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Title: Immunohistochemical and ultrastructural investigation of acinar cells in submandibular and sublingual glands of rats fed a liquid diet

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REVISED VERSION

ABSTRACT

In atrophic parotid glands induced by liquid diet, acinar cell apoptosis is increased while proliferative activity is reduced. This study aimed to clarify how liquid diet affects submandibular and sublingual glands, including acinar cell apoptosis and proliferation. Seven-week-old male Wistar rats were fed either a liquid (experimental group) or pellet diet (control group) from 3 to 21 days, respectively. Submandibular and sublingual glands were weighed and examined histologically, ultrastructurally, and immunohistochemically using antibodies to cleaved caspase-3 (Casp-3) and 5-bromo-2'-deoxyuridine (BrdU). Weights of submandibular and sublingual gland from the experimental group were not significantly different from controls at any time point. Histological and ultrastructural characteristics of experimental acinar cells in both glands were normal. Acinar cells in control and experimental submandibular glands were positively stained with periodic acid Schiff (PAS) and weakly stained by alcian blue (AB). In control and experimental sublingual glands, mucous acinar cells were PAS-positive and strongly AB-positive. Although Casp-3- and BrdU-positive acinar cells were identified in both glands in the experimental group, their labeling indices were not significantly different from controls. In conclusion, liquid diet in rats does not induce atrophic alterations to acinar cells, including apoptosis and proliferative activity in submandibular and sublingual glands.

1. Introduction

Increasing intake of soft foods, a feature of current dietary habits, is thought to negatively influence the craniofacial region (Hanihara et al., 1981; Varrela, 1992). To investigate these influences, an experimental model of feeding a liquid diet to animals is widely used. Parotid glands from liquid-fed animals become atrophic, acinar cells are reduced in size (Hand and Schneyer, 1964; Wilborn and Schneyer, 1970; Hand and Ho, 1981; Scott and Gunn, 1991; Takahashi et al., 2012) and acinar cell numbers decrease in the atrophic parotid glands (Johnson, 1982; Scott et al., 1990; Takahashi et al., 2012). Despite an increasing accumulation of knowledge regarding parotid glands of liquid-fed animals, few reports have investigated other salivary glands such as the submandibular and sublingual glands. Of these studies, some (Kim, 1990; Kuntsal et al., 2003), but not all (Ekstrom, 1973; Mansson et al., 1990; Nakamura, 1997; Kurahashi and Inomata, 1999) reported that submandibular glands of rats fed a liquid diet showed atrophy. Although Mansson et al. (1990) and Kurahashi et al. (1999) demonstrated that liquid diet induced the atrophy of sublingual glands, other reports showed no atrophy of sublingual glands (Ekstrom, 1973; Scott and Gunn, 1991; Nakamura 1997). Therefore, the induction of atrophy in submandibular and sublingual glands by liquid diet is controversial.

Previous studies showed that apoptosis is important for atrophy of parotid glands induced by duct-ligation (Walker and Gobe, 1987; Scott et al., 1999) and regression after withdrawal of isoprenaline administration (Chisholm et al., 1995). Recently, we also

demonstrated increased apoptosis and reduced proliferative activity of acinar cells in parotid glands of rats fed a liquid diet (Takahashi et al., 2012). However, no studies have investigated apoptosis and proliferation of acinar cells in the submandibular and sublingual glands of liquid-fed rats. Under some pathological conditions such as duct-ligation and isoprenaline administration, the results in parotid glands are similar to those in submandibular glands (Takahashi et al., 2000; Chisholm and Adi, 1995), while the same phenomena do not necessarily occur in duct-ligated sublingual glands (Takahashi et al., 2002). Thus, it should not be assumed that increased acinar cell apoptosis and reduced acinar cell proliferation occur in submandibular and sublingual glands of rats fed a liquid diet, as is observed in the parotid glands.

The purpose of this study was to clarify whether liquid diet induced atrophic alterations to acinar cells in submandibular and sublingual glands, such as apoptosis and proliferation of acinar cells. This was achieved through histological, immunohistochemical, and ultrastructural analysis of submandibular and sublingual glands from rats fed a liquid diet.

2. Materials and Methods

2.1. Animal experiments

This study used 32 male Wistar rats aged 7 weeks, weighing 220-240 g (Hokudo, Japan). Control rats were given a normal pellet diet and experimental rats were given a liquid diet, prepared daily by mixing two parts of water to one part of a powdered diet prepared from the normal pellet diet. Body weights of rats were measured daily. Rats were perfused with 4% buffered paraformaldehyde (pH 7.4) under pentobarbital general anesthesia on days 3, 7, 14, or 21. Four control and four experimental animals were used at each time point. Rats in both groups were deprived of food for fasting 12 h before perfusion. Rats were intraperitoneally injected with 25 mg/kg body weight BrdU 1 h prior to perfusion.

