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Cell growth in arginine- and ornithine-deprived medium and conversion of glutamate to ornithine and arginine in rat hepatoma cells

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Summary. A subline growing in medium without arginine and ornithine was established from a rat Reuber hepatoma cell line (R-Y121B·cho). The subline designated R-Y117B·cho was able to grow in glutamine, arginine and ornithine-free, glutamate-supplemented medium. Arginine synthesis from glutamate requires four urea cycle enzymes and another two enzymes, glutamate semialdehyde dehydrogenase and ornithine aminotransferase. Since R-Y121B·cho cells have all the urea cycle enzymes, two other enzyme activities were determined. The activities of ornithine aminotransferase and glutamate semialdehyde dehydrogenase were similar in R-Y117B·cho and its parental R-Y121B·cho cells, but R-Y117B·cho cells had higher conversion of glutamate to arginine than parental cells.

Key words: Amino acids – Arginine – Cultured cell – Glutamate semialdehyde dehydrogenase – Ornithine – Ornithine aminotransferase

Introduction

Although arginine is synthesized from ornithine through citrulline and arginosuccinate, it is an essential amino acid in cultured cells. Most cultured cells can utilize citrulline as a precursor for arginine, but can not utilize ornithine. In a few cases, the cell growth in the arginine-free medium containing ornithine was reported in rat hepatoma cultures and hepatocyte-hepatoma hybrid cells (Yamamoto and Niwa, 1993). Ornithine is synthesized from glutamate through glutamate-γ-semialdehyde by glutamate semialdehyde dehydrogenase (L-glutamate-γ-semialdehyde: $NADP^+$ oxidoreductase, EC 1. 2. 1. 41) and ornithine aminotransferase (L-ornithine-2-oxo glutarate δ-aminotransferase, EC 2. 6. 1. 13) as shown Fig. 1. But, no cell line was reported to grow in arginine- and ornithine-free medium.

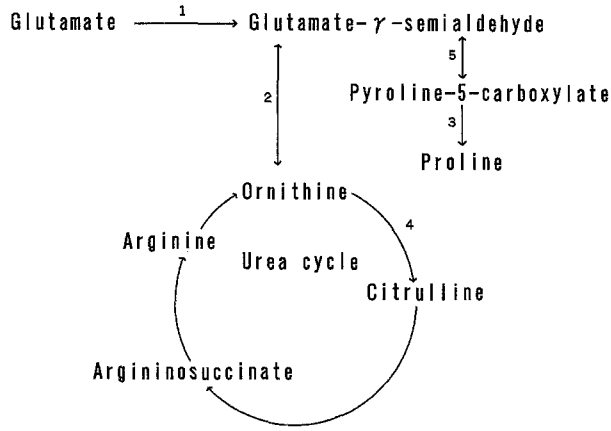

Fig. 1. Pathway of arginine and proline synthesis. The enzymes catalyzing the respective reactions are indicated by the numerals as follows: *1* glutamate semialdehyde dehydrogenase (EC 1. 2. 1. 41); *2* ornithine aminotransferase (ED 2. 6. 1. 13); *3* pyrroline-5-carboxylate reductase (EC 1. 5. 1. 2); *4* ornithine carbamoyltransferase (EC 2. 1. 3. 3). Step *5* is spontaneous

We have previously reported that R-Y121B cells derived from a rat Reuber hepatoma cell line, H4-II-E, have been grown serially in arginine-deprived and ornithine-supplemented medium (Niwa et al., 1980). From the R-Y121B cells, a choline- and inositol-prototroph was also established and named R-Y121B·cho (Yamamoto et al., 1985). R-Y121B cells and R-Y121B·cho cells can synthesize arginine from ornithine. In this paper, we examined whether these cultured cells synthesize arginine from glutamate and grow in arginine- and ornithine-free medium.

