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Cytosuppressive Effect of Bromocriptine on Human Prolactinomas: Stereological Analysis of Ultrastructural Alterations with Special Reference to Secretory Granules[1]

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Running title: Bromocriptine-Treated Prolactinomas

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Abstract

To ascertain the mechanisms of bromocriptine in lowering serum prolactin (PRL) levels and reducing cell size of human prolactinomas, stereological analysis at electron microscope level was performed on 6 adenomas treated with bromocriptine (10 mg/day for 2 weeks) and 4 untreated adenomas. The bromocriptine treatment significantly decreased all the major organelles involved in PRL synthesis when expressed in absolute volume per single tumor cell, although it decreased only Golgi apparatus when expressed in relative volume within the cells. Secretory granules, lysosomes and lipid droplets increased in relative volume but not in absolute volume in bromocriptine-treated adenomas. Consequently, the bromocriptine decreased the volume of individual tumor cell to approximately 60% of that of untreated tumor cells. Unexpectedly, exocytosis of secretory granules increased significantly in the bromocriptine-treated adenomas in spite of remarkable decrease in serum PRL levels. This appears contradictory to the current view that a decrease in serum PRL levels with a concurrent increase in the intracellular PRL levels by the bromocriptine treatment results from the inhibition of exocytosis of secretory granules. The secretory granules of bromocriptine-treated adenomas may contain a little amount of PRL, as suggested by a culture study reporting degradation of PRL by bromocriptine.

Introduction

Bromocriptine not only lowers serum prolactin (PRL) levels but also reduces tumor size of human prolactinomas (1-3). Recently, Gen *et al*. (4) and we (5) have suggested that the size reduction of human prolactinomas by bromocriptine treatment results from the reduction in size of individual tumor cell as well as the reduction in number of tumor cells secondary to cell necrosis. This implies that bromocriptine has a cytosuppressive effect and possibly a cytocidal effect on human prolactinomas, which causes reduction in cell size and cell necrosis, respectively. However, no more than the phenomenon has been found on the cytocidal action. The cytosuppressive action has been suggested to consist of several sequential processes including a decrease in exocytosis of secretory granules (3, 6). However, the hypothesis comes from observations mostly on normal pituitaries of experimental animals by *in vitro* studies (7-15). It has not been established that the hypothesis is applicable also to human prolactinomas, at least partly because of the absence in appropriate animal models for human prolactinomas. Bromocriptine is inactive for rat transplantable pituitary tumors (16, 17), although some ergot derivatives with vasoconstrictive action are potent to reduce the tumor size (16, 18). The present study was undertaken to ascertain the mechanism of cytosuppressive action of bromocriptine on human prolactinomas, by analyzing quantitatively the ultrastructural alterations.

There are only 5 studies including ours, which have quantitatively demonstrated the cytosuppressive effect of bromocriptine on human prolactinomas (5, 6, 19-21). A study by

Tindall et al. (19) is the sole one which has analyzed volumetric changes of cytoplasmic organelles involved in PRL synthesis at electron microscope level. The number of tumors they analyzed were only 2, and their application of morphometric method appears somewhat short of appropriateness. In the present study, 6 prolactinomas treated with bromocriptine were analyzed stereologically in comparison with 4 control tumors. The procedures employed were based on a well-established stereological method, by which the cytoplasmic organelles could be expressed in relative abundance within the cells as well as in absolute value per an average single cell. Among the findings obtained in the present study, the most interesting one was concerned in the exocytosis of the secretory granules (release of the granules from the cells). In spite of remarkable decrease in serum PRL levels, a significant increase in number of exocytosis in bromocriptine-treated adenomas was found, which seems contradictory to the current view concerning the mechanism of bromocriptine. This will be discussed later.

