Abstract: Silent information regulator 2 homolog 1 (SIRT1) inhibits oxidative injury and has anti-inflammatory effects. SIRT1 may be involved in healing of periapical periodontitis; however, SIRT1 expression in periapical periodontitis lesions has not been investigated. This study evaluated SIRT1 expression and a marker of oxidative stress—8-hydroxy-2’-deoxyguanosine (8-OHdG)—in periapical granulomas. First, we used real-time polymerase chain reaction to determine whether U-937 monocytes express SIRT1. U-937 cells treated with the SIRT1 activator resveratrol exhibited the highest SIRT1 mRNA level after 6-h incubation. By contrast, treating cells with the SIRT1 inhibitor sirtinol returned SIRT1 expression level to that of the control. In addition, immunocytochemical analysis using cytospin specimens showed that U-937 cells co-expressed SIRT1 and Ki-67. Dual-color immunofluorescence imaging showed that round cells in periapical granulomas co-expressed SIRT1 and 8-OHdG; however, neither was expressed in healthy gingival tissues. The number of 8-OHdG-expressing cells was significantly greater than the number of SIRT1-expressing cells. Our findings suggest that macrophages express SIRT1 and that wound healing in periapical granulomas is enhanced by a SIRT1-mediated reduction in the level of oxidative stress.

Keywords: 8-OHdG; oxidative stress; periapical granulomas; periapical periodontitis; SIRT1; U-937 cells.

Introduction
Patients with periapical periodontitis may develop poor appetite or sleeplessness because of symptoms such as throbbing pain, occlusal pain, and gingival swelling. Supporting bone resorption around the apex is a characteristic finding on radiography. Periapical periodontitis is caused by oral microorganisms. Virulence factors of Gram-negative bacteria, such as lipopolysaccharide (LPS), induce production of cytokines and matrix metalloproteinases (1,2), which leads to tissue damage and apical bone resorption. Furthermore, enzymes secreted by Gram-positive bacteria, including hyaluronidase and collagenase, destroy cells and tissues (3). Previous studies of immune reactions in periapical periodontitis focused on the mechanisms of inflammation (4,5); anti-inflammatory factors and pathways have received little attention (6).

Silent information regulator 2 homologue 1 (SIRT1) is a member of the sirtuin family of class III histone deacetylases (7). Sirtuins deacetylate enzymes, transcription factors, and heat-shock proteins and are involved in antiaging mechanisms, calorie restriction, DNA repair, and inhibition of inflammation (8-10). Clinically, SIRT1
is involved in recovery from inflammatory conditions such as rheumatoid arthritis (11). In addition, SIRT1 suppresses the NF-κB signaling pathway, thereby acting as an anti-inflammatory agent in persons with atherosclerosis, a chronic inflammatory disease (12).

Interestingly, the SIRT1 activator resveratrol inhibits ICAM-1 expression in endothelial cells (13), Th2 cytokine expression in mast cells (14), and interleukin (IL)-1β-induced expression of matrix metalloproteinase-13 and IL-6 in articular chondrocytes (15). In addition, resveratrol decreases nitric oxide synthesis by hepatocytes (16), which suggests that SIRT1 activation by resveratrol ameliorates inflammation. More importantly, resveratrol may have therapeutic potential in periapical periodontitis and as a pharmacological agent.

Oxidative stress is caused by an imbalance between the production of free radicals and their detoxification through neutralization by antioxidants. Free radicals modulate apoptosis and damage cells and tissues, and oxidative stress is involved in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis (20,21). In a rat model of chronic kidney disease, reduced SIRT1 activity in peritoneal-derived macrophages suggested induction of oxidative stress and inflammation in tissues (22). SIRT1 also reduces oxidative stress and mitochondrial dysfunction in endothelial cells, thus inhibiting apoptosis (23,24). Taken together, the evidence indicates that, in the presence of inflammation, SIRT1 reduces oxidative stress and induces wound healing.

Macrophages and endothelial cells are important in wound healing and inflammation (25-28). Interestingly, SIRT1 regulates macrophage differentiation and proliferation; however, decreased SIRT1 expression inhibits macrophage proliferation (29). Thus, we hypothesized that macrophages in periapical lesions play a role in wound healing by ameliorating oxidative stress via a mechanism involving SIRT1.

