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Periodontitis-induced lipid peroxidation in rat descending aorta is involved in the initiation of atherosclerosis

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Background and Objective: Periodontitis is a risk factor for the development of atherosclerosis. Recent studies indicate that oxidative mechanisms, including lipid peroxidation, are involved not only in periodontitis but also in atherosclerosis. Lipid peroxidation may play an important role in the pathogenesis of atherosclerosis, particularly during its earliest stages. The purpose of this study was to investigate the relationship between lipid peroxidation induced by periodontitis and the initiation of atherosclerosis.

Material and Methods: Sixteen rats were randomly divided into two groups of eight rats each. Periodontitis was ligature-induced for 4 wk in the experimental group, whereas the control group was left untreated. After the experimental period, the mandibular first molar regions were resected and then subjected to histological analysis and measurement of hexanoyl-lysine expression as an indicator of lipid peroxidation. Descending aorta was used for measuring the levels of hexanoyl-lysine, reactive oxygen species and lipid deposits, and for real-time polymerase chain reaction microarray analysis. The level of hexanoyl-lysine was also measured in serum.

Results: In the experimental group, the levels of hexanoyl-lysine in periodontal tissue and serum increased. Only aorta samples in the experimental group showed lipid accumulation, with increased expression of hexanoyl-lysine, reactive oxygen species and oxidative stress-related genes (including nitric oxide synthases 2 and 3), whereas the superoxide dismutase 1 gene level was down-regulated.

Conclusion: In a ligature-induced periodontitis rat model, increased lipid peroxidation was found in serum and aorta as well as in periodontal tissue. Atherosclerosis-related gene expression and histological changes were also stimulated. Periodontitis-induced lipid peroxidation in the aorta may be involved in the early stage of atherosclerosis.

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The most recent data from the World Health Organization mortality database (67 countries) show that the all-cause mortality rates are strongly related to atherosclerotic cardiovascular disease (1). However, atherosclerotic cardiovascular disease is not clearly associated with the classical risk factors (i.e. hypertension, smoking,

hypercholesterolemia and diabetes) in more than one-third of patients dying from it (2–4), which suggests other contributory mechanisms. There is increasing evidence of a systemic inflammatory process in patients with atherosclerotic cardiovascular disease and corresponding animal models (2–6), with the progression of atherosclerosis mirroring that of a chronic inflammation (7). While the contributory role of inflammation in cardiovascular disease is generally accepted, and numerous reviews exist on the development of atherosclerosis (8–11), the initiating factors leading to the inflammatory injury remain speculative. Many studies have demonstrated that cardiovascular diseases and periodontitis are associated and possibly causally linked (12–15). Two prevailing hypotheses may explain the relationship between periodontitis and cardiovascular disease: first, periodontal bacteria may have a direct effect on the vasculature (16,17); and, second, local inflammation causes an enhanced inflammatory response at distant sites without the spread of the infectious agent (18).

The production of reactive oxygen species is the essential pathogenic mechanism for diseases associated with phagocytosis, as the host defense against bacterial pathogens (19,20). However, when reactive oxygen species overwhelm the cellular antioxidant defense, such conditions increase tissue injury and result in increased oxidative stress (21,22). Recent studies indicate that oxidative mechanisms, including lipid peroxidation, are involved in periodontitis (23–25). Periodontitis in humans and in animal models increases the level of local or systemic malondialdehyde (23,24), which is an end product of lipid peroxides and a marker of lipid peroxidation (26). Lipid peroxidation is also involved in the pathogenesis of atherosclerosis (27–29), and circulating malondialdehyde, indicating lipid peroxidation, increases in the atherosclerotic model (30). Lipid peroxidation may be the key factor that explains a causal relationship between atherosclerosis and periodontitis. The purpose of this study was to investigate the relationship between lipid peroxidation induced by rat periodontitis and the initial stage of an atherosclerotic lesion, and to profile in the aorta, by using a new array system, the gene-expression pattern associated with atherosclerosis. In this study, hexanoyl-lysine was used because it is a novel lipid hydroperoxide-modified lysine residue formed in the early stages of lipid peroxidation (31).

