Proteome analyses of human macrophages exposed to low cytotoxic IC90 copper (2+) ions

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Dental noble alloys often contain copper (Cu). Eluted metal ions sometimes irritate oral tissues. The most eluted ions are Cu ions. The purpose of this study was to investigate the effects of low cytotoxic (IC90, 100 $\mu\text{mol/L}$) Cu ions on macrophages by proteome analyses consisting of two-dimensional (2D) electrophoresis and matrix-assisted laser desorption/ionization —time of flight (MALDI-TOF) mass spectrometry. The analyses revealed that stimulation with IC90 Cu ions for 1 day caused the macrophage to significantly increase five specific protein spots. Mascot peptide mass finger-print matching suggested that four of them were attributed to 70 kDa heat shock protein 1A/1B (HSP70). HSP70 expression was verified by expressions of corresponding HSPA1A and HSPA1B mRNAs of the macrophage in quantitative real-time PCR analyses. It was concluded that by producing abundant HSP70, the macrophage protected itself against intracellularly intruding cytotoxic Cu ions that might un-fold and crosslink cellular proteins.

Keywords: Macrophage, Proteome analysis, Copper ions

INTRODUCTION

Dental noble alloys often contain Cu¹⁾. Cu (2+) ions are most eluted from these noble dental alloys when exposed to hostile environments such as lactic acid²⁾ secreted by oral micro-organisms and electrical polarization between two different alloys³⁾. The quantities of Cu (2+) ions might be very minute⁴⁾, but their effects on oral tissues have not been well examined⁵⁾. It is also known that macrophages play a key role in the body's self defense reaction against Cu (2+) ions and Cu (2+) ion-bound proteins⁶⁾. Although there are many reports to evaluate cytotoxic effects of high dose Cu (2+) ions on fibroblasts and monocytes⁷⁾, those of low-quantity Cu (2+) ions on macrophage are sparse⁸⁾.

Cu has both advantageous and disadvantageous effects on the human body, dependent upon the quantities of Cu (2+) ions⁹⁾. Trace amounts of Cu (2+) ions (usually, 24 nmol/L in human serum¹⁰⁾) are required to maintain human life such as for synthesis of super-oxide dismutase, the detoxification enzyme of active oxygen⁹⁾. On the other hand, excess amounts of Cu (2+) ions, however, produce free radicals dose-dependently, damaging cell membranes and DNA, and leading to cell death^{11,12)}. It has been further reported that Cu (2+) ions might cause allergies in certain circumstances¹³⁾.

The proteome analysis (*i.e.* 2D electrophoresis+MALDI-TOF mass spectrometry) is relatively a new

sophisticated technique to evaluate biological phenomenon of cells at the protein expression levels¹⁴⁾. This technology has not been, however, well used in the cytotoxicity evaluation of metallic ions, yet.

The purpose of this study was, therefore, to compare and analyze protein expressions by human macrophage [phorbol-12-myristate-13-acetate (PMA)-differentiated THP1 cells¹⁵⁾] cultured for a day in two culture media, *i.e.*; a control medium without Cu (2+) ions and a test medium supplemented with low-cytotoxic (IC90) Cu (2+) ions by proteome analyses so that the effects of IC90 Cu (2+) ions on human macrophage could be better understood. To confirm specifically up-regulated protein expressions of the macrophage when subject to IC90 Cu (2+) ions, expressions of corresponding mRNAs of the macrophage were further examined by quantitative real-time RT-PCR analyses¹⁶⁾.

MATERIALS AND METHODS

Preparation of culture media with Cu (2+) ions

Copper (II) chloride dihydrate (min. 99% pure) (Jyunsei Chemical, Tokyo, Japan) was dissolved in super-pure water at the concentration of 0.2 mol/L (Cu (2+) ion stock solution, pH=3). It was sterilized by 0.22 μm membrane filtration. By diluting this stock solution, media containing Cu (2+) ions with concentrations of 0, 20, 50, 100, 200, 500 and 1,000 $\mu\text{mol/L}$ were prepared. The pH of all fresh media was maintained at around 7.2.

Fig. 1 The cell morphology of macrophages (PMA-differentiated THP-1 cells). Bar size=100 μ m.

