Functional and Histochemical Analysis on Pancreatic Islets of APA Hamsters with SZ-Induced Hyperglycemia and Hyperlipidemia

Atsushi TAKATORI, Etsuko NISHIDA, Toshiaki INENAGA, Keiko HORIUCHI, Seiji KAWAMURA, Shin-ichi ITAGAKI, and Yasuhiro YOSHIKAWA

Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, the University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Abstract: To clarify how Syrian hamsters of the APA strain (APA hamsters) keep a diabetic condition for a long period, the functional and histochemical changes in the pancreatic islets of diabetic APA hamsters were examined. By glucose tolerance test, no glucose-induced insulin secretion was seen in the diabetic APA hamsters. By immunohistochemistry, it was revealed that at 24 hr after SZ-injection, the number of islets had decreased and that remnant islets had become markedly smaller. The islets had hardly any insulin-immunoreactive cells and consisted of cells stained by anti-glucagon and somatostatin antibodies. One, three and six months after SZ-injection, a small number of cells with vacuolative changes, which were positive for PAS staining, were observed in most islets and the vacuolated cells were stained mainly by anti-insulin antibody. In addition, a number of PCNA-positive cells were observed, especially in the periphery of the vacuolated cells, while TUNEL-positive cells were not detected. This data suggests that β-cells proliferating as a result of the replication of the resident β-cells in islets had fallen into degeneration and necrosis by a stress, such as the glycogen deposition in hyperglycemia and hyperlipidemia. Consequently, secretion of insulin was maintained at low levels, which allowed the hamsters to live without insulin therapy in the diabetic condition for over 6 months.

Key words: APA hamsters, diabetes mellitus, histochemistry, pancreatic islet, streptozotocin

Introduction

Diabetes mellitus (DM) is a universal health problem estimated to affect more than 140 million people worldwide. The causes of DM are complicated and are linked with obesity, lack of exercise, hyperlipidemia and so on. For the study of DM, there are various animal models, which are either chemically induced or spontaneously developing diabetic animals. Streptozotocin (SZ) is widely used to induce diabetes mainly in rats and mice based on its specific toxicity to pancreatic islet β-cells [3–5, 11, 12, 15, 20, 22]. SZ-treated rats and mice, however, offer few advantages for the investigation of the complications of diabetes, because

(Received 3 April 2001 / Accepted 19 May 2001)
Address corresponding: A. Takatori, Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, the University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
although SZ induces acute DM the complications develop very slowly in these animals.

On the other hand, it is known that hyperglycemia and hyperlipidemia can be evoked in Syrian hamsters of the APA strain (APA hamsters) by a single injection of SZ without severe toxic effects [8], and that the diabetic condition is maintained for at least 6 months [10, 25]. Studies have also demonstrated that APA hamsters develop diabetic complications similar to human diabetic diseases, such as atherosclerosis [24–26] and nephropathy [7, 10], which are leading causes of death among diabetic patients. However, little is known about the pathological characteristics of the pancreatic islets of APA hamsters with diabetic complications. Therefore, we examined the functional and histochemical changes in the pancreatic islets of APA hamsters at the acute and chronic stages of diabetes.

**Materials and Methods**

**Animals:** Male APA hamsters were purchased from Japan SLBC (Shizuoka, Japan) and maintained under controlled conditions (temperature, 24 ± 2 C; humidity, 55 ± 5%) in plastic cages with sterilized wood shavings for bedding. They were fed commercial diets, CMF (Oriental Yeast Co. Ltd., Tokyo, Japan), with tap water *ad libitum*. Diabetes was induced with a single injection of freshly prepared SZ (Sigma, St Louis, MO, USA) in 0.1 M citrate buffer, pH 4.5, at a dose of 30 mg/kg body weight at 2 months of age, under fasting from 6 hr before to 18 hr after injection. Five hamsters each at 24, 48 and 72 hr, and 1, 3 and 6 months after SZ-injection (SZ24H, SZ48H, SZ72H, SZ1M, SZ3M and SZ6M, respectively) were euthanized by exsanguination under ether anesthesia and their pancreata were removed. The same number of age-matched APA hamsters which were injected with citrate buffer alone, served as controls at each time point. This experiment was conducted according to the guidelines of the Animal Care and Use Committee of Graduate School of the Agricultural and Life Sciences, the University of Tokyo.

**Blood biochemistry:** Serum glucose and cholesterol levels of all animals were colorimetrically measured using the Glucose ClII-test and Cholesterol E-test kit (WAKO pure chemicals Co. Ltd., Tokyo, Japan), respectively.

