Epithelial Cell Hyperproliferation Induced in the Exocrine Pancreas of Mice by a Western-Style Diet

Lexun Xue, Kan Yang, Harold Newmark, Dennis Leung, Martin Lipkin*

Background: Pancreatic cancer is a common cause of mortality in the United States, with an estimated 27,800 people dying of the disease in this country in 1996. Epidemiologic studies have suggested that Western diets containing high fat, high protein, and low calcium contents are associated with increased incidence of pancreatic cancer. Purpose: We investigated whether a Western-style diet containing increased fat content and decreased calcium and vitamin D contents would induce epithelial cell hyperproliferation (excess cell duplication) or hyperplasia (excess cell accumulation) in the pancreas, as was previously demonstrated in the colon and mammary gland. Methods: C57BL/6J mice at 4 weeks of age were randomly assigned to one of two groups of 14 mice each. One group received the control diet ad libitum, and the other group was given the Western-style diet ad libitum. After 6, 9, and 15 weeks on the diet, four or five mice per group were infused with 5-bromo-2'-deoxyuridine (BrdU) for 72 hours by use of subcutaneously implanted Alzet osmotic pumps. The mice were then killed, and the pancreas of each mouse was removed. In the exocrine pancreas with ductal secretion, the duct system (including interlobular and intralobular ducts and centroacinar [i.e., centroacinar] regions—cancer-prone regions in certain rodent models and in humans) and acini per mouse in the Western-style diet group was similar to that in the control diet group during the entire feeding period (P = .76, .32, .93, and .42, respectively). Statistically significant higher BrdU-labeling indices of the ductal interlobular and intralobular epithelial cells were seen in mice fed the Western-style diet than in mice fed the control diet during the entire observation period (P = .014 and .016, respectively). There was no statistically significant difference (P = .098) between both diet groups in the BrdU-labeling indices of the centroacinar epithelial cells. Conclusions: A Western-style diet induced pancreatic epithelial cell hyperproliferation in mice, further suggesting that increased fat content and decreased calcium and vitamin D contribute to the development of pancreatic neoplasms. [J Natl Cancer Inst 1996;88:1586-90]

Carcinoma of the pancreas is a common cause of mortality in Western countries. It has been estimated that approximately 25,300 new patients with pancreatic cancer will be detected and 27,800 people will die of this disease in the United States in 1996 (1). Obviously, carcinoma of the pancreas remains one of the most important human cancers because reliable methods of early disease detection and effective treatment are lacking (2). Moreover, several dietary constituents that have been implicated as causative factors include high dietary fat and protein intake (3-5) and low calcium intake (6).

Previous studies from our laboratory (7-10) have demonstrated that feeding
mice a Western-style diet containing increased fat and phosphate contents and low contents of calcium and vitamin D resulted in epithelial cell hyperproliferation (excess cell duplication) and hyperplasia (excess cell accumulation) in both the colon and mammary gland. Several studies in rodents and epidemiologic studies in humans have associated high fat consumption (11-14) and decreased calcium intake (6) with pancreatic carcinogenesis, although the cause of pancreatic cancer is still not well defined.

The exocrine pancreas is a compound acinar gland consisting of a branching duct system and clusters of glandular acini. An acinus consists of a single layer of pyramid-shaped cells with the narrow apical ends bordering the lumen. Nuclei are located in the basal region of the acinar cells. The duct system includes the following:

(a) Interlobular ducts: These large ducts are located between the lobules and are lined by cuboidal to columnar epithelium.

(b) Intralobular ducts: These are intermediate ducts where exocrine secretory droplets collect; they are lined by low cuboidal epithelium. These ducts connect with the larger interlobular ducts, which further lead to major excretory channels.

(c) Centroacinar cells: Also called centroductular cells, these cells are small, flattened epithelial cells and constitute the beginning of the duct system (15).

A pump-infusion method was developed using 5-bromo-2'-deoxyuridine (Brdu) to study the pancreas utilizing a monoclonal antibody against Brdu (16). This technique is a reliable means of measuring early proliferative changes in pancreatic epithelial cells induced by the Western-style diet.

We investigated whether a Western-style diet containing increased fat content and decreased calcium and vitamin D contents would induce epithelial cell hyperproliferation or hyperplasia in the pancreas, as was previously found in the colon and mammary gland.

Materials and Methods

Diets

The diets were based on a semipurified rat and mouse diet developed by the American Institute of Nutrition (AIN-76A) (17). The control diet was the complete form of the diet that meets known nutritional requirements for rats and mice and that supports their growth and development comparable to the best cereal-based diets now available. A modified form of the diet was used as an experimental diet: simulating a Western-style human diet, it contained increased levels of fat and phosphate and decreased levels of calcium and vitamin D. Details of the Western-style diet are given in Table 1.

