Iron Chlorin e_6 Scavenges Hydroxyl Radical and Protects Human Endothelial Cells against Hydrogen Peroxide Toxicity

Je-Wook Yu,[a] Sung-Sik Yoon,[b] and Ryung Yang*,[a]

Department of Biotechnology, Yonsei University,[a] 134 Shinchon-dong, Seodaemoon-gu, Seoul 120–749, Korea and Department of Biological Resources and Technology, Yonsei University,[b] 234 Maeji-ri, Heungup-myon, Wonju 222–710, Korea. Received March 12, 2001; accepted May 11, 2001

Iron chlorin e_6 (FeCe6) has recently been proposed to be potentially antimutagenic and antioxidative. However, the antioxidant property of FeCe6 has not been elucidated in detail. In this study, we investigated the ability of FeCe6 to scavenge hydroxyl radical and to protect biomolecules and mammalian cells from oxidative stress-mediated damage. In electron spin resonance (ESR) experiments, FeCe6 showed excellent hydroxyl radical scavenging activity, whereas its iron-deficient molecule, chlorin e_6 (Ce6) showed little effect. FeCe6 also significantly reduced hydroxyl radical-induced thiobarbituric acid reactive substance (TBARS) formation and benzoate hydroxylation in a dose-dependent manner. The rate constant for reaction between FeCe6 and hydroxyl radical was measured as $8.5\times10^{10}\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}$ by deoxyribose degradation method, and this value was much higher than that of most hydroxyl radical scavengers. Superoxide dismutase (SOD) activity of FeCe6 was also confirmed by ESR study and cytochrome c reduction assay, but its *in vitro* activity appeared to be less efficient in comparison with other well-known SOD mimics. In addition, FeCe6 appreciably diminished hydroxyl radical-induced DNA single-strand breakage and protein degradation in Fe-catalyzed and Cu-catalyzed Fenton systems, and it significantly protected human endothelial cells against hydrogen peroxide (H_2O_2) toxicity. These results suggest that FeCe6 is a novel hydroxyl radical scavenger and may be useful for preventing oxidative injury in biological systems.

Key words iron chlorin e_6 (FeCe6); antioxidant; hydroxyl radical scavenger; hydrogen peroxide toxicity; oxidative stress

Reactive oxygen species (ROS), inevitably produced from various biological reactions, have been implicated in the pathogenesis of many diseases.[1—3] Although endogenous antioxidant defense systems protect cellular homeostasis from the toxic effects of these oxygen metabolites,[1,4] ROS are able to induce severe injury under oxidative stress conditions in which the balance between prooxidant and antioxidant is disrupted like inflammation[5] and ischemia-reperfusion injury.[6,7] With a sudden increase in the intracellular oxidant level, bacteria rapidly cope with these circumstances by transcription of several antioxidant genes, sensitive to a specific oxidant, whereas mammalian cells lack these adaptive defenses.[4,8] Mammalian cells are thus more susceptible to a sudden increase in ROS level and more adaptable to the beneficial effects of therapeutic antioxidants. Hydroxyl radical is the most reactive substance among several toxic oxygen metabolites, and it is able to attack most organic compounds in biological systems.[9] Accordingly, it is considered to be an important subject to seek an efficient hydroxyl radical scavenger for therapeutic purposes and many compounds have been examined for their scavenging abilities toward hydroxyl radical.[10]

Chlorophyll, abundant in green vegetables, has been the topic of considerable interest for its potential antimutagenic role based on the fact that the antimutagenic activity of certain vegetables is strongly correlated with their chlorophyll content.[11] However, the water-insoluble property of chlorophyll has led many investigators to use several water-soluble derivatives in studies on its biological effect. Chlorophyllin (CHL), widely studied as a chlorophyll derivative, has been shown to inhibit the mutagenicity of various chemicals.[12,13] Although its precise antimutagenic mechanism is not clear, its antioxidant property has been proposed to be one possible mechanism.[14—16] Previous reports demonstrated that commercial CHL consists of a few kinds of copper chlorophyll derivatives.[15,17] Among them, two chlorin compounds, copper chlorin e_6 (CuCe6) and copper chlorin e_4 (CuCe4), were suggested to be the major components responsible for the antioxidant activity of CHL.[15] This finding presented the possibility that some chlorin derivatives may be potent antioxidants.

Chlorin e_6 (Ce6)-related molecules have been studied mainly for clinical photodynamic therapy because of their photosensitizing and tumor-localizing properties, but little information is available on their antioxidant activities.[18] Sodium iron chlorin e_6 (FeCe6), as shown in Fig. 1, has shown not only antimutagenicity to benzo[α]pyrene[19] and heterocyclic amine[20] but also SOD mimicking activity.[20,21] Recently, FeCe6 protected natural killer cell activity against ROS-mediated inactivation[22] and inhibited lipid peroxidation in rat fetal brain[23]; no detailed antioxidant ability of FeCe6 has yet been established, however. In this study, we sought to evaluate the ROS-scavenging ability of FeCe6 especially for hydroxyl radical. Since the toxicity of iron is lower than that of copper or manganese[24] and several iron complexes showed considerable superoxide dismutating[25,26] and peroxynitrite scavenging activities,[27] it would be of great interest to investigate iron porphyrin compound as an oxygen free

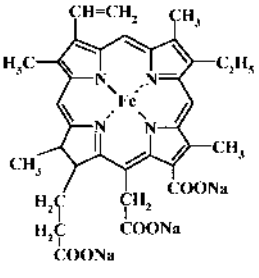

Fig. 1. Structure of Iron Chlorin e_6-Na_3 (FeCe6)

* To whom correspondence should be addressed. e-mail: yangr@yonsei.ac.kr

radical scavenger. Here, we present evidence that FeCe6 is a potent hydroxyl radical scavenger.

