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Copper-1,10-phenanthroline-induced apoptosis in liver carcinoma Bel-7402 cells associates with copper overload, reactive oxygen species production, glutathione depletion and oxidative DNA damage

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Abstract

The mechanism of cytotoxicity on liver carcinoma Bel-7402 cells induced by copper-1,10-phenanthroline, $Cu(OP)_2$, has been studied. Cell viability and apoptotic rate were examined in cells treated with $Cu(OP)_2$ or Cu^{2+} alone. It was found that the apoptosis induced by $Cu(OP)_2$ could not be induced by Cu^{2+} or OP alone in our experimental conditions. Total copper content in cells was measured by atomic absorption spectrophotometry, and the abnormal elevation of intracellular copper transported by lipophilic OP ligand may play the role of initial factor in the apoptosis, which caused subsequent redox state changes in cells. Intracellular levels of reactive oxygen species (ROS) were detected by fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA). Reduced (GSH) and total glutathione (GSSG + GSH) were determined by High-performance liquid chromatography (HPLC) after derivatization, and the ratios of GSH/GSSG were subsequently calculated. The overproduction of ROS and the decreased GSH/GSSG ratio were observed in cells which represented the occurrence of oxidative stress in the apoptosis. Oxidative DNA damage was also found in cells treated with $Cu(OP)_2$ in the early stage of the apoptosis, and it suggests that the activation of DNA repair system may be involved in the pathway of the apoptosis induced by $Cu(OP)_2$.

Introduction

Apoptosis is an important mechanism of controlled cell depletion in response to both physiological and toxic stimuli (Gerschenson & Rotello 1992). Chemical agents which stimulate the intracellular generation of reactive oxygen species (ROS) can lead cells to apoptosis (Young *et al.* 2003). Intracellular excessive production of ROS can induce oxidative stress which has been suggested to play a critical role as a common mediator in apoptosis (Buttke & Sandstrom 1994; Jabs 1999). ROS can interact with intracellular macromolecules such as proteins, membrane lipids and nucleic acids. Oxidative attack of proteins can lead to oxidation of amino acid residue side chains, formation of protein–protein cross-linkages, and oxidation of the protein backbone resulting in protein fragmentation (Barbara & Stadtman 1997; Dean *et al.* 1997). Cell membrane is another target of ROS, and generally the effects of lipid peroxidation are to decrease membrane fluidity, increase the leakiness of the membrane, and inactivate membrane-bound enzymes, leading to complete loss of membrane integrity (Gaetke & Chow 2003). Exposure of DNA to ROS, especially hydroxyl radical (·OH), has been reported to cause DNA to both single- and double-strand breaks, bases modification and DNA–protein crosslinks, which induce genome toxicity and mutation (Halliwell & Aruoma 1991).

Copper has been recognized as an essential trace metal for living organisms, and its role as a

cofactor for crucial enzymes has been well established (Pulg & Thiele 2002). Copper deficiency in humans usually leads to several diseases (Danks 1988). Whereas, the metal becomes toxic to cells at its concentration surpasses certain natural level, and this is called copper overload (Theophanides & Anastassopoulou 2002). The excessive copper can promote damage to cellular molecules and structures through free radicals formation such as superoxide anion ($O_2^{\cdot -}$) and ·OH (Galaris & Evangelou 2002). The metal chelator phenanthroline (OP) together with copper will form a stable complex copper-1,10-phenanthroline, $Cu(OP)_2$, which has nuclease activity in the presence of reducing agents and molecular oxygen. It can bind tightly to the minor groove of DNA and induce the cleavage via ·OH formed by the catalysis of copper (Sigman *et al*. 1979). DNA fragmentation has been detected in isolated cell nuclei treated with $Cu(OP)_2$ (Burkitt *et al*. 1996). $Cu(OP)_2$ is known to promote ·OH formation from reducing agents and molecular oxygen by redox-cycling and is therefore considered to be a suitable agent for the stimulation of ROS formation (Dizdaroglu *et al*. 1990).

We have reported that $Cu(OP)_2$ induced G_1-phase specific apoptosis in liver carcinoma cell line Bel-7402 (Zhou *et al*. 2002). While the mechanism of the cytotoxicity of $Cu(OP)_2$ has not been well elucidated, especially the role of OP ligand and the biochemical changes in cells during the early stage of the apoptosis. The following experiments were undertaken to examine the possible pathway of the apoptosis induced by $Cu(OP)_2$, and we investigated its effect on cells in order to gain insights into the function of metal complex and ROS in cell apoptosis.

Materials and methods

Materials

Cupric sulfate, phenanthroline, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), *ortho*-phthalaldehyde (OPA) were purchased from Sigma. 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), RNase A, Triton X-100, phenylmethyl sulfonylfluouride (PMSF), ethidium bromide (EB), bovine serum albumin (BSA), dithiothreitol (DTT), N-lauroyl sarcosine Na salt (SLS) and dimethyl sulfoxide (DMSO) were all purchased from Amresco. All other chemicals and reagents were of the highest quality and obtained from standard commercial sources. Stock solutions of $Cu(OP)_2$, $CuSO_4$ and OP were prepared using water filtered through a Milli-Q water system (Millipore) and sterilized before they were used.

