Alteration of Pancreatic Endocrine Cell Patterns and Their Secretion during Pancreatic Carcinogenesis in the Hamster Model

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ABSTRACT

Proliferation of endocrine cells was found to occur during early, i.e., first 12 weeks, exocrine pancreatic carcinogenesis after 6 weekly treatments of Syrian hamsters with the pancreatic carcinogen N-nitrosobis(2-oxopropyl)amine (BOP). Cells containing insulin (Ins), glucagon (Glu), and somatostatin (Som) were noted in all stages of tumor development and were present in adenocarcinomas and in metastases to the liver. Some of the cancer cells were of amphicrine (hybrid) type, i.e., produced both mucin and endocrine substances. Measurement of these hormones revealed a significant decrease in plasma Ins during early stages of carcinogenesis with concomitant increase of Ins level in pancreatic juice at 12 weeks after 6 weekly BOP treatments. Plasma Glu and Som were not changed. The changes noted, particularly in relation to Ins, suggest that proliferation of endocrine cells in pancreatic carcinogenesis may be associated with alterations in hormone secretion.

INTRODUCTION

Pancreatic cancer, which has become a common international health problem, is characterized by an extremely poor prognosis and lack of response to therapeutic approaches (1, 2). There are as yet no methodologies for early detection of the disease for possible screening of populations at high risk (3–9). The presently existing tumor-associated antigens, such as CA 19-9, DU-PAN-2, carcinoembryonic antigen, etc., are useful for monitoring the disease, but their efficacy in early cancer detection has remained disappointing. Therefore, more specific and sensitive methods are required. Our recent studies in the hamster pancreatic cancer model, which in clinical, biological, and morphological aspects closely resembles the human disease (10–14), have provided data which seem promising for developing early diagnostic modalities.

We have identified Ins and growth hormone-like substances in the pancreatic juice of untreated hamsters (15). This finding was not surprising, as pancreatic hormones, including Ins, Glu, Som, and EGF-like substances, have been found in the pancreatic juice of several species, including humans (16–21). In a later study, we noted the proliferation of Ins, Glu, and Som cells simultaneous with that of ductal/ductular cells in early pancreatic carcinogenesis (12). This phenomenon (which imitates the embryonic development of the pancreas) persisted throughout the carcinogenesis process, and endocrine cells were found in almost all induced cancers (10–14).

The current study was designed to quantitate the proliferation of endocrine cells during pancreatic carcinogenesis and to determine if changes in the endocrine cell population were associated with alteration of hormone levels in plasma and pancreatic juice.

MATERIALS AND METHODS

Animals

Male Syrian hamsters from the Eppley Colony were housed in groups of five in plastic cages on granular cellulose bedding (Bed-O-Cobs, The Anderson Cob Division, Maumee, OH), kept under standard laboratory conditions, and given Wayne Lab Blox (Allied Mills, Chicago, IL) and water ad libitum.

Carcinogenesis Experiments

Experiment 1: Pattern of Endocrine Cells during Pancreatic Carcinogenesis. Male Syrian hamsters were treated weekly for 6 weeks with BOP (10 mg/kg s.c.) and sacrificed in groups of three to four hamsters 12 and 33 weeks after the last BOP injection. Similar numbers of hamsters were used as controls. The pancreases of these animals were processed for immunohistochemistry for demonstration of Ins, Glu, and Som as described below.

Experiment 2: Hormone Levels in Plasma and Pancreatic Juice. This experiment was designed after the results of Experiment 1 were evaluated. Male hamsters were treated weekly for 6 weeks with BOP (10 mg/kg s.c.). Four, 8, and 12 weeks after the last BOP injection, pancreatic juice (stimulated by secretin/CCK-OP) and plasma were examined in groups of 20 animals per interval for the presence of Ins, Glu, and Som and compared to age-matured controls. The levels of all three hormones were determined in plasma, but the amount of juice obtained was too small to allow for measurement of Som.