MATERIALS AND METHODS

Chemicals FeCe6 and Ce6 were obtained from Porphyrin Products Inc. (Logan, UT, U.S.A.). H_2O_2 was purchased from Fluka (Steinheim, Switzerland). CHL, ferricytochrome c, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), xanthine, xanthine oxidase, benzoic acid, 2-deoxyribose, bovine albumin, mannitol, catalase, SOD (bovine liver), 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), ascorbic acid and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were of analytical grade and all buffers were used after treatment with chelex 100 resin for removal of trace metal.

ESR Spectrometry Hydroxyl radical and superoxide anion were detected using DMPO as a spin trapping agent by ESR technique.[28] For the formation of DMPO-OH spin adducts, a reaction mixture (100 μl) contained 10 mM H_2O_2, 90 mM DMPO and chlorin compounds in 50 mM potassium phosphate buffer containing 0.1 mM EDTA, pH 7.8. The reaction was initiated by UV-irradiation (λ=254 nm) to the mixture for 90 s, and 2 min later ESR spectra of DMPO-OH adducts were recorded using a JEOL JES-TE 200 ESR spectrometer (JEOL, Tokyo, Japan). Superoxide anion was generated from the xanthine oxidase reaction[29] and was detected as a DMPO-OOH adduct. A reaction mixture contained the following reagents at these final concentrations: 0.43 mM xanthine, 90 mM DMPO and chlorin compounds in the same buffer as mentioned above. The scanning was started exactly 2 min after the addition of xanthine oxidase (16 mU ml^{-1}) to the mixture. The inhibition rate was determined on the basis of peak height. Instrumental settings were standardized as follows: microwave power, 4 mW; microwave frequency, 9.18 GHz; modulation frequency, 100 kHz; modulation width, 0.1 mT; scan range, 10 mT; sweep time, 1 min; and time constant, 0.1 s.

Determination of Hydroxyl Radical Scavenging Activity Hydroxyl radical scavenging activity was determined by the inhibition rate of TBARS and benzoate-hydroxylated product formation according to previous methods with some modifications.[9,30] The Fenton reaction was used for generation of hydroxyl radical in both assays. In the deoxyribose degradation method, the reaction was started by the addition of H_2O_2 at the final concentration of 0.5 mM to a reaction mixture containing 0.05 mM $FeSO_4$, 0.05 mM EDTA, 0.05 mM ascorbic acid, 1.5 mM 2-deoxyribose and tested compounds at appropriate concentrations in 20 mM potassium phosphate buffer (pH 7.2). The reaction mixture (2 ml) was incubated at 37 °C for 1 h, followed by the addition of both 1% (w/v) TBA solution (1 ml) and 2.8% (w/v) TCA solution (1 ml), and the mixture was heated at 100 °C for 10 min. The amount of TBARS, deoxyribose-degraded products, was measured by the absorbance at 532 nm. Rate constants for reaction of tested compounds with hydroxyl radical were calculated from the slope obtained from a plot of 1/A *versus* sample concentration, where A is the absorbance at 532 nm in the presence of tested compound, using $k_{\text{deoxyribose}}=3.1\times10^9$ M^{-1} s^{-1} as described previously.[30] To induce benzoate hydroxylation by hydroxyl radical, H_2O_2 (50 μl) at the final concentration of 3 mM was added to a reaction mixture (200 μl) containing the following reagents at these final concentrations: 0.2 mM $FeSO_4$, 0.2 mM EDTA, 1 mM benzoate and tested compounds in 50 mM potassium phosphate buffer (pH 7.2) on 96 well fluoroplates (Nunc, Roskilde, Denmark). After incubation at room temperature for 10 h, the fluorescence of each well was measured using a Varian Cary Eclipse fluorescence spectrophotometer (Varian Inc., Mulgrave, Australia) at excitation and emission wavelengths of 300 nm and 406 nm, respectively.

Determination of Superoxide Scavenging Activity SOD-like activity was determined by the inhibition rate of superoxide-catalyzed ferricytochrome c reduction.[29] Briefly, xanthine oxidase (8 mU ml^{-1}) was added to a reaction mixture containing 10 μM xanthine, 10 μM ferricytochrome c, 100 U ml^{-1} catalase and tested compounds in 50 mM potassium phosphate buffer (pH 7.8). The reduction of cytochrome c^{3+} was then followed at the wavelength of 550 nm for 3 min. IC_{50} values were calculated from the slope, obtained from a plot of $(v_o/v_i)-1$ *versus* sample concentration, at $(v_o/v_i)-1=1$, where v_o is the reduction rate of cytochrome c in the absence of tested compound and v_i is the reduction rate of cytochrome c in the presence of tested compound, as described previously.[31] The catalytic rate constants (k_{cat}) were calculated by the following equation: $k_{cat}=k_{cyt\,c}\times$[cytochrome c^{3+}]/IC_{50}, using $k_{cyt\,c}=3.0\times10^6$ M^{-1} s^{-1}.[32]

