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Growth Inhibition and Apoptosis Induction of Gastric Cancer Cells by Copper (II) Glycinate Complex

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Abstract The *in vitro* cytotoxic effects of newly synthesized copper (II) glycinate complex were investigated in two gastric cancer cell lines of SNU484 and SNU638 cells. The complex inhibited the growth and decreased the viability of both gastric cancer cells in a dose-dependent manner. Gastric cancer cells treated with the complex exhibited the features of apoptosis, as demonstrated by fragmentation of chromosomal DNA, activation of caspase-3-like enzyme, and cleavage of poly[ADP-ribose] polymerase (PARP). With the treatment of copper (II) glycinate complex, the active form of caspase-3 was observed in SNU484 cells, but not in SNU638 cells, indicating that an alternative pathway of apoptosis might have been triggered in SNU638 cells. In conclusion, copper (II) glycinate complex induces apoptosis of SNU484 and SNU638 gastric cancer cells, and it is suggested that novel copper (II) glycinate complex is highly active against human gastric cancer cells.

Key words: Copper (II) glycinate complex, apoptosis, caspase, gastric cancer

Apoptosis is an active process of gene-directed cellular self-destruction after the cells come in contact with a certain stimuli. It has been known that impairment of apoptosis is related to immortality and carcinogenesis of cells [26]. The induction of apoptosis in tumor cells is essential to eradicate tumor cells and, therefore, the developments of new chemotherapeutic agents strongly rely on apoptosis of tumor cells.

Apoptosis is induced by a variety of stimuli such as chemicals, chemotherapeutic agents, and radiation [14, 22]. Extracellular signals are received by the cells through the surface receptors, Fas, tumor necrosis factor-α, and transforming growth factor-β [2, 5, 8, 20, 27]. Endogenous signals trigger the release of cytochrome *c* from mitochondria [19]. Several pathways exist to transduce the apoptotic signals to the cell death machinery. Caspases is a family of cysteine proteases that plays a central role in the apoptotic pathway [1, 9, 21, 23, 25]. Regarding the apoptotic stimulation, multiple caspases are sequentially activated and ultimately break the cellular structures by recognition and cleavage of specific substrate proteins, which eventually leads to apoptotic morphologies.

Gastric cancer is the most common malignant tumor among Korean women. The patients who are suffering from advanced gastric cancer are treated with chemotherapy and/or radiation therapy. Several chemotherapeutic agents such as cisplatin, 5-fluorouracil, mitomycin C [10, 12, 15, 22], and natural products such as urishiol [3], chitosan [13], and *Anemarrhena asphodeloides* [26] have been reported to induce apoptosis in gastric cancer cell lines. However, chemotherapeutic agents often have severe side effects and it is necessary to develop new chemotherapeutic agents which specifically kill tumor cells. We recently synthesized copper (II) glycinate complex as a chemotherapeutic agent. Elucidating the mechanisms by which copper (II) glycinate complex cause cell death is essential to further improve this metal complex as a chemotherapeutic agent. The goal of this study was to evaluate the cytotoxic effects of copper (II) glycinate complex against gastric cancer cells and the mechanisms by which copper (II) glycinate complex kill gastric cancer cells.

Materials and Methods

Preparation of Copper (II) Glycinate Complex

Copper sulfate and glycine were used as copper (II) and aminoacidate sources to synthesize copper (II) aminoacidate

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complex [4]. Copper sulfate and glycine were separately dissolved in sterile distilled water at room temperature, and the glycine aqueous solution was slowly added to the copper sulfate aqueous solution. The mixture was constantly stirred for 30 min, and then pH was adjusted to 8.0 with ammonia water. This copper (II) glycinate complex was used for the following experiments without further purification or drying. The color of copper (II) glycinate complex was clear dark blue.

Cell Lines and Culture Conditions

Two gastric cancer cell lines of SNU484 and SNU638 were used. Cell lines were purchased from the Korean Cell Line Bank (Seoul National University, Seoul, Korea). SNU484 cells were originated from a primary gastric cancer in a 53-aged male, which was diagnosed as a poorly differentiated adenocarcinoma. SNU638 cells were obtained from the pleural exudates of a 48-aged male who was diagnosed as a poorly differentiated adenocarcinoma of stomach. Both SNU484 and SNU638 cells carried the mutated *p16* gene [16, 18]. The cells were maintained in RPMI 1640 (GIBCO Laboratories, Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, Utah, U.S.A.), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were cultured in a 5% CO_2 incubator at 37°C. For the induction of cell death, cells were seeded in new dishes and grown to 80% of confluent monolayers.

