TREATMENT OF NITROSAMINE-INDUCED PANCREATIC TUMORS IN HAMSTERS WITH ANALOGS OF SOMATOSTATIN AND LUTEINIZING HORMONE-RELEASING HORMONE

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Contribution by A. V. Schally, October 27, 1986

Abstract Pancreatic ductal adenocarcinoma was induced in female Syrian golden hamsters by injecting N-nitrosobis(2-oxopropyl)amine (BOP) once a week at a dose of 10 mg per kg of body weight for 18 weeks. Hamsters were then treated with somatostatin analog d-Phe-Cys-Tyr-d-Trp-Lys-Val-Cys-Trp-NH₂ (RC-160) or with [6-d-tryptophan]luteinizing hormone-releasing hormone ((d-Trp)₆-LH-RH) delayed delivery systems. Microcapsules of somatostatin analog RC-160, designed to release a dose of 5 μg/day, were injected twice a month and microcapsules of [d-Trp]₆-LH-RH, calculated to liberate 25 μg per day, once a month. After 18 weeks of BOP administration, the hamsters were divided into three groups of 10–20 animals each. Group I consisted of untreated controls, group II was injected with RC-160, and group III was injected with [d-Trp]₆-LH-RH. A striking decrease in tumor weight and volume was obtained in animals treated with [d-Trp]₆-LH-RH or with the somatostatin analog RC-160. After 45 days of treatment with either analog, the survival rate was significantly higher in groups II and III (70%), as compared with the control group (35%). The studies, done by light microscopy, high-resolution microscopy, and electron microscopy, showed a decrease in the total number of cancer cells and changes in the epithelium, connective tissue, and cellular organelles in groups II and III treated with the hypothalamic analogs as compared to controls. These results in female hamsters with induced ductal pancreatic tumors confirm and extend our findings, obtained in male animals with transplanted tumors, that [d-Trp]₆-LH-RH and somatostatin analogs inhibit the growth of pancreatic cancers.

New cases of pancreatic carcinomas diagnosed in the United States number 25,500 annually and cause >24,000 deaths per year (1). The most common type of pancreatic cancer is the duct cell carcinoma, which constitutes about 90% of the pancreatic exocrine tumors. The lack of knowledge about its etiology, the increasing incidence of pancreatic cancer, and the poor results from the different methods of treatment have created a need for a better animal model of the human disease (2–5). In recent years, various animal models of experimental pancreatic cancer have been developed (3, 6–10). The Syrian golden hamster has become the model of choice since, anatomically, it has lobulation of the pancreas comparable to that of humans and tumors can develop spontaneously or may be induced by carcinogenic agents. These tumors are morphologically and histologically similar to human pancreatic cancer (4–10).

Several classes of chemical substances have been used to induce experimental pancreatic cancer. Nitrosamines (4, 5, 10–12) are more specific for the induction of ductal carcinoma, while azaserine (8–10, 13, 14) will induce only the acinar type of tumors. Administration of large doses of the carcinogen N-nitrosobis(2-oxopropyl)amine (BOP) has been shown to induce carcinomas of the pancreas, lungs, liver, and kidneys, but with low-dose regimens, pancreatic tumors can be selectively produced (10, 12). The incidence of the pancreatic tumors induced by BOP in the Syrian golden hamster model is >90%.