Carcinogen, BOP was synthesized in our Institute as described (22), dissolved in physiological saline shortly before use, and given s.c.

Collection of Pancreatic Juice and Plasma. A perfusate solution containing secretin and CCK-OP was made up as follows: (a) vials of Secretin-Kabi were reconstituted in 7.5 ml of saline yielding a stock solution of 819 nm, (b) CCK-octapeptide (Kinevac, Squibb) was reconstituted as 0.6 ml solution in saline, (c) the perfusate solution contained 0.1 ml CCK-OP stock solution, 0.6 ml secretin stock solution, and 19.3 ml BSA/saline per 20 ml. Hamsters were anesthetized with nembutal (80 mg/kg body weight i.p.). A femoral venous catheter was inserted for the perfusion solutions. The common bile duct was ligated at the ampulla and at the liver hilum and cannulated between these ligatures with PE 10 tubing to collect pancreatic juice. A bolus of 0.5–cc perfusate solution was initially give i.v., followed by a continuous infusion of 0.8 cc/h. Pancreatic juice was collected into iced, covered tubes containing 20 ml of saline (100 ml KIU/ml). Following juice collection, blood was drawn from the inferior vena cava, and the plasma separated by centrifugation in tubes containing 0.1 ml trisylol. Specimens were frozen at −70°C prior to radioimmunoassay.

Radioimmunoassay. Ins, Glu, and Som were all performed by double-antibody radioimmunoassay in the Core Ligand Laboratory, National Diabetes Research and Training Center, University of Michigan, Ann Arbor, MI, with the following antibodies:

- Insulin. The anti-pork Ins serum (Ann Arbor) reacts with Ins and proinsulin. Pork Ins labeled with 125I is employed as the tracer and human Ins as the standard. Second antibody is used to separate the bound and free fractions. The sensitivity limit is 0.08 mU/ml, intrassay variability 8.4%, and interassay variability 6.4% (23). Iodination was performed by the modified method of Freychet et al. (24).

- Glucagon. The anti-beef/pork-Glu serum (G9-M, Ann Arbor) reacts with the carboxyl terminal of the Glu molecule and has negligible cross-reactivity with Glu-like materials present in the intestinal mucosa (25). Beef-pork Glu is used as 125I-labeled tracer (New England Nuclear) and standard. Separation of the bound and free fractions is accomplished using second antibody (Ann Arbor). The limit of sensitivity is 2.0 pg/
tube, the interassay variability 10.2% with the intraassay variability at 7.5%. Iodination was performed by the method of Brower et al. (26).

Somatostatin. A dextran-coated charcoal separation radioimmunoassay using Ann Arbor antibody “Big Wig” OS1184 was produced in rabbit. The methodology is modified from Gerich et al. (27). The limit of sensitivity is 1.6 pg/tube with the intra- and interassay variability at 5.3 and 9.8%, respectively. Samples are not extracted prior to analysis.

Immunohistochemistry. Pancreases were fixed in Bouin’s solution for 6 h, processed for histology by conventional methods, and embedded in paraffin. Tissues were cut in serial secretions and examined by antibodies against Ins, Glu, and Som (Immunon, Lipshaw, Detroit, MI) by ABC techniques using commercially available kits (Immulok Inc., Carpinteria, CA). We have previously shown that the antibodies provided by the kits react very well with hamsters’ pancreatic endocrine cells (12). Control slides were prepared similarly but either without treatment with primary antibody or after absorption of antibodies with the relevant hormones.

Histological Examination. Tissues were examined microscopically at 40 × magnification. In each section 10 islets at different, but assigned areas (duodenal lobe, body, and tail of gastric lobe, body and tail of splenic lobe) were evaluated for determination of the ratio of α:ß:δ cells by counting the cells stained with the given antibody and cells which were unstained. The average value of 10 islets was then considered as the representative value for those specimens.

