in vivo *21*: 835-840 (2007)

Rapid Changes in Amino Acid and Polyamine Metabolism during Copper-induced Cell Death of Human Gingival Fibroblast

HIROSHI SAKAGAMI[1], TAKASHI YAMAZAKI[2], HIROYUKI ONUKI[2], ATSUSHI YAMAZAKI[3], YASUSHI HIBINO[3], KEN HASHIMOTO[1], YUMIKO KANDA[4], SHIRO KUNII[4], YOSHIKO YOKOTE[5], HIROSHI NAKAJIMA[3] and JUN SHIMADA[2]

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

Abstract. *There are very few studies on the interaction between dental alloys and oral tissues. The effect of direct contact with copper (Cu) on the cellular function of human gingival fibroblast (HGF) derived from the periodontal tissues was investigated. When HGF cells were inoculated onto a Cu plate, the viability of HGF cells immediately declined. This was accompanied by vacuolization and chromatin condensation near the nuclear membrane. The intracellular concentration of spermidine and spermine declined, whereas that of putrescine slightly increased. Amino acid analysis of the medium revealed that glutamine was consumed at the greatest rate, amounting to more than half of the total amino acid consumption. Contact with the Cu plate resulted in the complete elimination of glutamine utilization and a simultaneous increase in the production of most amino acids, possibly due to enhanced proteolysis. This was accompanied by a time-dependent increase in the consumption of cystine, possibly due to oxidative reactions, and the enhanced production of glycine and glutamic acid. These data suggest that the contact with the Cu plate induced non-apoptotic cell death in HGF cells, which was tightly coupled with a rapid dysfunction of amino acid and polyamine metabolism.*

Dental alloys have been reported to occasionally induce allergic reactions in the oral cavity (1) by the metal ions released from the alloys (2, 3). The metal ions may be incorporated into the cells, possibly *via* metal transporter-mediated endocytosis (4, 5). Numerous studies have shown cytotoxic activity and tissue-damaging activity of metal extracts (6-8). However, the biological impact of contact with metals is unclear. We recently developed an assay system to investigate the cytotoxicity induced in cultured cells by direct contact with metals (9). Using this system, we found that direct contact with a copper (Cu) plate induced non-apoptotic cell death, characterized by a smear pattern of DNA fragmentation and minor caspase activation, and vacuolization in a human promyelocytic leukemic cell line (HL-60), whereas other metals such as gold, silver and palladium were essentially inactive (9). Here, whether direct contact with Cu has a similar impact on cultured human gingival fibroblasts (HGF) derived from periodontal tissues was investigated.

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 2792758, Fax: +81 49 2855171, e-mail: sakagami@dent.meikai.ac.jp

Key Words: Copper plate, gingival fibroblast, cytotoxicity, vacuolization, glutamine, amino acid, polyamine.

Materials and Methods

Materials. The following materials were obtained from the companies indicated: Cu (99.99%, 20x20x0.5 mm) (Tokuriki Honten, Co, Japan); Dulbeccos's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS), RPMI-1640 medium, actinomycin D (Sigma Chem. Co., St. Louis, MO, USA); trichloroacetic acid (TCA) (Wako Pure Chem Co., Tokyo, Japan).

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

Cell culture. HGF cells were prepared from periodontal tissues according to the guideline of the Meikai University Ethics Committee (No. 0206), after obtaining informed consent from the patients, and cultured in DMEM supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere. HGF cells were harvested by detaching them from the culture plate with

