Cytocidal Effects of Bromocriptine, Somatostatin Analog, and Heat on Growth Hormone-Secreting Pituitary Adenoma *In Vitro*

Keiichi Kawamoto, PhD, Tohru Uozumi, MD, Katsuaki Sakoda, MD, Kazutoshi Mukada, MD, Kaoru Kurisu, MD, and Takashi Yano, MD

The effects of bromocriptine (BC), a somatostatin analog (SMS), and heat on the secretion of growth hormone (GH) and prolactin (PRL), and on the morphologic features of human GH-secreting pituitary adenoma were studied *in vitro*. The treatment with BC, SMS, or heat (41.5°C and 42.5°C) markedly suppressed the secretion of GH and PRL from the adenoma cells and reduced the number of cells immunoreactive with GH or PRL. The combined treatment with BC and heat induced a marked reduction in the number of GH and PRL cells consistent with the effect on the secretion of GH and PRL. These results suggest that BC, SMS, and heat treatments produced the cytotoxic effects on pituitary adenoma cells, and that the simultaneous treatment of BC and heat enhanced this effect. *Cancer* 1992; 69:2688–2696.

The dopamine agonist bromocriptine (BC) reduces excessive prolactin (PRL) secretion in patients with PRL-secreting pituitary adenomas and inhibits growth hormone (GH) secretion in some patients with acromegaly. This agent also is effective in reducing PRL adenoma size.[1–4] A somatostatin analog (SMS), SMS 201-995, has been shown to have a marked inhibitory effect on GH secretion and on tumor growth in acromegaly.[5–7]

The *in vitro* effects of BC on PRL secretion[8,9] and the morphology of cells in PRL adenoma[10–13] have been examined by many investigators. However, the cytotoxic effects of BC on GH pituitary adenomas have not been fully documented,[14] and the effects of SMS on cytotoxicity and cell morphology remain unknown.

Hyperthermia is well known to have a preferential cytotoxic effect on certain tumors, and when combined with chemotherapy agents, hyperthermia has been shown to enhance significantly their cytotoxic effects on neoplastic cells.[15–21] Despite extensive experimental studies on hyperthermia, no results have been reported on benign tumors, such as pituitary adenomas. Thus, the current study examines the cytocidal effects of BC, SMS, heat alone, and heat in combination with BC or SMS on GH-secreting pituitary adenomas *in vitro*. To achieve this, primary cultures of pituitary adenoma cells were maintained in polylysine-coated culture dishes.

Materials and Methods

Monolayer Culture of Pituitary Adenoma Cells

Pituitary adenomas were surgically removed by the transsphenoidal approach in 11 previously untreated patients (7 with acromegaly and 4 with PRL-secreting adenomas). PRL adenoma tissues (Patients 1, 2, 3, and 4) primarily were used to determine culture conditions. The tissue was immediately placed into Ham's F_{12} medium containing 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, and was divided into fragments approximately 1 mm in size. Tissue was incubated with Dispase (600 to 1000 PU/ml medium; Grodo Shusei Co., Ltd., Tokyo) for 1 hour at 37°C with gentle magnetic stirring. The single cell suspension was transferred to a plastic tube and centrifuged at 150 $\times$ *g* for 10 minutes. The cell pellet was resuspended in the medium described at the concentration of 2 to 3 $\times$ 10^5 cells/ml medium, and placed onto plastic or polylysine-coated plastic culture dishes. For polylysine coat-

From the Department of Neurosurgery, Hiroshima University School of Medicine, Hiroshima, Japan.

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Address for reprints: Keiichi Kawamoto, PhD, Department of Neurosurgery, Hiroshima University School of Medicine, 1-2-3, Kasumi, Minami-Ku, Hiroshima 734, Japan.

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ing, the method of Pettman *et al.*[22] was used. The culture dishes were incubated at 37°C in an atmosphere of 95% air and 5% carbon dioxide (CO_2).

Agents and Heat Treatments

For each experiment, at least four replicate cell cultures were maintained simultaneously for the controls and variables. BC and SMS used in the current study were gifts from Sandoz Ltd. (Basel, Switzerland). BC was dissolved in ethanol plus tartaric acid. Equal amounts of tartaric acid and ethanol (final concentration, 0.04%) were added to the other experimental media and to the controls. Heat treatments (6, 12, and 24 hours or 6 days) of cultured cells were performed by using a temperature-gradient CO_2 incubator (Advantec Toyo Co., Ltd., Tokyo, Japan). The pH of the media overlaying the cells was between 7.2 and 7.4 during treatment. Agent-containing or free media were changed daily during 6-day treatments. After treatments, the medium was replaced every 2 days for 14 days to remove excess agents.

