Morphological Effects of Diabetes on the Granular Ducts and Acini of the Rat Submandibular Gland

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KEY WORDS Salivary gland, Diabetes, Insulin, Electron microscopy, Streptozotocin

ABSTRACT Effects of experimental diabetes on rat submandibular glands have been documented, but earlier reports suggested that diabetes caused an extensive cellular degeneration and a replacement of the parenchymal cells by fibrous connective tissue. Such observations, however, are difficult to reconcile with the relatively normal physiological responsiveness of the gland (Anderson and Suleiman, 1989). This study, therefore, reexamined the histological, histochemical, and ultrastructural effects of streptozotocin-induced diabetes on rat submandibular gland. The tissues were examined at 3 weeks, and 3 and 6 months after the induction of diabetes, and compared with glands from age-matched controls by both light and electron microscopy. Light microscopically, the proportional volumes of the acini and granular ducts remained constant in control rats at about 46% and 39% respectively. In diabetic animals the volume density of the acini increased progressively to 62%, whereas that of the granular ducts decreased to 25%. The diameter and number of granular ducts were reduced in diabetic animals, but acinar cell area was only affected 6 months after the induction of diabetes. Ultrastructurally, there was an accumulation of lipid in the acinar cells and, with increasing duration of diabetes, the number of autophagic structures in both the acini and the granular ducts increased. Although there was evidence of some cellular degeneration it was never extensive. Morphometry showed that the volume density of secretory granules within the acini cells was unaffected, but there was a significant reduction in the volume density of secretory granules within the granular ducts. Thus, in the rat submandibular gland the greatest effect of streptozotocin-induced diabetes was to cause hypertrophic changes in the cells of the granular ducts. The relative contributions of a direct effect of insulin insufficiency and the hyperglycemic effects of diabetes, however, are not known. © 1984 Wiley-Liss, Inc.

INTRODUCTION

The term diabetes mellitus refers to a group of metabolic disorders that are clinically and genetically heterogeneous, but which share the common feature of glucose intolerance. The key defect that leads to the onset of insulin-dependent diabetes mellitus (IDDM) is the destruction of the β-cells of the pancreas, and therefore the loss of insulin producing capacity. Insulin is an important anabolic hormone that plays a significant role in regulating several metabolic pathways, including carbohydrate metabolism and glycogen storage, fatty acid synthesis, amino acid transport and the synthesis of DNA, RNA, and protein.

During the past several years, numerous investigations have demonstrated the effects of experimental diabetes on the structure and function of the major salivary glands of the rat, including the submandibular gland (Anderson, 1983; Anderson and Garrett, 1986; Anderson et al., 1989; Hand and Weiss, 1984; High et al., 1985; Liu and Lin, 1989a; b, Reutervig et al., 1986; Takai et al., 1983). Liu and Lin (1989a) were amongst the first to study the structural changes in the rat submandibular gland following the induction of diabetes. Alloxan diabetes and subsequent insulin treatment were shown to markedly affect the growth and development of the submandibular gland. Significant decreases in gland weight and granular duct diameter were measured, but no discernable histological differences in the acinar portion of the gland were reported. Nevertheless, when indirectly measured as a change in the number of nuclei per unit area, acinar cell size appeared to be smaller in diabetic glands. Similar findings were subsequently reported by High et al. (1985) and Reutervig et al. (1986). Finally, a reduction in the number and diameter of the granular ducts also has been noted in a spontaneous animal model, the db/db diabetic mouse (Hanker et al., 1980).

At the ultrastructural level, the most striking morphological effect of diabetes is the rapid accumulation of lipid within the acinar cells of the salivary and submandibular glands (Anderson, 1983; Anderson and Garrett, 1986; Hand and Weiss, 1984; Reutervig et al., 1986).
### TABLE 1. Effects of diabetes on body and subcutaneous gland weights

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>Body Weight</th>
<th>Gland Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2 Weeks</td>
<td>350 ± 9*</td>
<td>254 ± 12</td>
</tr>
<tr>
<td>Diabetic</td>
<td>2 Weeks</td>
<td>352 ± 7*</td>
<td>239 ± 8*</td>
</tr>
<tr>
<td>Control</td>
<td>3 Months</td>
<td>507 ± 9</td>
<td>229 ± 72*</td>
</tr>
<tr>
<td>Diabetic</td>
<td>3 Months</td>
<td>205 ± 18*</td>
<td>229 ± 19*</td>
</tr>
<tr>
<td>Control</td>
<td>6 Months</td>
<td>650 ± 27*</td>
<td>298 ± 33*</td>
</tr>
<tr>
<td>Diabetic</td>
<td>6 Months</td>
<td>356 ± 24*</td>
<td>259 ± 35*</td>
</tr>
</tbody>
</table>

*Mean ± S.E.M.  

