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Title: Involvement of apoptosis and proliferation of acinar cells in atrophy of rat parotid glands induced by liquid diet

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Abstract

Parotid glands of experimental animals fed a liquid diet are reported to show atrophy (Hall and Schneyer 1964; Wilborn and Schneyer 1970; Hand and Ho 1981; Scott et al. 1990; Scott and Gunn 1991). To clarify whether apoptosis and proliferation of acinar cells participate in atrophy of rat parotid glands induced by liquid diet, rats were fed a liquid diet and compared to pellet-fed controls. Parotid glands were removed at 3, 7, 14 or 21 days, weighed, and examined using transmission electron microscopy (TEM), and studied immunohistochemically for cleaved-caspase-3 (Casp-3), a marker of apoptotic cells, and 5-bromo-2'-deoxyuridine (BrdU), a marker for proliferating cells. Body weights of experimental rats fed liquid diets were not significantly different from controls fed pellet diets; however weights of experimental parotid glands were smaller than those of controls. In the experimental parotid glands, structures like apoptotic bodies were histologically observed in acini at each time point; more Casp-3-positive acinar cells were identified in experimental parotid glands than in the controls on days 3, 7, and 14. Experimental glands showed fewer BrdU-positive acinar cells at each time point. TEM confirmed typical apoptotic acinar cells in the atrophic glands. These findings suggest that increased acinar cell apoptosis and reduced acinar cell proliferation occur in atrophic parotid glands of rats fed a liquid diet.

Keywords; liquid diet, apoptosis, cell proliferation, parotid gland, atrophy

Introduction

Mastication is important both for preparing of a food boluses for swallowing and digestion and for maintaining oral health and enhanced salivation (Kawamura 1972). Although modern diets feature soft foods which do not require extensive mastication (Murakami et al. 2007), an easily chewable diet is thought to have unfavorable effects on the craniofacial region (Hanihara et al. 1981; Varrela 1992). Many experimental studies using laboratory animals have investigated these effects; influence of liquid diet has been confirmed in the craniofacial skeleton (Ito et al. 1988; Yamamoto 1996) and masseter muscles (Maeda et al. 1990; Kitagawa et al. 2004). Parotid glands also reportedly show atrophic alterations in animals fed a liquid diet. In atrophic parotid glands induced by liquid diet, shrinkage of acinar cells, in which secretory granules decrease, has been morphologically observed (Hall and Schneyer 1964; Wilborn and Schneyer 1970; Hand and Ho 1981; Scott et al. 1990; Scott and Gunn 1991) and reductions in parotid gland amylase activity have been shown by biochemical analysis (Hall and Schneyer 1964; Hand and Ho 1981; Johnson 1982; Scott and Gunn 1994).

Apoptosis is reportedly common in atrophy of exocrine glands. For example, epithelial cell numbers decrease by apoptosis during involution of the lactating mammary glands after weaning (Walker et al. 1989). Acinar cells apoptose in the atrophic pancreas induced by duct obstruction (Walker 1987), and similar observations are reported in atrophy of parotid glands following duct ligation (Walker and Gobe 1987; Scott et al 1999). However, the role and manifestation of apoptosis in atrophy of parotid glands of rats fed liquid diets

have received little attention. Involvement of acinar cell proliferation, which regulates cell population together with apoptosis, is unclear as well.

To establish whether apoptosis and mitotic cell proliferation of acinar cells were involved in liquid diet-induced atrophy of parotid glands, atrophic parotid glands of rats fed a liquid diet were examined, using immunohistochemistry for cleaved-caspase-3 (Casp-3) and for 5-bromo-2'-deoxyuridine (BrdU) and transmission electron microscopy (TEM). This study is important to contribute to clarifying the mechanism of atrophy of salivary glands, playing significant roles to maintain the oral cavity environment.

Materials and Methods

All animal experimentation of this study was conducted with the approval of 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. Seven-week-old male Wistar rats (Hokudo Co. Ltd., Sapporo, Japan) were divided into control and experimental groups. Control rats were fed a pellet diet; experimental animals were fed a liquid diet, prepared by mixing two parts of water with one part of a powdered diet of the above pellet diet for 3, 7, 14, or 21 days (n=4 for the control and experimental groups at each time point). All animals were weighed daily during the experimental period. Both diets were removed for a 12-hour fasting period prior to perfusion. One hour after administration of BrdU at a dose of 25 mg/kg body weight by intraperitoneal injection, the animals were perfused with 4% paraformaldehyde buffered at pH 7.4 with 0.1M phosphate buffer under pentobarbital anaesthesia; parotid glands were then removed. A small portion of each left gland was set aside for ultrastructural examinations. The right glands were weighed and immersed in the same fixative for 24 hours. After fixation, the whole right glands were tangentially embedded in paraffin and were serially sectioned to obtain maximum surface. Several paraffin sections were stained with hematoxylin and eosin (HE).