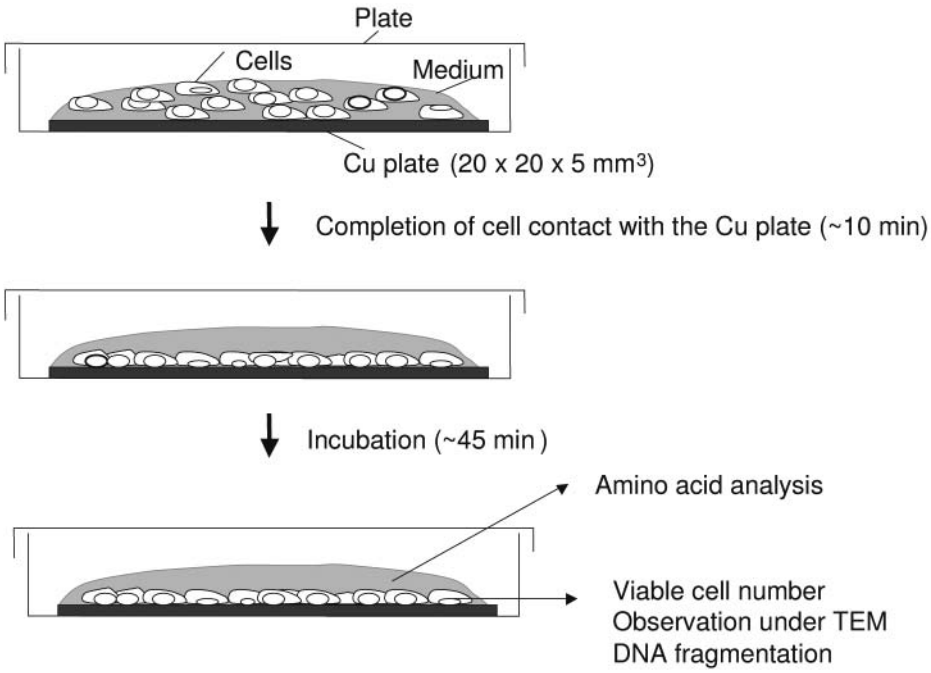

Figure 1. *Experimental scheme.*

0.25% trypsin-0.025% EDTA in phosphate-buffered saline (PBS) without Mg or Ca (PBS(–)) and subcultured at a 1:4 split ratio once a week, with one medium change in between. Since HGF cells have a limited lifespan, ceasing proliferation at a 20 population doubling level (PDL) (10), the cells at 9-12 PDL were used in the present study. Human promyelocytic leukemic HL-60 cells (supplied by Prof. Nakaya, Showa University) were cultured in RPMI-1640 medium supplemented with 10% FBS (11).

Cytotoxicity induced by direct contact with a Cu plate. HGF cells were trypsinized and resuspended at cell density of $2x10^6$/mL in fresh culture medium. Five hundred μL of HGF cells ($1x10^6$) were inoculated onto the Cu plate (placed in a 3.5 cm dish) and incubated for 15, 30 or 45 min at 37˚C in 5% CO_2 (Figure 1). Cells were recovered from the Cu plate by gentle pipetting and washing with a total of 0.5 mL of PBS(–). The viable cell number was determined by cell counting with a hemocytometer after staining with 0.15% trypan blue dye and correction for the dilution factor of 4 (due to PBS washing and trypan blue addition). Cells were collected by centrifugation for DNA fragmentation assay and fine structure observation under electron microscopy. The supernatant was used for amino acid analysis.

Assay for DNA fragmentation. Cells were washed once with PBS(–), lysed, digested with RNase A and proteinase K, and DNA was then prepared and applied to 2% agarose gel electrophoresis, as described elsewhere (11). DNA marker (Bayou Biolabs, Harahan, LA, USA) and DNA from apoptotic HL-60 cells induced by UV irradiation (12) 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).

