Cytolytic Effect of 6-Hydroxydopamine on Neuroblastoma Cells

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INTRODUCTION

Sympathetic neuroblasts are lethally injured by 6-OHDA when this analog is administered to newborn animals during a critical period of the growth and development of the sympathetic nervous system (1, 2).

In adult animals, the lesions are restricted to the nerve terminals, while the cell body of the sympathetic neurons is not affected (13, 14). With the use of a proper dosage of 6-OHDA in newborn mice, rats, and chickens, it has been possible to obtain the complete and permanent destruction of the sympathetic ganglia (2, 9). The selectivity of the toxic effect elicited by 6-OHDA is due to the specific uptake of this dopamine analog by the sympathetic cells; once it has entered the immature cells, the analog undergoes rapid oxidation and brings about cell death. It was of interest, therefore, to explore the possibility of inducing similar irreversible injuries in the neoplastic cells of sympathetic origin, namely in neuroblastoma cells. There is, in fact, evidence that this neoplasm is still reminiscent of the tissue of origin and exhibits the capacity of uptake and metabolism of catecholamines and their precursors. Goldstein (6) has shown that human neuroblastoma cells cultured in vitro retain the capacity of rapidly incorporating norepinephrine-3H or its precursor, dopamine-3H. The turnover of these catecholamines was more rapid in the tumor cells than in fetal human sympathoblasts. More recently, a transplanted mouse tumor (C-1300), believed to be a neuroblastoma, has been established in tissue culture where it undergoes differentiation. The cells are able to synthesize catecholamines, as shown by the high levels of the enzymes involved in their biosynthetic processes (3) and by the conversion of tyrosine into dopamine and norepinephrine (12).

In this paper, we report on the effects of 6-OHDA on both human and mouse neuroblastoma cells cultured in vitro and on the growth of transplanted neuroblastoma in mice. The results obtained indicate that the dopamine analog produces a specific cytotoxic effect on these cells similar to that observed in immature sympathoblasts; the limited uptake capacity of the tumor cells seems, however, to be rate limiting for the carcinolytic effect of the drug in vivo.

MATERIALS AND METHODS

The C-1300 mouse neuroblastoma (originally obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine) was maintained by transfer in A/J mice. A total of 60 mice, weighing 16 to 18 g each, were given s.c. injections of a cell suspension of this tumor. One or two weeks later, the animals were divided into tumor-control and tumor-test groups. Tumor size and body weight in experimental and control mice were recorded every 2 to 3 days. At the end of each experiment, the animals were sacrificed, and the tumors were removed and fixed for histological examination.

Tissue culture lines of the mouse neuroblastomas were established with Medium 199 (supplemented with 10% calf serum and 5% fetal calf serum) or Medium F/10 (supplemented with 20% calf serum). Similar media had been used in previous investigations on these tumors (3, 12). Tissue culture lines of human neuroblastoma, kindly provided by Dr. M. Goldstein, were subcultured in Medium 199, supplemented with 25% calf serum. HeLa cells and mouse sarcoma 37 cultured in the same medium were used as controls. For autoradiographic experiments, subcultures of neuroblastoma cells, in small Falcon dishes with a coverslip on the bottom were pulsed with 5 or 10 μCi/ml of dopamine-3H or 5 μCi of norepinephrine-3H (Amersham, England) for 15 min. The cultures were rinsed in 3 changes of Hanks' solution and fixed in 2% glutaraldehyde in Hanks' solution for 1 hr. After fixation,
coverslips were mounted on slides and covered with autoradiographic emulsion (Eastman Kodak Co., Rochester, N. Y.).

The preparations were developed after storage at 4° for 7 days, stained with hematoxylin and eosin, rinsed, and dried. The original 6-OHDA was kindly given by Merck Sharp and Dohme Research Laboratories, Merck and Co., Rahway, N. J., and subsequently was purchased from Aldrich Chemical Co., Milwaukee, Wis. Solution of 6-OHDA was freshly prepared every day by dissolving the drug in acidified 0.9% NaCl solution or in a 0.2% ascorbic acid solution.

