J Periodont Res 2009; 44: 43–51

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2007.01060.x

Oxidative stress causes alveolar bone loss in metabolic syndrome model mice with type 2 diabetes

T. Ohnishi[1], K. Bandow[1], K. Kakimoto[1], M. Machigashira[2], T. Matsuyama[2], T. Matsuguchi[1]

[1]Division of Biochemistry and Molecular Dentistry, Department of Developmental Medicine, Kagoshima University, Graduate School of Medical and Dental Sciences, Kagoshima, Japan and [2]Department of Periodontology, Kagoshima University, Graduate School of Medical and Dental Sciences, Kagoshima, Japan

Ohnishi T, Bandow K, Kakimoto K, Machigashira M, Matsuyama T, Matsuguchi T. Oxidative stress causes alveolar bone loss in metabolic syndrome model mice with type 2 diabetes. J Periodont Res 2009; 44: 43–51. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard

Background and Objective: Alveolar bone loss is caused by a host response to periodontal pathogens, and its progression is often enhanced by systemic conditions such as insulin resistance.Alveolar bone dehiscence has been observed in KK-A^y mice, which are metabolic syndrome model mice with type 2 diabetes. The aim of this study was to investigate inducements responsible for alveolar bone dehiscence in the KK-A^y mice.

Material and Methods: The expression of endothelial nitric oxide synthase in the mandibles of mice was detected using immunohistochemical staining and the reverse transcription–polymerase chain reaction. After administration of *N*-acetylcysteine, an antioxidant, to KK-A^y mice, alveolar bone loss and the expression of endothelial nitric oxide synthase protein in gingival keratinocytes and of hydrogen peroxide concentrations in plasma, were analyzed. The effect of hydrogen peroxide on endothelial nitric oxide synthase expression in keratinocytes was examined using cultured keratinocytes.

Results: The expression of endothelial nitric oxide synthase was decreased in gingival keratinocytes from KK-A^y mice compared with gingival keratinocytes from control mice. Administration of *N*-acetylcysteine to the mice restored endothelial nitric oxide synthase expression in the gingival keratinocytes, suppressed the alveolar bone loss and decreased the hydrogen peroxide concentrations in plasma without the improvement of obesity or diabetes. *In vitro*, stimulation with hydrogen peroxide decreased the expression level of endothelial nitric oxide synthase in cultured keratinocytes, which was restored by the addition of *N*-acetylcysteine.

Conclusion: Reactive oxygen species, such as hydrogen peroxide, are responsible for the alveolar bone loss accompanied by decreased endothelial nitric oxide synthase expression in KK-A^y mice. Therefore, we propose a working hypothesis that the generation of oxidative stress is an underlying systemic condition that enhances alveolar bone loss in periodontitis occurring as a complication of diabetes.

Tetsuya Matsuguchi, Division of Biochemistry and Molecular Dentistry, Department of Developmental Medicine, Kagoshima University, Graduate School of Medical and Dental Sciences, 35-1 Sakuragaoka 8, Kagoshima, Japan
Tel: +81 99 275 6131
Fax: +81 99 275 6138
e-mail: tmatsugu@denta.hal.kagoshima-u.ac.jp

Key words: diabetes; metabolic syndrome; endothelial nitric oxide synthase; reactive oxygen species

Accepted for publication October 16, 2007

The metabolic syndrome is characterized by a cluster of related cardiovascular metabolic risk factors, including various combinations of glucose tolerance, hypertension and hyperlipemia. The metabolic syndrome was initially described as a disorder of insulin resistance (1), which is closely associated with type 2 diabetes, and is also called the insulin resistance syndrome by the European Group for the Study of Insulin Resistance (2). The

development of obesity generally precedes the onset of insulin resistance in a person with the metabolic syndrome. KK-A^y mice, established as a type 2 diabetes mouse model accompanied by obesity (3), are considered to be an appropriate mouse model of the syndrome because the mice develop insulin resistance and cardiovascular risk factors such as hypertension, hyperlipemia and glucose tolerance (4,5).

The metabolic syndrome is associated with oxidative stress in various tissues. The accumulation of fat in adipose tissue increases the generation of oxidative stress (6). The mechanical stress to vascular cells, which is caused by hypertension, stimulates superoxide generation by nicotinamide adenine dinucleotide phosphate-oxidase through protein kinase C activation (7). Activity of protein kinase C is also augmented by increased diacylglycerol levels in many tissues from diabetic model animals, and neutrophils from diabetic patients produce more superoxide than neutrophils from normal subjects (8,9). Simultaneously, a high glucose condition occasionally increases the generation of mitochondrial superoxide (10).

Periodontal disease, which affects at least 50% of adults worldwide, occasionally develops as one of the complications of diabetes. The relationship between diabetes and periodontal disease has been evaluated in numerous reports. Patients with poor glycemic control have an increased risk for periodontitis (11,12). Periodontitis is caused by a host response stimulated by pathogenic microflora 'dental plaque' on the tooth (13). The presence of the dental plaque as a pathogen is requisite for the development of periodontitis, and the host response to oral bacteria destroys the periodontal tissues, including alveolar bone (14,15). When periodontitis develops, the local production of interleukin-8 and granulocyte–macrophage colony-stimulating factor recruits and activates neutrophils, leading to the increased production of reactive oxygen species in the periodontal tissues (16–18). On the other hand, the direct relationship between reactive oxygen species and bone metabolism was described in a study reporting that antioxidant ameliorates ovariectomy-induced bone loss in normal mice (19).

In the present study, we performed histological analyses of the periodontal tissues from a metabolic syndrome model mouse strain, KK-A^y, and found alveolar bone loss in the mandibles. We then examined the protein expression of endothelial nitric oxide synthase in the periodontal tissues of the KK-A^y mice, as deficiency of endothelial nitric oxide synthase has been reported to cause a defect in bone formation *in vivo*, suggesting that endothelial nitric oxide synthase regulates bone formation (20,21). We found significantly decreased levels of endothelial nitric oxide synthase in the gingival epithelium of KK-A^y mice, which was restored by the administration of an antioxidant, *N*-acetylcysteine, *in vivo*. As the production of nitric oxide is reported to be regulated by the expression level of endothelial nitric oxide synthase under high glucose conditions (22), we examined the effect of *N*-acetylcysteine administration on the expression of endothelial nitric oxide synthase in the gingiva of KK-A^y mice. In consequence, we proposed a hypothesis that that alveolar bone loss in a mouse model of the metabolic syndrome is caused by oxidative stress through the decreased expression of endothelial nitric oxide synthase in the gingival epithelium.

