Dopamine Responsiveness of Human Prolactinoma Cells as Determined by the Reverse Hemolytic Plaque Assay

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Abstract. The responsiveness *in vivo* to dopamine of prolactin (PRL) secretion in patients with prolactinoma was compared with that *in vitro* of single cells obtained from the same prolactinomas by surgical operations. Six patients with prolactinoma showing various degrees of hyperprolactinemia were challenged by bromocriptine suppression test (2.5 mg, peroral) before operation. Bromocriptine administration caused a decrease in the serum PRL concentration ranging 24–95% and there was no correlation between the basal PRL level and bromocriptine-induced inhibition. Monodispersed pituitary cells obtained from the prolactinomas by operation were subjected to a reverse hemolytic plaque assay for PRL to determine PRL secretion at the single cell level under basal conditions as well as in response to dopamine. The percentage of plaque-forming cells under basal conditions ranged 15–55% among the prolactinomas. The percentage of plaque-forming cells and plaque area were decreased in a dose-dependent manner by 10^{-7} – 10^{-5} M dopamine for the pituitary cells obtained from some adenomas but not for those from other adenomas. When the inhibition rates *in vitro* due to 10^{-5} M dopamine in these two parameters were compared with the inhibition rate *in vivo* in the serum PRL concentration due to bromocriptine, it was found that there was a significant correlation between them. These results show that the reverse hemolytic plaque assay can be used to determine *in vitro* responsiveness to dopamine of PRL secretion from single prolactinoma cells. We conclude that 1) the relative abundance of PRL-secreting cells varies among prolactinomas; and 2) there is a correlation between *in vivo* and *in vitro* responsiveness of prolactinoma cells to dopaminergic inhibition.

Key words: Reverse hemolytic plaque assay, Prolactinoma, Dopamine, Bromocriptine

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DOPAMINE secreted from tuberoinfundibular neurons acts on the anterior pituitary gland as a prolactin (PRL)-release inhibiting factor [1]. On the basis of the dopamine action, bromocriptine is widely used to treat human prolactinomas [2, 3]. Although the serum PRL level and adenoma size are successfully decreased by bromocriptine treatment in most prolactinomas, this treatment is ineffective in some prolactinomas [3–7]. These two

types of dopamine-sensitive and resistant prolactinomas are distinguished by histopathological and computerized tomographic features [5, 8].

In the past years, most studies have revealed a function of endocrine cells as a amount of hormone secretion secreted into blood or medium. However, techniques such as the reverse hemolytic plaque assay (RHPA) [9] and cell immunoblot assay [10–12] have recently been developed to quantify hormone secretion from single endocrine cells in culture. These techniques have proven to be a reliable means for analyzing hormone secretion at the single cell level [13, 14].

In the present study, we determined the responsiveness to dopamine of PRL secretion of anterior

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pituitary cells obtained from patients with prolactinoma by using an RHPA. Furthermore, it was investigated whether the PRL responsiveness *in vitro* to dopamine of pituitary cells from each adenoma as determined by the RHPA correlates with that *in vivo* which has been determined by bromocriptine suppression test before surgical operations.

Materials and Methods

Subjects

Six patients with PRL-secreting adenoma, aged 19–57, were studied (Table 1). The patients revealed hyperprolactinemia of various degrees and all four females had amenorrhea and two males had visual disturbance. They showed no change in the serum concentrations of other anterior pituitary hormones. All of them were confirmed to have macroadenomas by computerized tomography. The patients were challenged by bromocriptine suppression test to determine the responsiveness *in vivo* of PRL secretion to dopamine before surgical operations. They were given perorally bromocriptine (2.5 mg) and bled before and 1, 2, 3, 4, 6, 12 and 24 h after. Serum PRL was measured by radioimmunoassay at SRL Laboratories (Tokyo, Japan).

Cell dispersion

Adenoma tissues were removed by the transphenoidal route and minced with a razor blade

into small fragments. The fragments were incubated with shaking at 37 °C for 90 min in Dulbecco's Modified Eagle's Medium with 20 mM HEPES buffer (DMEMH) containing 0.4% collagenase and 0.005% deoxyribonuclease (Sigma, St. Louis, USA). During incubation, trituration was performed every 15 min with a flame-polished siliconized Pasteur pipette. The fragments were then mechanically dispersed in Ca^{2+} , Mg^{2+} -free Hanks' solution containing 0.02% EDTA until monodispersed cells were obtained. The monodispersed cells were resuspended at a cell density of 3×10^5 cells/ml in DMEMH containing 0.1% BSA.