Experimental and clinical findings suggest that it might be possible to develop a hormonal therapy for cancer of the pancreas based on somatostatin analogs (15–17). Somatostatin and some of its analogs exert an inhibitory action on the exocrine and endocrine pancreas and suppress the release of insulin, glucagon, and the secretion or action of gastrin, secretin, cholecystokinin, and VIP. Secretin and cholecystokinin have been shown to produce hyperplasia and hypertrophy of the exocrine pancreas in addition to stimulating pancreatic secretion of enzymes and bicarbonate (17–20). Gastrin can also produce pancreatic hypertrophy. Studies on ductal pancreatic adenocarcinoma cell lines have demonstrated that gastrointetinal hormones stimulate the growth of these malignant cells (21). In addition, the presence of intracellular receptors for estrogen and androgen in pancreatic cells indicates that sex hormones could exert some influence on the growth of normal and malignant pancreatic cells (22, 23). These findings suggest that pancreatic adenocarcinoma might be sensitive to both gastrointestinal and sex hormones. Preliminary clinical results support this view (22, 24). Previously, we showed that chronic administration of some somatostatin analogs or the agonist, [6-d-tryptophan]-luteinizing hormone-releasing hormone ((d-Trp)₆-LH-RH), inhibited the growth of transplanted pancreatic carcinomas in rat and hamster models (15, 16). In this study, we have selectively induced pancreatic tumors in Syrian golden hamsters by administration of low doses of BOP. We then followed tumor progression and morphological and histological changes in the tumors and other organs by light and electron microscopy during treatment with microcapsules of (d-Trp)₆-LH-RH and somatostatin analog RC-160.

MATERIALS AND METHODS

Fifty female Syrian golden hamsters (weighing 100–120 g) were obtained from NCI–Frederick Cancer Research Center (Frederick, MD). They were kept in a temperature-controlled room with a 12-hr light/12-hr dark schedule and received pelleted diet and water ad libitum. BOP (Ash Stevens,

Abbreviations: [d-Trp]₆-LH-RH, [6-d-tryptophan]luteinizing hormone-releasing hormone; BOP, N-nitrosobis(2-oxopropyl)amine; VIP, vasoactive intestinal peptide.

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Detroit, MI) in sterile NaCl solution was injected s.c. once a week for 18 weeks, at a dose of 10 mg per kg of body weight. Somatostatin analog RC-160 (D-Phe-Cys-Tyr-d-Trp-Lys-Trp-Cys-TRH-NH2) was synthesized by solid-phase methods (25). Microcapsules of [d-Trp6][LH-RH] (16, 26) and somatostatin analog RC-160 in poly(lactide-co-glycolide) were prepared by a phase-separation process by CytoTech (Martigny, Switzerland) and supplied by Debiopharm (Lausanne, Switzerland). Microcapsules of [d-Trp6][LH-RH] in aliquots of 35 mg were designed to release a dose of 25 μg per day for 30 days (16, 26). A prototype batch of microcapsules of RC-160 in the amount of 7.5 mg was calculated to liberate a dose of ~5 μg per day for 15 days. Microcapsules of [d-Trp6][LH-RH] and of RC-160 were weighed directly in disposable syringes and suspended in 0.7 ml of injection vehicle, containing 2% CM-cellulose and 1% Tween 20 in water. The suspensions of microcapsules of [d-Trp6][LH-RH] were injected s.c. once a month and RC-160 was injected every 15 days. The hamsters were observed daily and weighed once a week during the administration of BOP. After 18 weeks, all the hamsters had lost weight but none had died, so 5 of them were subjected to laparotomy to confirm the existence of tumors. The remaining hamsters were divided into three groups of 10–20 animals each. Group I consisted of untreated controls, group II was treated with RC-160, and group III was treated with [d-Trp6][LH-RH]. The therapy with hypothalamic analogs was continued for 45 days. Hamsters that died during the course of treatment were autopsied and organs were fixed in 10% buffered formalin for histological examination. At the conclusion of the experiment, the remaining hamsters were sacrificed by decapitation, trunk blood was collected, and serum was separated for RIA of progesterone and insulin.

The tumors were removed, cleaned, and carefully measured and weighed. For light microscopy, the tissues were fixed in 10% buffered formalin and then embedded in paraffin. Serial sections were cut and stained with either hematoxylin and eosin, Masson trichrome, or periodic acid Schiff. Appropriate tumor sections were selected for high-resolution light microscopy and electron microscopy examination. The latter specimens were fixed in paraformaldehyde and postfixed in osmium tetroxide according to standard methods. The specimens were then dehydrated, embedded in plastic, and polymerized overnight in an oven. Several sections (1–3 μm thick) were obtained using an MT2 or JB4 microtome and placed in sequence on glass slides. One section was stained with toulidine blue, while the others were processed for electron microscopy (27).

