Control of Rat C_6 Glioma Cell Proliferation: Uncoupling of the Inhibitory Effects of Hydrocortisone Hormone in Suspension and Monolayer Cultures

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ABSTRACT We undertook a comparative study of the effects of the hormone hydrocortisone (Hy) on C_6 glioma cells grown in monolayer and in suspension in cultures. We found Hy reversibly renders C_6 cells anchorage- and serum-dependent for their growth. In monolayer cultures, Hy was found to inhibit cell cycle traversing exclusively at G_1 phase. In agarose suspension, Hy was found to block colony development. Hy-resistant variants were selected and isolated in agarose suspension. Examination of these variants showed that cells selected for Hy-resistance in suspension can be Hy sensitive when anchored to a solid substrate. We conclude that resistance to Hy in suspension and resistance to it in monolayer culture are two independent phenotypes.

Cell transformation is characterized by loss of anchorage and serum dependence for growth (1, 2). The relationship between anchorage and serum dependence is still obscure. Serum dependence reflects the cells' growth requirements for hormones and growth factors (3, 4, 5). One interpretation for anchorage dependence assumes no qualitative difference between anchored cells and cells in suspension as far as the types of serum factors required; only greater amounts would be required by cells in suspension (6). Another interpretation is that cell-substrate and cell-cell interactions modulate non-transformed cells' response to growth factors and hormones that regulate their proliferation (7). Only a better understanding of the actions of hormones and growth factors in both suspension and monolayer cultures will allow the elucidation of this important problem.

The growth of C_6 rat glioma cells (8) is inhibited by glucocorticoids (9) and stimulated by pituitary peptide growth factors (10, 11, 12). Results described here show that hydrocortisone (Hy), a glucocorticoid hormone, reversibly renders C_6 cells anchorage- and serum-dependent for their growth. Variant clones selected for resistance to Hy in suspension cultures were not resistant when anchored to a solid substrate. The data point to differences between hormonal action in suspension and that in monolayer cultures.

MATERIALS AND METHODS

Cell Culture: Stock cultures were regularly kept in 5% fetal calf serum (FCS)–95% Dulbecco's modified Eagle's medium (DME) as previously described (13). The strain of rat C_6 glial cells (8) used here came originally from the American Type Culture Collection (CLL 107, Rockville, MD). Variant ST1 was isolated in this laboratory (14) by subjecting cultures to serum-free medium and collecting cells that rounded up as previously described (15).

Hormones and Growth Factors: Hydrocortisone was from Sigma Chemical Co. (St. Louis, MO). Pituitary growth factor was prepared by Dr. A. G. Gambarini, in our laboratory, according to procedures described in references 13, 16, and 17. The preparation used in this work consisted of the CM-1 fraction (16, 17) that contains several protein bands and both acid and basic active growth factors.

Determination of Labeling Index and Mitotic Index: Cells were seeded at 2×10^4 cells/cm^2 onto glass coverslips in 5% FCS-DME. For MI determination, cells were fixed with 10% trichloroacetic acid (TCA) stained with Giemsa and examined under light microscope; at least 10 microscopic fields and 2,000 cells were counted per coverslip. For labeling index (LI) determinations, at each time point cells were pulse-labeled (30 min) with methyl-[^{3}H]thymidine (1 μCi/ml; 50 Ci/mmol). Coverslips were extracted twice with 10% TCA, washed with 95% ethanol, air dried, and covered with Kodak AR-10 stripping film. Percent of labeled nuclei was estimated by counting a minimum of 10 microscopic fields and at least 1,000 cells per coverslip.

Growth in Agarose Suspension: Cells were plated at different densities in 35-mm dishes containing a layer of 0.6% agarose, 10% FCS-medium. Plating medium consisted of 0.3% agarose and 10% FCS. Liquid 10% FCS-medium (1 ml/dish) was added 24 h after plating and renewed, by gentle aspiration, every 2 to 4 d. Plating efficiency was determined at 21 d by counting macroscopic (1–3-mm diameter) colonies. In the presence of Hy, in addition to the macroscopic colonies, we eventually detected a background of microcolonies that could be scored only under a stereo-microscope. Since these microcolonies failed to develop into macrocolonies when longer cultivation periods were allowed (up to 40 d), they were not scored in plating efficiency assays.

The effects of hormones and growth factors on growth in suspension were assessed by addition to the liquid overlay medium. Pituitary fraction (5 μg/ml) was added every 2–3 d and Hy (250 ng/ml) every time the liquid overlay was renewed.

