Effects of Bromocriptine on Staining Indices of Ki-67 and Proliferating Cell Nuclear Antigen, and Nucleolar Organizer Region Number in Pituitary Adenomas

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Abstract

The effects of bromocriptine (CB-154) on the proliferative capacities of prolactinoma and somatotropinoma were investigated by immunocytochemical staining indices of proliferating cell nuclear antigen (PCNA) and Ki-67 (with MIB-1 antibody), and silver staining of nucleolar organizer region (NOR) number in histological sections. Patients with prolactinoma and somatotropinoma were divided into two groups: no preoperative treatment (control group), and treated with CB-154 for 2 weeks before adenomectomy (CB-154 group). The prolactinoma CB-154 group showed a significantly lower PCNA staining index (n = 6, 13.1 ± 2.0%) and Ki-67 staining index (n = 6, 0.2 ± 0.03%) than the control group (n = 4, 27.1 ± 2.1%; n = 8, 1.9 ± 0.5%; respectively) (p < 0.01). The somatotropinoma CB-154 group showed a significantly lower Ki-67 staining index (n = 5, 0.7 ± 0.07%) than the control group (n = 5, 1.2 ± 0.2%) (p < 0.05), but there was no significant difference in PCNA staining index (control: n = 5, 19.1 ± 2.8% vs. CB-154: n = 5, 20.2 ± 1.4%). However, variable intensities of PCNA staining between the cells were observed, resulting in an extraordinarily high staining index. NOR numbers did not vary significantly between the two prolactinoma groups (control: n = 4, 2.0 ± 0.3 vs. CB-154: n = 6, 1.7 ± 0.1) and two somatotropinoma groups (control: n = 5, 1.3 ± 0.1 vs. CB-154: n = 5, 1.4 ± 0.2). Ki-67 staining index with MIB-1 antibody in paraffin sections heated in a microwave oven is more reliable than PCNA staining index or NOR number for evaluating the proliferative capacities of pituitary adenomas. The immunocytochemical procedure with MIB-1 antibody was technically more feasible than that of Ki-67 in frozen section. The results suggest that CB-154 treatment has an anti-proliferative effect on prolactinomas and somatotropinomas.

Key words: prolactinoma, somatotropinoma, bromocriptine, Ki-67, proliferating cell nuclear antigen, nucleolar organizer region

Introduction

Bromocriptine (CB-154) administration in patients with prolactinoma decreases the serum levels of prolactin (PRL) and can reduce the size of the tumor. CB-154 probably causes both cytoplasmic atrophy of the tumor cells and cell death, which experimental evidence suggests is apoptosis. These cytosuppressive and cytoidal effects of CB-154 result in the reduction of the tumor size. CB-154 inhibits deoxyribonucleic acid (DNA) synthesis or mitotic activity in rat prolactinoma. However, significant suppression of the proliferative capacities of human pituitary adenomas by CB-154
has not been demonstrated. This study investigated whether CB-154 treatment for 2 weeks suppresses proliferation in both prolactinoma and somatotropinoma based on measurement of nucleolar organizer regions (NORs), staining indices of Ki-67 and proliferating cell nuclear antigen (PCNA).

Materials and Methods

I. Patients

This study included 10 patients with prolactinoma and 10 with somatotropinoma. The patients were divided into two groups (control and CB-154 groups). The prolactinoma control group consisted of four patients (1 male, 3 females; age 19-28 yrs) who did not receive CB-154 before adenomectomy. The prolactinoma CB-154 group included six patients (2 males, 4 females; age 24-58 yrs) who received CB-154 (10 mg/day) orally for 2 weeks before adenomectomy to make the adenoma fragile allowing easy surgical removal. There were five patients (2 males, 3 females; age 39-59 yrs) in the somatotropinoma control group and five (2 males, 3 females; age 28-50 yrs) in the CB-154 group. A further four prolactinoma (all females; age 25-27 yrs) and six somatotropinoma (3 males, 3 females; age 21-54 yrs) samples were added to the control groups for the Ki-67 immunostaining study only. Before treatment, patients with prolactinomas had serum PRL levels ranging from 114 to 12,000 ng/ml and serum growth hormone (GH) levels within normal limits. Patients with somatotropinomas had serum GH levels ranging from 22.5 to 260 ng/ml and serum PRL levels within normal limits. Tumor tissues obtained at adenomectomy were fixed with 10% formalin in phosphate buffer, embedded in paraffin, and sliced into sections for light microscopy examinations.