RHPA

The RHPA was done according to the method of Neill and Frawley [9]. Ovine RBC (oRBC) were obtained from Nippon Biotech (Japan). Protein-A-conjugated oRBC were prepared as described elsewhere [9] and used within 2 days. The assays were conducted in Cunningham incubation chambers ($22 \times 22 \times 0.1$ mm) constructed on poly-L-lysine (Sigma)-coated microscopic glass slides. Equal volumes of adenoma cell suspension and protein-A-coated oRBC (18%) were mixed and infused into the chamber, and the cells were allowed to attach for 1 h by 37 °C incubation. After incubation, the chambers were filled with DMEMH containing 10% fetal bovine serum, streptomycin (100 $\mu\text{g}/\text{ml}$), and penicillin G (100 U/ml) and cultured for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Immediately before assay, the chambers were washed with DMEMH containing 0.1% BSA and

Table 1. Serum PRL concentration before operation in 6 patients with prolactinoma

case	age	sex ^a	serum PRL (ng/ml)		% inhibition
			basal ^b	bromocriptine	
1	19	F	400	113	72
2	26	F	760	103	86
3	15	M	4500	750	83
4	57	M	1600	73	95
5	31	F	1200	490	59
6	26	F	62	47	24

a: F, female; M, male. b: Normal basal PRL concentrations are below 20 ng/ml. Patients were challenged by peroral administration of 2.5 mg bromocriptine and bled before and 1, 2, 3, 4, 6, 12 and 24 h after. Values are the nadir ones for serum PRL concentrations after bromocriptine administration.

then filled with a solution of DMEMH containing rabbit anti human PRL antiserum (DAKO A569) at a final dilution of 1:50. Various amounts of dopamine (10^{-8} – 10^{-5} M) were added to the solution containing PRL antiserum. After incubation with the antiserum and dopamine for 60 min at 37 °C in a CO₂ incubator, guinea pig serum (GIBCO Grand Island, USA) was added at a final dilution of 1:50 as a source of complement. After a 30 min incubation with guinea pig serum, the reaction was stopped by filling the chambers with 2% glutaraldehyde in saline.

The specificity of the assay was confirmed when no hemolytic plaque was found by omission of either anti-PRL or guinea pig serum and by replacement of anti-PRL with normal rabbit serum.

Quantitation and statistics

Adenoma cells in chambers were stained with Turk solution for microscopic analysis. When a minimum of two RBC layers were found around an adenoma cell, the cell was defined as a plaque former. A minimum of 300 cells were counted and scored positive or negative for plaque formation for each slide. At least 3 slides were counted in each cell preparation from patients. Differences between groups were statistically analyzed by Student's *t*-test or analysis of variance followed by Duncan's multiple range test [15].

Results

Six patients with prolactinoma had high serum

PRL concentrations ranging 62–4500 ng/ml (Table 1). In order to know the responsiveness *in vivo* of PRL secretion to dopaminergic inhibition, 2.5 mg bromocriptine was administered and the percent inhibition due to bromocriptine was determined by comparing the nadir of the serum PRL concentration after the administration with the basal concentration before it. The basal PRL concentration was decreased by bromocriptine administration of various degrees. The percent inhibition of the serum PRL concentration varied from 95 to 24% depending upon the patient. Cases 2, 3 and 4, cases 1 and 5, and case 6 were considered to be good, medium, and poor responders, respectively, to dopaminergic inhibition. There was no significant correlation between the basal level and percent PRL inhibition.

Monodispersed prolactinoma cells which had been obtained from pituitary adenomas were subjected to a RHPA for PRL in order to determine the basal secretion *in vitro* as well as secretion in response to dopamine. The percentage of plaque-forming cells at 1 h incubation without dopamine ranged 15–55% among patients from whom the prolactinoma cells were obtained (Table 2). The plaque area also varied 427–6629 μm^2 among the patients, showing a significant correlation with values for the percentage of plaque-forming cells of the corresponding patients ($P<0.05$). However, neither the percentage of plaque-forming cells nor the plaque area of individual patients correlated with their basal concentrations of serum PRL. Figure 1 shows that the percentage of plaque-forming cells and plaque area of individual patients was gradually decreased due to dopamine treatment.

Table 2. Effects of dopamine on PRL secretion from single prolactinoma cells

case	% of plaque-forming cells		plaque area (μm^2)	
	control	dopamine	control	dopamine
1	54.9 $\pm$ 1.7	25.0 $\pm$ 0.9 ^a	6629 $\pm$ 463	3685 $\pm$ 326 ^a
2	40.0 $\pm$ 2.3	0 ^a	4426 $\pm$ 1210	0 ^a
3	38.5 $\pm$ 1.9	5.3 $\pm$ 0.7 ^a	1790 $\pm$ 116	667 $\pm$ 64 ^a
4	18.3 $\pm$ 1.9	1.4 $\pm$ 0.2 ^a	3475 $\pm$ 478	1278 $\pm$ 358 ^a
5	15.7 $\pm$ 4.1	10.1 $\pm$ 1.5	879 $\pm$ 60	839 $\pm$ 51
6	15.2 $\pm$ 1.6	13.5 $\pm$ 0.3	427 $\pm$ 27	435 $\pm$ 29

Single pituitary cells obtained from patients were subjected to an RHPA for PRL. The pituitary cells were incubated for 1 h with either medium alone or medium containing 10^{-5} M dopamine. Values are the mean $\pm$ SEM.

a: significantly different from control ($P<0.05$).