Table 1. Diet composition*

<table>
<thead>
<tr>
<th>Ingredients per gram diet</th>
<th>Control</th>
<th>Western-style</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat (corn oil), mg</td>
<td>50 (13% of calories)</td>
<td>200 (40% of calories)</td>
</tr>
<tr>
<td>Calcium, mg</td>
<td>5 (1.4 mg/kilocalorie)</td>
<td>0.5 (0.11 mg/kilocalorie)</td>
</tr>
<tr>
<td>Phosphorus (as phosphate), mg</td>
<td>4 (1.1 mg/kilocalorie)</td>
<td>3.6 (0.8 mg/kilocalorie)</td>
</tr>
<tr>
<td>Calcium/phosphorus ratio</td>
<td>1.0</td>
<td>0.11</td>
</tr>
<tr>
<td>Vitamin D₃, IU</td>
<td>1.0 (0.3 IU/kilocalorie)</td>
<td>0.5 (0.11 IU/kilocalorie)</td>
</tr>
<tr>
<td>Kilocalories</td>
<td>3.6</td>
<td>4.5</td>
</tr>
</tbody>
</table>

*Because of the increased nutrient density of the Western-style diet, derived from the high fat content, all essential nutrients (protein, methionine, choline, vitamins other than vitamin D, and minerals other than calcium and phosphorus) are present at 20% higher levels in the Western-style diet than in the AIN-76A diet, added at the expense of sucrose, to give equivalent nutrient densities of essential nutrients. The units of vitamin D are international units (IU). See (7) for further details.

Mice and Treatments

Three-week-old female C57BL/6J mice were purchased from The Jackson Laboratory, Bar Harbor, ME. All the mice were housed at the animal facility of the Memorial Sloan-Kettering Cancer Center, which is accredited by the American Association for Accreditation of Laboratory Animal Care. Animal care was provided in accordance with guidelines outlined in "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 86-23, 1985).

Mice were housed four or five per cage in standard wire-top cages with sawdust bedding in a light-controlled (12 hours/day) room. After 1 week of acclimatization to the control diet, 28 mice at 4 weeks of age were equally randomly assigned to one of two groups: a control (n = 14) or a Western-style (n = 14) diet group. They were fed these diets ad libitum. All mice were given free access to water throughout the experiment. Mice were inspected daily for general state, including their physical activity, intake of food and drink, and stool characteristics. They were weighed weekly before and during the study. After 6, 9, or 15 weeks on the diets, four or five mice per group received Brdu (1.44 mg/mouse), which was administered continuously for 72 hours by implanted Alzet osmotic pumps (Model 1003D; Alza Corporation, Palo Alto, CA). The Alzet pumps (Model 1003D; Alza Corporation, Palo Alto, CA) were used to deliver 0.03% 3,3'-diaminobenzidine tetrahydrochloride solution (Sigma Chemical Co., St. Louis, MO) to the mice continuously for 72 hours by implanted Alzet osmotic pumps (Model 1003D; Alza Corporation, Palo Alto, CA). The Alzet pumps were implanted subcutaneously into the dorsal thoraco-lumbar areas in the anesthetized mice, and incisions were closed with nylon suture by use of aseptic technique. These pumps began continuous delivery at a constant rate (1 µL/hour) 4 hours after pump implantation. The mice were killed by cervical dislocation 76 hours after implantation. At necropsy, the pancreas of each mouse killed was removed and fixed in 80% ethanol solution for 24 hours and then in 55% ethanol solution. Tissues were embedded in paraffin; tissue sections were cut 4 µm thick, stained either with hematoxylin–eosin for histopathologic examination or immunohistochemically for Brdu, and analyzed without knowledge of the source of the specimens.