SIRT1 is expressed in dental pulp tissue (30-32); however, no study has examined the role of SIRT1 in periapical periodontitis. In addition, little is known of the effects of the SIRT1 activator resveratrol and the SIRT1 inhibitor sirtinol on SIRT1 expression by U-937 monocytes, which are derived from human myeloid leukemia cells. We evaluated the effects of resveratrol and sirtinol on SIRT1 expression by U-937 cells and determined expression levels of SIRT1 and 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative stress, in periapical granulomas.

### Materials and Methods

#### Cell culture

U-937 cells derived from histiocytic lymphoma were purchased from Health Science Research Resources Bank (Osaka, Japan). The cells (4 × 10⁶/well) were cultured with LPS (Escherichia coli 0111:B4-derived, 0.5 or 1.0 μg/mL; Sigma-Aldrich, St. Louis, MO, USA), resveratrol (10 or 50 μM; Sigma-Aldrich), and/or sirtinol (100 μM; Wako Pure Chemical Industries, Osaka, Japan) for 1, 2, 4, 6, 8, 10, 12, or 24 h at 37°C in an atmosphere containing 5% (v/v) CO₂.

#### Real-time polymerase chain reaction

SIRT1 mRNA levels were analyzed quantitatively by using real-time polymerase chain reaction (PCR). In brief, RNA was extracted with TRIZol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA was synthesized by using a PrimeScript RT Reagent Kit (TaKaRa Bio, Kusatsu, Japan) and then amplified by real-time PCR with a thermal cycler (Thermal Cycler Dice Real-Time System TP800; TaKaRa Bio). The PCR primers are shown in Table 1. SYBR Premix EX Taq (TaKaRa Bio) was used for amplification, and the SIRT1 mRNA level in each sample was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (G3PDH).

#### Dual-color immunocytochemistry

Cytospin specimens of cultured U-937 cells were prepared by using a cytocentrifuge and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). Then, we performed dual-color immunocytochemistry. In brief, cytospin specimens were treated with 10% (v/v) normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 60 min to block nonspecific binding and incubated with a rabbit monoclonal anti-human SIRT1...
antibody (1/100; Abcam, Cambridge, UK) and a mouse monoclonal anti-human Ki-67 antibody (1/100; Abcam) at 4°C overnight. After specimens were washed three times with PBS-Tween 20, a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (1/500; Abcam) and a rhodamine isothiocyanate (RITC)-conjugated goat anti-mouse IgG antibody (1/100; Abcam) were added, and specimens were incubated for 60 min. Nuclei were counterstained with 4’6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). The specimens were mounted in aqueous mounting medium (Perma Fluor; Thermo Fisher Scientific). We detected immunoreactive cells with a fluorescence microscope (Eclipse E600; Nikon, Tokyo, Japan) and obtained separate FITC and RITC images of the same field by changing filters. A normal rabbit IgG antibody (Cayman Chemical, Ann Arbor, MI, USA) served as a negative control (thus replacing the primary antibody).

Patients and sample collection
Seven patients with persistent periapical periodontitis (three men and four women; age range, 20-74 years) referred by general practitioners to the Department of Endodontics at Nihon University Dental Hospital were enrolled in this study. Periapical lesions were obtained during apicoectomy. The healthy control group (three men and seven women; age range, 20-57 years) comprised patients referred to our Department of Oral Surgery for extraction of impacted wisdom teeth. Healthy gingival tissues were obtained from those patients during tooth extraction. This study was approved by the Ethics Committee of the Nihon University School of Dentistry (EP2014-6) and was conducted in accordance with all requirements of the Helsinki Declaration. Written consent was obtained from all patients before sample collection.

Sample preparation and pathological examination
Periapical lesions and healthy gingival tissues were fixed with 10% (v/v) neutral-buffered formalin and embedded in paraffin. Sections (thickness, 5 μM) were prepared, stained with hematoxylin and eosin (HE), and examined under a light microscope. Five lesions were ultimately diagnosed as periapical granulomas.