The deparaffinized sections were immersed in 0.3% hydrogen peroxide for blocking the endogenous peroxidase. For Casp-3 immunohistochemistry, sections were boiled in 10mM tris/1mM EDTA buffer at pH 8.8 for antigen retrieval. Anti-Casp-3 rabbit polyclonal

antibody (Asp 175, Biocare Medical, Concord, CA) as the primary antibody and anti-rabbit swine polyclonal antibody (DakoCytomation, Glostrup, Denmark) as the secondary antibody were used. For BrdU immunohistochemistry, sections were pretreated with 0.1% trypsin and 3N HCl and rinsed thoroughly with phosphate buffered saline. Sections were then incubated with anti-BrdU mouse monoclonal antibody (Bu-20a, DakoCytomation), and next with biotinylated anti-mouse rabbit polyclonal antibody (DakoCytomation). Next, sections for both Casp-3 and BrdU were reacted with streptavidin-biotin horseradish peroxidase complex (DakoCytomation), placed in 3,3'-diaminobenzidine for visualization, and lightly counter-stained with Mayer's hematoxylin. Normal mouse or rabbit serum was substituted for the primary antibody as negative controls. Four sections stained with Casp-3 and with BrdU from each animal were used for quantification. Each section was divided into 10 parts with approximately equal area, and a field was chosen from each part without intention. In 10 fields, all acinar cells and immuno-positive acinar cells were counted at a magnification of x200 (ECLIPSE 80i, Nikon, Japan). The labeling index (percentage of acinar cells labeled) of each section was calculated from 10 fields; the mean of 4 sections was taken as representative value of that animal. Means and standard errors of means (SEM) were calculated for 4 control animals and for 4 experimental ones at each time point. All numerical data were subjected to the Mann-Whitney U-test. Values of $P < 0.05$ were considered statistically significant.

Specimens for TEM were immersed in 2% paraformaldehyde-1.25% glutaraldehyde buffered at pH 7.4 with 0.05M sodium cacodylate following perfusion. Samples were then post-fixed in 1% osmium tetroxide, stained en block with 4% uranyl acetate and embedded in

Epon 812. Ultrathin sections were cut and double-stained with 4% uranyl acetate and 0.1% lead citrate. Prepared sections were examined with a TEM (H-7100, HITACHI, Japan).

Results

Both the experimental animals and controls appeared healthy throughout the experimental period. Although the two groups showed no significant differences in body weights at any time (not shown), parotid gland weights of experimental animals decreased and were significantly smaller than those of controls at all time points (Fig.1).

Parotid glands of control rats showed normal morphology (Fig.2a). In experimental parotid glands, structures like apoptotic bodies in acini (Fig.2b), and atrophic acinar cells were observed (Fig.2c). However, ducts were histologically unchanged. Casp-3-positive reactions in acinar cells were seen rarely in controls (Fig.2d), but commonly in experimental parotid glands (Fig.2e, f). Casp-3 labeling indices for experimental acinar cells were significantly different from those of controls at days 3, 7, and 14, but not at day 21 (Fig.3a). More acinar cells showing BrdU-positive reactions were seen in control glands (Fig.2g) than in experimental ones (Fig.2h, i). BrdU labeling indices in experimental acinar cells were significantly lower than those of controls at every time point (Fig. 3b). Negative control sections for Casp-3 and BrdU showed no reaction.

The ultrastructural appearance of apoptosis of acinar cells was seen in experimental parotid glands at every time point. In some apoptotic acinar cells, nuclear chromatin aggregated into a sharply circumscribed dense mass abutting on the nuclear membrane; fragmentation of such nuclei and whorled rough endoplasmic reticulum (rER) were seen (Fig.4a). Partly degraded apoptotic bodies with nuclear fragments and rER were

phagocytosed by adjacent acinar cells (Fig. 4b).

Discussion

There were no differences in body weight of the experimental and the control animals at any point during the experimental period, showing that atrophic changes of parotid glands in rats fed a liquid diet were not caused by changes in the animals' general health.

Casp-3 has recently been employed as an immunohistochemical marker for apoptosis, since caspase-3 cleaved at apoptotic cascade activation affects structural alterations of apoptotic cells (Hughes and Gobe 2007); BrdU incorporated into DNA-synthesizing cells is a well known marker for cell proliferation (Gratzner 1982). Thus antibodies against Casp-3 and BrdU were used in this study.