For paraffin embedding, tissues were fixed overnight at room temperature in 4% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.2) containing 7.5% sucrose, washed in the same buffer without paraformaldehyde, and dehydrated through graded alcohols. Sections were cut at 5 μm and stained with hematoxylin and eosin (H&E), or with the polyclonalmcrimmonumize- 
aldazide-nitrile (DMAD) method for the demonstra- 
tion of tryptophan. For cryostat sections, a wedge of tissue was fixed in 2% paraformaldehyde-2% distilled glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 2-4 hours at 4°C, and then washed overnight in the same buffer. The tissue was rapidly frozen in isopentane cooled by liquid nitrogen and stored at −75°C. To demonstrate serine protease activity to the granules of the granular ducts, cryostat sections (8–10 μm) were mounted on cover- 
slips and incubated in a substrate mixture containing 0.2 mM 4-Methyl-7-phenylcoumarin-3-carboxylic acid (M-7-PCA) and 20 μM Fast Blue R in 0.15 M phosphate buffer (pH 6.5) for 1 hour at 37°C (Garrett, et al., 1982; Garrett, et al., 1986). The sec- 
tions were then treated with 1% CuSO4 for 3–5 min- utes to chelate the reaction product, washed in distilled water, cleared, and mounted in coverslip.

A third wedge of tissue was immersed in a glutaral- 
dehyde-parafomaldehyde mixture (Barnovsky, 1967), 
transsected into small cubes, and fixed for 2–4 hours at room temperature. The tissue was then rinsed overnight at 0–4°C in 0.05 M cacodylate buffer (pH 7.2) containing 7.5% sucrose. The tissue was then post-fixed in 1% OsO4, rinsed through graded alcohols and 
embedded in Araldite. Thick sections (1 μm) were 
stained with toluidine blue for light microscopical ex- 
samination. Thin sections were stained with uranyl acetate and lead and viewed in an electron microscopy.

### Materials and Methods

Male Wistar rats initially weighing 200–250 gms were used throughout these studies. Diabetes was in- 
duced with a single intravenous injection of streptozo- 
toxic (65 mg/kg). Age-matched animals served as con- 
trols. All animals were maintained on a 12 hour light- 
dark cycle, and allowed free access to food and water. Non-fasting serum glucose levels were determined, and only animals having glucose values greater than 15 mmol were considered diabetic.

### Tissue Fixation

Three weeks, 3 months, and 6 months after the in- 
duction of diabetes the animals were fasted overnight. Under anesthesia (pentoobarbital, 35 mg/kg i.p. fol- 
lowed by chloralose, 75 mg/kg i.v.), the subcutaneous glands were removed and weighed, and weights of tis- sues were prepared for morphological assessment. For stereological measurements tissues was always taken from the middle of the gland.

### Morphometry

A minimal sample size was determined for paraffin sections by finding the cumulative mean of a large sample, chosen randomly but studied systematically, using a Kontron Videoplan. A summation average graph was plotted, and the area point beyond which the graph started to oscillate within ± 5% of the same mean was considered to be the minimum number of fields to be studied. In the study of Dukull, Z. (1989) this was found to be 10 for granular ducts and 25 in the case of the acinar zone. 24 fields were taken (as the minimal sample size). For determination of the volume densities of acinar and granular ducts, 3,000 points were counted to ensure a standard error of 1%.

Intracellular volume densities were determined us- ing semithin and thin sections. Semithin sections (1 μm) were used to estimate the volume density of secre- 
tory granules within the granular duct. The thin slice sample size was 20 (determined as above). A 1 μm 
index grid was used for point counting on transversely 
cross-sectioned granular duct profiles. At the electron 
microscopic level, a block of tissue from each gland was 
chosen randomly. Thin sections were cut, and a total of four grids with 4 or 5 sections on each were 
stained. Twenty micrographs of indent dual acinar cells with a visible nucleus and which bordered a central lumen were used to determine the volume density of
TABLE 1. Effects of streptozotocin diabetes on the size and proportional volume densities of acinar and granular duct cells in the rat submandibular gland