Cell culture and treatment

Liver carcinoma cell line Bel-7402 was obtained from China Center for Typical Culture Collection (CCTCC). Cells were cultured in RPMI 1640 medium supplemented with 10% newborn bovine serum, 1%(w/v) glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All medium and serum were obtained from Hyclone. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells grew adhering to a substrate and 0.25% trypsin was used to separate cells from medium. The exponentially growing cells were treated as indicated in the following sections.

Cell viability assay

Cell viability was evaluated by MTT assay (Mosmann 1983). Cells were diluted to 1×10^5 cells/ml and plated at 100 μl per well in a 96-well microplate. For time dependent assay, cells were treated with 20 μM cupric sulfate or $Cu(OP)_2$ for 3 h and cultured subsequently for different time (0–20 h) in fresh medium; for dose dependent assay, cells were treated with cupric sulfate or $Cu(OP)_2$ in different concentrations (20–150 μM) for 3 h and cultured subsequently for 20 h. At the indicated time, cells were treated with MTT (5 mg/ml) and incubated continuously for 4 h at 37 °C in an incubator. The reaction was stopped by adding 100 μl DMSO, and the absorbance was measured at 490 nm. Results were expressed as percentage of the untreated controls, which were assumed as having 100% viability.

Quantitation of apoptosis

Cells were treated with 20 μM $CuSO_4$, 40 μM OP, or 20 μM $Cu(OP)_2$ for 3 h and cultured subsequently for 15 h in fresh medium. After incubation cells were collected and washed three times with phos-

phate buffer saline (PBS), then suspended in 75% ethanol at −20 °C overnight. Fixed cells were centrifuged and washed with PBS twice. For detecting DNA content, cells were stained in the dark with PI (50 μg/ml) and 0.1% RNAse A in PBS at 25 °C for 30 min. Stained cells were applied to a flow cytometer (Becton Dickinson). For each analysis, 10,000 events were recorded.

Measurement of copper

Cells were treated with 20 μM $CuSO_4$ or $Cu(OP)_2$ for 3 h. For intracellular remaining copper content determination, cells were incubated for further 12 h in fresh medium after treated with 20 μM $Cu(OP)_2$ for 3 h. Then cells were collected and washed three times with PBS. An aliquot was removed and lysed overnight with 10% (final concentration) HNO_3 at 4 °C. Total copper content in cells was measured with an atomic absorption spectrophotometer (AAnalyst 800, Perkin–Elmer). The calibration curve was created with an atomic absorption spectroscopy standard and a cell-free sample prepared in the same way was used as a blank. Another aliquot was used for protein concentration determination. The cell pellet was resuspended in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1 mM PMSF was added just before used). Protein concentration was determined using Bradford method (Bradford 1976), and BSA was used as a reference standard. The copper content was expressed as nmol/mg protein. To compensate for variations of absolute concentration of copper among different experiments, the results were converted to percentage of control values.

Measurement of intracellular ROS

For intracellular ROS measurement, the oxidation-sensitive fluorescent probe DCFH-DA was used. DCFH-DA was a stable nonfluorescent molecule that readily crossed cell membranes, and could be oxidized to highly fluorescent DCF with the presence of intracellular ROS (Curtin *et al.* 2002). Cells were treated with 20, 40, or 80 μM $Cu(OP)_2$ for 3 h, or treated with 20 μM $Cu(OP)_2$ for 3 h and cultured subsequently for 0, 4, 8, or 12 h in fresh medium. After incubation cells were collected and washed three times with PBS, then resuspended in PBS and incubated for 30 min at 37 °C with 10 μM DCFH-DA added. The DCF fluorescence was imaged by a fluorescence microscope (Leica) or measured at 488 nm excitation and 525 nm emission using a flow cytometer. For each analysis, 10,000 events were recorded.

GSH and GSSG determination

The determination of intracellular GSH and GSSG was modified according to Cereser (Cereser *et al.* 2001). Cells were treated with 20 μM $Cu(OP)_2$ for 3 h and cultured subsequently for 0, 2, 4, 6 or 8 h in fresh medium. After incubation cells were collected and washed three times with PBS. Then cells were homogenized with a homogenizer using PBS as the homogenized buffer. The cell homogenate was mixed with 10% (w/v, final concentration) trichloroacetic acid (TCA) to precipitate protein. The mixture was centrifuged at 10,000*g* for 10 min, and the supernatant was neutralized with sodium phosphate buffer (500 mM, pH 7.0). GSH content was determined by reaction with 5 mg/ml OPA for 5 min at room temperature. The derivatized samples were neutralized by addition of sodium phosphate buffer, and 20 μl were then injected into the HPLC system for reduced GSH determination. Total GSH was also evaluated by the present method by performing a reduction step of GSSG with DTT before protein precipitation, and the GSSG concentration was obtained from subtraction of the reduced GSH from the total GSH values. The HPLC separation of GSH–OPA adducts was achieved on a Zorbax Eclipse XDB-C18 column (150 × 4.6 mm) maintained at 25 °C, followed by fluorimetric detection at 420 nm with excitation at 340 nm.

