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Vitamin C intake inhibits serum lipid peroxidation and osteoclast differentiation on alveolar bone in rats fed on a high-cholesterol diet

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ABSTRACT

Objective: A high-cholesterol diet stimulates osteoclast differentiation, which may be induced by increased serum lipid peroxidation. The inhibition of serum lipid peroxidation by vitamin C may offer beneficial effects on osteoclast differentiation including increased expression of receptor activator of nuclear factor (NF)-κB ligand (RANKL) and NF-κB. This study investigated the effects of vitamin C intake on RANKL and NF-κB expression in periodontal tissue of rats fed a high-cholesterol diet.

Design: Twenty-four rats (8 weeks old) were divided into four groups: a control group (fed a regular diet) and three experimental groups (fed a high-cholesterol diet supplemented with 0, 1 and 2 g/l vitamin C/day) in this 12-week study. Vitamin C was provided by its addition to drinking water. As an index of serum lipid peroxidation, hexanoyl-lysine (HEL) level was determined by a competitive enzyme-linked immunosorbent assay method. Immunohistological analysis was performed to evaluate RANKL and NF-κB expression on the alveolar bone surface. The number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts was also counted.

Results: Feeding a high-cholesterol diet increased not only the serum HEL level but also the number of TRAP-positive osteoclasts on the alveolar bone surface, with an increase in RANKL and NF-κB expression on alveolar bone surface. Intake of vitamin C reduced the serum HEL level and osteoclast differentiation, with decreasing RANKL and NF-κB expression.

Conclusions: Vitamin C intake could suppress osteoclast differentiation, including RANKL and NF-κB expression on the alveolar bone surface, by decreasing serum lipid peroxidation in rats fed a high-cholesterol diet.

1. Introduction

Feeding a high-cholesterol diet increases the linear distance between the cemento-enamel junction (CEJ) and alveolar bone crest (ABC) in rats.[1] This indicates that a high-cholesterol diet is a risk factor for alveolar bone resorption. Because alveolar bone resorption is a major contributor to the progression of periodontal diseases,[2] it is feasible that a high-cholesterol diet is detrimental to periodontal health, supposedly through its stimulation of osteoclastic activity.

Animal studies have shown that feeding a high-cholesterol diet increases the serum level of lipid peroxidation.[3] Lipid

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peroxidation, which refers to the oxidative degeneration of lipids, plays a crucial role in the pathogenesis of various diseases, including bone resorption in osteoporosis.[4] Previously, an *in vitro* study demonstrated that lipid peroxides can stimulate expression of receptor activator of nuclear factor (NF)-κB (RANK) ligand (RANKL) in marrow haematopoietic cells,[5] which induces the differentiation of osteoclasts through NF-κB activation in osteoclast precursors.[6,7] Increases in serum lipid peroxidation, following feeding of a high-cholesterol diet, may promote alveolar bone resorption via up-regulation of RANKL and NF-κB expression in periodontal tissue.

Vitamin C is the main hydrophilic antioxidant in blood and acts primarily by scavenging superoxide and other reactive oxygen species.[8–10] Our previous study reported that vitamin C intake suppressed alveolar bone loss induced by a high-cholesterol diet.[11] We also demonstrated that anti-oxidative effect of vitamin C against gingival oxidative damage was useful to prevent alveolar bone loss in hyperlipidemic rats.[11] In that case, it is possible that vitamin C intake decreases gingival oxidative damage as a result of diminishment of lipid peroxidation in blood. However, this information remains unknown. In addition, it is unclear how vitamin C suppresses osteoclast differentiation following gingival oxidative damage induced by a high-cholesterol diet. In vitro studies have been suggested that vitamin C has an influence on RANKL and NF-κB expression.[12,13] Therefore, it is possible that vitamin C intake affects osteoclast differentiation through modulating RANKL and NF-κB expression.

