**In Vitro** Differentiation of Human Neuroblastoma Cells Caused by Vasoactive Intestinal Peptide

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**ABSTRACT**

Neuroblastoma, a tumor of the sympathetic nervous system, is the most common solid malignancy of childhood outside the central nervous system. Vasoactive intestinal peptide (VIP) is produced by some of these tumors, and elevated serum levels correlate with tumor cell differentiation and a favorable prognosis. It has previously been demonstrated that human neuroblastoma cell lines LA-N-5 and IMR-32 will differentiate in vitro when exposed to retinoic acid. It is now shown that VIP also induces in vitro differentiation of these neuroblastoma lines. LA-N-5 or IMR-32 cells were grown in the presence of different concentrations of VIP. Cell proliferation was suppressed, as measured by cell count, incorporation of [3H]thymidine, and measurement of the proliferation index. The degree of suppression correlated with the concentration of VIP, and the effect was indistinguishable, on a molar basis, from that seen when cells were treated with retinoic acid. Similarly, the morphological changes seen in the VIP-treated cells were the same as those seen in retinoic acid-treated ones. The effects of VIP on both cell lines, like those of retinoic acid, are reversible. The human neuroepithelioma line CHP-100, is much less sensitive to either agent. Vasoactive intestinal peptide is the first substance shown to cause differentiation of neuroblastoma cells in vitro which is also known clinically to have a specific association with that tumor. It is postulated that VIP may play a key role in the well-documented maturation of these tumors in vivo and in the normal development of the sympathetic nervous system. These findings may also have therapeutic implications for the management of this frustrating childhood malignancy.

**INTRODUCTION**

Neuroblastoma, a tumor of the sympathetic nervous system, is the most common solid malignancy of childhood outside the central nervous system. Despite dramatic advances in the field of pediatric oncology during the last quarter century, the overall cure rate for neuroblastoma has only been minimally improved, and "cures" appear to have more to do with the natural history of the disease than with the therapeutic interventions (1). It is well known that neuroblastomas often show histological maturation in vivo and on rare occasions may mature to a completely benign cell type or show spontaneous complete resolution (1). The biological mechanisms underlying these changes are unknown. This has underscored the significance of the observation that some neuroblastoma cell lines will differentiate in vitro in response to certain substances. The most commonly used agent has been retinoic acid (2, 3), but similar effects have been seen with other agents such as forskolin (4), phorbol esters (5, 6), dibutyryl cAMP (7), and nerve growth factor (8). The morphological changes seen in culture have been shown to correlate with other indicators of differentiation such as neurotransmitter synthesis (3, 6), increased acetylcholinesterase activity (9), changes in oncogene expression, especially N-myc (10), a decrease in the levels of the p53 protein (11), the presence of neurosecretory granules (6), and decreased tumorigenicity in nude mice (3).

Another important clinical observation is that there is a subset of patients with neuroblastoma with very high serum levels of VIP, a 28-amino acid compound originally identified in intestine but predominantly found in neurons (12). Secretory diarrhea is a part of this clinical syndrome, and most of these patients have a favorable prognosis (13). VIP appears to be produced by the tumor, and production has been correlated with tumor differentiation (14). It has not been determined, however, whether VIP is merely a passive marker or whether it actually contributes to the process of differentiation.

To date, numerous activities have been ascribed to VIP, but its actual physiological roles have not been precisely determined (12). It has been shown to have growth regulatory properties for certain cell types (15-17). It is also known to be associated with differentiation of some other types of tumor cells derived from nervous tissue (18, 19). It is produced by some neuroblastoma cells (20-22), which have also been shown to bear specific cell-surface VIP receptors (23), thus, suggesting an autocrine function. Because VIP levels have also been shown to be elevated in some children with neurogenic tumors who do not have the watery diarrhea syndrome, although to a lesser degree (24, 25), its potential importance is not just restricted to that small clinical subset.

We now show that vasoactive intestinal peptide can produce morphological differentiation and suppression of cell proliferation in neuroblastoma cell lines to a degree comparable to that seen with retinoic acid. VIP may be responsible for the in vivo maturation seen in neuroblastomas and may play an important role in the normal development of the sympathetic nervous system. Functional defects in this molecule, its metabolism, or its receptor could be responsible for some cases of neuroblastoma.

**MATERIALS AND METHODS**

Neuroblastoma Cell Lines. The human neuroblastoma cell line LA-N-5 (2) was originally obtained from Dr. Robert Seeger. Other neuroblastoma cell line IMR-32 (26) was purchased from the American Type Culture Collection (Rockville, MD). Both cell lines were maintained in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified 5% CO2 incubator.

Other Cell Lines. CHP-100 (27), a human cell line previously identified as a neuroblastoma, but now reclassified as a neuroepithelioma (28), was a gift from the cell bank of the Children's Hospital of Philadelphia. HL-60 (29), a promyelocytic leukemia line, and two breast carcinoma lines, MCF-7 (30) and ZR-75-1 (31), were purchased from the American Type Culture Collection. All three cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified 5% CO2 incubator.