**Glucose tolerance test:** At 1, 3 and 6 months after SZ-injection, APA hamsters fasted for 16 hr before the test were injected intraperitoneally with glucose (2.0 g/kg body weight). Blood samples were collected at 0, 30, 60 and 120 min after the glucose administration. Serum glucose levels were determined by the above-mentioned kit and immuno-related insulin concentrations were measured by an insulin ELISA kit (Morinaga Institute of Biological Science, Kanagawa, Japan).

**Histopathology:** Pancreata were fixed in 4% paraformaldehyde overnight and embedded in paraffin by a routine procedure. Consecutive 4 μm paraffin sections were cut and stained with hematoxylin and eosin (HE) for observing general morphological changes, and alkaline Congo red for detecting amyloid. Frozen sections of pancreas were stained with oil red O and periodic acid Schiff (PAS) for lipid and glycogen detection, respectively.

**Immunohistochemistry:** The primary antibodies used in this experiment were rabbit polyclonal antibodies against glucagon (diluted 1:75) and somatostatin (diluted 1:200), and guinea pig polyclonal antibody against insulin (diluted 1:200) purchased from Biomedica (Foster City, CA, USA). Immunostain was completed by the streptavidin-biotin-horseradish peroxidase complex (ABC/HRP; DAKO, Glostrup, Denmark) method and visualized using 3,3′-diaminobenzidine tetra-hydrochloride (DAB) plus hydrogen peroxide solution.

**Morphometry:** The whole pancreas and the total insulin-immunoreactive area in the sections were measured using a light microscope and microvidescope system with a colour image analyzer (SP500, Olympus, Tokyo, Japan). The measurement was done on 5 spots per one pancreas section from 5 hamsters in each group (25 spots per group).

**TUNEL method:** Sections were stained with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method using a kit (Apoptag: Oncor, Gaithersburg, MD, USA) for specific staining of DNA fragmentation and apoptotic body. Staining by the TUNEL method was carried out according to the manufacturer’s protocol.

**Evaluation of cell proliferation:** To determine the proliferating activity of β-cells, we performed immunostaining for proliferating cell nuclear antigen (PCNA). Sections were boiled for 5 min in 0.1 M
Table 1. Body weight, the nonfasting serum glucose and cholesterol levels of control and SZ-injected hamsters

<table>
<thead>
<tr>
<th>Stage of treatment</th>
<th>Body weight</th>
<th>Glucose</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>SZ</td>
<td>Control</td>
</tr>
<tr>
<td>24 hr</td>
<td>96.7±3.3</td>
<td>89.6±5.4</td>
<td>185.3±15.5</td>
</tr>
<tr>
<td>48 hr</td>
<td>97.2±3.1</td>
<td>90.9±3.9</td>
<td>141.2±23.5</td>
</tr>
<tr>
<td>72 hr</td>
<td>103.9±8.7</td>
<td>96.9±6.4</td>
<td>150.2±10.4</td>
</tr>
<tr>
<td>1 month</td>
<td>111.7±5.4</td>
<td>96.2±11.2**</td>
<td>160.2±50.7</td>
</tr>
<tr>
<td>3 months</td>
<td>138.1±6.9</td>
<td>122.9±12.8*</td>
<td>139.0±34.8</td>
</tr>
<tr>
<td>6 months</td>
<td>158.7±6.4</td>
<td>140.4±7.8**</td>
<td>133.3±39.6</td>
</tr>
</tbody>
</table>

Values are means ± SD. *p<0.05, **p<0.01 versus corresponding control hamsters.

citrate buffer (pH 6.0) to activate the antigen. After washing in phosphate-buffered saline (PBS), the sections were incubated with a mixture of anti-rat PCNA monoclonal mouse IgG (diluted 1:200; DAKO). Anti-PCNA antibody was detected using ABC/HRP method (DAKO) and developed with DAB.

Statistical analysis: Statistical analysis was carried out using the Student’s t-test.

Results

Physiological changes in the diabetic hamsters

Statistically significant depression of body weight gain was observed in the SZ-treated hamsters at 1, 3 and 6 months after injection when compared with untreated control animals (Table 1). Body weight of SZ-treated hamsters was approximately 90% of controls and increased parallel to controls until 6 months after injection. After SZ-injection, the nonfasting serum glucose level of the SZ-treated hamsters was 2–3 times as high as those of control animals throughout the experiment. Cholesterol concentrations of the SZ-treated hamsters were higher than those of the control animals from 48 hr to 6 months after SZ-injection.

