ABSTRACT

Groups of 15 female Syrian golden hamsters with N-nitrosobis(2-oxopropyl)amine-induced pancreatic cancers were treated for 2 mo with microcapsules of the luteinizing hormone-releasing hormone (LH-RH) antagonist [Ac-d-Nal(2),d-Phe(4)CH2.d-Pal(3),d-Cit(6),d-Ala(9)] LH-RH (SB-75) releasing 8 µg/day or with the microcapsules of the LH-RH agonist d-tryptophan-6-luteinizing hormone-releasing hormone (d-Trp-6-LH-RH) releasing 8 µg/day or 25 µg/day. Chronic treatment with SB-75 resulted in 70% inhibition of pancreatic tumor weight; d-Trp-6-LH-RH in doses of 8 µg/day and 25 µg/day produced 66% and 62% inhibition, respectively. The number of animals with pancreatic tumors was reduced by about 50% in each treated group. Tumorous ascites were found in seven control hamsters and in one hamster in each group treated with d-Trp-6-LH-RH but not in the group given SB-75. Reduction in serum luteinizing hormone levels and ovarian as well as uterine weights indicated that an inhibition of the pituitary-gonadal axis occurred during chronic SB-75 and d-Trp-6-LH-RH treatment. Membrane receptor assays showed a significant decrease of the concentration of binding sites for LH-RH in tumor cells after SB-75 or d-Trp-6-LH-RH treatment. Insulin-like growth factor I receptors, but not epidermal growth factor receptors, were down-regulated by d-Trp-6-LH-RH. SB-75 did not influence the concentration or the binding capacity of insulin-like growth factor I and epidermal growth factor receptors in the tumor cells. The inhibitory effect of chronic treatment with SB-75 and d-Trp-6-LH-RH on tumor growth was mediated by enhanced apoptosis (programmed cell death) induced by the change in hormonal environment. Apoptosis was also produced in hamsters with N-nitrosobis(2-oxopropyl)amine-induced pancreatic cancers by acute treatment (3 to 6 days) with high doses of d-Trp-6-LH-RH or SB-75. In view of its potency and an immediate powerful inhibitory effect, the LH-RH antagonist SB-75 might be considered as a possible new hormonal agent for the treatment of experimental pancreatic cancer.

INTRODUCTION

Agonist analogues of LH-RH and of somatostatin have been shown to inhibit the growth of various transplantable and induced, hormone-sensitive experimental animal tumors (1-8). A series of studies were carried out with these peptides on BOP-induced hamster pancreatic carcinoma (1, 5, 6, 9). We also pointed out that the tumor growth-inhibitory effect of these analogues was mediated by an enhanced rate of apoptosis (programmed cell death) (6-10). Recently an increase in survival time of patients with advanced pancreatic cancer after d-Trp-6-LH-RH therapy has been reported (11). Agonist analogues of LH-RH appear to decrease pancreatic cancer growth by eliminating the stimulatory effect of sex steroids and by direct effects on tumors (12). However, the agonistic analogues induce an initial stimulation of the release of gonadotropin and sex steroids which may cause a temporary flareup of the disease with undesirable clinical consequences. The use of new antagonistic analogues of LH-RH (13, 14), which cause an immediate inhibition of the pituitary-gonadal axis, would avoid the initial stimulatory effects on tumor growth. Studies carried out on tumor cell lines suggest that LH-RH antagonists may also have a direct inhibitory effect on tumor growth (15). This indicates the need to investigate the receptors for both LH-RH and growth factors in tumors.