Differentiating Agents. Synthetic vasoactive intestinal peptide (human, rat, porcine) was obtained from Peninsula Laboratories, Inc. [CANCER RESEARCH 50, 5177-5183. August 15, 1990]
NEUROBLASTOMA CELL DIFFERENTIATION CAUSED BY VIP

Human neuroblastoma cell lines LA-N-5 and IMR-32 were grown in the presence of varying concentrations of vasoactive intestinal peptide. Cell counts done after 8 days of treatment show that VIP suppresses cell proliferation in both of these lines (Figs. 1 and 2). The degree of suppression correlates with the concentration of VIP, and, on a molar basis, the effect is the same as that seen with retinoic acid. Comparable results are seen if either incorporation of tritiated thymidine or the proliferation index is used to monitor cell proliferation (Figs. 1 and 2). Because we have not seen cytotoxicity of either agent in this concentration range, the data suggest that cell division is more sensitive to inhibition than DNA synthesis and is, probably, blocked first.

Morphological differentiation was observed in both cell lines after treatment with VIP. The morphological features of RA-induced differentiation have been well described for both lines. For LA-N-5, these consist of the formation of large cellular aggregates (pseudoganglia) which are interconnected by long processes (2). VIP induced comparable changes in LA-N-5 cells at all concentrations tested (Fig. 3), and, as with RA, the changes were greatest at the highest concentrations (Fig. 4). The effects of retinoic acid on the morphology of IMR-32 cells are more variable. Sidell et al. (2) only observed cellular enlargement and vacuolization, but others have demonstrated that this line can also show pseudoganglion formation and neurite extension, although to a lesser degree than that seen with LA-N-MM.
NEUROBLASTOMA CELL DIFFERENTIATION CAUSED BY VIP

Fig. 3. Morphological appearance of the human neuroblastoma cell line LA-N-5 in the presence of (a) solvent control, (b) 1.0 μM VIP, and (c) 1.0 μM RA following 12 days of treatment. Cells were photographed utilizing an inverted, phase-contrast microscope (Nikon Diaphot). × 200.

Fig. 4. Morphological differentiation of LA-N-5 cells in the presence of increasing concentrations of VIP (•) or RA (○) as measured by neurite extension after 8 days of treatment. Points, mean values of quadruplicate cultures. SE = 4 for all values.

When VIP or RA was removed from the medium, cultures of either cell line resumed normal exponential growth quite rapidly (Fig. 8), and the established morphological differentiation disappeared. This indicates that the effect of both agents are reversible, although we cannot rule out the possibility that a small population of cells does undergo irreversible change. Previous studies which have looked at the reversibility of in vitro differentiation have not produced a consistent result (36, 37).

Could recovery of the cultures be due to resistant cells? This cannot be completely ruled out either, but it seems unlikely. Robson and Sidell (38), using long-term (up to 5 weeks) cultures of LA-N-5 in the presence of RA, have shown that only a very small number of cells continue to divide in these cultures and may, therefore, be resistant. In these long-term cultures, there is persistence of differentiation and no evidence of overgrowth by a population of resistant cells. Their results, coupled with our observations, especially the rapidity of recovery, convinces us that the effects of both agents on the majority of cells are reversible after a short course of treatment. It remains to be determined whether with longer treatment there is a point at which generalized, irreversible changes do occur.

Several other cell types were tested for their responsiveness to VIP. The neuroepithelioma cell line, CHP-100, has previously been reported to be relatively resistant to the effects of RA (2). Our data confirm that CHP-100 is less sensitive to RA than the two neuroblastoma lines (Fig. 9). It is also less sensitive to VIP, although the effects of VIP on cell growth were slightly more pronounced than those seen with RA. Unlike the earlier report (2), morphological changes were apparent at the highest concentration (10\(^{-6}\) M) of both agents. The major change was an enlargement of the cells with some process extension so that many of them took on the appearance of large ganglion cells. These morphological changes were more marked in the RA-treated cells (Fig. 10).

HL-60, a promyelocytic leukemia line, shows differentiation when exposed to retinoic acid, and this can be demonstrated by the ability of cells to reduce nitroblue tetrazolium (35). When cells were treated with VIP, there was no growth inhibition and no evidence of differentiation, indicating that the cell specificities of the two substances overlap but are not identical (Table 1).

MCF-7 and ZR-75-1, two breast carcinoma cell lines known to express VIP receptors (39), showed growth suppression to 57 and 69% of control values, respectively, at a VIP concentration of 1.0 μM. Interestingly, the growth of MCF-7 and ZR-75-B, a clone of ZR-75-1, has previously been shown to also be reversibly inhibited by retinoic acid (40). Differentiation in vitro has not been described for either of these lines, so it is unknown whether it can occur. In the absence of any known markers, the

5 (7, 36). We observed both types of morphological change within the same plate. VIP treatment produced changes in the IMR-32 cells which were qualitatively indistinguishable from those seen with RA (Figs. 5 and 6), although quantitatively slightly less (Fig. 7).
question of differentiation in response to VIP cannot be answered.

