Inhibition of Growth of Human Small Cell Lung Cancer by Bromocriptine

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ABSTRACT

Bromocriptine, a dopaminergic agonist, inhibited the growth of human small cell lung cancer (SCLC) implanted as tumor xenografts in athymic nude mice; the effect was dose dependent. In mice bearing a SCLC with ectopic vasopressin production, plasma levels of human vasopressin-associated neurophysin decreased concomitantly. Electron microscopy of tumor tissues revealed marked degenerative changes, including pyknosis, densely aggregated chromatin masses, and vacuolization of cytoplasm after bromocriptine treatment. When a SCLC cell line, NCI-H69, was grown in semisolid medium, bromocriptine inhibited its clonal growth in a dose-related manner. Coincubation with dopamine D2 receptor agonist, metoclopramide, or domperidone, completely blocked the inhibitory effect of bromocriptine. Receptor studies with a dopamine D2 receptor ligand, [3H]iodosulpride, showed high affinity binding sites on the membranes of SCLC cells. These results indicate that SCLC cells are enriched with dopamine D2 receptors, which may mediate the growth-inhibitory effect of bromocriptine on SCLC. Dopaminergic agonists may be useful in the medical treatment of SCLC.

INTRODUCTION

SCLC, unlike other lung cancers, expresses neuroendocrine properties, which include the presence of intracytoplasmic neurosecretory granules and its ability to secrete a variety of neuropeptides and ectopic hormones. The pathophysiologial significance of this peptide synthesis is not established; however, available evidence indicates that the peptides elaborated and secreted by SCLC may regulate its own growth in an autocrine or paracrine fashion (1, 2). The apparent dependence of growth on the secretion of autocrine growth factors then suggests the therapeutic potential of peptide antagonists with a broad range of inhibitory function in the treatment of SCLC.

Dopamine possesses a direct action on pituitary cells to inhibit the secretion of prolactin, growth hormone, ACTH, and thyrotropin. We and others have previously shown that dopamine or bromocriptine, a dopaminergic agonist, inhibits ACTH secretion from ACTH-producing SCLC cells in culture (3, 4). This finding supports the view that dopamine may exert an inhibitory effect on the secretory activity of SCLC. Further, cyproheptadine, an inhibitor of ACTH secretion from human pituitary corticotrophs (5), did not suppress ACTH release by SCLC, suggesting the specificity of dopamine in this regard (3). If SCLC growth is really regulated by multiple autocrine circuits, inhibition of the secretory activity by dopamine or dopaminergic agonists may lead to the disruption of the growth cycle of SCLC. To prove this hypothesis, we studied the effect of bromocriptine on the growth of SCLC in vivo and in vitro, and examined dopamine receptors on SCLC cells in the present study.

MATERIALS AND METHODS

SCLC Tissues. Four different SCLC tissues were used in this study. Three of the tissues (nos. 1–3) were obtained at autopsy from patients with SCLC and were serially propagated in nude mice. When incubated in vitro, tissue 1 produced and secreted vasopressin and neurophysin into the incubation medium, indicating that this SCLC was capable of producing vasopressin (6, 7). The other two tissues, on the other hand, were apparently devoid of ectopic hormone production. The fourth tissue (tissue 4) we used was NCI-H69, a human SCLC cell line, which was obtained from the American Type Culture Collection (Rockville, MD). NCI-H69 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO2 and 95% air at 37°C. Cultured cells were collected by low-speed centrifugation, resuspended in the culture medium, and injected s.c. into athymic nude mice. The tumors from the first implant generation were used as donor tumors. On light microscopy all four tumors showed the characteristics of intermediate cell type of SCLC.