All data are reported as the mean ± SEM. Statistical analyses were performed by using computer-assisted programs for Duncan’s new multiple range test or Student’s t test.

**RESULTS**

**Survival.** Hamsters injected weekly with BOP lost weight, but the survival rate was 100% after 18 weeks. During the 45 days of the treatment with [d-Trp6][LH-RH] or RC-160, a marked difference in survival rate was observed among the groups. The survival rate in the control group was 35%, but in the groups treated with either [d-Trp6][LH-RH] microcapsules or RC-160 it was 70%. During the last week of treatment, control hamsters showed a marked decrease in body weight; the animals that died showed the greatest weight losses.

**Gross Morphology.** All the hamsters in the control group developed pancreatic tumors after BOP. Metastasis was observed in the lungs in five animals (25%) and the liver in four animals (20%). Ten of these tumors were localized in the splenic portion (50%), whereas six were found in the hepatic portion (30%) and four were in both portions (20%) of the pancreas. One tumor was localized in the head of the pancreas, and the liver in that animal presented a greenish color and the gallbladder was enlarged. Tumor volume of the control group was 1360 ± 280 mm³ and tumor weight was 880.3 ± 120 mg (Table 1).

In the group treated with microcapsules of [d-Trp6][LH-RH], only one of the hamsters did not have a pancreatic tumor. Eight of these hamsters had tumors localized in the splenic portion (40%), five in the hepatic portion (25%), five in both portions (25%), and one was diffuse (5%). Metastases to the lungs were present in three of the hamsters (15%), while two had metastasis to the liver (10%). The reduction in tumor volume (324.7 ± 120 mm³; P < 0.01) and tumor weight (205.4 ± 70 mg; P < 0.001) in the group treated with [d-Trp6][LH-RH] was significant when compared with controls (Table 1).

In the group treated with somatostatin analog RC-160, five of the hamsters had pancreatic tumors localized in the splenic part (50%), while three had tumors in the hepatic part (30%) and two animals (20%) had diffuse tumors. There was a significant reduction in tumor volume (659.2 ± 220 mm³; P < 0.05) and tumor weight (430 ± 170 mg; P < 0.05) in hamsters treated with somatostatin RC-160 as compared with the control group. However, this response was about half of that observed with [d-Trp6][LH-RH] (Table 1).

In the group treated with [d-Trp6][LH-RH], progesterone levels (2.7 ± 0.1 ng/ml) were significantly decreased as compared with controls (4.79 ± 0.8 ng/ml; P < 0.01). Levels of insulin in the control group were 14.2 ± 3.2 microunits as compared to 23.5 ± 29 microunits/ml (NS) in the RC-160

**Table 1. The effects of treatment with somatostatin analog RC-160 and [d-Trp6][LH-RH] microcapsules on tumor volumes and weights and survival rate in Syrian golden hamsters with BOP-induced pancreatic cancer**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals, survival rate</th>
<th>Tumor volume, mm³</th>
<th>Tumor weight, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18 weeks</td>
<td>24 weeks</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>20 (100%)</td>
<td>7 (35%)</td>
<td>1360 ± 280</td>
</tr>
<tr>
<td>RC-160*</td>
<td>10 (100%)</td>
<td>7 (70%)</td>
<td>659 ± 220</td>
</tr>
<tr>
<td>[d-Trp6][LH-RH] 4</td>
<td>20 (100%)</td>
<td>14 (70%)</td>
<td>324 ± 120</td>
</tr>
</tbody>
</table>

Results are means ± SEM. The therapy was initiated after 18 weeks of treatment with BOP and continued for 45 days.