For determination of the number of each endocrine cell type (Ins, Glu, and Som) in each specimen, the following rule was used: an islet containing a particular endocrine cell was considered as 1 point, regardless of the number of the given endocrine cell in that islet. In extrainsular tissue, however, each type of endocrine cell (scattered between the exocrine cells) was counted as a point. For example, if in one microscopic field an islet contained 12 Glu cells (considered as 1 point only), one duct had 1 Glu cell (1 point) and two ductules 2 Glu cells (2 points), a total of 4 points was given. Endocrine cells occurring within carcinomas were not counted, since their numbers were usually very high. This type of evaluation was used because in carcinogen-treated hamster the number of endocrine cells was increased in the extrainsular tissue with no changes in the islets. A collection of five cells or less were regarded as extrainsular islet cells. For that reason other methods, such as linear scan could not be used. The ratio of α, β, and δ cells was calculated from counting each cell population in the appropriate sections of each pancreas by the above method; it does not refer to the cell ratios within the islet. For evaluation of each cell type, sections from the entire pancreas were used and evaluated twice and the mean of two values was regarded as representative.

Statistical Analysis. A Kruskal-Wallis nonparametric rank test was used to determine significant differences between the groups.

RESULTS

Experiment 1: Pattern of Endocrine Cells during Pancreatic Carcinogenesis. Focal or multifocal hyperplasia and hypertrophy of ductal and ductular epithelium were seen as early as 12 weeks posttreatment in all BOP-treated hamsters, and one of the three hamsters had a microscopic noninvasive cancer. At 33 weeks, in one out of three hamsters, there was an invasive adenocarcinoma which had metastasized into the liver. In addition, there were numerous hyperplastic and dysplastic ducts/ductules in all BOP-treated hamsters, an intraductal carcinoma and a ductular carcinoma in situ in one, and an invasive adenocarcinoma with metastasizes into the liver in another hamster. Partial ductal occlusion due to hyperplastic changes with small areas of atrophy were found in a few hamsters, and there was no correlation between the atrophic changes and the number of endocrine cells.

The number (points) of individual endocrine cells at 12 and 33 weeks are listed in Table 1. There were no changes in the distribution and number of endocrine cells in islets between treated and untreated hamsters. However, the number of extrainsular cells were increased in BOP-treated hamsters. The ratio of β:α cells remained stable (0.7-0.9) at each interval, whereas the ratio of β:δ sharply decreased at 33 weeks. The same was true for the α:δ ratio. An increased number of all three cell types were found in extrainsular tissue of treated hamsters at both intervals. At 12 weeks the number of Ins and Glu cells was greater than that of Som cells, whereas at 33 weeks, a dramatic increase of Som cells was found. The increase in the number of endocrine cells was mainly due to the appearance of single or a small group of immunoreactive cells within the hyperplastic ductal/ductular epithelium. At 33 weeks most hyperplastic lesions contained a large number of endocrine cells, some of which seem to reach the ductal lumen by a narrow neck. Note that in some ducts, Glu cells comprise about 50% of the cell population. Peroxidase-antiperoxidase (PAP) with anti-Glu serum (200 ×).

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Experimental intervals</th>
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<tr>
<td></td>
<td>12 weeks</td>
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<tr>
<td>Treated hamsters</td>
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<tr>
<td>β</td>
<td>173 ± 47°</td>
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<tr>
<td>α</td>
<td>196 ± 31°</td>
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<tr>
<td>δ</td>
<td>65 ± 34°</td>
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<tr>
<td>Ratio β:α</td>
<td>0.9</td>
</tr>
<tr>
<td>Ratio β:δ</td>
<td>2.7</td>
</tr>
<tr>
<td>Ratio α:δ</td>
<td>3.0</td>
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</table>

* Most of these endocrine cells were of extrainsular origin; data represent mean ± SD.

