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Increased Volumes of Mitochondria and Granular Endoplasmic Reticulum in Rat Glioma Cells Treated with Anti-tumor Agents[1] (39777)

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Introduction. Chemotherapy with DEX[5] and/or BCNU,[6] which readily penetrate the blood-brain barrier, offers one promising approach for the destruction of malignant cells or the inhibition of tumor cell proliferation within intracerebral gliomas (1). Therefore, these antineoplastic agents have been tested for their ability to produce either lethal cytotoxicity or sublethal suppression of cell proliferation in several *in vitro* glioma model systems (2-7).

We have shown that DEX elicits dose-dependent growth-inhibitory responses in rat glioma (strain C6) monolayer cultures (8). Additive inhibitory responses occur in C6 cultures treated sequentially with 1 μM DEX and 1 μM BCNU over a wide range of concentrations (9). Preliminary evidence suggests that additive ultrastructural changes in granular endoplasmic reticulum are produced in C6 cells during the enhanced growth-inhibitory response produced by 1 μM DEX and 1 μM BCNU (9).

This communication presents and analyzes further evidence that confirms and extends these initial observations. In addition, we also describe ultrastructural alterations in C6 cells exposed to 10 μM DEX and 10 μM BCNU.

Materials and methods. C6 cells (10) were grown as monolayer cultures in Ham's F10 medium (11) as described previously (8). Stock solutions (10 mM) of BCNU and DEX were prepared on the day of use by dissolving these drugs in 50% ethanol:50% phosphate-buffered saline (PBS; v/v, pH 7.4). After filtration (0.22 μm), the DEX stock solution was diluted into fresh culture medium at 37°. The stock BCNU solution was filtered and maintained at 0°. This drug solution was diluted into fresh medium at 37° just prior to treating the cells.

The experiments were initiated by inoculating Falcon tissue culture flasks (75 cm^2) each containing 13.5 ml of medium with 1.5 ml of a C6 cell suspension to achieve a cell density $>1.0 \times 10^4$ cells/cm^2. On the following day (designated Day 0), the cultures were divided into two groups. One group served as the control and received fresh medium containing 0.05% ethanol. The other group was treated with fresh medium containing 0.05% ethanol plus either 1 or 10 μM DEX. On Day 1, the control and treated cultures were each divided into two sets. One set from the control and the DEX-treated groups received 0.45 ml of fresh medium. The other sets from each group were treated with 0.45 ml of fresh medium containing BCNU to achieve a final concentration of either 1 or 10 μM in the culture medium.

Cell proliferation was monitored in control and drug-treated C6 cultures on Days 0, 1, and 2 by measuring mean cell densities (mean cell number/cm^2 ± SE). For each measurement, quadruplicate monolayers

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[5] DEX refers to dexamethasone (9-fluoro-11β, 17, 21-trihydroxy-16 α-methyl-pregna-1, 4-diene-3, 20-dione) which was supplied by Merck Sharp & Dohme Research Laboratories (West Point, Pa.).

[6] BCNU [1,3-bis(2-chloroethyl)-1-nitrosourea] was a generous gift from Dr. Milan Slavik, National Cancer Institute, Bethesda, Md.

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were washed three times with PBS and the cells were removed from the flask surface by the action of 0.25% trypsin. The suspended cells were counted with a hemacytometer. The Day 1 control mean cell density was subtracted from mean cell densities measured in control and drug-treated cultures on Day 2. The data were normalized in this manner to compare directly the growth-inhibitory responses produced by BCNU in DEX-pretreated cultures to the inhibitory responses elicited by BCNU in previously untreated C6 cultures. The ratio of the normalized experimental mean cell densities to the normalized control mean cell densities times 100 represents the percentage of control growth. The magnitude of a growth-inhibitory response is expressed as the difference between 100% control growth and the percentage of control growth that occurred in the drug-treated cultures.

