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Biological Impact of Contact with Metals on Cells

TAKASHI YAMAZAKI[1], ATSUSHI YAMAZAKI[2], YASUSHI HIBINO[2], SHAHEAD ALI CHOWDHURY[3], YOSHIKO YOKOTE[4], YUMIKO KANDA[5], SHIRO KUNII[5], HIROSHI SAKAGAMI[6], HIROSHI NAKAJIMA[2] and JUN SHIMADA[1]

[1]Division of Oral Maxillofacial Surgery and
[6]Division of Pharmacology, Department of Diagnostic and Therapeutic Sciences,
[2]Division of Dental Biomaterials Science, Department of Restorative and Biomaterials Sciences,
[3]Meikai Pharmaco-Medical Laboratory (MPL) and [5]Labolatory of Electron Microscopy,
Meikai University School of Dentistry, Sakado, Saitama 350-0283;
[4]Faculty of Science, Josai University, Saitama 350-0295, Japan

Abstract. *In order to investigate the in vivo effect of metals used in dentistry, we investigated the effect of direct contact with metal plates (20x20x0.5 mm^3) made of gold (Au), silver (Ag), copper (Cu) or palladium (Pd) on human promyelocytic leukemic HL-60 cells grown in RPMI1640 medium supplemented with 10% fetal bovine serum. When 0.5 mL of cell suspension was applied to the metal plates, cells were precipitated on the surface of the metal plate within 10 min. Contact with Cu induced a rapid decline of cell viability, the smear pattern of DNA fragmentation, and only minor activation of caspase-3. These effects were accompanied by a progressive decrease in the extracellular concentration of methionine, cysteine and histidine, with a corresponding increase in the concentration of methionine sulfoxide. Electron microscopy showed that contact with Cu induced vacuolization and cytoplasmic damage, prior to nuclear damage, without affecting the cell surface microvilli or mitochondrial integrity. Contact with the other metals did not induce such changes during the 3 h incubation, nor was any hormetic response (beneficial action at lower concentration) observed in the cells with any metals. Addition of N-acetyl-L-cysteine (4-5 mM) almost completely abrogated the Cu-induced cytotoxicity, whereas sodium ascorbate (0.1-0.5 mM) and catalase (6,000[1]-30,000 units/mL) were ineffective. Numerous serum proteins were adsorbed to the Ag plate, while bovine serum albumin was the major protein adsorbed to other metal plates. The present study suggests that direct contact with Cu induced non-apoptotic cell death by an oxidation-involved mechanism. The present model system may be applicable to the study of the interaction between cells and dental restorative materials.*

Correspondence to: Hiroshi Sakagami, Division of Pharmacology, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan. Tel: (+81) 49 285 5511, ex 336, 429, 690, Fax: (+81) 49 285 5171, e-mail: sakagami@dent.meikai.ac.jp

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Metals, such as gold (Au), silver (Ag), copper (Cu) and palladium (Pd) are important components of alloys used as restorative materials in dentistry. Dental alloys with low ionization tendency, or with passive film on their surface are clinically used. However, these alloys have been reported to induce allergic reactions in the oral cavity, though infrequently (1), which may be related to the stimulated release of metal ions from the alloys under acidic oral environments produced by inflammation, bacterial infection, and intake of soft drinks and coffee (2, 3). These metal ions may then be incorporated into the cells, possibly *via* metal transporter-mediated endocytosis (4, 5), but whether the target molecules are protein or DNA is unclear. Numerous studies have shown cytotoxic activity and tissue-damaging activity of metal extracts (6-8). However, no detailed study has been done using direct contact with metals. This urged us to construct an assay to investigate the interaction between metals and the oral environment. We investigated the effect of direct contact with four metal plates (Au, Ag, Cu, Pd) on the function of the human promyelocytic leukemic cell line HL-60, with a multi-potentiality to undergo either monocytic or granulocytic differentiation, apoptotic or autophagic cell death, depending on the types of inducers used. It has been reported that various toxicants, environmental hormones, radiations, and even lasers affect the cellular proliferation and functions in a bimodal fashion (so-called "hormesis"): a stimulatory (beneficial) effect at lower concentration and a cytotoxic (adverse) action at higher concentrations (9). We, therefore, investigated whether these metals induce similar bimodal effects on HL-60 cell growth.

