Immunocytochemical localization of insulin-immunoreactive cells in the pancreatic ducts of rats treated with trypsin inhibitor

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Summary. It is well known that soybean trypsin inhibitor exerts trophic effects on the exocrine pancreas, resulting in the hypertrophy of acinar cells. Some evidence also exists for hyperplasia in acinar tissue, the ductal epithelium and islet tissue. Rats maintained for 3 weeks on an oral administration of soybean trypsin inhibitor (200 mg/50 ml drinking water) were compared with untreated animals. Significant changes were noted in treated animals ($p < 0.01$). Trypsin inhibitor-treated rats showed an increase in pancreatic weight (2.33 ± 0.46 g). The volume ratio of acinar, islet and connective tissue as measured by the stereology point-count technique remained the same in both groups. Ductal tissue, however, exhibited an increase in volume ratio, $3.77 \pm 4.38\%$ per $2714 \mu\text{m}^2$ area of tissue, in trypsin inhibitor-treated animals. All tissue components showed an increase in the experimental animals: acinar (125%), islet (144%), ductal (325%) and connective tissue

(94%). Increased size of acinar cell nuclei, as measured by average cord length, $6.20 \pm 0.13 \mu\text{m}$, and a decreased nuclear density of acinar cells, 28 ± 4.74 per $150 \mu\text{m}^2$ area of tissue, indicated hypertrophic changes in these cells of the experimental animals. Using immunohistochemical localization and the point-count technique, a significant fraction of the total pancreatic volume in experimental animals was represented by ducts containing immunoreactive cells. The percent of volume ratio, $0.42 \pm 0.15\%$ per $2714 \mu\text{m}^2$ area of tissue, was calculated for ducts containing insulin-immunoreactive cells within their epithelium.

Key words: Soybean trypsin inhibitor, pancreatic duct cells, insulin immunoreactivity, pancreas, animal diabetes, experimental diabetes

The occurrence of insulin-containing cells within the pancreatic ductal epithelium has been reported under a variety of conditions in several species. Under normal conditions, post-natal pancreatic B-cell neogenesis via pancreatic ducts has been observed [1], but it is considered rare. In man, such occurrences have been noted under certain dietary regimens [2], in the pancreas of recent-onset Type 1 (insulin-dependent) diabetic patients [3], in sulphonylurea-treated diabetes [4], in cystic fibrosis of the pancreas [5], in pancreatic adenomas [6], and in familial multiple endocrine adenomatosis [7].

Under experimental conditions, ductular proliferation has been noted during regenerative phases in the islets of alloxan-treated animals [8–13], in steroid-treated [14, 15], and dextrose-injected animals [16], in subtotal pancreatectomies [17, 18], in normal and alloxan-diabetic rats subsequent to ductal ligation [19–21] and in tolbutamide-treated mice [22]. More recently, with immunohistochemical methods, immunoreactive insulin has been localized in the duct cells of neonatal rats treated with streptozotocin as they recovered from

experimental diabetes [23], and in the duct cells of pancreas from MtTW15 mammosomatotropic tumour-bearing rats [24].

It has been known for years that rats and chickens fed raw soybeans develop pancreatic enlargement and increased pancreatic enzyme secretion [25]. The effect of soybean feeding on the pancreas has been attributed to the heat-labile trypsin inhibitor contained in raw soybean. In a study by Yanatori and Fujita [26] comparing the pancreases of rats treated with raw soybean, trypsin inhibitor and subcutaneous injections of cholecystokinin-pancreozymin (CCK-PZ), it was shown that all three regimens produced hypertrophy of the exocrine pancreas. They observed that mitotic activity occurred not only in the acinar cells, but also in the ductal system, including the centroacinar cells and in the endocrine islets.

In the present experiments, we aimed to characterize duct cells immunohistochemically and to assess their quantitative change in rats treated with trypsin inhibitor.

Table 1. Body weight, pancreatic weight and pancreatic/body weight ratios.