Hormone Assay

The levels of GH and PRL in the media were measured by radioimmunoassay (RIA) using a double antibody method. Immunologic materials for RIA were supplied by the National Institute of Arthritics, Metabolism, and Digestive Disease and the National Pituitary Agency (Baltimore, MD). Radioiodinated human GH and PRL were purchased from Du Pont/NEN Research Products (Boston, MA). The intraassay and interassay coefficients of variation were less than 9%.

Morphologic Studies

For electron microscopic observations, cultured cells were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 hour. They were postfixed in 1% osmium tetraoxide for 1 hour, dehydrated, and embedded in Poly/Bed 812 (Polyscience Inc., Warrington, PA). Ultrathin sections, stained with uranyl acetate and lead citrate, were examined with an Hitachi H-7000 electron microscope (Tokyo).

To count the number of GH and PRL cells, hormone-secreting cells were identified by immunocytochemistry (avidin-biotin-peroxidase complex method). Preparation and fixation for immunocytochemistry were performed according to the methods described by Knigge *et al.*[23] The number of immunoreactive cells per unit area was calculated in each culture dish.

Statistical Analyses

Statistical analyses of differences were performed by Kruskal–Wallis H test followed by Mann–Whitney U test or Wilcoxon *t* test.

Results

Two PRL-secreting pituitary adenomas (Patients 1 and 2) were used to examine the effects of enzymatic cell dissociation on hormone secretion. PRL secretion of cultured cells rapidly increased during 9 to 12 days of culture, and this level was almost maintained until 15 to 18 days of culture. Thus, the subsequent experiments were begun from day 12 of culture.

Comparisons Between Use of the Polylysine-Coated Dish and the Nontreated Dish

The major problem in culturing pituitary adenoma cells is the poor adherence of these cells to plastic tissue culture dishes. Thus, we experimented with the use of polylysine-coated dishes in this study. Daily PRL secretion rates were compared between polylysine-coated culture dishes and nontreated culture dishes (Fig. 1).

PRL secretion from cultured cells plated on a nontreated dish significantly decreased on day 18, with a gradual decline to day 38 (Fig. 1, top). After BC treatment, PRL level in the medium significantly decreased after 24 hours, compared with the PRL level of the control. At day 20 (38 days of culture), after BC withdrawal, the PRL level was significantly lower in the treated cells than in the control cells, but the difference was less than that observed after 6 days of BC treatment (18 days of culture).

In contrast, PRL level in the medium plated on the polylysine-coated culture dish was temporarily depressed, but the value gradually increased and recovered to that of the initial control level at 38 days of culture (Fig. 1, bottom). Inhibitory effect of BC on PRL secretion was the same as that observed in the nontreated culture dish. Thus, the use of polylysine increased cell adherence and facilitated manipulation of the culture because of the tenacious attachment of cells.

Basal Levels of GH and PRL From Cultured Pituitary Adenoma Cells

Adenoma tissues obtained from seven acromegalic patients (Patients 5, 6, 7, 8, 9, 10, and 11) were used in the current study. Figure 2 indicates the mean value of GH and PRL in these cultured adenoma cells, plated on polylysine-coated dishes during the experimental period. The values of GH and PRL varied in different