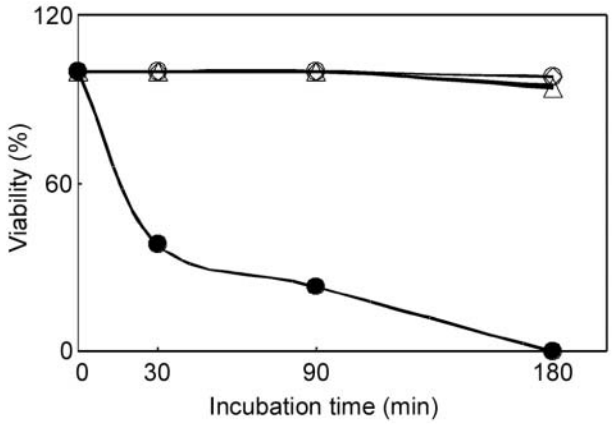

Figure 1. *Effect of contact with metal plates on cell viability. HL-60 cells (2x10^6/mL, 0.5 mL) were inoculated onto a plastic dish (control), or an Au (□), Ag (△), Cu (●) or Pd (◇) plate in the dish, and the viable cell number was determined at the times indicated thereafter, and expressed as % of control.*

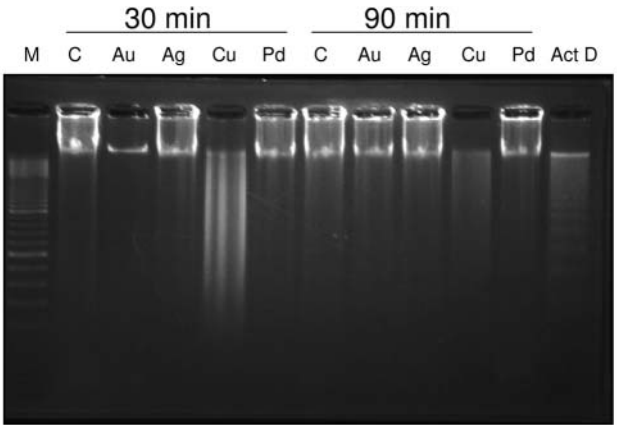

Figure 2. *Effect of contact with metal plates on the induction of DNA fragmentation. HL-60 cells were incubated for 30 or 90 minutes on a plastic dish (control), or the indicated metal plate. DNA was then extracted and applied to agarose gel electrophoresis. M, DNA marker; C, control; Act.D, actinomycin D (1 μg/mL).*

Since the adsorption of proteins to metal surfaces may affect the release of metallic ions from that surface and cellular adsorption to it (10), the binding of serum proteins to the metal surface, in comparison with the cytotoxicity of the metal was also investigated.

Materials and Methods

Materials. The following metals, chemicals and reagents were obtained from the indicated companies: Au, Ag, Cu, Pd plate (99.99%, 20x20x0.5 mm) (Tokuriki Honten, Co, Japan); fetal boving serum (FBS), RPMI1640 medium, actinomycin D (Sigma Chem. Co., St. Louis, MO, USA); trichloroacetic acid (TCA) (Wako Pure Chem Co., Tokyo, Japan); marker proteins for SDS-PAGE (M1: Cat. no. 161-0318; M2: Cat. no. 161-0324, BIO-RAD, Hercules, CA, USA).

Polishing of metal plate surface. All metal plates were polished using alumina-water slurry (micropolish, Buehler) (0.05 μm particle size). After polishing, surfaces were examined using scanning electron microscope (JSM-6360LV, JEOL, Japan) to confirm consistency of surface smoothness.

Cell culture. Human promyelocytic leukemic HL-60 cells were cultured in RPMI1640 supplemented with 10% heat-inactivated FBS under humidified 5% CO_2 atmosphere.

Cytotoxicity from direct contact with metal plate. Five hundred μL of HL-60 cells (2x10^6/mL) in RPMI1640+10% FBS were inoculated on the metal (placed in 3.5 cm dish) and incubated for 30, 90 and 180 min at 37˚C in 5% CO_2. Cells were recovered from the metal plates by gentle pipetting and washing with a total of 0.5 mL of phosphate-buffered saline without calcium and magnesium (PBS(–)). The viability of the cells were determined by cell counting with a hemocytometer after staining with 0.15% trypan blue dye. Cells were collected by centrifugation for DNA fragmentation assay,