Growth Studies in Vivo. Donor tumors were sectioned in pieces of 2-mm cubes and each piece was implanted s.c. into the left rear flank of female, athymic nude mouse at 7 weeks of age weighing between 20 and 22 g. Animals were randomized into control and test groups after the implantation. A group of 12 mice received a low-dose (0.5 mg/kg/day) or a high-dose (10 mg/kg/day) bromocriptine mesylate (Sandoz, Basel, Switzerland) in food. The control group consisted of 12 mice given normal food. Tumor sizes were measured weekly by a caliper and were expressed by a multiple of longitudinal and transversal lengths of tumors. When tumor xenografts grew and the largest tumor in each experiment reached a certain size (a multiple of longitudinal and transversal distances of tumors less than 600 mm3), all mice were sacrificed and tumors were removed. Tumors were weighed and histology was examined by light and electron microscopy.

Hormone Measurements. Blood samples were collected from individual mice by retroorbital puncture into heparinized pipets. Plasma was separated by centrifugation and stored at −20°C until assayed. Prolactin concentrations in the plasma were measured by radioimmunoassay with the use of materials kindly provided by Dr. A. F. Parlow of the Pituitary Hormones and Antiserum Center, Harbor UCLA Medical Center. Concentrations of human vasopressin-associated neurophysin were measured by radioimmunoassay as previously described in detail (8). The plasma from nude mice without tumor xenografts had no measurable neurophysin (less than 0.4 ng/ml) in this radioimmunoassay system.

Electron Microscopy. Tumor tissues were fixed with 2% glutaraldehyde, followed by postfixation with 1% osmium tetroxide. After dehydration with a graded ethanol series, the tissues were embedded in epoxy resin mixture and polymerized. Ultrathin sections were cut on a LKB 2088 microtome (Pharma- cia LKB Biotechnology Inc., Uppsala, Sweden), stained with uranyl acetate and lead citrate, and observed with a JEM 1200EX transmission electron microscope (Nihonbashi, Tokyo, Japan).

Clonogenic Assay. NCI-H69 cells were suspended in phosphate-buffered saline (pH 7.4) containing 0.25% trypsin and 0.02% EDTA, and disaggregated into a single cell suspension by drawing repeatedly through a Pasteur pipet. The cells were washed and viability was judged by trypan blue exclusion on a standard hemocytometer. Approximately 105 viable cells were resuspended in 1 ml of 0.3% agarose-RPMI 1640 medium including 5% fetal bovine serum with or without 3-fold concentrated bromocriptine, metoclopramide hydrochloride (Fujisawa Pharmaceutical Co., Osaka, Japan), or domperidone (Kyowa Hakko Co., Tokyo, Japan). The cells were then plated in 5 replicate 35-mm plastic dishes containing a 2-m1 base layer of 0.5% agarose in culture medium. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air for 14 days. Colonies with diameters of >120 μm were counted under a microscope.

Dopamine Receptor Study. SCLC tissues propagated in nude mice were stored at −70°C until analyzed. The tissues were sectioned while still frozen, and homogenized in 5 volumes of ice-cold 50 mm Tris-HCl buffer, including 1 mm EDTA (pH 7.4), using a glass-Teflon homogenizer. The homogenate was centri-
fuged at 600 × g at 4°C for 15 min, the supernatant was collected, and the low-speed pellet was treated as described above. Both supernatants were then pooled and centrifuged at 20,000 × g at 4°C for 20 min, and the pellet representing the membrane fraction was suspended in incubation buffer containing 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 1.5 mM MgCl2, 1.5 mM CaCl2, 5.7 mM ascorbic acid, and 200 units/ml aprotinin (pH 7.4). Protein concentrations were determined by BCA protein assay kit (Pierce, Rockford, IL).

Aliquots of the membrane fraction were incubated at room temperature for 1 h with [125I]iodosulpride (specific activity, 74 TBq/mmol; Amersham International, Amersham, United Kingdom) (9) in the incubation buffer (total incubation volume, 0.4 ml). Nonspecific binding was determined by incubation in the presence of 2 μM (−)-sulpiride. The incubations were terminated by centrifugation at 10,000 × g for 20 min. The supernatants were aspirated and the tips of the tubes containing the membrane pellets were counted in a gamma counter.

