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ORIGINAL ARTICLE

Effects of Porcine Pancreatic Enzymes on the Pancreas of Hamsters. Part 2: Carcinogenesis Studies

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ABSTRACT

Context Our previous study suggested that porcine pancreatic extract in hamsters with peripheral insulin resistance, normalizes insulin output, islet size and pancreatic DNA synthetic rate. It also inhibited the growth of human pancreatic cancer cells in nude mice. **Objective** To examine the potential value of the porcine pancreatic extract in controlling pancreatic carcinogenesis in this model, the present experiment was performed. **Design** Hamsters were fed a high fat diet and four weeks later when insulin resistance emerges, they were divided into two groups. One group received 1 g/kg BW of porcine pancreatic extract in drinking water and the other group received tap water. One week later, when insulin output normalizes in porcine pancreatic extract-treated hamsters, a single subcutaneous injection of N-nitrosobis-(2-oxopropyl) amine (BOP) at a dose of 40 mg/kg BW was given to all hamsters. The experiment was terminated at 43 weeks after the porcine pancreatic extract treatment. The number and size of pancreatic tumors, blood glucose, insulin, amylase and lipase levels, the average size of islets and the number of insulin cells/islets were determined. **Results** The incidence of pancreatic cancer was significantly lower in the porcine pancreatic extract group (P=0.043), as well as the plasma insulin level and the size of the islets in the porcine pancreatic extract group were significantly lower (P<0.001) than in the control group. No significantly differences were found in the glucose level between the groups. **Conclusion** These results show that porcine pancreatic extract has a potential to inhibit pancreatic cancer growth.

INTRODUCTION

The origin of pancreatic adenocarcinoma is still quite controversial. Although human ductal cells have been generally considered to be the cancer progenitor cells, the results from experimental models are conflicting. It has been claimed that acinar, ductal/ductular cells and islet cells serve as the cancer cell origin [1, 2, 3, 4, 5]. In the hamster model, which mimics the human disease, in morphological, biological, clinical, and molecular biological aspects tumors also derive from ductal and ductular cells [2, 3, 5, 6, 7, 8, 9, 10, 11, 12]. However, most tumors in this model develop within the islets, possibly from stem cells (precursor cells). In fact, the first alteration during pancreatic carcinogenesis is the development of intra-insular ductular structures, which undergo hyperplastic, metaplastic, and malignant alterations, which subsequently destroy the islets and invade the surrounding tissues even when they are only microscopic in size [2, 5].

Several studies in the hamster model indicated that stimulation of islet cell proliferation promotes, whereas inhibition of islet cell turnover inhibits pancreatic carcinogenesis [2, 13, 14, 15, 16]. The promoting effect of a high fat diet on pancreatic carcinogenesis was found to be related to the induction by the high fat diet of peripheral insulin resistance associated with hyperinsulinemia and islet cell proliferation [17]. Indeed, amelioration of peripheral insulin resistance by metformin, an antidiabetic drug, normalized the insulin level and the size of the islet and prevented pancreatic tumor formation [18]. These studies, therefore, pointed to the significant role of islets in pancreatic carcinogenesis.

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Key words Cell Proliferation; Glucose; Insulin; Pancreatic Neoplasms /prevention and control

Abbreviations PPE: porcine pancreatic extract

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Peripheral insulin resistance is a common feature during pancreatic carcinogenesis in the hamster model [17] as it is in humans [19]. More than 65% of pancreatic cancer patients develop peripheral insulin resistance, altered glucose tolerance or frank diabetes weeks or months prior to the clinical manifestation of the disease [20, 21, 22]. Although its mechanism is still obscure, it appears that islet cells are somehow involved in the neoplastic process early on. Hence, inhibition of peripheral insulin resistance and overproduction of insulin, which is known to stimulate cancer cell growth, would inhibit or even prevent cancer induction or growth. This possibility was validated by the aforementioned effect of metformin [18]. Because of the known toxicity of metformin, we were investigating other non-toxic and preferably naturally occurring substances.

In our pilot study using the pancreatic enzyme, which is widely used as a replacement therapy in patients with pancreatic insufficiency [23, 24, 25, 26, 27], we noticed that the porcine pancreatic extract (PPE) reduced the size of islets and the levels of insulin in otherwise untreated hamsters as described in the basic studies. This finding prompted us to investigate the effect of PPE in pancreatic carcinogenicity in hamsters with fat-induced peripheral insulin resistance.