Materials and Methods

Ten patients with prolactinomas were divided into two groups. Six patients were treated with 10 mg/day of bromocriptine (2.5 mg every 6 hours) for 2 weeks until the morning of surgery day (bromocriptine group), whereas 4 patients received no medication (control group). All the patients underwent transsphenoidal surgery. Tumor size was evaluated by high resolution CT scan (GE 8800 X2) and also by the amount of tumor tissues excised. Serum PRL levels before and after bromocriptine therapy were determined by radioimmunoassay.

Their clinical features and laboratory findings are summarized in Table 1.

The tumor tissues were fixed for electron microscopy in 3% glutaraldehyde buffered with 0.1 M, pH 7.4 s-collidine, postfixed in 1% osmium tetroxide, and embedded in Epon. Thin sections exhibiting silver interference color were stained with uranyl acetate and lead citrate, and viewed in a Hitachi 12 electron microscope at 100 kV.

Stereological analysis was performed by using a point-counting method at electron microscope level. Stereological procedures applied in the present study were almost the same as detailed previously (22, 23). In brief, 4 representative tissue blocks were chosen from 10-20 blocks in each tumor, based on the histological appearance of semithin sections stained with toluidine blue. Stereological analysis was carried out on 40 electron micrographs at a final magnification of x 7,500, and 40 more at x 32,000 for each of the tumors. Number of tumor cells analyzed roughly equaled 170 and 250 cells in each tumor of control group and bromocriptine group, respectively. These micrographs were taken randomly from 4 different blocks (10 micrographs per tissue block), except for tumor cells severely injured by bromocriptine therapy. Stereological parameters estimated in the present study included volume density, numerical density and surface density, i.e., volume, number and surface area of organelles per unit volume of the tumor cells. The lower magnification views were used to estimate volume density and numerical density of organelles except for the endoplasmic reticulum (ER), and also the surface density of plasma membrane and nuclear membrane of the tumor cells. The higher magnification views provided surface density and volume density

measurements of the ER. For volume density and numerical density, a transparent tripple-lattice (1:4:16) test sheet containing 1,728 test points in an area of 436 cm^2 was placed on the electron micrographs. To estimate numerical density of secretory granules, the actual mean diameter of the secretory granules was evaluated from the diameter of 300 profiles in section for each adenoma, using a method of Giger and Riedwyl (24). Exocytosis of secretory granules may occur in unigranular or multigranular form. The numerical density of the exocytosis was evaluated by counting the number of all the secretory granules which were present evidently outside the cells as well as in pockets of the plasma membrane. For the measurement of surface density, a coherent multipurpose grid containing 132 test points and 66 lines of 2 cm was used.

Primary data thus collected were applied to stereological formulae to estimate the different densities of organelles. Information on stereological theory and formulae is detailed elsewhere (24). All the values obtained were expressed in mean $\pm$ SEM. Significance in difference between the control group and bromocriptine group was examined by Student's *t* test.

Results

Serum PRL Levels and Tumor Size. Serum PRL levels in the patients before therapy ranged 62 - 12,000 ng/ml (normal values at our institute: < 30 ng/ml). Bromocriptine treatment for 2 weeks reduced the serum PRL levels of all the patients to less than 20% of those before treatment (Table 1). Three patients showed normalized serum PRL levels. A marked reduction in tumor size was observed by CT scan

in one patient (Patient 8; decrease in diameter from 14 to 11 mm), whereas the others failed to show reduction in tumor size. The reasons seem to be limitation in resolving power of CT, and an increase in amount of connective tissue within the tumor by bromocriptine treatment (5, 25).

Light and Electron Microscopy. As described previously (5), tumors treated with bromocriptine for 2 weeks showed the reduction in cell size and a variety of necrotic changes, the former being common to all the tumors and more prominent than the latter. The present study, however, is limited to the analysis of size reduction of tumor cells and their ultrastructural alterations. The nucleus became small with clumped chromatin and irregular contour, and the cytoplasm decreased noticeably in amount, compared to the control group (Fig. 1 vs. Fig. 2). Rough ER and Golgi apparatus reduced by bromocriptine treatment, the latter being more remarkable. Secretory granules increased in number in the treated tumors. Most tumors had uniform-sized, smaller granules, which were located predominantly in the peripheral cytoplasm. One tumor had exceptionally numerous granules varying in size from 140 to 800 nm. Curiously, exocytosis of the secretory granules was more frequently found in bromocriptine group than in control group (Fig. 3). The exocytosis in bromocriptine-treated adenomas was either unigranular or multigranular, while that in untreated adenomas was mostly unigranular.

