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# Fenton Reaction Is Primarily Involved in a Mechanism of (−)-Epigallocatechin-3-gallate to Induce Osteoclastic Cell Death

Hiroshi Nakagawa,* Masaaki Wachi,* Je-Tae Woo,† Masanori Kato,* Shinya Kasai,* Fuminori Takahashi,* In-Seon Lee,‡ and Kazuo Nagai†,[1]

**Department of Bioengineering, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan; †Department of Biological Chemistry, College of Bioscience and Biotechnology, Chubu University, 1200 Matsumoto-cho, Kasugai, Aichi 487-8501, Japan; and ‡Department of Food Science and Technology, College of Natural Science, Keimyung University, 1000 Shindang-dong, Dasu-ku, Taegu 704-701, Korea*

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**To propose candidates for the prevention or treatment of osteoporosis, we have screened compounds naturally in food for their ability to regulate the differentiation and function of osteoclasts. One of the major green tea flavonoids, (−)-epigallocatechin-3-gallate (EGCG), was found to induce apoptotic cell death of osteoclast-like multinucleated cells after 24 h treatment in a dose-dependent manner (25–100 $\mu$M), whereas osteoblasts were not affected. In the present study, we report for the first time a novel cell-death-inducing mechanism triggered by EGCG. The induction of apoptosis by EGCG was suppressed by pretreatment of catalase or calcitonin. It was also suppressed by Fe(III) and Fe(II) chelators. Furthermore, EGCG promoted the reduction of Fe(III) into Fe(II), and the combination of EGCG/Fe(III)/$H_2O_2$ induced single-strand DNA breakage in a cell free system. These results indicate that the Fenton reaction is primarily involved in EGCG-induced osteoclastic cell death.** 

***Key Words:*** **EGCG; Fenton reaction; osteoclast; apoptosis; caspase activation; DNA breakage.**

Osteoclasts are primary bone-resorbing cells that play a crucial role in bone remodeling (1, 2). Bone is resorbed mainly by multinucleated osteoclasts. These are formed by fusion of preosteoclasts (pOCs) (3), which are derived from hematopoietic stem cells in the presence of cytokines such as macrophage-colony stimulating factor (M-CSF) (4, 5) and receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) (6, 7). These cytokines are expressed by osteoblasts/stromal cells and modulate functions and survival of OCLs (2, 5, 7). These cytokines and signal from integrins induce the formation of polarized cell structures in osteoclasts that participate in bone resorption (8). After a period of bone resorption, some of osteoclasts die by apoptosis. Thus, regulations of these processes potentially result in control of bone resorption. Because enhanced bone-resorbing activity and/or recruitment of osteoclasts is responsible for the pathogenesis of bone diseases such as osteoporosis, agents that inhibit differentiation or induce apoptosis in osteoclasts could be applied as a prophylactic or therapeutic agent for treatment of such diseases.

For many years, it has been recognized that compounds in foods are beneficial to our health. Among these, flavonoids exhibit physiological and pharmacological properties such as antioxidative, antibacterial, antimutagenic, and antitumor activities (9–13). These polyphenolic compounds are found ubiquitously in foods of plant origin. More than 4000 kinds of these compounds have been described so far. Because of their low toxicity, some of flavonoids are thought to have potential to be developed into a new class of prophylactic or therapeutic agents against various kinds of disease. Many investigators have reported that flavonoids are effective in coronary heart disease, dermatitis, malaria, and cancer (12–14). However, only a few studies of flavonoids have been reported in connection to osteoporosis (15, 16). Flavonoids also exhibit radical scavenging (17, 18) or iron chelating (9) properties, but their precise mechanisms of action remain to be studied. Catechins are one of the major flavonoids present

Abbreviations used: EGCG, (−)-epigallocatechin-3-gallate; MNCs, multinucleated cells; OCLs, osteoclast-like MNCs; pOCs, preosteoclasts; M-CSF, macrophage colony-stimulating factor; RANK, receptor activator of nuclear factor-κB; RANKL, RANK ligand; TRAP, tartrate-resistant acid phosphatase; SOD, superoxide dismutase.

[1] To whom correspondence should be addressed. Fax: +81-568-52-6594. E-mail: knagai@isc.chubu.ac.jp.

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naturally in certain species of plants, including tea. Many physiological and pharmacological properties of catechins as well as other flavonoids have been reported. Among them, antitumor activity of catechins has been shown to be due to the induction of apoptosis. Some studies have reported that catechins induce activation of caspase-3 and -9 (19, 20), which play a central role in the initiation and execution of apoptosis (21, 22). The suppressive effect of catalase on EGCG-induced apoptosis indicates the involvement of $H_2O_2$ in this phenomenon (23, 24). However, the precise mechanism has yet to be elucidated.

In the present study, we screened natural compounds in foods that regulate the differentiation and the function of osteoclasts, and found that catechins inhibit bone resorption by inducing cell death of OCLs but not osteoblastic cells. Among the tested catechins, (−)-epigallocatechin-3-gallate (EGCG), a major component of green tea, was most effective. A previous study reported that (+)-catechin inhibited bone resorption (25), but the precise mechanism of action remained to be elucidated. In this paper, we first report that the hydroxyl radicals (˙OH) produced by Fenton reaction (26) induce activation of caspase-3 and cleavages of DNA in OCLs.

## MATERIALS AND METHODS

*Animal and chemicals.* Newborn Std.ddY mice and 6- to 9-week-old male Std.ddY mice were supplied by Japan SLC Co. (Hamamatsu, Japan). (−)-Epigallocatechin gallate (EGCG), Fast red violet LB salt, naphthol AS-MX phosphate, catalase and deferoxamine mesylate were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase, 1$\alpha$,25-dihydroxyvitamin $D_3$ [1$\alpha$,25$(OH)_2D_3$], prostaglandin $E_2$ ($PGE_2$), eel calcitonin, *o*-phenanthroline hydrochloride, and Hoechst 33258 were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dispase was obtained from Godo Shusei (Tokyo, Japan), pronase from Calbiochem (San Diego, CA), and collagen gel solutions (Cellmatrix, Type I-A) from Nitta Gelatin Co. (Osaka, Japan). Recombinant human M-CSF (Leukoprol) and recombinant human soluble form of RANKL (sRANKL) were commercial products of Welfide Co. (Osaka, Japan) and Pepro Tech EC Ltd. (London, U.K.), respectively. Protease inhibitor cocktail tablet (Complete) was purchased from Roche Ltd. (Mannheim, Germany). Acetyl-Asp-Glu-Val-Asp-$\alpha$-(4-methyl-coumaryl-7-amide) (Ac-DEVD-MCA) and z-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) were purchased from Peptide Institute Inc. (Osaka, Japan). pUC 19 DNA was obtained from Takara Shuzo Co., Ltd. (Shiga, Japan).