Statistical Analyses. Values in the text, table, and figures are expressed as the mean ± SE. The significance of differences was calculated by analysis of variance and Student’s t test, or by means of the χ² test with Yates’ correction. Values of P < 0.05 were considered to be significant.

RESULTS

Growth Studies in Vivo. To determine whether bromocriptine at doses used in the present study may show cumulative toxicities in animals, a group of 5 female, athymic nude mice at 7 weeks of age were fed 0.5 or 10 mg/kg bromocriptine daily for 15 weeks. No toxic side effects or premature deaths were observed in any of the mice. The mean body weights of these mice measured once a week were not significantly different from those of 5 control mice given normal food throughout the experiment.

Fig. 1 shows the growth curves of tumor xenografts after inoculation of four different SCLCs into nude mice. Most implanted mice developed tumors. The tumor take rates of SCLCs 1, 2, and 3 in the high-dose bromocriptine group were lower than in the control group, but the differences were not statistically significant. All tumors in control mice grew progressively with varying growth rates in individual SCLCs. Administration of bromocriptine at a dose of 10 mg/kg/day caused a significant inhibition of tumor growth in mice bearing SCLCs 1, 3, and 4. The mean tumor sizes in bromocriptine-treated mice at the end of experiments were 51% (no. 1), 46% (no. 3), and 47% (no. 4), respectively, of those in control mice. In accordance with this finding, the mean weights of tumors removed from these mice were significantly lower in the bromocriptine group than in the control group (Table 1). In the remaining one experiment using SCLC 2, the mean tumor sizes in bromocriptine-treated mice were smaller than in controls throughout the period of observation, but the differences were not statistically significant at almost all experimental stages. There was no significant difference between the mean tumor weight in the bromocriptine group and that in the control group in this experiment (Table 1). The actual body weights of mice at sacrifice in control and bromocriptine groups calculated by subtracting tumor weights from measured body weights did not differ significantly in all experiments.

To determine if this action of bromocriptine may be dose related, we tested the effect of a lower dose (0.5 mg/kg/day) of bromocriptine on tumor growth in mice implanted with SCLC 3. The low-dose bromocriptine was also effective in inhibiting tumor growth, although the magnitude of inhibition was less when compared to high-dose bromocriptine (Fig. 1). The mean tumor size in the bromocriptine group at the end of the experiment corresponded to 61% of that in the control group. Again, the mean tumor weight in bromocriptine-treated mice was significantly (P < 0.05) smaller than in control mice (Table 1). Similarly, the tumor growth-inhibitory effect was demonstrated by the low-dose bromocriptine in two separate experiments using mice bearing SCLCs 1 and 4 (data not shown).

When tested in vitro, SCLC 1 produced and secreted vasopressin. To explore whether bromocriptine may decrease vasopressin secretion...
with inhibition of tumor growth, we measured concentrations of human vasopressin-associated neurophysin in the plasma of mice bearing the tumor xenografts. The mean plasma neurophysin concentration (8.5 ± 3.6 ng/ml, n = 10) in mice treated with the high-dose bromocriptine at the end of the experiment was significantly (P < 0.05) lower than in control mice (24.5 ± 4.7 ng/ml, n = 12).

To ascertain that bromocriptine was absorbed and really caused biological effects in mice, mice bearing SCLC 3 were bled before sacrifice and prolactin concentrations in the plasma were measured. The mean plasma prolactin concentration in the high-dose bromocriptine group (7.3 ± 1.4 ng/ml, n = 11) was significantly (P < 0.05) lower than in the control group (44.7 ± 17.1 ng/ml, n = 12). The mean plasma prolactin concentration (27.8 ± 5.0 ng/ml, n = 12) in the low-dose bromocriptine group was lower than in the control group, although the difference was not statistically significant.