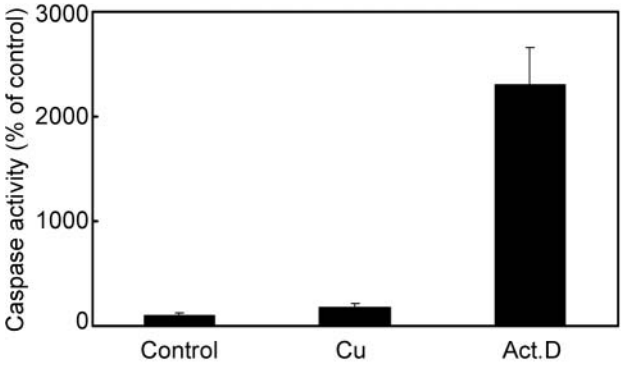

Figure 3. *Effect of contact with Cu plate on caspase-3 activity. HL-60 cells were incubated for 30 minutes in a plastic dish (control) or on a Cu plate, or for 6 h in plastic dish with 1 μg/mL actinomycin D, and the caspase-3 activity was determined by a substrate cleavage assay. Each point represents mean±S.D. from triplicate determinations.*

the determination of caspase activity and the observation of fine structure under electron microscopy. The supernatant was used for amino acid analysis. The serum proteins adsorbed on the metal surface were recovered using SDS sample buffer and then analyzed using SDS polyacrylamide gel electrophoresis.

Assay for DNA fragmentation. Cells were washed once with PBS(–) and lysed with 50 μL lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroyl-sarcosinate solution]. The solution was incubated with 0.4 mg/mL RNase A and 0.8 mg/mL proteinase K for 1-2 h at 50˚C. The lysate was mixed with 50 μL of NaI solution [7.6 M NaI, 20 mM EDTA-2Na, 40 mM Tris-HCl, pH 8.0], and then 100 μL of ethanol, and centrifuged for 20 min, at 20,000 xg. The precipitate was washed with 1 mL of 70% ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

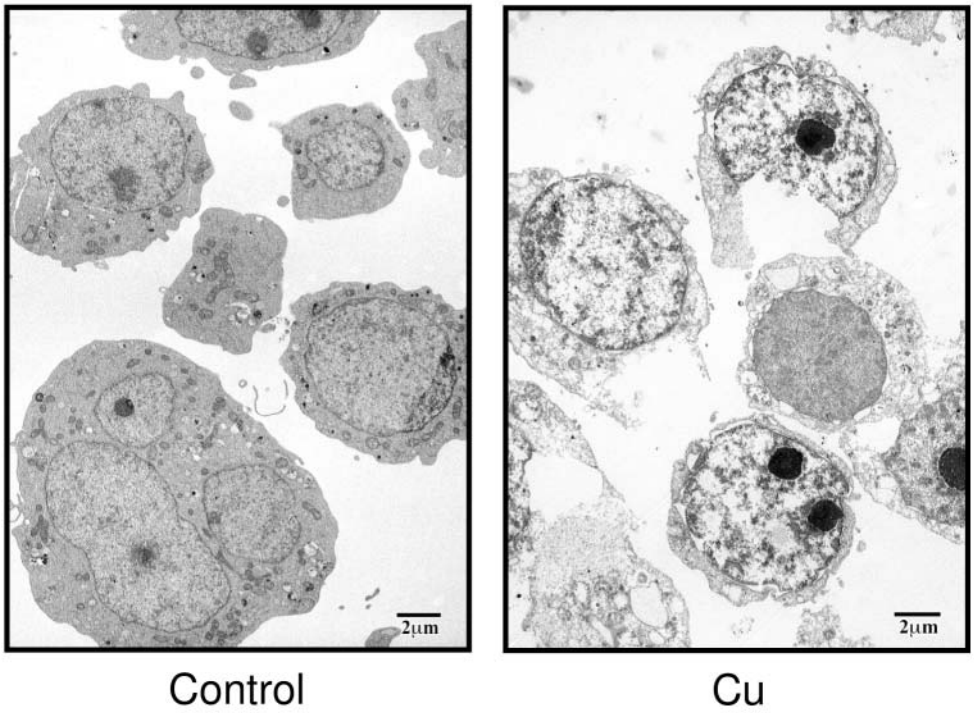

Figure 4. *Effect of contact with metal plates on the fine structures of cells. HL-60 cells were incubated for 30 min on a plastic dish (control) or on a Cu plate and examined under transmission electron microscopy.*

Samples (10-20 μL) were subjected to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). DNA marker (Bayou Biolabs, Harahan, LA, USA) and DNA from apoptotic cells induced by 1 μg/mL actinomycin D were run in parallel as a positive control. After staining with ethidium bromide, DNA was visualized using UV irradiation and photographed with a CCD camera (Bio Doc Inc., UVP, Upland, CA, USA) (11).