METHODS

Animals

Three to four-week-old Syrian Golden hamsters of both genders were purchased from SASCO Inc. (Wilmington, MA, USA). The animals were housed in plastic cages with commercial cage bedding and kept under standard laboratory conditions (temperature 20±2°C, 12-hour light/12-hour dark cycle). Hamsters had free access to pelleted food and tap water. For ethical consideration see part 1 of this report [28].

Porcine Pancreatic Extract (PPE)

The formula of the PPE and its preparation in water was reported earlier [20]. The dose of PPE was 1 g/kg body weight. The amount of PPE was adjusted weekly based on the body weights and water intake. PPE was administered freshly every 24 hours. For details please see part 1 of this report [28].

Diet

High fat diet was formulated as described [14, 17], prepared freshly, and stored in the cool-room for no longer than two weeks.

Experiment

After weaning, 124 hamsters of both genders were fed the high fat diet for life. Two weeks after the high fat feeding, when peripheral insulin resistance develops consistently, hamsters were randomly divided into two groups. One group (PPE group, n=64; 32 males, 32 females) received PPE in drinking water at a concentration of 1 g/kg body weight. Another group received tap water (control group, n=60; 30 males, 30 females). N-nitrosobis (2-oxopropyl) amine (BOP) (Nakarai tesqu, Kyoto, Japan) was given once subcutaneously at a dose of 40 mg/kg body weight one week after the initiation of PPE treatment, at a time when insulin level normalizes. All hamsters were closely observed during the study and examined periodically by abdominal palpation for the presence of tumors. The water intake was measured daily and food consumption and body weight weekly. Three males from each group were sacrificed at week ten, and two males and two females from each group at week 20 to examine pancreatic tissue for the presence of induced lesions. At week 24 and 27, one female from each control group, and one male from PPE group at week 29 were euthanized because of injuries. Between week 24 and 33, three hamsters (one from the control group, two from the PPE group) died of spontaneous disease. The remaining hamsters (25 males and 25 females in the control group and 26 males and 28 females in the PPE group) were euthanized at week 43 post-PPE treatment. Blood glucose and plasma insulin, amylase and lipase levels were assayed and the size of islets and the number of alpha and beta cells were determined.

Autopsy and Histology

A complete necropsy was performed in all hamsters and abdominal and thoracic tissues were examined for abnormalities. The pancreas, with and without tumors, liver, spleen, kidneys, and lungs were weighed. The pancreas was cut in 4 µm step sections (six sections/pancreas) and evaluated histologically by two pathologists in a blinded fashion. All hyperplastic, preneoplastic and neoplastic lesions were diagnosed according to our established criteria [5] and the number of each lesion was recorded and pancreatic tumors were measured in mm^3.

Determination of Islet Size

The islet size was measured in hematoxylin and eosin-stained slides. The diameter of approximately 200 randomly selected islets in splenic lobe was calculated by a micro scale using an Axiomat® microscope (Zeiss, Jena, Germany). The average size was considered to be the representative value for that pancreas (area: μm^2 = π x length a/2 x length b/2).

Determination the Number of Beta Cells and Alpha Cells in Islets

An immunohistochemical examination was carried out using the avidin-biotin-peroxidase complex (ABC) method [29]. Mouse anti-insulin monoclonal antibody and rabbit anti-glucagon polyclonal antibody (Zymed Laboratories Inc., South San Francisco, CA, USA) were utilized in the staining process. Double immunostaining (for insulin and glucagon) was performed as reported [30]. The number of beta cells and alpha cells in the approximately 200 islets was then counted randomly.

Table 1. Comparison of body weights (g). The amount of PPE intake (mg/animal) is also shown.

Group	Weeks				
	0	1-10	11-20	21-30	31-40
Control (n=50)	53±4	109±20	141±25	147±29	143±21
PPE (n=54)	54±4	104±21	141±28	146±27	140±22
PPE intake	-	*96±23*	*139±24*	*145±24*	*140±20*
P value	P=0.794	P=0.217	P=1.000	P=0.856	P=0.479

Data are shown as mean±SD
Statistical analysis was performed by using one-way ANOVA

Biochemical Assay

The blood was collected from the right ventricle by using a 21G syringe under anesthesia after an overnight fasting. The plasma was prepared using a BD Vacutainer™ (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The serum was also prepared and stocked at -20°C until analyzed. The plasma insulin level was assayed by using the Insulin Enzyme Immunoassay Kit (Cayman Chemical Co., Ann Arbor, MI, uSA), according to the manufacturer's instructions. Blood glucose levels were measured using an Accu-Check® (Roche Diagnostic Corporation, Indianapolis, IN, USA) blood glucose meter. Plasma amylase and lipase levels were assayed as reported [20].

