Protection by Antioxidants of Copper-induced Decline of Proliferation and SOD Activity

NAOTO KINOSHITA1,2, KEN HASHIMOTO2, TAKAHIKO YAMAMURA1, HIROSHI TERANUMA1, TOMOYA KOIZUMI1, KAZUE SATOH2,3, TADASHI KATAYAMA1 and HIROSHI SAKAGAMI2

1Department of Operative Dentistry and 2Department of Dental Pharmacology, Meikai University School of Dentistry, Saitama; 3Department of Anatomy, School of Medicine, Showa University, Tokyo, Japan

Abstract. The effect of Cu plate on the cellular function was investigated by two different methods: an extraction method (Method I) and a direct contact method (Method II). In Method I, the supernatant of the culture medium, which had been pre-incubated with Cu plate, was added to mouse macrophage-like Raw 264.7 cells. This supernatant dose-dependently inhibited the proliferation and nitric oxide (NO) production by lipopolysaccharide-stimulated Raw 264.7 cells. In Method II, human promyelocytic leukemia HL-60 cells in suspension were incubated with culture medium which contained Cu plate. The direct contact with Cu plate rapidly suppressed the proliferation and MnSOD and Cu/ZnSOD activities. The suppressed proliferation and SOD activity reverted to or exceeded the control level by sodium ascorbate, whereas N-acetyl-L-cysteine (NAC) only reactivated the proliferation, but not the SOD activity. ESR spectroscopy showed that contact with Cu plate slightly diminished the hydroxyl radical (generated by Fenton reaction), without affecting the intensity of NO (produced from NOC-7) and DPPH radical. The present study suggests that two representative antioxidants, such as sodium ascorbate and NAC, protect the cells from Cu-induced cytotoxicity via different mechanisms.

We have previously shown that metals modified the cytotoxic activity of antioxidants. CuCl and CuCl2 significantly enhanced the cytotoxic activity of sodium ascorbate and gallic acid (1, 2), whereas CoCl2, FeCl2 and FeCl3 inhibited the cytotoxic activity of these antioxidants (1-3). However, very few studies have dealt with the interaction between metals and cellular function, possibly due to the difficulty in accurately quantifying metal ions released into the culture supernatant and the inevitable degeneration of the medium components on contact with metals. We have recently reported that palladium (Pd)-alloy induced cytotoxicity against human cancer cell lines, without induction of internucleosomal DNA fragmentation, and that the cytotoxic activity of Pd-alloy was reduced by N-acetyl-L-cysteine (NAC) and sodium ascorbate (4). In contrast, a higher concentration of sodium ascorbate induced apoptotic cell death (characterized by internucleosomal DNA fragmentation) in human promyelocytic leukemia HL-60 cells (5), and enhanced the cytotoxic activity of Pd-alloy. ESR spectroscopy shows that Pd-alloy enhanced the intensity of ascorbate radical under slightly alkaline conditions (4). This data suggests the possible interaction between Pd-alloy and antioxidants.

To get more information about the effect of metals on the cellular function, we investigated the cytotoxicity of Cu plate, using two different methods (Methods I and II). First, the Cu plate was incubated with culture medium overnight in a 5% CO2 incubator to allow Cu ion release into the medium. This supernatant, which contained Cu ion, was then added to unstimulated or lipopolysaccharide (LPS)-stimulated mouse macrophage-like Raw 264.7 cells to measure its effects on the cellular proliferation and nitric oxide (NO) production (Method I). Secondly, HL-60 cells in suspension were incubated with culture medium which contained Cu plate, and then cell proliferation and the activity of two superoxide dismutases (SODs) (i.e., MnSOD, Cu/ZnSOD) were assayed (Method II). Using ESR spectroscopy, we also measured the radical scavenging activity of Cu plate against hydroxyl radical, NO and DPPH, which may also modify the cellular function.