Detection of DNA Cleavage and Protein Degradation by Hydroxyl Radical Fe-catalyzed and Cu-catalyzed Fenton reaction systems were used for induction of DNA single-strand cleavage and albumin degradation. Plasmid pEGFP DNA was prepared using a Promega Wizard plus SV minipreps DNA purification kit (Promega, Madison, WI, U.S.A.). DNA (0.5 μg) was incubated at 37 °C for 30 min with a reaction mixture containing 8 μM $FeSO_4$/8 μM EDTA/80 μM H_2O_2 or 50 μM $CuSO_4$/500 μM H_2O_2 with or without tested compounds in 50 mM potassium phosphate buffer, pH 7.4. The mixture was then analyzed by electrophoresis on a 1% agarose gel at 100 V for 2 h. To induce protein degradation, bovine albumin (8 μg) was incubated at 37 °C for 24 h with a reaction mixture containing 0.1 mM $FeSO_4$/0.1 mM EDTA/1 mM H_2O_2 or 0.1 mM $CuSO_4$/1 mM H_2O_2 with or without tested compounds in 50 mM potassium phosphate buffer, pH 7.4. After incubation, the same volume of sample buffer containing 2% (v/v) sodium dodecyl sulfate and 100 mM dithiothreitol was added to each reaction mixture. Then, the mixture was heated at 100 °C for 5 min and was electrophoresed on a 10% polyacrylamide gel.[33]

Cell Culture and Cell Viability Assay Human umbilical vein endothelial cells (HUVEC, ATCC CRL 1730) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were grown in Dulbecco's minimum essential medium (DMEM, Biowhittaker, Walkersville, MD, U.S.A.) supplemented with 10% fetal calf serum (Gibco, Grand Island, U.S.A.) at 37 °C under humidified air containing 5% carbon dioxide. Cell viability was measured by MTT colorimetric assay.[34] Briefly, cells were plated and grown to 80—90% confluence in 96 well plates (Nunc, Roskilde, Denmark), and incubated for 8 h with fresh culture medium containing appropriate chlorin derivatives. The medium was then replaced by H_2O_2-containing medium.

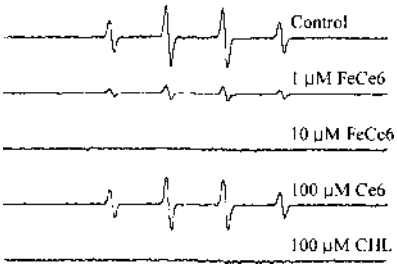

Fig. 2. Effects of FeCe6, Ce6, and CHL on DMPO-OH Formation

Each solution was transferred into a 100 μl capillary after appropriate treatments as described under Materials and Methods, and placed inside the ESR cavity. ESR spectra were recorded at room temperature.

After incubation with H_2O_2 for 4 h, MTT stock solution (5 mg/ml) was added to each well and incubated for an additional 4 h. Culture medium was then removed and the formed formazan was solubilized with DMSO. The absorbance of each well was measured by a microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) at 540 nm.

Statistical Analyses All values were expressed as means±S.D. of *n* observations. Data were statistically analyzed by unpaired Student's *t*-test after analysis of variance. *p*-values of 0.05 or less were considered significant.

RESULTS

Hydroxyl Radical Scavenging Effects As shown in Fig. 2, hydroxyl radical, generated in UV-photolysis of H_2O_2, was trapped by DMPO to form a stable DMPO-OH adduct (control). The signal intensity of DMPO-OH adduct was remarkably diminished by 1 μM FeCe6; the inhibition rate was approximately 77%. FeCe6 at higher concentrations than 10 μM completely inhibited DMPO-OH adduct formation, whereas its iron-deficient compound, Ce6 exhibited only 12% inhibition at a concentration of 100 μM. CHL, on the other hand, showed detectable hydroxyl radical scavenging effect in agreement with the previous finding that CHL dose-dependently scavenged hydroxyl radical in deoxyribose assay.[35] CHL reduced DMPO-OH formation with an inhibition rate of 37% at 10 μM (data not shown) and nearly 100% at 100 μM. These observations indicate that these chlorin derivatives may be potent hydroxyl radical scavengers and the metal ion-moiety of chlorin e_6 derivatives is important for this scavenging. To examine the ability of FeCe6 to scavenge hydroxyl radical in detail, we further tested its efficacy using 2-deoxyribose and benzoate as hydroxyl radical-trapping agents. Although it is controversial whether Fenton chemistry is a relevant hydroxyl radical-generating system *in vivo*, it is generally accepted that the majority of hydroxyl radicals are produced from metal-dependent reduction of H_2O_2 such as the Fenton reaction.[9] The Fe-catalyzed Fenton system was thus adopted as a hydroxyl radical source in our experiments. As shown in Fig. 3A, FeCe6 significantly inhibited the formation of TBARS, deoxyribose-degraded products. Likewise, FeCe6 diminished hydroxyl radical-induced benzoate hydroxylation in a dose-dependent manner (Fig. 3B). In both assays, hydroxyl radical scavenging activity of FeCe6 was much higher than that of mannitol, a well-known hydroxyl radical scavenger. In fact, hydroxyl radical is extremely reactive, and thus any molecule can react very quickly with it.[36]

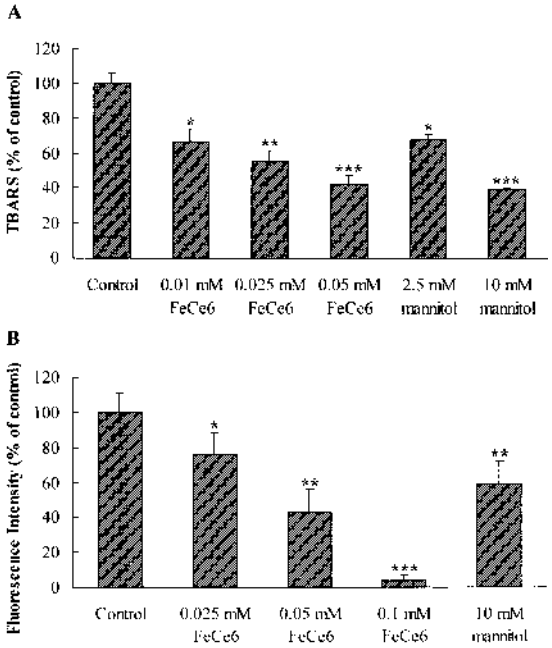

Fig. 3. Effects of FeCe6 and Mannitol on Deoxyribose Degradation and Benzoate Hydroxylation

