BIOCHEMICAL PHARMACOLOGY 73 (2007) 34–43

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

# Identification of two biologically crucial hydroxyl groups of (−)-epigallocatechin gallate in osteoclast culture

*Hiroshi Nakagawa* [a,b], *Keiji Hasumi* [b], *Masamichi Takami* [c], *Sachiko Aida-Hyugaji* [d], *Je-Tae Woo* [e], *Kazuo Nagai* [e], *Toshihisa Ishikawa* [a], *Masaaki Wachi* [a,*]

[a] *Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama, Kanagawa 226-8501, Japan*
[b] *Department of Applied Biological Science, Tokyo Noko University, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan*
[c] *Department of Biochemistry, School of Dentistry, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan*
[d] *Information Technology Center, Tokai University, 1117 Kitakaname, Hiratsuka, Kanagawa 259-1292, Japan*
[e] *Department of Biological Chemistry, College of Bioscience and Biotechnology, Chubu University, 1200 Matsumoto-cho, Kasugai, Aichi 487-8501, Japan*

ARTICLE INFO

*Article history:*
Received 19 June 2006
Accepted 5 September 2006

*Keywords:*
(−)-Epigallocatechin gallate
Ferric ion reduction
Hydrogen peroxide generation
Osteoclast
Quantum chemical calculation
SAR

*Abbreviations:*
CT, calcitonin
EGCG, (−)-epigallocatechin gallate
HOMO, highest occupied molecular orbital
LUMO, lowest unoccupied molecular orbital
OCL, osteoclast
PBS(−), PBS without $Ca^{2+}$ and $Mg^{2+}$
SAR, structure–activity relationship
SOD, superoxide dismutase
TRAP, tartrate-resistant acid phosphatase

ABSTRACT

(−)-Epigallocatechin gallate (EGCG) induces cell death of osteoclasts in an $Fe^{2+}$- and $H_2O_2$-dependent manner. In the present study, we further explore the cytotoxic mechanism of EGCG using four EGCG analogues. Molecules methylated at position 4′ in the B ring (EGCG-4′-O-Me) or at position 4″ in the D-ring (EGCG-4″-O-Me) showed markedly decreased cytotoxicity to osteoclasts, indicating that hydroxyl groups at these two positions of EGCG are crucial for inducing cell death of osteoclasts. EGCG-4′-O-Me also showed the lowest $Fe^{3+}$-reducing activity among five EGCGs. The $Fe^{3+}$-reducing activity of EGCG was enhanced under conditions whereby protonated EGCG levels were increased, indicating that the protonated status of EGCG was involved in the $Fe^{3+}$-reducing activity. The hydroxyl group at position 4″ in the D-ring was shown by quantum chemical calculation to be preferentially deprotonated among all of the hydroxyl groups in EGCGs. It was also shown that the highest occupied molecular orbital (HOMO) was localized to the B-ring of EGCGs, except for EGCG-4′-O-Me. We report here that the HOMO on the B-ring plays crucial roles in both the $Fe^{3+}$-reducing activity of EGCG and the cytotoxicity of EGCG to osteoclasts, while deprotonation of the hydroxyl group at position 4″ in the D-ring plays a supplementary role.



* *Corresponding author*. Tel.: +81 45 924 5770; fax: +81 45 924 5820.
E-mail address: mwachi@bio.titech.ac.jp (M. Wachi).
0006-2952/$ – see front matter 
doi:10.1016/j.bcp.2006.09.002

## 1. Introduction

Two major types of cells, osteoclasts and osteoblasts, are involved in bone metabolism. Osteoclasts are bone-resorbing multinucleated cells that become tightly attached to mineralized bone surfaces through their integrins and form resorption lacuna by secreting protons ($H^+$), proteases, and superoxide ($O_2^{\bullet -}$) through ruffled borders [1–3]. Osteoblasts are bone-forming cells that are located near to the surface of bone and produce cytokines such as macrophage-colony stimulating factor (M-CSF) [4] and receptor activator of nuclear factor-κB ligand (RANKL) [5], which are essential for osteoclast differentiation, function, and survival. Imbalance between bone-resorption and -formation is the key pathophysiological events in many metabolic bone disorders in adult humans, including osteoporosis, hyperparathyroidism, metastastic bone disease, and hypercalcemia in malignancy. Therefore, we have screened for natural compounds that suppress enhanced bone resorption. Among approximately 300 purified low molecular weight natural compounds, we previously found that one of the most extensively studied flavonoids (−)-epigallocatechin gallate (EGCG) induced apoptosis in osteoclasts [6].

We suggested that EGCG was a possible prophylactic or therapeutic agent for metabolic bone disorders [6] while it has been demonstrated *in vitro* to have a wide range of pharmacological properties such as antioxidative [7], antimutagenic [8], and cytotoxic effects [9–27]. Structure–activity relationship (SAR) analysis with (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and EGCG has been extensive [18–27], and has found two different substructures that primarily contribute to the cytotoxicity of the catechins. The pyrogallol moiety is crucial for cytotoxicity to human T-cell acute lymphoblastic leukemia Jurkat cells [18], Ha-*ras*-transformed JB6 30.7b cells [19], Ehrlich ascites tumor cells [20], human colon carcinoma LoVo cells [21], and human lymphoid leukemia Molt 4B cells [22,23], while the gallate moiety is crucial for cytotoxicity to human prostate cancer DU145 cells [24], human stomach cancer KATOIII cells [25] and MK-1 cells [26], and human myeloid leukemia U937 cells [27]. EGCG containing both gallate and pyrogallol moieties exhibited higher cytotoxicity than pyrogallol moiety-containing (−)-epigallocatechin or gallate moiety-containing (−)-epicatechin gallate in all of these studies. Although these two moieties are likely to act synergistically in EGCG, further detailed studies are required to determine the substructures of EGCG that are crucial for increased cytotoxicity.

EGCG has been found to cause several characteristic phenomena of apoptosis, such as cell cycle arrest [11–13], injury of DNA [11–17], and activation of caspases [15–18]. These represent a number of the molecular mechanism underlying the cytotoxicity of EGCG. We also proposed a new mechanism: EGCG triggers the Fenton reaction to form highly reactive hydroxyl radicals from $H_2O_2$ and $Fe^{2+}$ [$H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$], and the resulting hydroxyl radical induces apoptotic cell death in osteoclasts and Jurkat cells [6,18]. Furthermore, our previous studies demonstrated that EGCG exhibited both $H_2O_2$-producing and $Fe^{3+}$-reducing activities [6,18]. The $H_2O_2$-producing activity rather than $Fe^{3+}$ reduction played a crucial role in the cytotoxicity to Jurkat cells [18]. However, it has not yet been elucidated which activity is essential to the cell death of osteoclasts.

The present study was designed to elucidate the role of pyrogallol and gallate moieties in the cytotoxicity of EGCG, and to determine the substructures of EGCG that are crucial to the induction of osteoclast cell death and the contributions of the $Fe^{3+}$-reducing and $H_2O_2$-producing activities of EGCG.