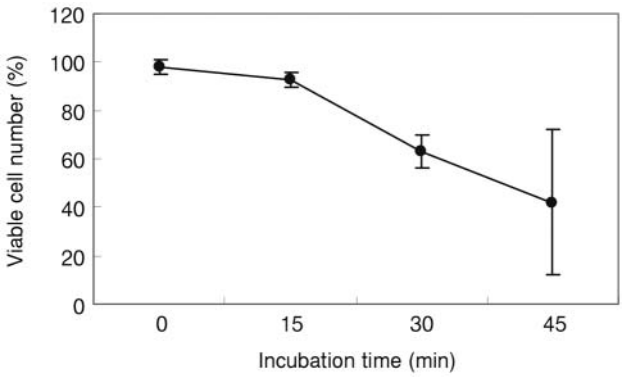

Figure 2. *Effect of contact with a Cu plate on cell viability. HGF cells ($2x10^6$/mL, 0.5 mL) were inoculated onto a Cu plate in a dish and the viable cell number was determined at the indicated times thereafter, expressed as % of the initial cell number. Each value represents mean±S.D. of three independent experiments.*

Electron microscopy. Cells were washed twice with PBS(–), fixed for 1 hour with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4˚C, post fixed for 1 h with 1% osmium tetraoxide-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 JEOL-1210 transmission electron microscope at an accelerating voltage of 100 kV (9).

Determination of polyamines. The cells were harvested by trypsinization, washed twice with PBS(–) and extracted with 10%

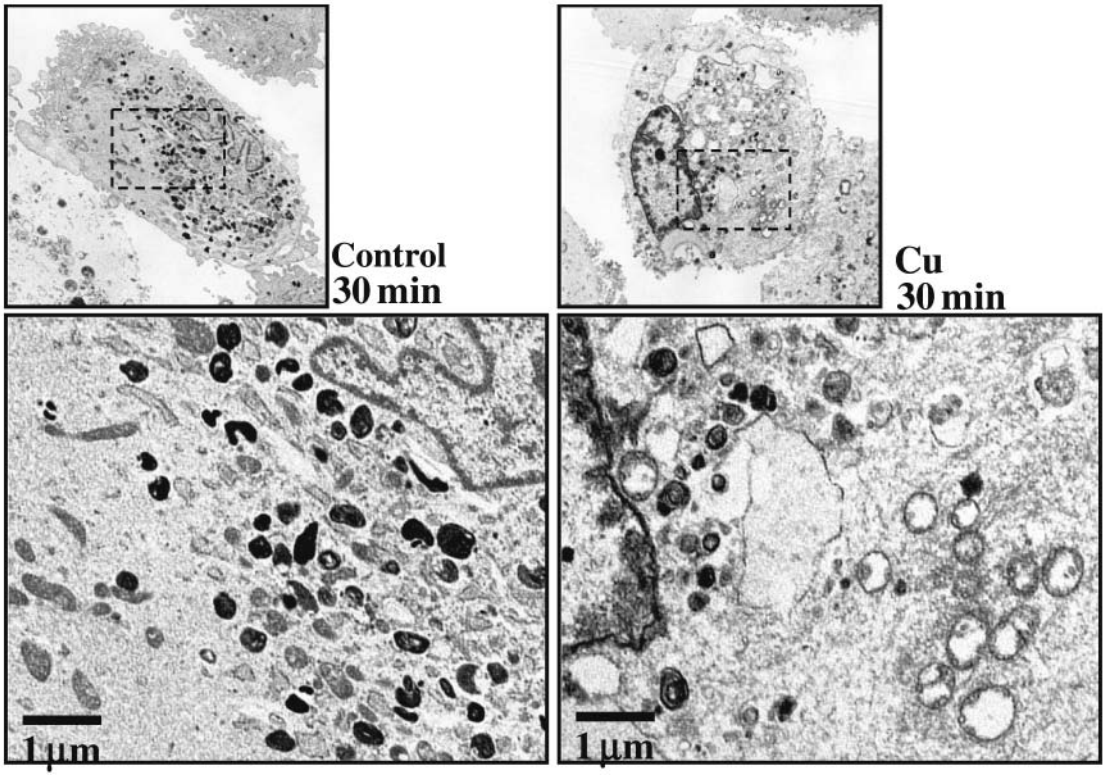

Figure 3. *Effect of contact with a Cu plate on the fine structure of cells. HGF cells were incubated for 30 min on a plastic dish (control) or on a Cu plate and collected by gentle pipetting so as not to damage the fine structure of the cells and examined under transmission electron microscopy. The areas surrounded by dotted lines are magnified. Scale bar: 1 μm.*