Cell differentiation of monocytic THP-1 to macrophage

Human monocytic cell line THP-1 (RCB 1189, Riken Cell Bank, Tsukuba, Japan) was cultured in RPMI1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 vol.% fetal bovine serum (Cat. No. 10099-141, Invitrogen, Carlsbad, CA, USA) and 2 vol.% antibiotics (penicillin-streptomycin-amphotericin, Cat. No. 15240-096, Invitrogen, Carlsbad, CA, USA) in a 5%CO₂ incubator at 37°C. THP-1 cells were differentiated into macrophages by culturing for 48 h in medium supplemented with 200 nM PMA (Sigma-Aldrich Japan, Tokyo, Japan) with a cell density of 10⁵ cells/mL. By PMA-differentiation, cells adhered on culture dishes. The cell morphology of the macrophages (PMA-differentiated THP-1 cells) is shown in Fig. 1.

Cell viability tests

After PMA-differentiation, media (1 mL) were removed from each well in 24-well microplates containing adhered cells (10⁵), and fresh media without PMA but with Cu (2+) ions of varying concentrations from 0 to 1,000 μ mol/L (1 mL) were added to each well and cultured for an additional 24 h. Cell viabilities of control macrophages and those exposed to Cu (2+) ions were evaluated ($n=6$; 3 wells for each Cu (2+) concentration $\times$ two repetitions of colorimetric assay) using Cell Counting Kit-8 (Doujin Chemical, Kumamoto, Japan) and a micro-plate reader (model 680, Biorad, Hercules, CA, USA.).

When cultured in IC90 concentration of Cu (2+) ions, the cell viability of macrophage drops to 90% relative to that of control macrophage (100%). The IC90 Cu (2+) concentration was regarded as low-cytotoxic to the macrophage examined in this study.

Proteome analyses

After PMA-differentiation, media (10 mL) were removed from two 100 mL culture dishes containing adhered cells (10⁶). Control medium without PMA and Cu (2+) ions and fresh test medium without PMA but with IC90 Cu

(2+) ions were then added to the dishes, respectively, and cultured for an additional 24 h. After washing twice with cold PBS (–) solutions, macrophages were collected with plastic cell scrapers and centrifugation. The proteome analyses in this study consisted of the following 2D-PAGE and mass spectrometry.

2D-PAGE

Proteins (cell pellets) (125 μ g) were dissolved in a 8-M urea buffer (7.8 M urea, 0.2 M thiourea, 1.9% Nonidet P40, 0.1 M dithiothreitol, and 0.01% bromophenol blue) containing 0.5% pharmalyte (pH3–9) (GE Healthcare Bio-Science, Piscataway, NJ, USA) and a protease-inhibitor cocktail (Sigma-Aldrich Co., Irvine, UK). Samples were electrically focused using an Immobiline DryStrip pH 3–10 NL and IPGphor (GE Health Bio-Science, Piscataway, NJ, USA) at 50 μ A per strip for 3 h at 20°C. For the second dimension, the strips were equilibrated in a 6-M urea buffer (50 mM Tris-HCl, pH 6.8, 6 M urea, 3% SDS, 50 mM dithiothreitol, and 0.01% bromophenol blue) and separated on 11% (w/v) polyacrylamide gels. The gels were then stained with Coomassie Brilliant Blue (CBB), and spots in gels were photographed by a digital camera. Quantitative analyses of protein spots were carried out with PDQuest (Biorad, Hercules, CA, USA.). To ensure reproducibility, every sample was analyzed in duplicate.