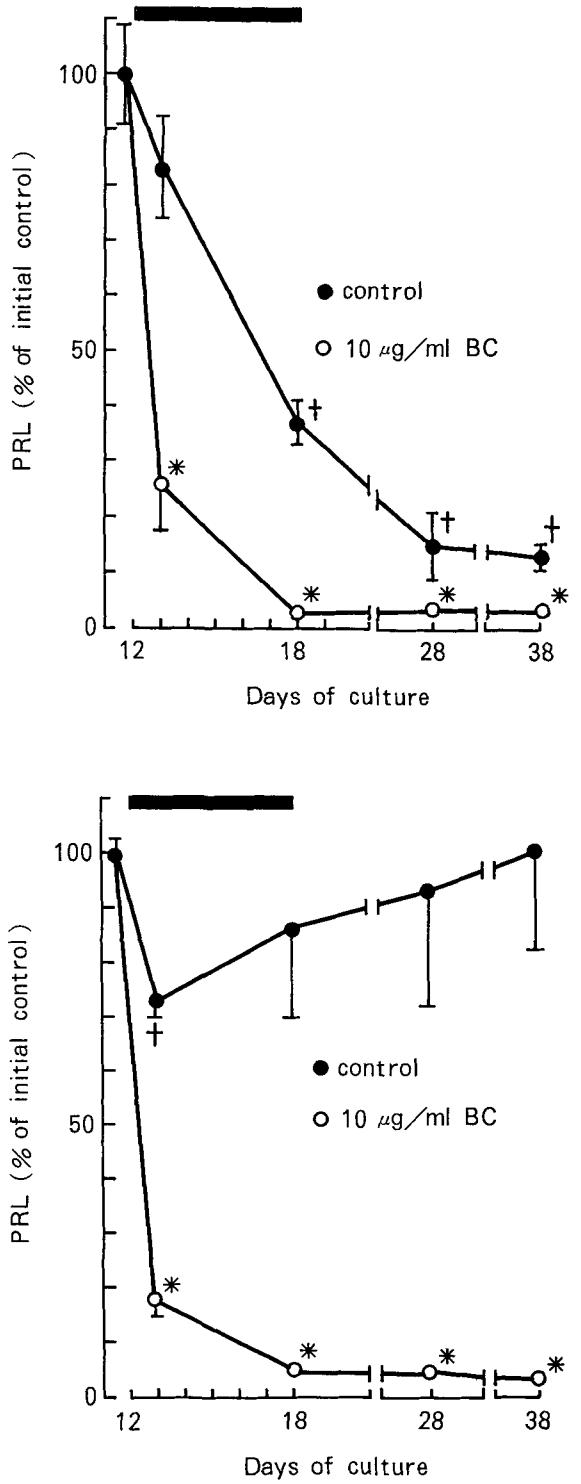

Figure 1. Comparisons between (Top) nontreated culture dishes and (Bottom) polylysine-coated culture dishes (Patient 3). PRL levels per 24 hours are expressed as percentages of initial control. Each circle depicts the mean ± standard error of four culture dishes. Horizontal bar indicates the period (6 days) of BC treatment. Daily medium changes were performed in control and BC during the period of treatment. Difference from initial control was + $P < 0.05$. Difference from matched control was *$P < 0.05$.

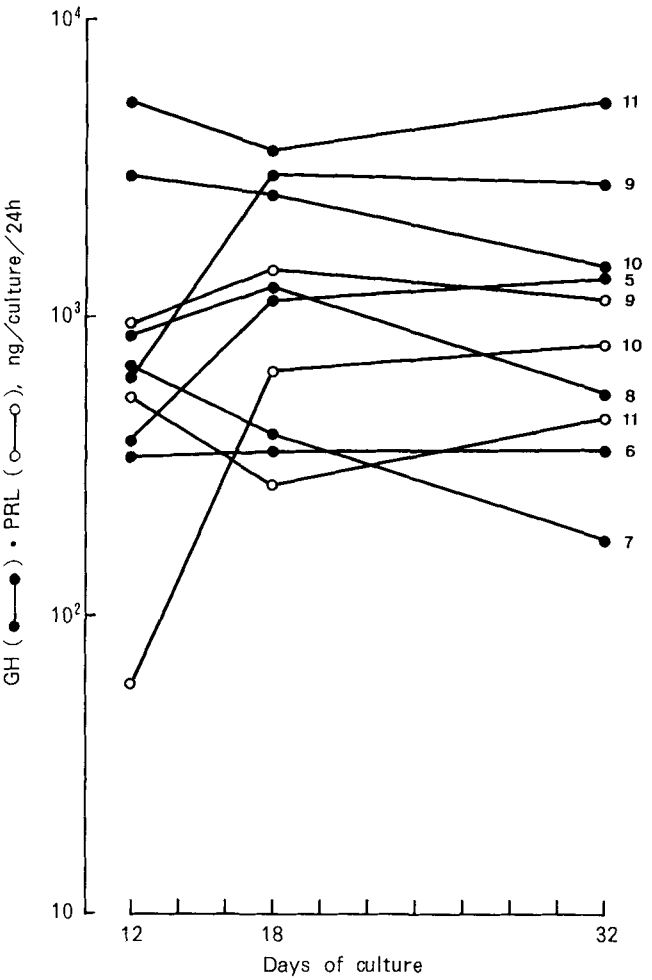

Figure 2. Mean daily secretion of GH and PRL from pituitary adenoma cells obtained from seven acromegalic patients (Patients 5 to 11). Each circle depicts the mean value of four to eight culture dishes.

adenoma tissue specimens. Thus, in the following studies, the mean hormone values in the controls were designated as 100% for comparisons.

