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A Microassay for Argininosuccinase in Cultured Cells

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A current difficulty with the prenatal diagnosis of inborn errors of metabolism is the relatively large numbers of cultured amniotic fluid cells necessary for most enzyme assays. Faced recently with the problem of diagnosing the fetus in a family at risk for recurrence of argininosuccinic aciduria, a rare autosomal recessive disease characterized biochemically by a deficiency of argininosuccinase (ASase) [1], we developed a simple microassay for ASase deficiency in cultured cells based on the uptake of ^{14}C-citrulline into protein. This indirect assay enabled us to distinguish fibroblasts normal, heterozygous, or homozygous for ASase deficiency, and to conclude tentatively that the fetus at risk was not affected with argininosuccinic aciduria.

MATERIALS AND METHODS

Cell Culture

Fibroblast lines were derived by standard procedures from skin biopsies and amniotic fluids. In the family referred for prenatal diagnosis of argininosuccinic aciduria, cell lines were derived from the father (line 318), mother (line 314), and two affected siblings (lines 315 and 319), as well as from the fetus (line 313) following amniocentesis at 15 weeks. Also available were lines derived from patients with argininosuccinic aciduria in three other families (lines 43, 47, and 199) [2; V. E. Shih, unpublished].

Cells were grown in Eagle's minimal essential medium or in arginine-free Eagle's medium (Grand Island), each supplemented with 15% fetal calf serum and nonessential amino acids. Since fetal calf serum contains about 43 μM arginine (V. E. Shih, unpublished), the latter medium was not strictly arginine-free, but it sufficed for the present studies.

^{14}C-Citrulline Uptake Assay

For the uptake assay, 10^5 cells in Eagle's medium were inoculated into 30 mm Falcon plastic petri dishes containing 22 mm cover slips. Then 48 hr later, when the cells were

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growing exponentially, the dishes were re-fed with 1 ml low-arginine medium supplemented with 1 μc/milliliter L-ureido-^{14}C-citrulline (3.5 mc/millimolar; New England Nuclear). After 4 hr or more incubation at 37° C, the cover slips were washed once with cold phosphate-buffered saline, extracted with cold 5% trichloroacetic acid (TCA) for 5 min, rinsed three times in cold 5% TCA and once in acetone, air-dried, and counted in a Nuclear Chicago model 680 liquid scintillation counter with an efficiency of 88%. All assays were performed in duplicate and included controls labeled in the same manner at 0° C, which were subtracted from the 37° C values. Replicate cover slips were trypsinized at the end of the labeling period, and the cell numbers were counted in a Coulter counter, Model A. Essentially all the ^{14}C-citrulline counts incorporated in 4 or 24 hr appeared to be in protein, since they were resistant to hot 5% TCA digestion (90° C for 20 min). They were assumed to be in arginyl residues, as Schimke [3] demonstrated for HeLa cells, since the urea cycle is incomplete in fibroblasts, which can neither degrade arginine to ornithine and urea nor citrulline to ornithine and carbamyl phosphate.

Enzyme Assay

Argininosuccinase was measured in extracts of confluent monolayers as the rate of urea synthesis from argininosuccinic acid in the presence of excess arginase, as previously described [2].

RESULTS AND DISCUSSION

Cultured fibroblasts normally require arginine for growth. This requirement cannot be satisfied by ornithine, since the cells lack ornithine transcarbamylase [4]; but it can be satisfied by citrulline, since fibroblasts contain both argininosuccinic acid synthetase (ASA synthetase) [4] and ASase [2]. On the other hand, fibroblasts deficient in either of these enzymes should not be able to utilize citrulline in place of arginine. This has already been demonstrated for an ASA synthetase–deficient line by Tedesco and Mellman [4], and in table 1 comparable data are presented for three ASase-deficient lines. In this and other experiments, the homozy-

TABLE 1

^{14}C-CITRULLINE INCORPORATION AND ASASE ACTIVITY IN SKIN FIBROBLASTS

Cell Line	ASase Genotype	Counts per Minute (10^5 Cells)	ASase (μmole/mg protein/hr)
16	+/+	1,195, 1,013	0.117*
23	+/+	1,180	...
288	+/+	1,695	...
314	+/−	949, 786	0.068
318	+/−	542, 364	0.074
43	−/−	116	0.002*
199	−/−	144	0.001
315	−/−	141, 109	0.010
319	−/−	36, 25	0.010

NOTE.—The counts represent TCA-precipitable material after 4 hr incubation with 1 μc/milliliter ^{14}C-citrulline, as described under Materials and Methods. Each number is an independent determination and represents the average of duplicate coverslips.