Material and methods

Animals

Sixteen male Wistar rats (8 wk of age) were housed in an air-conditioned room (23–25°C) with a 12-h light–dark cycle. They had free access to powdered food (MF; Oriental Yeast Co. Ltd., Osaka, Japan) and drinking water. All experimental procedures were performed in accordance with the Animal Research Control Committee of Okayama University Dental School.

Experimental design

The rats (n = 16) were divided into two groups of eight rats each. The control group had no treatment for 4 wk. Periodontitis was ligature induced for 4 wk in the experimental group. A 3/0 cotton ligature (Alfresa Pharma Co., Osaka, Japan) was placed in a submarginal position around the mandibular first molars (32).

Upon completion of the experimental period (4 wk), the animals were killed under general anesthesia and blood samples were collected from the heart for measuring the serum level of hexanoyl-lysine. For histological analysis, the mandibular first-molar regions were resected *en bloc* from each rat and then fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 1 d. The descending aorta was harvested, immediately frozen and kept at −80°C until processed for immunohistochemical analysis, measurement of oxidative stress, or real-time polymerase chain reaction (PCR) microarray analysis.

Analysis of periodontal tissues

After fixing in paraformaldehyde, the mandibular first-molar samples were decalcified by immersion in 10% tetrasodium-EDTA aqueous solution (pH 7.4) for 2 wk at 4°C. Formalin-fixed tissue samples were embedded in paraffin following dehydration with ethanol series (70, 80, 90 and 100%) and immersion in xylene. Bucco–lingual 4-μm sections were embedded in paraffin and stained with hematoxylin and eosin, or other stains, as described below.

Immunohistochemical staining for hexanoyl-lysine was performed using a Histofine Simple Stain MAX PO (M) kit (Nichirei Co., Tokyo, Japan). Briefly, deparaffinized tissue sections of the periodontal tissue were immersed for 30 min in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase activity. The sections were treated consecutively at 4°C with an anti-hexanoyl-lysine immunoglobulin G (Japan Institute for the Control of Aging, Shizuoka, Japan) (diluted 1:50) overnight and then with a secondary antibody (Fab) with peroxidase complex for 30 min. The color was developed by placing sections in a solution of 3,3′-diaminobenzidine tetrahydrochloride (DAB) in 50 mmol/L Tris–HCl buffer (pH 7.5) containing 0.001% hydrogen peroxide. The sections were counterstained with Mayer's hematoxylin. In addition, the specificity of staining of hexanoyl-lysine was established by the use of isotype antibody controls and by confirming inhibition of positive staining when the antibody was previously treated with standard hexanoyl-lysine solution (Japan Institute for the Control of Aging) in the immunohistochemical procedure.

A single examiner, blinded to the treatment assignment, performed the following histometric analyses using a light microscope. Using the periodontal tissue sections stained with hematoxylin and eosin, the degree of apical migration of the junctional epithelium and alveolar bone resorption were evaluated by measuring the distances between the cemento–enamel junction and the most apical portion of the junctional epithelium, and by measuring the distances between the cemento–enamel junction and the alveolar bone crest, using a microgrid at a magnification of ×200 (33). The

numbers of hexanoyl-lysine-positive fibroblasts and total fibroblasts in standard areas (0.1 mm × 0.1 mm each) adjacent to the cementum within the periodontal ligament (five serial areas from the top of the periodontal ligament) were determined at a magnification of ×400 (34), and the ratios of the number of hexanoyl-lysine-positive fibroblasts to the number of total fibroblasts were calculated to evaluate the density of hexanoyl-lysine-positive fibroblasts.

Measurement of serum hexanoyl-lysine

Blood was allowed to clot at 23–25°C, and serum was separated by centrifugation at 1500 *g* for 15 min. The level of hexanoyl-lysine in serum, as a marker of lipid peroxidation, was determined using an enzyme-linked immunosorbent assay kit (Japan Institute for the Control of Aging) (35).