## 2. Materials and methods

### 2.1. Animals and chemicals

Newborn Std.ddY mice and 6- to 9-week-old male Std.ddY mice were purchased from Japan SLC Co. (Hamamatsu, Japan). EGCG, (−)-epigallocatechin-3′-*O*-methylether gallate (EGCG-3′-*O*-Me), (−)-epigallocatechin-4′-*O*-methylether gallate (EGCG-4′-*O*-Me), (−)-epigallocatechin 3-(3″-*O*-methyl) gallate (EGCG-3″-*O*-Me), and (−)-epigallocatechin 3-(4″-*O*-methyl) gallate (EGCG-4″-*O*-Me) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Fast red violet LB salt, naphthol AS-MX phosphate, catalase from bovine liver, and D-sorbitol were from Sigma Chemical Co. (St. Louis, MO, USA). Collagenase, 1α,25-dihydroxyvitamin $D_3$ [1α,25$(OH)_2D_3$], *o*-phenanthroline hydrochloride, human recombinant superoxide dismutase (SOD), hydrogen peroxide ($H_2O_2$), and 0.1% (w/v) xylenol orange solution were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dispase and 0.25 M ammonium iron(II) sulfate solution were purchased from Godo Shusei (Tokyo, Japan) and Kanto Chemical Co., Inc. (Tokyo, Japan), respectively. Collagen gel solutions (Cellmatrix, Type I-A) were from Nitta Gelatin Co. (Osaka, Japan).

### 2.2. Preparation of osteoclasts

Murine tartrate-resistant acid phosphatase-positive multinucleated cells having more than four nuclei (OCLs, osteoclasts) were prepared from a coculture system as previously described [6,28]. In brief, primary mouse osteoblastic cells were obtained from calvariae of newborn ddY mice, and mouse bone marrow cells were from the femora and tibiae of 6- to 9-week-old male ddY mice. Bone marrow cells ($2 \times 10^7$ cells) and osteoblastic cells ($1 \times 10^6$ cells) were cocultured for 7 days in α-minimal essential medium (α-MEM) (Sigma Chemical Co., St. Louis, MO, USA) containing 10% (v/v) fetal calf serum (FCS) (Asahi Techno Glass Ltd., Chiba, Japan) on 100-mm tissue culture dishes (Corning Inc., Corning, NY) (15 ml/dish) precoated with 2.5 ml of 0.2% (w/v) collagen gel matrix. Half of the medium was changed after coculture for 2 and then 5 days. For the last 5 days, cells were cultured in the presence of 10 nM 1α,25$(OH)_2D_3$ and 1 μM $PGE_2$. Osteoclasts were formed within 7 days of culture and released from the dishes by treatment with 1.5 ml of 0.2% (w/v) collagenase and 0.1% (w/v) dispase. Cells were collected by centrifugation ($300 \times g$, 5 min) and placed into 96-well culture plates (Corning Inc.), where 180–270 of osteoclasts per well was observed.

### 2.3. Assay for cytotoxicity to osteoclasts

Osteoclast preparations were placed into 96-well culture plates. After preculture for 24 h, the cells were treated with vehicle [1% (v/v) methanol] or EGCGs [dissolved in methanol

such that the final concentration of methanol in culture medium was 1% (v/v)] for 24 h and stained for TRAP, a typical marker enzyme of osteoclasts. TRAP staining was carried out as described previously [28]. In brief, the cells were fixed with 3.7% (v/v) formaldehyde in phosphate-buffered saline without $Ca^{2+}$ and $Mg^{2+}$ [PBS(−)] for 15 min. After treatment with ethanol/acetone (1:1) for 1 min, the well surface was dried and treated with the TRAP staining solution [0.1 M sodium acetate buffer (pH 5.0) containing 50 mM sodium tartrate, 0.1 mg/ml naphthol AS-MX phosphate, and 1 mg/ml fast red violet LB salt] for 10 min. After the staining, the number of osteoclasts was counted. Data from quadruplicate cultures in one of three experiments were expressed as the mean ± S.D. of the percentage of the number of osteoclasts in control cultures.

### 2.4. Assay for cytotoxicity to osteoblastic cells

Primary osteoblastic cells ($4 \times 10^4$ cells/well) were placed into 96-well culture plates and precultured for 24 h. Then, the cells were cultured with vehicle [1% (v/v) methanol] or 100 μM catechins for 24 h. After the culture, the cells were incubated in 100 μl of 0.1% (w/v) crystal violet in PBS(−) for 15 min. After washing the cells with PBS(−), the dye incorporated into the cytoplasm of living cells was eluted with 100 μl of methanol, and then absorbance at 595 nm was measured. Viability of osteoblastic cells was expressed as a percentage of the absorbance in control culture. Data were expressed as the mean ± S.D. of quadruplicate cultures in one of three experiments.

### 2.5. Assay for $Fe^{3+}$-reduction

The activity of EGCG to reduce $Fe^{3+}$ were measured based on the previous method [6,18] with slight modification. In brief, EGCGs (1 μM) were added to 1 ml of working solution (5 mM *o*-phenanthroline in 1 mM $FeCl_3$), and the mixture was incubated at room temperature for 20 s. In another experiment, EGCG and EGCG-4′-*O*-Me (1 μM) were added to 300 μl of 1 mM $FeCl_3$-containing 100 mM acetate buffer (pH 3.6, 4.6 or 5.6). An equal volume of 5 mM *o*-phenanthroline solution was added after incubation at room temperature for 20 s, and the mixture was further incubated at room temperature for 20 s. After the incubation, absorbance of the $Fe^{2+}$-*o*-phenanthroline complex at 510 nm was immediately measured. $Fe^{3+}$-reducing activity of the catechins was assessed from the concentration of $Fe^{2+}$-*o*-phenanthroline complex measured in solutions, which was calculated from a standard curve generated by using known concentrations of $Fe^{2+}$-*o*-phenanthroline complex. This assay method was applicable to $Fe^{2+}$-*o*-phenanthroline complex solutions as low as 0.1 μM. Data were expressed as the mean ± S.D. of quadruplicate cultures in one of three experiments.

### 2.6. Measurement of the concentration of $H_2O_2$ in α-MEM

EGCGs (100 μM) were added to osteoclast cultures with or without 100 U/ml catalase, and then the cultures were placed at 37 °C under 5% (v/v) $CO_2$. After 15, 30, 60, 120, 240, and 360 min, the concentration of $H_2O_2$ in the culture was measured. $H_2O_2$-producing activity of catechins was also determined in cell-free medium, where EGCGs (100 μM) were added to 96-well culture plates filled with 100 μl of α-MEM without FCS and cells, and the plates were placed at 37 °C under 5% (v/v) $CO_2$. After 1 h, the concentration of $H_2O_2$ was measured by the ferrous ion oxidation-xylenol orange method with slight modification [18,29]. Briefly, medium (30 μl) was mixed with 300 μl of working solution [250 μM ammonium iron(II) sulfate, 25 mM $H_2SO_4$, 100 mM sorbitol, and 125 μM xylenol orange], followed by mixing and incubation at room temperature for 20 min. After incubation, absorbance of $Fe^{3+}$–xylenol orange complex at 595 nm was measured. The concentration of $H_2O_2$ was calculated from standard curve, which was obtained by determining $H_2O_2$ concentrations immediately after the addition of $H_2O_2$ into the culture medium. This assay method was applicable for determining $H_2O_2$ concentrations as low as 0.2 μM. Data were expressed as the mean ± S.D. of three independent experiments.

### 2.7. Quantum chemical calculation

The MOPAC2002 software (Fujitsu Ltd., Tokyo, Japan) was used for molecular orbital (MO) and molecular mechanics (MM) calculations in the present study. The structures of EGCG and its analogues (EGCG-3′-*O*-Me, EGCG-3″-*O*-Me, EGCG-4′-*O*-Me and EGCG-4″-*O*-Me) were determined by a conformation search using MM2 procedures and by following the semi-empirical MO method with AM1 parameters [30]. And then, the lowest unoccupied molecular orbitals (LUMOs), highest occupied molecular orbitals (HOMOs), and formation energies of EGCGs were quantum chemically calculated. The deprotonation energy of each hydroxyl group in EGCG was acquired by subtracting the formation energy of deprotonated EGCG from that of protonated EGCG.