*Preparation of OCLs.* Crude murine osteoclast-like multinucleated cells (OCLs) were prepared from a coculture system as previously described (27). Briefly, primary mouse osteoblastic cells were obtained from calvariae of newborn ddY mice. Bone marrow cells ($2 \times 10^7$ cells) and osteoblastic cells ($1 \times 10^6$ cells) were cocultured in $\alpha$-MEM containing 10% fetal calf serum, 10 nM 1 $\alpha$,25$(OH)_2D_3$ and 1 $\mu$M $PGE_2$ on 100-mm tissue culture dishes (Corning Inc., Corning, NY) (15 ml/dish) precoated with 2.5 ml of 0.2% collagen gel matrix. Half of the medium was exchanged every 2 days. OCLs were formed within 7 days of culture, and released from the dishes by treating with 1.5 ml of 0.2% collagenase and 0.1% dispase. Cells were collected by centrifugation (1000 rpm, 5 min) and placed on 96- or 6-well culture plates (Crude OCLs).

Purified OCLs were prepared by removing stromal cells with 0.002% EDTA and 0.02% pronase (28). After purification, the purity of OCLs in the culture was more than 80% (Purified OCLs) and the cells were cultured in the presence of 100 ng/ml sRANKL.

OCLs from preosteoclasts (pOCs) were prepared as previously described (3). In brief, bone marrow cells ($2 \times 10^7$ cells) and osteoblastic cells ($2 \times 10^6$ cells) were cocultured as mentioned above. After coculturing for 4 days, floating cells were removed and mononuclear cells attached to the osteoblastic cell layer were recovered as pOCs by gentle pipetting with fresh $\alpha$-MEM. Mononuclear cells were collected by centrifugation (1000 rpm, 5 min) and $3 \times 10^5$ cells were placed on 96-well culture plates. The purity of pOCs in this culture was about 50–60%. The pOC preparations placed on 96-well culture plates were cultured in the presence of M-CSF (20 ng/ml) and sRANKL (100 ng/ml) and OCLs were formed within 24 h of culture. The purity of the OCLs in this culture was more than 70% (OCLs from pOCs).

*Cell staining.* The OCL preparations were placed on 96-well culture plates. After preculturing for 24 h, the cells were treated with or without EGCG for 24 h, and stained for the tartrate-resistant acid phosphatase (TRAP), a typical marker enzyme of osteoclasts. TRAP staining was carried out as described previously (27).

In some experiments, cells were washed with phosphate buffered saline (PBS), and then treated with 0.1% crystalviolet for 5 min. After treatment, crystalviolet incorporated in the cells was eluted with MeOH, and absorbance at 595 nm was estimated.

Nuclei were stained with Hoechst 33258 (29). After fixation, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min. After treatment with 10 $\mu$M Hoechst 33258 for 10 min at room temperature, the morphology of nuclei in OCLs was visualized and detected under a fluorescence microscope (Carl Zeiss, Axioskop 2, Germany). In the present study, the images were obtained by using IPLab Spectrum Ver 3.2.4.

*Resorption pits staining.* Inhibitory effects of EGCG on pit-forming activity of OCLs were determined by the method previously reported (3). In brief, crude OCLs were placed on dentine slices (4 mm in diameter) and cultured with or without EGCG for 24 h. At the end of the culture period, the adherent cells were released from the dentine slices after freeze-thaw cycle at −20°C and room temperature. The slices were stained with Mayer's hematoxylin (Sigma Chemical Co.) to visualize resorption pits and the pits on the slices was observed under a microscope (Carl Zeiss, Axioskop 2, Germany). The images were obtained by using an IPLab Spectrum Ver 3.2.4.

*Assay for caspase-3-like protease activity.* Caspase-3-like protease activity in OCLs lysate was determined using Ac-DEVD-MCA, as the substrate (30). Purified OCLs placed on 6-well culture plates were lysed in lysis buffer [100 mM Tris-HCl (pH 7.5), 1 mM DTT, 1% Triton X-100, protease inhibitor cocktail] and 50 $\mu$g of protein was incubated with 20 $\mu$M Ac-DEVD-MCA at 37°C for 1 h. Fluorescence of the free aminomethylcoumarin was determined by excitation at 360 nm and emission at 460 nm using a CYTO FLUOR Multi-Well Plate Reader (PerSeptive Biosystems, MA).

*Fe(II) production.* To assess reduction of Fe(III) by EGCG, EGCG was added into 1 mM $FeCl_3$ solutions (500 $\mu$l) and incubated at room temperature for 20 s. After incubation, an equal volume of 1.2 mg/ml *o*-phenanthroline was added to the reaction mixtures, and the reactions terminated by addition of 3 volumes of deionized water. The absorbance at 510 nm was measured after dilution with deionized water.