Histological evaluation of aorta samples

Frozen sections (8 μm thick) were obtained from the aorta, embedded in Optimal Cutting Temperature compound (Tissue Tec; Miles, Naperville, IL, USA) and stained with oil red O to detect lipids (36). The percentage of the area of total aortic lumen occupied by lipids per section was calculated quantitatively using computer-assisted image-analysis software (WINROOF; Mitani Co., Fukui, Japan) (37). Three sections per rat were evaluated. The proximal aorta, which was dissected and opened longitudinally, was also stained with oil red O.

Immunohistochemical staining for hexanoyl-lysine in the aorta samples was performed using the same procedure employed for hexanoyl-lysine staining in periodontal tissues, as described above.

Reactive oxygen species (hydrogen peroxide) were detected using a staining procedure described previously (38). Briefly, cryostat sections were dried for 5 min, incubated with 0.1 mol/L of Tris–HCl (pH 7.6) for 5 min at 23–25°C, then incubated for 30 min in DAB solution (0.1 mol/L of Tris–HCl, pH 7.6, 0.5 mg/mL of DAB) at 37°C. The sections were counterstained with Mayer's hematoxylin.

Microarray expression analysis

Total RNA was isolated from the aorta biopsy samples, which were pooled from four control groups and four experimental groups, using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The isolated RNA was quantified by measuring the absorbance at 260 nm and the purity was determined by the 260/280 nm absorbance ratio. Samples with a ratio of > 1.8 were used in the procedure described below.

After removal of contaminating genomic DNA from total RNA samples (1 μg) by digestion with DNaseI, first-strand synthesis was performed with the RT^2 PCR array First Strand Kit (Superarray Bioscience Corporation, Frederick, MD, USA) (39). The samples were then screened to detect the expression of 84 genes related to endothelial cell biology by means of the RT^2 Profiler PCR Array System (Rat endothelial cell biology; Superarray Bioscience Corporation). Data of gene expression were confirmed using the RT-PCR-based gene profiling 'RT2 profiler system' (Superarray Bioscience Corporation). Each sample in the control and experimental groups was analyzed in duplicate.

Statistical analysis

The differences between the experimental and control groups were analyzed using the Mann–Whitney *U*-test. A *p*-value of < 0.05 was considered as statistically significant.

Results

No significant differences in weight gain or food consumption were detected between the two groups of rats during the experimental period.

The periodontal tissue samples in the experimental group exhibited apical migration of the junctional epithelium, alveolar bone loss and infiltration of inflammatory cells, including polymorphonuclear leukocytes (Fig. 1). The distance between the cemento–enamel junction and the alveolar bone crest, and the distance between the cemento–enamel junction and the most apical portion of the junctional epithelium were greater in the experimental group than in the control group.

In the periodontal ligament, some fibroblasts stained positive for hexanoyl-lysine. The ratio of hexanoyl-lysine-positive fibroblasts to total fibroblasts in the periodontal ligament in the experimental tissues was higher than that in the control group ($p = 0.001$) (Fig. 2).

The serum level of hexanoyl-lysine in the experimental group was significantly higher than that in the control group ($p < 0.001$) (Fig. 3).

Lipid deposition in the descending aorta was observed in the experimental group but not in the control group (Fig. 4). The percentage [mean (standard deviation)] of aortic lumen occupied by the lesion was 3.4 (0.7) in the experimental group.

Weak hexanoyl-lysine expression was detected in the descending aorta in the control group (Fig. 5A). By contrast, in the experimental group, an increase in hexanoyl-lysine expression was clearly evident (Fig. 5B). The expression of reactive oxygen species (hydrogen peroxide) in the aortic lumen increased in the experimental group compared with the control group (Fig. 5C,D).

Among the 84 genes tested on the array, 16 genes exhibited an increase of twofold or more, and 11 genes exhibited a decrease of twofold or more in the experimental group compared with the control group (Table 1). In the genes related to oxidative metabolism, the expression of nitric oxide synthases 2 and 3 was higher and of superoxide dismutase 1 was lower. Expression of xanthine dehydrogenase did not differ significantly between the experimental and control groups.