Mass spectrometry

Identifications of proteins separated on 2D-PAGE were performed by mass spectrometry as reported previously¹⁷. Briefly, protein spots were excised and reduced. Following alkylation, in-gel digestion was performed with sequential-grade trypsin (Promega, Madison, WI, USA) overnight at 37°C. Peptides were extracted with 50% (v/v) acetonitrile containing 5% (v/v) trifluoroacetic acid, and then the extract was concentrated using a Speed Vac (Thermo Fisher Scientific, Waltham, MA, USA). Mass spectra were recorded on a Voyager DE-Pro MALDI-TOF mass spectrometer (Applied Biosystems, Branchburg, NJ, USA). Matrix-related ions (α -cyano-4-hydroxycinnamic acid (Applied Biosystems Co., Foster City, CA, USA) (CHCA)) and trypsin autolysis products were used for internal calibration. Peptide mass fingerprinting analysis was performed using Mascot Search available at <http://www.matrixscience.com> (Matrix Science, Boston, MA, USA.). The conditions for search analyses were as follows: Database=SwissProt. 2010_008; Taxonomy=Homo Sapiens; Enzyme=Trypsin; Mass values=Monoisotopic; Protein Mass=Unrestricted; Peptide Mass Tolerance= $\pm$ 1.2Da; Peptide Charge State=1+; Max Missed Cleavages=1. Mascot Search results provided tables of several protein candidates which might match mass spectra of the searched spot with protein names, entry name and expectation values. Expectation value is the number of matches with equal or better scores that are expected to occur by chance alone. For a score that is exactly on the significance threshold ($p<0.05$), the expectation value is also 0.05. The lower the expectation value, the more significant the

score. In this study, the protein candidate of the highest possibility was presented for each protein spot.

Quantitative real-time PCR

Total RNAs were collected from two macrophages, *i.e.*; control and that subject to medium with IC90 Cu (2+) for 24 h, using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). The expressions of two genes (HSPA1A and HSPA1B) and one house-keeping gene (GAPDH) were confirmed by quantitative real-time PCR. PCR amplification of cDNA was performed by SYBR Premix ExTaq (Takara Bio, Tokyo, Japan). The primers for the real-time PCR were GGATAACGGCTAGCCTGAGGAG (the forward primer for HSPA1A gene); CTGGGAAGCCTTTGGGACAAC (the reverse primer for HSPA1A gene); TGGACTGTTGGGACTCAAGGAC (the forward primer for HSPA1B gene); GGGAACGAAACACCCTTACAGTATC (the reverse primer for HSPA1B gene); GCACCGTCAAGGCTGAGAAC (the forward primer for GAPDH); and TGGTGAAGACGCCAGTGGA (the reverse primer for GAPDH gene). After an initial denaturation at 95°C for 30 sec, a two-step cycle procedure was used (denaturation at 95°C for 5 sec, annealing and extension at 60°C for 30 sec) for 40 cycles in a Dice Real Time System TP800 (Takara Bio, Tokyo, Japan). Gene expression levels were normalized according to the level of GAPDH expression. Relative amounts (RQ values) of HSPA1A and HSPA1B mRNAs in each sample were calculated by $\Delta\Delta$ Ct method ($n=4$; 2 culture sets $\times$ 2 measurement repetition for each primer set). Data were presented as means $\pm$ standard deviation.

Statistics

Statistical analysis was performed by unpaired *t*-test.

RESULTS

Cell viabilities

Fig. 2 shows the dose-dependent cytotoxicity of Cu (2+) ions against macrophages examined. The IC90 concentration was found to be about 100 μ mol/L Cu (2+) ions. Cell viabilities of the macrophages exposed to Cu (2+) ions with concentrations of less than 100 μ mol/L Cu (2+) ions were not statistically significantly different ($p>0.01$) from those of control macrophages cultured in the medium without Cu (2+) ions. In other words, this IC90 concentration of Cu (2+) ions lay in the sub-cytotoxic region.

Fig. 2 Effects of Cu (2+) ion concentrations on cell viability of human macrophages (PMA-differentiated THP-1 cells) cultured for one day.

Fig. 3 2D-PAGE of extracts from human macrophages (PMA-differentiated THP-1 cells): (Left) control cells (coded as Cu (-)), (Right) cells exposed to sub-cytotoxic concentration (100 μ mol/L) Cu (2+) ions for one day (coded as Cu (+)).

Fig. 4 Mass spectra of protein spot 1 of macrophages exposed to IC90 (100 $\mu\text{mol/L}$) Cu (2+) ions (Fig. 3 (Right)). Note: Mass peaks derived from added CHCA matrix were eliminated from mass finger-print analysis.