**Electron Microscopy of Implanted Tumors.** Fig. 2 shows electron microphotographs of the tumors removed from mice implanted with SCLC 1. The tumors from bromocriptine-treated mice had marked degenerative changes, including pyknosis, densely aggregated chromatin masses, and vacuolization of cytoplasm. Administration of bromocriptine induced similar changes in tumor xenografts in mice implanted with SCLC 3 and 4. Degenerative changes were also observed, although less marked, in tumors removed from mice bearing SCLC 2, which responded poorly to the tumor growth-inhibitory effect of bromocriptine.

**Growth Studies in Vitro.** In a clonogenic assay, bromocriptine in concentrations as low as 0.1 nM significantly inhibited the growth of SCLC cell line NCI-H69 (Fig. 3A). Moreover, a dose-response relationship was observed between the concentrations of bromocriptine and the magnitude of inhibition of colony formation. When 1 μM metoclopramide, a dopamine D2 receptor antagonist, was coincubated with 100 nM bromocriptine, the inhibitory effect of bromocriptine on clonal growth was completely blocked (Fig. 3B). Dopamine D2 receptor blockade by domperidone was also effective in this regard. Metoclopramide or domperidone alone did not significantly affect SCLC colony formation.

**Dopamine Receptors on SCLC.** To determine whether the inhibitory effect of bromocriptine on SCLC growth may be mediated by dopamine receptors, we examined the in vitro binding of $[^{125}\text{I}]$iodosulpride, a selective ligand for dopamine D2 receptors (9), to membrane fractions prepared from SCLC. Specific $[^{125}\text{I}]$iodosulpride-binding sites were present in membranes from all four SCLC tissues. With 0.07 nM $[^{125}\text{I}]$iodosulpride, binding was linear with respect to protein concentrations (Fig. 4A). As expected, binding sites were the fewest in SCLC 2 that did not respond significantly to the growth-inhibitory effect of bromocriptine.

Fig. 4B shows saturation curve and Scatchard analysis with the use of $[^{125}\text{I}]$iodosulpride as a ligand and (-)-sulpiride as a competitor in the membrane fraction of SCLC 4. The specific binding of $[^{125}\text{I}]$iodosulpride to the SCLC membranes was monophasic and of high affinity. The estimated dissociation constant was 0.86 nM and the receptor density was 17.7 fmol/mg protein. High-affinity saturable $[^{125}\text{I}]$iodosulpride-binding sites were also demonstrated in membrane fractions derived from two other SCLC tissues (nos. 1 and 3). The dissociation constants were 0.72 and 0.93 nM, and the receptor densities were 83 and 10.7 fmol/mg, respectively. Because of a limited number of binding sites for $[^{125}\text{I}]$iodosulpride, accurate determination of kinetic data was not possible in the membrane fraction derived from SCLC 2.

**Table 1 Mean weights of tumor xenografts removed from mice in control and bromocriptine groups**

<table>
<thead>
<tr>
<th>Tumor and group</th>
<th>No. of mice</th>
<th>Tumor wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCLC 1 Control</td>
<td>12</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>SCLC 1 Bromocriptine (10 mg/kg/day)</td>
<td>10</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>SCLC 2 Control</td>
<td>10</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>SCLC 2 Bromocriptine (10 mg/kg/day)</td>
<td>12</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>SCLC 3 Control</td>
<td>12</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>SCLC 3 Bromocriptine (10 mg/kg/day)</td>
<td>11</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>SCLC 3 Bromocriptine (0.5 mg/kg/day)</td>
<td>12</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>SCLC 4 Control</td>
<td>12</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>SCLC 4 Bromocriptine (10 mg/kg/day)</td>
<td>12</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

* Mean ± SE.

* P < 0.05 versus control.