DISCUSSION

Our data indicate that, rather than being just a product of partially differentiated malignant cells, VIP has a role in initiating or promoting differentiation. If this is true, the group of patients with very high VIP levels may simply represent cases of receptor dysfunction associated with a normally functioning negative feedback mechanism, similar to the situation seen in many endocrine disorders when there is some degree of end-organ resistance. If VIP does play a fundamental role in the in vivo maturation of neuroblastoma cells, then it is likely that it also plays a role in the normal embryological differentiation of the adrenal medulla and the sympathetic nervous system, processes about which little is currently known. A similar suggestion has been made by Gozes (41), who believes that VIP may regulate differentiation in the brain. It would follow that abnormalities in the function of the VIP molecule or in the regulation of its gene expression would disrupt normal differentiation and could be responsible for some cases of neuroblastoma.

The concentrations of VIP used here are much higher than normal serum levels (<30 pmol/liter) (42) or even the serum levels seen in patients with VIPomas (60–600 pmol/liter) (42). However, because of the apparent autocrine nature of its function, the local VIP concentration would be critical rather than the serum level. Yamaguchi et al. (43) have found that local tumor concentrations can reach these levels.

VIP is known to raise intracellular cAMP levels, both in neuroblastoma cells (44) and in other cell types (17, 45–47), as do several other differentiating agents (4, 7). This may be responsible for some, if not all, of the observed effects. Evidence
serves to amplify the effect when cells are exposed to exogenous VIP. The same amplification may also be seen when cells are stimulated by nerve growth factor (57), which has variable responsiveness to VIP. Indeed, VIP receptors are found on numerous cell types (12, 39, 47), and reference has already been made to the growth regulatory effects that are often seen in cells (57, 60, 61), and some of the effects of these agents may require VIP as an intermediary. Interestingly, VIP synthesis is also stimulated by nerve growth factor (57), which has variable effects on the intracellular level of cAMP (56, 62, 63), and by phorbol esters, which do not appear to affect the levels (56, 57, 62).

VIP is also known to be produced in response to a rise in intracellular cAMP levels (57–59), and some neuroblastoma lines are known to release VIP in culture (20–22). This could serve to amplify the effect when cells are exposed to exogenous VIP. The same amplification may also be seen when cells are treated with other differentiating agents that raise cAMP levels. Indeed, forskolin and dibutyryl cAMP have both been shown to produce an increase in VIP levels in cultured neuroblastoma cells (57, 60, 61), and some of the effects of these agents may require VIP as an intermediary. Interestingly, VIP synthesis is also stimulated by nerve growth factor (57), which has variable effects on the intracellular level of cAMP (56, 62, 63), and by phorbol esters, which do not appear to affect the levels (56, 57, 60).

Neuroblastoma cells are obviously not unique in showing responsiveness to VIP. Indeed, VIP receptors are found on numerous cell types (12, 39, 47), and reference has already been made to the growth regulatory effects that are often seen in culture and which may represent a fairly generalized function of this peptide. However, VIP also has functions which are specific to certain tissues (12), such as its effect on ion transport in intestinal epithelia (64). Current evidence, including this study, suggests that in neural tissue VIP may have a role as a regulator of cell survival and differentiation (18, 19, 41, 65–67). Whether all types of neural tissue are sensitive to its effects at a later step, bypassing the need for increased cAMP levels.

The relationship between cAMP and RA is obviously still far from clear. Using a different cell line, the neuroblast subclone SK-N-SH-SY5Y, which is derived from the parent neuroblastoma line SK-N-SH, Yu et al. (56) have shown a 30% increase in cAMP levels as a result of RA treatment. More significantly, they have shown that RA treatment dramatically enhances the cAMP accumulation seen in response to prostaglandin E. This same effect was seen with two other neuroblastoma lines, IMR-32 and Kelly, even though cAMP levels were unaffected in the former by retinoic acid alone and were actually decreased slightly in the latter.

The full mechanisms of action of VIP and RA, and the relationship between them, if any, are not yet known. Preliminary results from our laboratory suggest that they act synergistically. If VIP functions primarily by raising the intracellular concentration of cAMP, this would be in agreement with the findings of Lando et al. (4), who have shown synergy between RA and other cAMP-elevating agents.
or whether only certain subtypes are remains to be determined.

Vasoactive intestinal peptide is the first substance found to produce differentiation of neuroblastoma cell lines in vitro which is also known clinically to have a specific association with that tumor. As such, further investigation of its function is warranted and could have therapeutic implications for the management of this frustrating childhood malignancy.

ACKNOWLEDGMENTS

The authors gratefully acknowledge B. J. Kerns for performing the immunohistochemical analyses and D. R. Pittman for preparation of this manuscript.

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