The m/z values of mass spectra for spot 1 selected for mass finger-print matching were 904.3582, 907.1787, 909.2883, 923.3241, 927.3923, 982.501, 996.5181, 998.4699, 1004.3709, 1007.4778, 1017.5318, 1033.5178, 1035.4429, 1051.6236, 1065.4886, 1082.4700, 1133.6103, 1151.6403, 1154.5071, 1165.6650, 1179.4991, 1211.4879, 1230.5597, 1265.6576, 1279.6747, 1296.6168, 1334.7549, 1448.7607, 1475.6844, 1488.7319, 1505.7124, 1568.6597, 1584.8081, 1590.6809, 1638.7976, 1769.7884, 1774.7767, 1790.812, 1818.7959, 1819.7710, 1835.7621, 1851.8433, 1873.8348, 1940.8551, 1993.9435, 2092.9914, 2099.9604, 2144.9661, 2182.1830, 2185.0821, 2226.0880, 2227.1412, 2239.0532, 2243.0944, 2408.0742 and 2807.1858.

Proteome analyses of macrophages exposed to IC90 concentration Cu (2+) ions

Fig. 3 indicates 2D-PAGE of extracts from human macrophage (PMA-differentiated THP-1 cells); (Left) control cells (coded as Cu (-)) and (Right) cells cultured in the medium added with IC90 (100 $\mu\text{mol/L}$) Cu (2+) ions (coded as Cu (+)). Detailed comparisons between Cu (-) and Cu (+) revealed that five protein spots were significantly increased in Cu (+). Table 1 shows fold changes of five protein spots, defined by optical densities of test macrophage exposed to IC90 (100 $\mu\text{mol/L}$) Cu (2+) ions for one day (coded as Cu (+)), divided by those of control macrophages (coded as Cu (-)).

Figs. 4, 5, 6, 7 and 8 show mass spectra of protein spots 1, 2, 3, 4 and 5 of Cu (+) in Fig. 3, respectively. The m/z values of mass spectra for each spot selected for mass finger-print matching are displayed in each figure captions. Table 2 summarizes Mascot search (peptide finger-print identification) results of protein name, entry

Fig. 5 Mass spectra of protein spot 2 of macrophages exposed to IC90 (100 $\mu\text{mol/L}$) Cu (2+) ions (Fig. 3 (Right)).

The m/z values of mass spectra for spot 2 selected for mass finger-print matching were 996.4847, 1051.5910, 1109.5084, 1110.4963, 1111.4928, 1165.6237, 1183.5845, 1197.5926, 1205.5447, 1315.5331, 1329.5828, 1465.7520, 1488.7401, 1542.6892, 1803.7865, 2169.9827, 2531.2580, 2662.1643, 2769.2863, 2786.2988, 2807.2917 and 2981.4147.

name and expectation values (p) for each protein spot. The identifications revealed that four protein spots (spots 2, 3, 4 and 5) were attributed to heat shock protein 70 kDa 1A/1B while protein spot 1 arose from U5 small nuclear ribonucleoprotein 200 kDa helicase. Identifications of spots 3, 4 and 5 were very reliable due to very low expectation values ($p < 0.01$), while those of spots 1 and 2 were less trustable because of relatively large expectation values.

Quantitative real-time PCR analyses of macrophages exposed to IC90 concentration Cu (2+) ions

Fig. 9 shows relative quantities (RQ) of GAPDH, HSPA1A and HSPA1B mRNAs of control macrophage (coded as Cu (-)), and test macrophage exposed to IC90 Cu (2+) ions for 1 day (coded as Cu (+)). Both RQs of HSPA1A and HSPA1B mRNA of the latter test macrophage (Cu (+)) were significantly greater than those of control macrophage (Cu (-)), respectively ($p < 0.01$).

DISCUSSION

Eluted metallic ions from dental noble alloys might be not only Cu ions, but also Au, Ag, and Zn ions²⁾. Although the effects of all these metallic ions on macrophage must be considered in the future, the effects of Cu ions on macrophage was considered in this study as a first step.

Fig. 6 Mass spectra of protein spot 3 of macrophages exposed to IC90 (100 $\mu\text{mol/L}$) Cu (2+) ions (Fig. 3 (Right)).

