CLONAL DIFFERENCES IN GLUTAMINE SYNTHETASE ACTIVITY OF HEPATOMA CELLS

Effects of Glutamine and Dexamethasone

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Most hepatomas contain only traces of glutamine synthetase (GS) although they originate from liver which has high levels of the enzyme (1–3) The HTC cell line which was derived from Morris Hepatoma 7288C retained certain characteristics of liver cells, notably the induction of tyrosine amino transferase (4, 5), while other liver characteristics, among them GS activity, could not be detected[1] Since HTC cells in culture apparently required glutamine for growth[1], the question arose whether the genetic information necessary for the synthesis of GS was absent or was present but for some reason was not expressed The present report shows that clones which produce glutamine synthetase and can survive without glutamine can be isolated from the HTC cell population Some of the properties of these clones are outlined

MATERIALS AND METHODS

Media

"Growth medium" was Swim's 77 medium (Grand Island Biological Co , Berkeley, Calif) containing 0.5 g/liter $NaHCO_3$, 0.05 M Tricine (Calbiochem, Los Angeles, Calif.), 5% (v/v) calf serum, 5% (v/v) fetal calf serum (Grand Island Biological Co), and 2 mM glutamine. "Growth medium without glutamine" contained dialyzed serum and 0.5 mM asparagine plus 0.5 mM glutamic acid instead of glutamine.

"Cloning medium" consisted of Swim's 77 medium containing 1.47 g/liter $NaHCO_3$, 0 02 M Tricine, 5% (v/v) calf serum, 5% (v/v) fetal calf serum, 2 mM glutamine, and 1 mM sodium pyruvate. "Cloning medium without glutamine" was made with dialyzed serum and contained 0.5 mM glutamic acid and 0.5 mM asparagine instead of glutamine.

[1] Tomkins, G. M., unpublished data.

Mass Culture

Cells were grown in suspension culture in complete growth medium as previously described (4, 6).

Cloning Procedure

An appropriate number of cells was suspended in 9 0 ml of cloning medium at 37°C in a 17 × 100 mm capped, sterile plastic tube (Falcon Plastics 2057, Falcon Plastics, Division of BD Laboratories, Inc., Los Angeles, Calif.). The cell suspension was then mixed rapidly with 1 0 ml of 3% (w/v) agar (Difco Laboratories, Inc., Detroit, Mich , purified) kept liquid at 47°C Tubes were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for 2–6 wk When selective medium was used, not more than 10^5 cells were added per tube.

Isolation of Clones

Single colonies were withdrawn from the soft agar with an 18 gauge needle, attached to a 1 ml syringe inserted from the top of the tube

Clones WT2 and WT17 were isolated from the stock HTC cell population in complete cloning medium. Clone GM22 was isolated in glutamine-free cloning medium from stock HTC cells which had been treated with 2×10^{-5} M *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) for 2 hr at 37°C and subsequently grown in complete growth medium for 3 days.

Analytical Methods

Tyrosine aminotransferase was assayed by the method of Diamondstone (7) adapted to the autoanalyzer. Protein was determined according to Lowry et al. (8).

Glutamine synthetase was determined by the following modification of the glutamotransferase assay (3, 9). About 10^7 cells were washed with 50 ml of 0.1 M sodium chloride buffered with 0.05 M sodium phosphate, pH 7.6, and sonicated with 0.5–1.0 ml of 0.01 M imidazole HCl, pH 6.6 by three 10-sec bursts of a Bronwill Biosonic sonicator (Bronwill Scientific, Inc., Rochester, N. Y.) at 20% output. The reaction mixture for enzyme assay (1 ml) contained 120 mM glutamine, 0.1 mM adenosine triphosphate (ATP), 10 mM Na_2HAsO_4, 50 mM imidazole, 30 mM hydroxylamine HCl, 1 mM $MnCl_2$; final pH 6.2, adjusted with HCl. After incubation for 30–60 min at 37°C the reaction was stopped with 0.75 ml of 8.75 (w/v) trichloroacetic acid, 0.875 N HCl, and 1.75% $FeCl_3$. The protein precipitate was removed by filtration and the absorbance at 500 mμ of the filtrate was compared with a standard curve prepared with glutamyl γ-hydroxamic acid (Sigma Chemical Co., St. Louis, Mo.). One unit is defined as the amount of enzyme which produces 1 μmole of glutamyl hydroxamic acid per hour. We assume that this glutamotransferase reaction adequately represents the GS activity of the cell extract (cf. 9).