*In vitro DNA damage assay.* The *in vitro* DNA damage assays was performed as previously described with minor modification (31). Reaction mixtures (20 $\mu$l) contained pUC19 plasmid DNA (155 ng) in distilled water. To assess activity of EGCG to induce DNA damage, the reaction was carried out in the presence or absence of EGCG, $H_2O_2$ (1 mM) and $FeCl_3$ (100 $\mu$M). After incubation for 30 min at room temperature, the reaction was stopped adding the iron chelator 2,2′-dipyridyl to a final concentration of 5 mM. DNA was extracted from the reaction mixtures with phenol/chloroform (1:1) followed by

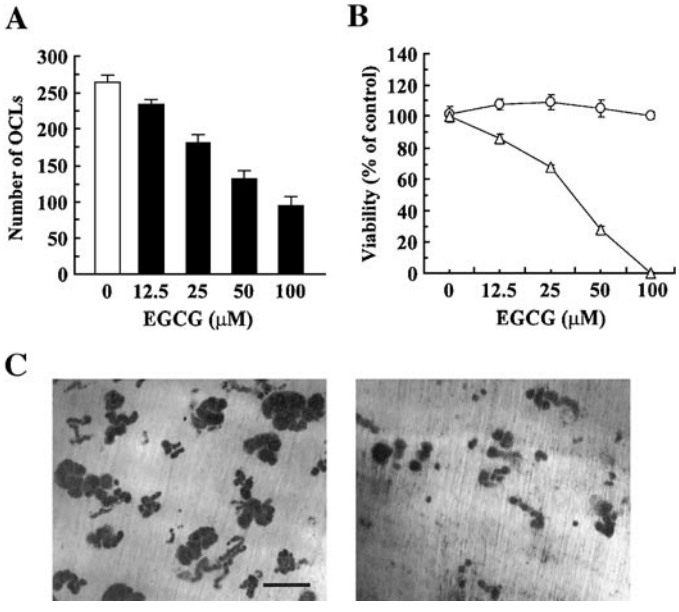


**FIG. 1.** Dose-dependent decrease in the number of OCLs by EGCG. (A) Crude OCLs were placed on 96-well culture plates. After culture for 24 h, they were treated with or without the indicated concentrations of EGCG for 24 h. After treatment, cultures were stained for TRAP, and then TRAP(+)MNCs (OCLs) were counted. (B) Osteoblastic cells (circle) and OCLs from pOCs (triangle) were treated with or without the indicated concentrations of EGCG for 24 h. OCLs from pOCs were cultured in the presence of M-CSF (20 ng/ml) and sRANKL (100 ng/ml). After treatment, OCLs from pOCs and osteoblasts were stained with TRAP or crystalviolet, respectively. (C) Resorption pits formed on dentine slices by crude OCLs cultured with (right) or without (left) 100 $\mu$M EGCG for 24 h were stained with Mayer's hematoxylin (means ± S.D.; $n$ = 4). Bar = 100 $\mu$m.

chloroform/isoamyl alcohol (24:1). Supercoiled plasmid DNA and nicked DNA were separated by electrophoresis in 0.8% agarose gel, and ethidium bromide-binding DNA was detected by using a COMPACT B.I.S (Amersham Pharmacia Biotech, Buckinghamshire, UK). Images were obtained using Adobe Photoshop 5.0 Limited Edition.

## RESULTS

*EGCG decreased the number of OCLs remaining on plates.* We have screened natural compounds in food for their activity on osteoclasts. We found that catechins decreased the number of OCLs remaining on plates in the presence of osteoblastic cells. Among the tested catechins [(−)-catechin gallate, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, (−)-epigallocatechin gallate (EGCG)], EGCG was the most effective (data not shown). The number of OCLs treated with EGCG for 24 h decreased in a dose-dependent manner (12.5–100 $\mu$M) (Fig. 1A). The number of OCLs began to decrease after 12 h treatment with 100 $\mu$M EGCG (data not shown). Although survival of OCLs is dependent on osteoblastic cells/stromal cells in the physiological condition, EGCG did not affect the survival of osteoblastic cells in this experimental condition (Fig. 1B). EGCG also reduced the number of OCLs in the absence of osteoblastic cells (Fig. 1B). The number of OCLs remaining was less than 10% of control at 100 $\mu$M EGCG. These results suggest that EGCG directly affects OCLs. We next examined the effects of EGCG on pit-forming activity of OCLs on dentine slices since EGCG also decreased the number of OCLs cultured on dentine slices (data not shown). OCLs placed on dentine slices formed resorption pits within 24 h (Fig. 1C, left panel). EGCG inhibited pits formation in size, but not in number at the same concentration that reduced the number of OCLs (Fig. 1C, right panel).

*EGCG induces osteoclastic cell death.* Because several studies have reported that EGCG induce apoptosis in tumor cells, we wished to determine if the decrease in the number of OCLs by EGCG results from apoptosis. In OCLs treated with EGCG, caspase-3 activity increased in a dose-dependent manner. The increase was completely suppressed by a synthetic pan-caspase inhibitor (Z-VAD-FMK) (Fig. 2A). The decrease of the number of OCLs by EGCG was partially suppressed by Z-VAD-FMK (Fig. 2B). A dramatic increase of caspase-3 activity was observed after 4 h treatment with 100 $\mu$M EGCG. The increase of this protease activity was transient, and maximum activity was seen after 8 h treatment (data not shown). We next examined morphological change of nucleus, a typical indication of apoptosis, in OCLs treated with 100 $\mu$M EGCG for 14 h. As shown in Fig. 2C, 100 $\mu$M EGCG induced nuclear disintegration, seen as fragmentation of nuclei with condensed chromatin. These results indicate that EGCG induces apoptosis in OCLs via increased caspase activity. However, incomplete suppression of the EGCG-induced cell death by Z-VAD-FMK suggests that other caspase-independent pathways may also be involved in EGCG-induced osteoclastic cell death.

*$H_2O_2$ and EGCG-produced Fe(II) is involved in EGCG-induced osteoclastic cell death.* Previous studies reported that EGCG-induced apoptosis in tumor cells is suppressed by the catalase (23, 24). It is recognized that bone-resorbing OCLs generate superoxide and convert it to hydrogen peroxide ($H_2O_2$) by a SOD-like enzyme in the plasma membrane (32–35). Superoxide generation is suppressed by calcitonin (36, 37). We examined the involvement of $H_2O_2$ in EGCG-induced osteoclastic cell death. Pretreatment of OCLs with 100 U/ml catalase or 10 nM calcitonin for 1 h significantly suppressed EGCG-induced cell death and activation of caspase-3 (Figs. 3A–3C). These results indicate that $H_2O_2$, probably generated from superoxide, is involved in EGCG-induced caspase activation and cell death. Complete suppressions by calcitonin of caspase-3 activation and cell death suggest that $H_2O_2$ also plays a role in other pathways in addition to the caspase-dependent pathway. We next tried to identify

