A Human Neuroblastoma Cell Line with an Altered Ornithine Decarboxylase*

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A human neuroblastoma cell line (Paju) was resistant to 10 mM difluoromethylornithine, a concentration at which the growth of all mammalian cells normally stops. Ornithine decarboxylase from Paju was very resistant to inhibition by difluoromethylornithine in vitro (Kᵢ = 10 μM compared to 0.5 μM for mouse kidney ornithine decarboxylase). After purification, apparently homogenous Paju ornithine decarboxylase was inactivated with [3H]difluoromethylornithine and analyzed by polyacrylamide gel electrophoresis. Under denaturing conditions it was found to have an altered molecular structure, i.e., two nonidentical subunits of M₄ = 55,000 and 60,000. Another unusual feature of Paju ornithine decarboxylase was its long half-life in vivo (T½ = 8 h compared with 36 min in human HL-60 promyelocytic leukemia cells). The disappearance of immunoreactive protein was only slightly slower than the loss of catalytic activity. The long half-life of Paju ornithine decarboxylase was not shared by adenosylmethionine decarboxylase. The Paju karyotype did not contain double minute chromosomes or any large homogenously staining region such as that seen in a mouse lymphoma cell mutant that is resistant to difluoromethylornithine and overproduces ornithine decarboxylase (McConlogue, L., and Coffino, P. (1983) J. Biol. Chem. 258, 12083–12086).

Ornithine decarboxylase (EC 4.1.1.17) catalyzes the decarboxylation of ornithine to putrescine in the initial step of polyamine biosynthesis (1, 2). It has been suggested that this pathway is controlled by rapid changes in the level of ornithine decarboxylase, since the enzyme has a very short half-life (3) and its amount appears to be controlled directly by the rapid modulation of its rate of synthesis (2, 4, 5) and degradation (2, 6–8).

Recently a method has been reported (6, 9, 10) for specific labeling of ornithine decarboxylase with radioactive difluoromethylornithine (an enzyme-activated irreversible inhibitor of ornithine decarboxylase) (11). The labeling method has facilitated studies of the amount and molecular structure of ornithine decarboxylase in various systems (6, 10, 12, 19), where the enzyme is present in very low quantities even after its induction (14, 15). In this paper we demonstrate the use of [3H]difluoromethylornithine in studying ornithine decarboxylase in a human neuroblastoma cell line which shows both altered amounts and an altered structural form of ornithine decarboxylase.

EXPERIMENTAL PROCEDURES

Materials—[5-3H]Difluoromethylornithine (7.8 Ci/mmol) was purchased from New England Nuclear, and L-[1-14C]ornithine, S-adenosyl-L-[carboxy-14C]methionine (62 mCi/mmol), and L-[14C]methionine (286 mCi/mmol) were from Amersham. Unlabeled α-difluoromethylornithine was a generous gift from Merrell-Dow, Cincinnati, OH. Other biochemicals were obtained from Sigma.

Paju Cell Line—The neuroblastoma cell line was established from the pleural fluid of a sixteen-year-old girl who had a wide-spread metastatic tumor. She received several cytostatic treatments with different drugs, which did not inhibit the growth of the tumor. The tumor cells have now been cultivated for eight months and they grow predominantly in suspension cultures with a few adherent cells. The addition of retinoic and of neural growth factor causes a differentiation characterized by surface adherence and the acquisition of ganglion cell morphology with slender dendritic processes. This differentiation and other cytobiological properties will be reported in detail elsewhere. Both Paju cell line and HL-60 promyelocytic leukemia cells (16) were grown in RPMI-1640 medium supplemented with 10% fetal calf serum.

Purification of Ornithine Decarboxylases—Omnithine decarboxylase from the kidneys of 150 mice was purified as described earlier (14). Paju ornithine decarboxylase was purified using the same method as described for mouse kidney ornithine decarboxylase (14) from 5.8 g of cells (wet weight) grown in a volume of 6.2 RPMI medium. The purification yielded a homogenous preparation of the enzyme as judged by the binding of [3H]difluoromethylornithine by the enzyme (9). The extent of purification was 1,200-fold and the specific activity of the purified Paju ornithine decarboxylase was 1,520,000 nmol/30 min/mg of protein. Antiserum against mouse kidney ornithine decarboxylase was produced as described earlier (6, 10).

Preparation of [5-3H]Difluoromethylornithine-labeled Ornithine Decarboxylases—The purified preparations were treated with [3H]difluoromethylornithine as described in Ref. 6 except that for Paju ornithine decarboxylase 20 μM of [3H]difluoromethylornithine was needed to obtain enough radioactive ornithine decarboxylase for gel electrophoresis.

Immunotitration—Immunotitration of ornithine decarboxylase from Paju and HL-60 cells were carried out by incubating varying amounts of antiserum (raised in rabbit against mouse kidney ornithine decarboxylase) or control serum with enzyme in a volume of 200 μl for 30 min at room temperature. To the mixture was then added 100 μl of a 10% suspension of staphylococci. Finally, the mixture was centrifuged at 45,000 × g for 30 min, and the supernatant was used to determine the activity of ornithine decarboxylase. In the half-life studies, where the amount of immunoreactive protein was determined after the addition of cycloheximide, it was shown that protein synthesis was totally inhibited as judged by the incorporation of [14C]methionine into protein.