Determination of Cell Growth

The growth inhibition of gastric cancer cells was measured by using the Premix WST1 cell proliferation assay system (TaKaRa Shuzo Co. Ltd., Shiga, Japan). WST1 is a tetrazolium salt and cleaved to formazan dye by the succinate-tetrazolium reductase, which exists in mitochondrial respiratory chain that is active only in viable cells. The dark red formazan dye formed by metabolically active cells was quantitated by measuring its absorbance. SNU484 and SNU638 cells were seeded at the concentration of 2.0×10^5/ml in 96-well microplates. Copper (II) glycinate complex was added to the wells of the microplate at a range of 0.04 to 40 μg/ml. The growth inhibition of the cells was measured at 450 nm 3 h after treating WST1. For each cell line, the same concentration of copper (II) glycinate complex was placed in 4 wells per experiment. The growth inhibition of each cell line was measured with the WST1 assay on 0, 6, 12, 24, and 48 h.

Trypan Blue Exclusion Assay

The viability of gastric cancer cells was determined by using the trypan blue exclusion assay. After treated with copper (II) glycinate complex for 48 h, the cells were harvested and stained with 0.4% trypan blue. Trypan blue-stained and -unstained cells were counted by using a hemocytometer [17].

Measurement of DNA Fragmentation

After cells were treated with copper (II) glycinate complex for 48 h, the adherent and detached cells were harvested and analyzed for DNA fragmentation [11, 17]. Briefly, the detached cells were collected by centrifugation at 1,000 ×g for 5 min at 4°C, washed with ice-cold PBS, and resuspended in lysis buffer [0.5% Triton X-100, 5 mM Tris (pH 7.4), 20 mM EDTA]. The adherent cells were washed with PBS and lysed directly by adding lysis buffer to the dish. The lysates were collected and kept on ice for 15 min. After centrifugation at 12,000 rpm for 15 min at 4°C, the supernatants were incubated with RNase A (5 μg/ml) at 37°C for 1 h, followed by 1 h incubation at 50°C with proteinase K (200 μg/ml). After extraction with phenol/chloroform, DNA was precipitated with ethanol and 3 M sodium acetate. Fragmented DNA was analyzed on 1.5% agarose gel.

Determination of Caspase-3-Like Enzymatic Activity

Following the treatment with copper (II) glycinate complex, cells were lysed in lysis buffer [50 mM Tris (pH 7.5), 0.03% NP-40, 1.0 mM of DTT] for 30 min on ice. After centrifugation at 12,000 rpm for 30 min at 4°C, protein concentration in the supernatant containing cytosolic extracts was quantitated by following the Bradford assay kit (Life Science Co., CA, U.S.A.). For the analysis of caspase-3-like enzymatic activity, the synthetic tetrapeptide substrate, ac-DEVD-*p*NA (Enzyme System Product Co., CA, U.S.A.), was used. The lysates (20 μg) were incubated with 0.2 mM of ac-DEVD-*p*NA in a total volume of 0.1 ml. Assays were performed in duplicate, and the results were presented as the average increase in absorbance at 405 nm.

Western Blot Analysis

Cells were lysed in lysis buffer [10 mM of Tris (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, phenylmethylsulfonylfluoride (10 μg/ml), aprotinin (10 μg/ml), leupeptin (10 μg/ml), 5 mM phenanthroline, and 28 mM benzamidine-HCl] for 30 min on ice. The lysates were cleared by centrifugation at 12,000 rpm for 30 min, and protein concentration was quantitated by using the Bradford assay kit. Supernatant containing 30 μg of proteins was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 8% or 12% gel, and bands were then transferred to a polyvinylidene difluoride membrane (Millipore Co.). The membrane was reacted with primary antibodies, followed by treatment with horseradish peroxidase-conjugated secondary antibody. The bands were detected by enhanced chemilluminescence kit (Amersham Life Science Co., Buckinghamshire, U.K.) according to the manufacturer's instructions. Primary antibodies against poly[ADP-ribose] polymerase (PAPR, Enzyme System products Co.), caspase-3 (Santa Cruz Co., CA, U.S.A.), and β-actin (Santa Cruz Co.) were applied at optimized concentrations.

RESULTS

Growth Inhibition of Gastric Cancer Cells by Copper (II) Glycinate Complex

To determine the growth inhibition of gastric cancer cells, SNU484 and SNU638 cells were treated with the range of 0.04 to 40 μg/ml of copper (II) glycinate complex for 48 h. Copper (II) glycinate complex inhibited the growth of SNU484 and SNU638 cells in a dose-dependent manner (Fig. 1). When both gastric cancer cells were treated with 2.5 μg/ml or higher concentrations of copper (II) glycinate complex, the growth of both gastric cancer cells was completely inhibited at 6 h of the experiment.