Single-cell gel electrophoresis (SCGE)

The assay method used was based on that of Collins (Collins 2004). All analyses were carried out under reduced light conditions. Cells were treated with 20 μM $Cu(OP)_2$ for 3 or 6 h, or treated with 40 μM $Cu(OP)_2$ for 3 h. Then cells were collected and washed twice with cold PBS, and then suspended in melted 0.75% low-melting-point agarose in PBS and spread over slides that had been precoated with 1% agarose. Cells were then lysed overnight at 4 °C in 10 mM Tris, pH 10.0, containing 2.5 M NaCl, 100 mM EDTA, 1% SLS, 10% DMSO and 1% Triton X-100. Slides were immersed in 300 mM NaOH, 1 mM

EDTA, pH 12.0, for 20 min to allow the DNA to unwind, prior to electrophoresis in the same buffer for 20 min at 20 V and 200 mA. Immediately following electrophoresis slides were removed and the pH 12.0 buffer was neutralized by three 5-min washes in 0.4 M Tris, pH 7.5. Slides were then stained with 2 μg/ml EB and images were captured by a fluorescence microscope. Percent of DNA in the tail was calculated by Comet Assay Software Project (CASP) (Krzysztof 2003).

Data analysis

Data are expressed as mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) with a Student–Newman–Keuls follow-up test. A P-value < 0.05 was considered significant.

Results

Inhibition of cell viability caused by $Cu(OP)_2$ but not by Cu^{2+}

In order to examine the role of OP ligand in the cytotoxicity of $Cu(OP)_2$, the time and dose dependency of the cell viability of Bel-7402 cells treated with $Cu(OP)_2$ or Cu^{2+} alone were evaluated by MTT assay, as shown in Figure 1. Treatment of Bel-7402 cells with 20 μM $Cu(OP)_2$ for 3 h followed by incubation in fresh medium for 20 h decreased the cell viability to approximately 50%. In contrast, treatment of cells with 20 μM $CuSO_4$ for the same time did not cause inhibition of the cell viability, as shown in Figure 1a. The dose dependency is shown in Figure 1b, which demonstrates that the cell viability decreased with the concentration of $Cu(OP)_2$ increased from 20 to 150 μM, while $CuSO_4$ up to 150 μM had no effect on the cell viability.

Apoptosis induced by $Cu(OP)_2$ but not by Cu^{2+} or OP

Apoptotic rate of cells was examined by stained with PI and counted by a flow cytometry, as shown in Figure 2. The apoptotic rate after 15 h incubation was chosen referring to our previous reports (Zhou *et al.* 2002). The results indicated that neither Cu^{2+} nor OP alone induced significant

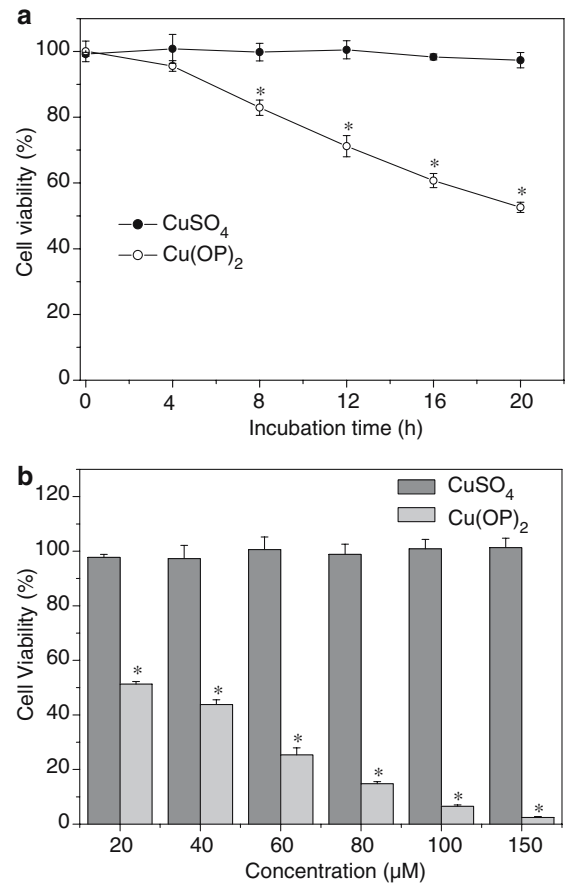

Figure 1. The time and dose dependency of the cell viability in Bel-7402 cells treated with $CuSO_4$ or $Cu(OP)_2$. (a) Cells treated with 20μM $CuSO_4$ or $Cu(OP)_2$ for 3 h and incubated for 0, 4, 8, 12, 16 or 20 h in fresh medium without $CuSO_4$ or $Cu(OP)_2$. (b) Cells treated with 20, 40, 60, 80, 100 or 150 μM $CuSO_4$ or $Cu(OP)_2$ for 3 h and incubated for 20 h in fresh medium. Results were obtained from three separate experiments. $^*P < 0.05$ vs. $CuSO_4$-treated cells.

apoptosis in Bel-7402 cells, and the apoptotic rates of cells treated with them were about 2% and 3%, respectively. Treatment of cells with $Cu(OP)_2$ induced significant increase of the apoptotic rate, approximately 17% after 15 h incubation.