Detailed experimental conditions are as described under Materials and Methods. (A) The amount of TBARS was represented as % of control value, calculated as follows: (OD_{532} in the presence of each sample/OD_{532} in the absence of sample)×100. All data were expressed as means±S.D. (n=3). Asterisks indicate significant differences from control. (* p<0.05, ** p<0.01, *** p<0.005) (B) Fluorescence intensity was represented as % of control value. All data were expressed as means±S.D. (n=4). Asterisks indicate significant differences from control. (* p<0.05, ** p<0.005, *** p<0.001).

Table 1. Rate Constants for Reaction of Several Compounds with Hydroxyl Radicals Determined by Deoxyribose Assay

Compound	Rate constant ($M^{-1} s^{-1}$)*a)*
Ce6	1.3×10^{10}
FeCe6	8.5×10^{10}
CHL	1.8×10^{10}
Mannitol	3.3×10^{8}

a) Triplicate assays were performed for each concentration and the errors in rate constant were within 10%.

To compare the efficacy of FeCe6 with other hydroxyl radical scavengers, we determined rate constants for reaction of chlorin compounds with hydroxyl radical by deoxyribose degradation method (Table 1). Second-order rate constant of FeCe6 was measured as $8.5\times10^{10}\,M^{-1}\,s^{-1}$, which was approximately 250 times as high as that of mannitol in our assays. It was also found that Ce6 and CHL more readily react with hydroxyl radical than mannitol on the basis of rate constants.

Superoxide Scavenging Effects To evaluate the ability of chlorin derivatives to catalyze the dismutation of superoxide, we tested their superoxide scavenging activity by ESR technique and cytochrome c reduction assay. In ESR study, superoxide radical, generated from the xanthine/xanthine oxidase system, formed DMPO-OOH adduct in the presence of DMPO as shown in Fig. 4 (control). FeCe6 reduced the signal intensity of DMPO-OOH adduct with the inhibition rate of 33% and 81% at the concentration of 10 μM and 100 μM, respectively. CHL showed the inhibition rate of 45% at 100 μM, but the inhibitory effect of Ce6 on DMPO-OOH for-

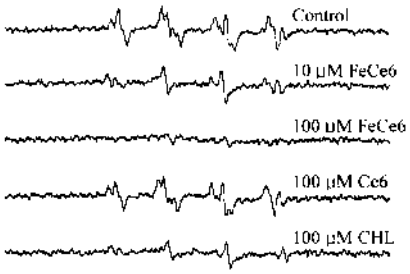

Fig. 4. Effects of FeCe6, Ce6, and CHL on DMPO-OOH Formation

ESR spectra were recorded at room temperature. Experimental procedures are as described in detail under Materials and Methods.

Table 2. SOD-like Activities of Several Compounds by Cytochrome c Reduction Assay

Compound	IC_{50} (μM)[a]	k_{cat} ($M^{-1}\,s^{-1}$)	% of SOD activity[b]
Ce6	169.51	1.8×10^5	—
FeCe6	12.25	2.4×10^6	0.13
CHL	20.29	1.5×10^6	0.08
SOD	0.016	1.9×10^9	100

a) IC_{50} values mean the concentration that causes 50% inhibition of the cytochrome c reduction. Triplicate assays were performed for each concentration and the errors in IC_{50} were within 10%. *b*) % of SOD activity was based on k_{cat} value.

mation was not significant. None of these chlorin compounds interfered with xanthine oxidase activity at tested concentrations (data not shown). These observations indicate that FeCe6 apparently scavenges superoxide anion in agreement with the previous reports.[20,21] To confirm the SOD mimicking activity of FeCe6, we evaluated its activity using the cytochrome c reduction method.[29] As shown in Table 2, FeCe6 exhibited an IC_{50} value of 12.25 μM and the catalytic rate constant for dismutation of superoxide of $2.4\times10^6\,M^{-1}\,s^{-1}$. Its relative SOD activity was higher than CHL, but not as excellent as several well-established SOD mimics, which exhibited approximately 0.1—3% of SOD activity.[25,26,37,38] Considering that the spontaneous dismutation of superoxide has a rate constant of $2\times10^5\,M^{-1}\,s^{-1}$,[1] we reasoned that Ce6 is not active in catalyzing the dismutation of superoxide in cytochrome c reduction assay. These results suggest that FeCe6 may be a potent SOD mimicking molecule and that iron-moiety of FeCe6 may be essential for its SOD activity.

Effect of FeCe6 on Hydroxyl Radical-Induced Impairments of DNA and Protein Hydroxyl radical is likely to cause severe damage to cellular biomolecules.[1] Here, we examined the effect of FeCe6 on hydroxyl radical-induced alterations of plasmid DNA and albumin. As shown in Fig. 5, single-strand breakage of plasmid DNA was observed when plasmid DNA was incubated with iron-catalyzed (lane 2) or copper-catalyzed Fenton reagents (lane 7). It is known that hydroxyl radical attacks the phosphodiester backbone of covalently closed circular (CCC) supercoiled DNA (form I) and consequently cleaves one DNA strand to produce relaxed circular DNA (form II). This DNA cleavage was appreciably diminished by treatment with FeCe6 at a concentration of 50 μM (lanes 3, 8) or 100 μM (lanes 4, 9), whereas mannitol at these concentrations showed a slight protective effect on DNA breakage in the Fe-catalyzed system (lanes 5, 6), but no effect in the Cu-catalyzed system (lanes 10, 11). Since free copper ions preferably bind macromolecules, such as DNA