This study was approved by the Laboratory Animal Committee of Hokkaido University (Approval No. 09-0009) and complied with the Guide for the Care and Use of Laboratory Animals of Hokkaido University.

2.2. Histology

The right submandibular and sublingual glands were excised, weighed, and placed in 4% buffered paraformaldehyde (pH 7.4) for 24 h. The fixed tissue was processed routinely and embedded in paraffin for histological and immunohistochemical examination. Paraffin sections were stained with hematoxylin and eosin (HE), periodic acid Schiff (PAS), or alcian blue (AB) (pH 2.5). In the HE sections, the area of at least 1000 acinar cells in randomly

selected fields at a magnification of x200 were measured, using the image-analysis system (DS-L2, Nikon, Japan).

2.3. Immunohistochemistry

Caspase-3. The endogenous peroxidase in sections was blocked by 0.3% hydrogen peroxide and antigen retrieval was performed using 10 mM Tris/1 mM EDTA buffer (pH 8.8) at 100°C. Then sections were incubated with anti-caspase-3 (Casp-3) rabbit polyclonal antibody (Asp 175, Biocare Medical, CA, USA, 1:50 dilution), anti-rabbit swine polyclonal antibody (DakoCytomation, Denmark, 1:100 dilution), and streptavidin-biotin horse-radish peroxidase complex (DakoCytomation) in turn. Peroxidase activity was visualized using 3, 3'-diaminobenzidine tetrahydrochloride, and sections were lightly counterstained with hematoxylin.

BrdU. Sections were pretreated with 0.1% trypsin at 37°C and 3 N HCl following endogenous peroxidase blocking. Anti-BrdU mouse monoclonal antibody (Bu-20a, DakoCytomation, 1:50 dilution) was the primary antibody and anti-mouse rabbit polyclonal antibody (DakoCytomation, 1:50 dilution) was the secondary antibody. Immunoreaction was developed and sections were counterstained as mentioned above.

Normal rabbit or mouse serum was substituted for the primary antibody as negative controls. In the immunostained sections, at least 1000 acinar cells were counted in randomly chosen fields at a magnification of x200 using an ECLIPS 80i microscope (Nikon), and labeling indices of Casp-3 and BrdU were calculated.

2.4. *Transmission electron microscopy*

The left submandibular and sublingual glands were immersed in 2% buffered paraformaldehyde-1.25% glutaraldehyde (pH 7.4) for 2 h after perfusion and then rinsed with 0.05M sodium cacodylate buffer (pH 7.4). Tissues were then post-fixed in 1% osmium tetroxide, stained *en block* with 4% uranyl acetate, and embedded in Epon 812. Ultrathin sections were double stained with 4% uranyl acetate and 0.1% lead citrate and examined with a JEM-1400 electron microscope (JOEL, Japan).

2.5. *Statistical analysis*

Numerical data were expressed as mean and standard error of the mean (SEM) for four control animals or four experimental animals at each time point. Statistical analysis was performed using the Mann-Whitney *U*-test, and values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Weights of bodies and glands

Body weights of rats fed a liquid diet (n=4) increased as normal from the beginning of the experiment and were not significantly different from controls (n=4) at all days over the experimental period (Fig. 1). Rats fed a liquid diet or pellet diet did not show symptoms such as diarrhea, and their general condition appeared to be good throughout the experimental period. The wet weights of submandibular and sublingual glands in the experimental rats were not significantly different from those in controls regardless of the experimental day examined (Fig. 2A, B).

3.2. Histological observations

Control submandibular glands at each time interval measured had similar histology. The secretory endpieces were composed of pyramidal seromucous acinar cells (Fig. 3A), containing strong PAS- (Fig. 3B) and weak AB-positive stained secretory granules (Fig. 3C). Granular duct cells showed PAS-positive (Fig. 3B), but AB-negative staining (Fig. 3C). In experimental submandibular glands, the shape and histochemical staining of acinar cells was unchanged during the experimental period and were similar to those of controls (Fig. 3D-F). There was no difference in the histology of control sublingual glands regardless of the feeding periods. Acini were mostly composed of mucous cells with clear cytoplasm (Fig. 3G), and serous demilunes were arranged around the mucous epithelium. Mucous acinar cells were

PAS-positive (Fig. 3H) and showed strong positive staining for AB (Fig. 3I). Histology of experimental sublingual glands showed normal tissue morphology and was similar to controls at each time point examined (Fig. 3J-L). The quantitative histological analysis showed that the areas of acinar cells in the control were not significantly different from those in the experimental groups at every time point in submandibular (Fig. 4A) and sublingual glands (Fig. 4B).