Correspondence to: Naoto Kinoshita, c/o Prof. Hiroshi Sakagami, Department of Dental Pharmacology, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan. Tel: (+81) 49-279-2758, 2759, Fax: (+81) 49-285-5171, e-mail: sakagami@dent.meikai.ac.jp; Naoto13@aol.com

Key Words: Cu plate, cytotoxicity, antioxidant, radical, SOD, ESR.
Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco’s modified Eagle medium DMEM (GIBCO BRL, NY, USA); fetal bovine serum (FBS), RPMI1640 medium, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), nitro blue tetrazolium (NBT) (Sigma Chem. Co., St. Louis, MO, USA); diethylenetriaminepentaacetic acid (DETAPAC) (Wako Pure Chem. Ind., Osaka, Japan); 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 1-hydroxyl-2-oxo-3-N-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7) and 2-(4-carboxyphenol)-4,4,5,5-tetramethylimidazoline-1-oxo-3-oxide (carboxy-PTIO) (Dojin, Kumamoto, Japan). Cell culture. Raw 264.7 and HL-60 cells were cultured in DMEM and RPMI 1640 medium, respectively, supplemented with 10% heat-inactivated FBS under humidified 5% CO₂ atmosphere. Assay for cytotoxic activity. Cu metal (weight, 0.416±0.022 g, diameter, 6.098±0.005 mm; thickness, 2.225±0.206 mm; purity, 99.94%) (Hitachi Cable, Yokohama, Japan) was immersed in 70% ethanol (5 minutes) and then in 99% ethanol (5 minutes) and sonicated by waterbath type sonicator (UC-0515, Ueno Seisakusho). After evaporation of ethanol, the metal was applied to the cells by the following two methods. Near confluent Raw 264.7 cells were treated for 24 hours with 100 µL of various concentrations of the supernatant of the medium (96-microwell plate: MICROTEST™ 96, Becton Dickinson Labware, Franklin Lakes, NJ, USA), which had been pre-incubated with the Cu plate in 2 mL of DMEM+10%FBS in 24-well plate (MULTIWELL™ 24 well, Becton Dickinson) for 24 hours (Figure 1A). The medium was discarded, and the cells were washed once and further incubated for 4 hours at 37°C with 0.1 mL of fresh medium containing 0.2 mg/mL MTT. After removal of the medium, the cells were lysed with 0.1 mL of DMSO. The absorbance at 540 nm of the cell lysate, which roughly represents the relative viable cell number, was then determined (Method I, Figure 1A).
HL-60 cells (1 x 10^6/mL) were incubated for 2 hours in the culture medium (RPMI1640+10% FBS-treated) containing the Cu plate. The cells were then pelleted by centrifugation and incubated for another 2 hours in fresh medium without or with the antioxidants. Cells were pelleted and incubated for 1 hour with 0.1 mL of fresh medium containing 0.2 mg/mL MTT. After removal of the medium by centrifugation, the cells were lysed with 0.1 mL of DMSO. The absorbance at 540 nm of the cell lysate was then determined after transfer into the 96-microwell plate (Method II, Figure 1B).

**Assay for NO production.** Near confluent Raw 264.7 cells were incubated for 24 hours with various concentrations of the supernatant of the medium which had been pre-incubated with Cu plate. The extracellular concentration of NO was quantified with Griess reagent, using the standard curve of NO-2 (6).

**Assay for superoxide dismutase (SOD) activity.** Two types of SOD, MnSOD and Cu/ZnSOD, were assayed for their enzyme activities on acrylamide gel electrophoresis (4). The cells were lysed in 1% Triton X-100, 0.25 M sucrose and 10 mM Tris-HCl, pH 7.4 and used as the enzyme source. Cell lysate (equivalent to 5 x 10^5 cells) was applied to each lane of 9% polyacrylamide gel electrophoresis. After electrophoresis for 60 minutes (20 mA), the gel was stained with 4 mg/ml NBT for 15 minutes. The gel was then illuminated overnight. During illumination, the gel became uniformly blue except for the bands where MnSOD and Cu/ZnSOD proteins migrate (7). The gels were washed in water and subjected to image processing by scanner (UMAX, Astra 1200S), and analyzed on a Macintosh (Power Macintosh 7600/120) computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, USA, part number PB95-500195GEI).