In this study, the LH-RH antagonist SB-75 (13), free of edematogenic effects, was administered to Syrian golden hamsters with BOP-induced carcinomas of the exocrine pancreas. The effect of SB-75 treatment was compared with that of the LH-RH agonist d-Trp-6-LH-RH.
received the following treatment: Group 1, injection vehicle only (BOP controls); Group 2, SB-75 microcapsules (13.3 mg/animal on Days 0, 21, and 42); Group 3, D-Trp-6-LH-RH microcapsules (36 mg/animal on Days 0 and 30); and Group 4, D-Trp-6-LH-RH microcapsules (12 mg/animal on Days 0 and 30). Other hamsters in groups of 3 received s.c. injections of analogues dissolved in 10% polyvinylpyrrolidone in 0.9% NaCl solution as follows: Group 5, 100 µg of SB-75/animal daily for 3 days before the termination of the experiment; Group 6, 50 µg of D-Trp-6-LH-RH/animal daily, for 3 days before sacrifice; and Group 7, 50 µg of D-Trp-6-LH-RH/animal daily for 6 days before sacrifice. The experiment was terminated 8 wk after the first treatment with the microcapsules or vehicle in Groups 1 to 4 or 4 to 7 wk after the first s.c. injection in Groups 5 to 7, which corresponded in all cases to 32 wk from the start of the BOP treatment.

Pathological Procedures. During the 8-wk treatment period, pancreata and livers of animals that died were processed for histology. At the end of the eighth week, the hamsters were exsanguinated under methoxyflurane (Metofane; Pitmann-Moore, Washington Crossing, NJ) anesthesia. Body weights were measured, and any ascites was removed and measured. The average weight of all pancreas as well as the average weight of the tumorous pancreata (based on histology) in each group was recorded. Pancreas, liver, uterus, and ovaries were removed, cleaned, and weighed. Pancreatic and liver tissue was fixed in 4% paraformaldehyde for 24 h, dehydrated in acetone and lead citrate and viewed in a JEM 100 B electron microscope (JEOL, Tokyo, Japan).

Receptor Assays. Only tumors weighing more than 0.2 g could be processed for receptor measurement. Membrane preparation, radioiodination of peptides, and receptor binding of D-Trp-6-LH-RH, EGF, and IGF-I were performed as previously described (19-21). Recombinant human IGF-I for receptor assay was kindly provided by Genentech, Inc. (San Francisco, CA). Recombinant human EGF was purchased from Amgen Biologicals (Thousand Oaks, CA). The LIGAND-PC computerized curve-fitting program of Munson and Rodbard (22) was used to determine the types of receptor binding, dissociation constants (KΔ), and the maximal binding capacity of receptors (Bmax).

Serum LH and IGF-I Levels. Serum LH and IGF-I levels were determined using LH-RP-2 and UKB-487 reference preparations provided by the National Hormone and Pituitary program of NIDDK.

Statistical Analyses. Statistical analyses were performed by the two-sided Student t test and the Fisher exact test.

RESULTS

Body, Ovarian, and Uterine Weights and Liver Pathology. The body weights of the animals increased by about 50% during the experiment. No differences were seen between the control and treated groups in this respect. A significant decrease in the weight of ovaries and uteri was seen in the treated groups, compared with the control (Table 1).

Serum LH and IGF-I Levels. Serum LH was decreased significantly after the treatment with SB-75. Serum IGF-I increased slightly after D-Trp-6-LH-RH therapy (Table 1).

Tumor Pathology. Five control animals died because of pancreatic cancer during the treatment period. Three of the hamsters treated with SB-75 and 3 treated with 8 µg/day of D-Trp-6-LH-RH also died. In both of these groups, 2 of the 3 hamsters had pancreatic tumors, and one showed preneoplastic lesions such as various types of ductal proliferation in the pancreas (23). The livers of all hamsters were cirrhotic, with occasional cystic dilation of the bile ducts, due to BOP treatment.

At the termination of the experiment, the average weight of the pancreata of hamsters treated with BOP increased more than 6-fold compared with the pancreata in normal hamsters, which was highly significant. The pancreata of the control BOP-treated hamsters weighed 2.17 g in average. The average pancreatic weight of the SB-75-treated animals was 0.53 g. D-Trp-6-LH-RH treatment resulted in an average pancreatic weight of 0.52 g for the 8-µg/day dose and 0.60 g for the 25-µg/day dose (Table 1). The diameters of the individual nodules varied from 2 to 10 mm, but sometimes the nodules were confluent, making it impossible to recognize the size. Because of the multinodularity, the weight of the tumorous pancreas was recorded, on the basis of histological examination, when pancreatic cancers were detected histologically.