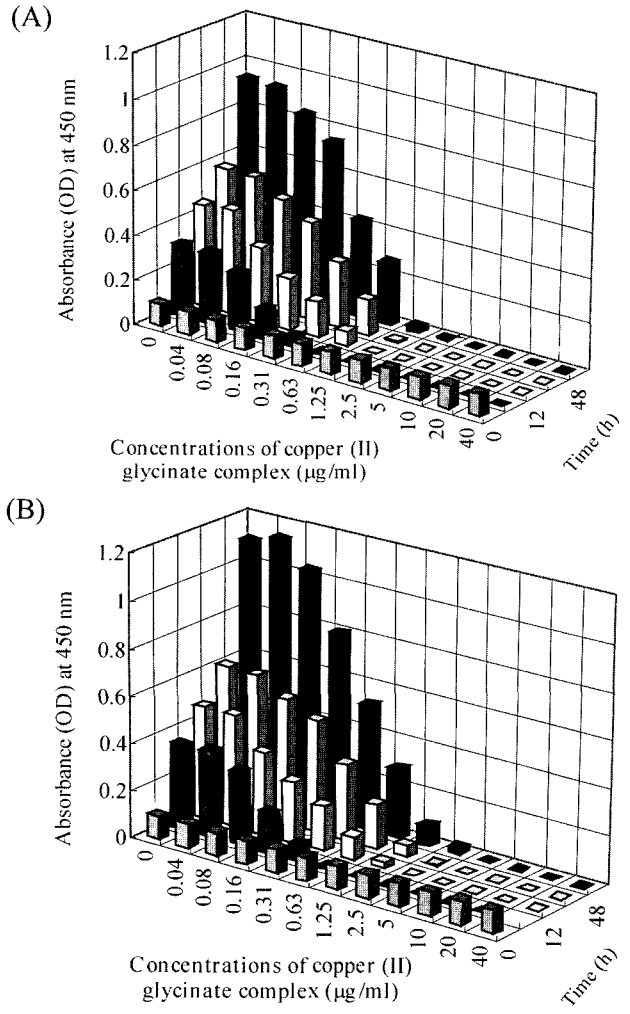

Fig. 1. Growth inhibition of SNU484 (A) and SNU638 cells (B) treated with copper (II) glycinate complex.
Growth inhibition of the cells was determined by using WST1 assay. Both SNU484 and SNU683 cells were treated with copper (II) glycinate complex for 48 h. Values represent the average of four wells from a representative experiment.

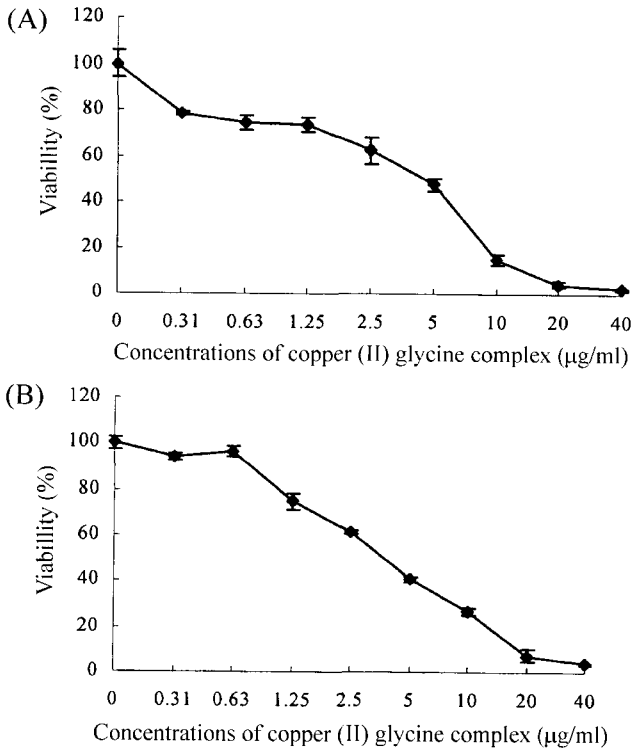

Fig. 2. Effect of copper (II) glycinate complex on the viability of SNU484 (A) and SNU638 cells (B).
Cells were treated with copper (II) glycinate complex for 48 h and the viability of cells was determined by the trypan blue exclusion assay. Values represent the mean±SD of three independent experiments.