TCA. After centrifugation for 5 minutes at 10,000 xg, the deproteinized supernatant was collected and stored at –40˚C. The polyamines (putrescine $NH_2(CH_2)_4NH_2$, spermidine $NH_2(CH_2)_4NH(CH_2)_3NH_2$, and spermine $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$) in the supernatant were determined after dansylation using an HPLC system (SHISEIDO CAPCELL PAK C_{18}), detected by excitation at 337 nm and emission at 521 nm), as described elsewhere (13).

Determination of free amino acids. Culture supernatant (medium fraction) was mixed with an equal volume 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 subject to a JEOL-JLC500/V amino acid analyzer and amino acids were detected using the ninhydrin reaction (9, 11). The amino acid concentration was corrected for the dilution factor of 4 (due to PBS wash and TCA addition).

Results

We previously reported that cells sediment at a speed of 0.042 cm/min and that approximately 3 min was required for all the cells to achieve contact with the metal surface (9). Contact with the Cu plate resulted in the rapid decline of cell viability (Figure 2). After 30 or 45 min, the viable

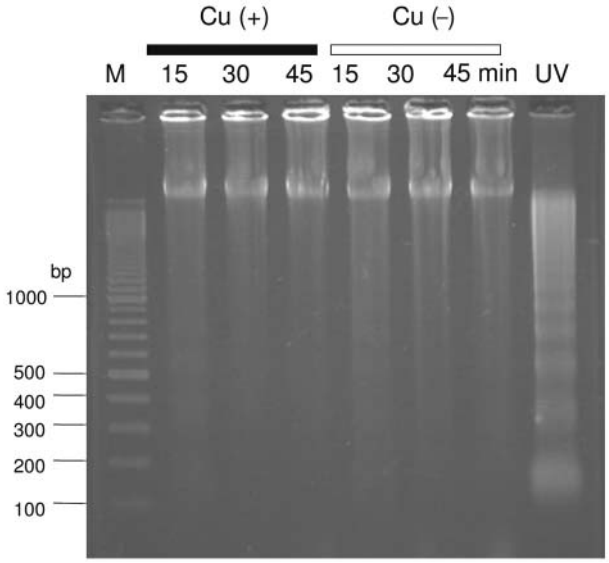

Figure 4. *Effect of contact with a Cu plate on the induction of DNA fragmentation. HGF cells were inoculated onto a Cu plate and incubated for 13, 30 or 45 min. Cells were recovered by gentle pipetting and DNA was then prepared and applied to agarose gel electrophoresis. M, DNA marker; UV, DNA of UV-irradiated HL-60 cells (positive control).*