Discussion

Rats in whom periodontitis was induced exhibited higher lipid peroxidation in the serum and aorta,

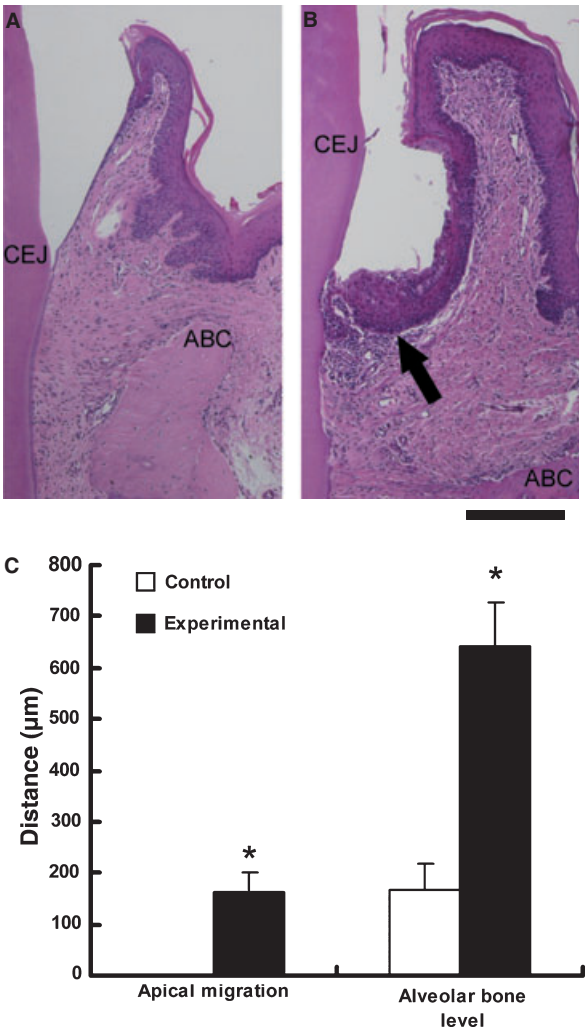

Fig. 1. Pathological changes in rat periodontal tissue. While the control group showed no pathological changes (A), the experimental group showed alveolar bone resorption, apical migration of junctional epithelium (arrow) and inflammatory cell infiltration in the connective tissue adjacent to the junctional epithelium (B) (hematoxylin and eosin staining). Distances between the cemento–enamel junction and the apical portion of the junctional epithelium (apical migration), and between the cemento–enamel junction and the alveolar bone crest (alveolar bone level) were larger in the experimental group than in the control group (C) (mean ± standard deviation). ABC, alveolar bone crest; CEJ, cemento–enamel junction. Scale bar = 200 μm. *$p < 0.001$, compared with the control group (Mann–Whitney *U*-test).

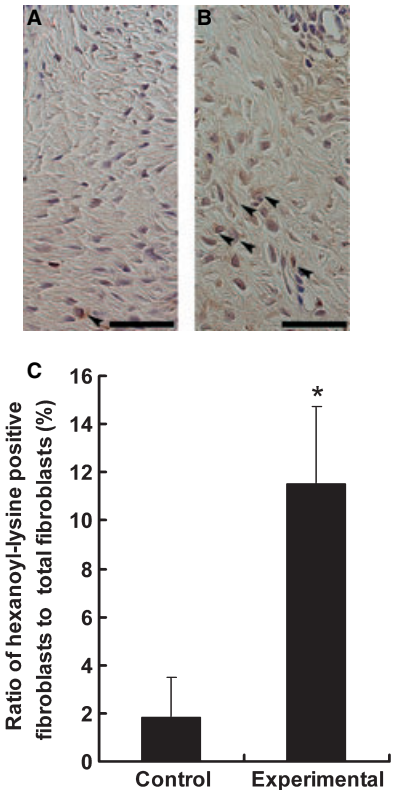

Fig. 2. Expression of hexanoyl-lysine in rat periodontal tissue. Many hexanoyl-lysine-positive cells were observed in the fibroblasts, and representative photographs of hexanoyl-lysine-positive fibroblasts (arrowheads) in the control (A) and experimental (B) groups are shown. The ratio of hexanoyl-lysine-positive fibroblasts to total fibroblasts (mean ± standard deviation) in the experimental group was significantly higher than that in the control group (C). *$p = 0.001$, compared with the control group (Mann–Whitney *U*-test).

