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Dopamine-β-Hydroxylase Activity in Mouse Neuroblastoma Tumors and in Cell Cultures

(5-bromodeoxyuridine/catecholamines/norepinephrine)

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ABSTRACT **Dopamine-β-hydroxylase activity is present in mouse neuroblastoma C-1300 tumors. The activity is proportional to the weight of the tumor. Serum activity is markedly increased in mice that bear the tumors. Treatment of mice with 5-bromodeoxyuridine causes marked inhibition of tumor growth and decrease of dopamine-β-hydroxylase activity in the serum. The histochemical studies reveal that 1–5% of the cells in mouse C-1300 neuroblastoma tumors contain catecholamines and that catecholamine-containing processes terminate mainly around blood vessels of the tumor. Dopamine-β-hydroxylase is present in clonal neuroblastoma cell lines. The cell line with the greater tendency to form axon-like processes has a higher activity of this enzyme.**

Dopamine-β-hydroxylase (hydroxylase) (EC 1.14.2.1) is found in normal animals only in adrenal glands and in adrenergic neurons (1, 2). It catalyzes the hydroxylation of dopamine to norepinephrine in the only physiologically significant pathway for the formation of this neurotransmitter. We now present data on the activity of this enzyme in mouse C-1300 neuroblastoma tumors and in cultured cell lines derived from this tumor.

METHODS

The C-1300 mouse neuroblastoma, originally obtained from the Jackson Memorial Laboratory, Bar Harbor, Me., was maintained by subcutaneous or intramuscular transfer in A/J mice (16–18 g). In some experiments, tumors were obtained by intramuscular injection of cell suspensions from cultured clonal lines (about 2×10^6 cells) into A/J mice. The tumor size and body weight were recorded daily.

Three kinds of neuroblastoma cells were maintained in culture: an uncloned cell line obtained from an explanted subcutaneous tumor; cultures of cells derived from Neuro-2a, a clone established by Klebe and Ruddle (3); (designated herein Neuro-2a-G); and cultures from clone N-18 established by Seeds *et al.* (4) (designated N-18-G). The cells were grown in Eagle's basal medium supplemented with 10% calf serum in either glass or plastic T-flasks. Cells adhered firmly to the bottoms of the flasks; the proportion of cells showing definite, usually fine, axon-like processes could readily be determined under phase-contrast microscopy with a ×10 objective. At intervals of 1–2 weeks, the cells were subcultured after exposure to 0.05% trypsin (Difco 1:250) in phosphate-buffered saline (pH 7.2) for 2–3 min at room temperature (5, 6). Trypsin was then inactivated by the addition of 0.5 volume of calf serum, and the cells were centrifuged and replated. The morphology of cultured cells and their growth characteristics have not appreciably changed during the year in which the experiments were performed. After trypsinization and centrifugation, cells (about 0.1 ml) were washed 3-times with 10 ml of tyrosine and phenylalanine-free Eagle's medium containing 10% calf serum and twice with saline solution; cells were then sonicated and aliquots were taken for enzyme assay. Cell numbers were determined in duplicate with the hemocytometer; after dilution in 0.4% trypan blue, 95–98% of cells regularly excluded the dye.

Hydroxylase in tumor, cell cultures, and plasma was assayed (7–9) by a sensitive procedure. In the first of coupled enzymatic reactions, tyramine is converted by the enzyme to octopamine; in the second reaction the octopamine is further converted by added phenylethanolamine: *N*-methyl transferase (EC 2.2.1.–) to *N*-methyl octopamine. The first reaction was done for 20 min and the second for 30 min (8). Tumors to be used for hydroxylase assay were homogenized in 5 mM Tris buffer (pH 6.8) containing 0.1% Triton X-100 and, the homogenates were centrifuged at 10,000 × *g* for 10 min. Aliquots of the supernatant containing 0.1–0.2 mg of tissue in 0.1–0.2 ml were assayed. For assay of the enzyme in serum, aliquots of 0.05–0.1 ml of serum (diluted 1:10 with water) were used, and for assay in cultured cells, 0.1- to 0.2-mg aliquots of the homogenate (50–100 μg of protein) were used. In all incubations, tyramine (0.4 μmol) served as a substrate for the enzyme and *S*-adenosyl-L-[*methyl*-^{14}C]methionine (43 mCi/mol) served as methyl donor.

In some experiments the enzymatically formed products were identified as acetylated derivatives by paper chromatography (10) in the "C" solvent system of Bush (11).

