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Hyperlipidemia is involved in apoptosis in rat submandibular glands

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ABSTRACT

Objective: The aim of the present study was to investigate the effects of hyperlipidemia on histological changes and apoptosis in submandibular glands using apolipoprotein E (apoE)-deficient rats.
Design: Histopathological findings related to induced apoptosis in the submandibular glands were compared between apoE-deficient rats (n = 6; male; age, 16 weeks) and the corresponding wild-type rats (n = 6).
Results: ApoE-deficient rats showed significantly higher plasma levels of oxidized low-density lipoprotein (LDL), total cholesterol, very LDL and LDL, and lower plasma levels of high-density lipoprotein when compared to control rats ($P < 0.05$). Lipid deposition in the submandibular gland was observed in apoE-deficient rat group and in none of the control group. Significant increases in vacuolization and apoptosis in acinar cells were observed in apoE-deficient rats, as compared to control rats ($P < 0.05$). The number of active caspase-3-positive cells was also higher in the apoE-deficient rat group when compared with the control group ($P < 0.01$).
Conclusions: According to our results, hyperlipidemia induced apoptosis in apoE-deficient rat submandibular glands. Oxidized LDL generation in case of hyperlipidemia may trigger off a reaction of apoptotic acinar cells with vacuolization in the submandibular glands.

1. Introduction

Salivary glands play essential roles through production of saliva, which contributes to the maintenance of oral and gastrointestinal tract function. However, salivary gland function is impaired by aging, inflammation, medication and autoimmune diseases (Nederfors, 2000). The impairment of salivary gland function is associated with a large variety of debilitating oral symptoms, including xerostomia, increased susceptibility to dental caries, oral mucosal infections, poor oral health and reduced digestion of starch by amylase (Ogawa et al., 2013; Vissink, Jansma, Spijkervet, Burlage, & Coppes, 2003). Thus, understanding the mechanisms of salivary gland dysfunction is important for maintaining oral and systemic health.

Hyperlipidemia is characterized serologically by elevated levels of plasma triglyceride and cholesterol, particularly low density lipoprotein (LDL). In patients with hyperlipidemia, a close relationship between high plasma lipid levels and salivary gland dysfunction has been reported (Lukach, Maly, Zini, & Aframian, 2013). In addition, salivary flow is impaired among patients with hyperlipidemia, and these patients have salivary gland swelling (Izumi et al., 2000). From these observations, hyperlipidemia appears to contribute to development of salivary gland dysfunction.

Hyperlipidemia may directly influence disease induction and indirectly modulate inflammation and immune response (Shamshiev et al., 2007). Accumulation of lipids has various biological effects involving inflammation, cell proliferation, immune response and cell apoptosis (Augé et al., 1998; Bourcier, Sukhova, & Libby, 1997; Cominacini et al., 2000). In particular, since apoptosis is involved in salivary glands dysfunction (Choi, Park, Kim, Lim, & Kim, 2013a; Choi, Park, Kim, Lim, & Kim, 2013b), better understanding the effects of hyperlipidemia on apoptosis would be important to see through the relationship between hyperlipidemia and salivary glands dysfunction. However, no literature is available with regard to the effects of hyperlipidemia on apoptosis in salivary glands.

The apolipoprotein E (apoE)-deficient animal model is accepted to induce experimental hyperlipidemia (Bagavant et al., 2011). In the

Abbreviations: ApoE, apolipoprotein E; HDL, high-density lipoprotein; LDL, low-density lipoprotein; oxLDL, oxidized LDL; TUNEL, TdT-mediated dUTP Nick-End Labeling; VLDL, very LDL

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present work, we hypothesized that hyperlipidemia may induce apoptosis in salivary glands. Therefore, the purpose of this study was to investigate the effects of hyperlipidemia on histopathological changes and apoptosis in submandibular glands using apoE-deficient rats.

2. Material and methods

2.1. Animals

Control (wild-type) and hyperlipidemic (apoE-deficient) rats (all Sprague-Dawley background; n = 6 each; age, 15 weeks) were obtained from Sigma Laboratory (St. Louis, MO). All experimental procedures were performed in compliance with guidelines approved by the Animal Research Control Committee of Okayama University (OKA-2011004). Animals were maintained under standard conditions and were given free access to food (MF; Oriental Yeast Co. Ltd., Osaka, Japan) and drinking water. After observation for one week, collection of blood, sacrificing of rats, and submandibular gland isolation were performed as described previously (Irie et al., 2011).

2.2. Analysis of blood samples

Blood samples were collected from the heart at age 16 weeks. Blood was allowed to clot at room temperature, and plasma was separated by centrifugation at 1500 × *g* for 15 min. Levels of plasma oxidized LDL (oxLDL) were measured using an enzyme-linked immunosorbent assay kit (Cusabio Biotech Co., Ltd., Wuhan, China) (Ekuni et al., 2012). The cholesterol and phospholipid profiles in plasma lipoproteins were analyzed using a gel permeation high-performance liquid chromatography system at Skylight Biotech (Akita, Japan) (Miida et al., 2008). We quantified individual subfractions using best curve fitting analysis, assuming that the particle sizes of all subfractions followed a Gaussian distribution. Particle sizes for individual subfractions were determined as 30–80 nm (very LDL [VLDL]), 16–30 nm (LDL) and 8–16 nm (high-density lipoprotein [HDL]).