II. NOR number

The sections (4-6 µm thick) were deparaffinized, immersed in a mixture of ethanol and acetic acid (3:1) for 5 minutes, and rehydrated in 70% ethanol for 5 minutes. The sections were rinsed in distilled water, and immersed in a mixture of one volume of 2% gelatin in 1% aqueous formic acid and two volumes of 50% aqueous silver nitrate solution for 40 minutes in the dark at room temperature. The sections were rinsed in distilled water and mounted. The NORs were counted by the method previously described. After changing the focus, the maximum number of NORs per cell was counted. In all cases, the number of NORs was counted in more than 200 cells and the mean number of NORs per cell was calculated.

III. Immunocytochemical examination

The sections (4-6 µm thick) were deparaffinized. Immunocytochemistry with MIB-1 antibody (Immunotech, Marseilles, France) used deparaffinized sections treated in citrate buffer (10 mM, pH 6.0) for 10 minutes at 500 W in a microwave oven. The sections were then treated with 0.3% hydrogen peroxide in methanol for 20 minutes to inactivate endogenous peroxidase. The sections were then incubated with 1% normal goat serum (for PRL and GH) or 1% normal horse serum (for PCNA and MIB-1) for 10 minutes, then incubated overnight at 4°C (according to Gerdes et al.) with a polyclonal anti-PRL or anti-GH antibody (Dako Corp., Carpinteria, Cal., U.S.A.; diluted peroxidase-anti-peroxidase kit), or a monoclonal anti-PCNA antibody (Oncogene Science, Inc., Uniondale, N.Y., U.S.A.) diluted to 0.05 µg/ml or MIB-1 diluted to 5 µg/ml. The sections were washed three times in phosphate buffer saline, and incubated with a secondary antibody (1:400; Vector Laboratories, Inc., Burlingame, Cal., U.S.A.) for 1 hour and followed by 1 hour incubation with avidin-biotin-horseradish peroxidase (HRP) complex (Vector Laboratories, Inc.). The histochemical localization of HRP was visualized with 3,3'-diaminobenzidine and hydrogen peroxide. The sections were lightly counterstained with hematoxylin. Normal pituitary gland tissue was used as a negative control for immunocytochemistry of PCNA and Ki-67.

PCNA or Ki-67 staining index was calculated as the percentage of immunopositive nuclei in the total number of nuclei counted in each section. More than 1000 cells were counted in several fields for each specimen. All immunopositive nuclei were counted irrespective of the intensity of staining.

IV. Statistical analysis

Student’s t-test or Mann-Whitney U test was used to determine the statistical significance. A p value less than 0.05 was considered to be statistically significant. Values are expressed as mean ± SEM.

Results

I. Clinical and histopathological effects

CB-154 treatment for 2 weeks reduced the serum PRL levels to less than 20% of those before treatment in the prolactinoma patients, and serum GH levels to 30-60% of those before treatment in the somatotropinoma patients. Computed tomography revealed the reduction of the tumor size of prolactinomas but not somatotropinomas.

The prolactinoma CB-154 group showed reduc-
tion in cell size and a variety of necrotic changes. Cell death was occasionally seen in the tumor nests. Fibrosis occurred in the perivascular space and also in the tumor nests, suggesting replacement fibrosis due to cell death. The volume of the stromal tissue increased to more than 2 times that of untreated tumors. The nuclei became smaller with clumped chromatin and irregular contours, and the amount of cytoplasm decreased noticeably, compared with the control group. The rough endoplasmic reticulum and Golgi apparatus were reduced remarkably. The number of secretory granules within a cell increased by 2.5 times, and the exocytosis of secretory granules increased 4 times, compared with the control group.