Histology, immunohistochemistry with Casp-3, and TEM in this study showed acinar cells were removed by apoptosis in atrophic parotid glands of rats fed a liquid diet. Although early liquid diet studies reported atrophic acinar cells undergoing necrosis (Wilborn and Schneyer 1970) and degenerating acinar cells (Hand and Ho 1981), those studies were performed before the notion of apoptosis was widely accepted; therefore, several of the reported necrotic or degenerating acinar cells might have been apoptotic acinar cells. Taken together with other reports that acinar cell apoptosis occurs in parotid gland atrophy induced by different conditions (Walker and Gobe 1987; Chisholm et al. 1995; Scott et al. 1999), acinar cell apoptosis appears to be essential to atrophic changes of salivary glands, regardless of the cause of atrophy. In this study, there were no differences in Casp-3 labeling index of acinar cells at day 21 of control and experimental groups, which suggests that parotid gland

atrophy gradually approaches a stable condition at 3 weeks of a liquid diet.

Stimulation of parasympathetic nerve activates acinar cell proliferation in parotid glands (Burlage et al. 2009); acetylcholine levels, which are proportional to parasympathetic nerve stimulation, become low in parotid glands made atrophic by liquid diet (Nakamura 1997). Considering these, reduced proliferative activity of acinar cells in atrophic parotid glands in our study could be due to decreased parasympathetic nerve stimulation caused by low masticatory stimulation. As the biological processes of apoptosis and cell proliferation are closely related (Chisholm et al. 1995), increased apoptotic acinar cells might be induced by decreased parasympathetic stimulation as well. Nakamura (1997) reported that wet weight and acetylcholine levels of parotid glands recovered to those of controls when animals were reintroduced to solid diets. Therefore, apoptosis and proliferation of acinar cells might return to control levels in such case. However, further study is necessary to clarify this question.

Increased apoptosis and decreased proliferation of acinar cells in the present study suggest that liquid diet leads to fewer acinar cells, causing rat parotid glands to atrophy. Although no studies report directly counting cells in this experimental model, Scott et al. (1990) speculated that acinar cells decrease because parotid gland DNA content is reduced in liquid diet-caused atrophy (Johnson 1982). Our data strongly support this speculation, and suggest that depletion of acinar cells is a factor in decreased parotid gland weight. Previous studies that use histomorphometric analysis (Scott et al. 1990; Scott and Gunn 1991) and TEM observations (Wilborn and Schneyer 1970; Hand and Ho 1981) have already shown that reduced parotid gland weight to be caused by the shrinkage of acinar cell; implying that liquid

diet-caused parotid gland atrophy results from both fewer and smaller acinar cells.

In conclusion, the present study shows liquid diet to promote apoptosis and reduce proliferation in parotid gland acinar cells of rats, leading to atrophy the parotid glands.

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References

- Burlage FR, Faber H., Kampinga HH, Langendijk JA, Vissink A, Coppes RP (2009) Enhanced proliferation of acinar and progenitor cells by prophylactic pilocarpine treatment underlies the observed amelioration of radiation injury to parotid glands. *Radiother. Oncol.* 90: 253-256.
- Chisholm DM, Adi MM, Ervine IM, Ogden GR (1995) Cell deletion by apoptosis during regression of rat parotid sialadenosis. *Virchows Arch.* 427, 181-186.
- Gratzner HG (1982) Monoclonal antibody to 5-bromo-and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science* 218, 474-475.
- Hall HD, Schneyer CA (1964) Salivary gland atrophy in rat induced by liquid diet. *Proc. Soc. Exp. Biol. Med.* 117, 789-793.
- Hand AR, Ho B (1981) Liquid-diet-induced alterations of rat parotid acinar cells studied by electron microscopy. *Arch. Oral Biol.* 26, 369-380.
- Hanihara K, Inoue N, Ito G, Kamegai T (1981) Microevolution and tooth to denture base discrepancy in Japanese dentition. *J. Anthropol. Soc. Nippon* 89, 63-70.
- Hughes J, Gobe G (2007) Identification and quantification of apoptosis in the kidney using morphology, biochemical and molecular markers. *Nephrology* 12, 452-458.
- Ito G, Mitani S, Kim JH (1988) Effect of soft diets on craniofacial growth in mice. *Anat. Anz.* 165, 151-166.
- Johnson DA (1982) Effect of a liquid diet on the protein composition of rat parotid saliva. *J.*

Nutr. 112, 175-181.