**Assay for radical intensity.** Cu plate was added to 200 µL of the respective buffer system and determined in an open system as described below. The radical intensity of the test sample was determined with flat cell at 25°C, using ESR spectroscopy (JEOL JES RE1X, X-band, 100 kHz modulation frequency). Instrument settings: center field, 336.0±5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT; gain, 500; time constant, 0.1 seconds; scanning time, 2 minutes. The radical intensity was defined as the ratio of peak height of these radicals to that of MnO (8).

For the determination of hydroxyl radical produced by the Fenton reaction (total volume: 200 µL)[(1 mM FeSO_4 + 0.02 mM DETAPAC) 50 µL, 1% DMPO 20 µL, sample 50 µL, 1 mM H_2O_2 30 µL, PB 50 µL], the microwave power, gain and scanning time were changed to 5 mW, 320 and 2 minutes, respectively. The radical intensity was determined with a flat cell 40 seconds after mixing.

For the determination of NO radical, sample was added to the reaction mixture of 20 µM carboxy-PTIO and 50 µM NOC-7 in 0.06 M phosphate buffer, pH 7.4. The microwave power and gain were changed to 5 mW and 250, respectively. The NO radical intensity was determined by a flat cell, and defined as the ratio of peak...
height of the 1st peak of carboxy-PTI (indicated by arrows in Figure 5B), which was produced by the reaction of NO (derived from NOC-7) and carboxy-PTIO, to that of MnO (6).

For the determination of DPPH radical, DPPH dissolved in ethanol (100 μM) was mixed with various concentrations of sample. After 40 seconds, the mixture was subjected to ESR spectroscopy (9).

Results

Effect of culture supernatant of Cu plate on Raw264.7 cells.
The culture supernatant of Cu plate was prepared by preincubating the Cu plate in DMEM supplemented with 10% FBS, and collected by centrifugation. When mouse macrophage-like Raw 264.7 cells were treated with this supernatant, their proliferation (determined by MTT method) slightly, but dose-dependently, declined (Figure 1A). The supernatant alone did not induce any detectable amount of NO production by Raw264.7 cells (Figure 2). On the other hand, addition of LPS potently induced the NO production (25 μM) by Raw 264.7 cells. The addition of the supernatant of Cu plate slightly, but dose-dependently, diminished the LPS-stimulated NO production (Figure 2). Since the decline of NO production by the supernatant and that of proliferation were superimposable with each other, the decline of NO production by Cu plate supernatant is probably due to its cytotoxicity.

Effect on HL-60 cells of direct contact with Cu plate.
We found that culture supernatant of Cu plate decreased the cell proliferation only by 30%. To enhance the effect of Cu plate, we explored the alternative method (Method II, Figure 1B). This method enabled us to evaluate the effect of Cu plate more rapidly and accurately. When HL-60 cells

Figure 4. Changes in Mn- and Cu/Zn-SOD activities during contact with Cu plate. HL-60 cells (1 x 10⁶/mL) were incubated for 2 hours without or with Cu plate [either untreated (A, B) or sandblasted (C, D)]. After centrifugation, the pelleted cells were incubated for a further 2 hours with the indicated concentrations of sodium ascorbate (VC) (A, C) or NAC (B, D). Mn- and Cu/Zn-SOD were then extracted from the cells and separated on polyacrylamide gel electrophoresis, and their activity was determined. Each bar represents mean±S.D. from 3 independent experiments.
were directly exposed to Cu plate-containing medium, the cell proliferation rapidly declined. The effect of Cu plate was slightly enhanced by sandblast treatment of the Cu plate (compare A and C, B and D in Figure 3). In the presence of antioxidants, either sodium ascorbate (vitamin C) or NAC, the cell damaging effect of Cu plate was significantly reduced. The protective effect of ascorbate (Figure 3A, C) was more potent than NAC (Figure 3B, D).

Contact with Cu plate led to the decline of MnSOD and Cu/ZnSOD (Figure 4). MnSOD was more sensitive to Cu plate than Cu/ZnSOD (Figure 4). Sandblast treatment of the Cu plate further enhanced its inhibitory effect on MnSOD activity, but not Cu/ZnSOD activity (compare A and C, B and D in Figure 4). The decline of these two enzyme activities returned to, or even exceeded, the control level on addition of sodium ascorbate, regardless of sandblast treatment of Cu plate (compare with A and C) (Figure 4). On the contrary, NAC failed to reanimate the SOD activity, without or with sandblast treatment (compare B and D) (Figure 4).