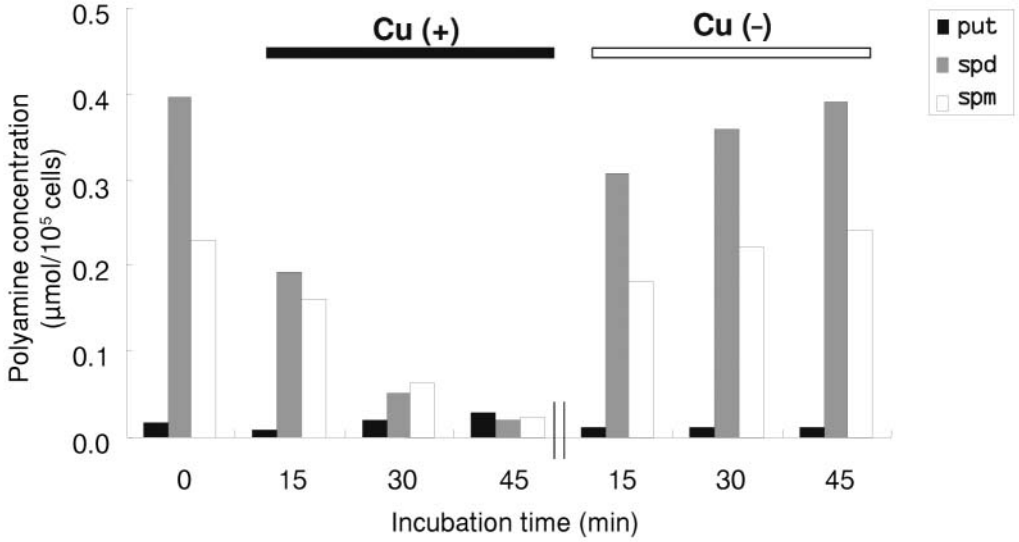

Figure 5. *Effect of contact with a Cu plate on the intracellular concentration of polyamines. HGF cells were inoculated onto a plastic (control) or Cu plate and incubated for 15, 30 or 45 min. Cells were then collected by gentle pipetting and the intracellular concentration of polyamines was then determined by HPLC. Put, putrescine; Spd, spermidine; Spm, spermine.*

cell number declined to 63% and 42% that of the control, respectively. It should be noted that HGF cells were more resistant to contact with Cu, as compared with HL-60 cells (9). Electron microscopy showed that contact with the Cu plate induced vacuolization and chromatin condensation near the nuclear membrane in HGF cells (Figure 3). Contact with the Cu plate failed to induce internucleosomal DNA fragmentation, a hallmark of apoptosis, in HGF cells (Figure 4), while resulting in the rapid decline of the intracellular concentration of spermidine and spermine, but an increase in that of putrescine (Figure 5).

Amino analysis revealed that, in the absence of a Cu plate, glutamine was consumed at the greatest rate among amino acids tested (112 nmole/mL/15 min, amounting to 57.4% of the total amino acid consumption), followed by isoleucine (9.7%), leucine (7.7%), arginine (6.2%), valine (4.1%) and serine (3.1%) (Figure 6). Consumption of lysine, histidine, tyrosine, phenylalanine, cystine, methionine and threonine was much less. On the other hand, alanine, glycine and glutamic acid were the major amino acids produced during incubation.

Contact with the Cu plate resulted in the complete elimination of glutamine utilization within 15 min and a slight increase in the production of most amino acids, possibly due to enhanced proteolysis. This was accompanied by the time-dependent increase in the consumption of cystine (21, 40 and 63 nmole/mL at 15, 30 and 45 min, respectively), possibly due to oxidative reactions. The production of glycine and glutamic acid during 45 min was increased by 53 and 68 nmole/mL, respectively, whereas the increase in alanine production was marginal (Figure 6).

Discussion

The present study demonstrated that contact with a Cu plate induced non-apoptotic cell death in HGF cells, characterized by the formation of vacuoles and lack of internucleosomal DNA fragmentation, confirming our previous finding that contact with Cu induced non-apoptotic cell death in HL-60 cells (9). There are at least three types of cell death: apoptosis (type I programmed cell death), autophagy (type II programmed cell death) and necrosis. The vacuolization and increased production of amino acids (possibly due to proteolysis in autophagosomes) induced by contact with the Cu plate suggests the occurrence of autophagy (14, 15). Further investigation of autophagy-specific marker, such as the accumulation of LC3 (Atg8) in autophagosomes is under way.

Within 15 minutes after the contact with Cu, the interruption of glutamine consumption was observed, as was the elevated production of glutamic acid and glycine, and the decline of spermidine and spermine. Since glutamine is a major energy source (16), as well as glucose, the interruption of glutamine consumption might seriously affect cell survival. Glutamic acid is known to be involved in neuronal cell death (17), and the elevation of this amino acid may trigger the cell death in HGF cells. Glycine is known as an inhibitory amino acid (18) and therefore the elevation of glycine may also affect the cellular function.