Copper overload in cells induced by $Cu(OP)_2$

Total copper content in Bel-7402 cells treated with Cu^{2+} or $Cu(OP)_2$ was measured by atomic absorption spectrometry. As shown in Figure 3, treatment of cells with 20 μM $Cu(OP)_2$ for 3 h significantly increased intracellular total copper content, which was approximately 14 folds to untreated control, and the high copper content re-

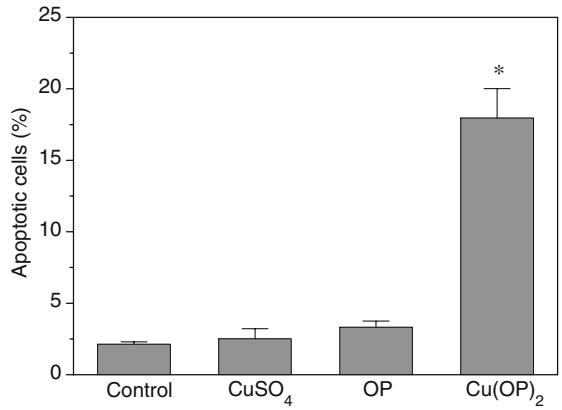

Figure 2. Apoptotic rate of Bel-7402 cells induced by $CuSO_4$, OP, or $Cu(OP)_2$. Cells were untreated or treated with 20 μM $CuSO_4$, 40 μM OP, or 20 μM $Cu(OP)_2$ for 3 h and incubated in fresh medium for 15 h. Percentage of apoptotic cells was measured by flow cytometry. Results were obtained from three separate experiments. $^*P < 0.05$ vs. untreated, $CuSO_4$-, and OP-treated cells.

mained in cells after 12 h incubation with fresh medium. Treatment of cells with 20 μM $CuSO_4$ only increased the copper content in cells very slightly, which was about 1.5 folds to untreated control. The results indicated that the significant increase of the copper content in cells which is called copper overload was induced by $Cu(OP)_2$ treatment.

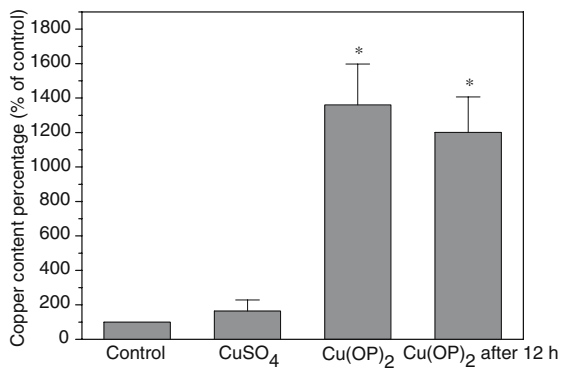

Figure 3. Copper content in Bel-7402 cells measured by atomic absorption spectrometry. Cells were untreated, or treated with 20 μM $CuSO_4$ or $Cu(OP)_2$ for 3 h. For intracellular remaining copper content determination, cells were incubated for further 12 h in fresh medium after treated with 20 μM $Cu(OP)_2$ for 3 h. The results were expressed as percentage of untreated control values, and were obtained from three separate experiments. $^*P < 0.05$ vs. untreated and $CuSO_4$-treated cells.

Intracellular ROS production induced by $Cu(OP)_2$

Production of ROS in Bel-7402 cells after treatment with $Cu(OP)_2$ is shown in Figure 4. It was demonstrated that the fluorescence intensity of DCF significantly increased in cells treated with $Cu(OP)_2$ compared with untreated control, as shown in Figure 4A. The fluorescence intensity enhanced as the concentration of $Cu(OP)_2$ increased from 20 to 80 μM. The fluorescence intensity in cells treated with 20 μM $Cu(OP)_2$ was examined by flow cytometry at different time, as shown in Figure 4B and C. The fluorescence intensity increased continuously during the incubation after treatment with $Cu(OP)_2$, with a peak at around 8 h. The results demonstrated that $Cu(OP)_2$ enhanced intracellular ROS production in Bel-7402 cells.

GSH/GSSG ratio decreased in cells after $Cu(OP)_2$ treatment

GSH is a low molecular weight antioxidant which is abundant in mammalian cells, and GSH/GSSG ratio represents the redox state in cells (Schafer & Buettner 2001). The ratio of GSH/GSSG in untreated control cells kept at around 30:1 during the incubation (data not shown). Treatment with 20 μM $Cu(OP)_2$ for 3 h resulted in significantly decrease of the GSH/GSSG ratio from approximately 30:1 to 7:1 in Bel-7402 cells, and the ratio kept at the low levels when cells were incubated for different time after treatment, as shown in Figure 5. The low GSH/GSSG ratio reflected the redox state in cells, which changed according to the oxidative stress induced by $Cu(OP)_2$.