Materials and methods

Cell and cell culture

The rat hepatoma cell line R-Y121B·cho (Yamamoto et al., 1985) was a subline of Reuber hepatoma cell line H4-II-E (Pitot et al., 1964). The R-Y121B·cho was maintained in arginine, glutamine, tyrosine, choline- and inositol-deprived biotin- and ornithine-added Eagle's minimum essential medium (MEM; Eagle, 1959) in which there is no serum or lipid. All cell lines were grown in rubber-stoppered two-ounce glass bottles which were incubated at 37°C with renewal of the culture medium every 2 to 3 days. Cell suspensions were made by scraping off monolayer cells with a silicon-rubber policeman, and splitting them between two new bottles. The compositions of experimental media used growth experiments are shown in Table 1. For growth experiments, the cells (5×10^4) were planted in 16-mm wells (24-well culture plate, Corning 25820, Corning, NY) in 1 ml arginine-free medium. On day 1, the medium was replaced with ornithine medium or glutamate medium. Four samples were harvested at 3-day intervals. The growth rate was determined by measuring cell protein according to Lowry et al. (1951).

[^{14}C]-Glutamate incorporation

R-Y121B·cho cells and R-Y117B·cho cells (see Results) were incubated in 5 ml of glutamate medium with [^{14}C] L-glutamate (37 kBq/ml) at about 80% confluence. The cells were harvested at 24 hours, washed twice with a phosphate buffer saline without calcium

Table 1. Amino acid supplements in experimental media

	Arg	Orn	Glu
arginine-free medium	−	−	−
glutamate medium	−	−	+
ornithine medium	−	+	−

Basic medium is arginine, glutamine, tyrosine, choline, and inositol-derived and biotin-added Eagle's minimum essential medium (Eagle, 1959).

and magnesium (Dulbecco and Vogt, 1954) and suspended in 0.4 ml of water. The cell suspensions were then hydrolyzed in 6N HCl for 48 hours at 110°C. Amino acids derived from the protein were separated by two-dimensional TLC on silica gel 60F-254. The solvent system consisted of butanol:acetate:H_2O (2:1:1, by vol) in the first dimension and phenol: H_2O (3:1, by vol) in the second dimension. The amino acids were made visible by exposure to ninhydrin. The arginine, glutamate and proline areas were scraped from the plate and counted for radioactivity.

Enzyme assay

Glutamate semialdehyde dehydrogenase activity was assayed by a modification of a published method (Kramer et al., 1983). The assay mixture contained 0.1 M Hepes buffer (pH 7.4), 20 mM $MgCl_2$, 8.5 units phosphocreatine kinase, 3 mM ATP, 15 mM creatine-phosphate, 0.2 mM NADPH, 1 mM [^{14}C] L-glutamate (37 kBq/μmol), 1 mM gabaculine, 0.05% NP-40 and 0.5–2 mg cell protein in a total vol of 850 μl. The mixture was incubated at 20 °C for 30 min. The reaction was terminated by adding of 0.15 ml of the stop solution, 1.5 M sodium glycine, pH 10/1 M KOH (8: 7, by vol). Then 50 μl of 240 mM $NaBH_4$ dissolved in 5 mM NaOH was added. After 20 min at room temperature 50 μl of 200 mM proline was added. The mixture was boiled for 5 min and then centrifuged at 3,000 × g for 10 min. The supernatant was applied with a Pasteur pipette to an AG1-X8 (acetate) column (0.7 × 4 cm) and the column was rinsed three times with 1 ml of H_2O. All the eluate from the column was collected, 15 ml of scintillant was added and the radioactivity was determined.

Ornithine aminotransferase activity was measured by a modification of the method of Matsuzawa et al. (1968). The assay mixture contained 50 mM phosphate buffer (pH 8.0), 20 mM ornithine, 10 mM α-keto glutalate, 20 μM pyridoxal phosphate and 0.1–0.6 mg cell protein in a total volume of 1 ml. The mixture was incubated at 37°C for 20 min. The reaction was stopped by adding 0.5 ml of ethanol and 0.5 ml of 10% TCA followed by 0.5 ml of o-aminobenzaldehyde in ethanol. After 30 min at room temperature, the assay mixture was centrifuged to remove precipitated protein and the optical density of the supernatant was measured at 443 mμ. The molar extinction coefficient (ε) of 2.71 × 103 was used to calculate the amount of the product, pyroline-5-carboxylate.