* Data taken from Shih et al. [2]. The average value for seven normal lines was 0.133 μmole/milligram protein per hour, with a range of 0.096–0.191 [5].

gous deficient lines from four unrelated kindreds all appeared "leaky," in that they incorporated from 3% to 12% as much ^{14}C-citrulline as normal fibroblasts in 4 hr and from 12% to 19% as much in 24 hr (data not shown). Direct enzyme assay also indicated a low level of ASase activity, although this activity was at the limit of detection by the spectrophotometric method used. Two heterozygous lines incorporated about half as much ^{14}C-citrulline as normal cells, although they showed considerable variation on repeated testing. The uptake and incorporation of ^{14}C-citrulline into protein, therefore, appears to distinguish all three fibroblast genotypes, namely, those normal, heterozygous, and homozygous with respect to ASase deficiency.

Normal amniotic fluid cells contain about half as much ASase activity as normal skin fibroblasts [5], and they incorporated about half as much ^{14}C-citrulline as fibroblasts (table 2). Cells (line 313) from the fetus which prompted the present

TABLE 2

^{14}C-CITRULLINE INCORPORATION AND ASASE ACTIVITY IN AMNIOTIC FLUID CELLS

Cell Line	ASase Genotype	Counts per Minute (10^5 Cells)	ASase (μmole/mg protein/hr)
95	+/+	782	0.068
320	+/+	584	0.095
331	+/+	596	...
313	(Present case)	403, 368	0.111

NOTE.—See table 1 and Materials and Methods for details.

study were examined late in their lifespan when they were dividing slowly. The rate of incorporation of ^{14}C-citrulline into protein was in the low normal or heterozygous range, while direct enzyme assay of these senescent cells gave a value higher than that expected for normal amniotic fluid cells earlier in the lifespan (table 2). The pregnancy was not interrupted, and a normal female infant was delivered at term. Cord blood analysis for ASase established the heterozygous state (G. J. Billmeier, S. V. Molinary, and R. S. Wilroy, unpublished).

This ^{14}C-citrulline uptake assay has advantages for prenatal diagnosis in terms of simplicity and the relatively small number of cells needed. The standard spectrophotometric assay requires at least 10^7 cells [2]. A more sensitive microassay has been described by Schimke, but preparation of ^{14}C-guanidino-L-argininosuccinic acid is necessary, and a concentrated cell extract is recommended [3]. In our uptake assay, only 10^5 cells were used per petri dish; thus a total of about 6×10^5 cells were needed for duplicate controls and experimental dishes. More recently, a second label, ^{3}H-leucine, has been included, with the results being expressed as the ratio of incorporation of ^{14}C-citrulline to that of ^{3}H-leucine. This double-label method reduces the total number of cells required (because cell counts on duplicate dishes are unnecessary) and corrects for variation in the rate of protein synthesis, easily

produced by various culture and cell conditions. Nonetheless, while the uptake assay appears promising, further data on its reproducibility and on the range of values for normal, heterozygous, and homozygous ASase-deficient amniotic fluid cells are needed before this assay can replace the direct measurement of ASase activity in the prenatal diagnosis of argininosuccinic aciduria.

SUMMARY

A simple microassay was developed for argininosuccinase in cultured cells based on the uptake of ^{14}C-citrulline into protein. The assay was employed in the prenatal diagnosis of argininosuccinic aciduria in cultured amniotic fluid cells from a fetus at risk for inheriting the disorder.

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