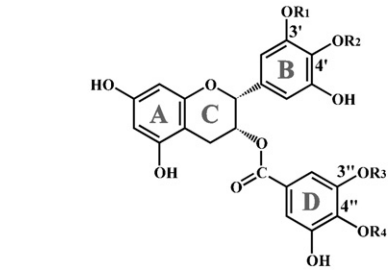
### 2.8. Statistical analysis

Statistical significance of differences was determined by using Student's *t*-test.

## 3. Results

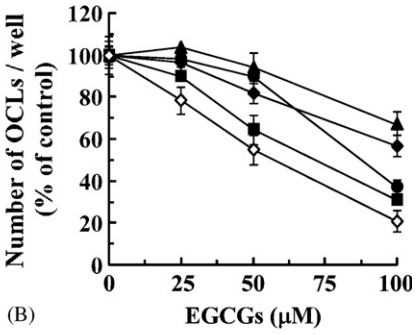
### 3.1. Involvement of hydroxyl groups in EGCG in induction of osteoclast cell death, reduction of $Fe^{3+}$, and production of $H_2O_2$

To determine the structural requirements for EGCG to induce cell death of osteoclasts, we performed a SAR analysis with four EGCG analogues (EGCG-3′-*O*-Me, EGCG-3″-*O*-Me, EGCG-4′-*O*-Me and EGCG-4″-*O*-Me) in which one of the hydroxyl groups in the B- or D-ring is methylated. All analogues showed cytotoxicity to osteoclasts, where no statistical significant difference was observed between $EC_{50}$ value of EGCG and those of its analogues ($EC_{50}$: EGCG = 58 ± 14 μM; EGCG-3′-*O*-Me = 87 ± 2.2 μM; EGCG-3″-*O*-Me = 70 ± 11 μM; EGCG-4′-*O*-Me = > 100 μM; EGCG-4″-*O*-Me = > 100 μM) (Fig. 1B). However, all analogues showed significantly reduced cytotoxicity to osteoclasts at 100 μM, compared to EGCG. The cytotoxic effects of EGCG-4′-*O*-Me and EGCG-4″-*O*-Me were about half as much as that of EGCG (Fig. 2A). Osteoclasts require the

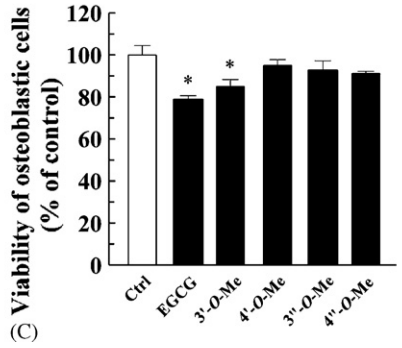



| EGCGs | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| EGCG | H | H | H | H |
| 3'-*O*-Me | $CH_3$ | H | H | H |
| 4'-*O*-Me | H | $CH_3$ | H | H |
| 3''-*O*-Me | H | H | $CH_3$ | H |
| 4''-*O*-Me | H | H | H | $CH_3$ |

(A)




(B)




(C)

**Fig. 1 – Cytotoxic effects of EGCG and its analogues on osteoclasts and osteoblastic cells. (A) Structures of EGCG and its analogues. (B) Cytotoxicity of EGCGs on osteoclasts. Osteoclasts placed into 96-well culture plates were cultured in the presence of EGCG-3′-O-Me (closed circle), EGCG-4′-O-Me (closed triangle), EGCG-3″-O-Me (closed square), EGCG-4″-O-Me (closed diamond), or EGCG (open diamond) (25, 50 and 100 μM). After culture for 24 h, the osteoclasts were stained for TRAP, and then counted (mean ± S.D. of quadruplicate cultures). (C) Effect of EGCGs on viability of osteoblastic cells. Osteoblastic cells placed into 96-well culture plates were cultured in the presence of EGCG and its methylated analogues (100 μM). After culture for 24 h, their cytotoxic effects on the viability of osteoblastic cells were determined. Data were expressed as the mean ± S.D. of quadruplicate cultures in one of three experiments. *P < 0.01 as compared with control (Ctrl). 3′-O-Me: EGCG-3′-O-Me; 4′-O-Me: EGCG-4′-O-Me; 3″-O-Me: EGCG-3″-O-Me; 4″-O-Me: EGCG-4″-O-Me.**

existence of osteoblasts to sustain their viability in this experiment. Although EGCG and EGCG-3′-*O*-Me significantly reduced viability of osteoblasts, other analogues showed no cytotoxicity to osteoblasts at 100 μM (Fig. 1C). These results indicate that the hydroxyl groups at positions 4′ and 4″ in EGCG play crucial roles in the induction of cell death of osteoclasts.

In cell-free medium, the low-cytotoxicity analogues EGCG-4′-*O*-Me showed the lowest $Fe^{3+}$-reducing activity and the lowest $H_2O_2$-producing activity among the five EGCGs tested (Fig. 2B and C). These results indicate that the hydroxyl groups at position 4′ in EGCGs play crucial roles in both $Fe^{3+}$-reducing and $H_2O_2$-producing activities. However, another low-cytotoxicity analogue, EGCG-4″-*O*-Me, had an $H_2O_2$-producing activity comparable with that of EGCG, which showed high cytotoxicity (Fig. 2C). Thus, it is suggested that $Fe^{3+}$-reducing activity rather than $H_2O_2$ production is involved in the cytotoxicity of EGCGs to osteoclasts.

### 3.2. *Cytotoxicity of exogenous $H_2O_2$ and SOD to osteoclasts*

To determine the contributions of the $Fe^{3+}$-reducing and $H_2O_2$-producing activities of EGCG to the induction of osteoclasts cell death, we next examined whether EGCG produced $H_2O_2$ in osteoclast culture as well as in cell-free medium. Levels of $H_2O_2$ increased after the addition of EGCG to osteoclast culture (Fig. 3A). However, the increase was transient and the levels decreased dependent on time, from the maximum observed level of approximately 20 μM $H_2O_2$ (Fig. 3A). Furthermore, EGCG-4′-*O*-Me having the lowest $H_2O_2$-producing activity generated 1.51 ± 0.44 μM $H_2O_2$ at 1 h after the addition. No $H_2O_2$ was generated in the presence of 100 U/ml catalase, an enzyme that catalyses the elimination of $H_2O_2$. In contrast, exogenously added $H_2O_2$ at concentrations exceeding 125 μM induced cell death of osteoclasts in a dose-dependent manner (Fig. 3B). These results suggest that the levels of $H_2O_2$ produced by EGCG in osteoclast culture are insufficient to induce cell death.