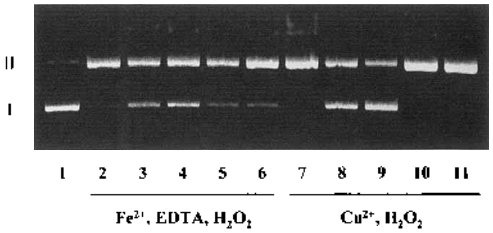

Fig. 5. Effects of FeCe6 and Mannitol on Plasmid DNA Breakage

Plasmid pEGFP DNA was incubated with reaction mixtures containing buffer alone (lane 1), Fe^{2+}/EDTA/H_2O_2 (lanes 2—6) and Cu^{2+}/H_2O_2 (lanes 7—11) in the absence of tested compound (lanes 1, 2, 7); in the presence of 50 μM Fe-chlorin (lanes 3, 8), 100 μM Fe-chlorin (lanes 4, 9), 50 μM mannitol (lanes 5, 10) and 100 μM mannitol (lanes 6, 11). Form I and form II represent covalently closed circular supercoiled DNA and relaxed circular DNA, respectively. Experimental conditions are as described in detail under Materials and Methods.

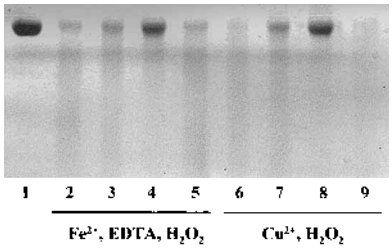

Fig. 6. Effects of FeCe6 and Mannitol on Bovine Albumin Degradation

Bovine albumin was incubated with reaction mixtures containing buffer alone (lane 1), Fe^{2+}/EDTA/H_2O_2 (lanes 2—5) and Cu^{2+}/H_2O_2 (lanes 6—9) in the absence of tested compound (lanes 1, 2, 6); in the presence of 50 μM Fe-chlorin (lanes 3, 7), 100 μM Fe-chlorin (lanes 4, 8) and 100 μM mannitol (lanes 5, 9). Experimental conditions are as described in detail under Materials and Methods.

and protein, hydroxyl radical, generated *via* the Cu^{2+}/H_2O_2 system, would rather attack adjacent biomolecules immediately than be released freely.[9,39] On the contrary, iron-EDTA complexes are likely to produce free hydroxyl radical, preventable by hydroxyl radical scavenger, owing to the open structure of Fe-EDTA complexes.[39] For this reason, hydroxyl radical scavenger generally could not protect macromolecules against hydroxyl radical-mediated damage in the Cu^{2+}/H_2O_2 system. Nevertheless, FeCe6 protected plasmid DNA under Cu-catalyzed Fenton system (Fig. 5, lanes 8, 9), and this finding verified its excellent hydroxyl radical-scavenging ability. Similar results were also obtained from hydroxyl radical-induced degradation of bovine albumin, as shown in Fig. 6. This porphyrin-like compound considerably protected albumin against hydroxyl radical at a concentration of 100 μM not only in the Fe^{2+}/EDTA/H_2O_2 system (lane 4) but in the Cu^{2+}/H_2O_2 system (lane 8), whereas 100 μM mannitol showed no protective effect (lanes 5, 9).

Protective Effect of FeCe6 on Mammalian Cell Death Induced by Hydrogen Peroxide To examine whether FeCe6 would act as a potent antioxidant in mammalian cells, we tested its effect on cell viability in the presence of H_2O_2. While H_2O_2 *per se* is a stable nonradical species and is nontoxic at physiological concentrations, it is able to generate highly reactive hydroxyl radical through Fenton or Haber-Weiss reaction.[36,40] Moreover, H_2O_2 can easily diffuse across biological membrane, but superoxide or hydroxyl radical generally has difficulty in crossing the membrane.[39] For this reason, H_2O_2 was employed as an intracellular oxidative stress inducer instead of Fenton system in our assay. Cell via-

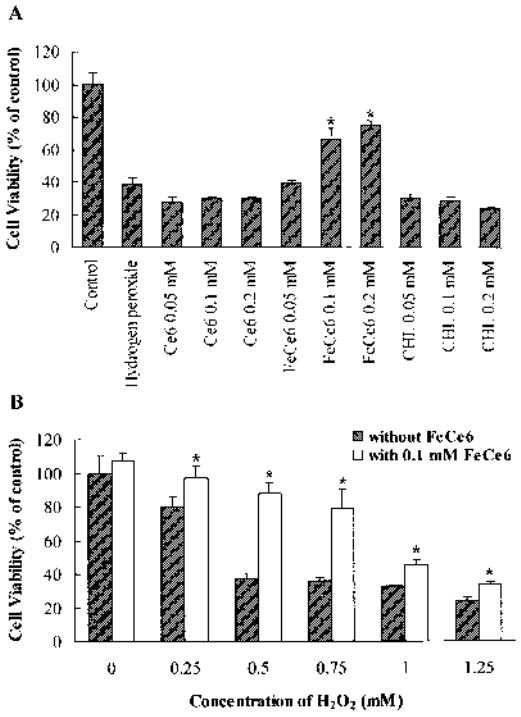

Fig. 7. Protective Effect of FeCe6 on Human Endothelial Cell Death Induced by H_2O_2