Figs 1 and 2

Fig. 3

Stereological Analysis. Stereological values were expressed in two ways, those per cm^3 of tumor cells and those per an average single tumor cell (Table 2). The former represents a relative abundance of

Table 2

the organelles in tumor cells, regardless of cell size. The latter represents an absolute value in an average single cell. When expressed per cm^3 of tumor cells, bromocriptine increased surface area of the nuclear membrane. This was consistent with the irregular contour of the nucleus. Relative abundance of Golgi area (the cytoplasm in which the Golgi apparatus predominated) decreased, while that of other major organelles involved in PRL synthesis remained unchanged. Secondary lysosomes (lysosomal structures engorging and digesting other cytoplasmic organelles to be disposed) and lipid droplets increased in volume. On the other hand, when expressed in absolute values per single tumor cell, major organelles (nucleus, rough ER, smooth ER, Golgi area, and mitochondria) decreased in volume or surface area. Particularly, the Golgi area decreased remarkably in volume.

Table 3

The diameter of secretory granules was not different on average between the bromocriptine group and the control group (Table 3). The granules increased in volume as well as in number in unit volume of bromocriptine-treated adenoma cells, but their increase in absolute volume per cell was not significant. Unexpectedly, exocytosis of the secretory granules increased remarkably in number. Frequency of crinophagy of the secretory granules (segregation and digestion within the lysosomal structures) remained unchanged.

Discussion

Mechanisms of bromocriptine in lowering serum PRL levels and reducing tumor size of human prolactinomas seem currently to be suggested as follows, based on numerous investigations using mostly

non-neoplastic pituitary tissues of experimental animals. Dopaminergic substances inhibit exocytosis of secretory granules containing PRL, resulting in a decrease in serum PRL concentrations with a concurrent increase in the intracellular levels of PRL (7-11, 14). This process is presumed to be linked to changes in adenylate cyclase activity (13), phosphatidylinositol concentrations (15), or most probably free Ca^{2+} concentration (12, 26). It is suggested that sustained rise in intracellular PRL concentrations reduces PRL synthesis by an intracellular feedback mechanism leading to inhibition of DNA synthesis (9, 14) and mitotic activity (11). Reduced PRL synthesis seems to result in reduction of subcellular organelles involved in it and subsequent reduction in size of tumor cells.

The present study demonstrated quantitatively the cytosuppressive effect of bromocriptine treatment for 2 weeks on human prolactinomas. Some 250 cells in each of 6 treated tumors were analyzed at electron microscope level, and compared with 4 untreated tumors. The most interesting finding was a significant increase in exocytosis of the secretory granules in bromocriptine-treated adenomas. The final administration of bromocriptine was done in the morning of the surgery day, 4 to 6 hours before tumor excision under the inspection of a nurse. It has been known that serum PRL levels reach minimum at 7 hours after oral administration of a single 2.5-mg dose of bromocriptine, although serum bromocriptine levels has already decreased to approximately a half of the maximum (27). Therefore, the increase in number of exocytosis is unlikely to be a rebound phenomenon due to a decrease in serum concentration of bromocriptine.