Reagents

L-[U-^{14}C] Glutamate was supplied by ICN Biomedicals Inc (Costa Mesa, CA) and was purified by cation and anion exchange chromatography. AG1-X8 and AG50W-X8 (200–400 mesh) were the products of Bio-Rad Laboratories (Richmond, CA). Creatine phosphokinase type I and gabaculine were the products of Sigma Chemical Co (St Louis, MO). All amino acids were purchased from Ajinomoto Co. (Tokyo, Japan) and vitamins were obtained from Nutritional Biochemicals Co (Cleveland, OH). All other chemicals were from Wako pure Chemical Industries (Tokyo, Japan). All culture media were prepared in our laboratory as described previously (Yasumura et al., 1978).

Results

Growth of R-Y121B·cho cell in arginine-free medium

Growth curves for the R-Y121B·cho in the arginine-free medium, ornithine medium and glutamate medium are shown in Fig. 2. The cells were able to grow in ornithine medium with population doubling times of 4 days. No cell growth occurred in either the arginine-free medium or the glutamate medium.

Establishment and growth characteristics of R-Y117B·cho cells

On April 1, 1988, R-Y121B·cho cells were plated at a density of 2×10^5 cells/ml in a 2-ounce glass bottle with glutamate medium. The culture medium was changed at about 4- or 7-day intervals. R-Y121B·cho cells proliferated slowly and were subcultured on August 5, 1988. In glutamate medium the cells were passaged 8 times at intervals of about 3 or 4 months. The passage intervals of the cells were gradually decreased until they were subcultured on about a 2 or 3 week schedule. The cells were designated R-Y117B·cho.

As shown in Fig. 3, R-Y117B·cho cells appeared similar in morphology to R-Y121B·cho cells. Fig. 4 shows the growth curves of R-Y117B·cho cells in arginine-free medium, ornithine medium and glutamate medium. R-Y117B·cho cells grew in ornithine medium or glutamate medium. In arginine-free medium, no cell growth occurred. The cells were grown in glutamate medium with a population doubling time of 5 days, whereas the population doubling time in ornithine medium was about 4.5 days.

[^{14}C] glutamate incorporation

To confirm that R-Y117B·cho cells really synthesize arginine from glutamate, cells were labeled with [^{14}C] glutamate. Parental R-Y121B·cho cells were used

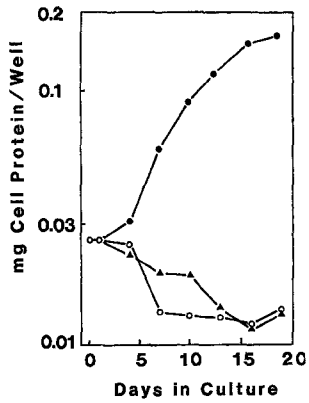

Fig. 2. Growth of R-Y121B·cho cells in medium without arginine. R-Y121B·cho cells grown in arginine-, glutamine-, tyrosine-, choline- and inositol-deprived and biotin- and ornithine-supplemented MEM (ornithine medium) without serum, protein and lipid were suspended in arginine- and ornithine-free media (○) at a density of 5×10^4 cells/ml, and each culture well was inoculated with 1 ml of the cell suspension. On day 1 the medium was replaced with a testing medium containing ornithine (●) or glutamate (▲)

as control cells. Table 2 shows the incorporation of the radiolabeled glutamate into arginine and proline in R-Y117B·cho cells. On the other hand, R-Y121B·cho cells incorporated [^{14}C] glutamate into arginine at a negligible level.

Enzyme activities

The activities of glutamate semialdehyde dehydrogenase and ornithine aminotransferase in R-Y121B·cho and R-Y117B·cho cells were measured in cell homogenates. As shown in Table 3, the enzyme activity of glutamate semialdehyde dehydrogenase and ornithine aminotransferase in R-