Effects of BC, SMS, and Heat Alone or Their Combinations on the Secretion of GH and PRL

Six-day treatments with BC significantly decreased GH and PRL secretion from cultured cells of the patients with pituitary adenoma. At 14 days after BC withdrawal, GH and PRL levels in cultured media rebounded to varying degrees but were statistically less than those of control values, except for the GH secretion of Patient 11 (Table 1). Inhibitory effect of SMS on GH and PRL secretion also was observed during treatment, but at 14 days after SMS withdrawal, a significant decrease in hormone secretion was detected in GH from GH adenoma (Patients 5, 6, and 7) and in PRL

Table 1. Growth Hormone and Prolactin Secretion at 14 Days After the Withdrawal of 6-Day Agents or Heat Treatments

		BC (μg/ml)			SMS (nM)				Heat (°C)		
Tumor	Control	*0.1*	*1*	*10*	*1*	*10*	*100*	*1000*	*40.5*	*41.5*	*42.5*
PRL adenoma											
Patient 4	100 ± 17.8	—	—	5.6 ± 7.8*	—	—	—	—	94.4 ± 13.3	—	ND
GH adenoma											
Patient 5	100 ± 0.9	20.3 ± 0.7*	—	—	54.6 ± 9.1*	34.6 ± 6.4*	23.6 ± 4.6*	—	—	—	—
Patient 6	100 ± 20.0	—	750 ± 11.0*	—	—	—	—	24.7 ± 6.8*	—	—	—
Patient 7	100 ± 7.5	—	70.0 ± 4.2*	—	—	—	—	48.1 ± 2.0*	—	—	—
Mixed adenoma											
Patient 9											
GH	100 ± 6.8	72.7 ± 2.3*	46.9 ± 4.0*	62.5 ± 3.7*	—	—	—	—	49.2 ± 11.9*	31.3 ± 15.6*	ND
PRL	100 ± 4.7	65.3 ± 3.4*	58.5 ± 5.0*	42.4 ± 3.4*	—	—	—	—	81.4 ± 1.7*	59.2 ± 12.7*	ND
Patient 10											
GH	100 ± 7.2	25.0 ± 6.9*	22.5 ± 1.8*	32.5 ± 2.4*	60.0 ± 12.0*	95.0 ± 26.2	110 ± 9.5	—	117.5 ± 28.6	37.5 ± 14.3*	—
PRL	100 ± 6.6	30.9 ± 11.2*	22.1 ± 18.6*	11.0 ± 5.8*	25.0 ± 21.8*	26.5 ± 19.7*	18.4 ± 6.6*	—	27.6 ± 2.8*	22.1 ± 12.1*	—
Patient 11											
GH	100 ± 8.3	—	126.9 ± 6.0	88.7 ± 12.0	—	—	84.8 ± 20.0	62.6 ± 18.3*	82.1 ± 12.0*	40.4 ± 6.5*	20.5 ± 12.3*
PRL	100 ± 12.3	—	16.2 ± 2.7*	8.3 ± 1.7*	—	—	68.1 ± 8.3*	27.7 ± 6.6*	57.9 ± 4.7*	65.6 ± 1.7*	ND

Values are mean ± standard error of mean (n = 4). The mean values of control per 24 hours were designated as 100%.

ND: not detectable; GH: growth hormone; PRL: prolactin; SMS: somatostatin analog; BC: bromocriptine.

* Differences from control: $P < 0.05$.

from mixed adenoma (Patients 10 and 11) (Table 1). In contrast, GH secretion from mixed adenoma was inhibited only in the dishes treated with a concentration of SMS (1 nM in Patient 10 and 1000 nM in Patient 11).

Six-day heat treatments (40.5°C, 41.5°C, and 42.5°C) also markedly suppressed the secretion of GH and PRL. The effect of heat treatment on the secretion of GH and PRL persisted 14 days after transfer to temperatures of 37°C, although GH and PRL values rebounded in varying amounts (Table 1). However, GH and PRL values in cultured media of cells heated to 42.5°C were far less than those detected by RIA, except for GH values in Patient 11.

The combined treatment with heat (40.5°C and 41.5°C) and BC had a significantly greater GH and PRL inhibitory effect than did separate treatments (Table 2). The effects of the combination of BC and heat treatments of 41.5°C were the same as those observed in heat exposure of 42.5°C alone, and after 14 days of treatment neither GH nor PRL were detected in the culture media of Patient 9. However, the combined treatment with heat and SMS failed to enhance the inhibitory effect on the secretion of GH and PRL, except on PRL secretion in culture dishes heated at 41.5°C (Table 2).