as well as in periodontal tissue, than control rats. The aorta samples in the experimental group showed accumulation of lipids, increased reactive oxygen species (hydrogen peroxide) production and changes of atherosclerosis-related gene expression, as described below. The reactive oxygen species (such as lipid peroxides) produced as a result of periodontitis may diffuse into the blood from the site of inflammation, as reported in rat periodontitis where diffusion of lipid peroxides into plasma occurred (24). The level of circulating lipid peroxides increased in atherosclerosis (30). These data support the hypothesis that local inflammation causes an enhanced inflammatory response at distant sites without the spread of the infectious agent (18), and lipid peroxidation by periodontitis may be an initiating factor leading to inflammatory injury in the early stage of atherosclerosis.

Atherogenesis starts with lipid insudation into the vessel wall, where it is trapped in the intima. The endothelium above the point where the lipid is trapped becomes dysfunctional, resulting in monocytes rolling and then adhering to the endothelium, prior to migrating into the intima, resulting in macrophage collection (40). Oxidative stress, such as increased lipid peroxidation and hydrogen peroxide expression in the experimental group, may be harmful to the vasculature if oxidative processes are out of control, as seen in endothelial dysfunction and subsequent atherosclerosis (41). Lipid peroxidation may be important in the

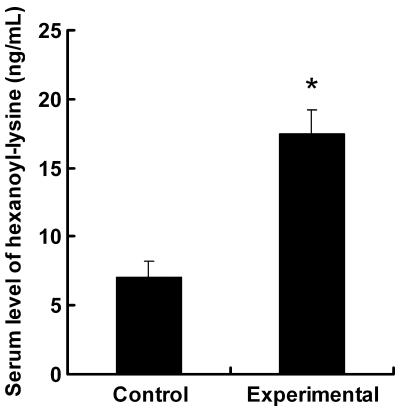

Fig. 3. Serum levels of hexanoyl-lysine in the control and experimental groups. Bars represent the mean ± standard deviation ($n = 8$). *$p < 0.001$, compared with the control group (Mann–Whitney *U*-test).