Material and methods

Animals

Seven-week-old male KK-A^y and C57BL/6 mice were purchased from Clea Japan Inc. (Tokyo, Japan). These mice were housed in a room under controlled temperature (23 ± 2°C) and had free access to water and normal food (CRF-1) (Charles River Laboratory Japan, Yokohama, Japan), with or without 1.2% *N*-acetylcysteine, until the age of 20 wk. Four to six mice were used in each group for the following experiments. Glucose levels in urine were semiquantitatively analyzed using Pretest™ (Wako Pure Chemical Industry, Osaka, Japan). The concentration of glucose in mouse sera was measured using glucose™ (Trinder; Sigma, St Louis, MO, USA). Mandibles were dissected from male C57BL/6 and KK-A^y mice of 7 and 20 wk of age, and treated with 4 M guanidium hydrochloridefor 10 d to remove soft tissues or fixed with 3.3% formaldehyde for 2 d. Animal experiments were conducted in accordance with the institutional guidelines for the care and use of laboratory animals. Plasma levels of hydrogen peroxide were measured using an Amplex Red hydrogen peroxide assay kit (Invitrogen Corp., Carlsbad, CA, USA) (6).

Immunohistochemistry

Dissected mandibles from control and KK-A^y mice were fixed and decalcified with 0.4 M EDTA, containing 1% formaldehyde, for 3 wk. Sections were prepared from paraffin-embedded blocks. After the treatment with or without proteinase K at 37°C for 20 min to retrieve the epitope, sections were blocked with 1% bovine serum albumin for 1 h at room temperature and then incubated with 2 μg/mL of antibody to endothelial nitric oxide synthase (C-2) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 1% bovine serum albumin-containing phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(−)] for 24 h at 4°C. After three washes with PBS(−), the sections were incubated with anti-rabbit IgG in 1% bovine serum albumin-containing PBS(−) for 24 h at 4°C. After a further three washes, color development was performed with diaminobentizine. Counterstaining was performed using hematoxylin and eosin.

Reverse transcription–polymerase chain reaction

Total RNA was extracted from the gingiva dissected from the mandibles of KK-A^y and control mice at 20 wk of age. Reverse transcription was performed using oligo dT, as previously described (23). Subsequent amplification for the detection of mouse endothelial nitric oxide synthase mRNA was carried out using the polymerase chain reaction (PCR) over 35 cycles of

95°C for 30 s, 55°C for 30 s and 72°C for 60 s using endothelial nitric oxide synthase primers that were designed based on the sequence of cDNA for mice endothelial nitric oxide synthase (NM008713). The primers used were 5′-CCTTCCGCTACCAGCCAGA-3′ and 5′-CAGAGATCTTCACTGCATTGGCTA-3′, generating a 105-bp fragment (24). β-Actin was also amplified as an internal control over 25 cycles under the conditions described previously (23). The primers used were 5′-GTGGGCCGCTCTAGGCACCAA-3′ and 5′-CTCTTTGATGTCACGCACGATTTC-3′ (25). The PCR products were analyzed by 1.8% (w/v) agarose-gel electrophoresis, and the intensities of the bands were analyzed using BIO-PROFIL (Vilber Lourmat, Marne-La-Vallee, France). The relative values of endothelial nitric oxide synthase mRNA by reverse transcription-PCR (RT-PCR) were normalized with respect to β-actin mRNA.

Stimulation of Pam 212 cells by hydrogen peroxide

Pam 212 cells, a mouse keratinocyte cell line from the American Type Culture Collection (Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's minimal essential medium, containing 10% fetal calf serum, and then stimulated with various concentrations of hydrogen peroxide for 5, 12, or 24 h. Proteins were extracted from the stimulated cells using PLC lysis buffer (10mM Tris-HCl, pH 7.5, 5mM EGTA, 150mM NaCl, 1% Triton X-100, 10% glycerol and 1mM sodium vanadate) containing protease inhibitors.

Assessment of alveolar bone loss by digital histomorphometry

Mouse mandibles were treated with 4 M guanidine hydrochloride for 1 wk to remove the soft tissue. Measurement of the alveolar bone loss areas located on a proximal root of the first molar was performed using a stereomicroscope with a digital camera system (SMZ-2T and digital sight DS-L; Nikon Corp., Tokyo, Japan) and a periodontal probe as an indicator of length. The mandible bone images were captured under ×17 magnification. We determined two apical positions of interdental septa between the first and the second molars, and interradicular septa between a buccal proximal root and a buccal mesial root of the first molar, of mandibular bone. The two apical positions were connected by a straight line. Then, we drew a curved line on the edge of the alveolar bone at the position of first molar. We determined dimensions of the area framed by the straight line and the curved line on the edge. More specifically, alveolar bone loss was determined as a triangular area located on the proximal root of the first molar, which is surrounded by the alveolar bone between the first and second molars of the lower jaw, and interradicular bone between the buccal distal and buccal mesial roots.

Western blot analysis

Gingiva and brain (as a positive control) were removed from C57BL/6 mice and lysed by homogenization in PLC buffer. Extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to western blotting analysis with antibody to endothelial nitric oxide synthase.

Results

Alveolar bone loss of mandibles from KK-A^y mice.

We maintained KK-A^y and control mice until 20 wk of age on a normal food diet. The mean weight of KK-A^y mice was 45 ± 2.0 g (standard deviation), which was approximately 1.5 times higher than the mean weight of control mice. We dissected mandibles from these mice and removed soft tissues using guanidium hydrochloride. Bone dehiscence was apparently observed in alveolar bones from all the KK-A^y mice examined, whereas the location of molars and the size of mandible bones of KK-A^y mice were similar to those of their normal counterparts (Fig. 1A).