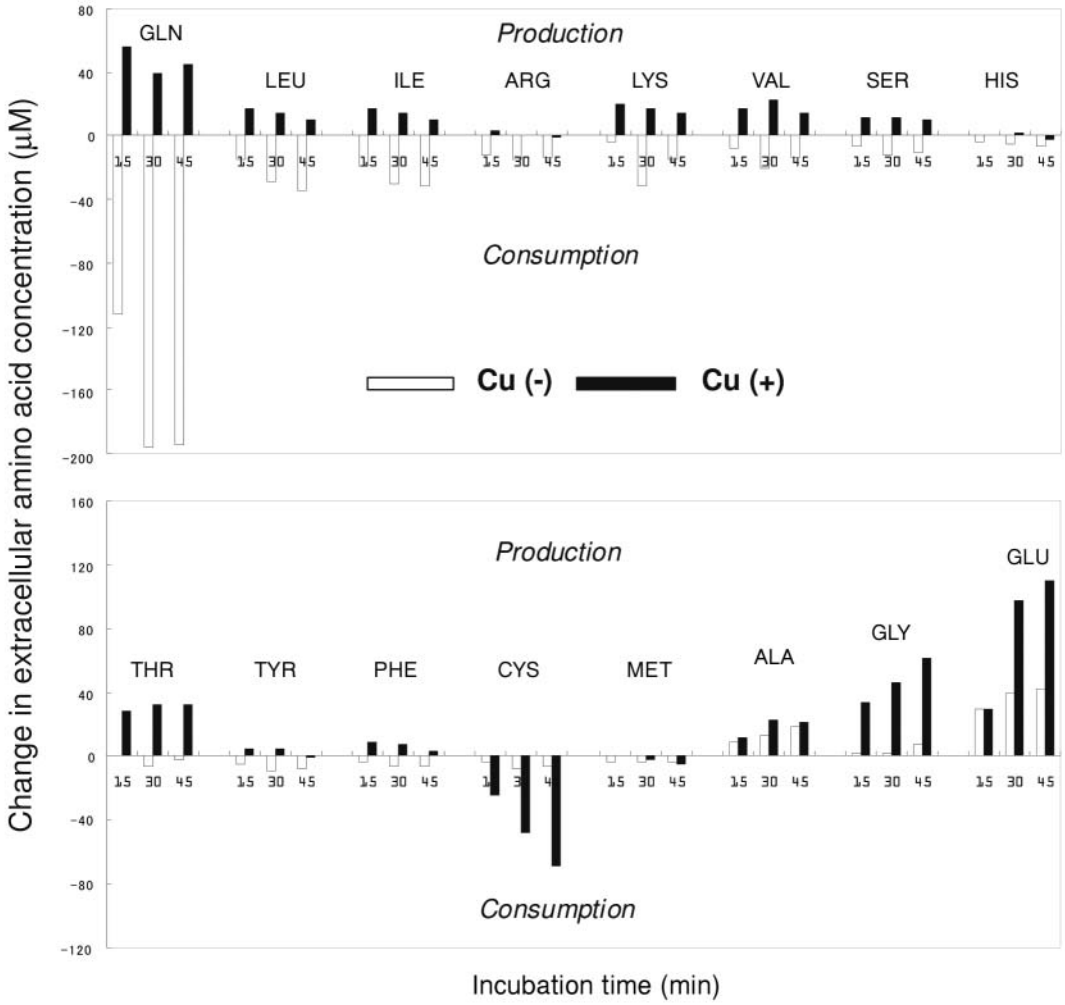

Figure 6. *Effect of contact with a Cu plate on amino acid utilization. HGF cells were inoculated onto a plastic dish (control) or a Cu plate, and incubated for 15, 30 or 45 min. The TCA-soluble fraction of the cell culture medium was then applied to an amino acid analyzer. The ordinate represents the changes in the concentration of each amino acid in the culture medium during 15, 30 or 45 min.*