In spite of remarkable decrease in serum PRL levels, the number of exocytosis in the bromocriptine-treated adenomas increased to more than 4 times much as that in the untreated adenomas. This finding appears contradictory to the current suggestion concerning the mechanism of action of bromocriptine, as mentioned above. It has well been established that secretory granules contain anterior lobe hormones (28) and are released outside the cells by means of exocytosis (29, 30). Correlation of plasma PRL levels with the ultrastructure of human prolactinomas showed that the plasma PRL levels were inversely proportional to the number of secretory granules within the cells and paralleled the frequency of the exocytosis (31). Therefore, it should be reasonable to suppose that the decrease in serum PRL levels induced by bromocriptine treatment results from the decrease in number of exocytosis of the secretory granules. However, there seems no report which has obviously demonstrated the decrease in exocytosis by bromocriptine treatment, in either human prolactinomas or prolactinomas of experimental animals. An observation by Ectors _et al_. (32) seems only one substitute which suggests the decrease in exocytosis by bromocriptine. In _in vitro_ study with normal rat pituitary, they observed that ergocornine treatment for 18 hours decreased the exocytosis, and increased secretory granules within the cells. Their findings, however, seem somewhat short of persuasion because of mere survey observation but not quantitative analysis. The discrepancy between their findings and ours may be due to the differences in ergot derivatives examined, pituitary tissues used, treatments (_in vitro_ or _in vivo_) used, and particularly the duration of treatment.

Meanwhile, Dannies and Rudnick (33) reported the degradation of PRL molecule by bromocriptine. Their study with primary culture using dispersed normal pituitary cells showed that bromocriptine did not change the total amount of PRL for the first 8 hours, though release inhibition and intracellular accumulation were observed. However, bromocriptine reduced greatly the total amount of PRL after 4 days of the treatment because of the decrease in extracellular release and the same levels of intracellular PRL as the controls. At this time, synthesis of new PRL was only partly attenuated, but the stability of PRL was shown to decrease in bromocriptine-treated pituitary cells in culture. These results suggest that secretory granules in mammotrophs treated with bromocriptine for more than 4 days contain a little amount of PRL, and that the increase in exocytosis of the secretory granules does not contribute to the elevation of serum PRL levels. The secretory granules in mammotrophs have been shown to contain dopamine (34), glycosaminoglycans and glycoproteins (35) in addition to PRL. Bromocriptine may alters the composition of secretory granules. Demonstration of the decrease in PRL concentrations in secretory granules treated with bromocriptine is under investigation. It seems that bromocriptine decreases serum PRL levels by causing inhibition of exocytosis and consequent intracellular accumulation of the secretory granules in the early stage of treatment. However, in later period of treatment, mechanisms other than currently supposed in lowering serum PRL levels and reducing tumor size may occur. This should be clarified in future studies.

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Table 1 Clinical, endocrinological and radiological findings

Group	Patient No.	Age	Sex	Symptoms (duration: year or month)	Serum PRL (ng/ml) Before CB[a]	On CB	Tumor size before CB (diameter: mm)
Control group	1	24	F	A-G syndrome[b] (4 y)	114	-	4
	2	28	F	A-G syndrome (5 y)	152	-	12
	3	24	F	A-G syndrome (3 y)	300	-	12
	4	19	M	Visual disturbance (6 m), Impotence (3 m)	1,450	-	32
CB group	5	33	F	A-G syndrome (11 y)	62	12	3
	6	26	F	A-G syndrome (4 y)	721	10	9
	7	24	F	A-G syndrome (5 y)	268	5	12
	8	25	F	A-G syndrome (6 y)	1,480	62	14
	9	58	M	Impotence (24 y), Galactorrhea (24 y)	12,000	100	31
	10	50	M	Impotence (13 y), Visual disturbance (4 y), Nausea, Vomiting (2 y)	11,040	97	48

a Bromocriptine.

b Amenorrhea-Galactorrhea syndrome.