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Selection of Hy-resistant C_6 Variants in Agarose Suspension: For purposes of variant selection and of estimating the rates at which Hy-resistant variants arise, cell densities no higher than 10^4 cells per plate were used. Colonies grown in the presence of Hy for 21 d were individually picked with a Pasteur pipet. We gently washed each colony to eliminate surrounding agarose by pipeting in liquid medium. The colony was transferred to a test tube containing fresh liquid medium, and the cells were dissociated by vigorous pipeting and plated at low cell density for recloning in solid substrate. The rate at which Hy-resistant clones arise in C_6 cultures was estimated by taking freshly isolated, sensitive clones, plating them in Hy-containing agarose suspension (10^4 cells per 35-mm dish), and counting the macroscopic colonies that developed.

Characterization of Variants: All variants were analyzed by karyotyping and by the presence of functional β-receptors. Karyotype analysis included G and C banding according to references 18 and 19. Chromosome identification and counts were carried out in enlarged pictures taken at 400 magnification under a Zeiss photomicroscope, by comparison with the Committee Standard Karyotype for *Rattus norvergicus* (20). Our strain (39–44 chromosomes per cell; modal number 42) is characterized by the presence of marker chromosome t (6q, 8q) which is likely to have arisen by a Robertsonian translocation between chromosomes 6 and 8. Among the clones isolated, the number of chromosomes per cell ranges from 36 to 44 with modal number from 40 to 42. All clones display that t (6q, 8q) marker, usually in monosomy. We have not found specific cytogenetic markers among the variants isolated. C_6 and all variant lines that originated from it presented a few small acrocentric chromosomes that lack specific banding patterns. Functional β-adrenergic receptors, an important physiological marker of the C_6 parental line (21, 22), were assayed in the variants by scoring, under phase-contrast microscope, the cell shape change caused in glial cells by cAMP (10^{-5}, 10^{-4}, and 10^{-3} M) and catecholamines (10^{-7}, 10^{-6}, and 10^{-5} M). Clones P_2, P_5, P_7, P_{11}, and ST1 responded to isoproterenol, noradrenaline, and cAMP, ST1 being the most responsive clone.

RESULTS

Hy Inhibits Growth of Glioma Cells Both in Agarose Suspension and in Monolayer Cultures

C_6 glioma cells grow in agarose suspension cultures (80% plating efficiency [PE] at 2×10^2 cells per dish). This colony growth is reversibly blocked by Hy (zero PE at 2×10^2 cells per dish; see Table I). Growth can be resumed by the addition of a pituitary growth factor preparation (20% PE) or hormone withdrawal (80% PE).

Hy also inhibits growth of C_6 cells in monolayer cultures. This growth inhibition is manifested by reduction in both the growth rate and the saturation density (Table II). The overall result of Hy action on the growth control pattern of C_6 cells is to reversibly restore anchorage and serum and density dependence.

Hy Effect Maps at G_1 Phase of the Cell Cycle

In monolayer, the effect of Hy could be traced to a reduction in the cell cycle traverse rate exclusively at G_1 phase.

TABLE I

Growth in Agarose Suspension of Hydrocortisone-resistant and Hydrocortisone-sensitive Variants of C_6 Glioma Cells: Effect of Hydrocortisone

Cell type	Plating efficiency (%) −Hy	Plating efficiency (%) +Hy	No. of experiments
C_6 (parental)	60 ± 20	0	6
P_2	75 ± 5	30 ± 4	2
P_5	20 ± 3	20 ± 2	2
P_7	75 ± 5	75 ± 6	2
P_{11}	46 ± 3	43 ± 4	2
ST1	70 ± 10	0	4

2×10^2 cells were plated per 35-mm dish following the protocol detailed in Methods. Values (SEM) are averages of the indicated number of experiments.

TABLE II

Growth in Monolayer of Hydrocortisone-resistant and Hydrocortisone-sensitive Variants of C_6 Glioma Cells: Effect of Hydrocortisone

Cell type	Doubling time (h) −Hy	Doubling time (h) +Hy	Saturation density (cells/cm² × 10^5) −Hy	Saturation density (cells/cm² × 10^5) +Hy
C_6 (parental)	14	32	5.2	1.2
P_2	17	34	2.6	0.8
P_5	16	16	5.0	5.0
P_7	14	14	4.7	3.6
P_{11}	17	28	3.0	0.6
ST1	14	27	4.5	1.0

10^5 cells were plated per 35-mm dish (5% FCS medium) in the absence or presence of hydrocortisone (250 ng/ml). Cells were allowed to grow until saturation density was reached, counting two plates every 24 h. Saturation densities and doubling times were estimated graphically from the growth curves. The values are averages of two independent experiments, performed with the same batch of medium and serum, in which deviations in results were no greater than 10%.