Dual-color immunofluorescence imaging
Dual-color immunofluorescence imaging was performed as described previously to identify cells expressing SIRT1 and 8-OHdG (33). In brief, paraffin tissue sections of periapical granulomas or healthy gingival tissues were stained with a rabbit monoclonal anti-human SIRT1 antibody and a mouse monoclonal anti-human 8-OHdG antibody (1/100; Abcam) as primary antibodies, followed by incubation with an FITC-conjugated goat anti-rabbit IgG antibody and an RITC-conjugated goat anti-mouse IgG antibody (1/100; Abcam) as secondary antibodies. Nuclei were counterstained with DAPI. SIRT1-, 8-OHdG-, and DAPI-positive cells were counted, and the proportions of SIRT1- and 8-OHdG-positive cells among DAPI-positive cells were calculated.

Statistical analysis
Statistical analyses were performed with SPSS version 15.0 (SPSS, Chicago, IL, USA). The Tukey-Kramer test or Mann-Whitney U test was used to compare differences between groups. A P value of <0.05 was considered to reflect statistical significance.

Results
Effects of resveratrol and sirtinol on SIRT1 expression by U-937 cells
U-937 cells were cultured with LPS (0.5 or 1.0 μg/mL) and/or resveratrol (10 or 50 μM), and SIRT1 mRNA level was determined by real-time PCR. Treatment with LPS or resveratrol increased SIRT1 mRNA level in a dose-dependent manner (Fig. 1A, B) after incubation for 6, 12, and 24 h. Indeed, combined treatment with LPS and resveratrol resulted in the highest SIRT1 mRNA level (Fig. 1C). Treatment with 1.0 μg/mL LPS or 50 μM resveratrol for 6 h resulted in the highest SIRT1 mRNA level.

Next, U-937 cells were treated with LPS, resveratrol, or sirtinol for 1, 2, 4, 6, 8, or 10 h. SIRT1 mRNA level peaked after 6-h incubation with LPS (1.0 μg/mL) or resveratrol (50 μM; Fig. 2A, B), but sirtinol (50 μM) did not affect SIRT1 mRNA level (Fig. 2C). Combined treatment with LPS and resveratrol for 8 h resulted in the highest SIRT1 mRNA level (Fig. 2D). In contrast, combined treatment with LPS, resveratrol, and sirtinol resulted in a SIRT1 mRNA level similar to that of the control group (Fig. 2E).

Dual-color immunocytochemistry
Dual-color immunofluorescence staining was used to analyze cytospin specimens of cultured U-937 cells for SIRT1 and Ki-67 expression. The control group showed no detectable SIRT1 or Ki-67 expression. The control group showed no detectable SIRT1 or Ki-67 expression (Fig. 3A-D). Treatment of U-937 cells with resveratrol (Fig. 3L) resulted in higher SIRT1 and Ki-67 protein levels than did treatment with LPS (Fig. 3E-H). Treatment with sirtinol (Fig. 3M-P) did not increase SIRT1 or Ki-67 protein levels (Fig. 3I-J).
not affect the protein level of SIRT1 or Ki-67. Combined treatment with LPS and resveratrol resulted in the highest SIRT1 and Ki-67 protein levels (Fig. 3Q-T). By contrast, combined treatment with LPS and sirtinol had no effect on SIRT1 or Ki-67 protein levels (Fig. 3U-X).

Pathological examination of periapical lesions and healthy gingival tissues
Surgically excised periapical lesions (n=7) and healthy gingival tissues (n=5) were examined pathologically with HE staining and evaluated as described by Nair (34). Five of the periapical lesions contained granulomatous tissue that harbored numerous capillaries and infiltration of inflammatory cells. No epithelial cells were evident in any of the lesions, which were therefore diagnosed as periapical granulomas (Fig. 4A). Healthy gingival tissues exhibited epithelial cell layers and fewer inflammatory cells (Fig. 4B) than did periapical granulomas.