Fig. 1. Hyperplastic ducts and ductules in the early stages of pancreatic carcinogenesis in the Syrian hamster. The black-stained cells populating the ducts/ductules are Glu cells, some of which seem to reach the ductal lumen by a narrow neck. Note that in some ducts, Glu cells comprise about 50% of the cell population. Peroxidase-antiperoxidase (PAP) with anti-Glu serum (200 ×).
The increase in the number of Ins and Glu cells seems to be a marker for the early event, whereas appearance of Som cells reflected a more advanced stage of tumor development.

Although most endocrine cells were found at the base of ductal/ductular epithelium, many of them reached the lumen by tiny cytoplasmic portions (Figs. 1 and 2). In one of the three cases examined at 33 weeks, over 30% of the cells populating ductules and numerous cells along the entire length of hyperplastic common duct epithelium were comprised of Som cells (Fig. 2).

In one hamster with a large invasive adenocarcinoma a remarkably large number of Som cells were identified intermingled with goblet cells. These cells were also found in the metastatic foci of this cancer into the liver (Fig. 3). In this case many 5 cells seemed to have both mucin and endocrine granules (Fig. 3). There were also numerous endocrine-like cells which were either faintly stained or unstained and seemed to present immature endocrine cells.

Unlike Som cells, Ins or Glu cells were not found in either the common duct epithelium or the intestine. Som cells were detected in various numbers scattered throughout the duodenal mucosa. However, their numbers did not differ between the treated and control hamsters.

Experiment 2: Hormones in the Serum and Pancreatic Juice. In this experiment, plasma and pancreatic juice levels of Ins, Glu, and Som were examined every 4 weeks after the conclusion of BOP treatment. Because BOP-treated hamsters develop pancreatic atrophy and duct occlusion, it was only possible to collect pancreatic juice during the early stages of carcinogenesis 4, 8, and 12 weeks after BOP treatment. Subsequently, the volume of pancreatic juice fell to unusable levels. Even in the early stages of carcinogenesis, a sufficient volume of pancreatic juice could be obtained only to measure Ins and Glu.

Histologically, as in Experiment 1, proliferation of ductal/ductular cells was found as early as 4 weeks in a few hamsters, but was more frequent at 8 weeks and was pronounced at 12 weeks. At 8 weeks hyperplastic ducts and ductules occurred in most hamsters and at 12 weeks all hamsters had hyperplastic lesions and three had ductular carcinoma in situ. We did not evaluate the number of each endocrine cell type because surgery and treatment with secretin-CCK are known to stimulate their secretion and hence could have masked their real numbers and patterns.

Table 2 summarizes the levels of hormones found in the plasma and pancreatic juice of BOP-treated and untreated hamsters.

Plasma levels of Ins, Glu, and Som did not change significantly with age in control animals. In BOP-treated animals, there was a significant decrease in plasma insulin levels at 12 weeks when compared to its levels at 4 weeks (\( P < 0.01 \)) while at the same time the pancreatic juice levels of Ins increased twofold. The difference in the levels of Ins in the pancreatic juice was, however, not significant. Plasma Glu and Som levels did not differ significantly between control and BOP-treated animals. In pancreatic juice in both BOP-treated and control groups, there were high levels of reactivity to anti-Glu, and this reactivity was significantly greater in BOP-treated animals.
The assay of Glu in pancreatic juice revealed large amounts of reactivity to anti-Glu, which is probably an artifact from nonspecific interference in the radioimmunoassay (16). Nevertheless, the data in Table 2 clearly show that whatever substances in the juice cross-react with anti-Glu used, their concentrations are considerably higher in BOP-treated hamsters than in controls. Identification of these substances requires further studies.

Som levels could not be examined in the pancreatic juice due to the limited amount of the material obtained. Clearly, additional experiments with improved methodology to detect hormone concentrations are needed. Examination of Som in pancreatic juice would be more useful than its level in plasma, because the latter is of extrapancreatic origin and may not reflect its pancreatic origin and because juice levels of Som have been noted to rise in conditions associated with hyperplasia of Som cells (17).

