

Intraperitoneal injection of Nrf2 activator inhibited calvarial bone destruction in mice

Ken Tanaka¹, Hiroyuki Kanzaki^{1,2*}, Satoshi Wada¹, Yuta Katsumata¹, Tsuyoshi Narimiya¹ and Hiroshi Tomonari¹

¹Department of Orthodontics, School of Dental Medicine, Tsurumi University, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama, Kanagawa 230-8501, Japan

²Tohoku University Hospital, Maxillo-oral Disorders, Sendai, Japan

Abstract

Background: Aging-related bone and cartilage destructive diseases, such as rheumatoid arthritis, are characterized by excessive bone resorption by osteoclasts. Osteoclastogenesis is mainly regulated by RANKL, which used reactive oxygen species (ROS) for the intracellular signaling. Previously we discovered that RANKL attenuates anti-oxidation via inhibition of transcriptional regulation of Nrf2-mediated anti-oxidant enzymes, and augments intracellular ROS signaling. In this research, we compared several anti-oxidant, such as Nrf2 activators, and non Nrf2-activating molecules, for the inhibitory effects of *in vitro* osteoclastogenesis and *in vivo* bone destruction.

Material and methods: RAW 264 cells were stimulated with RANKL in the presence of Catechin, Epigallocatechin-Gallate (EGCG), Sulforaphane, or vitamin C. Nrf2 promoter reporter assay was also performed. Using the calvarial bone destruction mouse model, which induced by repeated LPS injections, we compared inhibitory effect of the intraperitoneal injection of catechin, EGCG, or Sulforaphane on bone destruction using microCT images. Results: RANKL-mediated osteoclastogenesis from RAW 264 cells were blocked by SFN and EGCG. Transcriptional activity of Nrf2 were induced by SFN and EGCG. *In vivo* bone destruction were also inhibited by SFN and EGCG. Conclusions: In conclusion, the molecule which has Nrf2 activation property successfully inhibited bone destruction, which signifying Nrf2 activator would be therapeutic drug for bone destructive diseases.

Abbreviations: Nrf2: nuclear-factor E2-related factor 2; RANKL: receptor activator of nuclear factor kappa beta ligand; ROS: reactive oxygen species; EGCG: Epigallocatechin-Gallate; LPS: lipopolysaccharide; SFN: Sulforaphane; CT: computed tomography; QOL: quality of life; IL: interleukin TNF- α : tumor necrosis factor alpha; VC: vitamin C; FBS: fetal bovine serum; TRAP: tartrate resistant acid phosphatase; SD: standard deviation; ARE: antioxidant response element; HO1: heme oxygenase 1; GCS: γ -glutamylcysteine synthetase.

Introduction

Japan is in a super-aged society in which 1 in 4 persons in the Japanese population is elderly [1]. Therefore, prevention of an aging-related bone destructive diseases such as rheumatoid arthritis, osteoporosis, and periodontitis is in demand from the point of view of maintaining QOL, social aspects, and medical expenses. As to the pathogenesis of osteoarthritic cartilage destruction, not only cartilage but also bone and synovium play key roles in progression of disease by producing pro-inflammatory cytokines such as IL-1 and TNF- α [2]. Elevated inflammatory cytokines induce matrix metalloproteinase production in site, which resulted in the matrix degradation in articular cartilage [3]. In addition, production of reactive oxygen species (ROS) are induced during articular cartilage destruction. These pro-inflammatory cytokines and ROS favor osteoclastogenesis directly and indirectly, which promote subchondral bone resorption and further destruction of joint tissue [4].

Bone destruction is generally caused by the dysregulated activation of osteoclasts [5], with augmented production of osteoclastogenic cytokines such as RANKL, TNF- α , and interleukins [6]. Among these osteoclastogenic cytokines, RANKL is thought to be an essential factor

for osteoclastogenesis [7]. RANKL uses multiple intracellular signaling pathways, which includes reactive oxygen species (ROS) [8]. Not only work as an intracellular signaling molecule, but also ROS exhibits cytotoxicity against cells [9]. Cells have several protective mechanisms against these oxidative stressors, accordingly [10]. One of the major cellular anti-oxidation is the transcriptional induction of anti-oxidant enzymes by nuclear-factor E2-related factor 2 (Nrf2) [11].