Oxidative DNA damage in cells after $Cu(OP)_2$ treatment

Comet assay is a very sensitive assay which will detect both single- and double-strand DNA breaks (Collins 2004). The percent of tail DNA content has been used to represent the degree of DNA damage (Krzysztof 2003). A typical control nucleus with no tail of degraded DNA is shown in Figure 6a. Treatment of cells with 20 μM $Cu(OP)_2$ for 3 h induced DNA damage which was shown by the ''comet tail'', and the percent of tail DNA increased from 1.43% of control to 11.39%, as shown in Figure 6b. Treatment of cells with

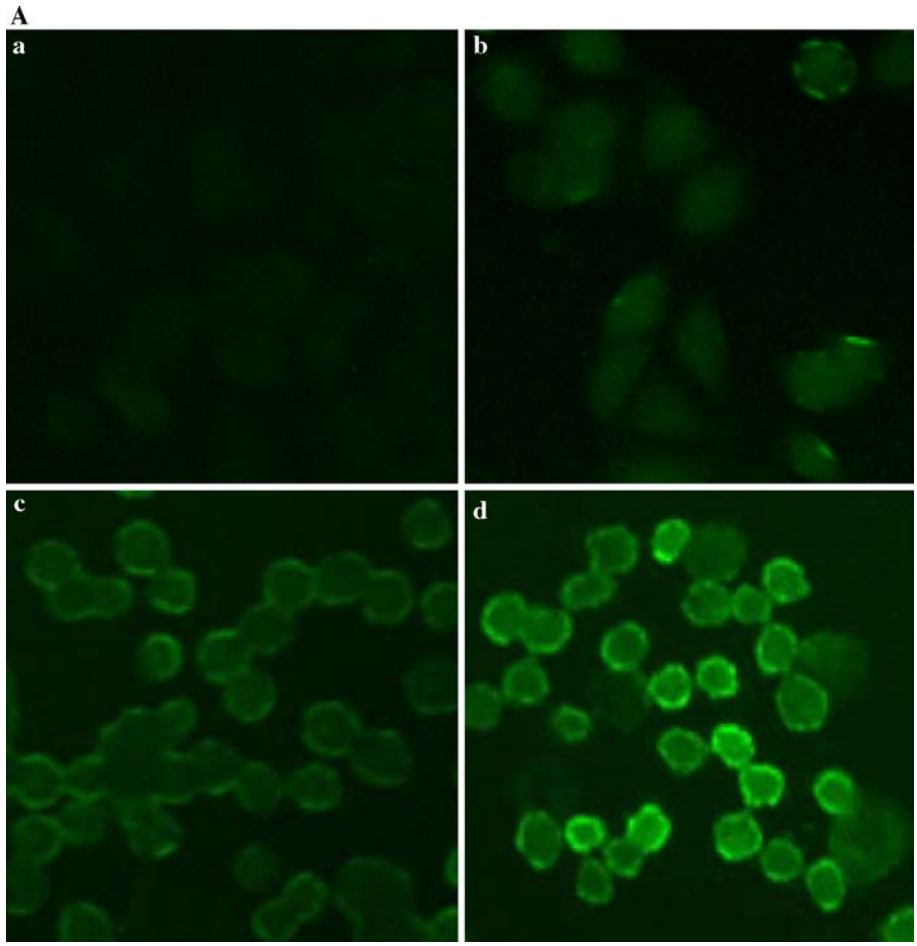

Figure 4. Intracellular ROS production was measured after $Cu(OP)_2$ treatment. (A) Cells were untreated (a) or treated with 20 (b), 40 (c), or 80 μM $Cu(OP)_2$ (d) for 3 h, and DCFH-DA was added to the cells, which were then further incubated for 30 min. Images were obtained on a fluorescence microscope. (B) Cells were untreated (a) or treated with 20 μM $Cu(OP)_2$ for 3 h and incubated in fresh medium for 0 (b), 4 (c), 8 (d) or 12 h (e), then stained with DCFH-DA and the fluorescence intensity was measured by flow cytometry. An experiment representative of three is shown. (C) ROS levels are defined as the percentage between the mean fluorescence of $Cu(OP)_2$-treated and untreated cells. Results were obtained from three separate experiments. $^*P < 0.05$ vs. untreated control cells.

40 μM $Cu(OP)_2$ for 3 h or 20 μM $Cu(OP)_2$ for 6 h enhanced the degree of DNA damage, and the percents of tail DNA were 29.18% and 20.02% respectively, as shown in Figure 6c and d.