Enhanced Effects of Combination of BC and Heat on GH Secretion

To determine the conditions for an enhanced effect on GH secretion after BC and heat treatments, the effects of combinations of various doses of BC and of various exposure times at temperatures of 42.5°C were examined (Fig. 3). GH secretion from cultured cells treated with various concentrations of BC decreased at an additional 14 days after treatments. Heat treatments at 42.5°C for 6 hours, 12 hours, and 24 hours also were effective.

The enhanced effects of heat in combination with BC on GH secretion were obtained only under the condition of 24-hour heat treatment (Fig. 3). The different concentrations of BC used in this study did not alter these effects.

Electron Microscopic Study

Control samples were composed of cells that were rich in secretory granules, with well-developed, rough endoplasmic reticula, scattered mitochondria, and Golgi apparatus. Some of these samples contained fibrous bodies around the centrioles, suggesting GH adenoma cells. The characteristics of cultured cells were not different from those observed in tumor tissue (Fig. 4, top left).

Table 2. Growth Hormone and Prolactin Secretion at 14 Days After the Withdrawal of 6-Day Combined Treatment With Agents and Heat

Tumor	Control	Heat + BC (μg/ml)						Heat + SMS (nM)	
		40.5°C			41.5°C			40.5°C	41.5 °C
		0.1	*1*	*10*	*0.1*	*1*	*10*	*100*	*100*
Mixed adenoma									
Patient 9									
GH	100 ± 6.8	35.9 ± 4.7*	34.3 ± 16.4*	27.3 ± 8.6*	ND	ND	ND	—	—
PRL	100 ± 4.7	41.5 ± 0.6*†	38.1 ± 1.8*†	35.6 ± 1.1*	ND	ND	ND	—	—
Patient 11									
GH	100 ± 8.3	—	30.7 ± 5.7*†	—	—	10.7 ± 3.1*†	—	88.2 ± 9.4	32.9 ± 5.7*
PRL	100 ± 12.3	—	7.2 ± 1.2*†	—	—	2.3 ± 0.1*†	—	64.8 ± 6.3*	15.8 ± 3.4*†

ND: not detectable; GH: growth hormone; PRL: prolactin; SMS: somatostatin analog; BC: bromocriptine.

* Differences from control = $P < 0.05$.

† Differences from matched heat (°C), BC (μg/ml), or SMS (nM) treatment (Table 1) = $P < 0.005$.

Values are mean ± standard error of mean (n = 4). The mean values of control per 24 hours were designated as 100%.