RESULTS

About 1% of the cells of the stock HTC cell population survived and formed colonies in cloning medium without glutamine supplemented with 10^{-6} M dexamethasone (Table I). In the absence of dexamethasone, on the order of 0.1% of the cells formed colonies.

The high frequency of cells auxotrophic for glutamine suggested that the stock HTC cell population might be heterogeneous and contain some cells capable of surviving glutamine deprivation and others not able to survive. To examine this question, a sample of the stock HTC cell population was cloned in medium containing glutamine, and 12 colonies were selected at random. After growth of each in mass culture in the presence of glutamine, cells of each presumptive clone were tested for their ability to survive in glutamine-free medium. Various clones differed markedly in their ability to survive glutamine deprivation (Table I). One clone (WT2) had a much higher cloning efficiency (about 10%) in glutamine-free medium plus dexamethasone than the original HTC population while two others (e.g. WT17, Table I) had a much lower efficiency (0.1% or less, depending to some extent on the density of the inoculum). The other clones isolated had cloning efficiencies ranging between these extremes (e.g. WT19, Table I). The widely differing abilities of clones WT2 and WT17 to survive without glutamine were maintained after more than 30 generations in complete growth medium.

TABLE I

Cloning Efficiency of HTC Cell Clones in Glutamine-Free Medium Plus Dexamethasone

	Number of colonies per tube		
	10^5 cells/tube	5×10^2 cells/tube	5×10^2 cells/tube
HTC population	++*	42	3
Clone WT2	++++*	383	60
Clone WT19	+*	31	1
Clone WT17	159	0	0

Cells of clones WT2, WT17, and WT19 were grown in monolayer in 75-cm^2 T flasks and were washed with 10 ml of cloning medium without glutamine before suspension in the same medium. Samples containing the number of cells indicated were cloned in cloning medium without glutamine plus 10^{-6} M dexamethasone as described in Materials and Methods. HTC population cells were taken from the stock spinner culture and treated similarly.

* Colonies were too numerous to count. Pluses indicate relative frequencies.

Cells of clone WT17 had a low basal GS (glutamotransferase) specific activity which was not increased on transfer of the cells to glutamine-free medium with or without dexamethasone (Fig. 1).

On the other hand, when cells of clone WT2 were transferred from medium containing glutamine to medium without glutamine, GS specific activity increased about 10-fold within 4 days (Fig. 1). Dexamethasone accelerated the rate of increase of GS activity. In presence of the steroid, GS activity reached a maximum at about 20 times the initial level in 4 days. Cells of clones isolated from the HTC cell population in glutamine-free medium attained even higher maximum levels of GS than WT2 cells. Fig. 2 shows the kinetics of accumulation of GS activity in cells of such a clone, GM22. When glutamine was removed from the medium the GS specific activity increased up to 15-fold, reaching a maximum in 4 days. Addition of dexamethasone simultaneously with the removal