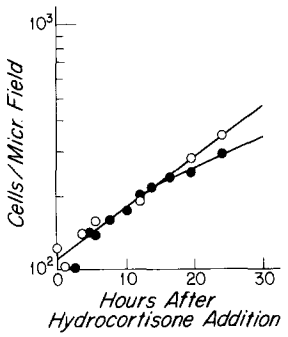

FIGURE 1 Effect of hydrocortisone treatment on ST1 cells' growth rate: 5×10^3 cells/cm² were seeded onto glass coverslips in 15-cm diameter petri dishes and 5% FCS medium. When exponential growth was reached, hydrocortisone (250 ng/ml) was added and coverslips were withdrawn periodically, fixed, and stained with Giemsa's. 10 microscopic fields were counted per coverslip (two coverslips/point). O, control, no addition. ●, hydrocortisone addition.

Addition of Hy to exponentially growing cells leads to a biphasic growth curve (Fig. 1). 14–15 h after hormone addition, the doubling time increases from 15 to 24 h. This hormonal effect could be explained by an inhibition of cells' progress through the G_1 phase of the cell cycle. Data supporting this proposition were obtained in kinetic studies following Hy addition to C_6 cells (ST1 variant) exponentially growing in high (5%) serum medium. LI and mitotic index (MI) are shown in Figs. 2 and 3, respectively. LI remained constant for the first 4.5 to 5.0 h of hormone treatment (Fig. 2), whereas MI took longer to drop, i.e., 14–15 h. Therefore, the two gates of the cell cycle, namely, entry into S phase and mitosis, detected reduction in cell cycle progress and in a sequential manner (first, a decrease in S phase entry and, ~10 h later, a diminution in mitosis frequency).

When the medium of exponentially growing cells was changed to low (0.2%) serum medium plus Hy, the LI also remained constant for 4.5 to 5.0 h (Fig. 2). However, after this initial constant period, LI dropped continuously, reaching negligible levels 12 h after reduction of serum concentration and hormone addition. Reduction of serum concentration to 0.2%, per se, also reduces LI but relatively high levels are maintained (19% after 24 h). These results indicated that the glucocorticoid hormone completely halts the cell entry into S phase in medium containing low serum levels.

Altogether, these data are consistent with the glucocorticoid hormone's acting by exclusively inhibiting cells' progress through G_1 phase. The exact time point, before S phase, at

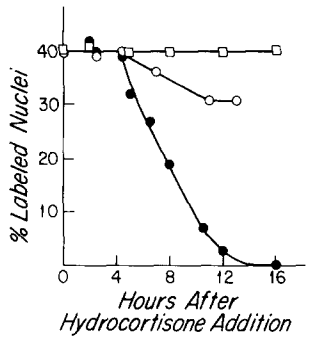

FIGURE 2 Effect of hydrocortisone on the LI of exponentially growing ST1 cells: Cells plated as in Fig. 1. Hydrocortisone (250 ng/ml) was added at zero time, and 30-min pulses of [^{3}H]thymidine (1 μCi/ml; 50 Ci/mmol) were carried out periodically thereafter. LI was determined as described in Materials and Methods. The graph is a composite of 3 independent experiments. □, control, no additions. ○, hydrocortisone addition. ●, medium changed to 0.25% FCS medium plus hydrocortisone.

which cells are affected by the hormone cannot be determined from these results because it should be equal to 4.5 h (time interval with constant LI in Fig. 2) minus the unknown latency period for hydrocortisone effective action.

Agarose Suspension Cultures Used to Isolate Hy-resistant C_6 Variants

We reasoned that agarose suspension cultures would be useful for selection of C_6 variants capable of escaping the Hy growth block. The selection of Hy-resistant variants was based on always taking the largest colonies from suspension cultures kept in the presence of Hy (see Materials and Methods).

Several colonies were isolated by a single selection step in 250 ng/ml Hy. Clones P_2, P_5, P_7, and P_{11} proved to be Hy-resistant variants, as indicated by the plating efficiency results of Table I. Thus in a single selection step it is possible to obtain stable Hy-resistant variants from the C_6 line.