Assay for caspase-3 activity. Cells were washed with PBS(–) and lysed in lysis solution (MBL, Nagoya, Japan). After standing for 10 min on ice, the cells were centrifuged for 20 min at 15,000 xg and the supernatant was collected. Lysate (50 μL, equivalent to 100 μg protein) was mixed with 50 μL 2 x reaction buffer (MBL) containing a substrate for caspase-3 (DEVD-*p*NA (*p*-nitroanilide)). After incubation for 4 h at 37˚C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured using a microplate reader (Multiskan, Biochromatic, Labsystem, Osaka, Japan) (11).

Determination of free amino acids. Culture supernatant (medium fraction) of control and treated cells was mixed with an equal evolume of 10% TCA and stood on ice for 30 min. After centrifugation for 5 min at 10,000 xg, the deproteinized supernatant was collected and stored at –40˚C. The supernatants (20 μL) were analyzed with an LC-300 amino acid analyzer (JEOL) and amino acids were detected using the ninhydrin reaction. The concentration of each amino acid at 30, 90 or 180 min after contact with each metal plate was expressed as % of control (without metal plate). The concentration of each amino acid in DMEM + 10% FBS is described in our previous papers (12, 13).

Electron microscopy. Cells were washed twice with PBS(–), fixed for 1 h with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4˚C, dehydrated, and then embedded in Araldite 502 (CIBA-GEIGY, Basel, Switzerland; NISSHIN EN Co., Ltd., Tokyo, Japan). Fine sections were stained with uranyl acetate and lead citrate, and then observed under a JEM-1210 transmission electron microscope (JEOL) at an accelerating voltage of 100 kV (14).

Assay for protein adsorption. The proteins were recovered from the metal plates using SDS sample buffer (0.05 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS-0.005% bromophenol blue, 0.6% 2-β mercaptoethanol), boiled and applied to the 12% polyacrylamide gel electrophoresis (11). After electrophoresis, the gels were stained with Coomassie brilliant blue, de-stained and photographed.

Results

Effects on cell viability. First, the sedimentation rate of HL-60 cells was determined. HL-60 cells were resuspended at 10^8/mL in PBS(–) and then applied to the top of a 10-cm layer of culture medium in a 15-mL centrifugation tube. The cells were found to be sedimented at a speed of 0.042 cm/min (data not shown). We inoculated 0.5 mL of HL-60 cell suspension ($2x10^6$ cells/mL) onto the metal plates (2 x 2 cm^2), making the approximate height of the cell suspension 0.125 cm (0.5 cm^3 / 2 x 2 cm^2). We estimate that the time required for all the cells to reach the metal surface should