Since growth potential and polyamines are tightly coupled (19), the decline of spermine and spermidine on contact with the Cu plate is not surprising. However, it was surprising that the intracellular concentration of putrescine was not reduced, but rather slightly elevated. This further supports the notion that Cu-induced cell death is non-apoptotic, since most apoptosis-inducers cause a rapid decline in the level of putrescine in HL-60 cells (20, 21).

The present study demonstrated that contact with a Cu plate stimulated the consumption of cystine, a dimer of cysteine with antioxidant potential, in the culture medium, suggesting the occurrence of oxidation. This may explain why antioxidants such as *N*-acetyl-L-cysteine (NAC) significantly protected the cells from Cu-induced injury (9). We recently found that during cell death induction by Ag and Pd, glutamine utilization was considerably disrupted, but the consumption of cystine was not observed (22). This suggests that the enhanced consumption of cystine is specific to Cu, whereas the disruption of glutamine utilization is a general phenomenon associated with the effects of all metals.

It has been reported that various toxicants, environmental hormones, radiation and even lasers can affect the cellular proliferation and functions in a bimodal fashion: a stimulatory (beneficial) effect at lower and a cytotoxic (adverse) effect at higher concentrations (strengths) (23). However, we did not observe such a hormetic response of cells upon exposure to Cu during the 45 min incubation. The cytotoxic action of Cu was very rapid and thus an investigation of the possible biochemical changes occurring at earlier stages on contact with the Cu plate should be performed to finally answer the question whether hormesis is involved in the present system or not.

References

1 Mehulic M, Mehulic K, Kos P, Komar D and Katunaric M: Expression of contact allergy in patients with oral diseases undergoing prosthodontic therapy. Minerva Stomatol *54*: 303-309, 2005.

2 Celebic A, Baucic M, Stipetic J, Baucic I, Miko S and Momcilovic B: Ion release from gold/platinum dental alloy: could release of other elements be accountable in the contact allergy attributed to the gold? J Mater Sci Mater Med *17*: 301-305, 2006.

3 Benemann J, Lehmann N, Bromen K, Marr A, Seiwert M, Schulz C and Jockel KH: Assessing contamination paths of the German adult population with gold and platinum. The German Environmental Survey 1998 (GerES III). Int J Hyg Environ Health *208*: 499-508, 2005.

4 Lam-Yuk-Tseung S and Gros P: Distinct targeting and recycling properties of two isoforms of the iron transporter DMT1 (NRAMP2, Slc11A2). Biochemistry *45*: 2294-2301, 2006.

5 Okafor CC, Haleem-Smith H, Laqueriere P, Manner PA and Tuan RS: Particulate endocytosis mediates biological responses of human mesenchymal stem cells to titanium wear debris. J Orthop Res *24*: 461-473, 2006.

6 Wataha JC, Hanks CT and Craig RG: The *in vivo* effects of metal cations on eukaryotic cell metabolism. J Biomed Mater Res *25*: 1133-1149, 1991.

7 Sjorgren G, Sletten G and Dahl JE: Cytotoxicity of dental alloys, metals and ceramics assessed by millipore filter, agar overlay, and MTT tests. J Prosthet Dent *84*: 229-236, 2000.

8 Kinoshita N, Yamamura T, Teranuma H, Katayama T, Tamanyu M, Negoro T, Satoh K and Sakagami H: Interaction between dental metals and antioxidants, assessed by cytotoxicity assay and ESR spectroscopy. Anticancer Res *22*: 4017-4022, 2002.

9 Yamazaki T, Yamazaki A, Hibino Y, Chowdhury SA, Yokote Y, Kanda Y, Kunii S, Sakagami H, Nakajima H and Shimada J: Biological impact of contact with metals on cells. In Vivo *20*: 605-612, 2006.