Discussion

Apoptosis induced by metals and their complex with chelating agents has been reported previously (Ma *et al*. 1998). It has been reported that $Cu(OP)_2$ induced G_1-phase specific apoptosis in liver carcinoma cell line Bel-7402 (Zhou *et al*. 2002). While whether the apoptosis can be induced by Cu^{2+} or OP alone has not been elucidated. In this research, we have examined the inhibition of the cell viability by $Cu(OP)_2$ or Cu^{2+} alone by time and dose dependency. It was found that treatment with Cu^{2+} alone (20–150 μM) for a short term (3 h) had no effect on the cell viability in our experimental conditions. From the results of DNA content analysis by flow cytometry we found out that neither Cu^{2+} nor OP alone has the ability to induce apoptosis in Bel-7402 cells, while the apoptotic rate increased significantly when Cu^{2+} and OP were both added to the medium. It was shown that OP ligand may play an important role in the cytotoxicity of $Cu(OP)_2$ complex. Copper ions have been reported to induce cytotoxicity in some cell lines such as hepatocyte (Pourahmad

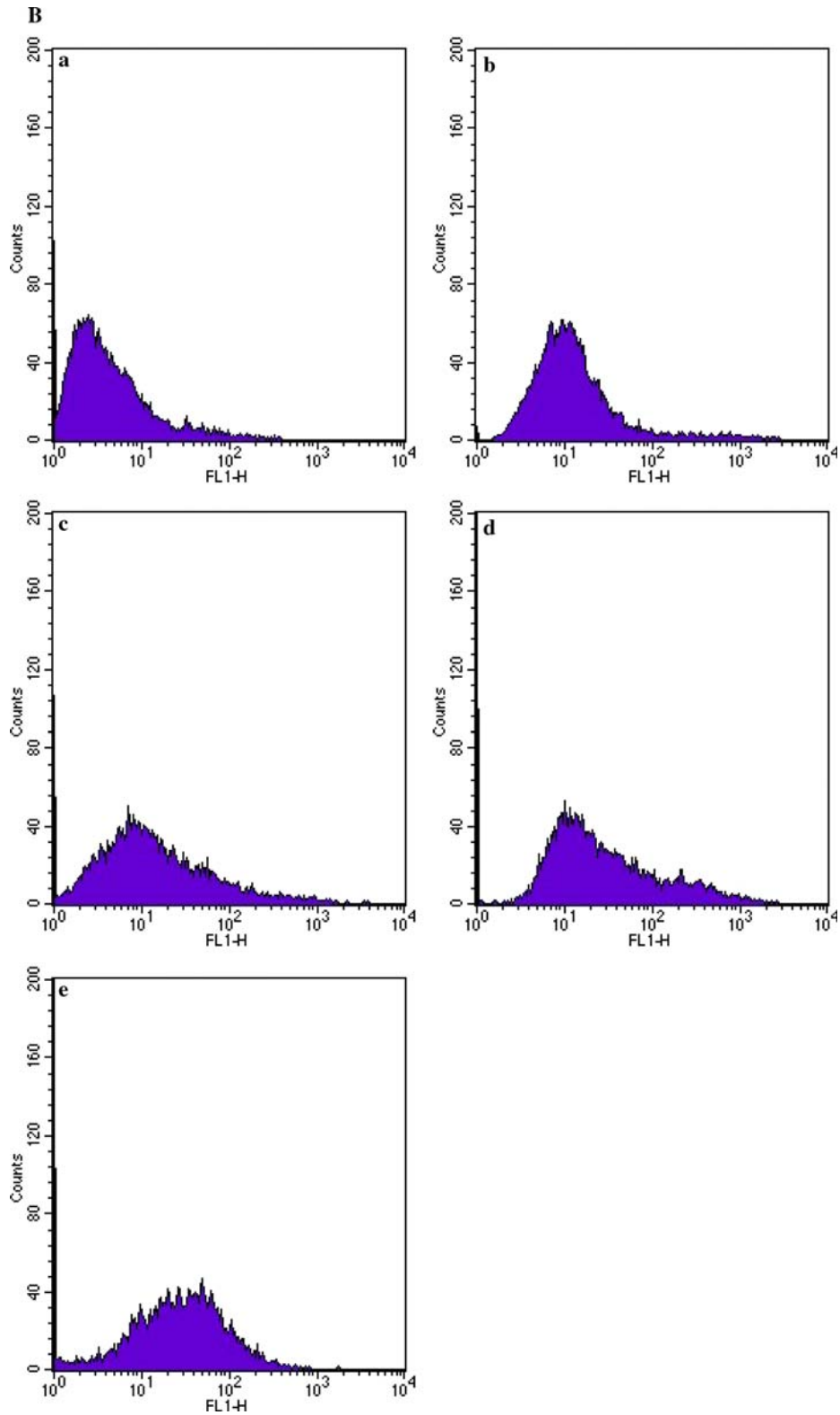

Figure 4. Continued

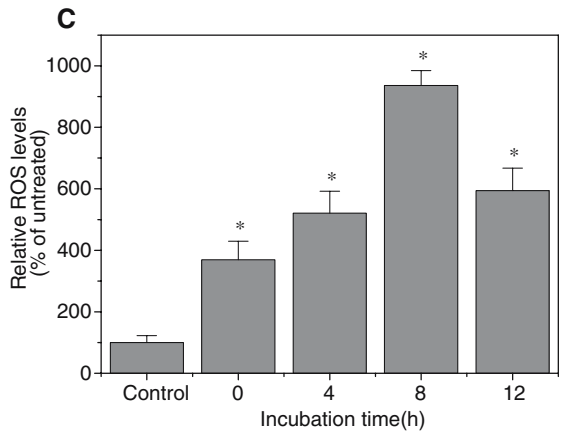

Figure 4. Continued

et al. 2001) and human peripheral blood mononuclear cells (Singh *et al*. 2006). Nevertheless, copper ions do not necessarily promote apoptosis. In dendritic cells copper ions (100–300 μM) failed to induce changes in gene expression characteristic of apoptosis (Linder 2001). These potential effects of copper are likely to be promoted only in special circumstances, such as when chelating agents with specificity for copper can bring the charged ions across cell membrane and enter cells and tissues, or when excessive copper accumulates in cells which is called copper overload (Theophanides & Anastassopoulou 2002). To explore the initial factors in the apoptosis induced by $Cu(OP)_2$, total copper content in cells treated with $Cu(OP)_2$ or Cu^{2+} were measured, and the results indicated that the copper content increased significantly in the cells treated with $Cu(OP)_2$, while treatment with Cu^{2+} alone only increased the copper content in cells very slightly. Cu^{2+} cannot easily cross cell membrane according to its positive charge and hydrophilicity. Biological uptake of copper relies on the presence of copper transporters named copper ATPase (ATP7A), the mutant gene in Menkes disease (Ravia *et al*. 