We next performed histological analyses of the first molar and the periodontal tissues of mandibles from control and KK-A^y mice. On hematoxylin and eosin staining of the frontal serial sections of the mandibles, the gingival epithelial attachment to the surface of teeth was found to be located at the cemento–enamel junction of both mesial and distal edges of the first molars in both strains of mice (Fig. 1C). By contrast, apical migration of the gingival epithelial attachment was observed at the buccal proximal root of the first molars of KK-A^y mice (the lower fifth panel from the left in Fig. 1C) compared with that of the control mice (Fig. 1C). Although the alveolar bone loss and apical migration of gingival attachment in the KK-A^y mice were similar to the typical histological findings of periodontitis, inflammatory responses, such as the invasion of inflammatory cells, were absent in the periodontal tissues of KK-A^y mice (Fig. 1C).

The glucose levels in urine from KK-A^y mice were usually > 500 mg/dL after 8 wk of age, whereas they were always under 50 mg/dl for control mice (data not shown). Increases of blood glucose levels were apparent in KK-A^y mice from 9 wk of age (Fig. 2A, lower panel), which was consistent with the high urine glucose levels in KK-A^y mice. These data indicated that diabetes was not apparent at 7 wk of age, when little loss of alveolar bones was observed in KK-A^y mice (Fig. 2B). Alveolar bone loss was quantified by measuring the area of bone dehiscence located on the proximal root of the first molar (Fig. 2C) because the loss of attachment of gingival epithelium was localized on the proximal root (Fig. 1B). Quantification of bone loss confirmed that the alveolar bone loss progressed after the development of diabetes (Fig. 2B,D).

Reduction of endothelial nitric oxide synthase expression in gingival keratinocytes of KK-A^y mice

As endothelial nitric oxide synthase is involved in bone remodeling and its expression is reported to be down-regulated under diabetic conditions (22), we examined endothelial nitric oxide synthase expression in the periodontal tissues of KK-A^y mice. The endothelial

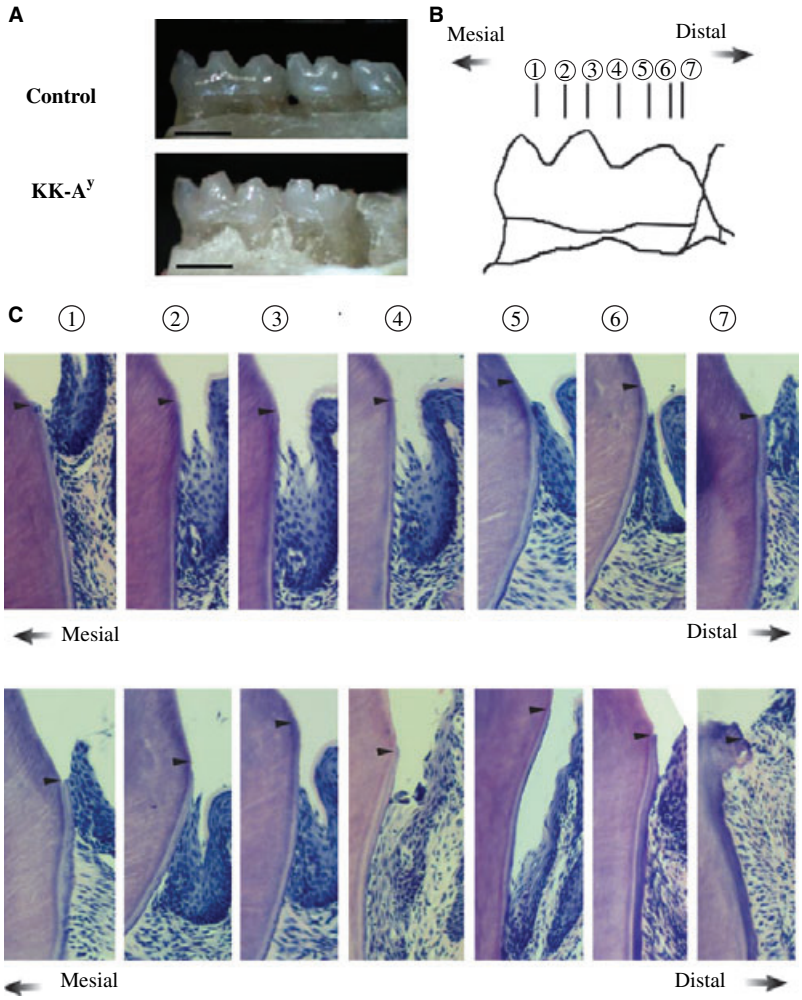

Fig. 1. Periodontal tissues in diabetic model mice. (A) Mandibles were extracted from male control and KK-A^y mice at 20 wk of age, and the soft tissues were removed. Bars indicate the length of 1 mm. (B) Location of the histological section of periodontal tissues in (C). (C) The extracted mandibles were decalcified with 0.5 M EDTA. Serial sections of the first molars and the periodontal tissues from control (upper panel) and KK-A^y mice (lower panel) were stained with hematoxylin and eosin. Arrowheads indicate the cemento–enamel junction.

nitric oxide synthase protein expression level in gingival keratinocytes of KK-A^y mice was significantly lower than in the gingival keratinocytes of control mice, whereas endothelial nitric oxide synthase was expressed at similar levels in the endothelial cells of both mouse strains (Fig. 3A–D). In order to confirm this finding, we then performed partially quantitative analysis of endothelial nitric oxide synthase mRNA in gingiva of both mouse strains by RT-PCR (Fig. 3E). The endothelial nitric oxide synthase mRNA expression levels in gingival specimens (lanes 6 and 8) from KK-A^y mice were higher than those in the other specimens from KK-A^y mice. The gingival specimens probably contain other cell types, such as endothelial cells, in which endothelial nitric oxide synthase expression was similar between KK-A^y and control mice. Although RNA samples were isolated from gingival tissues, including endothelial cells, we still found a statistically significant decrease of endothelial nitric oxide synthase mRNA expression in the gingivae from KK-A^y mice compared with those from control mice (Fig. 3F).