Glucose tolerance test

The diabetic hamsters at 1, 3 and 6 months after SZ-injection showed significantly higher serum glucose levels than those of control hamsters at each measurement after glucose administration (Fig. 1). Blood glucose level in control animals reached a peak at 30 min which was lower than 400 mg/dl (on average) after intraperitoneal glucose administration, then decreased rapidly to the normal level. However, in the diabetic hamsters, the blood glucose level increased until 60 min, 800 mg/dl (on average), and decreased gradually thereafter (120 min, 600 mg/dl). No apparent glucose-induced insulin secretion was seen in the SZ-injected hamsters (Fig. 1). Therefore, SZ-injected hamsters were thought to maintain their diabetic conditions for 6 months after SZ-injection.

Histopathology

Each age-matched control hamster showed normal observation as shown in Fig. 2a (age-matched to SZ24H). The size of islets in SZ24H hamsters was smaller and the number of islets in the pancreas was fewer than those of age-matched controls, and in the central part of the islets, degenerated cells and infiltrated inflammatory cells were observed (Fig. 2b). On the sections of SZ1M, 3M and 6M, the islets, which showed some vacuolar degeneration, were still small in size and consisted of cells with various sized nuclei. The vacuolated cells and the neighboring region were not stained by oil red O or Congo red, but they were positively stained by PAS (Fig. 3), which reacted slightly positive in islets of control animals (data not shown). Accordingly, it was thought that glycogen [1] but not lipid or amyloid was deposited in the islets of SZ-injected hamsters.

Immunohistochemistry

Consecutive sections of pancreata were immunohistochemically stained with anti-insulin, glucagon and somatostatin antibodies. As shown in Fig. 4, in control hamsters, insulin-immunoreactive cells occupied the central portion of the islet, which was surrounded by 1–2 cell layers of glucagon- or somatostatin-immunoreactive cells. The characteristic of age-matched control APA hamsters’ islets was not
Glucose (mg/dl)  

IRI (pg/ml)  

1M

3M

6M

Time after injection

Fig. 1. Glucose tolerance test by intraperitoneal injection of glucose in APA hamsters treated with SZ. IRI, immunoreactive insulin. Data are means ± SD.

changed throughout the experiment.

The islets of SZ-injected hamsters had hardly any insulin-immunoreactive cells and consisted of cells stained by anti-glucagon and somatostatin antibodies. Insulin-immunoreactive cells were scattered in the islet and glucagon- or somatostatin-immunoreactive cells were distributed all over the islet. The vacuolated cells observed in HE stained sections were stained mainly by anti-insulin antibody and partly by anti-somatostatin antibody. The periphery of the vacuolated cells was also stained by anti-insulin antibody.
**Morphometry**

To estimate how insulin-positive cell mass was disordered, the sections stained with anti-insulin antibody were subjected to a quantitative analysis. As shown in Fig. 5, the insulin-positive area decreased to 0.5% against the whole pancreas area as early as at 24 hr after SZ-injection, whereas the control pancreas showed 2.4%. The population of β-cells thereafter remained very low level until 6 months after SZ-injection (0.3–0.5% in SZ24, 48 and 72H and –0.2% in SZ1, 3 and 6M, respectively).

**TUNEL method**

To clarify the fate of the degenerated cells in the islets whether they fell into necrosis or apoptosis, they were examined by TUNEL staining (Fig. 6a and c). The results revealed that a few TUNEL-positive cells were seen in the islets of SZ24H (Fig. 6a) and SZ48H hamsters but not in the islets of SZ72H–6M. No TUNEL-positive cells were detected in the islets of age-matched control hamsters (data not shown).

▲ **Fig. 2.** Islets of Langerhans in pancreas of control (a) and SZ-treated APA hamsters. The degenerated cells and infiltrated inflammatory cells are seen in islets of SZ24H (b). Islets of SZ1M (c) and 6M (d) hamsters are still small and show some vacuolar degeneration. HE, × 200.

▲ **Fig. 3.** Histological appearance of islets of SZ6M hamsters. SZ6M islets are not stained by oil red O (a) or Congo red (b) but are positively stained by PAS (c). a–c, ×250.
Fig. 4. Immunostaining of islets of control (a–c), SZ1M (d–f) and SZ6M (g–i) APA hamsters by anti-insulin (a, d, g), glucagon (b, e, h) and somatostatin (c, f, i) antibodies. a–i, × 180.