Apoptosis of Gastric Cancer Cells by Copper (II) Glycinate Complex

To determine the viability rate of gastric cancer cells, the cells were treated with the range of 0.31 to 40 μg/ml of copper (II) glycinate complex for 48 h and then stained with trypan blue. The viability of SNU484 and SNU638 cells was decreased in a dose-dependent manner (Fig. 2). Both gastric cancer cells were less than 10% viable at the concentration of 20 μg/ml. To determine whether the death of gastric cancer cells was due to apoptosis, DNA from the cells was isolated and analyzed by using the gel electrophoresis. Chromosomal DNA from SNU484 and SNU638 cells which were treated with 40 μg/ml of copper (II) glycinate complex exhibited DNA breakdown with faint apoptotic laddering in increments of approximately 200 bp, which correlated with the approximate size of a nucleosome (Fig. 3), indicating that both gastric cancer cells were undergoing apoptosis by the complex.

Activation of Caspase and Degradation of Caspase Substrate in Gastric Cancer Cells

The induction of apoptosis is mediated through ordered series of events, such as the activation of caspases followed

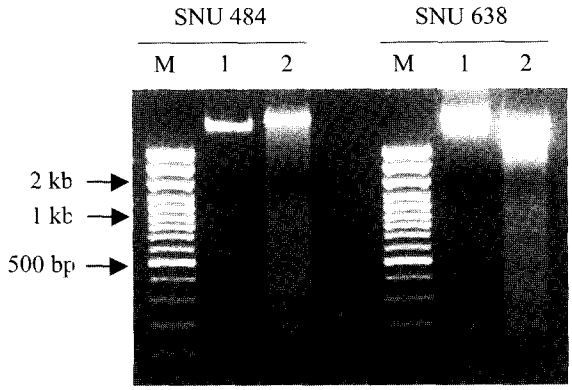

Fig. 3. Analysis of internucelosomal DNA fragmentation of SNU484 and SNU638 cells.
Cells were cultured with medium alone (lane 1) or with 40 μg/ml of copper (II) glycinate complex (lane 2) for 48 h and then DNA was extracted from the cells. Extracted DNA was electrophoresed on 1.5% agarose gel.

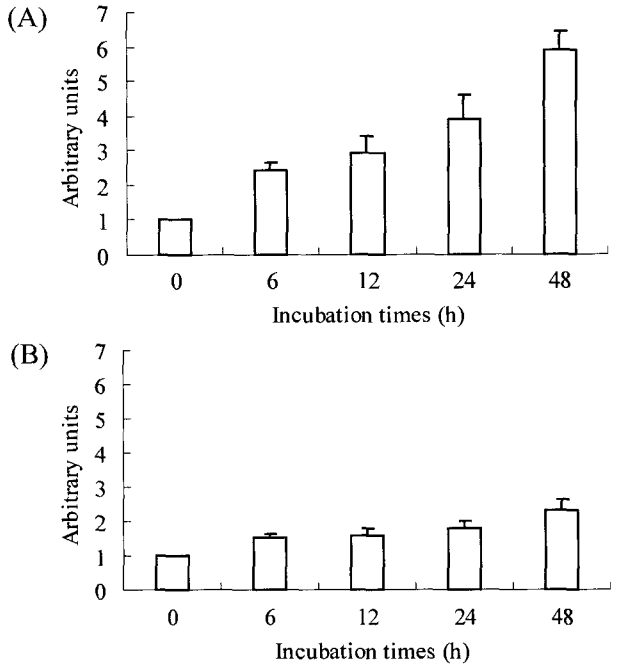

Fig. 4. Analysis of caspase-3-like enzymatic activity in SNU484 (A) and SNU638 (B) cells.
Cells were treated with 40 μg/ml of copper (II) glycinate complex for the indicated times. The caspase-3-like activity was measured as absorbance at 405 nm. Values represent the mean±SD of three independent experiments.

by its substrate degradation. To investigate the activation of caspases in gastric cancer cells treated with copper (II) glycinate complex, caspase-3-like enzymatic activity was determined. Thus, SNU484 and SNU638 cells were treated with 40 μg/ml of copper (II) glycinate complex for 48 h. The caspase-3-like activity of both cancer cells was increased 5.9-fold and 2.3-fold, respectively, compared with the control cells (Fig. 4). To confirm the activation of caspase-3 in the complex-treated gastric cancer cells, the cleaved forms of caspase-3 and nuclear enzyme PARP, a common substrate for multiple caspases [7], were detected by the Western blot. Caspase-3 is synthesized as a 32 kDa precursor, which is cleaved to 17 kDa and 12 kDa subunits on activation. In the SNU 484 cells, a 32 kDa precursor form was decreased and a 17 kDa subunit appeared 24 h after being treated with copper (II) glycinate complex (Fig. 5). In consistence with this finding, a 116 kDa PARP was also cleaved to a 85 kDa subunit under the same condition. The active form of caspase-3 was not observed in the SNU638 cells, although PARP was cleaved to a 85 kDa subunit.