Inhibition of endothelial nitric oxide synthase expression in keratinocytes by reactive oxygen species

Reactive oxygen species play a causal role in insulin resistance (26). The production of reactive oxygen species, such as superoxide and hydrogen peroxide, was reported to increase in type 2 diabetic model mice (6). To explore the possible involvement of reactive oxygen species in the regulation of endothelial nitric oxide synthase expression in keratinocytes, we stimulated a mouse keratinocyte cell line, Pam 212 with hydrogen peroxide. We detected a 135-kDa molecule as endothelial nitric oxide synthase in mouse brain and gingiva extracts, and in keratinocytes, by western blot analysis followed by probing with the endothelial nitric oxide synthase antibody. A 90-kDa molecule in keratinocytes appeared to be nonspecifically recognized by the endothelial nitric oxide synthase antibody. We found that hydrogen peroxide significantly decreased endothelial nitric oxide synthase expression in a time-dependent manner (Fig. 4). The maximum reduction (26%) was observed at 8 h and the expression level remained decreased at least until 24 h after the hydrogen peroxide stimulation (Fig 4).

Pretreatment with an antioxidant, *N*-acetylcysteine, abrogated the decrease of endothelial nitric oxide synthase by hydrogen peroxide (Fig. 4C). To examine if the antioxidant restored the endothelial nitric oxide synthase expression in gingival keratinocytes of KK-A^y mice *in vivo*, we maintained KK-A^y and control mice on an *N*-acetylcysteine -containing diet from 8 wk of age. After 12 wk of the *N*-acetylcysteine-containing diet, the lower jaws were extracted and the decalcified sections were stained with endothelial nitric oxide synthase antibody. The administration of *N*-acetylcysteine-containing food restored endothelial nitric oxide synthase expression in gingival keratinocytes (Fig. 5). In addition, we measured hydrogen peroxide levels in plasma to confirm reduction of the reactive oxygen species level *in vivo* by the administration of *N*-acetylcysteine. The levels of hydrogen peroxide in the plasma of KK-A^y mice were significantly higher than in control mice and were decreased by the administration of *N*-acetylcysteine-containing food. (Fig. 6A).

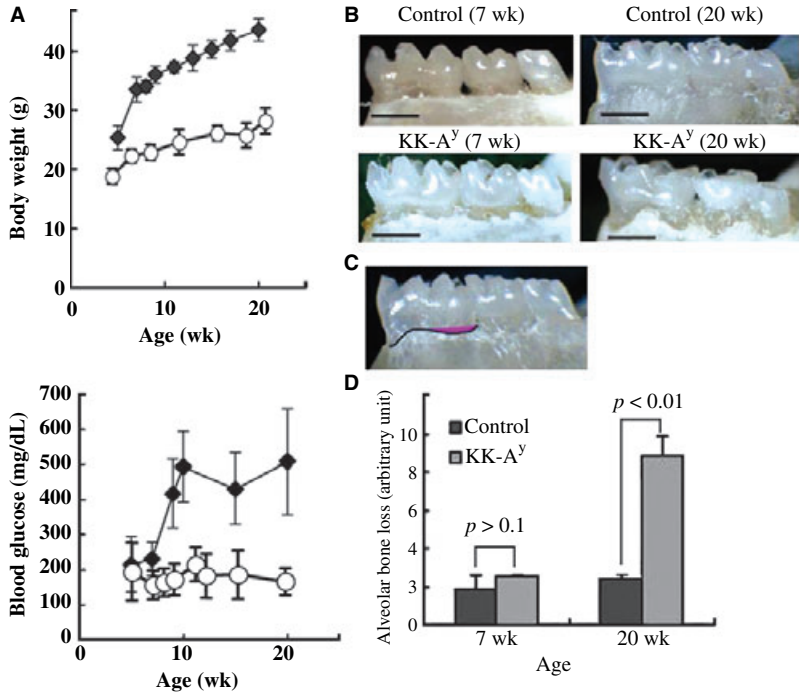

Fig. 2. Progress of alveolar bone loss and increase of blood glucose levels. (A) Body weights (upper panel) and blood glucose levels (lower panel) of control mice and KK-A^y mice were measured at the time-points indicated. (B) Mandibles were extracted from male control or KK-A^y mice at 7 and 20 wk of age, and the soft tissues were removed. Bars indicate the length of 1 mm. (C) The edge of alveolar bone is indicated as a black line and the purple area defines the alveolar bone loss. (D) Alveolar bone loss at the proximal areas of the first molars was measured in control and KK-A^y mice at 7 and 20 wk of age, as described in the Material and methods (results are given as means ± standard deviation, $n = 4$).

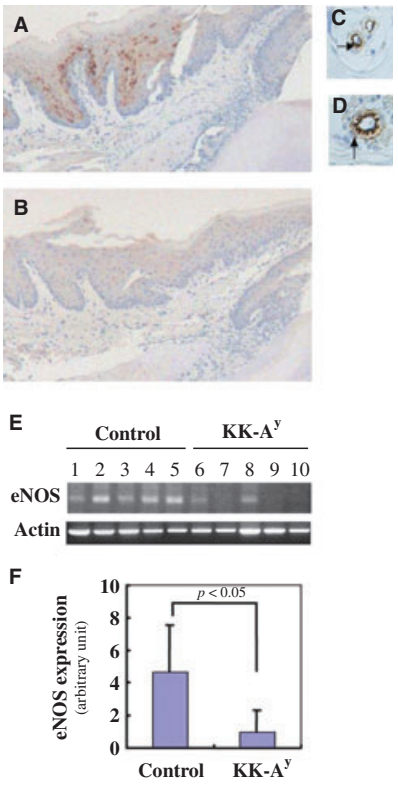

Fig. 3. Endothelial nitric oxide synthase expression in gingiva from control or diabetic mice. Mandibles were extracted from male control (A, C) or KK-A^y (B, D) mice at 20 wk of age. After treatment with (C, D) or without (A, B) proteinase K, the mandibles were decalcified with 0.5 M EDTA. The sections were stained with endothelial nitric oxide synthase antibody, as described in the Material and methods. Arrows indicate endothelial cells. (A and B, magnification ×64; C and D, magnification ×320.) (E) Total RNA extracted from individual periodontal tissues of control (lanes 1–5) or KK-A^y (lanes 6–10) mice was reverse transcribed and amplified using the polymerase chain reaction (PCR). The reverse transcription-PCR products were subjected to electrophoresis and stained with ethidium bromide. (F) The intensities of the bands of endothelial nitric oxide synthase in (E) were analyzed using BIO-PROFIL, and relative values of endothelial nitric oxide synthase mRNA were normalized with respect to those for β-actin. eNOS, endothelial nitric oxide synthase.