Previously we discovered that RANKL attenuates anti-oxidation via inhibition of transcriptional regulation of nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated anti-oxidant enzymes, and augments intracellular ROS signaling [12]. Induction of anti-oxidant enzymes by Nrf2 activation subsequently inhibits osteoclastogenesis and *in vivo* bone destruction by local injection [13-17]. Inversely, augmented osteoclastogenesis and bone destruction was observed with Nrf2 deficiency [18-22]. Taken together, this shows that Nrf2-dependent anti-oxidant enzymes play a critical role in the regulation of bone destruction. More specifically, Nrf2 activation would be therapeutic treatment for bone destructive diseases.

Besides the induction of anti-oxidant enzymes by Nrf2 activation, direct ROS scavenging by anti-oxidant such as vitamin C (VC), also

***Correspondence to:** Hiroyuki Kanzaki, D.D.S., Ph.D., Associate Professor, Department of orthodontics, School of Dental Medicine, Tsurumi University, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama, Kanagawa pref., 230-8501, Japan, Tel: +81-45-580-8507; Fax: +81-45-573-9599; E-mail: kanzaki-h@tsurumi-u.ac.jp

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contribute the management of ROS [23]. Though previous report revealed that ROS-scavenging vitamin E, which has no Nrf2-activating property, failed to inhibit bone destruction [24], it still remains unclear whether there is obvious difference of inhibitory effect for bone destruction between Nrf2 activator and ROS scavenger.

In this research, we compared inhibitory effect for bone destruction by systemic administration of Nrf2 activator and ROS scavenger using mice.

Materials and methods

Chemicals

Catechin was purchased from Cayman Chemical, Ann Arbor, Michigan, and dissolved in ethanol. Epigallocatechin-Gallate (EGCG) was purchased from Kurita-Kogyo, Tokyo, Japan, and dissolved in ethanol. Sulforaphane (SFN) was purchased from Toronto Research Chemicals, Toronto, ON, and dissolved in dimethylformamide. Vitamin C (ascorbic acid) was purchased from Sigma-Aldrich, St. Louis, MA, USA.

Cells

Mouse monocytic Cell-line RAW264.7 were obtained from the Riken-BioResource Center (Tsukuba, Japan).

Cell culture

RAW264.7 were cultured in α -modified Eagle's medium (Wako-Pure Chemical, Osaka, Japan) containing 10% fetal bovine serum (FBS; Thermo Scientific, South Logan, UT) supplemented with antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin). All cells were cultured at 37°C in a 5% CO₂ incubator.

Osteoclastogenesis assay

Cells were plated onto 96-well plates (10³ cells/well) in triplicated in the presence or absence of recombinant soluble-RANKL (sRANKL; 100 ng/ml; Wako Pure Chemical) with or without catechin (100 μ M), EGCG (10 μ M), vitamin C (50 μ g/mL) and SFN (10 μ M). These concentration of factors were maximum inhibitory concentration we tested and no cytotoxicity were confirmed at the concentration (data not shown). After culture, cells were stained for TRAP using an acid-phosphatase kit (Sigma, St. Louis, MO). Dark-red multinucleated cells (≥ 3 nuclei) were counted as TRAP-positive multinucleated cells.

Nrf2 promoter reporter assay

Cells were transiently transfected with an Nrf2 promoter reporter plasmid (Cignal Antioxidant Response Reporter; Qiagen, Germantown, MD) using the X-tremeGENE HP DNA transfection reagent (Roche Applied Science, Indianapolis, IN, USA). Cells were then stimulated with catechin (100 μ M), EGCG (10 μ M), Vitamin C (50 μ g/mL), and SFN (10 μ M) for 24h, and cell lysates were prepared using lysis buffer containing long half-life luciferase substrate (Pikka-gene LT7.5; Toyo B-Net, Tokyo, Japan). Firefly luciferase activities were measured with a Synergy HTX Multi-Mode plate Reader (BioTek Japan, Tokyo, Japan).