3.3. Immunohistochemical observations

Casp-3-positive acinar cells were rarely observed in experimental submandibular and sublingual glands similar to controls (Fig. 5A, C, E, G). In some sections, all acinar cells were negative for Casp-3 staining. The labeling indices of Casp-3 for acinar cells are shown in Fig. 6. Casp-3 labeling indices of submandibular and sublingual glands were less than 0.1% in both groups and there was no significant difference between the experimental and control groups at each time point measured. BrdU-positive staining was frequently observed in acinar cells from both glands in both groups (Fig. 5B, D, F, H). The labeling indices of BrdU for acinar cells in both glands from both groups were around 1%, and the mean indices of BrdU were not statistically significant between groups (Fig. 7).

Negative control sections for Casp-3 and BrdU showed no staining.

3.4. Ultrastructural observations

Acinar cells in submandibular glands from controls had a round nucleus situated

basally and a well-developed rough endoplasmic reticulum was observed around the nucleus. The cytoplasm of acinar cells was filled with electron-lucent granules with relatively little substructure (Fig. 8A). Acinar cells from control sublingual glands contained many electron-lucent secretory granules, and a flattened nucleus and rough endoplasmic reticulum was observed at the basal area of the cell (Fig. 8B). Ultrastructural characteristics of acinar cells from the experimental submandibular (Fig. 8C) and sublingual glands (Fig. 8D) were similar to controls and were unchanged throughout the experimental period. Apoptosis and necrosis of acinar cells were not observed in submandibular and sublingual glands from experimental rats.

4. Discussion

Previous studies suggested that changes in tissues of animals fed a liquid diet were not caused by changes in general health, because body weight loss or a general deterioration in health was not observed (Hall and Schneyer, 1964; Johnson, 1982; Mansson et al., 1990; Scott et al., 1990; Kurahashi and Inomata, 1999; Takahashi et al., 2012). In the present study, the body weights of experimental rats increased constantly and were not significantly different from those of controls, confirming the findings of previous studies.

Whether submandibular and sublingual glands of liquid-fed rats become atrophic is controversial. In the current study, the weights of the submandibular and sublingual glands of liquid-fed rats did not decrease, showing both glands were not atrophic macroscopically. Then we examined this problem from a standpoint of apoptosis and proliferation of acinar cells.

Immunohistochemistry of Casp-3 and BrdU in the present study did not demonstrate that promotion of acinar cell apoptosis and inhibition of acinar cell proliferation took place. Therefore it is first shown from the point of view of cell number control that liquid diet feeding induces no atrophy in submandibular and sublingual glands. These results also suggest no differences in acinar cell numbers between the two groups. Kim (1990) investigated submandibular glands of mice fed a liquid diet and reported that cell numbers decreased from reduction of DNA content, which is inconsistent with the present study. This may be due to the method of analysis used in his study. Because the whole submandibular glands were analyzed biochemically, changes of other types of cells including mesenchymal

cells may effect on his data. Although the BrdU labeling indices for acinar cells were higher than Casp-3 in submandibular and sublingual glands at all time points, the weights of the glands were stable over the experimental period in the current study. This relation is not necessarily contradictory, because the periods labeled for BrdU and Casp-3 are different and it is impossible to compare directly these labeling indices.

In this study, histological and ultrastructural examination of submandibular and sublingual glands of liquid-fed rats determined no morphological changes such as acinar cell size or nature of secretory granules were observed. Our results in sublingual glands coincided with a previous report (Scott and Gunn, 1991). However, some studies have observed acinar cell shrinkage (Kim, 1990; Scott and Gunn, 1991), acinar cell degeneration, and secretory granule reduction (Kuntsal et al., 2003) in submandibular glands. It is difficult to explain the differences among these studies, and it would be unsuitable to consider these depend on the differences of species such as rat and mouse. Because the atrophic changes were observed in rats (Scott and Gunn, 1991; Kuntsal et al., 2003) as well as in mice (Kim, 1990).

We demonstrated that a liquid diet did not induce atrophy in submandibular and sublingual glands, although parotid glands became atrophic under the same experimental conditions (Takahashi et al, 2012). This agreed with previous findings in submandibular (Ekstrom, 1973; Mansson et al., 1990; Kurahashi and Inomata, 1999) and sublingual glands (Ekstrom, 1973; Scott and Gunn, 1991) in studies using the same experimental conditions. When atrophy of submandibular (Scott and Gunn, 1991) and sublingual glands (Mansson et al., 1990; Kurahashi and Inomata, 1999) was observed, it was very slight despite the induction

of severe atrophy in the parotid glands. Taken together, this suggests that parotid glands might be affected more strongly by a liquid diet than the submandibular and sublingual glands.