![Fig. 2. Electron microphotographs of the tumors (SCLC 1) removed from control mice (A) and bromocriptine-treated mice (B). × 3000.](image-url)
DISCUSSION

The foregoing results demonstrate that bromocriptine inhibits the growth of SCLC implanted as tumor xenografts in athymic nude mice in a dose-related manner. Since bromocriptine at doses used in this study did not show any toxic side effect in mice, the observed inhibitory action of bromocriptine on SCLC growth may be occasioned by dopaminergic stimulation per se. In accordance with this view, the clonal growth of SCLC cell line NCI-H69 was inhibited by bromocriptine in a dose-dependent fashion, and this effect was completely reversed by dopamine D₂ receptor blockade with metoclopramide or domperidone. Moreover, high-affinity dopamine D₂ receptors were present on the membranes of SCLC cells. As expected, the receptor density was low in one SCLC that responded poorly to the growth-inhibitory effect of bromocriptine.

Further evidence to support the direct action of bromocriptine on SCLC was obtained by electron microscopy of tumor tissues. Tumors removed from mice showed selective degenerative changes after bromocriptine treatment. We conclude from these results that SCLC cells are enriched with dopamine D₂ receptors, which may mediate the inhibitory action of bromocriptine on SCLC growth. Whether this growth-inhibitory effect of bromocriptine may be confined to SCLC, or may be observed also in other types of tumors is unknown. In view of the finding that dopamine receptors are not present in human tumoral cell lines devoid of the characteristics of the amine precursor uptake and decarboxylation system (10), the former view may be more likely.

Dopamine acts directly on pituitary cells to inhibit hormone secretion. Based on this observation, dopaminergic agonists are now...
widely used in the medical management of functioning pituitary adenomas. In addition to inhibition of prolactin release, dopaminergic agonists cause pituitary tumor regression in the majority of patients with prolactinoma. Although the antimitotic action of bromocriptine has been demonstrated in rat pituitary lactotrophs stimulated by estrogen (11) and in rat or human pituitary tumor cells (12, 13), a number of reports suggest that pituitary tumor regression induced by bromocriptine is largely due to lactotroph cell size reduction with a rapid involution of rough endoplasmic reticulum and Golgi apparatus (14). This contrasts to the marked degenerative changes observed in SCLC tissues after bromocriptine treatment in the present study. The mechanisms underlying the inhibition of SCLC growth by bromocriptine, therefore, may be different from those involved in pituitary tumor shrinkage in patients with prolactinoma.

What then is responsible for the bromocriptine-induced inhibition of SCLC growth? A large body of evidence indicates that the production of autocrine growth factors by SCLC cells may play an important role in the abnormal growth regulation characteristic of malignant cells (1, 2). Bombesin or gastrin-releasing peptide, the mammalian counterpart of bombesin, stimulates the proliferation of SCLC cells both in vivo and in vitro (15–17). A monoclonal antibody against bombesin, or bombesin analogues that bind to bombesin receptors on SCLC cells without activation of signal transduction, can inhibit growth of xenografted SCLC in nude mice and the clonal growth of SCLC cells in vitro (18, 19). The recent study shows, in addition, that other neuropeptides including bradykinin, galanin, neurotensin, cholecystokinin, and vasopressin stimulate growth of SCLC cells in a clonogenic assay (20). In view of these observations, dopamine D2 receptor activation may have suppressed, as in pituitary cells, the production and secretion by SCLC of these autocrine growth factors with a resultant inhibition of SCLC growth. The concentrations of human vasopressin-associated neurophysin in the plasma of mice bearing a SCLC with ectopic vasopressin production decreased after bromocriptine treatment. This indicates that vasopressin secretion by the tumors was in fact inhibited by bromocriptine (6, 7), although the possibility that decreased vasopressin secretion may be secondary to the reduction of tumor sizes could not be completely excluded.

An alternative explanation for the inhibition of SCLC growth by bromocriptine may be the blockade of generation of early signals in the mitogenic response of SCLC cells by dopamine D2 receptor stimulation. Dopamine D2 receptors are negatively coupled to adenylate cyclase (21), which may be one of the early signals in a complex signaling pathway, and new approaches to the treatment of SCLC.
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