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>Acinar Cells</th>
<th>Granular ducts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control(S)</td>
<td>3 weeks</td>
<td>608 ± 24*</td>
<td>48 ± 1</td>
</tr>
<tr>
<td>Diabetic(S)</td>
<td>3 weeks</td>
<td>801 ± 60</td>
<td>56 ± 1</td>
</tr>
<tr>
<td>Control(G)</td>
<td>3 months</td>
<td>900 ± 27</td>
<td>64 ± 2</td>
</tr>
<tr>
<td>Diabetic(G)</td>
<td>3 months</td>
<td>161 ± 32</td>
<td>34 ± 1</td>
</tr>
<tr>
<td>Control(G)</td>
<td>6 months</td>
<td>949 ± 68</td>
<td>62 ± 3</td>
</tr>
<tr>
<td>Diabetic(G)</td>
<td>6 months</td>
<td>613 ± 61*</td>
<td>31 ± 1</td>
</tr>
</tbody>
</table>

*Mean ± S.E.M.

**Acinar = acinar cells; control = control animals; diabetic = diabetic animals; Group = Group assigned.

RESULTS

The effects of diabetes on body weight and submandibular gland weight are shown in Table 1. Mean acinar and ductal weights in control animals ranged from 15 to 35 mg. The induction of diabetes resulted in a typical retardation of growth, and at all time points both body weight and gland weight were significantly less in diabetic than in control animals. Gland to body weight ratio (mg/g) however, was not affected by diabetes, 0.73 in control and 0.77 in diabetic rats.

Light Microscopy

Paraffin sections of control glands stained with hematoxylin and eosin showed normal histological features with a regular distribution of parenchyma, acini and granular ducts, within a connective tissue stroma (Fig. 1A). Araldite sections (1 mm) revealed that the cytoplasm of the acinar cells was filled with pale, mucous-like granules, whereas that of the granular ducts contained variously dense, serous-like secretory granules of varying size that occupied the apical two thirds of the cell (Fig. 1C). In diabetic animals, paraffin and Araldite sections clearly demonstrated a reduction in size and in number of the granular ducts (Fig. 1B). As early as 3 weeks after the induction of diabetes, the granular content of these structures was already affected, but the extent of the reduction varied from animal to animal (Fig. 1D).

At 6 months the duration longer of diabetes was reflected in the increased severity of the structural changes, particularly in the granular ducts. Sections stained with the substrate D-Val-Leu-Arg-MNA, which demonstrates the proteases in the granular ducts, illustrated that these structures were considerably reduced in size and in number compared with either control or 3 week diabetic animals (Fig. 2A, B).

Electron Microscopy

All control glands showed normal morphological features as described previously (Garrett, et al., 1991). The acinar cells were relatively uniform in size, and contained electron-lucent secretory granules that filled most of the cytoplasmic volume (Fig. 3). Nuclear, endoplasmic reticulum, and Golgi apparatus were all located in the basal onethird of the cell. The granular ducts (Fig. 5) were easily distinguished from the striated ducts by the presence of numerous electron dense secretory granules of variable size.

Ultrastructurally, the primary changes seen at 3 weeks were in the acinar cells (Fig. 4). Intracellular lipid accumulation was observed in the basal region of many acinar cells, and in addition there was an increased prominence of the rough endoplasmic reticulum, and a variable coalescence of serous granules. Nevertheless, the majority of the acinar cells appeared normal and showed no discernable pathological changes. The cells of the granular ducts also appeared to be normal, and the changes detected at the light microscopical level could not be readily verified electron microscopically.

At 3 and 6 months (Figs. 6, 7, 8), the abnormalities were more striking than at 3 weeks. The reduction in the size, as well as the content of the granular ducts, was now readily apparent (Fig. 6). In the acinar cells, lipid droplets were still evident, but the most conspicuous finding was the presence of autophagic vacuoles, and the pooling of serous granules (Figs. 7, 8). Nevertheless, most of the acinar cells appeared to be relatively normal. Autophagic vacuoles were observed in the granular ducts, although not as frequently as in the acinar cells.

Morphometry

Paraffin sections were used to determine the volume densities of acini and granular ducts (Table 2). In control animals the proportional volumes for these two
Fig. 1. Light micrographs of control and 3-week diabetic rat submandibular glands. A: Paraffin section of a control submandibular gland stained with H&E showing large and frequently distributed granular duct (G) among the more intensely staining acinar cells. B: Paraffin section of a normal submandibular gland stained with H&E demonstrating a reduction in the size of the granular duct (G). In contrast, no discernible changes in the acinar cells are seen. C: Paraffin section of a control submandibular gland stained with toluidine blue showing the variability in granular duct diameter and secretory content. D: Paraffin section of a diabetic submandibular gland stained with toluidine blue showing a reduction in secretory granules content of the granular duct. 