The somatotropinoma CB-154 group showed an increase in stromal tissue volume, but no apparent size reduction of cells or intracellular organelles. Single cell death was occasionally observed in a few somatotropinomas. The number of secretory granules within a cell and the frequency of exocytosis were the same in both somatotropinoma groups.

II. NOR number

NORs were seen as dark dots in the nuclei of the tumor cells. The numbers, size, and localization of NORs varied among the nuclei. Both prolactinoma and somatotropinoma cells usually contained one or two large, and round NORs.

In the prolactinoma control group, most cells contained one or two NORs, while some had a large NOR and some had more than two. In the prolactinoma CB-154 group, most cells had a single large NOR. However, there was no significant difference in NOR number between the control (2.0 ± 0.3) and CB-154 (1.7 ± 0.1) groups. In both somatotropinoma control and CB-154 groups, most cells contained a single large NOR. There was no significant difference in NOR number between the control (1.3 ± 0.1) and CB-154 (1.4 ± 0.2) groups.

III. Immunocytochemical examinations

PRL was localized in most tumor cells of the prolactinoma control group, although the staining intensity varied from cell to cell. Tumor cells in the prolactinoma CB-154 group were generally stained more intensely. GH was not expressed in any of the tumor cells. GH was expressed in the cells of both somatotropinoma control and CB-154 groups. The staining intensity was varied among the tumor cells. PRL was not expressed in any of the tumor cells.

Many nuclei were stained with varying intensities in both prolactinoma and somatotropinoma (Fig. 1). It was sometimes difficult to distinguish the positive from negative cells. The prolactinoma control group demonstrated many nuclei of cells positively stained for PCNA (27.1 ± 2.1%). The prolactinoma CB-154 group showed a significantly lower staining index (13.1 ± 2.0%) than the control group (p < 0.01). Both the somatotropinoma control and CB-154 groups demonstrated many nuclei positive for PCNA. There was no significant difference in staining index between the control (19.1 ± 2.8%) and CB-154 (20.2 ± 1.4%) groups.

There was very little discrete staining for Ki-67 of nuclei in both prolactinoma (Fig. 2) and somatotropinoma. In the prolactinoma control group (n = 8), the staining index ranged widely from 0.8% to 5.2% (1.9 ± 0.5%). The prolactinoma CB-154 group showed a consistently low staining index (0.2 ± 0.03%). There was a significant difference between the two groups (p < 0.01). The staining index of the somatotropinoma control group (n = 11) ranged from 0.1% to 2.7% (1.2 ± 0.2%) and was
significantly higher than that of the somatotropinoma CB-154 group (0.7 ± 0.07%) (p < 0.05).

Discussion

The proliferative capacity of the pituitary adenomas has been studied by several immunocytochemical methods. Monoclonal antibodies against bromodeoxyuridine (BrdU), a thymidine analog incorporated by DNA synthesis, can be used to evaluate the S-phase fraction of the cell cycle.\textsuperscript{25,26,34} Ki-67,\textsuperscript{16,19,26,34} DNA polymerase alpha,\textsuperscript{17} and PCNA\textsuperscript{15} are nuclear antigens, all expressed in the late G\textsubscript{1}, S, G\textsubscript{2}, and M phases, but not in the G\textsubscript{0} phase. The NOR number has also been considered to correlate with cellular proliferation of pituitary adenomas.\textsuperscript{34-36} Although the BrdU method is considered to be the most reliable to evaluate the proliferative potential, it requires preoperative infusion of BrdU to patients, which may have toxic or mitogenetic effects.\textsuperscript{21} This may be why BrdU labeling index has not been measured in human pituitary adenomas treated with CB-154. Our study used NOR number, and PCNA and Ki-67 staining indices in paraffin sections to evaluate the proliferative capacities of pituitary adenomas.