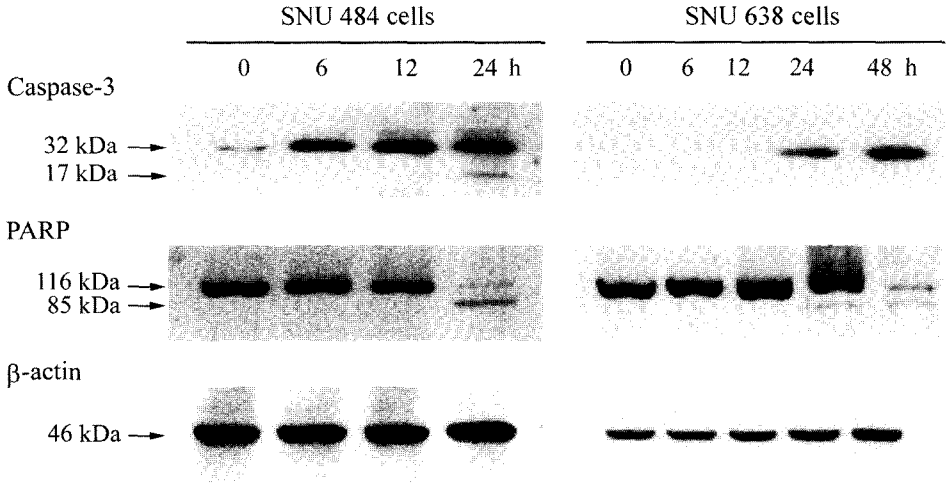

Fig. 5. Western blot analysis of caspase-3 and PARP in SNU484 and SNU638 cells.
Cells were treated with 40 μg/ml of copper (II) glycinate complex for the indicated times. Cell lysates were resolved on 8% or 12% polyacrylamide gel and immunoblotted with anti-caspase-3, anti-PARP, and anti-β-actin antibodies.

DISCUSSION

Metal chelating agents such as cobalt, copper, iron, manganese, ruthenium, and zinc, were found to be active against a large number of microorganisms by capturing the metallic ions necessary for metabolism of microorganisms [4]. Moreover, *in vitro* study has revealed that certain metal chelating agents were active against some tumor cells [6, 10, 15, 24]. These finding suggest that certain metal chelating agents can be used for the treatment of tumors in humans. Among metal-based chemotherapeutic agents, drugs based on endogenous metals may be less toxic, and this leads to development of copper-based drugs [15]. Many copper-based anticancer complexes have been developed, and cisplatin is one of the most commonly used chemotherapeutic agents for treating malignant tumors [6, 10, 24].

In this study, we evaluated the growth inhibition and apoptotic cell death of two gastric cancer cell lines by newly synthesized copper (II) glycinate complex. Copper (II) glycinate complex inhibited the growth and induced apoptosis of both SNU484 and SNU638 cells in a dose-dependent manner. The caspase-3-like enzymatic activity of SNU 484 cells was significantly increased by copper (II) glycinate complex. Consistent with this finding, the cleaved form of caspase-3 and PARP was observed. These results indicated that caspase-3 was activated in SNU484 cells undergoing copper (II) glycinate complex-induced apoptosis. On the other hand, an active form of caspase-3 was not observed in SNU638 cells during 48 h of observation, although the caspase-3-like enzymatic activity of SNU638 cells was also slightly increased. These results indicated that copper (II) glycinate complex triggered an alternative pathway of apoptosis in the SNU638 cells. The question of whether apoptosis of gastric cancer cells occurred via release of cytochrome *c* or binding of copper (II) glycinate complex to surface receptors is under investigation. In addition to gastric cancer cells tested, copper (II) glycinate complex also effectively killed many tumor cells, such as laryngeal carcinoma cells (HEp-2 cells), cervical carcinoma cells (HeLa cells), leukemia cells (HL 60 cells), and hepatoma cells (SNU475 cells) *in vitro* (data not shown). These results indicate that copper (II) glycinate complex is active against a wide range of tumor cells.

Although the mechanism by which copper (II) glycinate complex induces apoptosis in gastric cancer cell lines is unclear, our findings that copper (II) glycinate complex actually induced apoptosis of gastric cancer cells suggest a possible role of the complex as a chemotherapeutic agent.

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