Histological examination of the pancreata showed that the number of carcinomas in the surviving BOP controls sacrificed at the termination of the experiment was 9. Only 6 carcinomas were found in the 12 SB-75-treated hamsters, 5 in the 12 animals treated with 8 µg/day of D-Trp-6-LH-RH, and 7 in the 15 hamsters given 25 µg/day of D-Trp-6-LH-RH. This means that altogether 14 pancreatic tumors developed in the 15 BOP control hamsters, but only 8 in the 15 SB-75-treated hamsters. Seven of 15 hamsters in each D-Trp-6-LH-RH-treated group had pancreatic tumors, which was significantly lower than in the control group (Table 1). The pancreata of the remaining animals showed preneoplastic lesions. The average weight of the tumorous pancreas (including both tumor weight and the weight in the rest of the pancreas) in the control group was 2.3 g. SB-75 therapy resulted in 70% inhibition of tumor weight. Treatment with D-Trp-6-LH-RH caused 66% and 62% inhibition for doses of 8 µg/day and 25 µg/day, respectively (Table 1). Seven of the 14 pancreatic cancers in the control animals showed an invasion toward the peritoneum and produced bloody ascites. Only one tumor in the group given 8 µg/day of D-Trp-6-LH-RH showed the same invasive growth (Table 1). The liver of the control and treated animals showed various degrees of atrophic cirrhosis with bile duct proliferation and cysts. In some cases, regardless of pancreatic tumors, 4 to 5 ml of clear ascites were found in the abdominal cavity, apparently due to liver cirrhosis. Histologically, the pancreatic tumors were adenocarcinomas (Fig. 1) with infiltrative ductal and papillary cystic elements as described earlier (5). A scanty fibrotic stroma was seen among the tumorous glandular structures. The mitotic index was between 1 and 2% in the pancreatic tumors of the control as well as of the treated group.

The histological examination of the tumors of animals treated for 3 or 6 days with 50 µg of D-Trp-6-LH-RH and for 3 days with 100 µg of SB-75 showed significant alterations when compared with the control. The majority of the tumor cells revealed the signs of apoptosis. These comprised the shrinkage of tumor cells, formation of apoptotic bodies in the cytoplasm, separation of tumor cells which underwent apoptosis from the neighboring cells, as well as karyopyknosis and karyorrhexis. Several tumor cells showed generalized cytoplasmic swelling (Fig. 2). The cytoplasmic organelles become compacted together in several tumor cells, retaining their structural integrity (Fig. 3). The percentage of tumorous glandular structures containing apoptotic cells (apoptotic index) was as high as 80 to 90% (Table 2).