In vivo bone destruction model

All animal study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Tsurumi University (No. 28A087). Animal experiments were performed in compliance with the Regulations for Animal Experiments and Related Activities at Tsurumi University. The calvarial bone destruction mouse model,

induced by repeated LPS injections, has been described previously [14]. Twenty five 7-week-old male BALB/c mice (Clea Japan, Tokyo, Japan) were used. Mice were randomly assigned to five groups (n = 5 each): control group, LPS group, LPS and SFN group, LPS and catechin group, and LPS and EGCG group. Control group received repeat calvarial PBS injections and latter four groups received repeat calvarial LPS injections (10 μ L of 1 mg/mL LPS) on days 1, 3, 5, 7, and 9. LPS and SFN group, LPS and catechin group, and LPS and EGCG group received repeat intraperitoneal injections of SFN (75 nmol), catechin (3 μ mol), and EGCG (0.3 μ mol) on days 1, 3, 5, 7, and 9, respectively. On day 11, mice were euthanized by cervical dislocation and cranial tissue samples were fixed overnight with 4% paraformaldehyde in PBS. Then soft tissue were defleshed from calvarial bone, and TRAP staining were performed. The photographs of the stained calvarial bone were then taken, and red channel were separated using Photoshop (Adobe Systems Co., Ltd., Tokyo, Japan). Percent of positive area, (number of pixels of red+ area/ number of pixels of analyzed calvarial bone area) were calculated using Image-J (National Institutes of Health, Bethesda, MD).

Statistical analysis

All data are presented as the mean \pm standard deviation (SD). Multiple comparisons (a priori comparisons) were performed using Tukey's test. P < 0.05 was considered to be statistically significant.

Results

SFN and EGCG strongly, but catechin weakly inhibited osteoclastogenesis *in vitro*

Firstly, inhibitory effects of SFN, EGCG, catechin, and VC on osteoclastogenesis were examined (Figure 1). SFN almost completely inhibited osteoclastogenesis (Figure 1c). EGCG strongly inhibited osteoclastogenesis (Figure 1d). But catechin weakly inhibited osteoclastogenesis (Figure 1e). VC had marginal inhibitory effects on osteoclastogenesis (Figure 1f). These results suggest that each anti-oxidant has different inhibitory activity on osteoclastogenesis.

Nrf2 promoter-luciferase reporter activity

We found that SFN induced antioxidant response element (ARE)-dependent transcription of the reporter gene, indicating increased transcriptional activity of Nrf2 (Figure 2). EGCG also induced reporter gene. However, the induction of the reporter gene by catechin was weak as compared to that of SFN and EGCG. Vitamin C marginally induced the expression of the reporter gene.

These results suggest that SFN and EGCG triggers but catechin and vitamin C cannot trigger Nrf2-mediated transcription of antioxidant enzymes in RAW 264.7 cells.

SFN and EGCG strongly, but catechin weakly induced the expression of anti-oxidant enzymes

We then examined the expression of anti-oxidant enzymes in RAW 264.7 cells. Experiment 1 clearly demonstrated that SFN strongly induced the expression of HO1 (Figure 3a). EGCG induced both HO1 and GCS expression in experiment 1 and 2 (Figures 3a and 3b). Catechin weakly induced GCS expression in experiment 2 (Figure 3b). On the other hand, vitamin C marginally induced both HO1 and GCS (Figures 3a and 3b). These results suggest that SFN and EGCG strongly, but catechin weakly induces Nrf2-mediated transcription of antioxidant enzymes in RAW 264.7 cells.