Figure 5. Effect of Cu plate on the intensity of hydroxyl (A), NO (B) and DPPH (C) radicals. The reaction mixtures were incubated for the indicated times and applied to ESR spectroscopy, and the intensity of DMPO-OH (A), NO (B) and DPPH (C) radical was expressed as % of initial. The 1st peak of carboxy-PTI, produced by the reaction of NO (derived from NOC-7) and carboxy-PTIO, is indicated by arrow (B). The ESR spectra at 0 (initial) and 30 minutes after the start of incubation with the reaction mixtures for the assay of radical scavenging are shown.
Effect of radical intensity. ESR spectroscopy showed (Figure 5) that contact with Cu plate slightly diminished the hydroxyl radical (generated by Fenton reaction) (A), without affecting the intensity of NO (produced from NOC-7) (B) and DPPH radical (C) during a 30-minute incubation period.

Discussion

We found that Cu plate supernatant was cytotoxic against Raw 264.7 cells and inhibited the NO production by LPS-stimulated Raw 264.7 cells. However, it was difficult to accurately determine the concentration of Cu ion released into the culture medium, and to prevent the denaturing of medium components. The decline of viable cell number may be derived from the direct cytotoxic action of Cu plate, the indirect effect of Cu-induced oxidation of medium components, or both of these possibilities. Therefore, we explored the Method II, which allowed us to investigate the effect of direct contact with a metal surface on cellular function in a much shorter time (4 hours). We found that contact with Cu plate caused a rapid decline in the cell proliferation. At present, it is unclear whether the cytotoxicity of Cu plate is derived from the direct contact between the cell and the Cu plate, or from the contact of the cells with the concentrated Cu ion released near the surface of the Cu plate. The proliferation-inhibitory effect of Cu plate was counteracted with antioxidants such as sodium ascorbate. The cytotoxic effect of Cu plate may be derived from its oxidation potential, since (i) mitochondrial MnSOD was more sensitive to Cu plate than cytoplasmic Cu/ZnSOD, and (ii) the declined proliferation and both MnSOD and Cu/ZnSOD activities were reactivated by sodium ascorbate, a popular antioxidant. It was unexpected that, although NAC recovered the cellular proliferation, it failed to reactivate the SOD activity. This suggests that NAC may exert its protective effect by blocking the SH groups in the proteins. Thus, sodium ascorbate and NAC may protect the cells from the Cu-induced injury via different mechanisms. We found that Cu plate has essentially no radical scavenging activity against hydroxyl radical, NO and DPPH radicals (Figure 5), suggesting that the radical scavenging activity of Cu plate is not a major factor to determine the cytotoxic activity of Cu. The slight decline of hydroxyl radical intensity by Cu plate may be due to the interaction of Cu and components of the assay buffer for the radical scavenging activity.

Many studies have been performed on the harmful effects of trace minerals such as Pd, Cu, Ti, Zn Ni, Cr and Hg, which are present in tooth crown restorative materials, using osteoblast, human dental pulp cells, human and mouse gingival fibroblast (10). The extents of the harmful effect of these metals, especially Cu and Zn, depend upon the concentration and the ratio of individual metals in the materials (11-13). Ti has been widely utilized for dental implants and has shown its osteo-integration and biocompatibility activities. However, these studies also suggest that the in vivo effects of tooth crown restorative materials including Ti may be modified by the surface property of these materials (smooth or coarse) (14-22). In dental practice, the surface of the tooth crown restorative material is usually polished so as to reduce the harmful effects to a minimum (4). This practice is supported by the present finding that sandblast-treated Cu plate showed higher cytotoxicity and inhibited more potently the MnSOD activity than polished Cu plate. The use of antioxidants such as vitamin C and NAC may further reduce such unfavorable effects.

Acknowledgements

This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (Sakagami, No. 14370607).

References


Received May 18, 2004
Accepted October 19, 2004