Fecal Fat and Urine Analysis

At autopsy, feces from the descending colon were examined qualitatively with Sudan IV for the presence of fat as reported [20]. Reagent strips of Multistix® 10SG (Bayer Corporation, Elkhart, IN, USA) were used as reported [20] for the qualitative detection of urinary glucose, ketone, and bilirubin.

ETHICS

Animals were managed and sacrificed in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines. The method of euthanasia was consistent with the recommendation of the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia.

STATISTICS

Data are reported as mean±SD or frequencies. Statistical analysis of scale variables was performed by using one-way ANOVA and Fisher's exact test was used to analyze 2x2 tables. The SAS statistical package (SAS Institute Inc., Cary, NC, USA) was used for data analysis. Two-tailed P values less than 0.05 were considered significant.

Table 2. Comparison of food consumption (g/week/hamster).

Group	Weeks			
	1-10	11-20	21-30	31-40
Control (n=50)	62.9±10.1	53.9±11.9	53.9±9.7	57.2±7.8
PPE (n=54)	61.1±11.2	53.9±13.0	51.3±9.5	56.5±7.2
P value	P=0.393	P=1.000	P=0.170	P=0.653

Data are shown as mean±SD
Statistical analysis was performed by using one-way ANOVA

Table 3. Comparison of water intake (mL/day/hamster).

Group	Weeks			
	1-10	11-20	21-30	31-40
Control (n=50)	19.9±1.7	24.6±1.2	27.8±3.6	33.1±1.4
PPE (n=54)	15.6±0.8	14.9±0.5	18.4±2.6	23.7±1.6
P value	P<0.001	P<0.001	P<0.001	P<0.001

Data are shown as mean±SD
Statistical analysis was performed by using one-way ANOVA

RESULTS

Weekly Body Weight, Food Consumption, and Water Intake

Before the beginning of the experiment, body weights were not significantly different between the two groups (Table 1). Between week one and week ten post-PPE treatment, however, the body weights in the PPE group were lower, but not significantly (104±21 g; P=0.217), when compared to the control group (109±20 g); in the subsequent weeks neither the body weights differed significantly between the groups, nor was the relative weight of the pancreas (PPE group: 0.27±0.05 g/100g; controls: 0.28±0.21 g/100g; P=0.735).
During the entire study period the food consumption was not statistically different between the two groups (Table 2); however, the food consumption between 21 and 30 weeks was significantly less in the PPE group than in the control group even if not reaching the significant level (P=0.170). The average daily water intake at each time point was significantly greater in controls than in PPE group (Table 3).

Blood Glucose and Insulin Levels

The glucose levels was not significantly different between the two groups (Table 4). However, the plasma insulin levels in the control group (0.29±0.05 ng/mL) were significantly higher than in the PPE group (0.16±0.17 ng/mL, P<0.001).

Plasma Amylase and Lipase Levels

The plasma amylase levels did not differ between the groups (Table 5). However, the plasma lipase levels, were significantly higher in the PPE group compared with the control group (P<0.001).

Fecal Fat and Urinary Analysis

Steatorrhea was more common in the control group than in PPE group (92.0% *vs*. 66.7%; P=0.002; Table 5), while no significant differences in urine data were found between the two groups (Table 5).

Table 4. Blood glucose and plasma insulin levels.

	Control	PPE	P value
Glucose (mg/dL)	86.0±26.9 *(n=50)*	82.6±19.8 *(n=54)*	P=0.462
Plasma insulin (ng/mL)	0.29±0.05 *(n=30)*	0.16±0.17 *(n=31)*	P<0.001

Data are shown as mean±SD
Statistical analysis was performed by using one-way ANOVA
The number of available data is shown within parentheses.

Table 5. Plasma amylase and lipase level and the incidence of the fecal fat.