The catecholamine-containing cells in the C-1300 tumors were localized with the histochemical fluorescence method (12).

RESULTS

Activity in Mouse Neuroblastoma Tumors and in Serum at Different Times after Implantation of the Tumor. Fig. 1 shows that tumors possess substantial hydroxylase activity and that in growing tumors the amount of enzyme is proportional to their weight, the activity/g of tumor being the same at day 14 as at day 21, although the size increased 3- to 4-times.

Hydroxylase activity in the serum of mice that bear tumors is markedly increased over that of control mice 2 weeks after implantation of the tumor (Fig. 1). There was no difference 1 week after implantation when the tumors first became palpable. The increase in enzyme activity in the serum is propor-

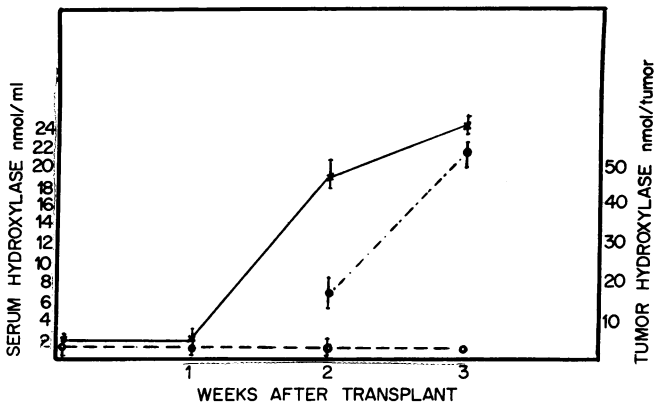

FIG. 1. Dopamine-β-hydroxylase activity in mouse neuroblastomas and in serum at different times after implantation. ×, activity of enzyme in serum of C-1300 mice; O, activity in serum of A/J mice; ●, activity in tumor tissue from C-1300 mice.

tional to the increase in size of the tumor, continuing from the 14th day to the 21st day after implantation.

In order to ascertain whether or not the elevated levels of serum hydroxylase activity are due specifically to the presence of the neural tumor that synthesizes and releases the enzyme or to stress generated by bearing of the tumor, we have also analyzed serum hydroxylase activities in mice with lymphomas. No hydroxylase activity was detectable in lymphomas, and the C57 C3H mice with lymphomas had the same serum hydroxylase activities as the controls.

The Effects of 5-Bromodeoxyuridine (BrdU) on Tumor Growth and on Hydroxylase Activity. Table 1 shows that treatment of mice with BrdU causes marked inhibition of tumor growth in the first 2 weeks after treatment. Concomitantly, the hydroxylase activity decreases in the tumor and serum of treated mice. Administration of BrdU was most effective for the first 2 weeks after treatment; subsequently tumor growth resumed.

Hydroxylase Activity in Clonal Cell Lines of Mouse Neuroblastoma C-1300 Tumors and in Tumors Derived from These Lines. The results in Table 2 shows that enzyme activity is higher in clonal cell line N-18-G than in line Neuro-2a-G. Table 2 also shows that the cells of the line with higher activity (N-18-G) have a greater tendency to produce axon-like processes than does the line with lower activity (Neuro-2a-G). The results in Table 3 show that tumors derived from mouse neuroblastoma cells maintained by continuous animal passage have a higher activity of hydroxylase than do tumors derived from neuroblastoma cells cultured for prolonged periods *in vitro* and then implanted into mice. The activity was significantly higher in tumors derived from cultured cell line N-18-G than in tumors from the cultured cell line Neuro-2a-G or from the uncloned cell line. Enzyme activity in the serum was also higher in mice bearing tumors derived from the C-1300 mouse neuroblastoma than in mice bearing tumors derived from the cultured cell lines.

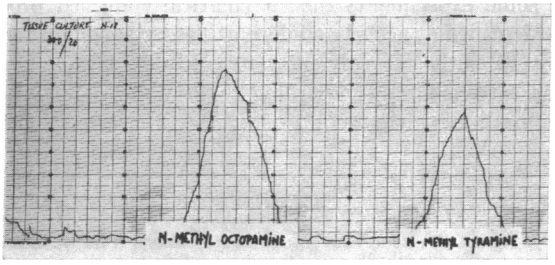

FIG. 2. A radiochromatogram of acetylated [^{14}C]amines isolated from incubations with homogenates of cultured cells (N-18-G line). Similar radiochromatograms were obtained from incubations with homogenates of C-1300 tumor tissues. The radioactive products obtained from six dopamine-β-hydroxylase assays were pooled and, after evaporation of the organic phase, the [^{14}C]amines were acetylated and chromatographed as described in the *text*. The nonradioactive standards used were acetylated *N*-methyl-octopamine and acetylated *N*-methyl-tyramine.