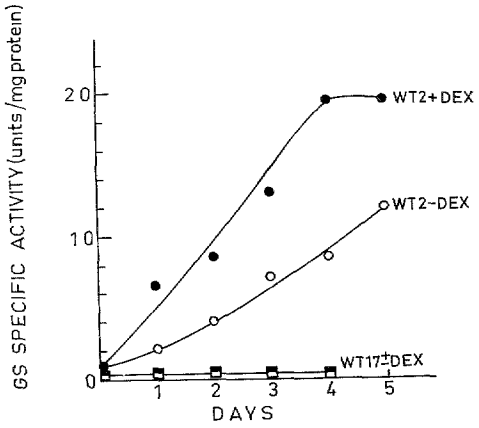

FIGURE 1 Effect of glutamine-free medium with or without dexamethasone on GS activity of WT2 and WT17 cells. About 10^8 clone WT2 cells grown in spinner culture in complete growth medium were sedimented and resuspended in 500 ml of growth medium without glutamine, containing 50 μg/ml neomycin and 2.5 μg/ml fungizone. After removal of a zero-time sample the suspension was divided into two equal portions, to one of which 10^{-6} M dexamethasone was added. Suspensions were maintained in spinner culture, and samples were removed daily for analysis of GS activity. Fresh medium was added when required to maintain cells at constant density. Cells of clone WT17 were grown and treated in the same way. ○——○, WT2; □——□, WT17 without glutamine; ●——●, WT2, ■——■, WT17 without glutamine plus dexamethasone.

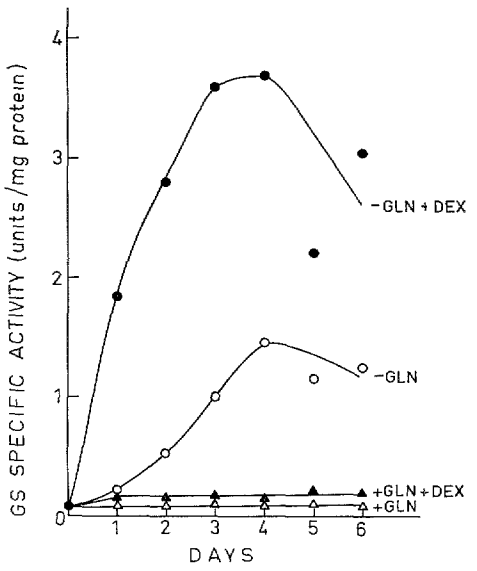

FIGURE 2 Effects of transfer to glutamine-free medium and/or dexamethasone addition on GS activity in GM22 cells. About 2×10^8 cells grown in spinner culture in complete growth medium were sedimented and suspended in 1 liter of growth medium without glutamine, containing 50 μg/ml neomycin and 2.5 μg/ml fungizone After removal of a zero-time sample the culture was divided into four equal portions, with the following additions. none, ○——○; 10^{-6} M dexamethasone, ●——●; 2 mM glutamine, △——△; 2 mM glutamine + 10^{-6} M dexamethasone, ▲——▲. Other details as for Fig 1

of glutamine greatly accelerated the increase in GS specific activity which rose up to 40-fold, reaching a maximum in about 3 days Maximum specific activity was maintained in some experiments but was followed by a fall in others (e.g Fig 2) Addition of dexamethasone in the presence of glutamine caused a two-fold increase in GS specific activity within a day (Fig 2)

In contrast to GS, tyrosine aminotransferase was inducible by dexamethasone in WT17 cells, as well as in WT2 and GM22 cells.

Addition of glutamine to cells maintained in glutamine-free medium plus dexamethasone caused a rapid decay of GS specific activity to about $\frac{1}{10}$ or less of its original level (Fig. 3) In short-term experiments GS activity decayed to half its original level about 5 hr after the addition of glutamine If dexamethasone was removed at the time of addition of glutamine, GS activity fell even lower to $\frac{1}{20}$th or less of the control level (Fig. 3). In contrast, removal of dexamethasoen without addition of glutamine caused a slower fall of GS specific activity which reached a steady-state level after several days

DISCUSSION

The results show that clones with different abilities to survive without glutamine could be isolated from the stock HTC cell population and that their properties were stable for many generations There seemed to be a correlation between the ability of clones to survive without glutamine and their ability to accumulate GS in glutamine-free medium A number of alternate explanations could account for the differences between the clones Each clone could be derived from a parent cell with a different degree of inducibility of GS If the character breeds true, then each clone would be homogeneous and composed of cells with the same inducibility of GS as the parent cell On the other hand, it is possible that the clones studied