	Control (n=50)	PPE (n=54)	P value
Amylase (U/L)	652±39	600±57	P<0.001 [a]
Lipase (U/L)	312±37	1,239±401	P<0.001 [a]
Fecal fat [c]	46 (92.0%)	36 (66.7%)	P=0.002 [b]
Urine data: [d]			
- Glucose	0	0	-
- Ketone	19 (38.0%)	25 (46.3%)	P=0.431 [b]
- Bilirubin	0	0	-

Plasma amylase and lipase levels are shown as mean±SD
[a] One-way ANOVA
[b] Fisher's exact test
[c] Incidence of steatorrhea [20]
[d] Incidence of presence of urinary glucose, ketone, and bilirubin [20]

Histology

Ten weeks after the PPE treatment, ductal hyperplasia was seen only in the control group. After 20 weeks, ductular proliferation was noticed in both groups. The first malignant lesions (carcinoma *in situ*) were seen at week 24 in one female of the control group. After 43 weeks of PPE treatment, the incidence of adenocarcinoma (P=0.043) and their size (P<0.001) were significantly lower in the PPE group, while no significant differences were found in the multiplicity of the tumors (Table 6). During the overall 43-week period of the study the ductular proliferation, ductal/ductular hyperplasia, dysplastic ducts/ductuls, periductal inflammation, and atrophy of acinar cells were not different between the two groups (Table 7).

Islet Size

The size of islets in control group was significantly larger than in the PPF group (P<0.001; Table 8). By comparing the islet sizes in tumor-bearing and non-tumor-bearing hamsters of both groups (Table 9) we found that in the control group with cancers the islets were significantly larger (35,861±9,191 μm^2) than in the corresponding PPE group (24,737±7,185 μm^2, P=0.018) as well as the islet sizes of control hamsters without cancer (32,310±8,866 μm^2) were much larger than those in the corresponding PPE group (26,224±5952 μm^2; P<0.001). No significant differences were found between cancer and non-cancer hamsters within the two groups (P=0.024 and P=0.575 in the control and PPE groups, respectively).

The Number of Beta ad Alpha Cells

The number of beta cells/islet in the control group (202±49) was significantly (P<0.001) higher than the PPE group (167±29) (Table 8). In each group of pancreatic cancer-bearing hamsters, the number of beta cells was higher (216±58 in the control group and 172±39 in the PPE group) than in those without cancer (184±34 in the control group and 160±10 in the PPE group; however, the data reached significant values in the control group only (P=0.020) but not in the PPE group (P=0.077). The number of alpha cells did not differ significantly between the groups in any of the experiments (data not shown).

Table 6. Incidence, multiplicity and size of adenocarcinomas.

	Control (n=50)	PPE (n=54)	P value
Adenocarcinoma	13 (26.0%)	6 (11.1%)	0.043 [a]
Multiplicity	0.34±0.64	0.15±0.99	0.251 [b]
Tumor size (mm)	0.88±0.55	0.56±0.38	0.001 [b]

Data are shown as frequencies or mean±SD
Adenocarcinoma: adenocarcinoma, microcarcinoma (carcinoma less than 1 mm in diameter), and carcinoma *in situ*.
Multiplicity: number of carcinoma lesion per animal
[a] Fisher's exact test
[b] One-way ANOVA

Table 7. Histological findings (multiplicity and incidence of non-neoplastic lesion in the pancreas).

	Control (n=50)	PPE (n=54)	P value
Ductular proliferation [a]	8.2±8.5	8.3±7.1	0.948 [b]
Ductal hyperplasia	33 (66.0%)	38 (70.4%)	0.677 [c]
Dysplastic ducts	3 (6.0%)	1 (1.9%)	0.349 [c]
Dysplastic ductuls	5 (10.0%)	5 (9.3%)	1.000 [c]
Periductal inflammation	30 (60.0%)	37 (68.5%)	0.416 [c]
Atrophy of acinar cells	6 (12.0%)	6 (11.1%)	1.000 [c]

[a] Ductular proliferation was evaluated as multiplicity and is shown as mean±SD
[b] One-way ANOVA
[c] Fisher's exact test

DISCUSSION

In our studies of the hamster pancreatic cancer model, islets were found to play an essential role in tumorigenesis [2, 3, 5, 6, 7, 8, 9, 10, 11, 12]. Similar situations seem to apply to human pancreatic cancer [12]. Although ductal cells are generally thought to be cancer progenitor cells, the development of altered glucose metabolism, including frank diabetes in the majority of pancreatic cancer cases shortly before the clinical detection of cancer, usually points to the involvement of islet cells in humans as well. This assumption was validated by significant alterations of islets in pancreatic cancer patients, especially in those with altered glucose tolerances [22]. The rare reports on the development of pancreatic cancer in type I diabetes, indicate that either insulin or the beta islet cells *per se* are fundamental in carcinogenesis. If so, improvement of insulin metabolic alterations could prevent, or at least hinder, pancreatic cancer development. Confirming this possibility, the anti-diabetic drug metformin prevented pancreatic cancer induction in the hamster model [18]. Since the toxicity of metformin is a concern, the identification of a non-toxic anti-diabetic compound is desirable. In a study to test the long-term effects of the pancreatic enzyme, we noticed that PPE reduced insulin levels and the sizes of islets in hamsters with peripheral insulin resistance. This finding prompted us to examine the effect of PPE in hamsters with peripheral insulin resistance, induced

Table 8. The islet size and the number of beta cells.