	Initial body weight (g)	Final body weight (g)	Pancreatic wet weight (g)	Pancreatic/body weight ratio %
Control rats (n=9)	147 ± 3.05	249 ± 4.85	1.10 ± 0.12	0.40 ± 0.13
Rats treated with trypsin inhibitor (n=9)	147 ± 3.18	243 ± 6.08	2.33 ± 0.46 ^a	0.95 ± 0.03

Results expressed as mean ± SEM. ^a Significantly different from control ($p < 0.01$)

Table 2. Average point-count ratios (%) of component tissues of the pancreas per 2714 µm² area of tissue

Tissue	Rats treated with trypsin inhibitor (n=3)	Control rats (n=3)
Acinar	89.7 ± 6.75	90.5 ± 7.60
Islet	1.68 ± 2.92	1.58 ± 4.14
Ductal	3.77 ± 4.38 ^a	2.04 ± 2.26
Connective tissue septa	3.52 ± 3.36	4.17 ± 3.41

^a Significantly different from control ($p < 0.01$)

Table 3. Mean number of acinar nuclei contained in 150 µm² area and mean cord length measured on 200 nuclei

	Nuclear density	Nuclear size (µm)
Rats treated with trypsin inhibitor (n=9)	28 ± 4.74 ^a	6.20 ± 0.13
Control (n=9)	51.4 ± 7.39	5.67 ± 0.08

^a Significantly different from control ($p < 0.01$)

Materials and methods

Animals

Eighteen male Sprague-Dawley rats (weight approximately 150 g) were divided equally into two groups. Group 1 was given, in lieu of ordinary water, a fresh solution of trypsin inhibitor ad libitum every 24 h and fed a normal rat chow diet. Group 2 received ordinary drinking water and a normal rat chow diet.

Trypsin inhibitor

Trypsin inhibitor (200 mg) from raw soybean (Type II-S, Sigma Chemical Company, St. Louis, Mo, USA) was dissolved in 50 ml of water with the pH adjusted to 7.0 with dilute NaOH as described by Yanatori and Fujita [26]. The inhibitory activity to the trypsin was commercially determined to be 1 mg/0.6 mg of trypsin. A trypsin inhibitor assay, based on the rate of hydrolysis of the substrate o-toluenesulphonyl-L-arginine methyl ester (TAME) as measured by the increase in absorbancy at 247 Å [27], indicated that there was no loss in inhibitory activity of trypsin inhibitor during the 24-h period.

Tissue preparation

After a period of 3 weeks, each rat was weighed, anaesthetized intraperitoneally (sodium pentobarbital, 0.1 ml/100 g body weight) and transcardially perfused with 0.9% saline. The pancreas of each animal was removed surgically, trimmed free of fat and lymph nodes, and the wet weight recorded. From the splenic region, a portion of tissue, approximately 1 cm² in size, was removed. These were fixed for 12 h in Bouin's solution while agitated (40RPM) on a rotator platform (Tek-Tator V, American Hospital Supply, Evanston, Ill, USA) and rinsed in

6 changes (2 h each) of 50% ethanol. They were dehydrated in graded ethanols, cleared in toluene, infiltrated in vacuo and embedded in Paraplast Plus (Monojet Scientific, St. Louis, Mo, USA). Each block of tissue was serially sectioned at 6 µm on an AO-Spencer rotary microtome (Model 820: American Optical, Buffalo, NY, USA).

Immunocytochemistry

The sections of each block were serially mounted on gelatinized microscope slides, deparaffinized in xylene, rehydrated and stained immunocytochemically for insulin, using the unlabelled antibody enzyme technique of Sternberger et al. [28]. The primary antiserum (guinea pig, anti-bovine insulin serum; GP-Ab28, University of Minnesota, Minneapolis, Minn, USA) was diluted (1:500) with phosphate buffered saline (0.2 M, PBS) at pH of 7.2, containing 1% normal sheep serum (Cappel Laboratories, Cochranville, Pa, USA). 50 µl of the primary antibody were applied to each section and incubated in a humid chamber overnight at 4°C. The specificity of the antiserum was demonstrated by pre-absorption (12 h) of diluted primary antiserum with insulin (100 µg/ml) and substitution of normal serum for antiserum. The tissues were rinsed in PBS for 15 min subsequent to the incubation and the secondary antibody applied. The secondary antibody (sheep, anti-guinea pig IgG, Cappel) was diluted 1:250 with PBS. After the sections were incubated for 1 h at room temperature, they were rinsed in PBS for 15 min and 50 µl of the peroxidase, anti-peroxidase (guinea pig PAP: Cappel) complex applied. These were incubated for an additional hour at room temperature and rinsed in PBS. The PAP chromagen was oxidized by placing the tissues in a solution of 3,3'-diaminobenzidine tetrahydrochloride (33 mg/100 ml PBS, DAB: Grade II, Sigma) to which 0.05% H₂O₂ was added until the reaction was complete as judged by microscopic inspection. The sections were washed in PBS, counterstained with Ehrlich's haematoxylin, dehydrated in graded ethanols, cleared in xylene and coverslipped with Histoclad (Clay Adams, Parsippany, NJ, USA).