TABLE 1. *Effect of treatment with BrdU on growth of C-1300 mouse neuroblastoma and on dopamine-β-hydroxylase activity in tumor and serum*

Treatment	Weight of tumor (g)	Hydroxylase activity: Tumor (nmol/tumor)	Hydroxylase activity: Serum (nmol/ml)
None	1.67 ± 0.40	297.3 ± 8.0	18.4 ± 2.5
BrdU	0.44 ± 0.08	70.7 ± 2.0*	3.6 ± 0.5*
None	2.23 ± 0.70	445.3 ± 12.7	19.5 ± 3.0
BrdU	0.32 ± 0.06	37.3 ± 2.4*	2.9 ± 0.4*

Mice were treated for 13 days with BrdU (3 mg/kg, intraperitoneally, in two daily doses), starting on day 4 after the transplantation. Values represent mean (±SE) of six mice.

* Significantly different from the untreated animals; $P > 0.01$, by Students *t*-test.

Identification of the Enzymatically Formed Products. Upon acetylation of the [^{14}C]amines formed during incubation of homogenates of C-1300 tumor tissues or of cultured cells from the N-18-G line, two radioactive compounds were obtained with the same chromatographic mobilities as authentic acetylated *N*-methyl-octopamine and *N*-methyl-tyramine (Fig. 2). From the blank samples (incubations with boiled tumor tissues or boiled culture cells) only one radioactive [^{14}C]amine was isolated. This had the chromatographic mobility of authentic acetylated *N*-methyl-tyramine, the product that results from *N*-methylation by phenylethanolamine-*N*-methyl-transferase of the substrate tyramine.

TABLE 2. *Dopamine-β-hydroxylase activity in mouse neuroblastoma cell culture lines**

Cell line	% Differentiated cells	Hydroxylase activity† (nmol/mg protein)
N-18-G	60–70	0.60 ± 0.07
Neuro-2a-G	10–20	0.15 ± 0.03

* Neuroblastoma cells in logarithmic growth were assayed on the seventh day of incubation. T-flasks of 70-cm^2 surface area were inoculated with about 5 × 10^5 cells in 20 ml of medium. At harvest, about 3 to 4 × 10^6 cells were obtained from both lines.

† Values are expressed as mean ±SE of five experiments.

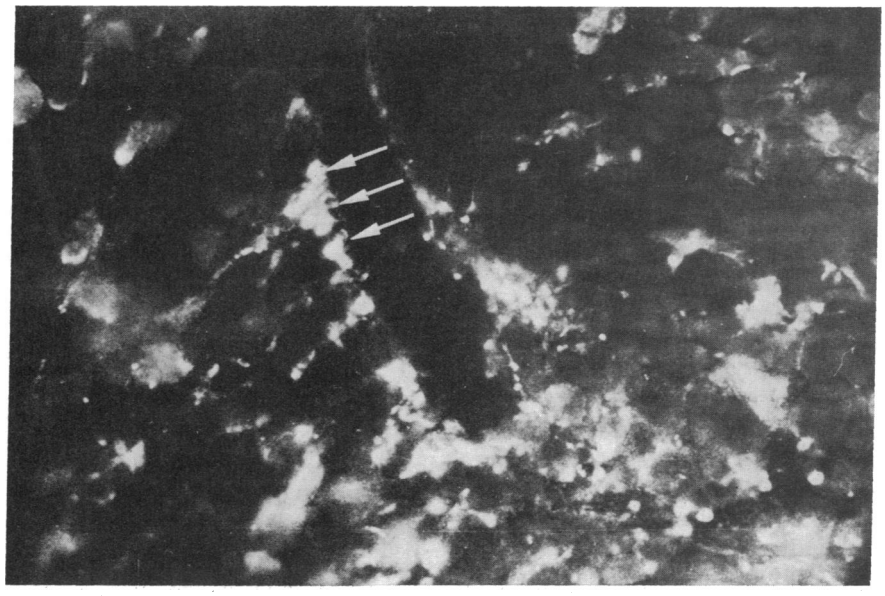

FIG. 3. Localization of catecholamine-containing cells in mouse C-1300 neuroblastoma tumors. ×160.