To investigate the origin of $H_2O_2$, we used superoxide dismutase (SOD), an enzyme that generates $H_2O_2$ from $O_2^{\bullet-}$, and calcitonin, a hormone that suppresses bone resorption [31]. SOD enhanced the cytotoxic activity of EGCG to osteoclasts (Fig. 4A and C) while it showed cytotoxicity to neither osteoclasts nor osteoblastic cells (Fig. 4B and C). Although statistical significant difference was not provided between $EC_{50}$ value of EGCG and EGCG + SOD ($EC_{50}$: EGCG = 52 ± 6.6 μM; EGCG + SOD = 41 ± 5.3 μM), SOD significantly enhanced the cytotoxicity of EGCG to osteoclasts at 50 and 100 μM (Fig. 4A). It is known that bone-resorbing osteoclasts generate superoxide [2,3], and that superoxide generation is suppressed by calcitonin. Calcitonin (10 nM) suppressed the cytotoxicity of EGCG to osteoclasts in the presence of SOD as shown in Fig. 4C as well as in its absence as reported in our

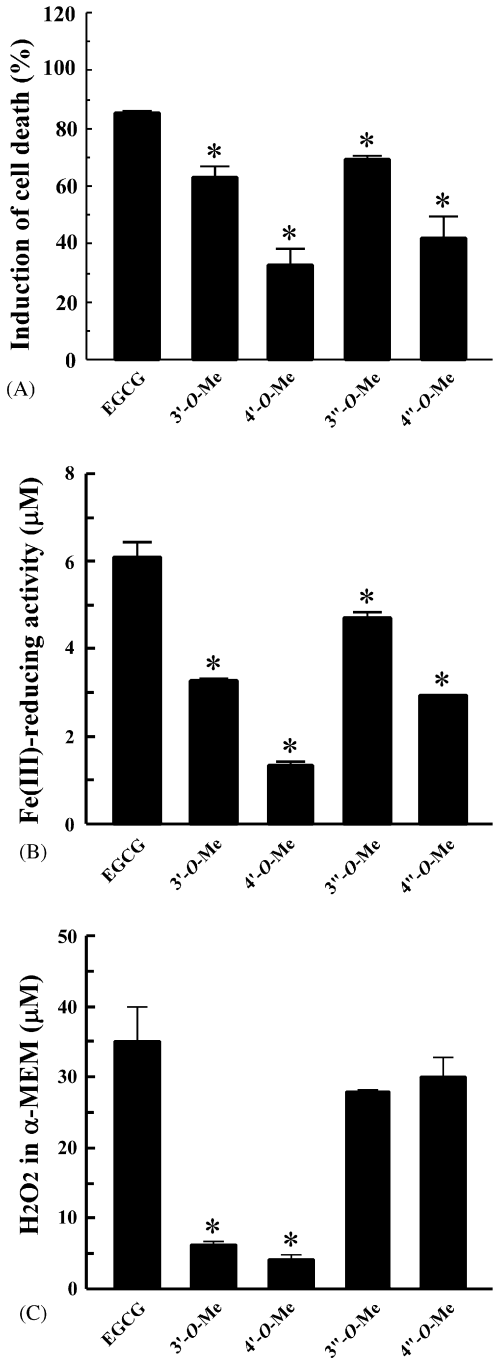



**Fig. 2 – SAR analysis of EGCGs. (A) Inhibitory effects of EGCGs on the survival of osteoclasts. Osteoclasts placed into 96-well culture plates were cultured in the presence or absence of EGCGs (100 μM). After culture for 24 h, the numbers of osteoclasts stained for TRAP were counted, and the inhibitory effects of EGCGs on the survival of osteoclasts were determined. Data are expressed as the inhibition of cell survival (%) compared with the control (mean ± S.D. of quadruplicate cultures). (B) $Fe^{3+}$-reducing activity of EGCGs. Each compound (1 μM) was added to 1 ml of working solution (5 mM o-phenanthroline and 1 mM $FeCl_3$). After incubation at room temperature for 20 s, the absorbance at 510 nm was measured immediately. (C) $H_2O_2$-reducing activity of EGCGs. Each compound (100 μM) was added to 100 μl of α-MEM without FCS. After incubation at 37 °C under 5% (v/v) $CO_2$ for 1 h, the concentration of $H_2O_2$ was measured. Data are expressed as the mean ± S.D. of quadruplicate cultures in one of three experiments. *P < 0.01 as compared with EGCG. 3′-O-Me: EGCG-3′-O-Me; 4′-O-Me: EGCG-4′-O-Me; 3″-O-Me: EGCG-3″-O-Me; 4″-O-Me: EGCG-4″-O-Me.**

previous study [6]. These results suggest that superoxide generation by osteoclasts is required for the induction of osteoclast cell death by EGCG.

### 3.3. *pH-dependent $Fe^{3+}$-reducing activity of EGCG*

It is recognized that bone-resorbing osteoclasts secrete protons onto the bone surface, where an acidic microenvironment (resorption lacuna, pH 3.0 or less) is thus created [32,33]. Since osteoclasts showed higher sensitivity to EGCG than osteoblastic cells (Fig. 1B and C), we examined whether low-pH conditions such as those in resorption lacunae would affect the $Fe^{3+}$-reducing activity of EGCG. As shown in Table 1, the $Fe^{3+}$-reducing activities of EGCG and EGCG-4′-O-Me were modulated by pH. At pH 3.6, the activities were about two-fold higher than those at pH 5.6, which indicates that the $Fe^{3+}$-reducing activity of EGCGs prefers acidic pH. This suggests that the protonation status of the hydroxyl groups of EGCG affects its $Fe^{3+}$-reducing activity in osteoclast culture.

### 3.4. *Quantum chemical calculation of structures of EGCG and its analogues*

To elucidate the chemical reaction(s) involved in $Fe^{3+}$ reduction and $H_2O_2$ production by EGCG, we performed quantum chemical calculations of their structures. Frontier orbital theory indicates that the lowest and the highest energy-containing molecular orbitals (lowest unoccupied molecular orbital, LUMO, and highest occupied molecular orbital, HOMO) play crucial roles in electrophilic reactions and nucleophilic reactions, respectively. Thus, we determined the localization of LUMO and HOMO in the structures of EGCGs. LUMO and HOMO localized to the D- and B-ring of EGCG, respectively (Fig. 5). The same localization pattern was expected in EGCG-3′-O-Me, EGCG-3″-O-Me, and EGCG-4″-O-Me. However, EGCG-4′-O-Me differed from these four EGCGs, in that LUMO and HOMO were localized to the D-ring and A-ring, respectively (Fig. 5). The hydroxyl group at position 4′ causes localization of HOMO to the B-ring. These results also suggest that HOMO on the B-ring plays a crucial role in the $Fe^{3+}$-reducing activity and cytotoxicity of EGCG.

Since the protonation status of the hydroxyl groups of EGCG affects its $Fe^{3+}$-reducing activity, we calculated the deprotonation energy for all hydroxyl groups of EGCGs. The hydroxyl group at position 4″ in the D-ring of EGCG, EGCG-3′-O-Me, and EGCG-3″-O-Me required the lowest energy for deprotonation (Fig. 5). However, the hydroxyl group at position 5″ was shown to be preferentially deprotonated in the low-cytotoxicity analogue EGCG-4″-O-Me, in which the hydroxyl group at position 4″ is methylated. Therefore, it was suggested that preferential deprotonation of hydroxyl groups at position