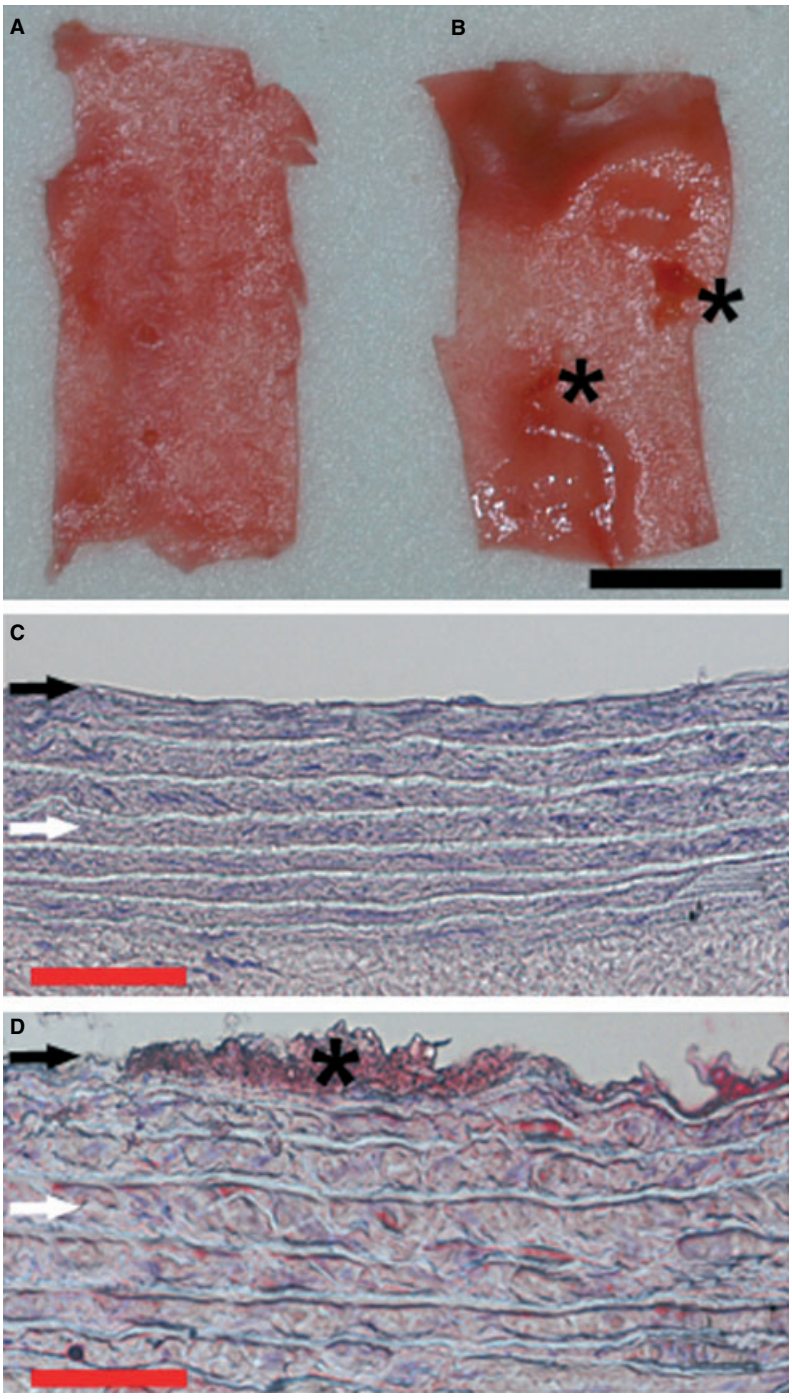

Fig. 4. Representative results of lipid deposition in the descending aorta stained with oil red O. Lipid deposition (asterisk) was observed in the experimental group (B, D) but not in the control group (A, C). Black arrows, intima; white arrows, media; black scale bar = 1 mm; red scale bar = 50 μm.

pathogenesis of atherosclerosis, particularly in its earliest stages (27). Hydrogen peroxide potentially oxidized low-density lipoprotein that increases the adhesion of monocytes to the endothelium, the transformation of macrophages into foam cells and the impairment of endothelium-dependent vasorelaxation (42–44).

The levels of mRNA expression of nitric oxide synthases 2 and 3 were enhanced in the experimental group. It is reported that nitric oxide synthase 2 in the endothelium may lead to oxidative stress and endothelial dysfunction (41), and nitric oxide synthase 2 may become a peroxynitrite generator, leading to a dramatic increase in oxidative stress (45). The redox equilibrium between nitric oxide and oxidative stress has a profound impact on the expression of genes in the vessel wall, which is related to the progression and vulnerability of atherosclerotic lesions as well as to parameters of inflammation and cell apoptosis (41). Total nitric oxide production might be enhanced because nitric oxide is not only produced by nitric oxide synthase 2, but also by nitric oxide synthase 3, in macrophages and other cell types in the atherosclerotic plaque (46).

The *Sod1* gene expression level was down-regulated in the experimental group. Superoxide dismutases are major cellular defense systems that are active against superoxide in all vascular cells (41). Superoxide dismutase 1 is a potentially important mediator of the prevention of oxidized low-density lipoprotein accumulation within atherosclerotic plaques (47). The low level of superoxide dismutase 1 may contribute to the initiation of atherosclerosis by periodontitis.