After measuring the mean cell densities on Days 1 and 2, the cell suspensions from quadruplicate control and experimental cultures were pooled and prepared for electron microscopy as described previously (9). At least 64 photomicrographs of centrally sectioned control and drug-treated cells were taken by random field selection with a Philips EM 301 electron microscope. These photomicrographs were enlarged to a magnification of 15,000 diameters and were analyzed morphometrically employing the stereologic techniques described by Weibel and Bolender (12). A transparent test pattern, consisting of 224 points 1 cm equidistant from each other, was placed randomly over each photomicrograph to cover the cell section completely. The points falling on the cytoplasm, nucleus, Golgi bodies, mitochondria, and granular endoplasmic reticulum were counted. Golgi bodies were counted only if the points fell on the parallel lamellae of this organelle as the peripheral vesicular portions were not always discernible. Volume fractions were determined by dividing the number of test points falling on the various cellular structures by the total number of test points per cell section. Mean volume fractions (±SE) were then calculated. The Student's *t* test (two-tailed) was used to determine whether differences between the mean volume fractions of control and drug-treated cells were significant statistically. Volume fractions of control cellular structures were taken as 100%. Increases or decreases of the volume fraction of these structures in drug-treated cells are expressed as the ratio of the experimental values to the control values times 100.

Results. Growth inhibition in C6 cultures treated with DEX and BCNU. When log phase C6 cultures were exposed to either 1 or 10 μM DEX on Day 0, cell densities on Day 1 did not differ significantly from controls (Table I; experiments A and B). As control cells continued to proliferate at log phase rates, cell densities in these treated cultures were significantly lower on Day 2. The growth-inhibitory responses produced by 1 and 10 μM DEX were 45 and 58%, respectively. When 1 or 10 μM BCNU was supplied to previously untreated C6 cultures on Day 1, the respective inhibitory responses were 64 and 73% on Day 2. In contrast, these responses increased to 88 and 98% when 1 or 10 μM BCNU was supplied on Day 1 to cultures that were pretreated since Day 0 with 1 or 10 μM DEX, respectively.

Ultrastructural alterations in control and drug-treated C6 cells. Figures 1–6 depict representative electronmicrographs of C6 cells from experiment B that were analyzed morphometrically. The resulting mean volume fractions of the cytoplasm and organelles in control and drug-treated cells from both experiments are given in Table II.

No significant differences in cytoplasmic volume fractions were observed between control and drug-treated cells in either experiment. A 13% decrease in the nuclear volume fraction occurred in C6 cells exposed to 10 μM DEX for 1 day. By Day 2, the nuclear volume fraction in these DEX-treated cells did not differ significantly from controls.

In experiment A, Golgi volume fractions were not measurable in control and DEX-treated cells on Day 1. Relative to controls on Day 2, Golgi volume fractions decreased significantly in cells treated for 2 days with 1 μM DEX and in cells exposed to 1 μM BCNU for 1 day. Similar decreases also occurred in cells exposed sequentially to 1 μM DEX and 1 μM BCNU. In experiment

TABLE I. SUPPRESSION OF RAT GLIOMA (STRAIN C6) CELL PROLIFERATION BY DEXAMETHASONE (DEX) AND 1,3-Bis(2-CHLOROETHYL)-1-NITROSOUREA(BCNU).

Experiment	Day	Drug treatment	10^4 × Mean cell density per square centimeter[a]	Control growth[b] (%)
A	0	None	1.9 ± 0.18[c]	
	1	None	4.0 ± 0.22	
		1 μM DEX	4.1 ± 0.17	
	2	None	8.2 ± 0.25	
		1 μM DEX	6.3 ± 0.11*	55**
		1 μM BCNU	5.5 ± 0.26*	36**
		1 μM DEX + 1 μM BCNU	4.5 ± 0.13*	12**
B	0	None	2.2 ± 0.03	
	1	None	4.9 ± 0.06	
		10 μM DEX	4.8 ± 0.09	
	2	None	9.7 ± 0.17	
		10 μM DEX	6.9 ± 0.10*	42**
		10 μM BCNU	6.2 ± 0.12*	27**
		10 μM DEX + 10 μM BCNU	5.0 ± 0.14*	2**

[a] $N = 4$.
[b] Calculated after subtracting Day 1 control mean cell density.
[c] Mean ± SE.
* $P < 0.025$.
** Significantly different from each other by analysis of variance.

B, Golgi volume fractions were measurable on Day 1; these fractions were similar in control and 10 μM DEX-treated cells. On Day 2, the Golgi volume fraction in 10 μM DEX-treated cells did not differ from controls. However, Golgi volume fractions decreased significantly in cells exposed to 10 μM BCNU alone and in cells treated sequentially with 10 μM DEX and 10 μM BCNU.

