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Acinar cell response to liquid diet during rats' growth period differs in submandibular and sublingual glands from that in parotid glands

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ABSTRACT

Continuously feeding a liquid diet to growing rodents strongly inhibits parotid gland growth, due to suppressed growth of acinar cells. This study investigated whether a liquid diet had a similar effect on submandibular and sublingual glands of growing rats. Rats were weaned on day 21 after birth and then fed a pellet diet in the control group and a liquid diet in the experimental group for 0, 1, 2, 4, and 8 weeks. Their submandibular and sublingual glands were excised, weighed, and examined histologically, immunohistochemically (using antibodies to 5′-bromo-2-deoxyuridine and cleaved caspase 3), and ultrastructurally. The submandibular glands did not significantly differ between the control and experimental groups at all tested points. Only at Week 8, acinar cell area and 5′-bromo-2-deoxyuridine-labeling index of acinar cells in sublingual glands were significantly lower in the experimental group than in the control group. These results show that a liquid diet during rats' growth period had no effect on acinar cells in their submandibular glands, and only a slight effect on acinar cells in their sublingual glands of growing rats, in contrast to the marked effect of a liquid diet on parotid glands.

1. Introduction

Soft foods are common in modern diets, but reportedly have unfavorable effects on oral tissues. In experimental rodent models, liquid or soft diets suppressed growth of the craniofacial bone (Watt and Williams, 1951; Ito et al., 1988; Enomoto et al., 2010), masseter muscle (Kiliaridis et al., 1988; Maeda et al., 1990; Miehe et al., 1999), and temporo-mandibular joint (Kiliaridis et al., 1999; Kato et al., 2015; Uekita et al., 2015). Their effects on salivary glands, particularly the parotid glands, have been widely investigated in mature animals. Such studies have concluded that liquid diets cause severe atrophy to parotid glands. Short-term liquid diets have been shown to reduce parotid gland weight (Hall and Schneyer, 1964; Ekstrom and Templeton, 1977; Scott et al., 1990; Scott and Gunn, 1991; Kurahashi and Inomata, 1999; Takahashi et al., 2012), secretion (Ekstrom and Templeton, 1977; Ito et al., 2001), and amylase activity (Hall and Schneyer, 1964; Hand and Ho, 1981; Johnson, 1984; Kurahashi and Inomata, 1999). In such atrophic parotid glands, acinar cell shrinkage (Hall and Schneyer, 1964; Wilborn and Schneyer, 1970; Hand and Ho, 1981; Scott et al., 1990; Scott and Gunn, 1991; Takahashi et al., 2012), decrease in acinar cell numbers (Johnson, 1982), decreased acinar cell replication (Takahashi et al., 2012), and increased acinar cell apoptosis (Takahashi et al., 2012; ElGhamrawy, 2015) have been observed.

Submandibular and sublingual glands, as well as parotid glands are major salivary glands, but these three major salivary glands differ in histological structure and physiological function; and the effects of liquid diet on submandibular and sublingual glands have been less studied than those of parotid glands. Therefore, whether submandibular and sublingual glands would respond in the same way to liquid diets as parotid glands was unclear. Some studies appear to show slight atrophy of the submandibular (Kuntsal et al., 2003) or sublingual glands (Mansson et al., 1990; Kurahashi and Inomata, 1999) whereas others found no effect on these glands by liquid diets (Ekstrom, 1973; Scott and Gunn, 1991; Takahashi et al., 2014). Whether atrophy of submandibular or sublingual glands is induced by the liquid diet is thus controversial, although many investigations consistently report that submandibular and sublingual glands are much less affected by liquid diets than parotid glands (Ekstrom, 1973; Scott and Gunn, 1991; Nakamura, 1997; Kurahashi and Inomata, 1999; Takahashi et al., 2012; Takahashi et al., 2014). These findings could imply that liquid diets affect parotid glands and the other salivary gland types differently.

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The aforementioned studies mainly focused on salivary glands of mature animals, but the effects of a liquid diet on growing salivary glands have received little or no attention. Our previous study revealed that the weight of parotid glands of growing rats fed a liquid diet increased much less than solid-fed rats. Histologically, acinar cell size in these parotids did not increase, and acinar cell proliferation was suppressed during the growing period. However, acinar cell apoptosis was not found as a factor in inhibition of parotid gland growth (Takahashi et al., 2015). Our previous study showed that parotid glands of both mature and growing rats were affected negatively by liquid diet and that atrophic induction differs between mature and growing parotid glands. However, the influence of liquid diets on the growth of other major salivary glands is still unclear.

The aim of this report was to clarify whether the growth of submandibular and sublingual glands was affected by a liquid diet. For this purpose, we examined histologically, histochemically, immunohistochemically, and ultrastructurally glands of growing rats that were fed a liquid diet. In histochemical analysis, we used periodic acid Schiff (PAS) and Alcian blue (AB) staining to demonstrate the full complement of tissue proteoglycans, because the mucous component of the submandibular glands reacts to PAS and the mucous component of the sublingual glands is strongly AB-positive (Cecchini et al., 2009), and they are useful to examine the nature of secretory granules in an acinar cell.