Fig. 5. β-cells area detected by anti-insulin antibody per pancreas: section evaluated by quantitative analysis. The evaluation was performed in the hamsters from SZ24H to SZ6M. Data are mean ± SD.

Cell proliferation
To know precisely why the diabetic conditions of SZ-injected hamsters is kept for so long, we submitted the sections to immunostaining for PCNA. As shown in Fig. 6b and d, PCNA-positive cells were detected in the islets of SZ-injected hamsters throughout the experiment. There were few PCNA-positive cells in the islets of control hamsters (data not shown).

Discussion
SZ has been reported to act as a β-cell toxic agent, causing necrosis or marked degenerative changes in the β-cells with nuclear pyknosis and cytoplasmic vacuolization, and to produce permanent diabetes in many species, including the rat, mouse, guinea pig, Chinese hamster, and monkey [3–5, 12, 20, 22] as well as the Syrian hamster [11, 15]. Most species indicate high acute mortality to SZ-injection, whereas Syrian hamsters show a high percentage of recovery from SZ-induced diabetes [9]. Accordingly, there are few suitable animal models for chronic diabetes and its complications.

As an animal model of human diabetes mellitus, Syrian hamsters have been shown to be more useful than
rats [6], because hamsters, like humans, have a common cholesteryl ester transfer protein activity [14]. B-100 that acts as the only apoprotein in hepatic very low density lipoprotein [2], and comparable levels of low density lipoprotein [9] in plasma as in humans. In addition, APA hamsters develop similar diabetic lesions to human diabetic complications, such as atherosclerosis and nephropathy, within a short period of time [7, 10, 24–26].

In this study, we showed the histology of the islets of SZ-injected APA hamsters and revealed how the animals maintain the diabetic conditions over 6 months. Histology suggests that the islets of APA hamsters were first injured by SZ-injection and then fell into apoptosis, as shown by the results of TUNEL staining (Fig. 6). SZ is well known to be a diabetogenic agent, which causes β-cell apoptosis by its radical generation [18, 19]. Therefore, the hamsters showed marked insulin degranulation in islets and a low level of serum insulin, which might lead to a high fasting blood glucose level and glucose intolerance.

In the islets with a long diabetic condition, some PCNA-positive cells, most of which were stained in the periphery of vacuolated cells, were also stained with anti-insulin antibody. This suggests that proliferation of β-cells may occur as a result of the replication of the resident β-cells in islets. However, the proliferated β-cells may have fallen into not apoptosis but degeneration and necrosis by a stress such as hyperglycemia and hyperlipidemia. This may be why there were few cells stained with both anti-insulin antibody and the TUNEL methods in the islets of chronically diabetic hamsters. The glycogen deposition would interfere with the organized production of insulin by β-cells, thus increasing the severity of the diabetic state [1, 16]. This replication and degeneration of β-cells kept a kind of dynamic equilibrium in the islets enabling a relatively small number of β-cells to be maintained in the diabetic APA hamsters with chronic diabetes. Consequently, secretion of insulin was maintained throughout the experiment though at a low level allowing the hamsters to live without insulin therapy in a diabetic condition for over 6 months.

Immunohistochemical study revealed that most of the cells in islets of SZ-injected hamsters contained glucagon or somatostatin in contrast to control animals’ islets which were consisted of mostly insulin-positive cells surrounded by a relatively small number of glucagon- and somatostatin-positive cells. Islets of SZ-injected hamsters were drastically decreased in insulin-positive
area and exhibited marked vacuolation of remaining cells. These results are similar to those of previous reports [13, 16], except we examined up to 6 months after SZ-injection. In this study, insulin-immunoreactive cells were distributed not only in the islets but also in the ductal epithelium of the exocrine glands (data not shown). An adult pancreatic β-cell is thought to have limited ability to regenerate but it has been demonstrated that the β-cell can regenerate from pre-existing β-cells in the islets or differentiate from precursor cells in the ductal epithelium in some animal models [17, 21, 23]. Since the number of insulin-positive cells in the ductal epithelium was very small compared to the number of islet β-cells in this study, there is little necessity to consider the β-cell differentiating from ductal cells into insulin secretion in the SZ-injected hamsters.

In conclusion, our present results show that SZ-injected APA hamsters show complications after chronic diabetes, because β-cells injured by SZ are not sufficiently restored to ameliorate the diabetic conditions.

References