Histochemical Localization of Catecholamine-Containing Cells in Mouse C-1300 Neuroblastoma Tumors. The fluorescence specific for catecholamines was observed in 1–5% of tumor cells 14–21 days after implantation. Some of these cells exhibited weak green fluorescence, while others showed strong green fluorescence that was localized in the cytoplasm. The cells had strong fluorescent processes that were relatively thick (2–3 μm) and seemed mainly to terminate around the very fine blood vessels of the tumors (Fig. 3).

DISCUSSION

This study shows that hydroxylase activity is present in mouse neuroblastoma C-1300 tumors, and that the activity is proportional to the size of the tumor. The activity in the serum of mice bearing C-1300 tumors is higher than in the serum of control mice. In separate experiments we have shown that 24–48 hr after surgical removal of tumors, the serum hydroxylase concentrations were reduced to almost the same values as in control mice (unpublished data). These findings demonstrate that the elevated serum hydroxylase activity is due to the secretion of the enzyme by the neural tumor.

A number of studies support the idea that the enzyme is released from sympathetic nerves together with catecholamines. The activity of hydroxylase in serum is increased in a stressful situation, in which catecholamine excretion is increased, and the activity is decreased by destruction of sympathetic nerve terminals (13). However, adrenalectomy in animals does not lower the activity of the hydroxylase in serum (unpublished data). The findings that catecholamine-containing processes of the tumor cells seemed to terminate mainly around very fine blood vessels may indicate that the enzyme and catecholamines are released from these processes into the circulation. The histochemical studies indicate that neuroblastoma C-1300 tumors contain a mixed population of cells and that only a small number of cells contain catecholamines. In tumors derived from passage of different cultured cell lines the number of catecholamine-containing cells may vary.

Hydroxylase activity in human serum rises progressively with age; infants under 1 year of age have activities at the lower limits of sensitivity of the assay (14, 15). Serum hydroxylase activities can have a meaningful evaluation in clinical studies only when compared with those of control subjects of the same age group. We have shown that the determination of serum hydroxylase activity could be of value in clinical diagnosis of neuroblastoma (16). Some neuroblastoma

TABLE 3. *Dopamine-β-hydroxylase activity in serum and in neuroblastoma tumors derived from different kinds of neuroblastoma cells*

Origin of tumor	Weight (g)	Hydroxylase activity		
		Serum, mmol/ml	Tumor	
			nmol/tumor	nmol/mg protein
C-1300 tumor cells	2.30 ± 0.07	13.6 ± 3.1	141.5 ± 22.5*	1.37
Uncloned culture line	0.95 ± 0.11	1.1 ± 0.2	9.7 ± 2.3	N.E.
N-18-G culture line	0.62 ± 0.06	2.2 ± 0.5	19.2 ± 1.8†	0.88
Neuro-2a-G culture line	0.87 ± 0.23	0.8 ± 0.2	10.4 ± 3.2	0.34

Values expressed as mean ± SE of seven mice.

* Significantly different from the tumors derived from the cultured cell lines; $P > 0.001$.

† Significantly different from the tumors derived from the uncloned culture line or from the Neuro-2a-G culture line; $P > 0.05$. N.E., not estimated.

patients have extremely high values of serum hydroxylase, and there is good correlation between serum hydroxylase activity and urinary excretion of the norepinephrine metabolite, vanillylmandelic acid; but not between serum hydroxylase activity and urinary excretion of dopamine (16). Various factors might contribute to the wide range of serum hydroxylase activities in neuroblastoma patients. One explanation might be that the ratio of cells containing dopamine to those containing norepinephrine–dopamine–β-hydroxylase varies in human neuroblastoma tumors.

Administration of BrdU to neuroblastoma-bearing mice results in a noticeable slowing of tumor growth in the first 2 weeks after treatment. We have shown that 6-hydroxydopamine retards tumor growth (17), but treatment with BrdU seems to be far more effective. BrdU induces process formation in mouse neuroblastoma C-1300 cells, and BrdU-treated cells more closely resemble mature neurons (18). BrdU can be incorporated into DNA in place of thymidine, and it has been argued that BrdU alters the phenotype of the cell by such substitution of DNA (19). Although the mechanism of the effects of BrdU on the growth of neuroblastoma is still obscure, it is conceivable that either the higher production of axon-like processes induced by BrdU or the incorporation of the BrdU into the DNA is responsible for its activity.

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