The m/z values of mass spectra for spot 3 selected for mass finger-print matching were 996.5327, 1109.5240, 1110.5167, 1165.6663, 1179.5595, 1183.6032, 1197.6459, 1205.5779, 1228.5809, 1261.6070, 1283.5864, 1299.5813, 1315.5581, 1318.5960, 1405.7230, 1417.7116, 1465.7747, 1474.7286, 1475.7104, 1487.6643, 1493.7085, 1580.7641, 1614.7512, 1638.7909, 1658.8061, 1675.6940, 1680.7949, 1687.8535, 1707.7220, 1803.8104, 1838.8772, 1993.9145, 2169.0227, 2265.1318, 2769.3006, 2786.3670 and 2981.4869.

Fig. 7 Mass spectra of protein spot 4 of macrophages exposed to IC90 (100 $\mu\text{mol/L}$) Cu (2+) ions (Fig. 3 (Right)).

The m/z values of mass spectra for spot 4 selected for mass finger-print matching were 907.2047, 982.5189, 996.5497, 1034.5910, 1051.6610, 1109.5380, 1110.5204, 1151.6485, 1165.6944, 1183.6122, 1197.6455, 1211.6398, 1228.5922, 1242.6514, 1253.5913, 1261.6097, 1279.7022, 1290.6502, 1315.5772, 1361.7118, 1398.7346, 1402.0257, 1446.7341, 1450.7233, 1462.7318, 1465.7520, 1481.7668, 1487.6891, 1580.7657, 1591.8738, 1592.9180, 1596.7673, 1614.8595, 1675.6975, 1680.7890, 1687.8718, 1694.8188, 1696.8074, 1716.7987, 1856.8930, 1981.9920, 1983.0119, 2055.9442, 2056.9375, 2142.0642, 2169.04389, 2309.2387 and 2786.3794.

Table 1 Fold change of five protein spots, defined by optical densities of test macrophage exposed to IC90 (100 $\mu\text{mol/L}$) Cu (2+) ions for one day (coded as Cu (+)), divided by those of control macrophages (coded as Cu (-)), expressed as mean values ($n=2$).

Spot number	First measurement	Second measurement	Average
1	5.86 times	8.84 times	7.35 times
2	1.27 times	3.17 times	2.22 times
3	5.27 times	6.13 times	5.70 times
4	2.81 times	2.90 times	2.86 times
5	5.06 times	3.16 times	4.11 times

Table 2 Mascot peptide mass finger-print identifications of five up-regulated protein spots of macrophages exposed to IC90 Cu (2+) ions.

Spot number	Protein name	Entry name	Expectation values (p)
1	U5 small nuclear ribonucleoprotein 200 kDa helicase	U520_Human	0.01
2	Heat shock 70 kDa protein 1A/1B	HSP71_Human	0.091
3	Heat shock 70 kDa protein 1A/1B	HSP71_Human	$6.4e^{-11}$
4	Heat shock 70 kDa protein 1A/1B	HSP71_Human	$3.2e^{-07}$
5	Heat shock 70 kDa protein 1A/1B	HSP71_Human	$7.0e^{-05}$

Fig. 8 Mass spectra of protein spot 5 of macrophages exposed to IC90 (100 $\mu\text{mol/L}$) Cu (2+) ions (Fig. 3 (Right)).

The m/z values of mass spectra for spot 5 selected for mass finger-print matching were 915.3590, 948.3692, 982.4706, 996.5056, 1051.5835, 1053.485554, 1109.4336, 1136.5066, 1151.5244, 1165.6711, 1169.5261, 1197.6461, 1200.6062, 1228.5689, 1235.5415, 1261.5989, 1283.6239, 1321.5961, 1417.7002, 1474.72920, 1487.6483, 1580.7457, 1581.76013, 1614.7609, 1658.7681, 1675.6671, 1680.7787, 1687.8389, 1691.7111, 1696.7492, 1702.7568, 1717.7844, 1981.9330, 2093.0140, 2264.0484 and 2265.0756.