Systemic administration of SFN and EGCG strongly, but catechin weakly attenuated calvarial bone destruction *in vivo*

Finally, we examined whether systemic administration of SFN, EGCG, and catechin inhibit LPS-induced bone destruction at calvaria (Figure 4). Repeat LPS injection at calvaria induced extensive bone destruction (G-2; Figure 4b). IP injection of SFN (G-3) and EGCG (G-5) successfully attenuated LPS-induced bone destruction (Figures 4c and 4e). However, IP injection of catechin (G-4) could not inhibit

LPS-induced bone destruction (Figure 4d). Calculation of the resorbed area clearly demonstrated statistical difference at G-3 and G-5 against G-2, respectively (Figure 4f). Consistent with the *in vitro* results, SFN exhibited stronger inhibition of bone destruction as compared to that of EGCG. There was no statistical difference between G-2 and G-4.

These results suggest that inhibitory effect on osteoclastogenesis and bone destruction closely related to the induction activity of Nrf2-mediated transcription.

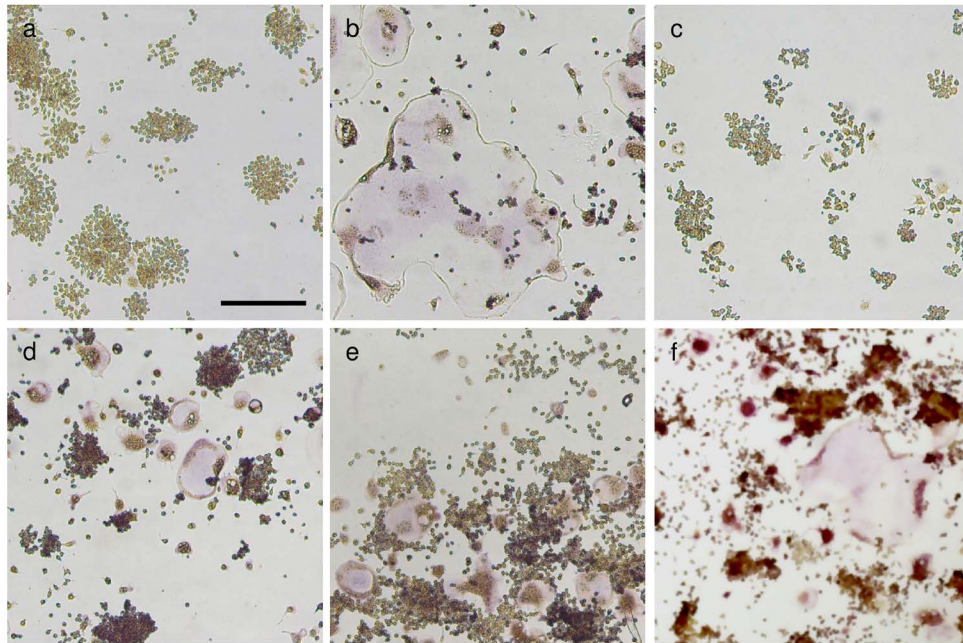


Figure 1. SFN and EGCG strongly, but catechin weakly inhibited osteoclastogenesis *in vitro*. Representative photographs of control (a), RANKL-treated (b), RANKL + SFN (c), RANKL + EGCG (d), RANKL + catechin (e), and RANKL + VC (E) are shown