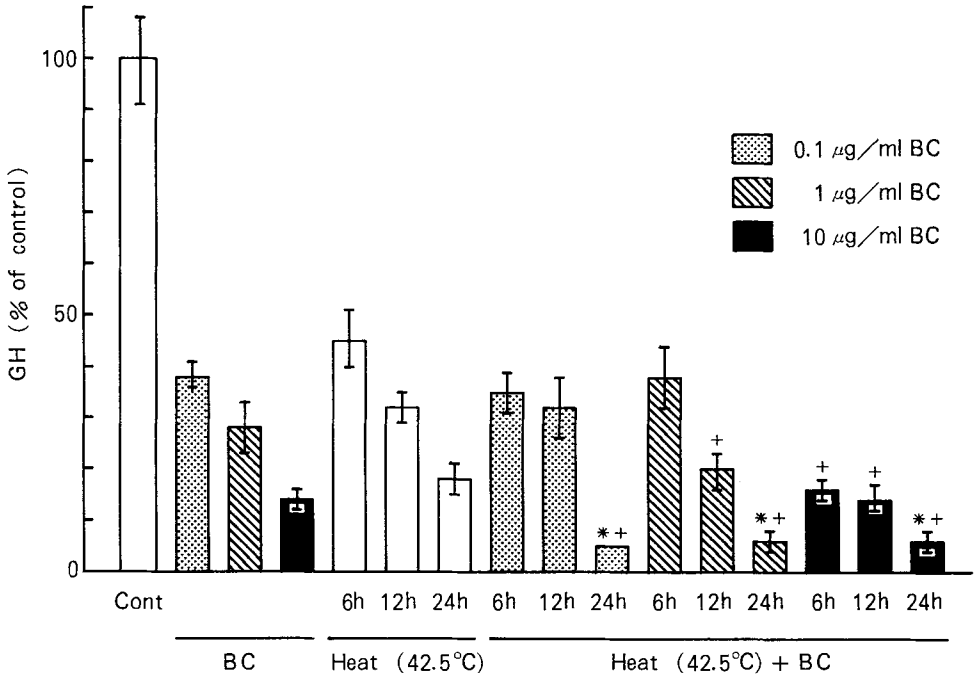

Figure 3. Effects of combination with heat (42.5°C) for 6, 12, or 24 hours and BC for 6 days on GH secretion in cultured pituitary adenoma (Patient 8). Each column depicts the mean ± standard error of four culture dishes at an additional 14 days after the withdrawal of treatments. GH secretion per 24 hours of every experimental group was significantly reduced as compared with that of controls. Difference from matched heat exposure time was $+P < 0.05$. Difference from matched dose of BC was $*P < 0.05$.

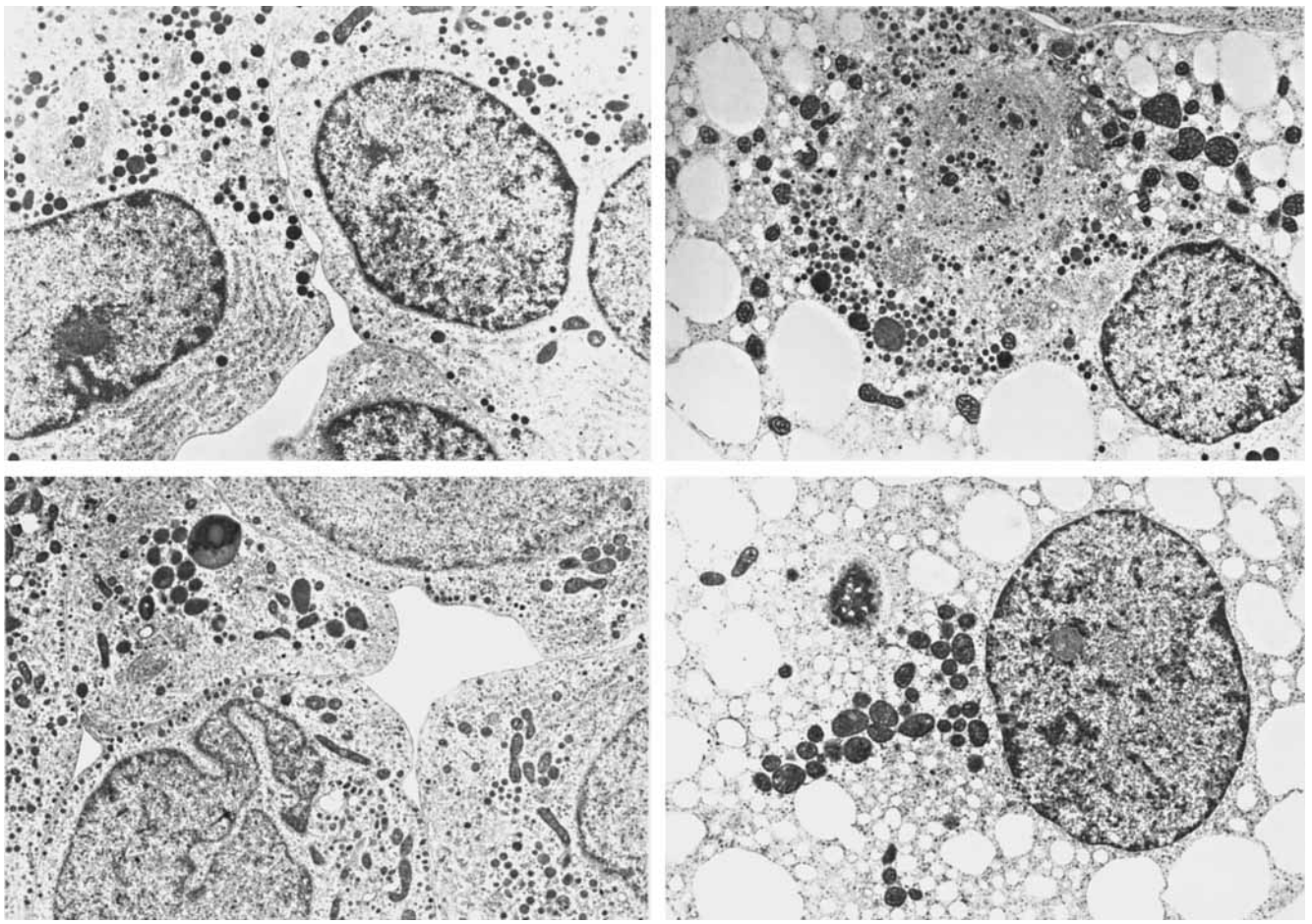

Figure 4. Electron micrographs in pituitary adenoma cells treated with agents for 6 days or heat for 2 days (Patient 10). Bar: 2 μm. (Top left): Control; (Top right): BC (1 μg/ml); (Bottom left): SMS (100 nM); (Bottom right): heat (41.5°C). Original magnification for all micrographs ×13,000.