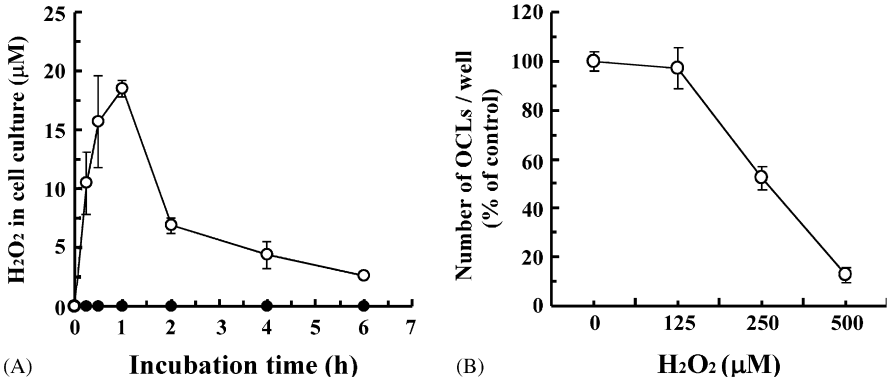



**Fig. 3 – Effect of exogenously added $H_2O_2$ on osteoclasts. (A) $H_2O_2$ generation by EGCG. EGCG (100 μM) was added to osteoclast cultures with (closed circle) or without 100 U/ml catalase (open circle). After incubation at 37 °C under 5% (v/v) $CO_2$ for 15, 30, 60, 120, 240, and 360 min, the concentration of $H_2O_2$ in the culture medium was measured. Data are expressed as the mean ± S.D. of three independent experiments. (B) Cytotoxicity of exogenously added $H_2O_2$ to osteoclasts. Osteoclasts placed into 96-well culture plates were cultured in the presence or absence of $H_2O_2$ (125, 250, or 500 μM). After culture for 24 h, the numbers of osteoclasts stained for TRAP were counted. Data are expressed as a percentage of the control (mean ± S.D. of quadruplicate cultures).**

4″ of EGCG played a crucial role in the $Fe^{3+}$-reducing activity of EGCG and its cytotoxicity to osteoclasts.

## 4. Discussion

EGCG is a natural compound that has a wide range of physiological functions [6–27]. We previously reported that EGCG induced apoptosis in osteoclasts. Since an $Fe^{2+}$ chelator o-phenanthroline and catalase suppressed it, we proposed a new mechanism of EGCG induced apoptosis in osteoclasts: EGCG triggers the Fenton reaction to generate highly reactive hydroxyl radicals from $H_2O_2$ and $Fe^{2+}$ [$H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$] [6]. Thus, we speculated that $Fe^{3+}$-reducing and/or $H_2O_2$-producing activity of EGCG contribute to its cytotoxicity to osteoclasts. In the present study, we explored the molecular mechanism of the cytotoxicity of EGCG using four EGCG analogues (EGCG-3′-O-Me, EGCG-3″-O-Me, EGCG-4′-O-Me and EGCG-4″-O-Me) based on their structures, $Fe^{3+}$-reducing activity, and $H_2O_2$-producing activity.

SAR analysis performed indicated that the magnitude of the $Fe^{3+}$-reducing activity of EGCGs was well correlated with the cytotoxicity to osteoclasts (EGCG > EGCG-3″-O-Me > EGCG-3′-O-Me > EGCG-4″-O-Me > EGCG-4′-O-Me). On the other hand,

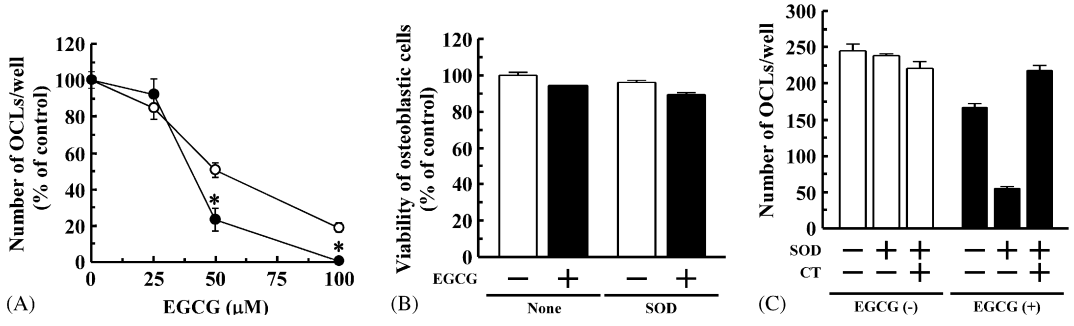



**Fig. 4 – Effects of SOD and calcitonin on cytotoxicity of EGCG. (A) Effect of SOD on cytotoxicity of EGCG to osteoclasts. Osteoclasts placed into 96-well culture plates were cultured with EGCG (0, 25, 50, or 100 μM) in the presence (closed circle) or absence (open circle) of 500 U/ml SOD. After culture for 24 h, the numbers of osteoclasts stained for TRAP were counted. Data are expressed as a percentage of the control (mean ± S.D. of quadruplicate cultures). $^*P < 0.01$ as compared with data in the absence of SOD. (B) Effect of SOD on viability of osteoblastic cells in the presence or absence of EGCG. Osteoblastic cells ($4 \times 10^4$ cells/well) placed into 96-well culture plates were cultured with or without 100 μM EGCG for 24 h. After culture, the cytotoxic effect of EGCG on the viability of osteoblastic cells was determined. Viability of osteoblastic cells was expressed as a percentage of the absorbance in the control culture. Data are expressed as the mean ± S.D. of quadruplicate cultures in one of three experiments. (C) Effect of calcitonin on SOD-enhanced cytotoxicity of EGCG to osteoclasts. Osteoclasts placed into 96-well culture plates were cultured with EGCG (100 μM) in the presence or absence of 500 U/ml SOD and/or 10 nM calcitonin. After culture for 24 h, the numbers of osteoclasts stained for TRAP were counted. Data are expressed as a percentage of the control (mean ± S.D. of quadruplicate cultures).**

**Table 1 – Behavior of EGCG and EGCG-4′-O-Me in various pH conditions**

| Compound | $Fe^{3+}$-reducing activity (μM)[a] | | |
|---|---|---|---|
| | pH 3.6 | pH 4.6 | pH 5.6 |
| EGCG | 2.33 ± 0.25 | 1.78 ± 0.17* | 0.98 ± 0.28* |
| EGCG-4′-O-Me | 1.09 ± 0.05** | 1.01 ± 0.14*,** | 0.45 ± 0.15* |

[a] $Fe^{3+}$-reducing activity was determined as described in Section 2. Data are expressed as the mean ± S.D. of three independent experiments.
* $P < 0.05$ as compared with pH 3.6.
** $P < 0.05$ as compared with EGCG.

the order of the $H_2O_2$-producing activity of EGCGs (EGCG > EGCG-3″-O-Me = EGCG-4″-O-Me > EGCG-3′-O-Me > EGCG-4′-O-Me) differed from that of their cytotoxicity to osteoclasts, which was consistent with reports from Miura et al. [34] and Akagawa et al. [35]. In addition, more than 125 μM $H_2O_2$ was required to induce osteoclast cell death and the maximum detectable $H_2O_2$ level was only about 20 μM when EGCG was added to an osteoclast culture. These indicate that $Fe^{3+}$-reducing activity rather than $H_2O_2$-producing activity is mainly involved in EGCG-induced cell death of osteoclasts. o-Phenanthroline may eliminate the free $Fe^{2+}$ provided by EGCG, which resulted in suppression of the cytotoxicity of EGCG to osteoclasts as indicated in Fig. 6. However, further studies are needed to determine the origin of $Fe^{3+}$ that allows EGCG to induce cell death of osteoclasts.