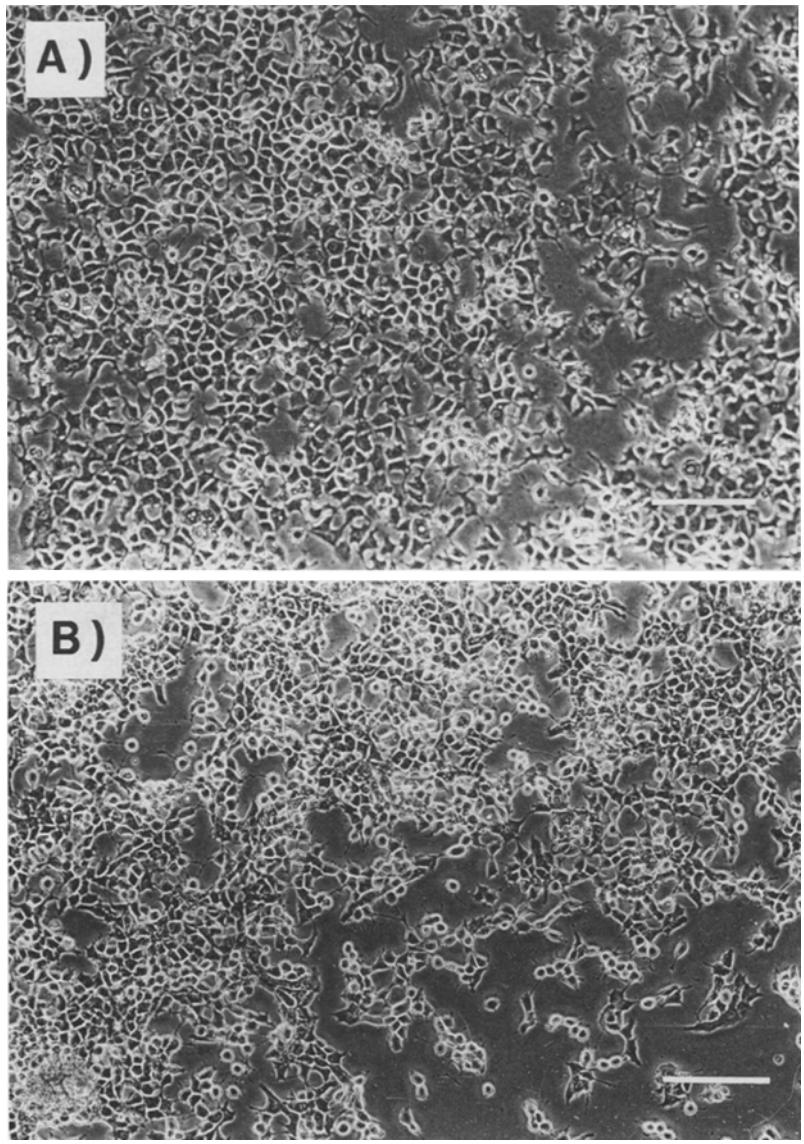

Fig. 3. Phase-contrast photomicrographs. **A** R-Y121B·cho; **B** R-Y117B·cho. Scale bar = 10μm

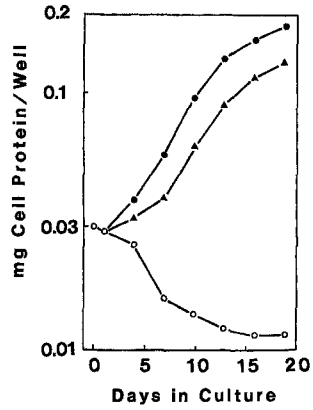

Fig. 4. Growth curves of R-Y117B·cho cells in medium without arginine. R-Y117B·cho cells were suspended and inoculated as described in Fig. 1. Arginine- and ornithine-free medium (○), arginine-free ornithine-supplemented medium (●) and arginine-free glutamate-supplemented medium (▲)

Table 2. Conversion of glutamic acid to arginine and proline

	Arginine	Proline	Glutamate
	$\times 10^4$ DPM/ day/mg cell protein		
R-Y121B·cho	0.02	8.07	1.23
R-Y117B·cho	1.88	9.64	3.3

Cell monolayers were incubated with ^{14}C-glutamate for 24 hours. The radioactivity of arginine, glutamate and proline was measured in acid hydrolysates of cell protein. The data are the means for two experiments.

Table 3. Enzyme activity of R-Y121B·cho and R-Y117B·cells

	Glutamate semialdehyde dehydrogenase (n = 8)	Ornithine aminotransferase (n = 8)
	pmol/ min/ mg protein	nmol/ min/ mg protein
R-Y121B·cho	20.0 ± 2.5	46.3 ± 4.2
R-Y117B·cho	22.0 ± 6.4	59.5 ± 6.3

Y117B·cho cells was 22 pmol/min/mg cell protein and 59.5 ± 6.3 nmoles/min/mg cell protein, respectively. The activity of the enzyme was almost the same in both cell lines.