Gel Electrophoresis—Polyacrylamide gel electrophoresis under denaturing conditions (17) for detection of radioactive ornithine decarboxylase in gel slices was carried out as indicated in Ref. 14.

Chromosome Analysis—The chromosome preparations were made by conventional methods and stained by a modification of the Giemsa-binding method (18).

Uptake Studies of [3H]Difluoromethylornithine—HL-60 and Paju
cultures were incubated in the presence of 2.5 μM tr-[5-3H]difluoromethylornithine for 30, 60, and 120 min at 37 °C. Then the cells were centrifuged and washed twice with ice-cold 0.9% saline containing 25 mM unlabeled difluoromethylornithine.

Other Methods—Ornithine decarboxylase activity was determined as in Ref. 19 and that of adenosylmethionine decarboxylase as in Ref. 20. Protein was determined by the method of Bradford (21) using bovine serum albumin as standard.

RESULTS

The initial reason for this study was the finding that Paju cells grew for at least 6 days in 10 mM difluoromethylornithine, which is normally enough to stop the growth of mammalian cells (4). Subsequent studies (not shown) showed that DNA synthesis was not inhibited by 10 mM difluoromethylornithine. However, HL-60 (sensitive to difluoromethylornithine) (22) and Paju cells took up difluoromethylornithine at about the same rate (3.3 × 10^6 cpm and 3.1 × 10^6 cpm in 120 min for Paju and HL-60 cells, respectively), showing that the inability of difluoromethylornithine to inhibit the growth of Paju cells could not be explained by the decreased uptake of difluoromethylornithine.

Paju ornithine decarboxylase was more resistant than mouse kidney ornithine decarboxylase to inhibition by difluoromethylornithine in vitro when both enzymes were purified to apparent homogeneity and then tested with difluoromethylornithine. Fig. 1 shows that ornithine decarboxylase from Paju cells was very resistant to difluoromethylornithine, since 10 μM difluoromethylornithine was needed to produce 50% inhibition whereas only 0.5 μM difluoromethylornithine was needed in the case of mouse enzyme. Fig. 1 also shows that the degree of inhibition caused by difluoromethylornithine did not change during the purification of either Paju or mouse kidney ornithine decarboxylase, suggesting that Paju cell extracts do not contain any material that binds difluoromethylornithine and prevents it from reacting with ornithine decarboxylase, and that the relative insensitivity of purified Paju ornithine decarboxylase is not due to a change during purification.

During the growth of Paju cells, ornithine decarboxylase activities reached very high levels, sometimes up to 2,000 nmol/mg protein/30 min; 0, ornithine decarboxylase from 100,000 cells; (specific activity, 216 nmol/mg protein/30 min); (specific activity, 200 nmol/mg protein/30 min). DFM0, difluoromethylornithine.

![Fig. 1. Effect of the concentration of difluoromethylornithine on the inactivation of ornithine decarboxylase from Paju cells and mouse kidney. The enzyme was obtained from Paju cells and mouse kidney as described under "Experimental Procedures." The enzyme was incubated for 60 min at 37 °C in 25 mM Tris/HCl, pH 7.5, 5 mM dithiothreitol, 0.02% Brij-35, 0.04 mM pyridoxal phosphate, and the indicated concentrations of difluoromethylornithine. Ornithine decarboxylase activities were then assayed (19). The results shown are means of three determinations for each point. Activity is expressed as a percentage of that present in samples incubated without difluoromethylornithine. Decay of the activity in the absence of difluoromethylornithine was less than 10% as shown earlier (8). A, ornithine decarboxylase from 100,000 × g supernatant of mouse kidney (specific activity, 216 nmol/mg protein/30 min); B, homogenous mouse kidney ornithine decarboxylase (specific activity, 145,000 nmol/mg protein/30 min); C, ornithine decarboxylase from 100,000 × g supernatant of Paju cells, (specific activity, 200 nmol/mg protein/30 min); D, homogenous Paju ornithine decarboxylase (192,000 nmol/mg protein/30 min). DFM0, difluoromethylornithine.](image)

DISCUSSION

There are already established cell lines that are resistant to difluoromethylornithine or to its derivatives. In most cases of radioactivity, corresponding to molecular weight of 60,000 and 55,000, whereas mouse kidney ornithine decarboxylase gave only one band with a molecular weight of 54,000 as reported earlier (6, 14).

