Histological Analysis of the Sublingual Gland in Rats with Streptozotocin-Induced Diabetes

By

Masaki KAMATA¹, Masayori SHIRAKAWA², Kenichiro KIKUCHI³, Takanori MATSUOKA³ and Shigeo AIYAMA³

¹The First Department of Oral and Maxillofacial Surgery, School of Life Dentistry at Tokyo, ²General Dentistry 1 at Tokyo, ³Department of Histology, School of Life Dentistry at Tokyo, The Nippon Dental University

Key Words: Sublingual gland, Diabetic rat, Streptozotocin, Morphological changes, Light and electron microscopy

Summary: This study was designed to examine whether the sublingual gland parenchyma is influenced by the development of insulin-dependent diabetes mellitus. The sublingual glands of rats with streptozotocin-induced diabetes were examined by light and electron microscopy. In order to define the limiting membrane of mucous granules in more detail, samples processed by rapid freezing following by freeze-substitution in addition to chemical fixation were also prepared for electron microscopy. Light and electron microscopy showed vacuole-like structures considered to be lipid droplets in the cytoplasm of serous demilune cells, the largest reaching 4 μm in diameter. Electron microscopy of the chemically fixed samples revealed granule-like structures in addition to the mucous granules proper in the mucous cell cytoplasm. However, electron microscopy of the freeze-substitution fixed samples demonstrated no limiting membrane on the surface of the granule-like structures, although this was clearly observed on the surface of the mucous granules. Accordingly, the granule-like structures present in the mucous cell cytoplasm appeared to be lipid droplets. These findings suggest that the sublingual gland mucous cells become dysfunctional during the development of insulin-dependent diabetes mellitus, although to a slighter degree than the serous demilune cells.

It is well known that rats administered streptozotocin (STZ) develop diabetes mellitus¹⁴. It has been reported that STZ specifically damages the B cells that secrete insulin in the islets of Langerhans, thus giving rise to hypoinsulinemia⁹. Hypoinsulinemia is accompanied by hyperglycemia in addition to hyperglycemia⁹. Patients with xerostomia due to diabetes mellitus suffer from a decrease in saliva secretion. Therefore, analysis of the structure of salivary glands in patients with xerostomia has attracted special attention. The salivary glands of rats with STZ-induced diabetes have also been studied to clarify any disease-related changes in gland structure¹–³,⁶,⁷,¹²,¹³,¹⁵,¹⁷). Some studies have shown that the acinar cells of the glands in rats with STZ-induced diabetes possess lipid droplets of various sizes in the cytoplasm¹–³,⁶,¹²,¹³,¹⁵,¹⁷). In addition, other reports have demonstrated atrophy and degeneration of the acinar cells and a decrease in the diameter of secretory granules²,³,¹³,¹⁷). On the other hand, it has been reported that the sublingual gland parenchymal cells do not show any morphological change, or that only the serous demilune cells develop lipid droplets¹,¹²). Such differences among previous studies of the sublingual gland seem to be due to the lack of data on this gland, in comparison with the submandibular and parotid glands. The present study was designed to examine in more detail the fine structure of the parenchymal cells of the sublingual glands in rats with STZ-induced diabetes.

Materials and Methods

Animals

All animal experiments followed the Guidelines for the Care and Use of Laboratory Animals in the Odontology Section of Biological Science, Nippon Dental University School of Dentistry Research Center. Ten 8-week-old rats were used. All the rats were maintained at an ambient temperature of 23°C with a 12-h light-dark cycle. Animals were fasted overnight and diabetes was induced by a single intravenous injection of streptozotocin (60 mg/kg) in cold physiological saline (adjusted to pH 4 with cit-
ric acid). Control animals were fasted and received physiological saline only. Serum glucose levels were determined prior to removal of the glands, using a colorimetric system. Only animals with glucose levels greater than 300 mg per cent were considered suitably diabetic.

**Light microscopy**

Seven weeks after the induction of diabetes, control and diabetic animals were anaesthetized with pentobarbital (40 mg/kg). The sublingual glands were dissected out, prefixed by fixation in a mixture of glutaraldehyde and paraformaldehyde in 0.05 M cacodylate buffer (pH 7.4), and post-fixed in cacodylate-buffered 1% osmium tetroxide. All fixed samples were dehydrated with ethanol, and embedded in an Epon-Araldite mixture. Samples were then cut into sections 2 μm thick and stained with toluidine blue for light microscopy.