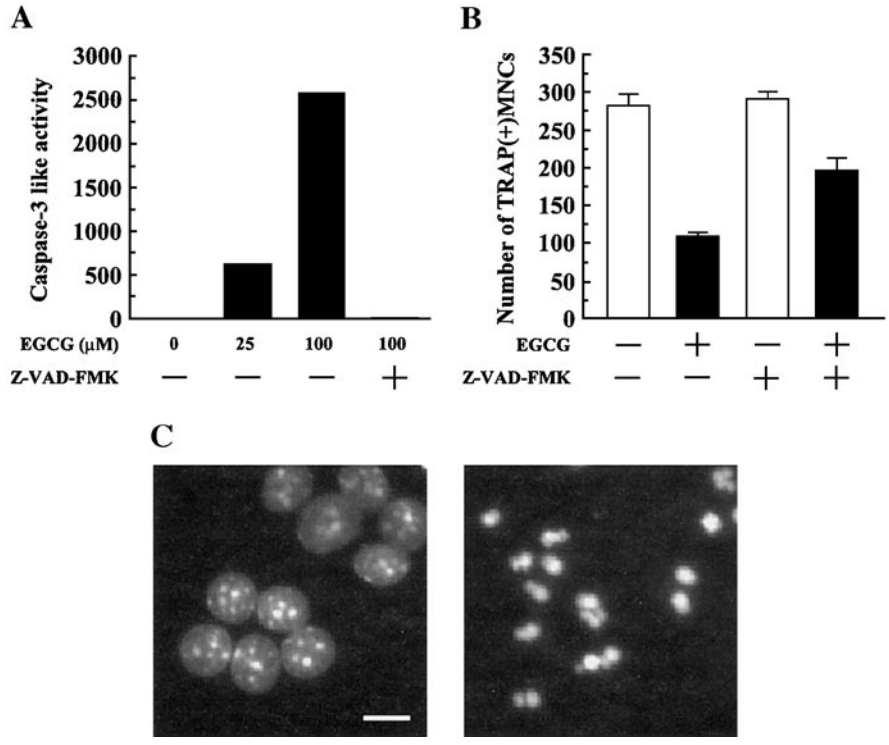


**FIG. 2.** Induction of caspase-dependent apoptosis in OCLs by EGCG. (A) Purified OCLs cultured with 100 ng/ml sRANKL for 2 h were treated with or without 25 $\mu$M Z-VAD-FMK in the presence of 25 or 100 $\mu$M EGCG for 6 h. After treatment, caspase-3 activities in the cell lysates were measured. Similar results were obtained in two other experiments. (B) Crude OCLs pretreated with or without 25 $\mu$M Z-VAD-FMK for 1 h were incubated with 100 $\mu$M EGCG for 24 h. After treatment, cultures were stained for TRAP, and OCLs were counted. (C) Crude OCLs were treated with 100 $\mu$M EGCG for 14 h. After treatment, cultures were stained with Hoechst 33258 (means ± S.D.; $n$ = 4). Bar = 10 $\mu$m.

the factor participating in other pathways. During the assays, we found that EGCG induced reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT reagent) in a dose-dependent manner (data not shown). This activity was enhanced by the presence of serum. These results indicate the redox potentials of EGCG. Since EGCG chelates iron ion, which is present in serum, we examined whether iron ion is involved in EGCG-induced cell death. Pretreatment of OCLs with a Fe(III) chelator deferoxamine or a Fe(II) chelator *o*-phenanthroline for 1 h significantly suppressed EGCG-induced cell death (Fig. 4A). These chelators also suppressed activation of caspase-3 (Fig. 4B). Complete suppression of cell death by 40 $\mu$M *o*-phenanthroline suggested that Fe(II) plays a crucial role in EGCG-induced cell death. We next investigated whether Fe(III) was reduced to Fe(II) by EGCG. The complexes of Fe(II)-*o*-phenanthroline have a maximal absorption at 510 nm. After reaction of $FeCl_3$ and EGCG for 20 s, the absorbance at 510 nm increased in a dose-dependent manner (Fig. 4C). This effect was also observed at 1 $\mu$M EGCG. These results indicate that EGCG reduces Fe(III) and the resulted Fe(II) is involved in the increase of caspase activity and EGCG-induced cell death.

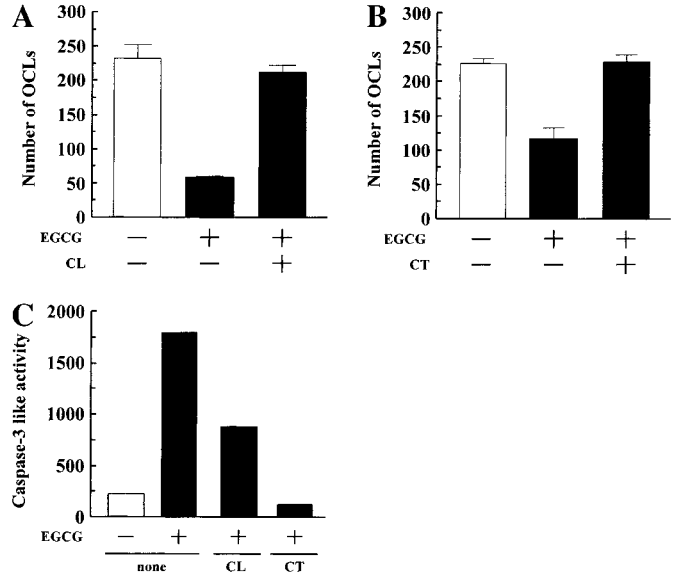


**FIG. 3.** EGCG-induced cell death was suppressed by catalase and calcitonin. Crude OCLs were pretreated with or without 100 U/ml catalase (CL) (A, C) and/or 10 nM calcitonin (CT) (B, C) for 1 h, respectively, and then treated with 100 $\mu$M EGCG for 24 h (A, B) or 6 h (C). After treatment, cultures were stained for TRAP (A, B) and caspase-3 activities in the lysates were measured. Similar results were obtained in two other experiments (means ± S.D.; $n$ = 4).