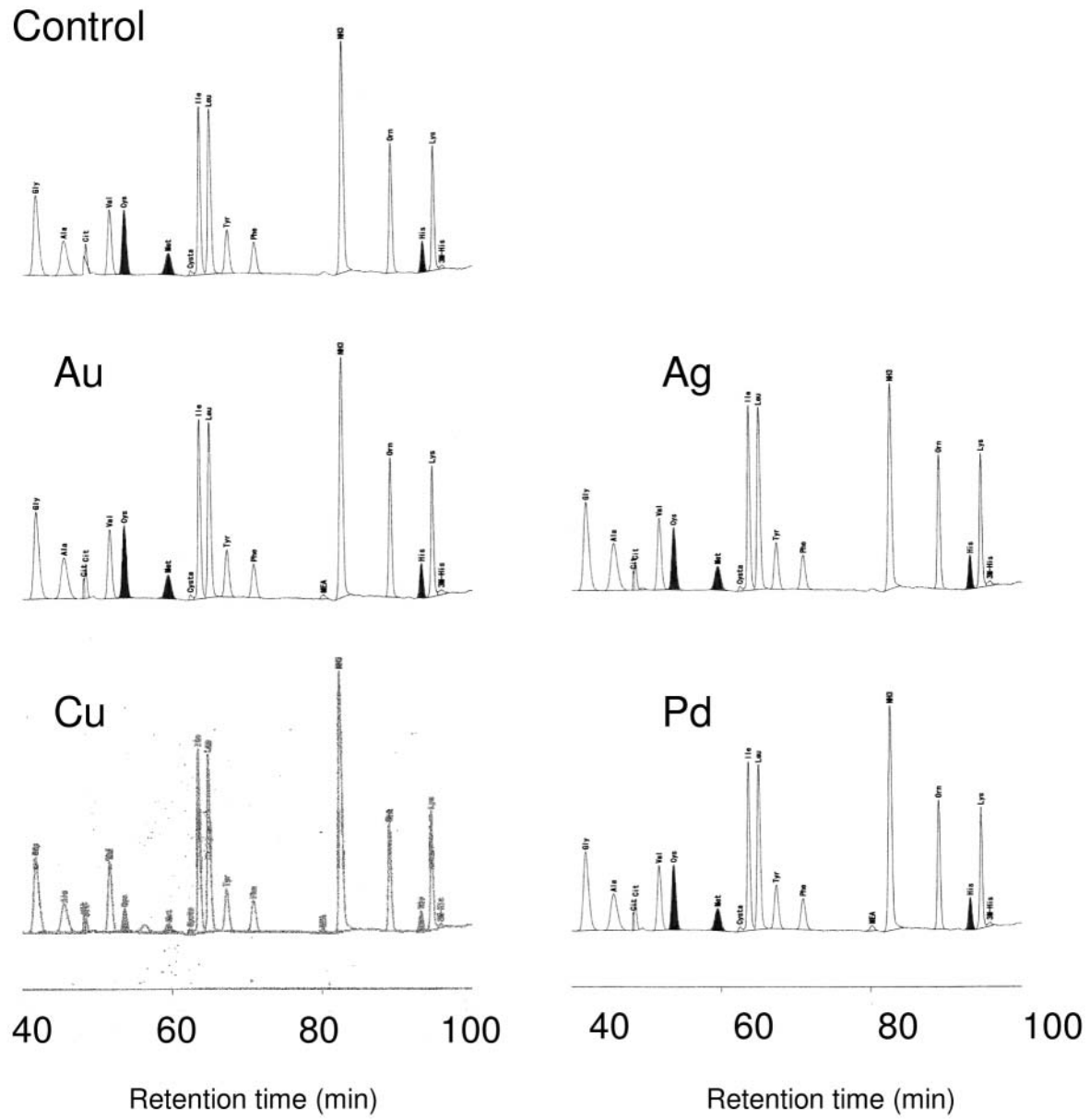

Figure 5. *Effect of contact with metal plates on the extracellular amino acid composition of HL-60 cells. HL-60 cells were incubated for 3 h on a plastic dish (control) or an Au, Ag, Cu or Pd plate. The TCA-soluble fraction of the cell culture medium was then applied to an amino acid analyzer. The ordinate represents the arbitrary unit of concentration of each amino acid. Cysteine (retention time = 52 min), methionine (retention time = 59 min) and histidine (retention time = 92 min) are indicated by blackened peaks.*

be approximately 3 min ([0.125 cm] / [0.042 cm/min]=2.98 min). Figure 1 shows that contact with the Cu plate resulted in the rapid decline of cell viability. Nearly all cells died within 30 min, as judged by the trypan blue staining. On the other hand, contact with other plates (Au, Ag, Pd) did not significantly affect the viability of the cells, at least over the time period studied (Figure 1). The recovery of the cells was nearly 100% in all plates.

Induction of non-apoptotic cell death. Figure 2 shows that contact with Cu plate produced a smear pattern of DNA fragmentation in HL-60 cells, in contrast to the internucleosomal DNA fragmentation induced by actinomycin D. Other metals produced neither a smear pattern nor internucleosomal DNA fragmentation (Figure 2). Contact with Cu increased the caspase-3 activity to a much lesser extent (only 2-fold), compared to that observed

Table I. *Effect of contact with metal plates on the concentration of amino acids in the culture medium after 30, 90 or 180 min incubation.*