Recovery of alveolar bone loss in KK-A^y mice fed an *N*-acetylcysteine-containing diet

We then examined the effect of an *N*-acetylcysteine-containing diet on the alveolar bone loss in KK-A^y mice. The defiscence of alveolar bone decreased in KK-A^y mice given an *N*-acetylcysteine-containing diet compared with the normal-diet counterparts. The bone loss areas in KK-A^y mice were decreased by approximately 30% as a result of eating *N*-acetylcysteine-containing food, which was still higher than those of control mice (Fig. 6A,B). *N*-Acetylcysteine not only has an antioxidant effect, but also an inhibitory effect on nuclear factor-κB, which is associated with osteoblast differentiation. According to these results, we presented a working hypothesis that reactive oxygen species are associated with alveolar bone loss.

Discussion

In the present study, alveolar bone loss was examined in mandibles from metabolic syndrome model mice with type 2 diabetes. The bone loss progressed after the development of diabetes in the mice, at about 8 wk of age, and the mice also showed a decrease of endothelial nitric oxide synthase in the gingival keratinocytes. Mice fed a diet containing *N*-acetylcysteine, an antioxidant, restored the bone loss. However, *N*-acetylcysteine is also reported to be an inhibitor for nuclear factors such as nuclear factor-κB (27). Nuclear factor-κB is activated during osteoclast differentiation by stimulation with receptor activator of nuclear factor-κB ligand. The present study could not disregard the possibility that inhibition of nuclear factor-κB activity by *N*-acetylcysteine inhibited the progression of alveolar bone loss. Therefore, we proposed a working hypothesis that alveolar bone dehiscence was implicated in the generation of reactive oxygen species.

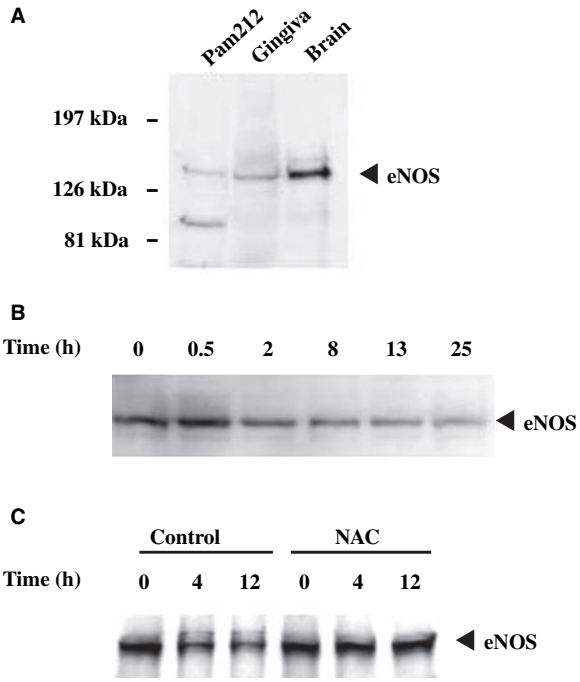

Fig. 4. Effect of hydrogen peroxide on endothelial nitric oxide synthase expression in keratinocytes. (A) Extracts from mouse gingiva and brain, accompanied by Pam 212 cell lysate (50 μg of each), were separated on a 7.5% polyacrylamide gel and subjected to immunoblotting with endothelial nitric oxide synthase antibody. (B, C) Pam 212 cells were cultured in Dulbecco's modified Eagle's minimal essential medium containing 10% fetal calf serum and then stimulated with 0.2 mM hydrogen peroxide in the presence (C) or absence (B, C) of 5 mM *N*-acetylcysteine for the indicated time. Aliquots of 200 μg of protein in cell lysates were analyzed by western blotting and detection with anti-endothelial nitric oxide synthase. eNOS, endothelial nitric oxide synthase; NAC, *N*-acetylcysteine.

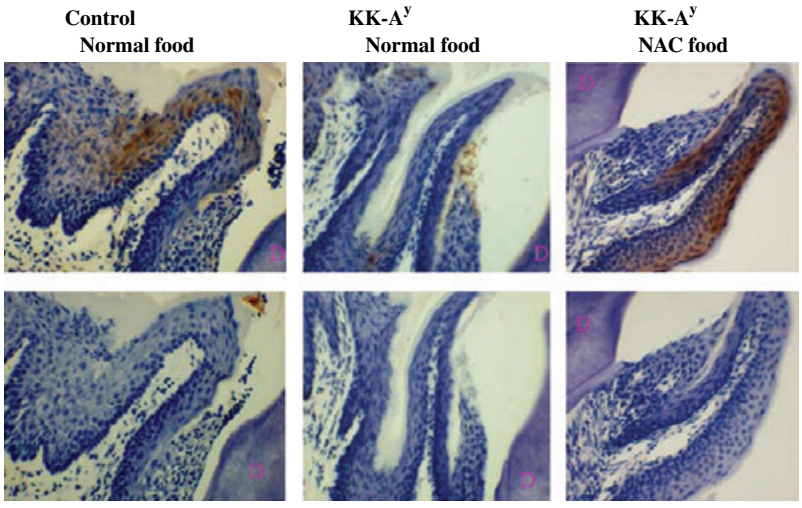

Fig. 5. Endothelial nitric oxide synthase expression in gingiva from control or diabetic mice. Control or KK-A^y mice at 8 wk of age were maintained on a normal diet or on a diet of *N*-acetylcysteine–containing food for 12 wk. Mandibles were extracted from male control and KK-A^y mice, and then decalcified in 0.5 M EDTA. The sections were stained with endothelial nitric oxide synthase antibody (upper panels) or nonimmunized rabbit IgG (lower panels), as described in the Material and methods. D, dentin; eNOS, endothelial nitric oxide synthase; NAC, *N*-acetylcysteine.