(A) Cells were incubated with 0.75 mM H_2O_2 or without H_2O_2 (control) after pretreatment with Ce6, FeCe6 and CHL at designated concentrations. Cell viability was expressed as % of control absorbance at 540 nm, calculated as follows: (OD_{540} in each group/OD_{540} in control group)×100. All data were expressed as means±S.D. (n=6). Groups pretreated with 100 μM and 200 μM FeCe6 were significantly different from the hydrogen peroxide group (*p<0.001). (B) Cells were incubated with various concentrations of H_2O_2 after pretreatment with medium alone (▨) or 100 μM FeCe6 (□). Cell viability was expressed as % of control value (in the absence of H_2O_2 and FeCe6), calculated as (A). All data were expressed as means±S.D. (n=8). Groups pretreated with FeCe6 were significantly different from each FeCe6-deficient group (*p<0.001).

bility was measured using MTT, which converts to an insoluble purple formazan by dehydrogenase enzymes, active only in living cells.[34] As shown in Fig. 7A, the incubation of 0.75 mM H_2O_2 for 4 h caused a decrease in cell viability to 39% of control. Pretreatment with FeCe6 at 100 μM and 200 μM significantly restored cell survival rate to 66% and 75% in spite of 0.75 mM H_2O_2 (p<0.001), while Ce6 and CHL showed no protective effect against H_2O_2 toxicity. In addition, 100 μM FeCe6 significantly protected human endothelial cells in the presence of 0.25—1.25 mM H_2O_2 (Fig. 7B, p<0.001), and FeCe6 at tested concentrations showed no cytotoxicity after 48 h incubation (data not shown). These results raise the possibility that FeCe6 can be used as an antioxidant drug for therapeutic purposes.

DISCUSSION

Various transition metal complexes have been investigated as a potent SOD mimetic, because transition metal is placed at the active site of natural SOD, such as Cu Zn-SOD, Mn-SOD and Fe-SOD.[41] In particular, much attention has been paid to metalloporphyrins, which demonstrated higher stability in the presence of metal chelators than other metal complexes.[41,42] A few metalloporphyrins have shown the prominent ability to catalyze the dismutation of superoxide and to protect mammalian cells and SOD-deficient strains against intracellular superoxide-mediated injury.[8,38,43] FeCe6, a derivative of porphyrin, also exhibited SOD-like activity in a very limited number of reports,[20,21] and prevented the injury associated with ROS in mammalian model systems.[22,23] In the present study, we focused on evaluating its hydroxyl radical scavenging activity, which has never been explored, by *in vitro* assay systems.

The spin trapping technique has been widely used for detection of ROS, for it had less interference than other methods using indicator molecules.[44] Thus, we preferentially evaluated hydroxyl radical scavenging activities by ESR technique. FeCe6 at micromolar concentrations reduced the signal intensity of DMPO-OH adduct (Fig. 2), and also showed prominent hydroxyl radical scavenging activity by deoxyribose degradation and benzoate hydroxylation method (Fig. 3). It has been reported that several hydroxyl radical scavengers exerted their effect by chelating metal ions, necessary for hydroxyl radical generation in the Fenton reaction, not by hydroxyl radical scavenging.[39,45] To exclude this possibility, a metal-free system, *i.e.* UV-photolysis of H_2O_2 was used for hydroxyl radical generation in ESR experiments. Therefore, the inhibitory effects of FeCe6 and CHL on DMPO-OH formation are considered to be the result of direct hydroxyl radical scavenging. According to the previous paper, some reagents which prevent hydroxyl radical generation by reacting with H_2O_2 might also be recognized as hydroxyl radical scavengers.[30] These reagents revealed a particular nonlinear competition plot in the deoxyribose method.[30] However, FeCe6 showed dose-dependent decreases in TBARS formation (Fig. 3A), suggesting that it competed with deoxyribose for hydroxyl radical. Likewise, FeCe6 did not decompose H_2O_2 as determined by monitoring of the absorbance of H_2O_2 at 240 nm in the presence of FeCe6 (data not shown). It is thus inferred that the scavenging effect of FeCe6 toward hydroxyl radical in our systems is not attributable to its reaction with H_2O_2.

All tested chlorin compounds demonstrated higher rate constants for reaction with hydroxyl radical than did mannitol, a well-known hydroxyl radical scavenger, in the deoxyribose degradation method (Table 1). Interestingly, Ce6 showed a slight inhibition of DMPO-OH formation, but a significant decrease in hydroxyl radical-catalyzed TBARS formation. Its low efficacy in ESR study can be explained as the difference in concentrations of DMPO and Ce6 in assay mixture. The rate constant for reaction between DMPO and hydroxyl radical is cited as $3.4\times10^9\,M^{-1}\,s^{-1}$ in the literature,[36] but DMPO was added at a concentration of 90 mM in our ESR experiments. Consequently, Ce6 at lower concentrations than 100 μM could not compete with DMPO, because the rate constant of Ce6 with hydroxyl radical was $1.3\times10^{10}\,M^{-1}\,s^{-1}$ in our experiments, which was only 3.8 times as high as that of DMPO. Especially, the rate constant for reaction between FeCe6 and hydroxyl radical was $8.5\times10^{10}\,M^{-1}\,s^{-1}$, calculated from the amount of TBARS, and this value was much higher than $1—9\times10^9\,M^{-1}\,s^{-1}$ of various hydroxyl radical scavengers.[30,36] These findings strongly suggest that FeCe6 is a novel hydroxyl radical scavenger. Considering that most biomolecules, including proteins, lipids and DNA, react with hydroxyl radical with rate constants of $10^{10}\,M^{-1}\,s^{-1}$ or less,[46] FeCe6 is expected to protect these molecules effectively against hydroxyl radical in biological systems.