**Electron microscopy**

Samples post-fixed in osmium tetroxide and embedded in Epon-Araldite were also used. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined using a Hitachi-H300 transmission electron microscope. In addition to these chemically fixed samples, samples processed by rapid freezing followed by freeze-substitution fixation were also prepared for electron microscopy. Small pieces of the sublingual glands were placed on a small aluminum disk set on the head of a rapid freezing device. The tissue pieces were then instantaneously frozen by smashing them against the polished surface of a copper block cooled with liquid helium. The frozen samples were fixed using freeze substitution by placing them in 4% osmium tetroxide in acetone at 4 °C overnight. After freeze-substitution, the tissue pieces were placed in 1% tannic acid in acetone at 4 °C for 2 h and then slowly brought back to room temperature. The samples were then embedded in Epon-Araldite after replacing the acetone with propylene oxide.

**Measurement of secretory granule size**

The sizes of the secretory granules in the mucous cells and serous demilune cells of the sublingual glands from the control and diabetic animals were assessed in electron micrographs of samples treated by chemical fixation. Three glands each from control and diabetic animals were used, respectively. For size measurement of the secretory granules in the mucous cells, i.e. mucous granules, two or three acini per gland were photographed at random. Each photograph was processed on a computer with the Scion Image program, and the cross-sectional area of 100 mucous granules in the acini was calculated. Then the average area of 300 mucous granules from three glands was computed for the control and diabetic animals, respectively. For size measurement of secretory granules in the serous demilune cells, i.e. serous granules, one or two serous demilune cells per gland were photographed at random. Each photograph was also processed on a computer with the Scion Image program, and the cross-sectional area of 10 serous granules in the serous demilune cells was calculated. Then, the average area of 30 serous granules from 3 glands was computed for the control and diabetic animals, respectively. In both mucous and serous granules, differences between means were analyzed for statistical significance using Student’s *t* test.

**Results**

**Light microscopy**

Light microscopy showed that the mucous cells had almost no differences in structure, size, or cell arrangement between the control and the diabetic animals. The serous demilune cells also showed hardly any differences between the two groups (Fig. 1a, b), although those in the diabetic animals revealed vacuole-like structures of various sizes in the cytoplasm (Fig. 1b). The size and number of the vacuole-like structures appeared to differ among the serous demilune cells. The intercalated and the striated ducts, of which the latter were predominant, also showed hardly any differences in size or cell arrangement between the control and the diabetic animals. Furthermore, neither of the groups appeared to possess vacuole-like structures in the ducts.

**Electron microscopy**

Electron microscopy demonstrated numerous electron-lucent secretory granules, i.e. mucous granules, in the mucous cells of both control and diabetic animals after chemical fixation. These granules appeared to show almost no difference in size and number between the control and the diabetic animals. However, the mucous cells of the diabetic animals showed granule-like structures that had slightly higher electron density than mucous granules, although were similar to mucous granules in terms of shape and size (Fig. 2). One to four granule-like structures were seen in each mucous cell. Serous demilune cells also contained granule-like structures similar to those present in the mucous cells. However, the former were usually larger than the latter, sometimes measuring up to 4 μm in diameter (Fig. 3). The large granule-like structures in the serous demilune cells appeared to corre-
spond to the vacuole-like structures revealed by light microscopy. Ultrastructurally, the serous demilune cells showed little difference between the control and the diabetic animals, except for the presence of granule-like structures in the glands of diabetic animals. The striated duct cells also showed little difference in the ultrastructure of cell organelles such as mitochondria between the control and the diabetic animals, in addition to the lack of any granule-like structures in both groups (Fig. 4).

The mucous cells of the sublingual glands from diabetic animals prepared by rapid freezing followed by freeze-substitution fixation also showed the granule-like structures in addition to numerous mucous granules in the cytoplasm, as was the case for cells subjected to chemical fixation (Fig. 5). However, granule-like structures in the samples processed by freeze-substitution fixation showed hardly any clear limiting membrane, even at high magnification (Fig. 6). On the other hand, the mu-
cous granules in the same samples clearly demonstrated a limiting membrane at high magnification (Fig. 7).

Measurement of secretory granule size

Average cross-sectional areas of 100 mucous granules from 3 glands in control animals were 0.84 μm², 0.79 μm² and 0.61 μm², respectively, with a mean value of 0.75 μm². The corresponding values for 3 glands in diabetic animals were 0.91 μm², 0.87 μm² and 0.99 μm², respectively, with a mean value of 0.92 μm². Student’s t test demonstrated no significant difference between the mean cross-sectional areas of the mucous granules from the controls and those from the diabetic animals.
The average cross-sectional areas of 10 serous granules from each of 3 glands in control animals were 0.59 μm², 0.43 μm² and 0.38 μm², respectively, with a mean value of 0.49 μm². The corresponding values for 10 serous granules in 3 diabetic animals were 0.50 μm², 0.38 μm² and 0.46 μm², respectively, with a mean value of 0.45 μm². Student’s t test also demonstrated no significant difference between the mean cross-sectional areas of the serous granules from the controls and those from the diabetic animals (Fig. 9).