Dopamine at concentrations of 10^{-7} – 10^{-5} M significantly decreased the two parameters in some patients ($P<0.05$) (cases 3 and 4) but had no effect on them in other patients (cases 5 and 6). The percentage of plaque-forming cells and plaque area treated with the maximal concentration of dopamine, 10^{-5} M, are individually shown in Table 2. When the percent inhibition of serum PRL due to bromocriptine was individually compared with the percent inhibition of the percentage of plaque-forming cells or plaque area due to dopamine, there were linear correlations between them ($y=1.33x-28.5$ and $y=1.26x-42.8$, respectively) (Fig. 2). The correlation coefficient was 0.94 for the inhibition of serum PRL and the percentage of plaque-forming cells ($P<0.01$), and 0.84 for the inhibition of serum PRL and plaque area ($P<0.05$).

Fig. 1. Dopamine inhibition of PRL secretion *in vitro* from pituitary cells obtained from individual patients with prolactinoma. The percentage of plaque-forming cells (lower panel) and plaque area (upper panel) were determined by the RHPA for PRL after 1 h incubation with various doses of dopamine. *Significantly different from no dopamine ($P<0.05$).

Discussion

To assess the secretory activity *in vitro* of human prolactinomas, we utilized RHPA in the present study. This is quite different from the methods used in previous studies [16–21]. The most important characteristic of this assay is that it is able to quantify hormone secretion from single endocrine cells in culture, which is difficult for a combination of conventional culture experiments and radioimmunoassay. However, another feature of RHPA is a simultaneous determination of the pro-

Fig. 2. Correlation of dopaminergic inhibition *in vivo* of PRL secretion of individual prolactinoma patients with that *in vitro* of PRL secretion of pituitary cells surgically obtained from them. For dopaminergic inhibition *in vivo*, serum PRL was determined before and after peroral administration of 2.5 mg bromocriptine to six patients with prolactinoma. For dopaminergic inhibition *in vitro*, the percent of plaque-forming cells (lower panel) and plaque area (upper panel) were determined after 1 h incubation with or without 10^{-5} M dopamine by the RHPA for PRL. Correlation coefficients in the lower and upper panels were 0.94 and 0.84, respectively.

portion of hormone-secreting cells to total cells and hormone secretion at the single cell level. These advantages of RHPA are fully appreciated in the present study for the analysis of PRL secretion. There are many studies with the RHPA that demonstrated basal as well as dopamine-inhibited PRL secretion in rats [9, 22, 23] but not in human subjects. Lloyd *et al.* [24] and Yamada *et al.* [25] measured only basal PRL secretion of human prolactinoma cells by means of the RHPA. Recovery of dispersed adenoma cells for 24 h in Cunningham chambers before being subjected to the RHPA made it possible to examine the effects of dopamine treatment on the plaque area and percentage of plaque-forming cells in the present study.

The percentage of plaque-forming cells after 1-h incubation without dopamine showed a great variation ranging 15–55% from patient to patient. This variation might be in part due to a difference in the rate of basal PRL secretion among the patients from whom prolactinoma cells were obtained. Determination of the maximal percentage of plaque-forming cells by longer incubation times would clarify this [26]. However, since the variation in the percentage of plaque-forming cells among patients seems too great to be accounted for solely by the difference in the secretion rate, the results rather suggest that the percentage of PRL-secreting cells differs from one prolactinoma patient to another. This view is well consistent with histopathological findings showing that the proportion of PRL-immunoreactive cells varied among prolactinomas [5, 8]. Furthermore, the present study demonstrates that the percentage of plaque-forming cells under basal conditions does not correlate with the basal level of serum PRL before operation.

A variation among prolactinomas was also observed for *in vitro* responsiveness of PRL secretion to dopamine. No more than 10^{-7} M dopamine significantly decreased both the plaque area and percentage of plaque-forming cells in pituitary cells

from some patients. These results are consistent with the results for the dopamine action in rats [16, 22]. However, even 10^{-5} M dopamine did not alter these two parameters in pituitary cells from some other patients (cases 5 and 6). The dopamine-resistant adenoma cells shown in the present study by means of the RHPA might be identical to those identified by histopathological and computerized tomographic aspects [5, 8]. The mechanism by which dopamine-resistant adenomas occur is still unknown. However, based on the finding that even normal rat pituitaries contain a minor subpopulation of lactotrophs that are unresponsiveness to dopamine [10, 23], it seems likely that normally-occurring dopamine-unresponsive lactotrophs proliferate and form a major population of dopamine-resistant adenomas.

The present study has shown that the rates of inhibition by dopamine of both the plaque area and plaque forming cells of each adenoma correlate well with the rate of inhibition of the serum PRL concentration by bromocriptine before operation. This result clearly indicates that *in vitro* responsiveness to dopaminergic inhibition of adenoma cells as determined by RHPA reflects their *in vivo* responsiveness which has been determined before operation. Probably adenoma cells would retain their *in situ* responsiveness to dopamine at least until 24 h after being removed from patients and cultured as in the present study. RHPA is therefore a useful *in vitro* technique for characterizing prolactinoma cells.

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