RESULTS

In a first series of experiments, the effect of 6-OHDA was investigated in tissue culture on cell lines of human and mouse neuroblastomas. In both sets of cultures, fibroblasts were intermingled with neoplastic cells. Subcultures in small Falcon dishes were incubated for 5 to 6 days in a CO2-conditioned thermostat. At the end of this period, most of the neoplastic cells had produced a long process, which had all the characteristics of an axon. Cells from human neuroblastoma had a small, ovoidal shape with opposite polar filaments. They aggregated in small clusters interconnected by processes emerging from these cells. Scattered among these cells were spindle-shaped cells, which exhibited a fibroblast-like appearance (Fig. 1, a and b). Cultures of mouse neuroblastomas are characterized by gigantic cells, probably polyploid, with multiple branchings and a very large nucleus. In order to test the effects of 6-OHDA, the tissue culture medium was replaced by 1 ml of Hanks' solution in each dish. A solution of 6-OHDA (freshly made in 0.2% ascorbic acid) was added to the experimental cultures at various concentrations (from 1 to 100 μg/ml). In controls, only ascorbic acid solution at the given concentration was used. The cultures were incubated for 60 min, and then the Hanks' medium was removed and replaced with fresh medium in both control and experimental dishes. The cultures were inspected every 12 hr at the inverted microscope, and the results were scored from 0 to 3+ according to the percentage of degenerating cells.

In order to ascertain whether the cytotoxic effects of 6-OHDA on human and mouse neuroblastomas were specific, cultures of HeLa cells and of mouse sarcoma 37 were treated similarly with 6-OHDA. Table 1 summarizes the results obtained.

The addition of 6-OHDA to the medium in neuroblastoma cultures at concentrations of 25 μg/ml or more results in marked degeneration of neuroblastoma cells. The cultures examined 24 hr after exposure to 6-OHDA show neoplastic cells detached from the dish surface and floating in the medium. Other cells are still attached but show signs of deterioration in the cytoplasm, such as vacuolization and granulation. The processes which exhibit all the morphological characteristics of nerve fibers are retracted and show swelling at their tips (Fig. 1, c and d). In contrast, the fibroblasts appear quite resistant to the action of the drug and do not show degenerative marks even in the presence of higher concentrations of 6-OHDA. Repeated exposure to 6-OHDA for 2 or 3 consecutive days results in the total disappearance of neuroblastoma cells while fibroblasts continue to grow. The toxic effect was more pronounced in the mouse than in the human neuroblastoma cell lines used in these experiments (Table 1).

HeLa cells and mouse sarcoma cells were not significantly affected by 6-OHDA under these experimental conditions.

The possibility that the observed toxic effects on neuroblastomas but not on other neoplastic or normal cells were due to a selective uptake of catecholamines by these cells was explored in radioautographic experiments. Subcultures of human and mouse neuroblastomas were pulse-labeled for 10 to 15 min with dopamine-3H (10 μCi/ml) and with norepinephrine-3H (5 μCi/ml). The cultures were then repeatedly washed, fixed in 2% glutaraldehyde, and processed for autoradiography.

Both mouse and human neuroblastoma cells showed a significant labeling over cell bodies and their processes after pulsing with catecholamine-3H. The intensity of labeling varied from cell to cell, ranging from a few sparse grains in the cytoplasm of some cells to intense labeling of the entire cell body in other cells (Fig. 2). Labeling of the cell processes was in all cases much lighter. When cultures were preincubated with 30 μg/ml of 6-OHDA 30 min before the addition of the labeled precursors, no labeling of the cells occurred. In all cultures, fibroblasts were only slightly labeled and were therefore clearly distinguishable from the labeled nerve cells.

In Vivo Experiments. Mice given injections of cell suspensions of C-1300 neuroblastoma were divided into control and test groups 1 week later. At this time, small tissue nodules were appreciable in the axillary region immediately beneath the skin. The 6-OHDA was injected i.m. twice a day at doses of 50 μg/g of body weight. Control animals received injections of the solvent. Tumor size and body weight were recorded every 2 to 3 days throughout the study. The results are shown in Chart 1. In control animals, the tumors grew quite rapidly in a constant manner, doubling in volume almost every 2 days. On the other hand, in treated animals the tumors grew at a much slower rate. Only in a small percentage of the total cases (2 out of 30) was there a complete regression of the tumor. As a crude measure of toxicity, animal weights were recorded throughout the experimental study. A slight but consistent loss in weight was found in treated mice but not in

Table 1

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<th>6-OHDA (μg/ml)</th>
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<th>HeLa cells</th>
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Chart 1. Tumor size (mm) in mice of control groups (○—○) and test groups (△—△). Body weight (in g/group of 5 mice) in control (○—○) and test (△—△) groups. The 6-OHDA treatment (50 μg/g twice daily) was started 1 week after transplantation.

controls; the final average weights (after 2 weeks of treatment) were about 10% less in the experimental group than in controls. Furthermore, the experimental animals, a few days after the beginning of treatment, showed less vitality than the controls. With higher doses of 6-OHDA (100 μg/g of body weight twice a day), the inhibitory effect on tumor growth was even more evident, particularly during the 1st week of treatment; however, a parallel increase in the toxicity of the drug was noticeable (Chart 2).