10 Satoh R, Kishino K, Morshed SRM, Takayama F, Otsuki S, Suzuki F, Hashimoto K, Kikuchi H, Nishikawa H, Yasui T and Sakagami H: Changes in fluoride sensitivity during *in vitro* senescence of human normal oral cells. Anticancer Res *25*: 2085-2090, 2005

11 Otsuki S, Morshed SRM, Chowdhury SA, Takayama F, Satoh T, Hashimoto K, Sugiyama K, Amano O, Yasui T, Yokote Y, Akahane K and Sakagami H: Possible link between glycolysis and apoptosis induced by sodium fluoride. J Dent Res *84*: 919-923, 2005.

12 Yanagisawa-Shiota F, Sakagami H, Kuribayashi N, Iida M, Sakagami T and Takeda M: Endonuclease activity and induction of DNA fragmentation in human myelogenous leukemic cell lines. Anticancer Res *15*: 259-266, 1995.

13 Sekine T, Takahashi J, Nishishiro M, Arai A, Wakabayashi H, Kurihara T, Kobayashi M, Hashimoto K, Kikuchi H, Katayama T, Kanda Y, Kunii S, Motohashi N and Sakagami H: Tumor-specificity and type of cell death induced by trihaloacetylazulenes in human tumor cell lines. Anticancer Res *27*: 133-144, 2007.

14 Broker LE, Kruyt FAE and Giaccone G: Cell death independent of caspase: A review. Clin Cancer Res *11*: 3155-3162, 2005.

15 Daido S, Yamamoto A, Fujikawa K, Sawaya R and Kondo Y: Inhibition of the DNA-dependent protein kinase catalytic subunit radiosensitizes malignant glioma cells by inducing autophagy. Cancer Res *65*: 4368-4375, 2005.

16 Zielke HR, Sumbilla CM, Sevdalian DA, Hawkins RL and Ozand PT: Lactate: a major product of glutamine metabolism by human diploid fibroblasts. J Cell Physiol *104*: 433-441, 1980.

17 Zhang Y and Bhavnani BR: Glutamate-induced apoptosis in neuronal cells is mediated *via* caspase-dependent and independent mechanisms involving calpain and caspase-3 proteases as well as apoptosis inducing factor (AIF) and this process is inhibited by equine estrogens. BMC Neurosci *7*: 49, 2006.

18 Lynch JW and Callister RJ: Glycine receptors: a new therapeutic target in pain pathways. Curr Opin Investig Drugs *7*: 48-53, 2006.

19 Seiler N and Raul F: Polyamines and apoptosis. J Cell Mol Med *9*: 623-642, 2005.

20 Sakagami H, Fujiwara E, Yokote Y, Akahane K, Asano K, Kochi M, Hara E and Shirahata A: Changes in intracellular concentration of amino acids and polyamines during apoptosis of HL-60 cells. Anticancer Res *20*: 265-270, 2000.

21 Nakamura C, Yasumoto E, Nakano K, Nakayachi T, Hashimoto K, Kusama K, Fukuda M, Sakashita H, Shirahata A and Sakagami H: Changes in intracellular concentrations of polyamines during apoptosis of HL-60 cells. Anticancer Res *23*: 4797-4804, 2003.

22 Onuki H, Sakagami H, Yamazaki T, Yamazaki A, Hibino Y, Yokote Y, Nakajima H and Shimada J: Disruption of amino acid metabolism in human myelogenous leukemic cell lines destined to die after contact with metal plates. In Vivo *21*: 841-846, 2007.

23 Calabrese EJ: Paradigm lost, paradigm found: The re-emergence of hormesis as a fundamental dose response model in the toxicological sciences. Env Poll *138*: 379-412, 2005.

Received February 21, 2007
Revised May 7, 2007
Accepted May 21, 2007