A few reports propose that FeCe6 possesses SOD mimick-

ing activity.[20,21] To confirm its potential ability, we measured SOD activity using ESR analysis and cytochrome c reduction method. As expected, FeCe6 dose-dependently decreased DMPO-OOH formation (Fig. 4) and inhibited superoxide-catalyzed cytochrome c reduction (Table 2). However, FeCe6 and CHL oxidized reduced cytochrome c (data not shown), and this phenomenon apparently overestimates the SOD activity of tested compounds as stated previously.[26] To solve this problem, catalase was added to the reaction mixture in our assay, because this reoxidation was strongly dependent on H_2O_2, produced from the dismutation of superoxide. Although relative SOD activity of FeCe6 was somewhat lower than that of other established SOD mimics as mentioned above (Table 2), FeCe6 efficiently scavenged superoxide radical, generated intracellularly by menadione, in T jurkat cells (data not shown) and preserved aconitase activity, which is regarded as a specific indicator of intracellular superoxide level,[8] against paraquat in A549 cells (data not shown). These facts support the possibility that FeCe6 may be used as a potent SOD mimic for the purpose of preventing superoxide-mediated injury as reported previously.[22,23]

Both Fe^{2+}/EDTA/H_2O_2 and Cu^{2+}/H_2O_2 systems were used for hydroxyl radical generation to induce oxidative DNA and protein damage, which have been postulated to be critical for aging.[32,47] As mentioned above, Fe-EDTA complexes release free hydroxyl radical to cause nonspecific damage to macromolecules. On the other hand, Cu^{2+}/H_2O_2 systems induce site-specific and more severe damage, generally not preventable by most hydroxyl radical scavengers.[39] Nevertheless, FeCe6 at micromolar levels appreciably diminished plasmid DNA cleavage and albumin degradation in the Cu^{2+}/H_2O_2 oxidation system, but mannitol failed to protect at the same concentration (Figs. 5 and 6). This result also verifies that the hydroxyl radical scavenging activity of FeCe6 is excellent.

H_2O_2 is a key metabolite in ROS biology and is increasingly proposed as a toxic intermediate in diverse pathophysiologies.[40] When we tested the protective effect of chlorin compounds on human endothelial cells against H_2O_2, only FeCe6 significantly restored cell viability even under severe oxidative stress conditions, *i.e.* millimolar concentrations of H_2O_2 (Fig. 7). CHL and Ce6 exhibited no protective effect in spite of *in vitro* hydroxyl radical scavenging effect. Similar results were also found in human lung carcinoma A549 cells (data not shown). It was proposed that catalase activity of some manganic porphyrins might be responsible for their protective effect against H_2O_2-induced endothelial cell injury.[42] On the other hand, nicergoline, an ergoline derivative, protected mammalian cells from H_2O_2 by up-regulation of intracellular catalase.[48] However, FeCe6 showed negligible H_2O_2 scavenging activity by *in vitro* catalase assay and it did not affect intracellular catalase or SOD activity after 10 h incubation of 100 μM FeCe6 to human endothelial cells (data not shown). Therefore, we considered that the protective mechanism of FeCe6 against H_2O_2 toxicity is not obvious but is probably attributable in part to the hydroxyl radical scavenging effect. Further study will be needed to elucidate the precise intracellular action of FeCe6.

In conclusion, our results show that FeCe6 contains excellent hydroxyl radical scavenging activity in addition to the previously reported SOD mimicking activity.[20,21] Moreover, FeCe6 protected cellular macromolecules and mammalian cells against oxidative stress-mediated injury, and these effects are thought to be the result of hydroxyl radical scavenging. FeCe6 is very stable like other metalloporphyrins and its iron-moiety is so tightly bound that it did not participate in the Fenton reation.[21] Furthermore, we observed that FeCe6 exhibited remarkable singlet oxygen ($^1O_2$) quenching ability as judged by the inhibition of 2,2-6,6-tetramethyl-4-piperidinol (TMP)-$^1O_2$ formation in ESR study (data not shown). These multifunctional antioxidant activities of FeCe6 suggest that it may be usable as a potent antioxidant for therapeutic purposes under oxidative stress conditions.

Acknowledgement This work was supported by Korea Research Foundation Grant (KRF-2000-042-00026).