Since it seemed that $H_2O_2$-producing activity of EGCG did not contribute mainly to its cytotoxicity to osteoclasts, we surmised that osteoclast-mediated $O_2^{\bullet-}$ secretion is responsible for it. To verify this speculation, we examined effects of SOD and calcitonin on cytotoxicity of EGCG. As expected, SOD enhanced the cytotoxicity of EGCG to osteoclasts, while it showed no cytotoxicity to osteoclasts. EGCG shows no cytotoxicity to osteoclasts cultured in the presence of calcitonin [6], which suppresses osteoclast-mediated $O_2^{\bullet-}$ secretion [6,31,36]. SOD-enhanced cytotoxicity of EGCG to osteoclasts was also suppressed by calcitonin. These results, taken together with our previous result that the cytotoxicity of EGCG was suppressed by catalase [6], support the following idea; exogenous SOD will produce $H_2O_2$ from osteoclast-secreted $O_2^{\bullet-}$, thereby enhancing the cytotoxicity of EGCG to osteoclasts. Since a SOD-like enzyme localizes on the plasma membrane of osteoclasts [2,3], $H_2O_2$ generated by the SOD-like enzyme from osteoclast-produced $O_2^{\bullet-}$ is likely to be involved in the cytotoxicity of EGCG to osteoclasts. As indicated in Fig. 6, catalase is likely to suppress the cytotoxicity of EGCG to osteoclasts eliminating the $H_2O_2$ produced by osteoclasts.

Since EGCG-4′-O-Me, which lacks a hydroxyl group at position 4′ in the B-ring, and EGCG-4″-O-Me, which lacks a hydroxyl group at position 4″ in the D-ring, showed the lowest and second-lowest $Fe^{3+}$-reducing activity and cytotoxicity to osteoclasts respectively, quantum chemical calculations were carried out to identify chemical properties of the two hydroxyl groups of EGCG. First, we determined the localization of LUMO

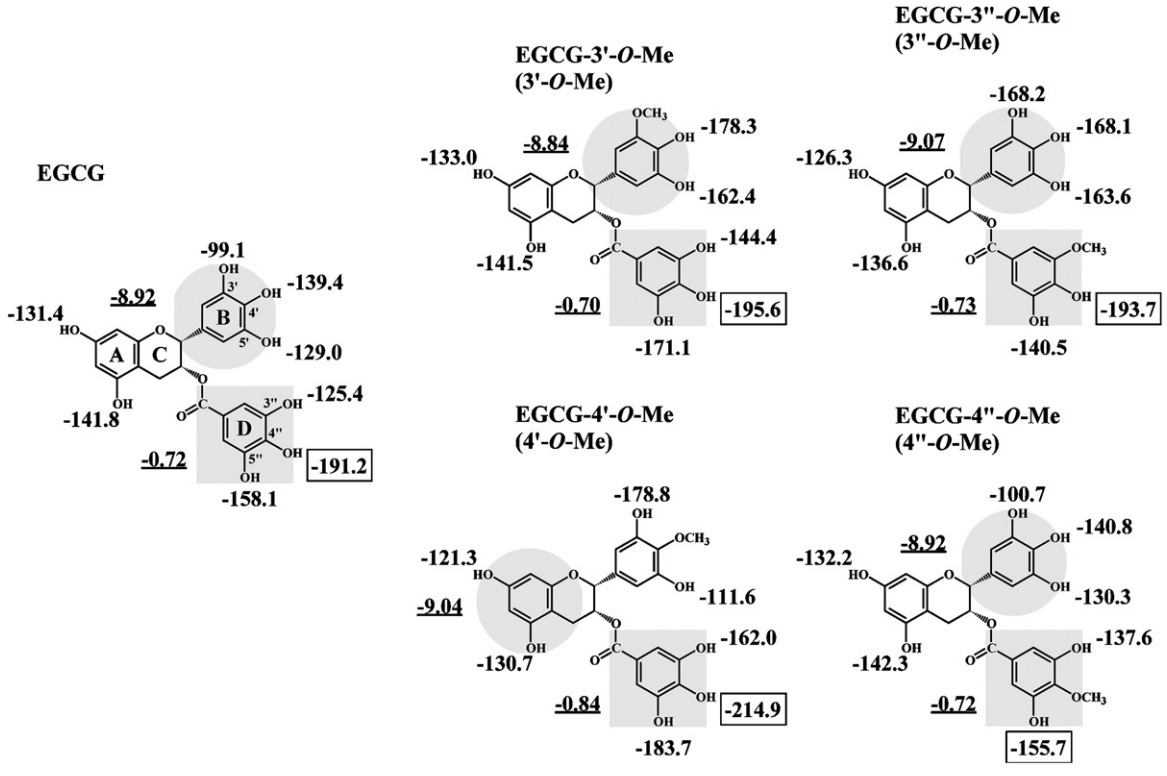



**Fig. 5 – Quantum chemical calculation. (A) LUMO, HOMO, and deprotonation energies of EGCGs. LUMO, HOMO, and deprotonation energies of EGCGs were acquired by quantum chemical calculation and are shown as numerical data (deprotonation energy, kJ/mol; LUMO and HOMO energy, eV). Data for LUMO and HOMO are underlined. Areas where LUMO and HOMO are localized are indicated as gray circular and square areas, respectively. Data for the lowest deprotonation energy are described in the open square box.**