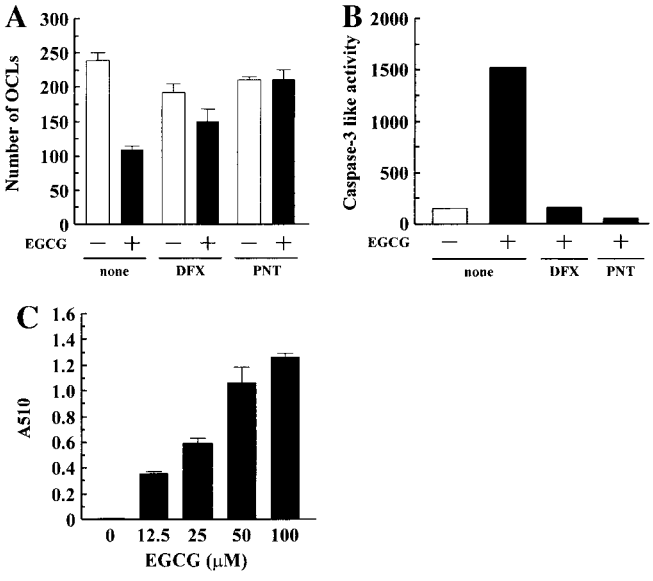


**FIG. 4.** Suppression of EGCG-induced cell death by a chelator for iron and reduction of Fe(III) to Fe(II) by EGCG. Crude (A) or purified (B) OCLs were pretreated with or without 100 $\mu$M deferoxamine (DFX) and 40 $\mu$M *o*-phenanthroline (PNT), respectively for 1 h, and then treated with 100 $\mu$M EGCG for 24 h (A) or 6 h (B). After treatment, MNCs stained with 0.1% crystal violet (OCLs) were counted (A) and caspase-3 activities in the lysates were measured (B). Similar results were obtained in two other experiments. (C) EGCG was added into 1 mM $FeCl_3$ solutions. After incubation at room temperature for 20 s, 1.2 mg/ml *o*-phenanthroline was added. Reduction of Fe(III) was determined by measuring absorbance at 510 nm (means ± S.D.; $n = 4$).

*EGCG induces the Fenton reaction and single-strand DNA breakage in a cell free system.* Fe(II) is one of the major substances that cause production of hydroxyl radical under physiological conditions. In this, the so called Fenton reaction, Fe(II) reacts with $H_2O_2$, and Fe(III) and hydroxyl radical are produced. Since the involvement of both Fe(II) and $H_2O_2$ in EGCG-induced cell death suggested the possibility that EGCG induces Fenton reaction and the resulting hydroxyl radicals cause the cell death, we examined whether EGCG induced the Fenton reaction in a cell free system. Hydroxyl radicals are powerful reactive oxygen species and directly cleave DNA. We evaluated the ability of EGCG to induce the Fenton reaction by assaying the single strand DNA breakage. Treatment of supercoiled pUC19 (SC) with Fe(III)/$H_2O_2$ resulted in the formation of nicked open circular molecules (OC). The amount of OC increased by addition of EGCG in a dose dependent manner (0.01–100 $\mu$M). Treatment with 100 $\mu$M EGCG caused extensive breakage of DNA. We detected smears of DNA bands in agarose gel (Fig. 5A, lane 5). As shown in Fig. 5B, both Fe(III) and H2O2 were required in this EGCG-induced DNA breakage. These results indicate that the combination of Fe(III), $H_2O_2$ and EGCG cause single-strand DNA breakage, and suggest that hydroxyl radicals produced by EGCG-induced Fenton reaction play a crucial role in the EGCG-induced osteoclastic cell death.

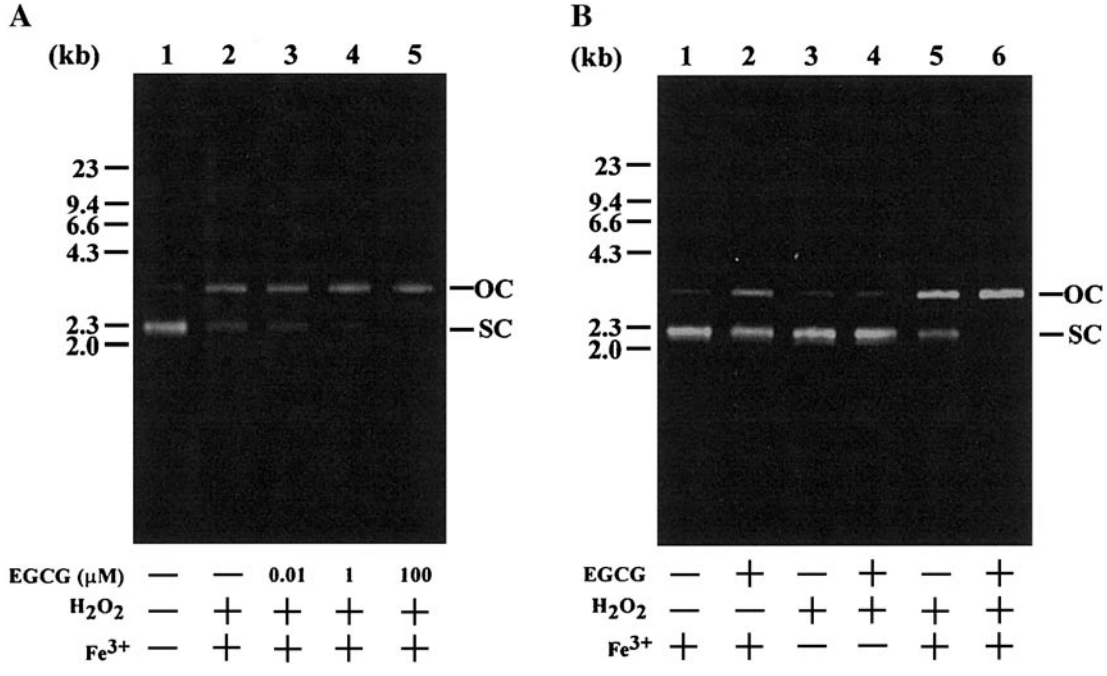


**FIG. 5.** EGCG cleaves single-strand DNA in the presence of Fe(III) and $H_2O_2$. Single-strand DNA (pUC19) was incubated at room temperature with or without EGCG (A; 0.01–100 $\mu$M, B; 100 $\mu$M) for 30 min with or without 1 mM $H_2O_2$ and 100 $\mu$M $FeCl_3$, respectively. After incubation, DNA was extracted and separated by electrophoresis in 0.8% agarose gel. OC, open circular molecules; SC, supercoiled molecules.