Immunohistochemistry

Tissue sections were heated at 58 °C or lower for about 40 minutes, deparaffinized in xylene, and then passed through graded alcohols. Subsequently, the sections were treated with 2 N HCl for 90 minutes at room temperature to denature double-stranded DNA and were incubated with 10% normal horse serum for 60 minutes at room temperature to eliminate nonspecific binding. The sections were incubated with 1:1500 anti-Brdu monoclonal antibody (Becton-Dickinson, Mountain View, CA) for 1 hour at room temperature and then overnight at 4 °C. After incubations with the primary antibody, sections were incubated with 1% biotinylated horse anti-mouse immunoglobulin G for 60 minutes at room temperature and then with the avidin–biotin–peroxidase complex (Vectastain ABC peroxidase kit; Vector Laboratories, Inc., Burlington, CA) for 45 minutes at room temperature. Finally, slides were mounted with 0.03% 3,3'-diaminobenzidine tetrahydrochloride solution (Sigma Chemical Co., St. Louis, MO) for about 45 seconds. Slides were rinsed three times with phosphate-buffered saline (pH 7.4) between the incubations described above. The sections were counterstained with hematoxylin, dehydrated, and cleared, and a cover slip was applied with crystal mount.

BrdU-positive cells were identified by the presence of brown to black pigment over their nuclei and were counted by light microscopy. Epithelial cell proliferation in the pancreas was expressed as labeling index, which was calculated as the ratio of the number of Brdu-labeled cells to the total number of pancreatic ductal or acinar epithelial cells counted.

Morphometry

Under 400x magnification, 1.25 × 10^6 µm² of pancreatic tissue was scored for each mouse. The
total number of acini and each of the three types of ducts (interlobular, intralobular, and centroacinar cells) was counted. In addition, the total number of pancreatic ductal or acinar epithelial cells and the number of BrdU-labeled cells were counted in each of the three types of ducts or acini.

**Statistical Analysis**

The data were analyzed by two-way analysis of variance (18). Mice randomly assigned to one of the two diet groups were compared with regard to the number of epithelial cells, the number of labeled cells, labeling index, and the number of ducts or acini. Separate comparisons were done for each type of duct and for acini. The total number of epithelial and labeled cells and the labeling index were measured separately for each duct or acinus of each mouse. Since the number of labeled cells (and labeling index) is zero in most ducts, analyses were performed on the average number of cells, labeled cells, and labeling indices over the ducts counted within a mouse. In the present experiment, since one cage of mice was used for each diet–time combination, we have used cage as a unit of analysis. Two-way analysis of variance was based on cage mean values; dietary group and the number of weeks of diet maintenance were used as factors. Two-way analysis of variance was also performed on the number of ducts or acini. Summary statistics were expressed as the means ± standard errors, except as otherwise stated. In all statistical analyses, a $P$ value of $\leq 0.05$ was considered significant. All $P$ values were generated from two-sided tests of statistical significance.

**Results**

**Diet Tolerance and Body Weights of Mice**

All the mice tolerated each of the two types of diets well. No mouse died during the 15-week feeding period. Average body weights of mice in the control and the Western-style diet groups did not differ statistically before ($P = .77$) or after ($P = .48$) the 15-week feeding period, suggesting that the caloric intake of the two diets was similar.

**Morphometric Study**

The number of pancreatic ducts (interlobular, intralobular, and centroacinar) and acini per mouse in the Western-style diet group was similar to that in the control diet group during the entire feeding period ($P = .76, .32, .93, .42$, respectively) (Table 2).

**Immunohistochemical Study**

The effects of feeding the Western-style diet for 6-15 weeks on epithelial cell proliferation of both the duct system and acini are shown in Table 2 and Fig. 1.

### Table 2. Effects of Western-style diet on epithelial cell proliferation of exocrine pancreas in mice

<table>
<thead>
<tr>
<th>Weeks on diet</th>
<th>Interlobular ducts*</th>
<th>Intralobular ducts*</th>
<th>Centroacinar cells*</th>
<th>Acini*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control diet</td>
<td>Western-style diet</td>
<td>Control diet</td>
<td>Western-style diet</td>
</tr>
<tr>
<td>6</td>
<td>22</td>
<td>44</td>
<td>14</td>
<td>48</td>
</tr>
<tr>
<td>9</td>
<td>25</td>
<td>43</td>
<td>11</td>
<td>44</td>
</tr>
<tr>
<td>15</td>
<td>37</td>
<td>96</td>
<td>20</td>
<td>70</td>
</tr>
</tbody>
</table>

$*_{Microscopically, the exocrine pancreas is a compound acinar gland that consists of clusters of acini with a single layer of pyramid-shaped cells and a branching duct system. The beginning of the duct system is the centroacinar cells characterized by small, flattened epithelial cells that are located in the lumen of some acini. The intermediate ducts located in the lobules are called intralobular ducts with low cuboidal epithelium. The larger ducts are intralobular ducts lined by cuboidal to columnar epithelium.}$