REFERENCES

1) Yu B. P., *Physiol. Rev*., **74**, 139—162 (1994).
2) Yu B. P., Yang R., *Ann. N. Y. Acad. Sci*., **786**, 1—11 (1996).
3) McIntosh L. J., Trush M. A., Troncoso J. C., *Free Radic. Biol. Med*., **23**, 183—190 (1997).
4) Harris E. D., *FASEB J*., **6**, 2675—2683 (1992).
5) Morcillo E. J., Estrela J., Cortijo J., *Pharmacol. Res*., **40**, 393—404 (1999).
6) Baker K., Marcus C. B., Huffman K., Kruk H., Malfroy B., Doctrow S. R., *J. Pharmacol. Exp. Ther*., **284**, 215—221 (1998).
7) Granger D. N., Rutili G., McCord J. M., *Gastroenterology*, **81**, 22—29 (1981).
8) Gardner P. R., Nguyen D.-D. H., White C. W., *Arch. Biochem. Biophys*., **325**, 20—28 (1996).
9) Kocha T., Yamaguchi M., Ohtaki H., Fukuda T., Aoyagi T., *Biochim. Biophys. Acta*, **1337**, 319—326 (1997).
10) Ueda J., Saito N., Shimazu Y., Ozawa T., *Arch. Biochem. Biophys*., **333**, 377—384 (1996).
11) Lai C., Butler M. A., Matney T. S., *Mutat. Res*., **77**, 245—250 (1980).
12) Chernomorsky S., Rancourt R., Virdi K., Segelman A., Poretz R. D., *Cancer Lett*., **120**, 141—147 (1997).
13) Chung W.-Y., Lee J.-M., Park M.-Y., Yook J.-I., Kim J., Chung A.-S., Surh Y.-J., Park K.-K., *Cancer Lett*., **145**, 57—64 (1999).
14) Ong T., Whong W.-Z., Stewart J., Brockman H. E., *Mutat. Res*., **173**, 111—115 (1986).
15) Sato M., Fujimoto I., Sakai T., Aimoto T., Kimura R., Murata T., *Chem. Pharm. Bull*., **34**, 2428—2434 (1986).
16) Kamat J. P., Boloor K. K., Devasagayam T. P. A., *Biochim. Biophys. Acta*, **1487**, 113—127 (2000).
17) Inoue H., Yamashita H., Furuya K., Nonomura Y., Yoshioka N., Li S., *J. Chromatogr*., **679**, 99—104 (1994).
18) Pandey R. K., Bellnier D. A., Smith K. M., Dougherty T. J., *Photochem. Photobiol*., **53**, 65—72 (1991).
19) Arimoto S., Kan-yama K., Rai H., Hayatsu H., *Mutat. Res*., **345**, 127—135 (1995).
20) Arimoto S., Inada N., Nakano H., Rai H., Hayatsu H., *Mutat. Res*., **400**, 259—269 (1998).
21) Kariya K., Nakamura K., Nomoto K., Kobayashi Y., Namiki M., *Cancer Biother*., **10**, 139—145 (1995).
22) Nakamura K., Matsunaga K., *Cancer Biother. Radiopharm*., **13**, 275—290 (1998).
23) Saito K., Maeda M., Yoshihara H., Amano K., Nishijima M., Nakamura K., *Biol. Neonate*, **77**, 109—114 (2000).
24) Nagano T., Hirano T., Hirobe M., *J. Biol. Chem*., **264**, 9243—9249 (1989).
25) Pasternack R. F., Halliwell B., *J. Am. Chem. Soc*., **101**, 1026—1031 (1979).
26) Weiss R. H., Flickinger A. G., Rivers W. J., Hardy M. M., Aston K. W., *J. Biol. Chem*., **268**, 23049—23054 (1993).
27) Crow J. P., *Arch. Biochem. Biophys*., **371**, 41—52 (1999).
28) Yaping T., Yunzhong F., Cunpu S., Wenmei S., Qinhui L., Menchang S., *Biochem. Biophys. Res. Commun*., **191**, 646—653 (1993).
29) McCord J. M., Fridovich I., *J. Biol. Chem*., **244**, 6049—6055 (1969).

30) Halliwell B., Gutteridge J. M. C., Aruoma O. I., *Anal. Biochem*., **165**, 215—219 (1987).
31) Batinić-Haberle I., Liochev S. I., Spasojević I., Fridovich I., *Arch. Biochem. Biophys*., **343**, 225—233 (1997).
32) Faulkner K. M., Liochev S. I., Fridovich I., *J. Biol. Chem*., **269**, 23471—23476 (1994).
33) Laemmli U. K., *Nature* (London), **227**, 680—685 (1970).
34) Alley M. C., Scudiero D. A., Monks A., Hursey M. L., Czerwinski M. J., Fine D. L., Abbott B. J., Mayo J. G., Shoemaker R. H., Boyd M. R., *Cancer Res*., **48**, 589—601 (1988).
35) Kim B.-Y., Yun C.-H., Chung A.-S., *Res. Commun. Biochem. Cell Mol. Biol*., **3**, 193—204 (1999).
36) Halliwell B., Gutteridge J. M. C., "Free Radicals in Biology and Medicine," 3rd ed., ed. by Halliwell B., Gutteridge J. M. C., Oxford University Press, Oxford, 1999, pp. 53—60.
37) Hardy M. M., Flickinger A. G., Riley D. P., Weiss R. H., Ryan U. S., *J. Biol. Chem*., **269**, 18535—18540 (1994).
38) Batinic-Haberle I., Benov L., Spasojevic I., Fridovich I., *J. Biol. Chem*., **273**, 24521—24528 (1998).
39) Halliwell B., Gutteridge J. M. C., *Arch. Biochem. Biophys*., **246**, 501—514 (1986).
40) Lord-Fontaine S., Averill D. A., *Arch. Biochem. Biophys*., **363**, 283—295 (1999).
41) Fridovich I., *Annu. Rev. Biochem*., **64**, 97—112 (1995).
42) Day B. J., Fridovich I., Crapo J. D., *Arch. Biochem. Biophys*., **347**, 256—262 (1997).
43) Day B. J., Shawen S., Liochev S. I., Crapo J. D., *J. Pharmacol. Exp. Ther*., **275**, 1227—1232 (1995).
44) Greenwald R. A., "CRC Handbook of Methods for Oxygen Radical," ed. by Thornalley P. J., Bannister J. V., CRC Press, New York, 1985, pp. 133—136.
45) Lopes G. K. B., Schulman H. M., Hermes-Lima M., *Biochim. Biophys. Acta*, **1472**, 142—152 (1999).
46) Gilbert D. L., Colton C. A., "Reactive Oxygen Species in Biological Systems: an Interdisciplinary Approach," ed. by Huie R. E., Neta P., Klewer Academic/Plenum Publisher, New York, 1999, pp. 33—73.
47) Sohal R. S., Agarwal S., Dubey A., Orr W. C., *Proc. Natl. Acad. Sci. U.S.A*., **90**, 7255—7259 (1993).
48) Iwata E., Miyazaki I., Asanuma M., Iida A., Ogawa N., *Neurosci. Lett*., **251**, 49—52 (1998).