Between Days 1 and 2, mitochondrial volume fractions (× 100) in *control* C6 cells increased from 2.8 to 4.7 in experiment A and from 3.5 to 4.9 in experiment B. Relative to controls on Day 1, volume fractions of these organelles increased 129% in 1 μM DEX-treated cells and 166% in cells exposed to 10 μM DEX (Figs. 1 and 2). On Day 2 of both experiments, the mitochondrial volume fractions in drug-treated cells did not differ significantly from controls.

Volume fractions of granular endoplasmic reticulum were not measurable on Day 1 in experiment A, but were measurable on Day 1 in experiment B. Relative to these Day 1 controls, a 158% increase in the volume fraction of this organelle was observed in cells treated with 10 μM DEX (Figs. 1 and 2). Relative to Day 2 controls, volume fractions of granular endoplasmic reticulum increased 189% in cells treated with 1 μM DEX for 2 days or 1 μM BCNU for 1 day. This volume fraction increased 267% in C6 cells treated sequentially with 1 μM DEX and 1 μM BCNU. When C6 cells were exposed to 10 μM DEX for 2 days, a 191% increase in the volume fraction occurred (Figs. 3 and 4), similar to that observed at the lower DEX concentration. In contrast, the granular endoplasmic reticulum volume fraction in 10 μM BCNU-treated C6 cells was 245% (Fig. 5); this effect was greater than that observed with 1 μM BCNU. The volume fraction of these organelles increased 300% in C6 cells treated sequentially with 10 μM DEX and 10 μM BCNU (Fig. 6).

Discussion. Combined growth-inhibitory responses occur when log phase C6 cultures are exposed sequentially to DEX and BCNU at concentrations of either 1 or 10 μM (Table I). Between Days 1 and 2 at the higher drug concentrations, C6 cell proliferation ceases completely.

Significant increases in the volume of granular endoplasmic reticulum are produced in C6 cells treated with either DEX or BCNU (Table II). The volume of these organelles increases still further in cells exposed sequentially to DEX and BCNU.