G.
Fig 2. Light micrographs of control and 3-month diabetic rat submandibular glands. Sections stained with D-Val-Leu-Lys and fast blue B to demonstrate proteinase activity on the cytoplasmic granules of the granular ducts (asterisk). A: Control submandibular gland demonstrating an intense reaction of the arylsulfatase over the granular ducts. x 200. B: Diabetic submandibular gland showing a considerably lower activity in the amount of arylsulfatase reactivity and in the number of granular ducts. x 200.
Fig. 3. Electron micrograph of a control rat submandibular gland showing a group of acinar cells. The cells are relatively uniform in size, and are packed with electron-lucent secretory granules. × 4,400.

Fig. 4. Electron micrograph of acinar cells from a 3-week diabetic submandibular gland, showing the deposition of lipid (L) in the acinar cells, but not the intercalated duct (I). Note the small, amorphous granules in the cells of the intercalated duct. Interface arrow. × 2,000.
Fig. 5. Electron micrograph of a control rat submaxillary gland. This represents a portion of a granular duct, consisting of cells containing variable numbers of secretory granules of varying electron density surrounding a central lumen (L). × 4,200.

Fig. 6. Electron micrograph of a portion of a granular duct and an acinar cell (A) from a 3-week diabetic rat submaxillary gland. The cells appear to contain fewer secretory granules than in control glands. × 4,600.
Fig. 7. Electron micrograph of a 3-month diabetic rat submandibular gland acinar cells showing lipid droplets (L), and prominent autophagic vacuoles (W). x 6,600.

Fig. 8. Electron micrograph of a 6-month diabetic rat submandibular gland acinar cell showing numerous degenerative structures and autophagic vacuoles (W). x 6,400.
TABLE 3: Effects of streptozotocin-diabetes on the proportional volume of secretory granules within acinar and granular duct cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>Volume of acini</th>
<th>Volume of ducts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control(S)</td>
<td>3 weeks</td>
<td>54 ± 2</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>Diabetic(S)</td>
<td>3 months</td>
<td>64 ± 2</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>Control(D)</td>
<td>3 months</td>
<td>60 ± 2</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>Diabetic(D)</td>
<td>6 months</td>
<td>65 ± 4</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>Diabetic(D)</td>
<td>6 months</td>
<td>65 ± 4</td>
<td>32 ± 2</td>
</tr>
</tbody>
</table>

* * * * * *

compartmental remained relatively constant, 48% and 36% respectively (giving a ratio of acini to granular ducts of 1.3:1). At all three time points after the induction of diabetes, the volume density of acini (growth) increased and that of the granular ducts (cellular density) was reduced. In diabetic rats, therefore, the ratio of acini to granular ducts increased to 2.7:1. No evidence of recovery was observed.

Acinar cell arm (um²) and granular duct diameter (um) were also measured (Table 2). In contrast to volume densities, mean acinar cell area was unaffected at 3 weeks and 3 months. A significant reduction (approximately 30%) in acinar area, however, was seen at 6 months. The average tubular diameter was similarly unaffected at 3 weeks, but at both 3 and 6 months there was a 30% reduction in granular duct size.

The intracellular volume density of secretory granules in the granular ducts was determined using semi-thin sections stained with toluidine blue, and that of secretory granules in the acinar cells was measured on electron micrographs (Table 3). With the possible exception of the 3 month time period, secretory granule volume density in the acinar cells was unaffected by diabetes. In contrast, the proportional volume of secretory granules in the granular ducts was reduced at all three time points. Although a significant reduction was observed at 3 weeks, a further reduction in volume density had occurred at 3 months. No further decrease in recovery were seen by 6 months.

DISCUSSION

The gross morphological effects of diabetes, including the reduction of both body and submandibular gland growth, are well documented (Anderson and Shapiro, 1976; High et al., 1969; Liu and Lin, 1969a, b) and the results of the present study, with one exception, are in agreement with previously published investigations. The gland to body weight ratios reported by Liu and Lin (1969a, b) and High et al. (1980) were increased slightly, but significantly, in diabetic rats compared with controls whereas we observed no such increase. This is in contrast to the effects of diabetes on the parotid gland in which there is a marked increase in the gland to body weight ratio (Anderson, 1983). Diabetic rats are hyperglycemic, and the difference between the relative weight gains of the parotid and submandibular glands may be related to their inherently differing responses to changes in mastication. Increased mastication results in parotid gland hypertrophy, which has not been observed in this study. However, the submandibular gland does respond to increased mastication, and the volume density of acini increased.