*Microcapsules liberating 5 μg per day administered every 15 days for 45 days.

†Significance was calculated by Duncan’s new multiple range test.

‡Significance was calculated by Student’s t test.

§Microcapsules liberating 25 μg per day administered every 30 days for 45 days.
treated group and 34.4 ± 0.6 microunits/ml ($P < 0.05$) in the animals given [d-Trp$^6$]LH-RH. Increased levels of insulin in the treated groups might reflect a better secretory activity of pancreatic islets, because of smaller tumor volume as compared to controls. Analog RC-160 inhibits insulin release much less than that of growth hormone and other hormones, and 15 days after the last injection of microcapsules the levels of insulin would be expected to recover.

**Microscopic Morphology.** All the tumors of the control group were well-differentiated ductal cell adenocarcinomas with columnar or cuboidal epithelial cells. These cells with hyperchromatic nuclei and pale cytoplasm showed areas of a typical papillary hyperplasia and goblet cell metaplasia (Fig. 1). These tumor cells were supported on a scant stroma showing an inflammatory reaction with an infiltration comprised of polymorphonuclear leukocytes. In the group treated with [d-Trp$^6$]LH-RH, a glandular pattern was present with the larger glands showing cuboidal or columnar epithelia without goblet cell metaplasia. Frequently, those glands formed cysts. The stroma tissue was increased with a proliferation of the interstitial cells (Fig. 2). Tumors treated with somatostatin analog RC-160 showed a glandular or cribriform pattern (Fig. 3). Sometimes these patterns were diffused and dispersed with atypical cells. Metastasis was found in the lungs and liver in all the groups, and the histological characteristics were the same as those of the pancreatic tumors cells. A tumor infiltration of the diaphragmatic muscle was found in one animal.

**Ultrastructural Study.** The tumoral cells in the control group were cuboidal or columnar with irregular apical surface projections, numerous microvilli, and dense glycocalyx. The lateral membranes had interdigitations with adjacent cells and junctional complexes near the luminal surface. The cytoplasm showed pleomorphic mitochondria with variable and irregular cristae and electron-dense bodies. Short cisternae of the granular endoplasmic reticulum free ribosomes, lipid droplets, and small Golgi complex were observed. In the apical cytoplasm, mucigen granules were found. The nucleus was irregular with prominent nucleoli and marginated chromatin (Fig. 4).

In the group treated with [d-Trp$^6$]LH-RH, an increase of the interdigitations with the adjacent cells of the granular endoplasmic reticulum and of the number and size of the mitochondria was observed. Mucigen granules were decreased. The nuclei were irregular with typical nuclear bodies and marginated chromatin. The perinuclear cisternae of some cells were distended (Fig. 5).

**DISCUSSION**

The high incidence of pancreatic tumors (98%) obtained in our study after the administration of the carcinogen BOP to Syrian golden hamsters suggests that this animal model is well-suited for the study of pancreatic carcinomas (10–12). The low incidence of tumors in other organs may be due to the dose regimen used (10–12). Furthermore, the metastases were found exclusively in lung and liver, and this differs from findings in other reports (10, 11). Adenomas were not encountered in this study, possibly because of the complete transformation of all the adenomas into adenocarcinomas during the total experimental time interval (10–12). Morphologically, the adenocarcinomas were very similar to human ductal adenocarcinoma with histological and ultrastructural characteristics previously reported by different authors (4, 10, 12).

The survival time of the hamsters after administration of BOP was >18 weeks. The survival rate at 24 weeks was
Figure 4. Electron micrograph of pancreatic adenocarcinoma from the control group showing numerous microvilli, dense glycocalyx, free ribosomes, pleomorphic mitochondria, and mucigen granules. The irregular nuclei have margined chromatin and prominent nucleoli. (×1200.)

Figure 5. Electron micrograph of the tumoral cells from the group treated with [d-Trp^6]LH-RH, showing increase of interdigitations, junctional complex near the luminal surface, and few mucigen granules. (×3550.)