	Concentration (% of control)											
	Au			Ag			Cu			Pd		
	30	90	180	30	90	180	30	90	180	30	90	180
Nonpolar												
Leu	98	98	98	95	102	112	99	103	110	92	102	98
lle	98	96	97	94	97	109	99	104	113	93	101	97
Gly	103	95	103	112	108	120	101	99	98	96	100	106
Val	99	97	98	97	103	113	99	103	105	95	100	100
Cys	98	96	102	94	97	98	**71**	**48**	**35**	94	98	99
Ala	105	103	ND	105	128	ND	103	91	ND	102	101	ND
Met	99	103	100	100	101	110	**85**	**62**	**42**	93	106	99
Phe	99	96	98	97	103	112	98	98	96	93	99	99
Polar, neutral												
Gln	100	99	95	97	104	113	100	105	111	93	101	97
Asn	97	97	97	97	104	104	98	98	92	93	99	99
Ser	107	100	ND	103	108	ND	102	112	ND	95	99	ND
Thr	97	96	ND	96	104	ND	100	112	ND	92	98	ND
Tyr	98	94	98	98	102	109	97	95	99	94	99	100
Polar, basic												
Arg	98	97	100	96	102	107	97	96	92	95	100	100
Lys	102	101	98	95	101	111	100	100	95	96	97	98
His	100	93	98	97	100	109	**93**	**84**	**63**	96	99	99
Glu	92	93	96	112	103	110	105	99	116	91	99	97

ND: not determined.

with the actinomycin D treatment (23-fold) relative to the control (Figure 3). Electron microscopy showed that contact with Cu induced vacuolization and cytoplasmic damage, prior to nuclear damage, without affecting the cell surface microvilli or mitochondrial integrity (Figure 4).

Link to oxidation reaction. Amino acid analysis of culture medium using an amino acid analyzer demonstrated that contact with Cu for 3 h resulted in the decline of the extracellular concentration of methionine (retention time = 59 min), cysteine (retention time = 52 min) and histidine (retention time = 92 min), and a corresponding increase in methionine sulfoxide (not shown) and an unknown substance (retention time = 56 min) (Figure 5). The decline of methionine, cysteine and histidine was time-dependent, whereas other amino acids did not change during the 3-h incubation (Table I). Contact with other metals did not induce any apparent changes in the concentration of any amino acids, including cysteine, methionine and histidine. Antioxidants such as *N*-acetyl-L-cysteine (NAC) (4-5 mM) significantly protected cells from the Cu-induced injury, whereas sodium ascorbate (0.1-0.5 mM) and catalase (6,000-30,000 units/mL) were ineffective (Table II).

Table II. *Effect of various antioxidants on Cu-induced cytotoxicity.*

Treatment	Viability (%)
None (Control)	100
Cu Plate	0
Cu Plate+NAC (4～5 mM)	71
Cu Plate+Sodium ascorbate (0.1-0.5 mM)	0
Cu Plate+Catalase (6,000-30,000 units/mL)	0

HL-60 cells were incubated for 30 min on a plastic dish without any antioxidant (control), or on a Cu plate, in the absence or presence of the indicated antioxidants, and the viable cell number was determined and expressed as % of control. Each value represents mean of triplicate determinations.

Adsorption of serum proteins to metal plates. Bovine serum albumin was the major protein adsorbed to Au, Cu and Pd plates, whereas numerous proteins were adsorbed to the Ag plate (Figure 6).

Discussion

The present study demonstrates that direct contact with a Cu plate induced non-apoptotic cell death in HL-60 cells.