In the tumors of the animals treated with SB-75 or with D-Trp-6-LH-RH for 2 mo, the signs of apoptosis and the consequences of the apoptotic process were detected. The latter consisted of desquamation of dead tumor cells into the glandular spaces and replacement of the tumor cells by presumably
Table 1 Effect of treatment with antagonist SB-75 and agonist D-Trp-6-LH-RH on body, pancreatic, pancreatic tumor, ovarian, and uterine weights, serum LH and IGF-I levels, number of animals with ascites, and number of animals with pancreatic tumors in Syrian golden hamsters with BOP-induced pancreatic cancers. Each group consisted of 15 animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body wt (g)</th>
<th>Pancreas wt (g)</th>
<th>Tumorous pancreas wt (g)</th>
<th>% of inhibition of tumorous pancreatic wt</th>
<th>Ovarian wt (mg)</th>
<th>Uterine wt (mg)</th>
<th>Serum LH (ng/ml)</th>
<th>Serum IGF-I (ng/ml)</th>
<th>AA*</th>
<th>AT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (BOP only)</td>
<td>151 ± 6.3</td>
<td>2.17 ± 0.52</td>
<td>2.3 ± 0.55</td>
<td>69 ± 4</td>
<td>535 ± 51</td>
<td>0.78 ± 0.04</td>
<td>287.7 ± 25.29</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SB-75 (8 μg/day)</td>
<td>148 ± 5.34</td>
<td>0.53 ± 0.09</td>
<td>0.7 ± 0.13 (a)</td>
<td>52 ± 5 (a)</td>
<td>453 ± 64</td>
<td>0.32 ± 0.043 (a)</td>
<td>259.2 ± 24.25</td>
<td>0 (c)</td>
<td>8 (c)</td>
<td></td>
</tr>
<tr>
<td>D-Trp-6-LH-RH</td>
<td>151 ± 5.8</td>
<td>0.52 ± 0.14 (a)</td>
<td>0.78 ± 0.31 (d)</td>
<td>44 ± 3 (a)</td>
<td>241 ± 24 (a)</td>
<td>0.64 ± 0.068 (d)</td>
<td>284.6 ± 20.37</td>
<td>1 (c)</td>
<td>7 (c)</td>
<td></td>
</tr>
<tr>
<td>D-Trp-6-LH-RH</td>
<td>145 ± 6.5</td>
<td>0.61 ± 0.15 (a)</td>
<td>0.88 ± 0.26 (d)</td>
<td>41 ± 3 (a)</td>
<td>209 ± 15 (a)</td>
<td>0.65 ± 0.046 (d)</td>
<td>331.4 ± 36.73</td>
<td>1 (c)</td>
<td>7 (c)</td>
<td></td>
</tr>
</tbody>
</table>

* AA, number of animals with tumorous ascites of 15; AT, number of animals with pancreatic tumors of 15.

* Mean ± SE.

* Significance versus the control (two-sided Student's t-test): (a), P < 0.005; (b), P < 0.01; (c), P < 0.05.

DISCUSSION

We have previously reported that LH-RH agonists inhibit the growth of experimental pancreatic cancers in rats and hamsters (1, 3, 5, 6, 9). This effect could be due to the induction of a state of deprivation of sex steroids, although Chester et al. (24) have shown that oophorectomy does not modify pancreatic carcinogenicity of BOP. Thus, direct effects of LH-RH agonists on pancreatic tumors are possible, as inferred previously (12, 20). This study demonstrates for the first time that the LH-RH antagonist SB-75 suppresses the growth of the BOP-induced pancreatic cancer cell membranes was higher in both treated groups as compared with the controls.

The affinity of D-Trp-6-LH-RH to the binding sites on pancreatic cancer cell membranes was consistent with the presence of a single class of receptors. The binding affinity was in the nanomolar range (Kd = 2.97 ± 0.92 nmol), and the binding capacity was 0.53 ± 0.19 pmol/mg of membrane protein. After treatment with D-Trp-6-LH-RH, IGF-I receptors were present in all control tumors. Interaction of 125I-IGF-I with binding sites was consistent with the presence of a single class of receptors. The binding affinity was in the nanomolar range (Kd = 2.97 ± 0.92 nmol), and the binding capacity was 0.53 ± 0.19 pmol/mg of membrane protein. After treatment with D-Trp-6-LH-RH, IGF-I receptors could be found in only 2 of 6 tumors investigated. Chronic treatment with D-Trp-6-LH-RH in a dose of 25 μg/day and particularly with SB-75 decreased the concentration of receptors (Bmax) for LH-RH on pancreatic cancer cell membranes. The affinity of D-Trp-6-LH-RH to the binding sites on cancer cell membranes was higher in both treated groups as compared with the controls.

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Analysis of the displacement of labeled EGF by unlabeled EGF suggests that, in control pancreatic cancer cells, labeled peptide was bound to one class of binding noncooperative membrane-binding sites with low affinity (Kd = 47.8 ± 12.8 nmol) and high capacity (Bmax = 4.08 ± 0.43 pmol/mg of membrane protein). In the group treated with SB-75 for 60 days, receptors for LH-RH were found in only 2 of 6 tumors investigated. Chronic treatment with D-Trp-6-LH-RH in a dose of 25 μg/day and particularly with SB-75 decreased the concentration of receptors (Bmax) for LH-RH on pancreatic cancer cell membranes. The affinity of D-Trp-6-LH-RH to the binding sites on cancer cell membranes was higher in both treated groups as compared with the controls.