Kawamura Y (1972) Recent advances in the physiology of mastication. In: Emmelin N,

Zotterman Y (ed) Oral physiology, 1st edn. Pergamon Press, Oxford, pp163-204.

Kitagawa Y, Mitera K, Ogasawara T, Nojyo Y, Miyauchi K, Sano K (2004) Alterations in

enzyme histochemical characteristics of the masseter muscle caused by long-term soft diet

in growing rabbits. Oral Dis. 10, 271-276.

Maeda N, Suwa T, Ichikawa M, Masuda T, Kumegawa M (1990) Effects of easily chewable

diet and unilateral extraction of upper molars on the masseter muscle in developing mice.

Acta Anat. 137, 19-24.

Murakami K, Sasaki S, Takahashi Y, Uenishi K, Yamaraki M, Hayabuchi H, Goda T, Oka J,

Baba K, Ohki K, Kohri T, Muramatsu K, Furuki M (2007) Hardness (difficulty of

chewing) of the habitual diet in relation to body mass index and waist circumference in

free-living Japanese women aged 18-22y. Am. J. Clin. Nutr. 86, 206-213.

Nakamura K (1997) Effects of short-term bulk or liquid diet feeding on the neurotransmitters

in the salivary glands and various sialogogue-induced salivation in mice. Jpn. J. Oral Biol.

39: 655-664.

Scott J, Berry MR, Gunn DL, Woods K (1990) The effects of a liquid diet on initial and

sustained stimulated parotid salivary secretion and on parotid structure in the rat. Arch.

Oral Biol. 35, 509-514.

Scott J, Gunn DL (1991) A comparative quantitative histological investigation of atrophic

changes in the major salivary glands of liquid-fed rats. Arch. Oral Biol. 36, 855-857.

- Scott J, Gunn DL (1994) Functional characteristics of atrophic parotid acinar cells from rats after liquid feeding. *J. Dent. Res.* 73, 1180-1186.
- Scott J, Liu P, Smith PM (1999) Morphological and functional characteristics of acinar atrophy and recovery in the duct-ligated parotid gland of the rat. *J. Dent. Res.* 78: 1711-1719.
- Varrela L (1992) Dimensional variation of craniofacial structures in relation to changing masticatory-functional demands. *Eur. J. Orthod.* 14, 31-36.
- Walker NI (1987) Ultrastructure of the rat pancreas after experimental duct ligation. I. The role of apoptosis and intraepithelial macrophages in acinar cell deletion. *Am. J. Pathol.* 126, 439-451.
- Walker NI, Gobe GC (1987) Cell death and cell proliferation during atrophy of the rat parotid gland induced by duct obstruction. *J. Pathol.* 153, 333-344.
- Walker NI, Bennett RE, Kerr JFR (1989) Cell death by apoptosis during involution of the lactating breast in mice and rats. *Am. J. Anat.* 185, 19-32.
- Wilborn WH, Schneyer CA (1970) Ultrastructural changes of rat parotid glands induced by a diet of liquid metrecal. *Z. Zellforsch.* 103, 1-11.
- Yamamoto S (1996) The effects of food consistency on maxillary growth in rats. *Eur. J. Orthod.* 18, 601-615.

Legends

Fig. 1 The wet weights of parotid glands in the experimental (open bars, n=4) and control (filled bars, n=4) rats. Error bars are SEM; asterisks (*) show statistically significant differences ($P < 0.05$) between experimental and control rats.

Fig. 2 Histology (HE) (a-c) and immunohistochemistry for Casp-3 (d-f) and for BrdU (g-i). Control-Day 7 (a, d, g), Experimental-Day 7 (b, e, h), and Experimental-Day 14 (c, f, i). Scale bars=25 μ m. Acinar cells in experimental parotid glands (c) are smaller than in control glands (a). A structure like an apoptotic body (arrow) is identified in an experimental gland acinus (b). There are more Casp-3-positive acinar cells (arrows) in experimental glands (e, f) than in control glands (d). BrdU-positive acinar cells (arrows) are more frequently seen in control glands (g) than in experimental glands (h, i).

Fig. 3 Acinar cell Casp-3 (a) and BrdU (b) labeling indices in experimental (open bars, n=4) and control (filled bars, n=4) rats. Error bars are for SEM; asterisks (*) show statistically significant differences ($P < 0.05$) between experimental and control rats.

Fig. 4 TEM. (a) Experimental-Day 3. Nuclear fragments (N) and whorled rough endoplasmic reticulum (arrows) are identified in an apoptotic acinar cell. Bar=2 μ m (b) Experimental-Day 21. Partly degraded apoptotic body (arrow) is phagocytosed by an acinar cell. Bar=2 μ m