Previous studies on endothelial nitric oxide synthasedeficient mice have revealed that endothelial nitric oxide synthase regulates bone formation by activating osteoblasts (20,21). Consistently, RT-PCR and immunohistochemical staining showed the expression of endothelial nitric oxide synthase in bone cells among three isoforms of NOS (28). However, neither the detailed quantification nor the physiological significance of endothelial nitric oxide synthase expression in osteoblasts has been clarified. In this study, we detected endothelial nitric oxide synthase protein in Pam 212, a mouse keratinocytic cell line, but not in MC3T3-E1, a mouse osteoblastic cell line, by western blot analysis (data not shown). These observations suggested that the expression of endothelial nitric oxide synthase in osteoblasts may be lower than in other cell types, such as keratinocytes. Thus, it is currently uncertain whether the bone defect in endothelial nitric oxide synthase knockout mice is a result of the loss of endothelial nitric oxide synthase in osteoblasts or other cell types.

Studies on endothelial nitric oxide synthase-deficient mice have revealed that endothelial nitric oxide synthase plays an important role in the prevention of ovariectomy-induced bone loss (20,21). The administration of *N*-acetylcysteine was reported to abolish ovariectomy-induced bone loss (19). In the present study, we found that *N*-acetylcysteine restored endothelial nitric oxide synthase expression in the keratinocytes of diabetic mice, accompanied with the reversal of alveolar bone loss. While the role of gingival keratinocytes in bone formation is unclear, considering these observations, the alveolar bone defect of diabetic mice may be associated with reduction of endothelial nitric oxide synthase expression in the adjacent gingival keratinocytes. In our present study, we also found that treatment with hydrogen peroxide decreased endothelial nitric oxide synthase expression in keratinocytes. Moreover, we found that *N*-acetylcysteine did not affect the ascorbic acid-induced activity of alkaline phosphatase and calcification of MC3T3-E1 cells (data not

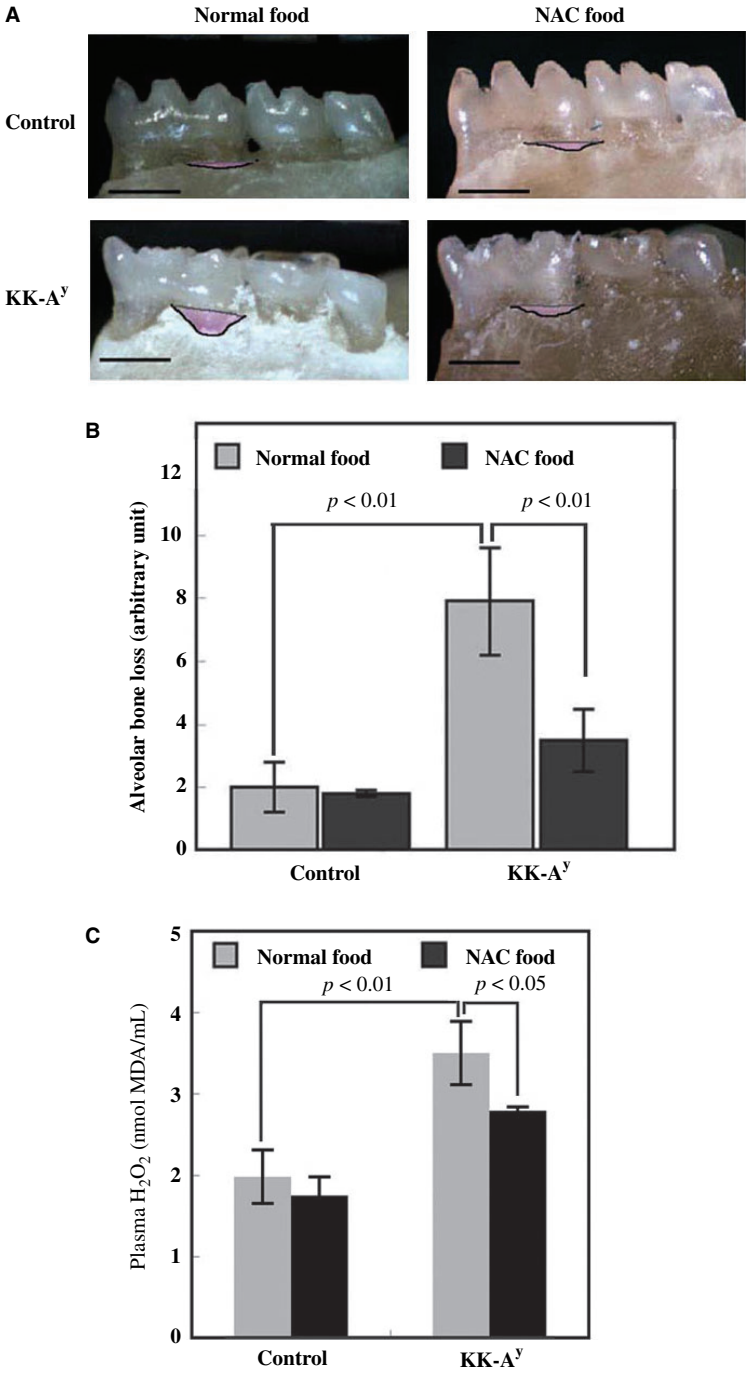

Fig. 6. Effect of an *N*-acetylcysteine–containing diet on alveolar bone loss and plasma hydrogen peroxide levels in diabetic mice. (A) Control or KK-A^y mice at 8 wk of age were maintained on a diet of normal or *N*-acetylcysteine-supplemented food for 12 wk. The hydrogen peroxide levels in plasma of KK-A^y and control mice were measured (A). Mandibles were extracted from male control (upper panel) and KK-A^y (lower panel) mice at 20 wk of age, and the soft tissues were removed. The bars indicate the length of 1 mm. The edge of alveolar bone is indicated as a black line and the purple area defines the alveolar bone loss. (B, C) Alveolar bone loss at the proximal areas of the first molars in control or KK-A^y mice was measured as described in the Material and methods (means ± standard deviation, $n = 6$). H2O2, hydrogen peroxide; MDA, malondialdehyde; NAC, *N*-acetylcysteine.