**Discussion**

The histological features of salivary glands in insulin-dependent diabetes mellitus has attracted the attention of investigators because of diabetic complications such as xerostomia and salivary gland enlargement\(^5\)\(^{10}\). However, there have been few reports on human salivary glands, as it is difficult to obtain sufficient materials. Studies of animals with diabetes induced by administration of STZ are therefore very useful in this respect\(^1\)\(^{11}\)\(^{14}\), and the salivary glands of such diabetic animals can be removed and examined. However, previous histological studies of salivary glands from rats with STZ-induced diabetes have focused mainly on the submandibular and parotid glands. Therefore, data on the sublingual gland are sparse, and moreover, some of the findings have been conflicting\(^1\)\(^{11}\)\(^{12}\). The reason for the limited number of reports would seem to be that the contribution of the sublingual gland to saliva secretion is the least among the three major salivary glands. Nevertheless, the sublingual gland is one of the main salivary glands, and therefore more detailed examination of histological changes due to diabetes in the gland would seem warranted.

**Light and electron microscopy**

Light microscopy showed vacuole-like structures with various sizes in the cytoplasm of the serous demilune cells, but not in the mucous cells. Electron microscopy revealed granule-like structures in the cytoplasm of serous demilune cells, ranging from secretory granule size to about 4 μm in diameter. Electron microscopy also revealed the granule-like structures with slightly higher electron density than mucous granules in the mucous cells, although they were similar in size. It has been reported that rats with STZ-induced diabetes show lipid droplets in the cytoplasm of the submandibular and parotid gland acinar cells, particularly, a striking feature in number and size in the parotid gland acinar cells\(^12\). However, with regard to the sublingual gland, the findings of previous studies have differed to some extent; some researchers have described that the acinar cells show almost no histological changes\(^15\), whereas others have observed lipid droplets in the serous demilune cells but no changes in the mucous cells\(^1\)\(^{11}\)\(^{12}\). Anderson and Garret\(^1\) have reported that the serous demilune cells in the sublingual gland and the acinar cells in the parotid gland of rats with STZ-induced diabetes produce lipid droplets positive for oil red O, and also that the lipid droplets present in both glands are similar in both size and number. The granule-like structures detected in the serous demilune cells in present study also appear to be lipid droplets, because they closely resemble the lipid droplets demonstrated by Anderson and Garret. Using electron microscopy, we also observed granule-like structures in the cytoplasm of mucous...
cells in the sublingual glands of diabetic rats. These were similar in shape and size to mucous granules proper, but had a slightly higher electron density. No previous report has described the appearance of lipid droplets in the mucous cells of the sublingual gland in STZ-diabetic rats. In order to determine whether the granule-like structures found in these mucous cells are, in fact, lipid droplets, samples subjected to freeze-substitution fixation were also observed by electron microscopy. Freeze-substitution fixation maintains the fine structure of tissues and cells more precisely than chemical fixation, and as a result retains the limiting membrane of mucous granules close to its original form. It has also been reported that electron microscopy demonstrates no limiting membrane on the surface of lipid droplets because it comprises only a single layer of phospholipids. In the present study, electron microscopy also revealed no limiting membrane on the surface of the granule-like structures, whereas a clear limiting membrane was evident on the surface of the mucous granules. Therefore, it seems likely that the granule-like structures in the cytoplasm of mucous cells in the sublingual gland of rats with STZ-induced diabetes are lipid droplets. The reason why previous studies failed to define these lipid droplets is probably because of their close similarity to mucous granules in terms of size, and also their low numbers.

**Measurement of secretory granule size**

Previous studies have shown that the secretory granules in parotid gland acinar cells of STZ-diabetic rats are decreased in size. Therefore we examined whether secretory granules in the sublingual glands of STZ-diabetic rats showed any change in size. We found that there were no significant differences in the mean diameter of serous and mucous granules between control and diabetic animals, suggesting that STZ-induced diabetes mellitus has hardly any influence on the size of secretory granules in the sublingual gland.

The accumulation of lipid droplets in the salivary gland acinar cells of diabetic animals has been attributed to a decrease in the amount of lipid used for formation of the limiting membrane, and that as a result, surplus lipids are stored as lipid droplets in the cytoplasm, owing to suppression of protein synthesis and secretory granule secretion in response to perturbed secretion of insulin. The present study has clarified that insulin-dependent diabetes mellitus causes the metabolism of the sublingual gland to become dysfunctional, leading to the accumulation of lipid droplets in both mucous cells and serous demilune cells.

**Acknowledgement**

We thank Ms. Sumie Sato, Department of Histology, School of Life Dentistry at Tokyo, The Nippon Dental University, for her skillful assistance.

**References**