be completed to clarify the mechanism by which E5166 induces differentiation of NB.

E5166 is one of the most potent reagents to induce morphological differentiation in various NB cell lines in vitro. It has equivalent differentiation activity to a similar concentration of all-trans-RA (10^{-5} M),^5 can be administered to patients p.o., and is 5 to 10 times less toxic than all-trans-RA (12, 13). Single p.o. administration of 40 mg/kg body weight of E5166 to rats can achieve more than a 1.7 × 10^{-5} M level of E5166 (the level used for in vitro differentiation) in blood, adrenal glands, and most body tissues for more than 48 h. As much as 200 mg/kg body weight of E5166 can be administered p.o. to rats over a relatively long period of time. The clinical usefulness of E5166 in NB is suggested by the morphological differentiation and the inhibitor of colony formation in soft agar in both KP-N-RT(LN) and SK-N-DZ cell lines. The drug may be useful in advance NB patients to eradicate residual tumor cells after surgery and chemotherapy.

ACKNOWLEDGMENTS

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REFERENCES

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