Histological study of various organs from both control and test animals showed no differences between treated and untreated mice. Histological study of the tumors stained with hematoxylin and eosin showed, on the contrary, extensive necrosis of tumors in experimental animals. This was far greater than the restricted area of necrosis in the central core of control tumors. In the experimental animals, necrosis was in fact present in all parts of the tumor (Fig. 3), sometimes surrounding areas with actively growing cells.

DISCUSSION

Selective destruction of neoplastic cells could be achieved if specific metabolic differences between neoplastic and normal cells were known, and one could exploit these differences by making use of chemical agents interfering with metabolic pathways essential to neoplastic but not to normal cells.

The results reported in this article indicate a possibly promising approach to this problem in the case of one particular cell line, which displays structural and metabolic characteristics of its cell of origin, the sympathetic adrenergic neuron.

The similarity between neuroblastoma cells and immature sympathetic nerve cells has been stressed in extensive histological and biochemical studies of this tumor in human infants and in vitro (7, 8, 10). Recently, remarkable structural and chemical similarities were also found between a mouse neuroblastoma grown in vitro and sympathetic nerve cells from which this tumor is presumed to originate (3, 12).

The present study is based on the discovery that a dopamine analog, 6-OHDA, selectively destroys immature sympathetic nerve cells while inflicting only restricted and reversible damages on the end terminals of differentiated sympathetic nerve cells. The lethal effects of this dopamine analog in immature adrenergic neurons result from the uptake and cytotoxic effects elicited by it in the sympathoblasts. Previous studies have shown that human neuroblastomas and ganglioneuromas are also able to incorporate, store, and metabolize catecholamines and their precursors (4, 5, 11). If neuroblastoma cells would prove to be as vulnerable to this amine analog as normal immature sympathetic nerve cells, irreversible damages to these neoplastic cells could be expected by feeding them with this agent. The results of the in vitro experiments reported above confirm this expectation. The addition to the culture medium of 6-OHDA results, as reported, in clear-cut cytotoxic effects in human and mouse neuroblastoma cells cultured in this medium, while normal fibroblasts or other neoplastic cells such as HeLa cells and cells from mouse sarcoma 37 are apparently not affected by this amine analog. The hypothesis that this toxic effect on mouse and human neuroblastoma cells might be due to the selective uptake of this dopamine analog by neoplastic cells received further support from autoradiographic experiments, which showed that labeled dopamine or norepinephrine are taken up by these cells in vitro. Labeling of the same cells was prevented by previous administration of 6-OHDA. This result can be explained as due either to the uptake of this cold analog, which is competitive with the labeled dopamine, or to damages inflicted by this analog to the storage sites.

In vivo, the administration of 6-OHDA resulted in complete remission of the tumor in only 2 out of 30 cases, while in all other cases a marked tumor regression was noticeable in the 1st week of treatment. The histological examination performed at the end of this period shows large areas of necrosis and cell degeneration throughout the neoplastic tissue. However, a decrease in the effectiveness of the treatment became apparent in subsequent days as the number of neoplastic cells increased. The low degree of differentiation and the rapid
growth rate of this highly malignant mouse tumor may account for these partial in vivo results. A more favorable response may obtain in human neuroblastoma which, at least in some cases, is characterized by a remarkable degree of cell differentiation and a parallel slow growth rate of the neoplastic tissue.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1. Living cultures of human neuroblastoma 24 hr in vitro (Nomarski interference microscope). a and b, small groups of ganglion-like cells (n) with long processes intermingled with spindle-shaped fibroblasts (f); c and d, cultures of human neuroblastoma 24 hr after exposure to 6-OHDA (50 μg/ml); arrows, nerve cells with short, swollen processes. In d, fibroblasts (f) in good condition are apparent.

Fig. 2. Cultures of human (a) and mouse (b) neuroblastoma pulse-labeled for 15 min with dopamine-³H. Numerous grains are seen in the cytoplasm of nerve cells.

Fig. 3. Histological sections at lower (a, b) and higher (c, d) magnification of mouse neuroblastoma from control (a, c) and 6-OHDA-treated (b, d) mice. Note extensive necrosis and cell degeneration in treated tumors.
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