In the present work, we hypothesized the inhibition of serum lipid peroxidation by vitamin C might suppress osteoclast differentiation through down-regulating RANKL and NF-κB expression. The purpose of the present study was to investigate the effects of vitamin C intake on RANKL and NF-κB expression on the alveolar bone surface in rats fed a high-cholesterol diet. In addition, it is known that osteoprotegrin (OPG) acts as a decoy receptor for RANKL to compete against RANK,[14] as well as inhibiting osteoclast formation by directly binding to an OPG ligand expressed on osteoblasts.[15] Therefore, OPG expression on the alveolar bone surface was also evaluated.

2. Materials and methods

2.1. Animals

Twenty-four male Wister rats (8 weeks old) were used in this study. Rats were housed two per cage in rooms maintained at 23–25 °C with 12-h light–dark cycles; the lights were turned off daily from 6:00 p.m. to 6:00 a.m. All animal experiments complied with guidelines approved by the Animal Research Control Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences.

2.2. Experimental design

Rats were allocated randomly using a random number table to one of four groups (one control and three experimental). In the experimental groups, rats were fed a diet containing 1% cholesterol (w/w) and 0.5% cholic acid (w/w) (Oriental Yeast Co., Tokyo, Japan) for 12 weeks.[11] The Control group was fed a regular diet for 12 weeks. One of the experimental groups (Cholesterol group) and the Control group were given distilled water, and the remaining two groups [Vitamin C (1 g/l) group and Vitamin C (2 g/l) group] received distilled water containing either 1 or 2 g/l vitamin C (Sigma Chemical Co., St. Louis, MO, USA).[11] Drinking water was prepared fresh daily.

2.3. Blood chemistry

At 12 weeks, blood samples were collected directly from the heart of 24-h-fasted rats. Blood was allowed to clot and serum samples were separated by centrifugation at $1500 \times g$ for 15 min. The concentration of hexanoyl-lysine (HEL) (an index of lipid peroxidation) was determined with an enzyme-linked immunosorbent assay kit (Japan Institute for the Control of Aging, Shizuoka, Japan).[16]

2.4. Histological and immunohistochemical analyses

After the experimental period, the animals were sacrificed under general anaesthesia. The left maxillary molar samples were resected from each rat and immediately fixed with 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4) for 1 day. The tooth and gingival samples were decalcified further with 10% tetrasodium-EDTA aqueous solution (pH 7.4) for 2 weeks at 4 °C. The paraffin-embedded bucco-lingal sections (4 μm) of each tooth were stained with haematoxylin and eosin or other immunohistochemical stains as described below.

RANKL, OPG and NF-κB were stained using a commercial kit (Histofine Simple Stain MAX PO; Nichirei Co., Tokyo, Japan). Polyclonal antibodies against RANKL (Santa Cruz Biotechnology, Santa Cruz, CA, USA), OPG (Santa Cruz Biotechnology) and NF-κB (Abcam, Cambridge, UK) were diluted to 1/100 in phosphate-buffered saline. The colour was developed with 3-3′-diamino benzidine tetrahydro-chloride. In addition, to identify osteoclasts, tartrate-resistant acid phosphatase (TRAP) activity was detected using the azo dye method.[17] Sections were counterstained with Mayer's haematoxylin.

Histological measurements were performed using a microscope at a magnification of 400 by one examiner, who was blinded to the treatment assignment. Tissue sections stained with haematoxylin and eosin were used to evaluate the degree of alveolar bone resorption. The linear distance between CEJ and ABC were measured.[1,18] RANKL-, OPG- and NF-κB-positive cells occurring along the edge of the alveolar bone surface, as well as total cells and TRAP-positive osteoclasts, were counted and reported as number/mm.[11]

2.5. Statistical analysis

Kruskal–Wallis test and nonparametric Tukey-type multiple comparisons were performed with the use of a statistical software package (SPSS 13.0J for Windows; SPSS Japan, Tokyo, Japan). Sample size for Kruskal–Wallis test was calculated by the nQuery Advisor (Statistical Solutions, Saugus, MA, USA), based on the results of our previous results.[1] As a result, sample size of 6 *per* group was required for detection of a