It is important to determine why submandibular and sublingual glands react differently to a liquid diet compared with parotid glands. Previous experimental studies investigating atrophy and development of salivary glands suggested that parasympathetic nerves are important for the maintenance and development of salivary glands. For example, parasympathectomy of salivary glands (Poat and Templeton, 1982; Hironaka et al., 2003; Carpenter et al., 2005) or damage to parasympathetic nerves by ligation (Harrison and Garrett, 1976; Harrison et al., 2001) induced salivary gland atrophy. Furthermore, cellular proliferative activity in salivary glands was enhanced by electrical stimulation of parasympathetic nerves (Schneyer et al., 1993) and administration of pilocarpine, a parasympathomimetic agent (Burlage et al., 2009). The afferent impulse induced by masticatory stimulus reaches the sensory nucleus in the brainstem and then is transmitted to the secretory center and to higher brain structures, which send efferent projections to the salivary nuclei (Pedersen et al., 2013). The secretory center of submandibular glands and sublingual glands is the superior salivary nucleus, from which their preganglionic parasympathetic fibers arise, accompanying the facial nerve. However, the secretory center of parotid glands is the inferior salivary nucleus, and its preganglionic parasympathetic fibers accompany the glossopharyngeal nerve (Young and Lennep, 1978). Therefore, it might be possible that one of the factors for the different reactions to a liquid diet is related to the difference in parasympathetic innervation between submandibular and sublingual glands and parotid glands. This speculation might be supported

by the fact that levels of acetylcholine and choline acetyltransferase activity, which determines acetylcholine synthesis, are down-regulated in parotid glands but unchanged in submandibular and sublingual glands of animals fed a liquid diet (Ekstrom, 1973; Nakamura 1997). However, it is difficult to confirm this only by the present study, and further studies will be necessary.

In conclusion, the present study demonstrated that rats fed a liquid diet did not develop atrophic alterations to acinar cells, such as apoptosis or proliferative activity in the submandibular and sublingual glands. This is in contrast to parotid glands of rats fed a liquid diet.

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Figure Legends

Fig. 1 The body weights of control rats fed a pellet diet (broken line, n=4) and experimental rats fed a liquid diet (solid line, n=4) were measured. There is no statistically significant difference in body weight (g) between the control and experimental groups at each day.

Fig. 2 The wet weights of submandibular (A) and sublingual (B) glands in control rats fed a liquid diet (filled bars, n=4) and experimental rats fed a liquid diet (open bars, n=4). The values are expressed as mean + SEM. No statistically significant difference was observed in wet weight (g) of submandibular and sublingual glands between the control and experimental groups.

Fig. 3 Histology on day 21. HE (A, D, G, J), PAS (B, E, H, K), and AB (C, F, I, L) staining of tissues are shown. Tissues were from control submandibular glands (A-C), experimental submandibular glands (D-F), control sublingual glands (G-I), and experimental sublingual glands (J-L). Scale bars = 30 μ m. The morphology and intensity of PAS and AB staining of acinar cells in experimental submandibular and sublingual glands were similar to those in control glands. Granular duct cells (arrows) showed positive PAS staining (B and E).

Fig. 4 Areas of acinar cells in submandibular (A) and sublingual glands (B) in control (filled bars, n=4) and experimental (open bars, n=4) groups. The values are expressed as mean+SEM.

There is no statistically significant difference in submandibular and sublingual glands between the control and experimental groups.

Fig. 5 Immunohistochemistry on day 21. Casp-3 (A, C, E, G) and BrdU (B, D, F, H) staining of tissues are shown. Tissues from control submandibular glands (A, B), experimental submandibular glands (C, D), control sublingual glands (E, F), and experimental sublingual glands (G, H). Scale bars = 30 μ m. Casp-3-positive acinar cells (A, C, E, G, arrows) and BrdU-positive acinar cells (B, D, F, H, arrows) were observed in submandibular and sublingual glands of both groups.

Fig. 6 Labeling indices of Casp-3 for acinar cells in submandibular (A) and sublingual glands (B) in control (filled bars, n=4) and experimental (open bars, n=4) groups. Bars represent the mean index and error bars represent SEM. The mean indices of experimental submandibular and sublingual glands were not significantly different from controls at all time points examined.

Fig. 7 Labeling indices of BrdU for acinar cells in submandibular (A) and sublingual gland (B) in control (filled bars, n=4) and experimental (open bars, n=4) groups. Bars represent the mean index and error bars represent SEM. No statistically significant difference was observed in mean indices of both glands between the control and experimental groups at all time points examined.

Fig. 8 Transmission electron microscopy on day 21. Tissues were from control (A, B) and experimental groups (C, D) and were from either submandibular (A, C) or sublingual glands (B, D). Scale bars = 2 μ m. Ultrastructural characteristics of acinar cells from experimental submandibular or sublingual glands were similar to control glands.