$†_{Mice on the control and Western-style diets after 6, 9, and 15 weeks were studied. For each mouse, 1.25 \times 10^9 \mu m^2 of pancreatic tissue was scored under 400× magnification. These values represent the total number of epithelial cells and 5-bromo-2'-deoxyuridine-labeled cells assayed for each group, respectively.}$

$‡_{Values = means ± standard errors (in parentheses). The two diet groups were compared with regard to number of epithelial cells, 5-bromo-2'-deoxyuridine (BrdU)-labeled cells, labeling index, and number of each of the three types of ducts or acini by use of two-way analysis of variance.}$

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Interlobular Intralobular Centroacinar Acini

Ducts Ducts Cells

6 Weeks 9 Weeks 15 Weeks

(0.18 0.10 0.06 .0.02 .0.00

Fig. 1. Effects of Western-style diet on cell proliferation (labeling index) in pancreas of C57BL/6J mice. At 4 weeks of age, mice were randomly divided into one of two groups (14 mice per group). One group was given control (AIN-76A) diet, and the other group received a Western-style diet. After feeding periods of 6, 9, and 15 weeks, four or five mice per group were continuously infused with 5-bromo-2'-deoxyuridine (BrdU) for 72 hours by subcutaneously implanted Alzet osmotic pumps. Mice were then killed, and the pancreas of each mouse was removed for histopathologic and immunohistochemical examinations as described in the "Materials and Methods" section. Values shown are means ± standard errors and reflect epithelial cell proliferation rate (labeling index) of the duct system (interlobular and intralobular ducts and centroacinar cells) and acini in the respective diet groups after 6, 9, and 15 weeks of diet administration. C = control diet group. S = Western-style diet group. Ordinate = BrdU-labeling index of epithelial cells.

There were no statistically significant changes in labeling index over time (P = .24).

Discussion

In the present study, the effects of feeding a Western-style diet (containing increased fat content and low levels of calcium and vitamin D) for 6, 9, and 15 weeks on the exocrine pancreas of female C57BL/6J mice were investigated by use of morphometry and BrdU immunohistochemistry. No statistically significant difference was observed in the number of pancreatic ducts or acini in the Western-style diet and control groups. However, statistically significant higher BrdU-labeling indices of epithelial cells in interlobular (P = .014) and intralobular (P = .016) ducts were seen in mice fed the Western-style diet compared with mice fed the control diet during the 6- to 15-week feeding period.

Ninety percent of human pancreatic cancers have been histopathologically classified as ductal epithelial cell adenocarcinomas (19). In addition, another study of human tumors (20) supports the likelihood that acinar cells are also involved in the development of ductal cell carcinoma of the pancreas; in mice and rats (15,21), tumors of the pancreas generally consist of cells that resemble acinar cells. In hamsters (21), neoplastic lesions of the pancreas are visible among both ductal and acinar cells, but tumors are almost exclusively ductal adenocarcinomas.

The cause of human pancreatic cancer is still unknown. Epidemiologic findings, however, have suggested that diets containing high levels of fat and protein (13,14) and low levels of calcium (6), i.e., traditional Western diets, are risk factors for pancreatic cancer. The Western-style diet in this study led to epithelial cell hyperproliferation of pancreatic ductal epithelial cells, as it previously did in the colon and mammary gland (7-10,22-25), further suggesting that diets with high fat content and low levels of calcium and vitamin D might have a role in human pancreatic carcinogenesis. In the colon, hyperproliferation induced by a Western-style diet has now been followed by the development of neoplastic colonic lesions without any chemical carcinogen, when mice were fed a Western-style diet throughout most of their life span (26). In humans, high dietary fat and protein (27,28) and low dietary calcium (6) have been associated with increased risk for pancreatic and colon cancer (29) and mammary cancer (30). Our earlier study (8) demonstrated that increasing dietary calcium levels without lowering dietary fat content also had a protective effect against epithelial cell hyperproliferation and hyperplasia in rodent colon.

In the present study, we were unable to assess precise mechanisms that might lead to a positive association of Western-style diet consumption and pancreatic epithelial cell hyperproliferation; however, high levels of fat in Western-style diets can stimulate release of gastrointestinal hormones, e.g., cholecystokinin, an important regulatory peptide for growth of the pancreas and secretion of the pancreatic juice (31). Some animal studies (32-34) have demonstrated that...
exogenous cholecystokinin, when administered to rats or hamsters, induces pancreatic hyperplasia and hypertrophy. In addition, low dietary calcium could enhance pancreatic epithelial cell hyperproliferation by mechanisms similar to its actions in the colon (7,8,35), including binding of fatty and bile acids and direct inhibition of hyperproliferation by calcium.

References


Notes

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