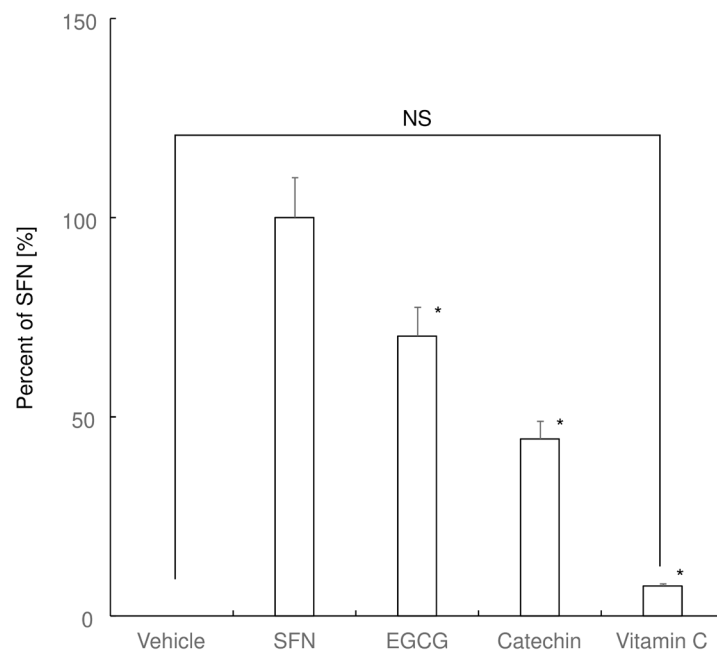


Figure 2. Nrf2 promoter-luciferase reporter activity. Relative activity of the Nrf2-responsive luciferase in RAW 264 cells against SFN-treated cells are shown. *P < 0.05 versus SFN. NS: no significant difference between the groups

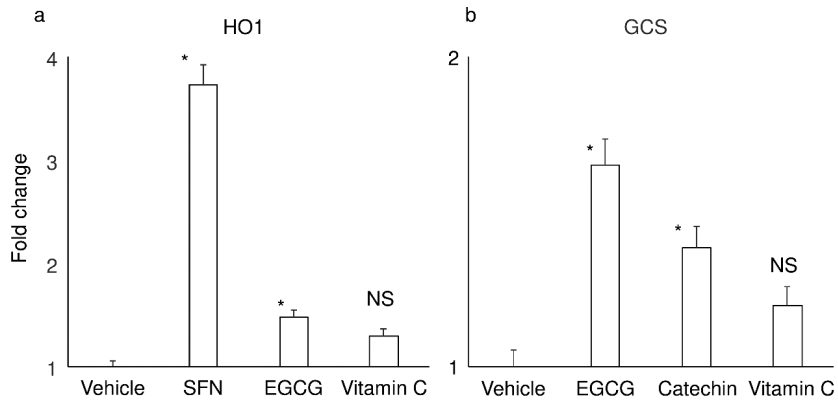


Figure 3. Realtime RT-PCR analysis for anti-oxidant enzymes. The results from independent experiments for the gene expression of heme oxygenase 1 (HO1) (a) and gamma-glutamylcysteine synthetase (GCS) (b) are shown. (n=3) *: $p < 0.05$ versus control (vehicle-treated). NS: not significant difference versus control

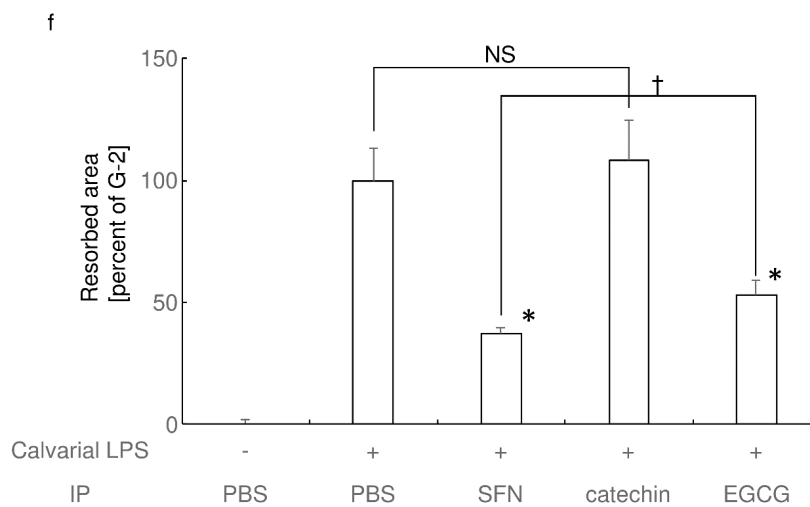
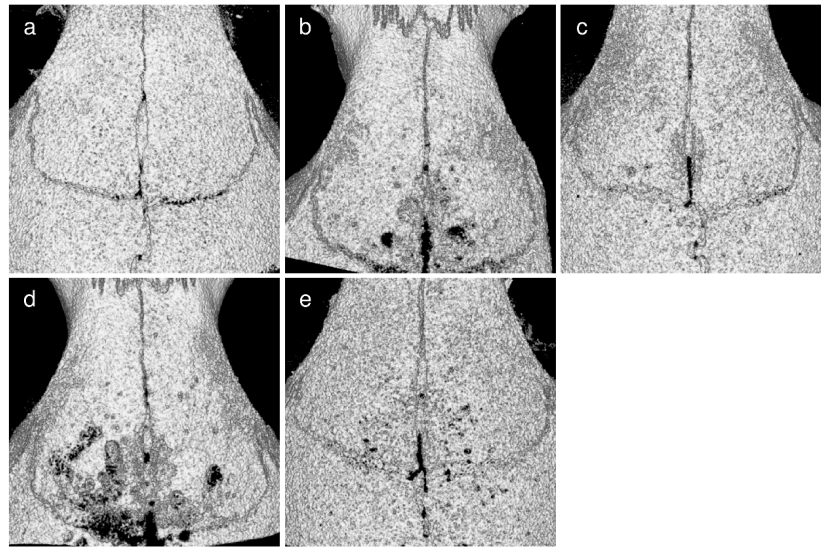