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Analysis of the displacement of labeled EGF by unlabeled EGF suggests that, in control pancreatic cancer cells, labeled peptide was bound to one class of binding noncooperative membrane-binding sites with high affinity (Kd = 1.91 ± 1.33 nmol) and low capacity (Bmax = 19.6 ± 15.2 fmol/mg of membrane protein). Treatment with SB-75 and D-Trp-6-LH-RH did not influence the binding of EGF to the receptors on pancreatic cancer cell membranes.

FIG. 1. BOP-induced, untreated pancreatic adenocarcinoma of a hamster. Note the absence of fibrosis. H & E, × 120.

FIG. 2. Apoptotic cells with pyknotic nuclei and swollen cytoplasm (arrow) in a glandular structure of a pancreatic tumor of an animal treated with 100 μg/day of SB-75 for 3 days. H & E, × 370.
Table 2. Apoptotic index (the ratio of glandular structures showing signs or the consequences of apoptosis) in BOP-induced pancreatic carcinomas of hamsters after treatment with SB-75 or D-Trp-6-LH-RH

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (μg/day/animal)</th>
<th>Duration of treatment (days)</th>
<th>Apoptotic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Vehicle only</td>
<td>60</td>
<td>2.6 ± 0.37°</td>
</tr>
<tr>
<td>SB-75</td>
<td>8</td>
<td>60</td>
<td>22 ± 1.1°</td>
</tr>
<tr>
<td>SB-75</td>
<td>100</td>
<td>3</td>
<td>89 ± 1.76°</td>
</tr>
<tr>
<td>D-Trp-6-LH-RH</td>
<td>8</td>
<td>60</td>
<td>24 ± 2.08°</td>
</tr>
<tr>
<td>D-Trp-6-LH-RH</td>
<td>25</td>
<td>60</td>
<td>21 ± 2.66°</td>
</tr>
<tr>
<td>D-Trp-6-LH-RH</td>
<td>50</td>
<td>3</td>
<td>86 ± 0.88°</td>
</tr>
<tr>
<td>D-Trp-6-LH-RH</td>
<td>50</td>
<td>6</td>
<td>86 ± 2.3°</td>
</tr>
</tbody>
</table>

* Mean ± SE.
* P < 0.005 versus control.

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pancreatic cancer in hamsters. This effect was significant and at least equal to that of D-Trp-6-LH-RH. Interestingly, the increase in the dose of D-Trp-6-LH-RH from 8 to 25 μg/day did not potentiate the tumor-inhibitory effect of this analogue. The regression of tumor growth was also manifested by a significant number of animals with tumorous ascites and by the lower number of hamsters which developed pancreatic cancer in each of the treated groups. Only early preneoplastic lesions (23) were observed in the remaining animals in the treated
The possible trigger for apoptosis may be a change in the hormonal environment or in the levels or activity of growth factors (28, 29). The alterations in serum LH levels and the decrease in the weights of ovaries and uterus in the treated groups show that the hormonal environment of the pancreatic tumor cells was changed upon the therapy. The results of the acute treatment with SB-75 or D-Trp-6-LH-RH show that such a change can be achieved with a high dose of the analogue within 3 to 6 days. Interestingly, D-Trp-6-LH-RH increased the apoptotic index after 3 days of treatment, when the levels of LH usually rise, showing that the transient initial stimulation of gonadotropin by this class of agonistic analogues does not interfere with this effect. It is also possible that the inhibitory effects of D-Trp-6-LH-RH or SB-75 were due, at least in part, to a direct action on the tumors. A direct action is assumed also from the fact that both peptides exerted essentially similar inhibitory action on pancreatic tumors, but the antagonist had less effect on uterine weights and LH levels than the agonist. The high percentage of apoptosis after acute treatment with SB-75 or D-Trp-6-LH-RH show that such inhibition of gonadotropin by this class of agonistic analogues does not interfere with the cell loss in the treated tumors. No differences were seen between the various treated groups in this respect.