The proportional volumes of the acini increased, and those of the granular ducts decreased in diabetic rats compared with those in controls. When measured directly, however, acinar cell size did not differ between control and diabetic rats until 6 months. In contrast, the diameter of the granular ducts decreased as early as 3 weeks. Liu and Lin (1969a, b) had reported a decrease in both acinar cell size and the diameter of the granular ducts within 21 days. Although the absolute values reported in this paper differ from those reported by High et al. (1980), the direction and magnitude of the changes were similar. We have found, however, that the proportion of granular ducts varies significantly between breeding colonies of the same strain (Garrett et al., 1981), and thus any comparison with previously published values must take strain differences into account.

Individual secretory granules within the acinar cells are not readily apparent at the light microscopic level with H&E staining, but the DMBB method, histochemical staining using D-Van-Lev-Ang-MNA and toluidine blue staining of Alaréto sections allowed distinct visualization of the granules in the ducts. The number of secretory granules per cell within the cells of the granular ducts appeared to be reduced, although this has not been confirmed stereologically. Nonetheless, the volume density of secretory granules was significantly decreased at all time points. These findings correlate well with the biochemical data reported by Jaffa et al. (1984a) that demonstrated a significant reduction in granular duct volume and number of proteinse present within the secretory granules of the granular ducts. At the light microscopical level, therefore, the most striking effects of diabetes appear to involve the granular ducts, but whether these effects reflect the influence of insulin in the ductal cells is unknown.

The development and maintenance of the granular ducts in the rat, as well as the level of some of the granular proteins, are known to be androgen dependent (Chievitz, 1977; Shaffer and Mahowald, 1954; Ekisilem and Nieri, 1968). Thus, the decrease in the size and in the proportional volume of secretory granules in the granular ductal cells may be due in part to a hypogonadism effect of diabetes (Murray, et al., 1981; Saset et al., 1988).

Enomowa (1972) has shown that protein-calorie malnutrition of sufficient severity to retard growth will also inhibit the development of the granular ducts in immature (5 to 8 weeks old) but not adult rats. The rats used in this study were 8 to 10 weeks old the granular ducts had already begun their development, and thus the generalized nutritional and metabolic disturbances characteristic of diabetes may not have contributed significantly to the observed changes in the structure or secretory granule content of the granular ducts.

Earlier reports on submandibular gland ultrastructure (Cutler et al., 1979; Takai et al., 1980) suggested

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that the induction of diabetes led to widespread degenerative changes, characterized by the accumulation of intracellular lipid and a pooling of secretory material, increased lysosomal activity, and the replacement of the parasympathetic cells by fibrous connective tissue. We have commented on the accumulation of lipid in diabetes (Anderson and Garrett, 1988), and in this study we also noted some pooling of secretory material in both the acinar and the granular duct cells, as well as an increase in the number of autophagic vacuoles and secondary lysosomal structures. However, in contrast to the reports by Takahashi et al. (1953) and Coutif et al. (1979), most of the acinar and granular duct cells in the present glands appeared to be relatively normal. Further, we found no evidence of fibrous degeneration even after 6 months, nor did we observe any acute morphological changes that were indicative of intracellular cellular damage. The morphological effects of diabetes on the acinar cells, and the significant atrophy of the granular ducts in the rat submandibular gland, therefore, seem to be more suggestive of extensive cellular degeneration and death than are those reported for the rodent (Andersen, 1983; Hand and Weiss, 1984).

Finally, recently obtained physiological data are not consistent with an extensive, fibrous degeneration of the submandibular gland. Although Takahashi et al. (1983) reported decreased submandibular responses to sympathomimetic and parasympathetic agonists, sympathetic nerve stimulation evoked normal secretory responses (measured as flow rate) in tissues which after the induction of diabetes, and an increase in flow rate after 3 months (Anderson and Subirian, 1989). Further, submandibular salivary flow rates upon parasympathetic nerve stimulation were actually increased at 5 weeks and no different from controls at 3 and 6 months (unpub. data).

ACKNOWLEDGMENTS

This work was supported by grants to A.H.S. by the Saudi Arabian Government and an O.R.S. grant, and to the Dental Research Institute of Dental Science for Dental Research (Grant DE 90695). Our thanks are due to Mr. R. and Mr. D. Bawley for technical help.

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