shown) suggesting that *N*-acetylcysteine may not directly stimulate the differentiation of osteoblasts. Considering the abovementioned importance of endothelial nitric oxide synthase in cell types other than osteoblasts, it is possible that the inhibitory effect of *N*-acetylcysteine on bone loss occurs through restoring the endothelial nitric oxide synthase expression in cell types other than osteoblasts.

It is of note that the form of the alveolar bone defect in KK-A^y mice was sphenoid-shaped, which is distinct from the bone defects induced by general periodontitis. The alveolar bone dehiscence means that a buccal bone defect is present at the locus adjacent to dental roots, which results in an occlusal force on the alveolar bone. In fact, occlusal wear was observed in the molar of lower jaws from the diabetic mice (Fig. 1A). Occlusal forces do not initiate periodontitis, but modify and progress the attachment loss and alveolar bone loss (29,30). In this study, we did not observe distinct inflammatory responses, such as the invasion of inflammatory cells, in the periodontal tissues of KK-A^y mice. The remodeling of alveolar bone, which responds to occlusal force in KK-A^y mice, might be reduced compared with that in control mice, because the bone remodeling in diabetic animals is reported to be decreased (31).

The expression of endothelial nitric oxide synthase in gingiva has been reported to be indirectly involved in alveolar bone-induced bone remodeling, leading to tooth movements (32). In the present study, the expression of endothelial nitric oxide synthase in the gingival tissues of orthodontically treated teeth was significantly greater than that of control teeth. In other reports, administration of a general inhibitor of nitric oxide synthase, but not of an indicible nitric oxide synthase-specific inhibitor, reduced the tooth movement by an orthodontic force (33,34), suggesting that the activity of endothelial nitric oxide synthase affects alveolar bone remodeling. These reports, along with our immunohistochemical staining results, in which endothelial nitric oxide

synthase expression is localized on the gingival epithelium, indicate the possibility that alveolar bone remodeling is affected by endothelial nitric oxide synthase activity in periodontal tissues such as gingiva.

Reduced expression of endothelial nitric oxide synthase has been reported in skin keratinocytes of other type 2 diabetic model mice with obesity, and is implicated as a mechanism for the impaired healing of diabetic patients (22). Our present data have indicated that the reduced endothelial nitric oxide synthase expression in keratinocytes occurs as a consequence of increased reactive oxygen species generation. It should be noted, however, that other mechanisms are conceivable for the reduced endothelial nitric oxide synthase expression. One possible candidate is tumor necrosis factor-α. Tumor necrosis factor-α, whose expression increases in type 2 diabetes, has been reported to down-regulate endothelial nitric oxide synthase expression (35,36). However, the plasma levels of tumor necrosis factor-α in control and KK-A^y mice were lower than 50 pg/mL and there was no significant difference between the tumor necrosis factor-α levels in these mice (data not shown). The second possible candidate is adiponectin. The expression of adiponectin, which up-regulates endothelial nitric oxide synthase expression, has been reported to be decreased in the obese KK-A^y mice (37). The third possible mechanism involves decreased insulin action. Insulin increases the expression of endothelial nitric oxide synthase so that endothelial nitric oxide synthase is produced at short intervals *in vivo* (38). Thus, decreased levels of insulin may lead to the reduced endothelial nitric oxide synthase expression observed in the KK-A^y mice with type 2 diabetes.

Reactive oxygen species are known to be one of the reasons for various complications of type 2 diabetes, including periodontitis. In the present study we found that an antioxidant, *N*-acetylcysteine, may exert an inhibitory effect on bone loss. *N*-Acetylcysteine is known be a popular supplement and beneficial in conditions characterized by an oxidative stress (39). However, *N*-acetylcysteine is also known to be an inhibitor for nuclear factor-κB, which is associated with osteoclast differentiation. Because the target molecules of *N*-acetylcysteine are still obscure, further investigation is necessary to prove that reactive oxygen species are associated with the alveolar bone in the diabetes model mice.

Acknowledgements

This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grants 17591943, 14571767 and 19390474).