2. Materials and methods

2.1. Animal model

The animal protocol of this study was approved by the Laboratory Animal Committee of Hokkaido University (Approval No. 14-0108); it complied with the Guide for the Care and Use of Laboratory Animals of Hokkaido University. After weaning at 21 days, 63 male Wistar rats (CLEA Japan Inc., Tokyo, Japan) were divided into control ($n = 35$) and experimental groups ($n = 28$). In the control group, each rat was given a pellet diet (25 g/day) for 0, 1, 2, 4, and 8 weeks; in the experimental group, each rat was given a liquid diet prepared daily by mixing 25 g powdered form of the pellet diet with 50 mL water for 1, 2, 4, and 8 weeks. At the end of the experimental period, rats were deprived of food for 12 h before perfusion.

2.2. Histological study

At each test point, five control and five experimental rats were perfused with 4% paraformaldehyde buffered with 0.1 M phosphate buffer (pH 7.4) under pentobarbital anesthesia at 1 h after administering 5′-bromo-2-deoxyuridine (BrdU) (Sigma-Aldrich, St. Louis, MO; 25 mg/kg body weight; intraperitoneal injection). The right submandibular and sublingual glands were then removed, weighed, immersed in the same fixative for 24 h, and processed for routine paraffin embedding. Sections were cut at 4 μm and stained with Hematoxylin and Eosin (HE), PAS, and AB (pH 2.5).

The image-analysis system (DS-L2, Nikon, Tokyo, Japan) was used to determine areas of individual acinar cells. Each HE-stained section was roughly divided into 5 parts to avoid uneven distribution of observation fields, and a field was chosen from the central area of each part without intention. In the 5 fields, approximately 1000 acinar cells were measured at an objective magnification of 40x. Three sections were used from each animal ($n = 5$), and the mean of the data from the 3 sections was calculated, as the representative value for that animal.

2.3. Immunohistochemical study

The sections prepared as described above were treated with 0.3% hydrogen peroxide in absolute methanol for 10 min after deparaffinization to quench endogenous peroxidase activity, in readiness for immunohistochemical staining. For BrdU stains to detect proliferating cells, sections were pretreated with 0.1% trypsin for 20 min at 37 °C and later with 3N HCl for 10 min at 37 °C for denaturation of the DNA double strand. The sections were then incubated with anti-BrdU mouse monoclonal antibody (Bu-20a, DakoCytomation, Glostrup, Denmark, 1:50 dilution) for 2 h, biotinylated anti-mouse rabbit polyclonal antibody (DakoCytomation, 1:100 dilution) for 1 h, and peroxidase labeling streptavidin (Histofine, Nichirei Bioscience, Tokyo, Japan) for 30 min. The antigen antibody reaction sites were visualized by 3, 3′-diaminobenzidine; sections were then lightly counterstained with Mayer's hematoxylin. To quantify acinar cell proliferative activity, we used 3 sections stained with BrdU from each animal. Each section was roughly divided into 5 parts to avoid unevenly distributed observation fields; a field was chosen from the central area of each part without intention. In the 5 fields, BrdU-positive acinar cells were counted per approximately 1000 acinar cells at an objective magnification of 40x. The labeling index (percentage of labeled acinar cells) of each section was then calculated. The average of the labeling indices of the 3 sections from each animal ($n = 5$) was used as the representative value of that animal.

In using cleaved caspase 3 (Casp-3) to detect apoptotic cells, the ready sections were boiled in 10 mM tris/1 mM EDTA buffer (pH 8.8) for 15 min as antigen retrieval treatment. After cooling, sections were reacted with anti-Casp-3 rabbit polyclonal antibody (Asp 175, Biocare Medical, Concord, CA, 1:20 dilution) overnight at 4 °C, biotinylated anti-rabbit swine polyclonal antibody (DakoCytomation, 1:100 dilution) for 1 h, and peroxidase labeling streptavidin for 30 min in turn. Visualization of immunoreaction and counterstaining were as described above. We did not calculate labeling indices for Casp-3-positive acinar cells, because Casp-3-positive cells were extremely rare in both control and experimental groups at all time intervals.

Negative control sections were incubated with normal mouse or rabbit serum instead of primary antibody for BrdU or Casp-3, respectively, and showed no reaction.

2.4. Statistical analysis

Numerical data such as body weights, gland weights, areas of individual acinar cells, and labeling indices of BrdU are shown as medians and ranges in box plots for five control animals and five experimental ones at each tested time point. To determine significant differences between control and experimental groups, the Mann-Whitney *U*-test was used (Ystat2008, Igakutosho, Tokyo, Japan). $P < 0.05$ was considered significant.

2.5. Ultrastructural study

At each tested time point, we perfused two control and two experimental rats with 2% paraformaldehyde-1.25% glutaraldehyde buffered with 0.1 M sodium cacodylate buffer (pH 7.4) under pentobarbital anesthesia. After perfusion, the excised right submandibular and sublingual glands were trimmed into small pieces and immersed in the same fixative for 2 h. The samples were then post-fixed in 1% osmium tetroxide, stained *en bloc* with 4% uranyl acetate, and embedded in Epon 812. Ultrathin sections were cut with a diamond knife on an ultramicrotome, stained with both uranyl acetate and lead citrate, and studied with a transmission electron microscope (JEM-1400, JOEL, Akishima, Japan).

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