## DISCUSSION

Enhanced bone-resorbing activity and/or recruitment of osteoclasts is strongly implicated in the pathogenesis of osteoporosis and other bone diseases. Here, we have screened natural compounds in foods and found that EGCG, one of catechins, induced osteoclastic cell death. Although (+)-catechin was previously reported to inhibit bone resorption (25), the mechanism has been unknown. In the present paper, we first demonstrated that the inhibitory effect of catechins resulted from the death of activated osteoclasts that was induced by oxidative stress produced via Fenton reaction. Some investigators have indicated that EGCG act on tumor cells but not on normal cells (38). We found that EGCG induces cell death in OCLs in a dose-dependent manner and has no significant effect on osteoblastic cells cultured with OCLs. This suggests that OCLs share some properties with tumor cells.

EGCG reduced the size of pit, but not their number at the same concentrations that induce the death of OCLs. When osteoclasts were cultured on dentine slices, pits were formed in just 2 h after (data not shown). The time required for EGCG to increase caspase activity in the OCLs was more than 4 h. This is why EGCG reduced the size of pit, but not the number. The increase of caspase-3 activity and its suppression by Z-VAD-FMK indicate that EGCG induces apoptotic cell death in OCLs as well as in tumor cells (11, 19, 20, 23, 24). EGCG-induced apoptotic cell death was confirmed by staining nuclei in OCLs with Hoechst 33258. However the EGCG-induced cell death was not completely suppressed by Z-VAD-FMK in spite of the complete suppression of the EGCG-induced caspase-3 activation. This suggests that other pathways, not mediated by the increase of caspase-3 activity be also involved in the EGCG-induced cell death.

Although the mechanism of EGCG-induced apoptosis remains to be elucidated, several studies have reported that it was suppressed by catalase (23, 24). We saw a similar suppression of apoptosis by catalase, and showed for the first time that iron chelators also suppress EGCG-induced caspase-3 activation and cell death in OCLs. The reduction of Fe(III) into Fe(II) by EGCG and the complete suppression of EGCG-induced caspase-3 activation and cell death by Fe(II) chelator suggest that Fe(II) plays a crucial role in EGCG-induced these phenomena. These results also indicate that both $H_2O_2$ and Fe(II) are required in EGCG-induced caspase-3 activation and cell death. It is generally accepted that hydroxyl radicals are generated by the reaction of $H_2O_2$ and Fe(II), the so-called Fenton reaction (26). It is difficult to detect hydroxyl radicals directly, but DNA breakage is easy to assay in a cell free system and is well correlated with the generated hydroxyl radical. *In vitro* assays of DNA damage showed that the single strand DNA breakage was induced by EGCG in a dose-dependent manner in the presence of both Fe(III) and $H_2O_2$. Although this action of EGCG conflicts with its antioxidative activity previously reported (10, 17, 18), it has also been reported that hydroxyl radical production is enhanced by flavonoids (39, 40). We confirmed the antioxidative activity of EGCG in the experiment performed at neutral and alkaline conditions where $FeCl_3$ is difficult to dissolve or when high concentration $H_2O_2$ (10 mM) was used (data not shown). These results suggest the possibility that EGCG enhances DNA breakage in the presence of ionized iron and that $H_2O_2$ and the resulting hydroxyl radicals directly induce DNA breakage.

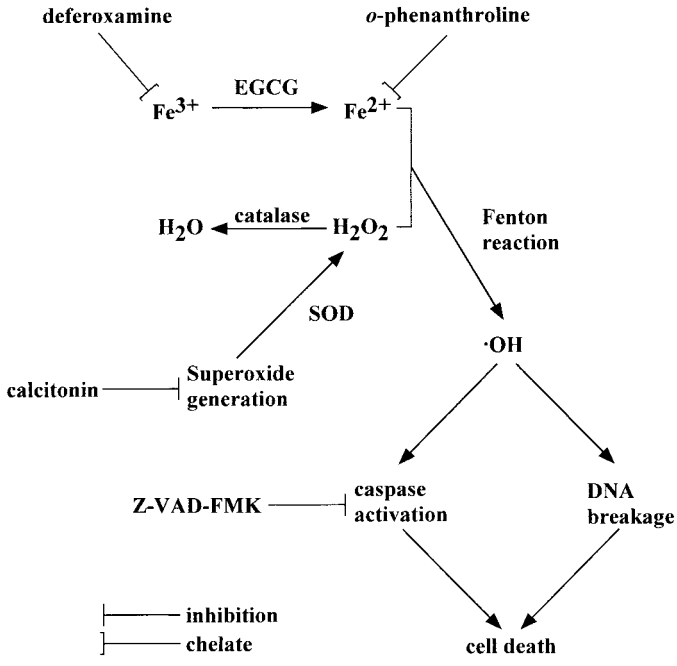


**FIG. 6.** Hypothetical mechanism of EGCG on osteoclastic cell death.

A requirement for both $H_2O_2$ and Fe(III) was shown both for induction of single strand DNA cleavage and EGCG-induced cell death. This indicates that EGCG promotes generation of hydroxyl radicals via the Fenton reaction in OCLs cultures and the resulting DNA breakage is involved in the EGCG-induced death of OCLs. This conclusion is also supported by the result that Z-VAD-FMK partially suppresses osteoclastic cell death, while completely suppressing the increase of caspase activity in OCLs. In Fig. 6, we propose that EGCG-generated hydroxyl radicals induce both caspase-3 activation and direct DNA breakage in OCLs, which cause cell death. Since $H_2O_2$ is generated from superoxide by SOD, the generation of $H_2O_2$ in OCLs would be promoted by the same mechanism. Although the precise mechanism of caspase-3 activation by EGCG remains to be elucidated, caspase-9 activation in tumor cells (20) suggests the possibility that

EGCG-induced hydroxyl radicals also induce disorder of the redox potential in mitochondria of OCLs.