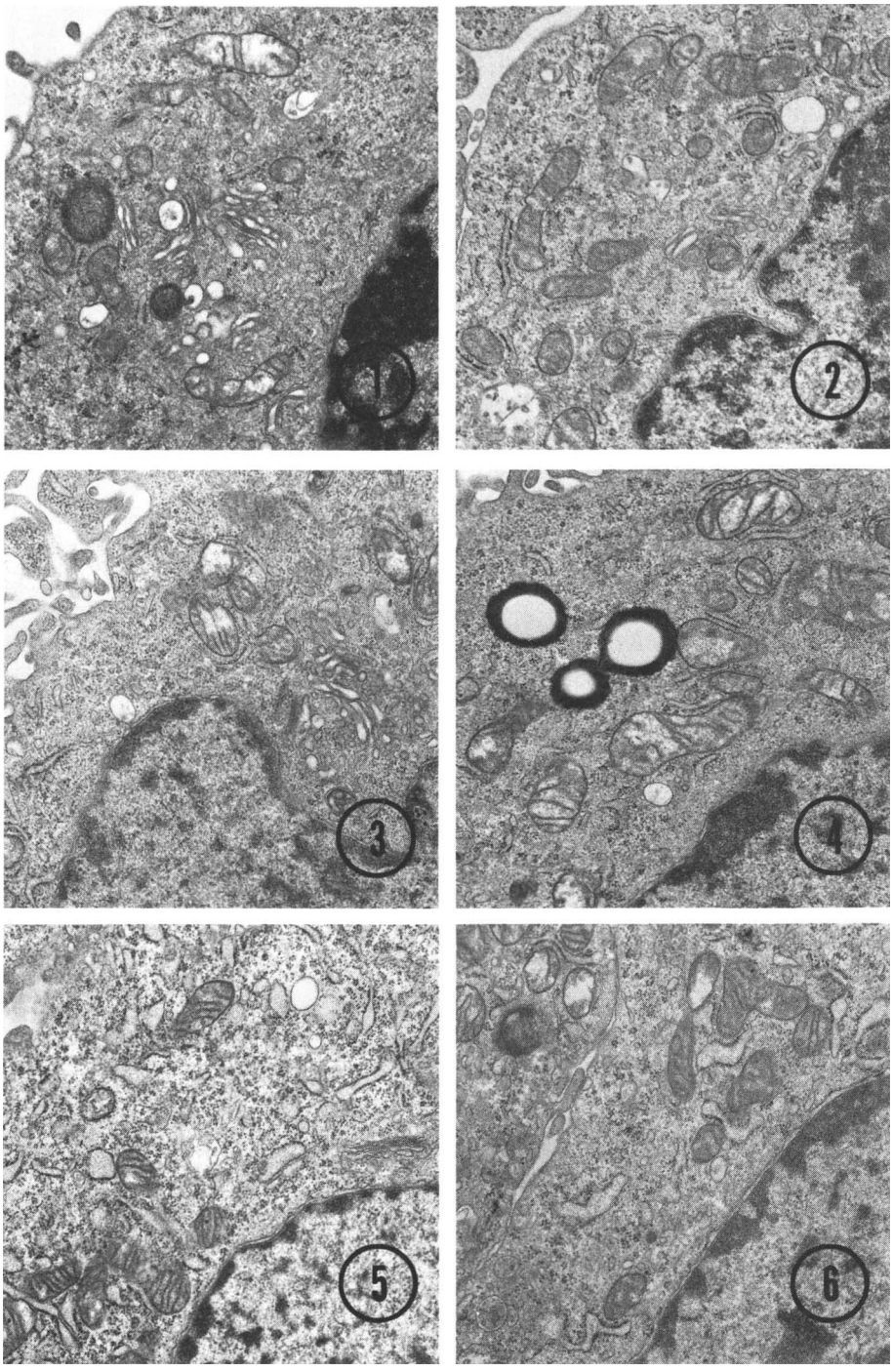

FIG. 1. A portion of a control rat glioma cell (strain C6) on Day 1 of experiment B. Mitochondria are not numerous compared to other cell types, and profiles of granular endoplasmic reticulum are sparse. A small Golgi body is present. × 15,000.

FIG. 2. A C6 cell treated with 10 μM DEX for 1 day. Relative to controls, mitochondrial volumes are increased and more profiles of granular endoplasmic reticulum are evident. × 15,000.

TABLE II. THE EFFECT OF DEXAMETHASONE (DEX) AND 1,3-Bis(2-CHLOROETHYL)-1-NITROSOUREA (BCNU) ON THE ULTRASTRUCTURE OF RAT GLIOMA CELLS (STRAIN C6).

Experiment	Day	Drug treatment	Volume fraction[a] × 100				
			Cytoplasm	Nucleus	Golgi bodies	Mitochondria	Granular endoplasmic reticulum
A	1	None	59.1 ± 1.3[b]	38.1 ± 1.5	–[c]	2.8 ± 0.3	–
		1 μM DEX	58.9 ± 1.5	37.5 ± 1.6	–	3.6 ± 0.3* (129%)[d]	–
	2	None	55.3 ± 1.0	38.4 ± 1.1	0.7 ± 0.1	4.7 ± 0.3	0.9 ± 0.1
		1 μM DEX	55.2 ± 1.9	39.1 ± 1.4	0.3 ± 0.1* (43%)	3.7 ± 0.4	1.7 ± 0.2* (189%)
		1 μM BCNU	54.3 ± 1.4	39.9 ± 1.6	0.2 ± 0.02* (29%)	3.9 ± 0.4	1.7 ± 0.2* (189%)
		1 μM DEX + 1 μM BCNU	53.2 ± 1.1	40.3 ± 1.1	0.2 ± 0.1* (29%)	3.9 ± 0.4	2.4 ± 0.3* (267%)
B	1	None	55.3 ± 1.0	39.3 ± 1.6	0.7 ± 0.2	3.5 ± 0.3	1.2 ± 0.2
		10 μM DEX	57.3 ± 1.3	34.2 ± 1.3* (87%)	0.8 ± 0.2	5.8 ± 0.3* (166%)	1.9 ± 0.2* (158%)
	2	None	54.4 ± 1.0	38.4 ± 1.1	1.2 ± 0.2	4.9 ± 0.3	1.1 ± 0.1
		10 μM DEX	55.3 ± 1.0	37.0 ± 1.0	1.2 ± 0.2	4.4 ± 0.3	2.1 ± 0.2* (191%)
		10 μM BCNU	55.5 ± 0.9	36.2 ± 0.9	0.7 ± 0.1* (58%)	4.9 ± 0.3	2.7 ± 0.2* (245%)
		10 μM DEX + 10 μM BCNU	54.0 ± 0.9	37.8 ± 1.1	0.4 ± 0.1* (33%)	4.5 ± 0.3	3.3 ± 0.2* (300%)

[a] $N = 64$.
[b] Mean ± SE.
[c] Not measurable.
[d] Percentage of controls represented as 100%.
* $P < 0.050$.