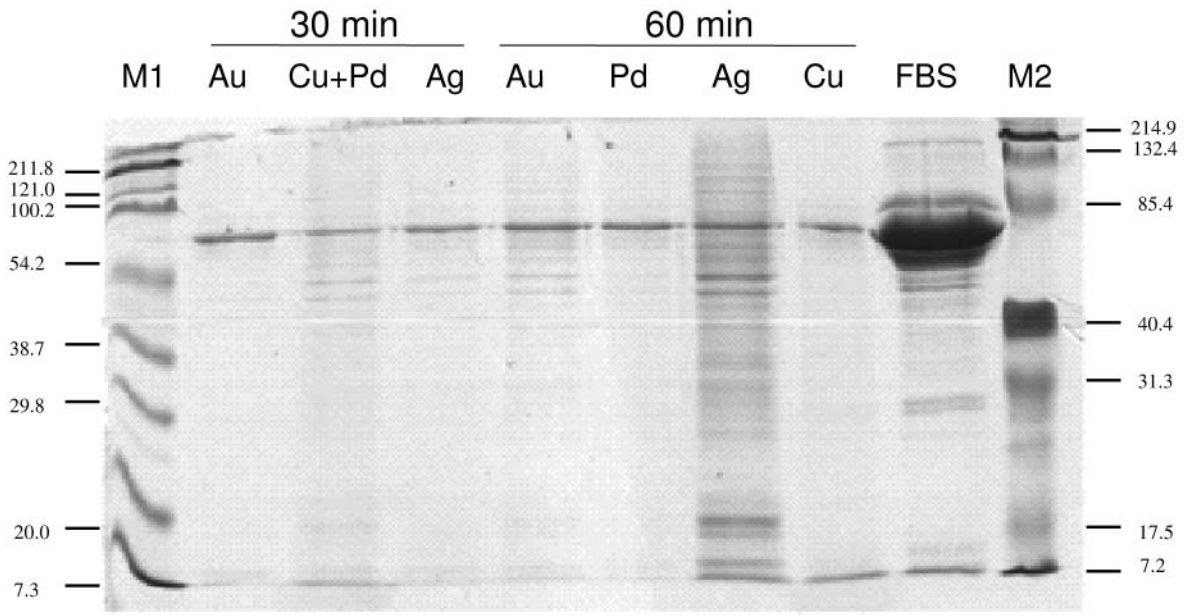

Figure 6. *Protein adsorption on metal plates. Cu+Pd indicates the combined proteins adsorbed onto both Cu and Pd plates. M1, M2: marker proteins with the molecular weight (kDa) indicated at both sides.*

This conclusion is based on the following evidences: (i) contact with Cu induced the loss of the membrane barrier (assessed by permeability of trypan blue stain) (Figure 1) and cytotoplasmic damage prior to nuclear damage (Figure 4); (ii) it induced little or no apoptosis-associated characteristics (*i.e.*, internucleosomal DNA fragmentation (Figure 2), increased caspase-3 activity (Figure 3), or loss of cell surface microvilli (15, 16) (Figure 4); (iii) it induced cell death rapidly (within 30 min), as compared with the apoptotic process that requires several hours for the activation of caspases and DNase (17); and, (iv) it induced the vacuolization (Figure 4), a marker of autophagy, a type II programmed cell death (18). At present, whether contact with a Cu plate induces autophagy is not conclusive. Further investigations of the expression of autophagy-specific markers, such as LC3 expression in autophagosome (19) and the effect of autophagy inhibitors are necessary to confirm this possibility.

The present study suggests that oxidation stress may be involved in the induction of cell death on contact with Cu. This is supported by our findings that: (i) the extracellular concentration of cysteine, methionine and histidine, known as easily oxidizable radical scavengers (20, 21), declined, while that of methionine sulfoxide (an oxidation product of methionine) was increased (Figure 5, Table I), and (ii) the cell death induced by contact with Cu was efficiently inhibited by NAC, a popular antioxidant (Table II). Two approaches are considered to reduce the cytotoxicity of Cu. The first approach is to add the protective substances. To achieve this, various antioxidants, such as saliva components and polyphenols (such as tannins, flavonoids and lignin), present in huge quantities in nature should be considered for their protective activity against Cu-induced cytotoxicity. Another approach would be to manufacture an alloy that has minimal amounts of Cu. For this second approach, we are planning to use human oral cells (such as gingival fibroblasts and periodontal ligament fibroblasts) as target cells, instead of HL-60 cells, to obtain greater insight into the interaction between dental alloys and the oral environment.

We did not observe the hormetic response of cells to any of the metals during the 3 h incubation. The cytotoxic action of Cu was rapid and an investigation of the possible biochemical changes occurring within 30 min contact with the Cu plate is needed. Whilst contact with the other metals (Au, Ag, Pd) was noncytotoxic during the 3 h incubation, however, the question remains, whether longer incubation with these metals induces cytotoxicity.

We found that serum albumin binds to Au, Cu and Pd plates (Figure 6). Whether the binding of serum proteins on the Cu plate modifies the cytotoxicity of Cu remains to be investigated. At present, it is not clear whether Cu acts from outside the cells (by an oxidation-involved mechanism) or from the inside of the cells by binding to cellular components. If the second possibility is correct, whether Cu enters the cells *via* the specialized transport system and attacks the DNA or modifies the gene expression should be investigated.

As far as we know, this is the first report on the biological impact caused by direct contact with a metal plate. The present system may be applicable for the study of the interaction between cells and various dental restorative materials in future.

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