Table 2 Stereological data on ultrastructural alterations in prolactinomas treated with bromocriptine (Mean ± SEM)

Organelle	Parameter	Per cm^3 of tumor cells			Per single tumor cell[a]		
		Control group (n=4)	CB[b] group (n=6)	Unit	Control group (n=4)	CB group (n=6)	Unit
Cell	Volume				1,510 ± 101	905 ± 114*	μm^3
	Surface	7,180 ± 560	7,780 ± 180	cm^2	1,080 ± 84	700 ± 90*	μm^2
Nucleus	Volume	24.6 ± 0.7	31.1 ± 3.1	%	370 ± 22	269 ± 29*	μm^3
	Surface	2,200 ± 120	3,200 ± 300*	cm^2	330 ± 31	280 ± 32	μm^2
Rough ER	Volume	6.1 ± 0.6	4.9 ± 0.5	%	93 ± 14	46 ± 9*	μm^3
	Surface	31,000 ± 3,500	24,000 ± 3,400	cm^2	4,800 ± 670	2,200 ± 310*	μm^2
Smooth ER	Volume	0.36 ± 0.07	0.22 ± 0.05	%	5.4 ± 1.1	2.0 ± 0.6*	μm^3
	Surface	2,000 ± 180	1,600 ± 320	cm^2	300 ± 31	151 ± 40*	μm^2
Golgi area[c]	Volume	3.0 ± 0.2	1.5 ± 0.4*	%	45 ± 3	16 ± 2*	μm^3
Mitochondria	Volume	3.7 ± 0.4	3.2 ± 0.3	%	56 ± 3	29 ± 4*	μm^3
Lysosome (primary)	Volume	0.24 ± 0.05	0.31 ± 0.05	%	3.7 ± 0.8	2.7 ± 0.5	μm^3
Lysosome (secondary)	Volume	0.22 ± 0.06	0.77 ± 0.17*	%	3.2 ± 0.8	7.0 ± 1.6	μm^3
Lipid droplet	Volume	0.05 ± 0.02	0.24 ± 0.07*	%	0.7 ± 0.3	2.2 ± 0.7	μm^3

a Values per single tumor cell were obtained by dividing those per cm^3 of tumor cells by the tumor cell number per cm^3 of tumor cells in each tumor, determined at light microscope level.
b Bromocriptine.
c Cytoplasmic area in which Golgi apparatus predominates.
* Significantly different from the control group ($p<0.05$).

Table 3 Effect of bromocriptine on secretory granules

	Parameter	Per cm^3 of tumor cells			Per single tumor cell		
		Control group (n=4)	CB[a] group (n=6)	Unit	Control group (n=4)	CB group (n=6)	Unit
Secretory granule	Diameter				241 ± 19	211 ± 8	nm
	Number	0.54 ± 0.15	2.17 ± 0.25*	$x10^{12}$	810 ± 220	1990 ± 310*	
	Volume	0.47 ± 0.15	1.08 ± 0.24*	%	7.1 ± 2.3	8.5 ± 1.6	μm^3
Exocytosis	Number	1.76 ± 0.34	13.7 ± 3.4*	$x10^{6}$,[b]	19 ± 5	80 ± 14*	
Crinophagy	Number	26.0 ± 1.2	36.9 ± 5.1	$x10^{9}$	39 ± 3	32 ± 5	

a Bromocriptine.

b Number of exocytosis is expressed per cm^2 surface area of tumor cells.