The histological investigation showed that the cell loss in the treated tumors was mostly due to apoptosis (25, 26). This indicates that the regressive effect on tumor growth was mediated by an enhanced rate of programmed cell death. The apoptotic index, the ratio of tumorous glandular structures containing apoptotic cells, appears to be a good indicator of the efficacy of hormonal treatment of pancreatic, breast, and prostate cancer, at least in the case of LH-RH analogues (10). The possible role of apoptosis in the inhibition of the development of pancreatic carcinoma from preblastomatous lesions (27) can be suggested also. In our previous studies we pointed out the importance of apoptosis in tumor regression after the treatment with the agonist D-Trp-6-LH-RH and the somatostatin analogue RC-160 (6, 10). The LH-RH antagonist SB-75 also appears to possess the ability to enhance programmed cell death in tumors.

Groups showing that the hormonal environment of the pancreatic cancer cell lines T3M4, PANC-1, COLO 357, and UACC-462 in tumors by LH-RH analogues (10, 15, 19–21, 30, 31). Specific, low-affinity, high-capacity receptors for D-Trp-6-LH-RH have been demonstrated by our group in cell membranes of BOP-induced pancreatic cancer (20). Receptors for D-Trp-6-LH-RH have been previously found in human pancreatic cancer specimens, but not in normal human pancreas, or in intact control hamsters (20, 30). In this respect, it is of interest that, in our experiments, apoptosis was seen only in pancreatic tumor cells of D-Trp-6-LH-RH- or SB-75-treated hamsters and not in the nontumorous pancreatic cells of the same animals. In our experiments, chronic treatment with D-Trp-6-LH-RH and, in particular, therapy with SB-75 decreased the binding capacity of receptors for LH-RH and produced their down-regulation. Down-regulation of receptors for LH-RH after treatment with SB-75 was also found in experiments with MXT mammary tumor of mice (31). It is possible that LH-RH antagonists, in addition to their main effect mediated by the creation of a state of sex steroid deficiency, may also operate by inducing desensitization of LH-RH receptors.

Specific receptors for IGF-I or EGF were previously shown in BOP-induced pancreatic adenocarcinoma and in human pancreatic cancer cell lines (20, 30, 32, 33). In our experiment, the number of EGF receptors per cell was calculated to be 5050 ± 3915 in the control group, 2582 ± 516 in the SB-75-treated group, and 4065 ± 383 in the D-Trp-6-LH-RH-treated group. The number of EGF receptors per cell in BOP-induced experimental cancer in the hamster was lower than in the pancreatic cancer cell lines T24, PANC-1, COLO 357, and UACC-462 (33). The concentration of EGF membrane receptors in BOP-induced cancer in hamsters is significantly lower than in human pancreatic cancer specimens (30). The \( K_d \) and \( B_{max} \) values for IGF-I and EGF binding to BOP-induced pancreatic cancer cells were not changed after chronic treatment with SB-75. After therapy with D-Trp-6-LH-RH, the number of samples showing IGF-I receptor binding was reduced (1 of 3) as compared with controls (6 of 6). Our experiments suggest that LH-RH agonists could suppress the growth of BOP-induced pancreatic cancers by effects which might involve IGF-I receptors. Changes in characteristics of LH-RH receptors after treatment with an LH-RH agonist or antagonist, together with tumor inhibition, suggest a direct effect of these analogues on BOP-induced pancreatic cancer in hamsters.

On the basis of our findings, the LH-RH antagonist SB-75...
can be considered as a potentially useful agent for the treatment of cancer of the exocrine pancreas. Its immediate inhibitory effects on the pituitary-gonadal axis and its probable direct effects on tumor cells suggest the merit of continued exploration of this potent LH-RH antagonist in the management of pancreatic cancer.

ACKNOWLEDGMENTS

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REFERENCES


Regression of Nitrosamine-induced Pancreatic Cancers in Hamsters Treated with Luteinizing Hormone-releasing Hormone Antagonists or Agonists

Bela Szende, Gordan Srkalovic, Kate Groot, et al.


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