In most cells treated with BC, extensive cytoplasmic vacuolization and dilation of mitochondria were revealed (Fig. 4, top right). In cells treated with SMS, the morphologic features were essentially unchanged. However, the secretory granules were moderately increased in number and size (Fig. 4, bottom left). In most of the cells heated at 41.5°C, cytoplasmic vacuolization and dilation of mitochondria were observed, and some of cells were destroyed (Fig. 4, bottom right). These profiles resembled those observed in BC treatment.

Immunocytochemical Study

Table 3 indicates the number of immunoreactive cells per unit area in each experimental group of two tumors. BC reduced the number of PRL cells to a greater extent than it did GH cells. The degree was generally dependent on the concentration of BC. Heat (for 6 days or 12 hours) also significantly reduced the number of immunoreactive cells. In culture dishes heated to 42.5°C for 6 days, no immunoreactive cells were encountered. Although SMS showed cytotoxic effects on GH- and PRL-secreting cells, the selectivity of this effect on different kinds of hormone-secreting cells was not as evident as that of BC (Table 3).

The combined treatment with BC and heat markedly reduced the number of GH and PRL cells, consistent with the effects of these treatments on hormone secretion. In particular, the combination of the dose of 0.1, 1, or 10 μg/ml BC and heat at 41.5°C for 6 days resulted in a complete cytotoxic effect. This finding accorded well with the results obtained by heat alone at 42.5°C.

Discussion

The use of polylysine-coated culture dishes enabled the long-term culture of pituitary adenoma cells. Thus, this method has great advantages in the evaluation of the morphology and hormone secretion of adenoma cells after chemotherapy and hyperthermia.

BC, SMS, and heat treatments caused significant decreases in the number of GH- and PRL-secreting adenoma cells and in the secretion levels of GH and PRL. These findings suggest that the decline in the hormone level is the result of damage to adenoma cells.

It has been well documented that BC has an inhibitory effect on the PRL level in patients with hyperprolactinemia and an antitumor effect on PRL adenoma.[1–4] Histologic observations of PRL adenoma tissue treated preoperatively with BC indicate that tumor shrinkage was caused by a direct cytotoxic effect on adenoma cells

Table 3. Number of Growth Hormone- or Prolactin-Immunoreactive Adenoma Cells Per Unit Area at 14 Days After the Withdrawal of 6-Day Treatments

Treatments	Patient 9		Patient 10	
	GH cells	*PRL cells*	*GH cells*	*PRL cells*
Control	100 ± 14	100 ± 14.7	100 ± 14.2	100 ± 12.2
BC (μg/ml)				
0.1	—	—	51.0 ± 14.8*	35.8 ± 7.9*
1	66.9 ± 5.8*	35.6 ± 1.6*	66.4 ± 21.1	37.2 ± 2.0*
10	54.9 ± 6.9*	39.6 ± 7.9*	37.3 ± 10.7*	29.4 ± 8.9*
Heat (°C)				
40.5	40.2 ± 5.7*	66.6 ± 5.0*	67.6 ± 20.0	55.5 ± 6.4*
41.5	0.4 ± 0.1*	6.0 ± 2.8*	54.4 ± 4.1*	47.0 ± 12.0*
42.5	0*	0*	—	—
42.5 (12 hr)	—	—	36.0 ± 9.1*	38.2 ± 1.4*
SMS (nM)				
1	—	—	35.9 ± 15.2*	40.1 ± 5.8*
10	—	—	58.8 ± 13.9*	29.8 ± 0.6*
100	—	—	79.2 ± 7.9	41.9 ± 9.0
Heat (°C) + BC (μg/ml)				
40.5 0.1	13.8 ± 3.1*	11.4 ± 5.7*	—	—
1	28.8 ± 7.7*	24.1 ± 9.9*	—	—
10	31.0 ± 0.2	33.9 ± 6.9*	—	—
41.5 0.1–10	0*	0*	—	—
42.5 (12 hr) 0.1	—	—	35.9 ± 17.6*	37.5 ± 10.3*

GH: growth hormone; PRL: prolactin; SMS: somatostatin; BC: bromocriptine.