Since McConlogue and Coffino (24) recently reported a mouse lymphoma cell line that was resistant to difluoromethylornithine because of the overproduction of ornithine decarboxylase due to amplification of ornithine decarboxylase gene (24-26), we studied the karyotype of Paju cells. Fig. 5 shows the chromosomal constitution of the Paju cell line. We could not find any homogenously staining region or double minute chromosomes indicative of gene amplification although some other interesting changes were seen. One of these is trisomy for chromosome 8. The chromosome carries the gene for c-myc oncogene (27). Our results indicate that the stabilization of Paju ornithine decarboxylase against intracellular degradation mechanism(s) is a very important factor which may explain its high intracellular level.
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**Fig. 2.** Catalytic activity and immunoreactive protein in Paju cells (A) and HL-60 cells (B) following the administration of cycloheximide (20 μg/ml) to the culture medium. Paju cells and HL-60 cells (grown to maximum density in suspension culture) were diluted to 5 x 10⁶ cells/ml 18 h prior to the addition of cycloheximide at zero time. At the indicated time, samples (5 x 10⁶ cells for Paju and 1 x 10⁶ for HL-60) were taken for the determination of ornithine decarboxylase activity and immunoreactive protein. Specific activities of ornithine decarboxylase at the start of the experiments were 1000 nmol/mg protein/30 min for Paju and 2.1 nmol/mg protein/30 min for HL-60. Immunoreactive ornithine decarboxylase was determined as described under “Experimental Procedures.” In parallel cultures it was shown that general protein synthesis was stopped after addition of cycloheximide as judged by the incorporation of 1-[U-¹⁴C]methionine in total protein in both cell lines. The decay lines of the activities and that of immunoreactive protein were computed by the least-squares method. A, 0, Paju ornithine decarboxylase activity; O, Paju immunoreactive ornithine decarboxylase. B, O, HL-60 ornithine decarboxylase activity; O, HL-60 immunoreactive ornithine decarboxylase. ODC, ornithine decarboxylase.

**Fig. 3.** Half-life of S-adenosylmethionine decarboxylase catalytic activity in Paju and HL-60 cells after the addition of cycloheximide to cultures. Details as in Fig. 2. Specific activities at the start of the experiments were 0.8 nmol/mg of protein/30 min for Paju and 1.1 nmol/mg of protein/30 min for HL-60. O, Paju enzyme activity; O, HL-60 enzyme activity.

**Fig. 4.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified [³H]difluoromethylornithine-labeled ornithine decarboxylase. The Paju ornithine decarboxylase and mouse kidney enzyme were prepared as described under “Experimental Procedures.” Portions of dialyzed radioactive protein solution were then subjected to polyacrylamide gel electrophoresis under denaturing conditions and the gel was then sliced into 2-mm slices and radioactivity was determined as described earlier (14, 15). The standard protein markers (arrows) were phosphorylase b (94,000), bovine serum albumin (64,000), ovalbumin (43,000), and carbonic anhydrase (30,000). DFMO, difluoromethylornithine; ODC, ornithine decarboxylase.

**Fig. 5.** Giemsa-banded karyotype of the Paju cell line. Arrows indicate approximate breaking points of the abnormal chromosomes; the arrowhead shows a marker chromosome (12a) placed separately.
the resistance is due to the increased synthesis of ornithine decarboxylase (24, 28, 29) although one cell line has increased ornithine decarboxylase level due to a slow degradation of enzyme protein (29, 30). However, studies with [3H]difluoromethylornithine did not reveal any changes in the structure of ornithine decarboxylase in this cell line compared to the parent hepatoma cell line (30). Thus, it appears that our report is the first to show an increased level of ornithine decarboxylase in a malignant cell type associated with change(s) in the molecular structure of the enzyme, revealed by the use of [3H]difluoromethylornithine. Our cell line is also interesting since there was no prior selection for its ability to grow in the presence of difluoromethylornithine, as has been used earlier to obtain difluoromethylornithine-resistant mouse lymphoma cell mutants (24–26). The Paju cell line was established directly from a patient who had received treatments with different cytostatic drugs, none of which, however, is known directly to inhibit polyamine biosynthesis. 1 So, it appears that difluoromethylornithine resistance can arise in vivo without exposure to drugs inhibitory to polyamine biosynthesis.

The striking change in ornithine decarboxylase from Paju cell is its nonidentical subunits, since all other ornithine decarboxylases studied at the molecular level have identical subunits (6, 14, 24, 30). Since the purification procedure used yielded a homogenous ornithine decarboxylase for mouse kidney enzyme, it is unlikely that the smaller subunit of Paju ornithine decarboxylase represents proteolytic degradation. One might speculate that this modification might lead to a prolonged half-life of ornithine decarboxylase in Paju cells. The increased stability is specific for ornithine decarboxylase in the sense that it is not shared by adenosylmethionine decarboxylase, which has a short half-life in various systems (2, 6, 23) and here showed a similarly short half-life in both Paju and HL-60 cells.

In both cell lines the half-life of the antigen was longer than its catalytic activity, in agreement with results for rat liver (31). This finding fits well with the idea that degradation of the enzyme molecule proceeds very rapidly once the molecule has lost its catalytic activity.

Difluoromethylornithine has been used experimentally in the treatment of cancer patients (32) in the hope of finding an effective anticancer drug. Our results show that cancer cells become resistant to difluoromethylornithine even without exposure to this drug. This indicates a limitation to the clinical usefulness of difluoromethylornithine since it is reasonable to predict that if the use of ornithine difluoromethylornithine becomes more popular, then more difluoromethylornithine-resistant cell lines will be found.

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