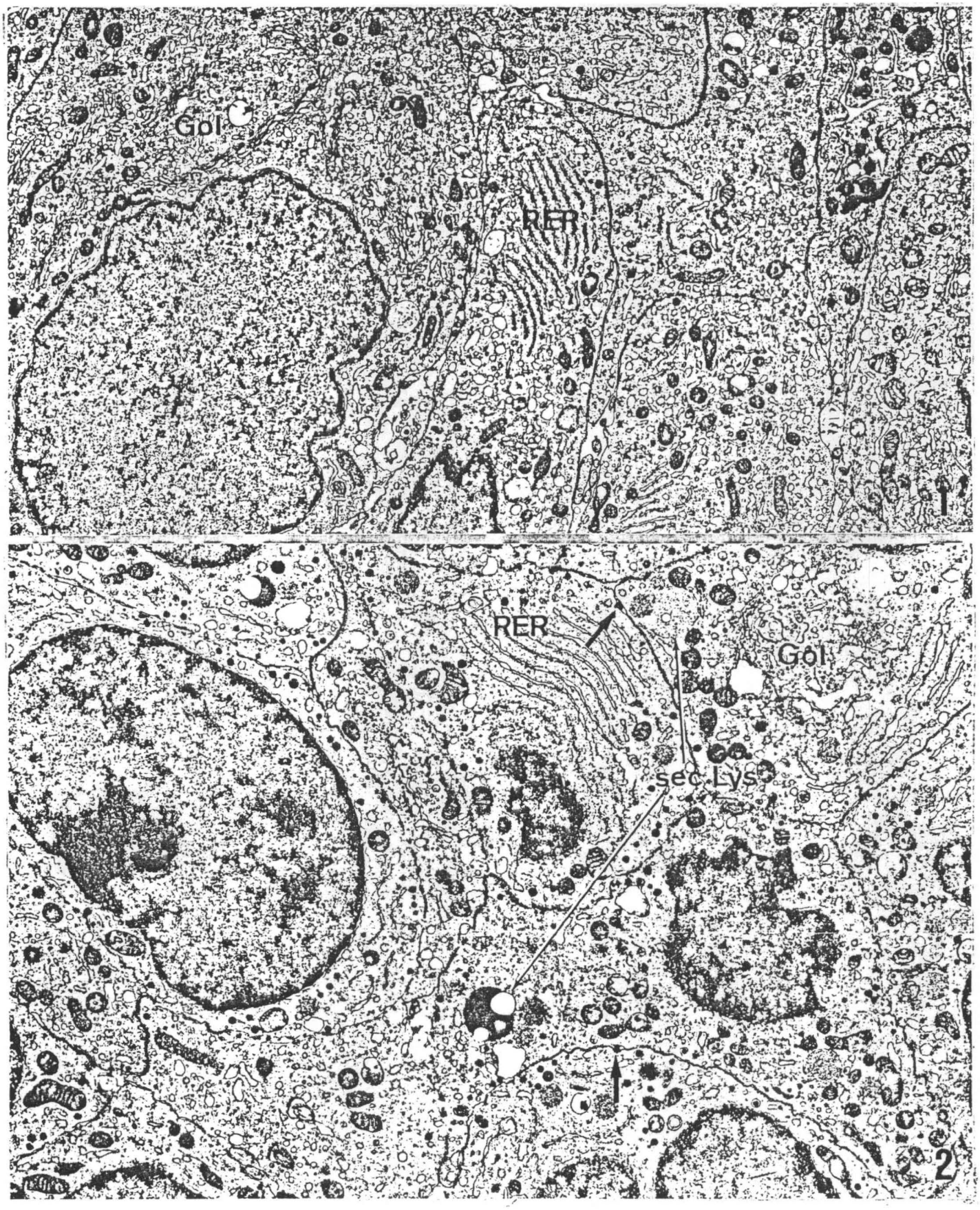
* Significantly different from the control group ($p<0.05$).

Legend for Figures

Fig. 1 Electron micrograph of an untreated prolactinoma showing well-developed rough endoplasmic reticulum (RER) and Golgi apparatus (Gol). Secretory granules were few in number. No exocytosis is seen in this micrograph. x 10,000.

Fig. 2 Electron micrograph of a bromocriptine-treated prolactinoma. Rough ER (RER) and Golgi apparatus (Gol) decreased in volume. Secretory granules increased in number. Arrows indicate exocytosis. sec Lys: secondary lysosomes. x 10,000.

Fig. 3 Electron micrograph from a bromocriptine-treated prolactinoma showing frequent exocytosis. Unigranular and multigranular exocytosis is indicated by single-arrows and double-arrows, respectively. x 16,000.


Gol
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