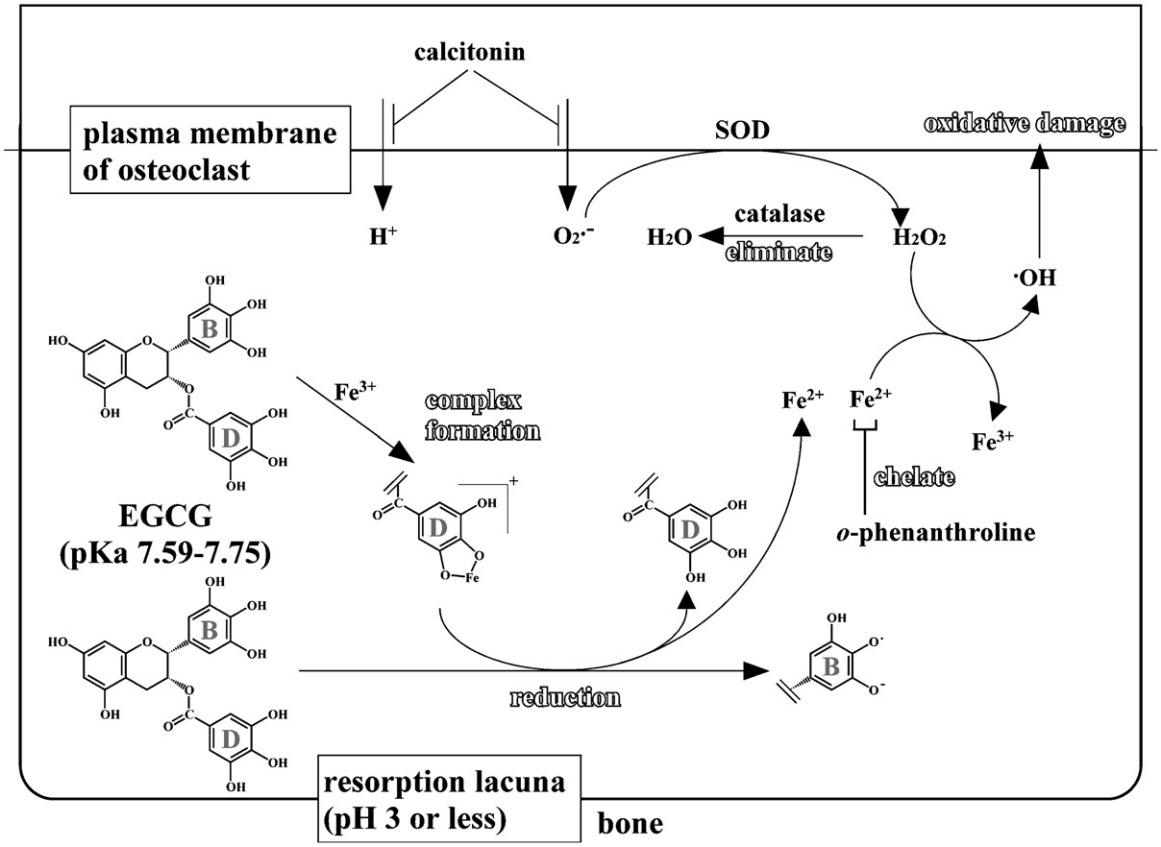



**Fig. 6 – Hypothetical molecular mechanism of EGCG in reducing $Fe^{3+}$ and inducing cell death of osteoclasts.**

and HOMO in the structures of EGCGs. It was indicated that LUMO was localized to the D-ring of EGCGs, and that HOMO to the B-ring, except for in EGCG-4′-O-Me, which showed the lowest $Fe^{3+}$-reducing activity. Mochizuki et al. reported that oxidized catechins have unpaired electron in the B-ring [37], which also suggests the highest reactivity of the B-ring. Thus, the hydroxyl group at position 4′ in the B-ring of EGCG will be required to localize HOMO on the B-ring, resulting in reduction of $Fe^{3+}$ and induction of cell death. Next, we calculated the deprotonation energy of each hydroxyl group in EGCGs. The results of the calculations indicated that the hydroxyl group at position 4″ in the D-ring showed the lowest energy among all of the hydroxyl groups in the EGCGs, except for EGCG-4″-O-Me. In EGCG-4″-O-Me, on the other hand, the hydroxyl group at position 5″ in the D-ring showed the lowest energy. Hynes et al. reported that gallic acid formed complex with $Fe^{3+}$ via its hydroxyl groups at positions 4 and 5 (or 3) [38]. van Acker et al. reported that catechol moiety in flavonoids was the main active site in their interaction with metal ion [39]. Thus, the hydroxyl group at position 4″ in the D-ring of EGCG may act as catechol moiety in conjunction with the hydroxyl group at position 3″ or 5″ in the ring as shown in Fig. 6. It is also suggested that deprotonation of the hydroxyl group at position 4″ in the D-ring of EGCG is an important structural change for binding and complex formation with $Fe^{3+}$.

Osteoclasts showed higher sensitivity to EGCGs than osteoblastic cells. Since calcitonin suppressed the cytotoxicity of EGCG to osteoclasts, it was speculated that the bone-resorbing activity of osteoclasts was needed for this high sensitivity of osteoclasts to EGCGs. In the present study, we examined the $Fe^{3+}$-reducing activity of EGCG in acidic conditions to estimate the behavior of EGCG at resorption lacunae, having a pH of 3.0 or less [32,33]. $Fe^{3+}$-reducing activity was observed at pH 3.6–5.6 and the highest activity was at pH 3.6. Taken together with our preliminary experimental result that $Fe^{3+}$-reducing activity of EGCG in the medium of osteoclast culture was extremely lower than that shown in Fig. 2B (data not shown), EGCG will behave as shown in Fig. 6 in an acidic microenvironment. The protonation level of hydroxyl group-possessing compounds increases with acidification of the solvent, and the larger part of such compounds is protonated when the pH is lower than the $pK_a$. Thus, the $pK_{a1}$ values of EGCG (7.59–7.75) [40,41] indicate that the principal forms of EGCG that exerts $Fe^{3+}$-reducing activity is protonated. Therefore, protonated EGCG is the principal form of EGCG that reduces $Fe^{3+}$ and exerts cytotoxicity on osteoclasts, where semiquinone radical will be involved as Mochizuki et al. [37] and Hynes et al. [38] reported using gallic acid and catechins.

An $Fe^{2+}$- and $H_2O_2$-dependent cytotoxic mechanism resulting from the Fenton reaction [$H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$] was previously proposed as the cytotoxic mechanism whereby EGCG induced cell death of osteoclasts [6]. The present study demonstrated that $Fe^{3+}$-reducing activity depending on the hydroxyl group at position 4′ in the B-ring and 4″ in the D-ring

of EGCG primarily contributed to the cytotoxicity of EGCG to osteoclasts. As shown in Fig. 1A, the B- and D-rings of EGCG comprise pyrogallol and gallate moieties, respectively. In our preliminary experiments, EGCG and (−)-epicatechin gallate, which have gallate moiety, showed about three-fold higher $Fe^{3+}$-reducing activity than (−)-epicatechin and (−)-epigallocatechin which have no gallate moiety (data not shown). Although further studies are required, we present here hypothetical roles of these two moieties: the pyrogallol moiety with high nucleophilicity allows the hydroxyl group at position 4′ of the moiety to reduce the $Fe^{3+}$ with which the catechol moiety in gallate moiety bound and formed complex (Fig. 6). Based on the mechanism proposed by Mochizuki et al. [37] and Hynes et al. [38], we now propose a mechanism as shown in Fig. 6. The hydroxyl group at position 4″ in the D-ring of EGCG binds and forms complex with $Fe^{3+}$, and the hydroxyl group at position 4′ in the B-ring of another EGCG advances the reduction of $Fe^{3+}$ to $Fe^{2+}$ in resorption lacunae, whereby the $Fe^{2+}$- and $H_2O_2$-dependent cell death of osteoclasts is induced.

## Acknowledgements

This work was partly supported by a Grant of the 21st Century COE Program from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