Figure 4. Systemic administration of SFN and EGCG strongly, but catechin weakly attenuated calvarial bone destruction in vivo Representative microCT image of control (a), LPS + PBS IP (b), LPS + SFN IP (c), LPS + catechin IP (d), and LPS + EGCG IP (e) are shown. (f) Percentage of the resorbed area in the cranial bone against G-2 (LPS + PBS IP) are shown. NS: no significant difference between groups. * $P < 0.05$ versus G-2, † $P < 0.05$ between the groups

Discussion

In this research, we examined the relationship between the Nrf2 activation and the inhibitory effects on osteoclastogenesis. Our results suggest that the molecule which induces stronger Nrf2 activation exhibited more extensive inhibitory effect on osteoclastogenesis and bone destruction. Exploration of the inductive activity of Nrf2 activation using Nrf2 promoter-luciferase reporter assay would be the novel simple tool for finding the drug for bone destructive diseases.

As to the pathogenesis of osteoarthritic cartilage destruction, not only cartilage but also bone and synovium play key roles in progression of disease by producing pro-inflammatory cytokines such as IL-1 and TNF- α [2]. In addition, synoviocytes in rheumatoid arthritis express RANKL and support osteoclastogenesis [25]. Furthermore, T cells in arthritic joint produce cytokines such as RANKL, interferon γ , and IL-17, which regulates osteoclastogenesis [6,26,27]. These elevated inflammatory cytokines induce matrix metalloproteinase production in site, which resulted in the matrix degradation in articular cartilage [3]. In addition, production of reactive oxygen species (ROS) are induced during articular cartilage destruction. These pro-inflammatory cytokines and ROS favor osteoclastogenesis directly and indirectly, which promote subchondral bone resorption and further destruction of joint tissue [4].

Bone destructive diseases are generally caused by the dysregulated activation of osteoclasts [5], we examined the direct effects of the molecules on osteoclast and osteoclast precursors. Our results support our hypothesis that Nrf2 activation more strongly inhibits osteoclastogenesis as compared to the inhibition of osteoclastogenesis by ROS scavenging. Consistent with our results, ROS scavenging vitamin E does not prevent bone loss in rats with ligature-induced periodontitis [24].

Although we focused on the direct effect of the molecules on osteoclast and osteoclast precursors, a possible indirect effect of the molecules on osteoclasts *via* osteoclastogenesis supporting cells should also be considered. Considering the cartilage/bone destructive site, osteoclastogenesis supporting cells such as osteoblasts, synoviocytes, and immune cells produces osteoclastogenic cytokines including RANKL [2-4,6,25,27]. We did not examine whether RANKL production was attenuated by the molecules or not, but it was reported that EGCG suppresses LPS-induced expression of RANKL in osteoblasts [28]. SFN also suppresses RANKL production in osteocytes [29]. Together, these Nrf2 activators inhibits RANKL production and indirectly attenuates osteoclastogenesis. In conclusion, the molecule which induces stronger Nrf2 activation could be used for the therapeutic drug for bone and cartilage destructive diseases.

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