The observed gene profile included atherosclerosis-related genes. We found that among the 84 genes analyzed, the expression of natriuretic

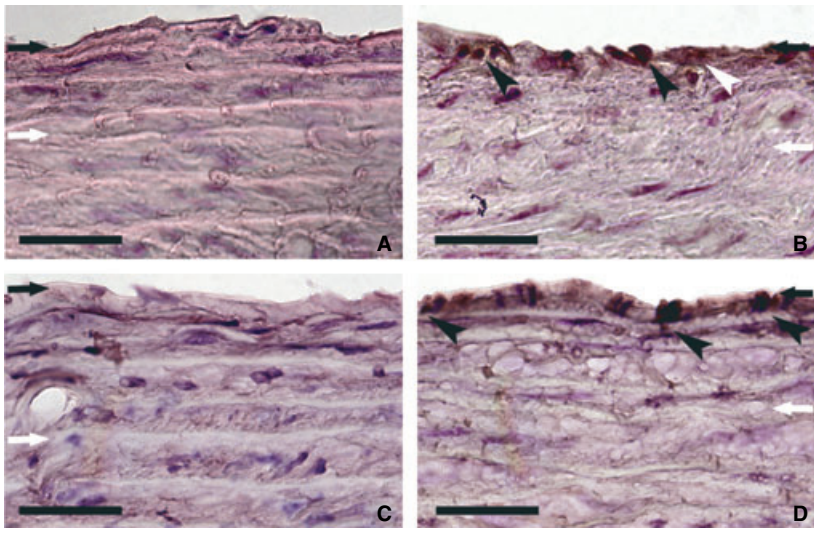

Fig. 5. Cross-sections of descending aorta in the control and experimental groups. Hexanoyl-lysine expression of endothelial cells (white arrowhead) or inflammatory cells (black arrowheads) in the experimental group (B) was more intense than in the control group (A). Reactive oxygen species (hydrogen peroxide) expression of inflammatory cells in the experimental group (D) (black arrowheads) was more intense than in the control group (C). Black arrows, intima; white arrows, media; scale bar = 50 μm.

Table 1. Fold change expression of genes related to endothelial cell biology in the experimental group compared with the control group

Description	Gene name	GenBank no.	Fold changes
Natriuretic peptide precursor type B	Nppb	NM_031545	31.3
Angiopoietin 1	Angpt1	NM_053546	8.5
Platelet/endothelial cell adhesion molecule	Pecam	NM_031591	7.8
Plasminogen activator, urokinase	Plau	NM_013085	6.8
Endothelial cell growth factor 1 (platelet-derived)	Ecgf1	NM_001012122	6.0
Tumor necrosis factor-related apoptosis-inducing ligand	Trail	NM_145681	4.4
Caspase 3, apoptosis related cysteine protease	Casp3	NM_012922	4.2
Chemokine (C-X-C motif) ligand 4	Cxcl4	NM_001007729	3.6
Cadherin 5	Cdh5	XM_226213	3.6
Interleukin 7	Il7	NM_013110	3.0
Nitric oxide synthase 2	Nos2	NM_012611	3.0
Chemokine (C-X-C motif) ligand 2	Cxcl2	NM_053647	2.4
Nitric oxide synthase 3	Nos3	NM_021838	2.3
FMS-like tyrosine kinase 1	Flt1	NM_019306	2.2
Bcl2-like 1	Bcl2l1	NM_031535	2.1
Angiotensin receptor 1b	Agtr1b	NM_031009	2.1
Serine (or cysteine) peptidase inhibitor, clade E, member 1	Serpine1	NM_012620	−7.9
Integrin alpha V	Itgav	XM_230950	−6.3
Tumor necrosis factor-α	TNF-α	NM_012675	−3.9
Fibronectin 1	Fn1	NM_019143	−3.6
Transforming growth factor beta 1	Tgfb1	NM_021578	−3.1
B-cell leukemia/lymphoma 2	Bcl2	NM_016993	−3.0
Fibroblast growth factor 1	Fgf1	NM_012846	−2.7
Matrix metallopeptidase 9	Mmp9	NM_031055	−2.4
E-selectin	Sele	NM_138879	−2.1
Tumor necrosis factor alpha-induced protein 3	Tnfaip3	NM_001024771	−2.0
Superoxide dismutase 1	Sod1	NM_017050	−2.0