Quantitation and stereology

Volume ratios. Each tissue component of the pancreatic sections, including acinar, ductal, islet and connective tissue septa, was quantitated using the stereology point-count technique to determine volume ratios. The determination of these volumes were based on the formulation as expressed by Delesse that the volume density of the various components within a sampling unit can be estimated on random sections by measuring the relative areas of their profiles [29]. Expressed as a formula, the area density (A_a) of profiles on sections is an unbiased estimate of the volume density (V_v) of the structures [30], or $A_a = V_v$. Furthermore, the number of test points contained within the parameter being measured (P_i) and the total number of test points (P_t) is also an estimate of A_a [31], or:

$$\frac{P_i}{P_t} = A_a = V_v$$

The serially mounted and immunocytochemically-stained tissue of 3 animals from each group was used for this quantitative analysis. Using an Olympus BHS microscope (Olympus Optical Company, Tokyo, Japan), three micrographs were taken at random within the microscopic field of each slide throughout the first 20 sections sampled at every fifteenth interval. This produced 60 micrographs per animal;

Fig. 1. A colour micrograph of pancreatic tissue from trypsin inhibitor-treated rats. The insulin antibody stain is clearly distinguishable from non-immunoreactive tissue. Insulin-immunoreactive duct cells (small white arrows) are shown and can be compared to the B cell immunoreactive stain contained in an adjacent islet (large white arrow). A small group of insulin-immunoreactive cells, resembling acinar-B intermediate cells, are shown adjacent to the islet (asterisk). Magnification $\times 25$

360 in total, which were individually placed on a magnetized digitizer tablet of a Zeiss Videoplan Image analyzer (Cark Zeiss, New York, NY, USA). A point-count grid, measuring 160 mm^2 and subdivided into 64 squares, was placed over each micrograph. Grid parameters and the magnification factor ($\times 60$) were entered into the computer in preparation for the data acquisition sequence of the stereology program. The "hits" of component tissues within an area measuring $2714\text{ }\mu\text{m}^2$ were recorded in each micrograph and their number automatically divided by the total number of possible hit points. The total values for each component tissue were averaged per group, compared statistically using the Student's t-test and expressed as mean percentage ratios. To assess the actual percentage of change in tissue components, the difference between the respective mean pancreatic weights of each group was multiplied by the percent of each tissue component and the gram weight increase divided by the control value.

Acinar quantitation. To measure further changes in acinar tissue, 5 light micrographs were taken of randomly selected microscopic fields of acinar tissue from serial slides prepared from each animal of both groups. The acinar cell nuclei, in a tissue area measuring $150\text{ }\mu\text{m}^2$, were counted. The totals were averaged and group differences calculated by means of the Student's t-test as described above. The size of acinar cell nuclei, based on the measurement of the maximum cord length, of 200 randomly selected nuclei from each group, was assessed from this same group of micrographs. The totals were averaged and the comparison of means calculated as previously described.

Duct quantitation

The same micrographs used to determine volume ratio of ductal tissue were subsequently used to calculate the volume ratio of ducts in which insulin-immunoreactive cells were present within their epithelium. At the time the ductal points were calculated, their hit points were marked on each micrograph. The photographed area of the original microscope slide was located and the ducts which represented original hits on the micrograph identified. Each duct was re-examined through the microscope and those containing insulin-immunoreactive cells marked on the corresponding ducts of the micrograph. The point-count ratio was then re-calculated using the volume ratio of ducts with insulin-immunoreactive cells, their fraction values averaged and compared to control values. Preliminary attempts to count individual duct cells and to measure the length of ducts were found unreliable and inconsistent.

Results

After treatment with the oral trypsin inhibitor, there were no differences in the appearance or weight-gain of the treated rats compared with the controls. The final mean body weight of the treated rats, $249 \pm 4.58\text{ g}$, was similar to that of the controls, $243 \pm 6.08\text{ g}$ (Table 1). There was, however, a significant increase in pancreatic weight in the trypsin inhibitor-treated animals. The mean pancreatic weight in the treated animals was $2.33 \pm 0.46\text{ g}$ and the pancreatic/body weight ratio, $0.95 \pm 0.03\%$ (Table 1).