Proliferation of endocrine cells with that of exocrine cells is not unique to hamsters and has been observed by us and by others (29–42) in over 80% of human pancreatic cancer specimens. We do not yet know whether endocrine cell proliferation in humans is associated with hormonal hypersecretion because of the lack of relevant clinical data. However, since over 80% of pancreatic cancer patients generally develop diabetes or hyperglycemia (45–52), it is possible that the situation in humans is similar to our experimental results, i.e., an altered serum hormone level during pancreatic carcinogenesis.

It must be pointed out that in the hamster model, endocrine cell proliferation appears to be prevalent during the early stages of cancer development and less prominent at advanced stages, possibly due to increasing dedifferentiation in growing tumors. The same appears to apply to the human situation. In one study (35) a higher incidence of endocrine cells was found in well-differentiated adenocarcinomas of the pancreas (82%) than in moderately differentiated (39%) and in undifferentiated types (18%). Therefore, the biological activity of endocrine cell proliferation may be clinically detectable at early stages of carcinogenesis and the correlation between the degree of tumor differentiation and presence of endocrine cells as tumor cell components may have prognostic value.

Further studies are necessary to further delineate the present findings, especially in view of the existing confusion relative to the relationship between diabetes and pancreas cancer.

REFERENCES

7. Levin, C. L., and Connelly, R. R. Cancer of the pancreas. Available epide-

### Table 2: Pancreatic hormone concentration in pancreatic juice and/or serum during pancreatic carcinogenesis

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Sample size</th>
<th>Plasma (µU/ml)</th>
<th>Juice ‡</th>
<th>Glucagon (ng/ml)</th>
<th>Plasma</th>
<th>Juice ‡</th>
<th>Somatostatin (pg/ml)</th>
<th>Plasma</th>
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<td></td>
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<td></td>
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<tr>
<td>BOP-treated hamsters</td>
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<td></td>
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<tr>
<td>4</td>
<td>17–18</td>
<td>5.95 ± 1.96</td>
<td>4.55 ± 1.40</td>
<td>0.525 ± 0.074</td>
<td>24.7 ± 6.0</td>
<td>47 ± 5</td>
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<tr>
<td>8</td>
<td>8–11</td>
<td>2.68 ± 2.51</td>
<td>4.98 ± 2.05</td>
<td>0.363 ± 0.094</td>
<td>35.3 ± 8.8</td>
<td>34 ± 7</td>
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<tr>
<td>12</td>
<td>12–18</td>
<td>2.51 ± 1.96</td>
<td>8.07 ± 1.67</td>
<td>0.462 ± 0.074</td>
<td>39.0 ± 6.9</td>
<td>49 ± 5</td>
<td></td>
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<tr>
<td>Control hamsters</td>
<td></td>
<td></td>
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<tr>
<td>4</td>
<td>11–12</td>
<td>6.60 ± 2.40</td>
<td>5.89 ± 1.67</td>
<td>0.407 ± 0.090</td>
<td>12.0 ± 7.5</td>
<td>60 ± 6</td>
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<tr>
<td>8</td>
<td>11</td>
<td>4.49 ± 2.51</td>
<td>2.47 ± 1.74</td>
<td>0.391 ± 0.094</td>
<td>18.3 ± 7.5</td>
<td>53 ± 6</td>
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<tr>
<td>12</td>
<td>6–7</td>
<td>3.96 ± 3.15</td>
<td>4.44 ± 2.19</td>
<td>0.234 ± 0.127</td>
<td>14.9 ± 9.4</td>
<td>52 ± 9</td>
<td></td>
<td></td>
</tr>
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</table>

* After BOP treatment.
† Number of animals from which both pancreatic juice and serum could be collected.
‡ Values were corrected for protein concentration; data are given in mean ± SD.
§ P < 0.01.
* P < 0.04. Comparison was made between the values with the same superscript letters (i.e., a with a, b with b).
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