Figure 6. Electron micrograph of the tumoral cells from the group treated with RC-160, showing irregular nuclei with prominent nucleoli and typical nuclear bodies. The cytoplasm exhibits an increase of organelles. (×1200.)

Figure 7. Detail of electron micrograph in Fig. 6, showing distended cisternae of granular endoplasmic reticulum and an increase of mitochondria and Golgi complex. (×5200.)

Increased after treatment with [d-Trp^6]LH-RH or with the somatostatin analog RC-160 to 70%, as compared with the 35% in the control group. In this study, the greatest reduction in tumor volume and weight was obtained after treatment with [d-Trp^6]LH-RH, in accord with our prior work in male rats and hamsters with transplanted tumors (16). Since chronic administration of [d-Trp^6]LH-RH inhibits the pituitary-gonadal axis and creates a state of sex-hormone deprivation, this suggests that pancreatic cancers may, at least in part, be sex-hormone sensitive (15, 16, 22, 24). In turn, this implies the existence of sex-hormone receptors in the hamster pancreatic tumoral cells similar to those found in humans and rats (22, 23). A significant reduction in tumor volume and weight was also obtained following treatment with the microcapsules of somatostatin analog RC-160. The batch used was an early prototype of RC-160 microcapsules, and a better inhibition of growth of pancreatic tumors might be obtained with improved batches of microcapsules. These results are in agreement with those obtained previously in hamsters and rats with pancreatic carcinomas following treatment with older (early) somatostatin analogs (16). The decrease in volume and weight of pancreatic tumors may be explained, in part, by the inhibitory action of somatostatin analogs upon the release or action of gastrointestinal hormones, some of which have been implicated in the stimulation of growth of normal and tumoral pancreatic cells (18–21). In addition, somatostatin and its analogs may act directly on tumor cells by suppressing cellular proliferation, inhibiting centrosomal separation, and nullifying growth stimulation induced by the epidermal growth factor (28). Other possible mechanisms of action of somatostatin analogs on pancreatic cancer have been also proposed. Somatostatin and its analogs may act directly by stimulation of dephosphorylation of membrane receptors (17, 28–30) and indirectly by inhibiting the secretion of growth hormone, which in turn may lead to a decrease in tumor autocrine growth factors (17).

The reduction in the tumoral cells and the increase in connective tissue observed in pancreatic tumors treated with [d-Trp^6]LH-RH are similar to those previously reported by our group in the Dunning rat prostatic cancer model following therapy with this analog (26). Comparable, but less intense, histological changes were found in pancreatic tumors treated with the somatostatin analog RC-160. A high degree of cellular pleomorphism and, sometimes, the presence of
undifferentiated patterns have recently been described (31). The results obtained in our ultrastructural study were analogous to those reported in other studies of pancreatic carcinomas induced by nitrosamines (32, 33). The increase of the number and pleomorphism of mitochondria and granular endoplasmic reticulum with distension of cisternae observed in the treated groups may be an attempt by tumoral cells to repair the damage caused by the hormonal therapy. These results in female hamsters with induced ductal pancreatic tumors confirm and extend our findings, obtained in male animals with transplanted tumors, that [d-Trp⁶]LH-RH and somatostatin analogs inhibit the growth of pancreatic cancers. These data may be useful in the development of hormonal therapy for pancreatic cancer based on somatostatin analogs and [d-Trp⁶]LH-RH.

We thank Dr. P. Orsolini (Cytotech-Debiopharm) for microcapsules of RC-160, Dr. E. Hoffman and Dr. M. Abad for their help in ultrastructural study, Martha Sampson for technical assistance, and Dr. Parvis Pour for valuable advice on BOP lesions. The work described in this paper was supported by National Institutes of Health Grants AM07467 and CA40077 (A.V.S.) and by the Medical Research Service of the Veterans Administration.