	Control (n=50)	PPE (n=54)	P value
Islet size (μm^2)	33,123±8,942	26,062±6,025	P<0.001
Number of beta cells	202±49	167±29	P<0.001

Data are shown as mean±SD
Statistical analysis was performed by using one-way ANOVA

either by feeding a high-fat diet [18, 30], or by the treatment with pancreatic carcinogen N-nitrosobis (2-oxopropyl) amine (BOP) [17].

Our short-term and long-term studies using PPE showed that PPE indeed influences the patterns and the function of islets. Reflecting the suppressive effect of PPE on beta cells, islet sizes were smaller and insulin levels lower than in the control group. Similar results were reported by Reddy *et al*. who found that the oral administration of mouse pancreatic extract reduced the incidence and delayed the onset of diabetes in the non-obese diabetic mouse model [31]. They hypothesized that pancreatic extract may influence the immunoregulatory cells. The mechanism involved in the effect of PPE on the hamster pancreas is presently obscure. The effect could be of a nutritional nature, as PPE-treated hamsters consumed less food and gained less weight, conditions which reflect controlled utilization of food associated with reduced beta cell activity. Another possibility is that PPE put the pancreas "in rest" through a feedback mechanism by reducing the cellular functions and the DNA synthesis.

Based on our experiences with metformin, we expected that PPE, by reducing the islet cell function, would inhibit pancreatic carcinogenesis. This did indeed happen. However, contrary to metformin, PPE did not prevent tumor induction. Because the incidence and multiplicity of cancer precursor lesions was not different between the PPE group and the control group, the lower cancer incidence in the PPE group indicated that PPE inhibited the progression of precursor lesions to cancer. This apparent lack of a therapeutic effect contrasts with the results of the experiment in mice, where PPE inhibited the growth of human pancreatic cancer cells and led to a significantly longer survived than those without PPE treatment [20]. However, it is presently unclear whether the effects of PPE depend on the dose and/or duration of the PPE treatment. Moreover, it is equally unclear whether the effects of PPE are related to the enzymes or to other yet unknown factors in this crude extract.

Nevertheless, the present study explicitly shows that PPE interferes with islet cell replication. Remarkably, the islet size differed in control hamsters with or without pancreatic cancer. The islet size in hamsters with cancer was significantly larger than in those without cancer, a possible indication that during carcinogenesis islet cells proliferate, as do ductal and ductular cells. Since the number of beta and alpha cells was not different between the control hamsters with and without cancers, the proliferation seems to occur in non-beta and non-alpha cells (precursor cells?). Similar results have been found in pancreatic cancer patients [12]. The results, confirms the prior studies outlined in part 1 of this report [28] that PPE slows down the function of both endocrine and exocrine function. Reasons for the increased level of lipase in the present study in contrast to the findings in the part 1 study [28] is obscure and requires further clarification. Nevertheless, PPE appear to have potential beneficiary effects on obesity, in pre-diabetic conditions associated with peripheral insulin resistance and in pancreatic cancer. Moreover, based on its effects, PPE could be used in a number of pancreatic diseases. Its inhibitory action of pancreatic enzyme secretion is suitable for the treatment of acute pancreatitis; its potential as pure uncontaminated pancreatic enzymes is certainly useful in any condition associated with pancreatic enzyme deficiency, including chronic pancreatitis and pancreatic cystic disease.

Table 9. Comparison of the islet sizes and the number of beta cell between with cancer and without cancer in each group

	Control	PPE	P value
Islet size (μm^2):			
- Cancer	35,861±9,191 [a]	24,737±7,185 [c]	0.018
- Non-cancer	32,310±8,866 [b]	26,224±5,952 [d]	<0.001
P value	*0.224*	*0.575*	
Number of beta cells:			
- Cancer	216±58 [a]	172±39 [c]	0.112
- Non-cancer	184±34 [b]	160±10 [d]	<0.001
P value	*0.020*	*0.077*	

Cancer: adenocarcinoma, microcarcinoma (carcinoma less than 1 mm in diameter), and carcinoma *in situ*.
Data are shown as mean±SD
Statistical analysis was performed by using one-way ANOVA
[a] n=13; [b] n=37; [c] n=6; [d] n=48

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Conflict of interest The authors have no potential conflict of interest

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