Discussion

The R-Y121B·cho cells were chosen for this study because they synthesized arginine from ornithine and grew in arginine-deprived and ornithine-added

medium (Niwa et al., 1980; Yamamoto et al., 1985). We isolated a subline, R-Y117B·cho, which grows continuously in glutamate medium (Fig. 4). R-Y117B·cho cells synthesized arginine and proline from glutamate (Table 2). Parental R-Y121B·cho cells synthesized proline from glutamate but did not synthesize arginine.

We next looked for the formation of ornithine from glutamate in cell homogenates. The first step in ornithine synthesis is the reduction of glutamate by ATP and NADPH to form glutamate-γ-semialdehyde (Fig. 1). This reaction is catalyzed by the glutamate semialdehyde dehydrogenase. The enzyme is located in a mitochondrial membrane of rat intestine (Wakabayashi et al., 1991). Several culture cells are known to convert glutamate to proline (Kao and Puck, 1967; Shen and Strecker, 1975) but were not known to convert glutamate to ornithine. The sublines derived from H4-II-E cells have this activity (Table 3). The enzyme activity was similar in both R-Y121B·cho and R-Y117B·cho cells. The activity of glutamate semialdehyde dehydrogenase in rat liver was about 1/650 of that found in duodenum (Wakabayashi et al., 1991) but, R-Y117·cho cells had about the 1/20 activity found in duodenum. The enzyme activity in our cultured cells was not changed in growing cells in ornithine medium or glutamate medium, although the activity in cell homogenates was inhibited by ornithine (Lodato et al., 1981) and proline (Valle et al., 1973).

The second step in ornithine synthesis is the transamination of glutamate-γ-semialdehyde and glutamate to ornithine. This reaction is catalyzed by ornithine aminotransferase which is a mitochondrial matrix enzyme widely occurring in mammalian tissues (Peraino and Pitot, 1963; Herzfeld and Knox, 1968). R-Y117B·cho cells had about 5-fold higher ornithine aminotransferase activity than that in liver (12.6 nmol/min/mg protein). Katunuma et al. (1965) and McGivan et al. (1977) have shown that ornithine aminotransferase in liver is involved in ornithine degradation rather than ornithine synthesis, but Herzfeld and Knox (1968) and Volpe et al. (1969) have suggested that this enzyme in liver functions primarily to provide ornithine for use in the urea cycle. Our results show that ornithine aminotransferase functions to provide ornithine for synthesis in the arginine. Jones (1985) stated that ornithine (arginine) and proline could be used for synthesis of glutamate in liver, and there was no conversion of glutamate back to ornithine and proline. Our results showed that labeled glutamate was metabolized to proline and arginine in R-Y117B·cho cells. The ornithine aminotransferase that forms ornithine from glutamate-γ-semialdehyde is reversible, although it proceeds solely toward glutamate-γ-semialdehyde. The glutamate-γ-semialdehyde is in chemical equilibrium with its enamine, pyrroline-5-carboxylic acid. The chemical equilibrium favors pyrroline-5-carboxylic acid formation (Schöpf and Steure, 1947), and the pyrroline-5-carboxylic acid is utilized as the precursor of proline. The reaction of citrulline synthesis through ornithine from glutamate takes place in mitochondria. The proline synthesis from pyrroline-5-carboxylic acid takes place in the cytosol of the cell. It is necessary for arginine synthesis that glutamate-γ-semialdehyde remain in the mitochondria and metabolize to citrulline via ornithine. It has been suggested

that compartmentation and coupling reactions may drive citrulline production. The enzyme activities of ornithine aminotransferase and glutamate semialdehyde dehydrogenase were similar in R-Y117B·cho and R-Y121B·cho cells (Table 3), but R-Y117B·cho cells had higher conversion of glutamate to arginine than R-Y121B·cho cells (Table 2). This may indicate that the concentration of glutamate semialdehyde in mitocondoria or the transport of glutamate and its metabolites across the mitocondorial membrane differs in these cells.

R-Y117B·cho cells could synthesize arginine from glutamate (Table 2), and had glutamate dehydrogenase (Yamamoto, unpublished data). This indicates that R-Y117B·cho cells could synthesize glutamate. The glutamate requirement of R-Y117B·cho cells should be caused by the limited biosynthetic capacity of glutamate.

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