The volume ratio of each component tissue of the pancreas remained consistent in both groups, except in the case of the ducts which was significantly higher in the treated rats, $3.77 \pm 4.38\%$ (Table 2). All tissue components from the treated rats, however, showed an increase when the percent of change was calculated: 125% (acinar), 144% (islet), 325% (ductal) and 94% (connective tissue septa). The assessment of acinar nuclear density revealed a significant decrease in the treated rats. The mean number of nuclei in experimental animals was 28 ± 4.74 or 54.6% of the control value (Table 3). The mean size of acinar cell nuclei was significantly larger in the treated rats, 6.20 ± 0.13 , or 9.3% larger than in control rats (Table 3). The point-count percent average of the volume ratio of ducts containing insulin-immunoreactive cells was significantly higher in the treated rats, $0.42 \pm 0.15\%$, than in the untreated rats $0.07 \pm 0.01\%$ ($p < 0.01$).

The microscopic appearance of trypsin inhibitor-treated tissue revealed a variety of ducts which contained insulin-immunoreactive cells. These cells, stained immunocytochemically with the oxidized dark brown chromagen of the PAP complex, were easily differentiated from the basophilic haematoxylin stain of adjacent duct cells and were identical to the pancreatic

Fig. 2a-f. Insulin-immunoreactive cells localized immunocytochemically within the ductal epithelium of trypsin inhibitor-treated rats. **a** A single insulin-immunoreactive cell within the epithelium of a small intralobular duct (white arrow). **b** A single insulin-immunoreactive cell (white arrow) within the epithelium of a large interlobular duct. **c** A single insulin-immunoreactive cell (white arrow) within the epithelium of a small interlobular duct. **d** Two insulin-immunoreactive cells (black arrows) found within the epithelium of two adjacent interlobular ducts. **e** An aggregate of several insulin-immunoreactive cells (black arrows) within an interlobular duct. **f** Small clusters of insulin-immunoreactive cells, as the one shown here (black arrow), were frequently encountered in close proximity with ducts. Magnification: a, c-f, $\times 50$; b $\times 60$

B cell stain of surrounding islets (Fig. 1). These cells were found singly in both intralobular (Fig. 2a) and small and large interlobular ducts (Fig. 2b-c), in discrete pairs (Fig. 2d), or as cellular aggregates within ductal epithelium (Figs. 1, 2e). Small clusters of insulin-immunoreactive cells were frequently found near large and small interlobular ducts (Fig. 2f). Large, irregular-shaped islets could be found in the tissue of trypsin inhibitor-treated animals which were less apparent in tissue from untreated rats. Although not included in the quantitative analysis, small clusters of insulin-immunoreactive cells resembling putative acinar-B intermediate cells observed by others in soybean-fed rats [32] were consistently encountered in tissue from treated rats, but only rarely in tissue from untreated rats (Fig. 1).

Discussion

In previous studies on soybean-fed and trypsin inhibitor-treated rats by Yanatori and Fujita [26] and Melmed et al. [32], duct cells were not characterized histologically and no immunohistochemical data was provided. Furthermore, no quantitation of these cells was available. In the present study, point-count quantitation supports the trophic effects of trypsin inhibitor on the pancreas and its component tissues. In addition, quantitation of nuclear density and size confirms hypertrophic changes in acini. Using immunohistochemical localization, significant numbers of ducts containing insulin-immunoreactive cells within their epithelium were found in trypsin inhibitor-treated animals.

While there is no existing evidence to support the trophic effects of trypsin inhibitor on ductal tissue of the pancreas, the present study suggests that this may be possible. The exact mechanism of this trophic influence, however, is unclear. It may be mediated, at least in part, by the gastrointestinal hormone, cholecystokinin-pancreozymin (CCK-PZ), which is released from the mucosal epithelium of the gut and is thought to effect the changes in acinar tissue of soybean-fed and trypsin inhibitor-treated animals [25].

The occurrence of insulin-immunoreactive cells within ductal epithelium of trypsin inhibitor-treated animals remains unexplained. It may suggest a hypothetical B-cell cytotropic response. While there was no change in the overall islet volume ratio, the percent of islet tissue increased in the treated rats. Further work is required to establish the significance of ductal cells exhibiting insulin immunoreactive.

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