* Differences from control: $P < 0.05$.

Values are mean ± standard error (n = 4). The mean number of cells in controls was designated as 100%.

or a reduction in cell volume attributable to a decrease in intracellular organelle size.[24–28] In contrast, BC rarely decreased GH level to within normal limits in patients with acromegaly and had no antitumor effect on GH adenoma, except in one patient.[29] In addition, histologic changes have not been seen in GH adenoma tissue after BC therapy. In regard to *in vitro* studies, Hassoun *et al.*[12] observed that BC decreased the cellular and nuclear surface area in PRL adenoma cells. In addition, Kabuto *et al.*[13] and Inoue *et al.*[14] reported the appearance of numerous intracellular vacuoles in BC-treated PRL and GH adenoma cells, respectively. They concluded that these numerous vacuoles originated from the dilated rough endoplasmic reticula.

In the current study, ultrastructural changes in the BC-treated cells were similar to the results reported by these other investigators. In addition, morphometric analyses by immunocytochemistry after BC withdrawal also revealed that BC has cytocidal effects on GH and PRL adenoma cells, although these effects were more pronounced on PRL adenoma cells than on GH adenoma cells. Despite the marked decrease in GH secretion during BC treatment, GH levels after BC withdrawal rebounded to the control level in one (Patient 11) of the cultured adenoma, but PRL level significantly decreased. However, in this instance it has not been determined whether BC caused some cytotoxic effects on GH adenoma cells.

Recently, SMS has been used as a potent and promising agent in the medical treatment of patients with acromegaly.[5–7] Long-term therapy resulted in a marked suppression of the GH level and a shrinkage of the pituitary adenoma mass, but no histologic changes suggesting cytotoxic effects have been detected in patients with GH adenoma.[30] We also showed that no adenoma cells showed degenerative features during SMS treatment, so the decrease in the number of GH and PRL adenoma cells after SMS withdrawal may be the result of a secondary effect caused by the prolonged inhibition of hormone secretion.

It has been well established by many experimental studies that hyperthermia alone or in combination with certain agents, such as antibiotics or alkylating agents, has a cytotoxic effect on exponentially growing tumors.[17–21] Several investigators have reported that nondividing cells under plateau phase conditions also showed thermosensitivity.[31,32] Pituitary adenomas used in this study rarely showed cell proliferation *in vitro*. Indeed, immunohistochemical studies using antibromodeoxyurdine and anti-Ki-67 monoclonal antibodies disclosed that the growth rate of pituitary adenomas *in vivo* was extremely low.[33,34] Thus, hyperthermia may be of potential benefit in treatment of pituitary adenomas. When heating was performed at temperatures of 40.5°C, 41.5°C, and 42.5°C for 6 days, GH- and PRL-immunoreactive cells significantly decreased, whereas heat exposure of 42.5°C resulted in a complete cytotoxic effect on the adenomas cells. Heat treatments at 42.5°C for 6, 12, or 24 hours also were effective in decreasing GH levels in the media and the number of GH and PRL adenoma cells.

In vitro studies, the plasma membrane, and intracellular organelle have been regarded as the primary targets involved in hyperthermic cell killing.[35–37] Ultrastructural changes in adenoma cells after mild heat exposure resembled those in BC-treated cells, which may operate in similar ways to damage the organelle, such as the rough endoplasmic reticula and mitochondria. Hyperthermia in combination with BC had a greater cytotoxic effect in all pituitary adenomas used in the current study compared with BC or heat treatment alone, whereas heat in combination with SMS there was no enhancement of the cytotoxicity. Although the reason for these differences in effects remains unknown, heat probably enhances the intracellular BC uptake by increasing the damage to and the permeability[38] of the tumor cell membrane. This may be supported by studies in which BC has been reported to have a nonreceptor-mediated action.[39–41] In terms of clinical application, the period of hyperthermia, in combination with BC, required is too long, so a temperature of 43.0°C or greater may be needed. Thus, combined treatment with hyperthermia and BC might improve the therapeutic effects in pituitary adenoma.

In conclusion, the current results suggest that the decline in GH and PRL levels after BC or heat treatment is the result of direct damage to the adenoma cells, and that combined treatment with heat and BC enhances the cytocidal effect.

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