In contrast to OCLs, EGCG had no effect on the osteoblastic cells. This indicates that the Fenton reaction may occur in OCLs but not in the culture medium or osteoblastic cells. An increase in intracellular cAMP level suppresses superoxide generation in OCLs (36, 37), resulting in inhibition of $H_2O_2$ generation. Catalase destroys $H_2O_2$ in culture media but not in the cells. Therefore, complete and partial suppression by calcitonin and catalase of EGCG-induced caspase-3 activation and cell death supports this model. Although further studies are required to elucidate the reason for sensitivity of OCLs to EGCG, numerous transferrin receptors in OCLs (41), expression of a SOD-related glycoprotein in OCLs (32, 33) indicate that high levels of iron and $H_2O_2$ exist in OCLs. The generation of superoxide in bone-resorbing OCLs (34, 35) and its suppression by calcitonin (36, 37) should result in higher sensitivity of bone-resorbing OCLs than that of other normal cells against EGCG. Recent study reported that TRAP facilitated hydroxyl radical formation in alveolar macrophages (42), suggesting that same mechanism as TRAP provide Fe(III) in OCLs. The Fenton reaction should easily take place in OCLs, especially in bone resorbing OCLs, and could be triggered by EGCG. A similar situation may also exist in tumor cells. Our results may also provide insights into the mechanism of action of EGCG on tumor cells.

In this study, we examined the *in vitro* effects of EGCG only. Whether EGCG exerts this activity *in vivo* is uncertain, but its low toxicity and the previous report that EGCG distributes in bone tissue (43) suggest its effectiveness *in vivo*. We propose that it may be possible to use EGCG and the reagents to induce Fenton reaction as a prophylactic or therapeutic agent for the treatment of osteoporosis.

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## REFERENCES

1. Suda, T., Takahashi, N., and Martin, T. J. (1992) Modulation of osteoclast differentiation. *Endocr. Rev.* **13,** 66–80.
2. Baron, R. (1989) Molecular mechanisms of bone resorption by the osteoclast. *Anat. Rec.* **224,** 317–324.
3. Takami, M., Woo, J. T., and Nagai, K. (1999) Osteoblastic cells induce fusion and activation of osteoclasts through a mechanism independent of macrophage-colony-stimulating factor production. *Cell Tissue Res.* **298,** 327–334.
4. Felix, R., Cecchini, M. G., Hofstetter, W., Elford, P. R., Stutzer, A., and Fleisch, H. (1990) Impairment of macrophage colony-stimulating factor production and lack of resident bone marrow macrophages in the osteopetrotic op/op mouse. *J. Bone Miner. Res.* **5,** 781–789.
5. Yoshida, H., Hayashi, S., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T., Shultz, L. D., and Nishikawa, S. (1990) The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* **345,** 442–444.
6. Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., Tomoyasu, A., Yano, K., Goto, M., Murakami, A., Tsuda, E., Morinaga, T., Higashio, K., Udagawa, N., Takahashi, N., and Suda, T. (1998) Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc. Natl. Acad. Sci. USA* **31,** 3597–3602.
7. Lacey, D. L., Timms, E., Tan, H. L., Kelley, M. J., Dunstan, C. R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C., Eli, A., Qian, Y. X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Guo, J., Delaney, J., and Boyle, W. J. (1998) Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* **93,** 165–176.
8. Duong, L. T., Lakkakorpi, P., Nakamura, I., and Rodan, G. A. (2000) Integrins and signaling in osteoclast function. *Matrix Biol.* **19,** 97–105.
9. Morel, I., Lescoat, G., Cogrel, P., Sergent, O., Pasdeloup, N., Brissot, P., Cillard, P., and Cillard, J. (1993) Antioxidant and iron-chelating activities of the flavonoids catechin, quercetin and diosmetin on iron-loaded rat hepatocyte cultures. *Biochem. Pharmacol.* **45,** 13–19.
10. Anderson, R. F., Fisher, L. J., Hara, Y., Harris, T., Mak, W. B., Melton, L. D., and Packer, J. E. (2001) Green tea catechins partially protect DNA from ˙OH radical-induced strand breaks and base damage through fast chemical repair of DNA radicals. *Carcinogenesis* **22,** 1189–1193.
11. Ahmad, N., Feyes, D. K., Nieminen, A. L., Agarwal, R., and Mukhtar, H. (1997) Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *J. Natl. Cancer Inst.* **89,** 1881–1886.
12. Harborne, J. B., and Williams, C. A. (2000) Advances in flavonoid research since 1992. *Phytochemistry* **55,** 481–504.
13. Jankun, J., Selman, S. H., Swiercz, R., and Skrzypczak-Jankun, E. (1997) Why drinking green tea could prevent cancer. *Nature* **387,** 561.
14. Ahmad, N., and Mukhtar, H. (1999) Green tea polyphenols and cancer: Biologic mechanisms and practical implications. *Nutr. Rev.* **57,** 78–83.
15. Blair, H. C., Jordan, S. E., Peterson, T. G., and Barnes, S. (1996) Variable effects of tyrosine kinase inhibitors on avian osteoclastic activity and reduction of bone loss in ovariectomized rats. *J. Cell Biochem.* **61,** 629–637.
16. Horcajada-Molteni, M. N., Crespy, V., Coxam, V., Davicco, M. J., Remesy, C., and Barlet, J. P. (2000) Rutin inhibits ovariectomy-induced osteopenia in rats. *J. Bone Miner. Res.* **15,** 2251–2258.
17. Robak, J., and Gryglewski, R. J. (1988) Flavonoids are scavengers of superoxide anions. *Biochem. Pharmacol.* **37,** 837–841.
18. Bors, W., and Saran, M. (1987) Radical scavenging by flavonoid antioxidants. *Free Radic. Res. Commun.* **2,** 289–294.
19. Islam, S., Islam, N., Kermode, T., Johnstone, B., Mukhtar, H., Moskowitz, R. W., Goldberg, V. M., Malemud, C. J., and Haqqi, T. M. (2000) Involvement of caspase-3 in epigallocatechin-3-gallate-mediated apoptosis of human chondrosarcoma cells. *Biochem. Biophys. Res. Commun.* **270,** 793–797.
20. Pan, M. H., Liang, Y. C., Lin-Shiau, S. Y., Zhu, N. Q., Ho, C. T., and Lin, J. K. (2000) Induction of apoptosis by the oolong tea polyphenol theasinensin A through cytochrome c release and

activation of caspase-9 and caspase-3 in human U937 cells. *J. Agric. Food. Chem.* **48,** 6337–6346.