2005). The membrane-permeable OP has the structure of aromatic macro ring and the lipophilicity of OP ligand can help to transport copper through biological membranes, which cause the excessive copper accumulation in cells. The slight increase of copper content in cells treated with Cu^{2+} alone may only performed by the copper transporters ATPase, while the significant increase of copper content in cells treated with $Cu(OP)_2$ may be the consequence of both copper transporters and OP ligand, and OP ligand may play the major role. The imbalance of redox state and intracellular circumstance may occur due to the highly reactive metal. It suggests that the abnormal elevation of the copper content in cells treated with $Cu(OP)_2$ may play the role of initial factor in the apoptosis.

Oxidative stress has been suggested as a mediator of apoptosis (Buttke & Sandstrom 1994) and copper is a highly redox-active metal which can cause ROS formation by a Harber–Weiss reaction (Theophanides & Anastassopoulou 2002; Linder 2001). Herein, a significant dose dependent increase of the ROS levels was observed in Bel-7402 cells at the early time following $Cu(OP)_2$ treatment, which may be induced by the high content of intracellular copper. Free radicals generated from the catalysis of copper ions with intracellular reductants may attack biological macromolecules and result in oxidative damage of cells (Galaris & Evangelou 2002; Zwart *et al*. 1999). The ROS levels increased continuously during the incubation after treatment with $Cu(OP)_2$, with a peak at around 8 h. During this time, ROS also act as signals to initiate a series of physiologic responses in cells, which may be the early events in the cascade of the apoptosis (Jabs 1999; Sun & Oberley 1996).

It is well established that GSH, which represents the major low molecular weight antioxidant in mammalian cells, plays a central role in the cellular defense against oxidative damage (Cotgreave & Gerdes 1998). Loss of GSH and oxidative

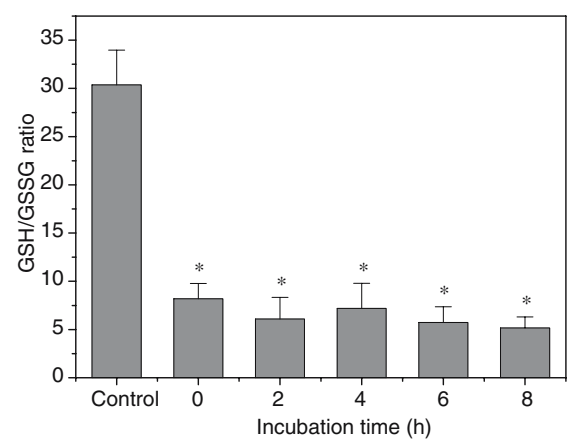

Figure 5. GSH/GSSG ratios in cells were determined after $Cu(OP)_2$ treatment. Cells were treated with 20 μM $Cu(OP)_2$ for 3 h and incubated in fresh medium for 0, 2, 4, 6 or 8 h. *$P < 0.05$ vs. untreated control cells.