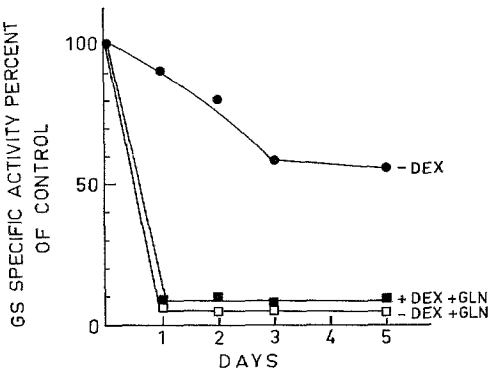

FIGURE 3 Effect of removal of steroid and/or addition of glutamine on GS activity of GM22 cells grown in glutamine-free medium plus dexamethasone. About 10^8 GM22 cells grown for 1 wk in growth medium without glutamine plus 10^{-6} M dexamethasone were washed with 100 ml of growth medium without glutamine, containing 2 5 μg/ml fungizone and 50 μg/ml neomycin, and resuspended in 1 liter of the same medium. After removal of a zero-time sample the suspension was divided into four equal portions containing, respectively, 10^{-6} M dexamethasone (control), no additions, ●——●; 2 mM glutamine, □——□; 2 mM glutamine + 10^{-6} M dexamethasone, ■——■. Data are expressed as per cent of control specific activity. Other details as for Fig. 1.

were heterogeneous and contained varying proportions of inducible and noninducible cells. Such a situation could arise, for example, if the clones differed in their rate of mutation to glutamine prototrophy and thus would accumulate different relative numbers of auxotrophic and prototrophic cells when grown under nonselective conditions. A third alternative is that cells of all clones have the potential to produce GS but that cells of clones with a low capacity to survive without glutamine are sensitive to sudden glutamine depletion in the medium and thus cannot produce GS under the test conditions used. Further work is required to distinguish between these alternatives

It is apparent, however, that the cells of the glutamine-dependent clone WT17, when seeded at high density, can give rise to glutamine-independent clones at quite a high frequency (Table I). Preliminary experiments indicate that cells of such subclones can continue to grow in glutamine-free medium Whether the relatively frequent conversion of glutamine-dependent to glutamine-independent cells represents a true genetic change or merely an alteration in gene expression (cf. 10, 11) remains to be seen. The results suggest that the loss of certain phenotypic properties from cultured cells may be reversible and that such a loss is therefore a reflection of alterations in regulation rather than being due to an irreversible loss of genetic information

In cells able to survive without exogenous glutamine, GS activity is apparently regulated by both glutamine and corticosteroids. It is not clear from the present data at what level these compounds control enzyme activity The doubling time of the cells was 24 hr while GS activity decayed to half its original level about 5 hr after adding glutamine. Thus, the drop in GS activity due to glutamine addition is too rapid to be accounted for simply by a shut off of enzyme synthesis followed by dilution but must involve inactivation or degradation of the enzyme

Other laboratories have shown that removal of glutamine from the medium enhances the GS (glutamotransferase) activity of HeLa cells (12) and L cells (13). Corticosteroids have also been shown to increase GS activity in L cells (14). The effects of both corticosteroids and glutamine on GS activity in L cells closely resemble those reported above for HTC cells Although, apart from similarity, there is no obvious connection with the behavior of tissue culture cells, it should be noted that corticosteroids induce GS in embryonic chick retina (15) while glutamine inhibits the induction of the enzyme (9)

It is uncertain whether the effects of glutamine and corticosteroids on HTC cells reflect similar regulatory mechanisms in normal liver. Even the maximum specific activities of GS attained by GM22 cells in the absence of glutamine and in the presence of dexamethasone were only 10–20% of the specific activity of GS in normal rat liver Claims that cortisol increases GS specific activity in neonatal liver (16) and hepatoma (17) but does not affect GS activity in adult liver (17) require confirmation. Dietary glutamine reportedly has no effect on hepatic GS activity (18).

SUMMARY

Clones derived from an established hepatoma cell line (HTC) differ in their abilities to survive without exogenous glutamine. Some clones isolated at random from the population had a high cloning efficiency (10%) in glutamine-free medium supplemented with dexamethasone while others cloned at a low efficiency (0 1%). The basis for

the variability of this character from one clone to another is not clear In cells able to survive without exogenous glutamine, dexamethasone increased glutamine synthetase activity while glutamine depressed it.

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