Dual-color immunofluorescence imaging
Periapical granulomas and healthy gingival tissues were examined by using dual-color immunofluorescence imaging with anti-human SIRT1 and 8-OHdG antibodies. SIRT1 and 8-OHdG were expressed by round cells in periapical granulomas (Fig. 4D, E), and merged images showed that some SIRT1-expressing cells also expressed 8-OHdG (Fig. 4F). The median proportions of SIRT1- and 8-OHdG-expressing cells, as indicated by DAPI-positive cells, were 16.5% and 29.9%, respectively (Fig. 4K), a significant difference. The percentage of co-expressing cells in periapical granulomas was 10.8%. Neither SIRT1 nor 8-OHdG was detected in healthy gingival tissues (Fig. 4H, I).

Discussion
This study evaluated the effects of resveratrol and sirtinol on SIRT1 expression by U-937 cells. Resveratrol is a plant-derived polyphenol and a SIRT1 activator. Interestingly, in animal models resveratrol inhibits diabetic nephropathy by enhancing angiogenesis (35). Treatment of fibroblasts with TNF-α resulted in production of proinflammatory cytokines such as IL-1β, IL-6, and inducible...
nitric oxide. However, resveratrol reduced expressions of these factors in a dose-dependent manner (36), which indicates that it has anti-inflammatory activity.

Treatment of U-937 cells with resveratrol increased SIRT1 mRNA level in a dose-dependent manner, with a peak at 6 h after incubation. Therefore, resveratrol likely functions early on SIRT1 activation. In addition, LPS treatment increased SIRT1 mRNA level in U-937 cells; however, sirtinol reduced this level to control levels. Thus, resveratrol may modulate SIRT1 activity in monocytes/macrophages and may be involved in suppressing LPS-mediated inflammation.

SIRT1 is involved in the proliferation and survival of macrophages, lymphocytes, and endothelial cells (29,37,38). In addition, suppression of SIRT1 expression inhibits the G1/S transition in the cell cycle (39). To assess the role of SIRT1 in cell proliferation, cytospin specimens of U-937 cells were subjected to dual-color immunofluorescence staining with anti-SIRT1 and anti-Ki-67 antibodies. Ki-67 is a nuclear factor that enhances proliferation of non-quiescent cells. Ki-67 is expressed in the G1, S, G2, and M but not G0 phases of the cell cycle and serves as a marker of cell proliferation (40). Treatment of U-937 cells with resveratrol resulted in a greater increase in SIRT1 and Ki-67 expression than did treatment with LPS; however, sirtinol did not increase the expression level of SIRT1 or Ki-67. Thus, SIRT1 may enhance macrophage proliferation. Combined treatment with LPS and resveratrol resulted in the highest SIRT1 and Ki-67 expression. By contrast, treatment with LPS and sirtinol did not affect expression of SIRT1 or Ki-67. These findings suggest that resveratrol induces SIRT1 expression in U-937 cells.

Resveratrol downregulates oxidative stress and reduces inflammation in persons with chronic disease or traumatic injury (41-43). The effects of SIRT1 on oxidative stress are essential in suppressing inflammation. Therefore, we analyzed expression levels of SIRT1 and 8-OHdG, a marker of oxidative stress, in periapical granulomas, which are rich in inflammatory cells. Dual-color
immunofluorescence imaging of periapical granulomas showed that SIRT1-expressing cells also expressed 8-OHdG. By contrast, healthy gingival tissues did not express SIRT1 or 8-OHdG. SIRT1 inhibits oxidative stress by upregulating superoxide dismutase 2 (44), and resveratrol reduces oxidative stress in macrophages (45). Therefore, we hypothesized that SIRT1 and 8-OHdG are co-expressed in periapical granulomas as a result of oxidative stress-mediated damage to inflammatory cells, which enhances the wound healing activity of these cells.

In conclusion, we found that resveratrol enhances proliferation of U-937 cells and induces SIRT1 expression and that inflammatory cells in periapical granulomas co-express SIRT1 and 8-OHdG. Therefore, we conclude that macrophages express SIRT1 and that healing of periapical granulomas is facilitated by the resulting reduction in oxidative stress.

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Conflict of interest
The authors have no conflict of interest regarding the authorship or publication of this article.

References