The NOR number has been reported to increase significantly with CB-154 treatment,\textsuperscript{34} and to decrease in prolactinoma treated with CB-154.\textsuperscript{36} Our study found no significant change in the NOR number between the control and CB-154 groups in both prolactinoma and somatotropinoma patients. These results suggest that the NOR number does not always correlate with the clinical aggressiveness or proliferative capacity of pituitary adenomas. NOR may reflect the effect of CB-154 on hormone production rather than the proliferative capacity.\textsuperscript{34}

PCNA is an auxiliary protein of DNA polymerase delta\textsuperscript{3} and is involved in DNA replication.\textsuperscript{4} The expression of PCNA is highest in the late G\textsubscript{1} and S phases, and rare in the M phase.\textsuperscript{5,7,37} PCNA immunoreactivity correlates with mitotic activity and tumor grade in solid human malignancies.\textsuperscript{30} The monoclonal anti-PCNA antibody has advantages over other widely used proliferative markers, because it does not require fresh and frozen tissue sections and, therefore, can be applied to routinely processed biopsy tissues.\textsuperscript{15} However, variations of staining intensity between cells were observed with this antibody in formalin-fixed paraffin-embedded tissues of pituitary adenomas (Fig. 1). Therefore, our result of PCNA staining index was extraordinarily high, as found in the previous report.\textsuperscript{15} These results suggest that PCNA staining index is not an appropriate cell cycle marker in pituitary adenomas. PCNA staining index may indicate other cellular activities in addition to proliferation.

Immunocytochemistry with Ki-67 antibody is a diagnostic technique for neoplasms of the nervous system, although requiring frozen tissues\textsuperscript{6} and a delay of less than 30 minutes between removal of the specimen and freezing.\textsuperscript{14} Previous studies have failed to demonstrate a decrease in Ki-67 staining index in pituitary adenomas after CB-154 treatment.\textsuperscript{19,34} In this study, visualization of antigen was attempted in formalin-fixed paraffin-embedded tissues heated in a microwave oven.\textsuperscript{13,35} This method was very simple and specific, and the positive nuclei were easily counted because only a few pituitary adenoma cells were stained (Fig. 2). We previously showed that Ki-67 staining index using MIB-1 antibody was well correlated with BrdU labeling index in transplanted human glioma in nude mice,\textsuperscript{20} but the PCNA staining index was not (data unpublished). This evidence suggests that Ki-67 staining index using MIB-1 antibody in paraffin sections is a reliable method for evaluation of proliferative capacity.

The numerous investigations into the effects of CB-154 on prolactinomas in both humans and experimental animals and non-neoplastic mammotrophs\textsuperscript{10,20,22-27} have not completely clarified the mechanism of the anti-tumor activity of CB-154. A sustained rise in intracellular PRL concentrations may reduce PRL synthesis by an intracellular feedback mechanism leading to inhibition of DNA synthesis\textsuperscript{21} and mitotic activity.\textsuperscript{21} However, such an anti-proliferative effect of CB-154 on human pituitary adenomas has not been demonstrated. The morphological examination of the tumors in this study found that CB-154 had a cytotoxic effect on both prolactinomas and somatotropinomas,\textsuperscript{23} resulting in tumor cell death and cytosuppressive effects, especially in prolactinomas.\textsuperscript{24,32} The cell death caused by CB-154 may be apoptosis based on electron microscopic study of animal pituitary tumors.\textsuperscript{9,18}

Our study indicated that CB-154 treatment for 2 weeks causes an anti-proliferative effect on human prolactinoma and somatotropinoma. Immunocytochemical measurement of Ki-67 staining index with MIB-1 antibody seems to be a convenient and reliable method of evaluating the proliferative potential of pituitary adenomas.

References

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