Fig. 9 Relative quantities (RQ) of HSPA1A and HSPA1B mRNAs of control macrophage (coded as Cu (-)), and test macrophage exposed to IC90 Cu (2+) ions for 1 day (coded as Cu (+)). Note: RQ values of Cu (-) and Cu (+) were calibrated against GAPDH mRNAs of each cells.

In dental materials community, the effects of Cu (2+) ions on human cells have been evaluated simply by measuring cell proliferation (*i.e.* cell viabilities)⁷, and the underlying mechanisms have not been well investigated. Only recently, cell death due to high concentration Cu (2+) ions was evaluated by oxidative stress and its induced apoptosis¹⁸. The effects of low dose Cu (2+) ions on macrophages have not been well investigated⁸, and related studies had been highly anticipated.

Proteome analysis consisting of 2D electrophoresis and MALDI-TOF mass spectroscopy has recently been used to clinically diagnose dental patients (*e.g.* those suffering from Sjögren's syndrome¹⁹), but its availability in dentistry is limited due to high cost and laborious handling. Proteome analysis was conducted in this study, because it was expected to unveil new biological phenomenon in the macrophage exposed to low-cytotoxic IC90 Cu (2+) ions at protein expression levels.

By proteome analysis, we confirmed that expression of one protein, heat shock 70 kDa protein 1A/1B (HSP70) was specifically up-regulated in the macrophage when exposed to IC90 Cu (2+) ions (Table 2). Identified HSP70 had four distinctive protein spots (protein spots 2, 3, 4 and 5). Such separation in one protein is not strange in MALDI-TOF mass spectroscopy because HSP70 could be partially degraded during the sample preparation, leading to production of separated protein spots. Since original HSP70 has an isoelectric point (pI) value of 5.5 and a protein molecular weight of 70053 Da²⁰, either one of protein spots 3 and 4 in Fig. 3 might be native HSP70. Identification of other one protein (protein spot 1) must only be treated as one possible choice with less reliability because of its high expectation value (Table 2).

The increase in the expression of HSP70 protein appeared to reflect the self-protection activities of macrophages against intracellularly invading Cu (2+) ions which damage proteins by unfolding and cross-linking. Heat shock proteins function as intra-cellular chaperones for other proteins²¹. They play an important role in protein-protein interactions such as folding and assisting in the establishment of proper protein conformation (shape) and prevention of unwanted protein aggregation²¹.

The increased HSP70 expression of the macrophage due to IC90 Cu (2+) ions was successfully confirmed by expressions of HSPA1A and HSPA1B mRNAs (Fig. 9). The HSP expression is encoded by expressions of HSPA1A and HSPA1B mRNAs²². The up-regulation of HSP70 mRNA due to Cu (2+) ions has already been reported as a biomarker of cytotoxicity of metal ions against HeLa S3 cells by Hashimoto *et al.*²³. We verified this up-regulation at the protein expression levels of human macrophages.

Finally, it might be worth to speculate reasons why IC90 Cu (2+) ions increased HSP70 of the macrophage at protein levels. Two sides are considered here. (1) Human cells have a unique transport system of Cu (2+) ions⁹ from extracellular region to intracellular one; and intracellularly liberated Cu (2+) ions can bind to, un-fold

and cross-link intracellular proteins. The resultant proteins might lose their proper function and be recognized as damaged ones. (2) In any human cell, H_2O_2 is routinely formed when super-oxide (O_2^-) from mitochondria is reduced. If excess Cu (2+) ions exist adjacent to H_2O_2 , OH^- radicals are formed by Fenton's reaction, which damage not only proteins but also lipids as oxidative stress¹¹⁾. For both (1) and (2) cases, HSP70 might play important roles to restore or eliminate intracellular proteins damaged by Cu (2+) ions of the macrophage as protective measures²¹⁾.

CONCLUSIONS

We found that macrophages cultured for one day in IC90 (100 $\mu\text{mol/L}$) Cu (2+) ions specifically increased one protein, namely heat shock 70 kDa protein 1A/1B (HSP70). The up-regulation of HSP70 expression was confirmed by expressions of corresponding HSPA1A and HSPA1B mRNAs.

It appeared that a proteome analysis might be useful for the cytotoxic evaluation of low concentration other metallic ions except Cu ions contained in dental alloys in the future.

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