peptide precursor type B was highest. Natriuretic peptide precursor type B is a well-established biomarker in chronic heart failure and its level of expression increases in line with advanced heart failure, indicating poor prognosis (48). Natriuretic peptide precursor type B may have an important role in the pathogenesis of atherosclerosis induced by periodontitis. It is reported that increases in platelet/endothelial cell adhesion molecules (49), cadherin 5 (50), urokinase plasminogen activator (51), tumor necrosis factor-related apoptosis-inducing ligand (52), chemokine (C-X-C motif) ligand 4 (53), chemokine (C-X-C motif) ligand 4 (54), FMS-like tyrosine kinase 1 (55), interleukin-7 (56) and caspase 3 (57), and decreases in transforming growth factor-β1 (58), tumor necrosis factor-α-induced protein 3 (59) and B-cell leukemia/lymphoma 2 (60), are associated with atherosclerosis. Our data are in agreement with these previous studies. However, tumor necrosis factor-α mRNA expression was down-regulated. Tumor necrosis factor-α has an important role in the pathology of atherosclerosis, and the presence of tumor necrosis factor-α has been demonstrated in human atherosclerotic plaques (61). Two possible explanations for the reason why tumor necrosis factor-α mRNA expression did not increase are (i) there is no marked induction of tumor necrosis factor-α mRNA and immunoreactivity in the aorta and other large arteries after injection of oxidized low-density lipoprotein (62) and (ii) feedback regulation may occur upon initiation of atherosclerosis by periodontitis.

The mean percentage of total aortic lumen occupied by lesions in this ligature-induce periodontitis model was 3.4% (standard deviation = 0.7), which was greater than the 0.62% (standard deviation = 0.13) reported in an apolipoprotein E knockout mouse model challenged with *Porphyromonas gingivalis* (37). These findings suggest that ligature-induced periodontitis also contributes to the initiation of atherosclerosis. However, the lesions in the aorta were not more extensive than those in other studies in which animals were fed on a high-fat

diet (37,63). In the future, a high-fat diet may be considered in the ligature model.

We used a ligature-induced periodontitis model because such a periodontal lesion mimics several features of human periodontitis, including inflammatory cell infiltration, loss of attachment and alveolar bone resorption (32,64,65). However, ligature-induced periodontal inflammation is an acute model of periodontitis and is not equivalent to the chronic disease in humans. It might be more reliable to use ligature along with application of bacterial pathogens (e.g. *P. gingivalis*) as a rat model that relates to human periodontitis (66,67).

The presence of putative pathogenic bacteria such as *P. gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Treponema denticola* and *Tannerella forsythensis* in a high percentage of atherosclerotic coronary arteries supports the concept that periodontal pathogens might be associated with the development and progression of atherosclerosis (17,68–70). However, some investigations could not detect periodontal bacteria in atheromatous plaques (71,72), suggesting that local inflammation causes an enhanced inflammatory response at distant sites without the spread of the infectious agent.

Direct and/or indirect mechanisms might be involved in the pathological changes of the descending aorta observed in the present study. Although bacterial data were not obtained in our study, it has been reported previously that the major bacterial components of plaque in the ligature model are actinomycetes and streptococci (73) and that bacterial infection contributes to the progression of ligature-induced periodontal inflammation (73–75).

In conclusion, periodontitis-induced rats showed greater accumulation of lipids in the aorta than did control rats, with increased hexanoyl-lysine levels, reactive oxygen species production and nitric oxide synthase expression, and inhibition of superoxide dismutase 1 expression. The results suggest that periodontitis-induced lipid peroxidation in the aorta may be involved in the early stages of atherosclerotic disease.

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