Cells resistant to Hy for growth in suspension arose spontaneously from freshly cloned C_6 cultures at an estimated rate of one variant in 10^4 cells per generation. Different from other Hy-sensitive C_6 sublines, clone ST1 has not yielded Hy-resistant cells even after many generations in culture.

Some Hy-resistant Variants, Selected in Agarose Suspension, Are Sensitive to Hy in Monolayer Cultures

The hormonal growth response of variants selected in suspension cultures was analyzed in cells attached to solid substrate. The unanticipated outcome was that cells selected for Hy-resistance in suspension cultures were not necessarily resistant when anchored to a solid substrate. Table II presents the results of doubling time and saturation density for the variants selected in suspension. Clones P_5 and P_7 proved to be also Hy-resistant in monolayer cultures. On the other hand, clones P_2 and P_{11} which are Hy-resistants in suspension behaved like the parental C_6 or the highly sensitive clone ST1.

DISCUSSION

Hy has been known to inhibit growth of rat C_6 glioma cells (9). More than growth inhibition, Hy reversibly renders C_6 cells anchorage (Table I) and serum and density dependent for growth (Table II and Fig. 2). These effects are observed at physiological concentrations (maximal activity at 50 ng/ml). Identical results are obtained with the synthetic glucocorticoid hormone, dexamethasone (maximal activity at 20 ng/ml). Progesterone is poorly effective whereas sex steroids (estradiol and testosterone) are totally ineffective (23, 24; Armelin, M. C. S. and H. A. Armelin, unpublished results). Therefore the effects reported seem to be specific for glucocorticoid hormones.

Kinetic studies (Fig. 1–3) have shown that in monolayer cultures Hy reduces C_6 cells' proliferation by inhibiting cell cycle progress exclusively at the G_1 phase. This is consistent with our previous observation that Hy increases the lag before DNA synthesis initiation (G_1 phase) in ST1 cells growth arrested by serum starvation and restimulated by serum readdition to the medium (14).

Others have reported glucocorticoids to increase the levels of cAMP in C_6 glioma cells (25). The effects we report for Hy were not accompanied by changes in the steady state levels of cAMP. We measured the intracellular cAMP levels of exponentially growing ST1 cells and obtained 9.5 (± 0.5 SEM) pmol/mg protein; 24 h of Hy treatment did not alter these levels. Furthermore, 24 h after reducing the serum concentration to 0.2%, cAMP levels increased to 20.5 (± 0.6 SEM) pmol/mg protein, irrespective to the presence or absence of Hy (M. C. S. Armelin and H. Armelin, unpublished results).

Analysis of the growth properties presented by the variant clones demonstrated that Hy resistance in suspension is not coupled to Hy resistance in solid substrate. Thus, clones P_2 and P_{11}, selected for Hy-resistance in suspension (Table I) were in fact sensitive to this hormone when anchored to a solid substrate (Table II). This does not prove that Hy acts by two completely independent mechanisms in suspension cultures and attached cells. But it suggests that, if a single central system coordinates Hy action, branching into different routes must occur at some point in order to account for the two independent phenotypes found.

In several instances the transformed state of a given cell line has been shown to be related to the constitutive production of a tumor growth factor by the cells (26). It is conceivable that Hy could be inhibiting the constitutive production of an autostimulatory tumor growth factor. This possibility seems rather unlikely for the following reason: at the time of Hy addition, exponentially growing cultures (as in Figs. 1–3) would have accumulated tumor factor in their medium and should not have been growth inhibited by Hy. Instead, cells were readily responsive to its inhibitory action as shown in the LI experiment in Fig. 2.

We are inclined to believe that Hy acts in C_6 cells, changing their transformed phenotype, by a more direct effect. It can be speculated that Hy may turn off an oncogene or modulate its expression. The accompanying paper describes alterations caused by Hy in C_6 cells which might be related to growth control mechanisms. Fibronectin deposition on the cells' surface and modifications in cytoskeleton functioning and architecture are among the effects described. Elsewhere, we also show that Hy reversibly renders C_6 cells growth dependent on high external Ca^{2+} concentrations (24).

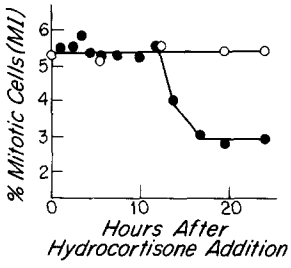

FIGURE 3 Effect of hydrocortisone on the MI of exponentially growing ST1 cells: Cells plated and treated as in Fig. 1. MI was determined as described in Materials and Methods. ○, control, no additions. ●, hydrocortisone addition.

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