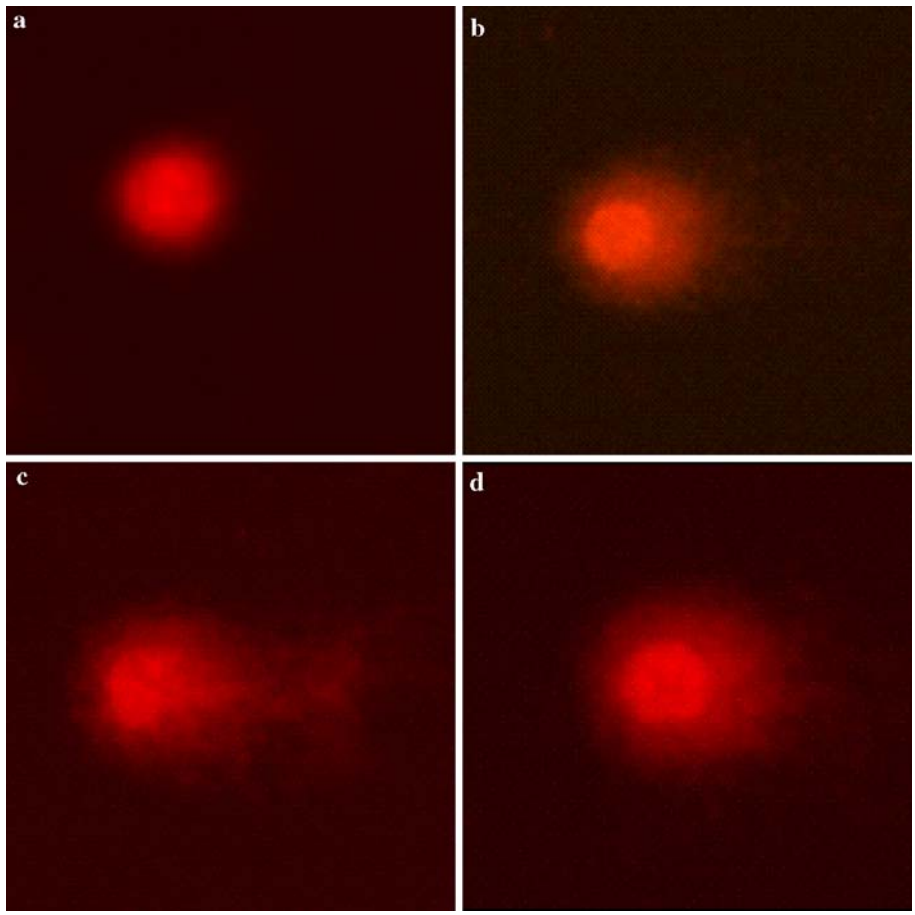

Figure 6. Images of individual nuclei following single-cell gel electrophoresis. Results are representative of those seen in different experiments. Cells were untreated (a), treated with 20 μM $Cu(OP)_2$ for 3 h (b), 40 μM $Cu(OP)_2$ for 3 h (c) or 20 μM $Cu(OP)_2$ for 6 h (d).

damage have been suggested to constitute early, possibly signalling events in apoptotic cell death (Schulz *et al*. 2000). Measurements of total GSH and/or GSSG levels have been used to estimate the redox environment of a cell. Many researchers estimate the redox state of the system by taking the ratio of GSH/GSSG (Schafer & Buettner 2001). It is known from the results that the high thiol depletion activity of $Cu(OP)_2$ decreased the intracellular GSH/GSSG ratio rapidly from approximately 30:1 to 7:1 after treatment for 3 h, and the ratio kept at the low levels around 5:1 in the further incubation. The antioxidant capacity of the cell, deduced from its GSH content and GSH/GSSG ratio, modulates the effect of apoptotic stimuli by reducing free radical production and by regulating transcription factors and protease activities (Klatt & Lamas 2000). Thus the increased ROS production and the decreased GSH/GSSG ratio caused by $Cu(OP)_2$ represented the change of the redox environment in cells which possibly induced the apoptosis by directly causing the intracellular macromolecules damage and modulating the redox state of signal molecules.

Considering the chemical nuclease activity of $Cu(OP)_2$, we examined the effect on cell nuclear DNA caused by $Cu(OP)_2$ using SCGE. DNA damage was found in the early stage of the apoptosis in Bel-7402 cells treated with 20 μM $Cu(OP)_2$ for 3 h, and the higher concentration of $Cu(OP)_2$ or the longer treatment enhanced the damage degree. The early effects on cells by $Cu(OP)_2$ not only include ROS production and GSH depletion, but involve oxidative DNA damage. The excessive copper in cells transported by OP ligand might still exist as the complex due to their high affinity. It

could cross the nuclear membrane and attack the nuclear DNA, which possibly resulted in single- and double-strand breaks, modified bases, and DNA–protein crosslinks (Toyokuni & Sagripanti 1996). DNA damage has been suggested to initiate cell cycle arrest and DNA repair system, and the early events in these pathways are highly conserved (Norbury & Zhivotovsky 2004). The cell cycle arrest may occur at G_1 to S phase transition, which is consistent to our previous reports (Zhou *et al*. 2002). DNA damage activates p53 that either trigger cell cycle arrest or apoptosis, and the apoptotic signaling cascade is initiated due to the expression of p53-dependent apoptotic genes (Chen & Shi 2002). Thus the activation of DNA repair system may play an important role in the pathway of the apoptosis induced by $Cu(OP)_2$.

Our data provide evidence for the involvement of copper overload, cellular redox state change and DNA damage in the apoptosis of Bel-7402 cells induced by $Cu(OP)_2$. The apoptosis pathway may be initiated by the excessive copper in cells transported by OP ligand, and the increased ROS production and the decreased GSH/GSSG ratio were induced by the catalysis of the redox-active copper with intracellular reductants. The damage on the biological macromolecules and the structures of Bel-7402 cells and the modification of the redox-sensitive signal molecules may both contribute to the induction of the apoptosis. DNA damage at the early time may also play an important role in the apoptosis initiation, which possibly activates the DNA repair system and the relevant signal molecules. The cell apoptosis may be the consequence of several factors, and the detailed molecular events will be explored in further experiments.

Acknowledgements

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