2.3. Histological analysis

For histological analysis, submandibular glands were resected *en bloc* from each rat and were immediately fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 1 day. Paraffin-embedded sections (4 μm) were stained with hematoxylin and eosin (HE).

In order to detect apoptotic cells in the submandibular glands, the TdT-mediated dUTP Nick-End Labeling (TUNEL) method was used. Briefly, sections were incubated with 2% H_2O_2 for 30 min, and were treated with 0.02 mg/mL proteinase K (Sigma Chemical Co., St. Louis, MO) for 10 min at room temperature. After rinsing, sections were incubated with a mixture of TdT enzyme and fluorescein-dUTP (Takara Bio Inc., Shiga Japan) for 60 min at 37 °C. Color was developed with 3,3′-diaminobenzidine tetrahydrochloride. Sections were counterstained with Mayer's hematoxylin.

Immunostaining of active caspase-3 was performed using a commercial kit (Histofine Simple Stain MAX PO; Nichirei Co., Tokyo, Japan). Monoclonal antibodies against active caspase-3 (Abcam, Tokyo, Japan) were diluted at 1/100 in phosphate buffered saline. Color was developed by placing sections in a solution of 3-3′-diaminobenzidine tetrahydrochloride. Sections were counterstained with Mayer's hematoxylin.

A single examiner (K. I.), blinded to the group assignment, performed the following histometric analyses using a light microscope (Olympus Co., Tokyo, Japan). Vacuolization of the submandibular gland was scored as follows: < 25% = 1+; 50% = 2+; < 75% = 3+; and > 75% = 4 (Ekuni et al., 2010). A mean value representing apoptotic cellsand active caspase-3-positive cells was determined at a magnification of ×400 in 15 randomly selected fields in each gland section (Nakamura-Kiyama et al., 2014). We evaluated intra-examiner reproducibility by double-scoring 10 randomly selected sections at two-week intervals. Intra-examiner agreement with active caspase-3-positive cells was greater than 80%.

2.4. Deposition of lipid in submandibular gland

Frozen sections (8 μm) were obtained from the submandibular gland, embedded in Optimal Cutting Temperature compound (Tissue Tec, Naperville, IL) and stained with oil red O in order to detect lipid deposition (Ekuni et al., 2012).

2.5. Statistical analysis

All data are expressed as means ± S.D. Comparisons between the hyperlipidemia and control groups were performed by independent *t*-test and Fisher's exact test using a statistical software package (SPSS 17.0J for Windows, SPSS Japan, Tokyo, Japan). $P < 0.05$ was considered to be statistically significant.

3. Results

No significant differences were observed between the control and hyperlipidemia groups with regard to food consumption, body weight or growth pattern during the 7-day period. There was also no significant difference in the submandibular glands weight between the two groups at 7 days.

Plasma levels of total cholesterol, VLDL cholesterol and LDL cholesterol were also higher in the hyperlipidemia group when compared to the control group ($P < 0.05$; Fig. 1). Plasma levels of oxLDL were also significantly higher in the hyperlipidemia group when compared to the control group ($P < 0.05$). On the other hand, plasma HDL cholesterol levels were lower in the hyperlipidemia group when compared to the control group ($P < 0.05$).

Lipid deposition in the submandibular gland was observed in hyperlipidemia group and in none of the control group (Fig. 2a, b). In addition, when compared with the control group, vacuolization of acinar cells was markedly detected in the hyperlipidemia group ($P < 0.05$; Fig. 2c, d, and Table 1).

The number of TUNEL-positive acinar cells was significantly higher in the hyperlipidemia group than in the control group ($P < 0.01$; Fig. 3d–f). To further confirm the involvement of apoptosis, we stained the submandibular gland with active caspase-3 antibodies. The number of active caspase-3-positive cells was higher in the hyperlipidemia group when compared with the control group ($P < 0.01$; Fig. 3a–c). Similar to the results with a TUNEL assay, the staining of active caspase-3 was less intense compared with that in the TUNEL assay.

In both groups, there were no obvious infiltrating inflammatory cells in the submandibular glands in HE-stained sections.

4. Discussion

This is the first known histopathological study to evaluate the effects of hyperlipidemia in rat submandibular glands. In this study, the apoE-deficient rats showed higher plasma levels of total cholesterol, LDL cholesterol and VLDL cholesterol, and lower plasma levels of HDL cholesterol when compared to control rats. These observations confirm that the apoE-deficient rats exhibited hyperlipidemia. The results also revealed lipid deposition in the submandibular gland was observed in the hyperlipidemia group and significantly greater levels of apoptosis and vacuolization in acinar cells in the hyperlipidemia group. Taken together, hyperlipidemia affects structural changes in rat submandibular glands.

Our histopathological findings showed significant increases in vacuolization, TUNEL-positive acinar cells and active caspase-3 in the hyperlipidemia group. Caspase-3 is a member of the caspase family, which are sequentially activated and play a central role in the