These combined ultrastructural changes accompany the combined growth-inhibitory responses produced by these antitumor agents. The lumina and cisternae of the dilated granular endoplasmic reticulum in drug-treated C6 cells are less electron dense than the surrounding cytoplasm, and ribosomes are attached to the internal surfaces (Figs. 4–6). These alterations could reflect modified protein metabolism which subsequently may result in the suppression of cell proliferation.

Mitochondrial volumes in control C6 cells increased 140–168% between Days 1 and 2. These unexpected increases may reflect physiological responses to the changing nutritional properties of the culture medium. Nevertheless, the mitochondrial volumes in DEX-treated C6 cells increase significantly relative to Day 1 controls. This effect occurs in log phase DEX-treated cells well before cell proliferation ceases. In addition, the mitochondrial density (i.e., the mean number of mitochondrial profiles per cell section) likewise increases on Day 1 in these treated cells relative to controls (9). Elevated mitochondrial volumes and densities in DEX-treated cells on Day 1 suggest that:

FIG. 3. A control C6 cell on Day 2 of experiment B. Mitochondrial volumes are increased relative to Day 1 controls. × 15,000.

FIG. 4. A C6 cell exposed to 10 μM DEX for 2 days. Profiles of dilated granular endoplasmic reticulum are evident. A cluster of lipid droplets can be seen. × 15,000.

FIG. 5. A C6 cell treated with 10 μM BCNU for 1 day (i.e., Day 2 of experiment B). Many profiles of dilated granular endoplasmic reticulum are apparent. Ribosomes are attached to the internal surfaces. × 15,000.

FIG. 6. Detail of a C6 cell pretreated with 10 μM DEX for 1 day and then exposed to 10 μM BCNU for an additional day (i.e., Day 2 of experiment B). These cells ceased proliferation between Days 1 and 2. Numerous granular endoplasmic reticulum profiles are dilated extensively. The matrices within the lumina and cisternae of these organelles are less electron dense than the surrounding cytoplasm. × 15,000.

(a) the number of mitochondria/cell increases, (b) preexisting mitochondria enlarge, or (c) both enlargement and increased numbers of mitochondria are produced by glucocorticoid action. Mitochondrial volumes in control and DEX-treated cells did not differ significantly on Day 2. These results suggest that glucocorticoid action in the treated cells accelerates the increase in mitochondrial volumes that occurs normally in control cells between Days 1 and 2.

Except for a slight decrease of the nuclear volume in cells exposed for 1 day to 10 μM DEX, nuclear volumes in treated C6 cells did not differ significantly from those in controls. Cytoplasmic volumes in drug-treated cells were also similar to controls. In most cases, Golgi bodies consisted of <1% of the total cellular volume. The inconsistencies in the Golgi volume measurements most likely reflect the sensitivity limits of the analytical procedures employed.

Taken together, these observations suggest that DEX and BCNU interfere with mechanisms that regulate the ultrastructure of granular endoplasmic reticulum. DEX, but not BCNU, appears to affect these mechanisms which regulate mitochondrial ultrastructure. These mechanisms may share common components with the molecular events that underlie the suppression of C6 cell proliferation by these antitumor agents.

Summary. The effects of DEX (1 and 10 μM) and BCNU (1 and 10 μM), alone and in combination, on the ultrastructure of rat glioma (strain C6) cells were investigated during the growth-inhibitory responses produced by these antitumor agents. Volume fractions of cellular structures were determined by morphometric analysis employing stereologic techniques. Relative to controls (100%), mitochondrial volumes increased 129–166% in C6 cells exposed to DEX for 1 day. Volumes of granular endoplasmic reticulum increased 189–245% in cells exposed to DEX for 2 days or BCNU for 1 day. When C6 cells pretreated with DEX for 1 day were exposed to BCNU for an additional day, granular endoplasmic reticulum volumes increased 267–300%.

These results suggest that the antitumor agents may interfere with common components of those mechanisms that regulate mitosis and the ultrastructure of mitochondria and granular endoplasmic reticulum in C6 cells.

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