## REFERENCES

[1] Suda T, Takahashi N, Martin TJ. Modulation of osteoclast differentiation. Endocr Rev 1992;13(1):66–80.
[2] Steinbeck MJ, Appel Jr WH, Verhoeven AJ, Karnovsky MJ. NADPH-oxidase expression and in situ production of superoxide by osteoclasts actively resorbing bone. J Cell Biol 1994;126(3):765–72.
[3] Darden AG, Ries WL, Wolf WC, Rodriguiz RM, Key Jr LL. Osteoclastic superoxide production and bone resorption: stimulation and inhibition by modulators of NADPH oxidase. J Bone Miner Res 1996;11(5):671–5.
[4] Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, et al. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature 1990;345(6274):442–4.
[5] Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998;93(2):165–76.
[6] Nakagawa H, Wachi M, Woo JT, Kato M, Kasai S, Takahashi F, et al. Fenton reaction is primarily involved in a mechanism of (−)-epigallocatechin-3-gallate to induce osteoclastic cell death. Biochem Biophys Res Commun 2002;292(1):94–101.
[7] Anderson RF, Fisher LJ, Hara Y, Harris T, Mak WB, Melton LD, et al. Green tea catechins partially protect DNA from •OH radical-induced strand breaks and base damage through fast chemical repair of DNA radicals. Carcinogenesis 2001;22(8):1189–93.
[8] Toering SJ, Gentile GJ, Gentile JM. Mechanism of antimutagenic action of (+)-catechin against the plant-activated aromatic amine 4-nitro-o-phenylenediamine. Mutat Res 1996;361(2–3):81–7.
[9] Yang GY, Liao J, Kim K, Yurkow EJ, Yang CS. Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. Carcinogenesis 1998;19(4):611–6.
[10] Ikigai H, Nakae T, Hara Y, Shimamura T. Bactericidal catechins damage the lipid bilayer. Biochim Biophys Acta 1993;1147(1):132–6.
[11] Ahmad N, Feyes DK, Nieminen AL, Agarwal R, Mukhtar H. Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. J Natl Cancer Inst 1997;89(24):1881–6.
[12] Ahmad N, Gupta S, Mukhtar H. Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor kappaB in cancer cells versus normal cells. Arch Biochem Biophys 2000;376(2):338–46.
[13] Gupta S, Hussain T, Mukhtar H. Molecular pathway for (−)-epigallocatechin-3-gallate-induced cell cycle arrest and apoptosis of human prostate carcinoma cells. Arch Biochem Biophys 2003;410(1):177–85.
[14] Naasani I, Oh-Hashi F, Oh-Hara T, Feng WY, Johnston J, Chan K, et al. Blocking telomerase by dietary polyphenols is a major mechanism for limiting the growth of human cancer cells in vitro and in vivo. Cancer Res 2003;63(4):824–30.
[15] Islam S, Islam N, Kermode T, Johnstone B, Mukhtar H, Moskowitz RW, et al. Involvement of caspase-3 in epigallocatechin-3-gallate-mediated apoptosis of human chondrosarcoma cells. Biochem Biophys Res Commun 2000;270(3):793–7.
[16] Hayakawa S, Saeki K, Sazuka M, Suzuki Y, Shoji Y, Ohta T, et al. Apoptosis induction by epigallocatechin gallate involves its binding to Fas. Biochem Biophys Res Commun 2001;285(5):1102–6.
[17] Chen C, Shen G, Hebbar V, Hu R, Owuor ED, Kong AN. Epigallocatechin-3-gallate-induced stress signals in HT-29 human colon adenocarcinoma cells. Carcinogenesis 2003;24:1369–78.
[18] Nakagawa H, Hasumi K, Woo JT, Nagai K, Wachi M. Generation of hydrogen peroxide primarily contributes to the induction of Fe(II)-dependent apoptosis in Jurkat cells by (−)-epigallocatechin gallate. Carcinogenesis 2004;25(9):1567–74.
[19] Chung JY, Huang C, Meng X, Dong Z, Yang CS. Inhibition of activator protein 1 activity and cell growth by purified green tea and black tea polyphenols in H-ras-transformed cells: structure-activity relationship and mechanisms involved. Cancer Res 1999;59(18):4610–7.
[20] Kennedy DO, Nishimura S, Hasuma T, Yano Y, Otani S, Matsui-Yuasa I. Involvement of protein tyrosine phosphorylation in the effect of green tea polyphenols on Ehrlich ascites tumor cells in vitro. Chem Biol Interact 1998;110(3):159–72.
[21] Tan X, Hu D, Li S, Han Y, Zhang Y, Zhou D. Differences of four catechins in cell cycle arrest and induction of apoptosis in LoVo cells. Cancer Lett 2000;158(1):1–6.
[22] Hibasami H, Achiwa Y, Fujikawa T, Komiya T. Induction of programmed cell death (apoptosis) in human lymphoid leukemia cells by catechin compounds. Anticancer Res 1996;16(4A):1943–6.
[23] Achiwa Y, Hibasami H, Katsuzaki H, Imai K, Komiya T. Inhibitory effects of persimmon (*Diospyros kaki*) extract and related polyphenol compounds on growth of human lymphoid leukemia cells. Biosci Biotechnol Biochem 1997;61(7):1099–101.
[24] Paschka AG, Butler R, Young CY. Induction of apoptosis in prostate cancer cell lines by the green tea component (−)-epigallocatechin-3-gallate. Cancer Lett 1998;130(1–2):1–7.
[25] Okabe S, Ochiai Y, Aida M, Park K, Kim SJ, Nomura T, et al. Mechanistic aspects of green tea as a cancer preventive: effect of components on human stomach cancer cell lines. Jpn J Cancer Res 1999;90(7):733–9.

[26] Kinjo J, Nagao T, Tanaka T, Nonaka G, Okawa M, Nohara T, et al. Activity-guided fractionation of green tea extract with antiproliferative activity against human stomach cancer cells. Biol Pharm Bull 2002;25(9):1238–40.
[27] Saeki K, Hayakawa S, Isemura M, Miyase T. Importance of a pyrogallol-type structure in catechin compounds for apoptosis-inducing activity. Phytochemistry 2000;53(3):391–4.
[28] Akatsu T, Tamura T, Takahashi N, Udagawa N, Tanaka S, Sasaki T, et al. Preparation and characterization of a mouse osteoclast-like multinucleated cell population. J Bone Miner Res 1992;7(11):1297–306.
[29] Nourooz-Zadeh J, Tajaddini-Sarmadi J, Wolff SP. Measurement of plasma hydroperoxide concentrations by the ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. Anal Biochem 1994;220(2):403–9.
[30] Dewar MJS, Zeobisch EG, Healy EF, Stewart JP. Development and use of quantum mechanical molecular models 76. AM1: a new general purpose quantum mechanical molecular model. J Am Chem Soc 1985;107(13):3902–9.
[31] Berger CE, Horrocks BR, Datta HK. cAMP-dependent inhibition is dominant in regulating superoxide production in the bone-resorbing osteoclasts. J Endocrinol 1998;158(3):311–8.
[32] Silver IA, Murrills RJ, Etherington DJ. Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. Exp Cell Res 1988;175(2): 266–76.
[33] Väänänen HK, Karhukorpi EK, Sundquist K, Wallmark B, Roininen I, Hentunen T, et al. Evidence for the presence of a proton pump of the vacuolar $H^+$-ATPase type in the ruffled borders of osteoclasts. J Cell Biol 1990;111(3):1305–11.
[34] Miura YH, Tomita I, Watanabe T, Hirayama T, Fukui S. Active oxygens generation by flavonoids. Biol Pharm Bull 1998;21(2):93–6.
[35] Akagawa M, Shigemitsu T, Suyama K. Production of hydrogen peroxide by polyphenols and polyphenol-rich beverages under quasi-physiological conditions. Biosci Biotechnol Biochem 2003;67(12):2632–40.
[36] Berger CE, Datta HK. Forskolin has a bimodal cAMP-independent effect on superoxide anion generation in isolated osteoclasts. Exp Physiol 2000;85(1):57–60.
[37] Mochizuki M, Yamazaki S, Kano K, Ikeda T. Kinetic analysis and mechanistic aspects of autoxidation of catechins. Biochim Biophys Acta 2002;1569(1–3):35–44.
[38] Hynes MJ, Coinceanainn MO. The kinetics and mechanisms of the reaction of iron(III) with gallic acid, gallic acid methyl ester and catechin. J Inorg Biochem 2001;85 (2–3):131–42.
[39] van Acker SA, van den Berg DJ, Tromp MN, Griffioen DH, van Bennekom WP, van der Vijgh WJ, et al. Structural aspects of antioxidant activity of flavonoids. Free Radic Biol Med 1996;20(3):331–42.
[40] Jovanovic SV, Hara Y, Steenken S, Simic MG. Antioxidant potential of gallocatechins A pulse radiolysis and laser photolysis study. J Am Chem Soc 1995;117: 9881–8.
[41] Kumamoto M, Sonda T, Nagayama K, Tabata M. Effects of pH and metal ions on antioxidative activities of catechins. Biosci Biotechnol Biochem 2001;65(1):126–32.