21. Hengartner, M. O. (2000) The biochemistry of apoptosis. *Nature* **407,** 770–776.
22. Faleiro, L., Kobayashi, R., Fearnhead, H., and Lazebnik, Y. (1997) Multiple species of CPP32 and Mch2 are the major active caspases present in apoptotic cells. *EMBO J.* **16,** 2271–2281.
23. Yang, G. Y., Liao, J., Kim, K., Yurkow, E. J., and Yang, C. S. (1998) Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. *Carcinogenesis* **19,** 611–616.
24. Yang, G. Y., Liao, J., Li, C., Chung, J., Yurkow, E. J., Ho, C. T., and Yang, C. S. (2000) Effect of black and green tea polyphenols on c-jun phosphorylation and $H_2O_2$ production in transformed and non-transformed human bronchial cell lines: Possible mechanisms of cell growth inhibition and apoptosis induction. *Carcinogenesis* **21,** 2035–2039.
25. Delaisse, J. M., Eeckhout, Y., and Vaes, G. (1986) Inhibition of bone resorption in culture by (+)-catechin. *Biochem. Pharmacol.* **35,** 3091–3094.
26. Stohs, S. J., and Bagchi, D. (1995) Oxidative mechanisms in the toxicity of metal ions. *Free Radic. Biol. Med.* **18,** 321–336.
27. Takahashi, N., Akatsu, T., Udagawa, N., Sasaki, T., Yamaguchi, A., Moseley, J. M., Martin, T. J., and Suda, T. (1988) Osteoblastic cells are involved in osteoclast formation. *Endocrinology* **123,** 2600–2602.
28. Tezuka, K., Sato, T., Kamioka, H., Nijweide, P. J., Tanaka, K., Matsuo, T., Ohta, M., Kurihara, N., Hakeda, Y., and Kumegawa, M. (1992) Identification of osteopontin in isolated rabbit osteoclasts. *Biochem. Biophys. Res. Commun.* **186,** 911–917.
29. Araki, T., Yamamoto, A., and Yamada, M. (1987) Accurate determination of DNA content in single cell nuclei stained with Hoechst 33258 fluorochrome at high salt concentration. *Histochemistry* **87,** 331–338.
30. Nagase, H., Fields, C. G., and Fields, G. B. (1994) Design and characterization of a fluorogenic substrate selectively hydrolyzed by stromelysin 1 (matrix metalloproteinase-3). *J. Biol. Chem.* **269,** 20952–20957.
31. Johnson, P. H., and Grossman, L. I. (1977) Electrophoresis of DNA in agarose gels. Optimizing separations of conformational isomers of double- and single-stranded DNAs. *Biochemistry* **16,** 4217–4225.
32. Oursler, M. J., Li, L., and Osdoby, P. (1991) Purification and characterization of an osteoclast membrane glycoprotein with homology to manganese superoxide dismutase. *J. Cell Biochem.* **46,** 219–233.
33. Collin-Osdoby, P., Li, L., Rothe, L., Anderson, F., Kirsch, D., Oursler, M. J., and Osdoby, P. (1998) Inhibition of avian osteoclast bone resorption by monoclonal antibody 121F: A mechanism involving the osteoclast free radical system. *J. Bone Miner. Res.* **13,** 67–78.
34. Steinbeck, M. J., Appel, W. H., Jr., Verhoeven, A. J., and Karnovsky, M. J. (1994) NADPH-oxidase expression and in situ production of superoxide by osteoclasts actively resorbing bone. *J. Cell Biol.* **126,** 765–772.
35. Darden, A. G., Ries, W. L., Wolf, W. C., Rodriguiz, R. M., and Key, L. L., Jr. (1996) Osteoclastic superoxide production and bone resorption: Stimulation and inhibition by modulators of NADPH oxidase. *J. Bone Miner. Res.* **11,** 671–675.
36. Berger, C. E., Horrocks, B. R., and Datta, H. K. (1998) cAMP-dependent inhibition is dominant in regulating superoxide production in the bone-resorbing osteoclasts. *J. Endocrinol.* **158,** 311–318.
37. Berger, C. E., and Datta, H. K. (2000) Forskolin has a bimodal cAMP-independent effect on superoxide anion generation in isolated osteoclasts. *Exp. Physiol.* **85,** 57–60.
38. Chen, Z. P., Schell, J. B., Ho, C. T., and Chen, K. Y. (1998) Green tea epigallocatechin gallate shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts. *Cancer Lett.* **129,** 173–179.
39. Canada, A. T., Giannella, E., Nguyen, T. D., and Mason, R. P. (1990) The production of reactive oxygen species by dietary flavonols. *Free Radic. Biol. Med.* **9,** 441–449.
40. Hiramoto, K., Ojima, N., Sako, K., and Kikugawa, K. (1996) Effect of plant phenolics on the formation of the spin-adduct of hydroxyl radical and the DNA strand breaking by hydroxyl radical. *Biol. Pharm. Bull.* **19,** 558–563.
41. Athanasou, N. A., and Quinn, J. (1990) Immunophenotypic differences between osteoclasts and macrophage polykaryons: Immunohistological distinction and implications for osteoclast ontogeny and function. *J. Clin. Pathol.* **43,** 997–1003.
42. Räisänen, S. R., Halleen, J., Parikka, V., and Väänänen, H. K. (2001) Tartrate-resistant acid phosphatase facilitates hydroxyl radical formation and colocalizes with phagocytosed *Staphylococcus aureus* in alveolar macrophages. *Biochem. Biophys. Res. Commun.* **288,** 142–150.
43. Suganuma, M., Okabe, S., Oniyama, M., Tada, Y., Ito, H., and Fujiki, H. (1998) Wide distribution of [$^3$H](−)-epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue. *Carcinogenesis* **19,** 1771–1776.