References

1. Reaven GM. Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes* 1988;**37:**1595–1607.
2. Balkau B, Charles M. European group for the study of insulin resistance (EGIR). Comment on the provisional report of the WHO consultation [letter]. *Diabet Med* 1999;**16:**442–443.
3. Nishimura M. Breeding of mice strains for diabetes mellitus. *Exp Anim* 1969;**18:**147–157.
4. Iwatsuka H, Shino A, Suzuoki Z. General survey of diabetic features of yellow KK mice. *Endocrinol Jpn* 1970;**17:**23–35.
5. Aizawa-Abe M, Ogawa Y, Masuzaki H *et al*. Pathophysiological role of leptin in obesity-related hypertension. *J Clin Invest* 2000;**105:**1243–1252.
6. Furukawa S, Fujita T, Shimabukuro M *et al*. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* 2004;**114:**1752–1761.
7. Touyz RM, Schiffrin EL. Reactive oxygen species in vascular biology: implications in hypertension. *Histochem Cell Biol* 2004;**122:**339–352.
8. Karima M, Kantarci A, Ohira T *et al*. Enhanced superoxide release and elevated protein kinase C activity in neutrophils from diabetic patients: association with periodontitis. *J Leukoc Biol* 2005;**78:**862–870.
9. Koya D, King GL. Protein kinase C activation and the development of diabetic complications. *Diabetes* 1998;**47:**859–866.
10. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* 2001;**414:**813–820.
11. Shlossman M, Knowler WC, Pettitt DJ, Genco RJ. Type 2 diabetes mellitus and periodontal disease. *J Am Dent Assoc* 1990;**121:**532–536.
12. Tsai C, Hayes C, Taylor GW. Glycemic control of type 2 diabetes and severe periodontal disease in the US adult population. *Community Dent Oral Epidemiol* 2002;**30:**182–192.
13. Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. *Lancet* 2005;**366:**1809–1820.
14. Williams RC. Periodontal disease. *N Engl J Med* 1990;**322:**373–382.
15. Genco RJ. Host responses in periodontal diseases: current concepts. *J Periodontol* 1992;**63:**338–355.
16. Guarnieri C, Zucchelli G, Bernardi F, Scheda M, Valentini AF, Calandriello M. Enhanced superoxide production with no change of the antioxidant activity in gingival fluid of patients with chronic adult periodontitis. *Free Radic Res Commun* 1991;**15:**11–16.
17. Tsai CC, Ho YP, Chen CC. Levels of interleukin-1 beta and interleukin-8 in gingival crevicular fluids in adult periodontitis. *J Periodontol* 1995;**66:**852–859.
18. Ren Y, Maltha JC, Van't Hof MA, Von Den Hoff JW, Kuijpers-Jagtman AM, Zhang D. Cytokine levels in crevicular fluid are less responsive to orthodontic force in adults than in juveniles. *J Clin Periodontol* 2002;**29:**757–762.
19. Lean JM, Davies JT, Fuller K *et al*. A crucial role for thiol antioxidants in estrogen-deficiency bone loss. *J Clin Invest* 2003;**112:**915–923.
20. Aguirre J, Buttery L, O'Shaughnessy M *et al*. Endothelial nitric oxide synthase gene-deficient mice demonstrate marked retardation in postnatal bone formation, reduced bone volume, and defects in osteoblast maturation and activity. *Am J Pathol* 2001;**158:**247–257.
21. Armour KE, Armour KJ, Gallagher ME *et al*. Defective bone formation and anabolic response to exogenous estrogen in mice with targeted disruption of endothelial nitric oxide synthase. *Endocrinology* 2001;**142:**760–766.
22. Stallmeyer B, Anhold M, Wetzler C, Kahlina K, Pfeilschifter J, Frank S. Regulation of eNOS in normal and diabetes-impaired skin repair: implications for tissue regeneration. *Nitric Oxide* 2002;**6:**168–177.
23. Ohnishi T, Suwa M, Oyama T, Arakaki N, Torii M, Daikuhara Y. Prostaglandin E2 predominantly induces production of hepatocyte growth factor/scatter factor in human dental pulp in acute inflammation. *J Dent Res* 2000;**79:**748–755.
24. Chu Y, Heistad DD, Knudtson KL, Lamping KG, Faraci FM. Quantification of mRNA for endothelial NO synthase in mouse blood vessels by real-time polymerase chain reaction. *Arterioscler Thromb Vasc Biol* 2002;**22:**611–616.

25. Tokunaga K, Taniguchi H, Yoda K, Shimizu M, Sakiyama S. Nucleotide sequence of a full-length cDNA for mouse cytoskeletal b-actin mRNA. *Nucleic Acids Res* 1986;**14:**2829.
26. Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature* 2006;**440:**944–948.
27. Allen RG, Tresini M. Oxidative stress and gene regulation. *Free Radic Biol Med* 2000;**28:**463–499.
28. Helfrich MH, Evans DE, Grabowski PS, Pollock JS, Ohshima H, Ralston SH. Expression of nitric oxide synthase isoforms in bone and bone cell cultures. *J Bone Miner Res* 1997;**12:**1108–1115.
29. Gher ME. Changing concepts. The effects of occlusion on periodontitis. *Dent Clin North Am* 1998;**42:**285–299.
30. Serio FG, Hawley CE. Periodontal trauma and mobility. Diagnosis and treatment planning. *Dent Clin North Am* 1999;**43:**37–44.
31. Liu Z, Aronson J, Wahl EC *et al*. A novel rat model for the study of deficits in bone formation in type-2 diabetes. *Acta Orthop* 2007;**78:**46–55.
32. D'Attillio M, Di Maio F, D'Arcangela C *et al*. Gingival endothelial and inducible nitric oxide synthase levels during orthodontic treatment: a cross-sectional study. *Angle Orthod* 2004;**74:**851–858.
33. Shirazi M, Nilforoushan D, Alghasi H, Dehpour AR. The role of nitric oxide in orthodontic tooth movement in rats. *Angle Orthod* 2002;**72:**211–215.
34. Hayashi K, Igarashi K, Miyoshi K, Shinoda H, Mitani H. Involvement of nitric oxide in orthodontic tooth movement in rats. *Am J Orthod Dentofacial Orthop* 2002;**122:**306–309.
35. Nilsson J, Jovinge S, Niemann A, Reneland R, Lithell H. Relation between plasma tumor necrosis factor-alpha and insulin sensitivity in elderly men with non-insulin-dependent diabetes mellitus. *Arterioscler Thromb Vasc Biol* 1998;**18:**1199–1202.
36. Anderson HD, Rahmutula D, Gardner DG. Tumor necrosis factor-alpha inhibits endothelial nitric-oxide synthase gene promoter activity in bovine aortic endothelial cells. *J Biol Chem* 2004;**279:**963–969.
37. Hattori Y, Suzuki M, Hattori S, Kasai K. Globular adiponectin upregulates nitric oxide synthaseproduction in vascular endothelial cells. *Diabetologia* 2003;**46:**1543–1549.
38. Kuboki K, Jiang ZY, Takahara N *et al*. Regulation of endothelial constitutive nitric oxide synthase gene expression in endothelial cells and *in vivo*: a specific vascular action of insulin. *Circulation* 2000;**101:**676–681.
39. Kelly GS. Clinical applications of *N*-acetylcysteine. *Altern Med Rev* 1998;**3:**114–127.