Anticancer activity of CopA3 dimer peptide in human gastric cancer cells

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INTRODUCTION

Antimicrobial peptides (AMPs) have been identified and characterized from most living organisms, including humans. AMPs have common features such as low molecular weight (less than 100 aa), net positive charge (cationic), an amphipathic structure, and they are heat-stable with low antigenicity (1). Moreover, AMPs exhibit broad-spectrum activity against various microbes together with a rapid mode of action of less than 100 minutes (2). AMPs are classified into five functional groups: anti-bacteria, anti-fungi, anti-virus, anti-parasites, and anti-cancer (3).

CopA3 is a homodimeric α-helical peptide derived from coprisin which is a defensin-like antimicrobial peptide that was identified from the dung beetle, Copris tripartitus. CopA3 has been reported to have anticancer activity against leukemia cancer cells. In the present study, we investigated the anticancer activity of CopA3 in human gastric cancer cells. CopA3 reduced cell viability and it was cytotoxic to gastric cancer cells in the MTS and LDH release assay, respectively. CopA3 was shown to induce necrotic cell death of the gastric cancer cells by flow cytometric analysis and acridine orange/ethidium bromide staining. CopA3-induced cell death was mediated by specific interactions with phosphatidylserine, a membrane component of cancer cells. Taken together, these data indicated that CopA3 mainly caused necrosis of gastric cancer cells, probably through interactions with phosphatidylserine, which suggests the potential utility of CopA3 as a cancer therapeutic.

Keywords: Antimicrobial peptide, Anticancer activity, CopA3, Necrosis, Phosphatidylserine

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Recently, AMPs have been featured as potential anticancer therapeutic agents. Anticancer activities of AMPs have been demonstrated against a broad range of cancer cell lines (4). AMPs are emerging as novel therapeutic alternatives that could reduce the detrimental side effects (toxicity and resistance) of conventional chemotherapeutics based on their mechanism of action and specificity. Two mechanisms of anticancer activity for AMPs have been proposed: necrosis via cell membrane damage and apoptosis via the mitochondrial pathway (5, 6). Interactions between AMPs and anionic cancer cell membrane surfaces initiate the process (7). Some important characteristics specific to cancer cells are exposure of phosphatidylserine (PS), increased number of sialic acid residues and heparan sulfate, changes in membrane fluidity and pH, and increased micravilli (7). Thus, this increase in the number of negative charges (from PS, sialic acid, and heparan sulfate) on the surface of cancer cells promotes the interaction with cationic AMPs to increase membranolytic activity during necrosis. In addition, the mitochondrial membrane is a major target of AMPs that have penetrated into the cytoplasm because of its high negative charge (i.e., cardiolipin) during apoptosis (7).

Cancer is one of the leading causes of death worldwide; lung, stomach, liver, colon, and breast cancer are the most frequently diagnosed cancers (8). Among them, gastric cancer has the fourth most incidences in the world, and gastric cancer occurs at a very high rate in East Asian countries (9). In Korea, the incidence of gastric cancer is second only to thyroid cancer; 30,092 people were diagnosed with gastric cancer in 2010, which accounted for 14.9% of total cancer cases (10). Surgical removal followed by localized radiotherapy and conventional chemotherapy are the primary regimens to treat the disease. Novel therapeutic regimens for the treatment of gastric cancer are being developed to improve efficacy as well as to reduce the side effects of conventional chemotherapeutics.

Previously, we have identified an insect defensin, named coprisin, which was isolated from the E. coli-challenged Dung beetle, Copris tripartitus (11). The synthetic peptide CopA3, an analogue derived from coprisin, was shown to decrease the cell viability of human leukemia cancer cells and induce apoptosis in AML-2, Jurkat, and U937 cancer cells via a caspase-independent pathway (12). CopA3 also demonstrated antimi-
brial activity against various microbes (13, 14). Here, we investigated the anticancer activity of the CopA3 peptide against human gastric cancer cells. We showed that the anticancer effect of CopA3 is mainly attributed to interactions with PS on the cell surface, which initiates necrosis in the cancer cells.

RESULTS

CopA3 reduced cell viability of gastric cancer cells
We investigated the effect of the synthetic peptide CopA3 on the cell viability of a human gastric cancer cell line (SNU-484, 601, 638, 668). Cancer cells were treated with various concentrations of CopA3 (10, 25, 50, and 100 μM) for 24 h, and cell viability was measured by the MTS assay. CopA3 decreased the viability of the gastric cancer cells in a dose-dependent manner (Fig. 1A). The IC50 of CopA3 for gastric cancer cells was about 20-50 μM. In contrast, Raw 264.7 cells were relatively resistant to CopA3 treatment, which suggests some specificity of CopA3 against the gastric cancer cells.

Effect of CopA3 on cancer cell membrane integrity
To delineate the cytotoxic mechanism of CopA3 on the gastric cancer cells, we determined the effect of pre-treatment with cancer cell-specific membrane components (ganglioside, heparin, and PS) on CopA3-induced reduced viability of the cancer cells. The viability of CopA3-treated cancer cells increased with increasing PS concentration in a dose-dependent manner (Fig. 2). The addition of ganglioside had less effect on cell viability in the presence of CopA3. The effect of the cancer cell membrane molecules on the antibacterial activity of CopA3 was also examined to determine the specific binding of CopA3 on the cancer cell surface. A constant amount of CopA3 (1 μg)

Fig. 1. Cell viability and cytotoxicity of human gastric cancer cells after CopA3 treatment. (A) Cell viability was measured by the MTS assay after 24 h incubation with indicated amount of the peptides. (B) After 24 h incubation with the peptide, LDH activity from each cell was detected using the LDH release assay. Each symbol represents the mean value from triplicate experiments and the error bars depict standard deviations.

Fig. 2. Effect of anionic cancer cell-surface molecules on cell viability. Viability assay of gastric cancer cells was performed by mixing various amounts of ganglioside, heparin, or phosphatidylserine with 25 μM CopA3. Data are expressed as means ± standard deviations from three individual measurements. Statistical analyses were performed as described. ***P < 0.001, **P < 0.01, *P < 0.05, compared to the non-treated control cells.
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was incubated at different concentrations of ganglioside, heparin, PS, phosphatidylcholine (PC), phosphatidylethanolamine (PE), or sphingomyelin, and then the mixture was tested for antibacterial activity in the radial diffusion assay (Fig. 3A). The antibacterial activity of CopA3 was significantly reduced as the amount of heparin and PS increased in the mixture. In contrast, the other cell membrane components did not affect the antibacterial activity of CopA3. In addition, we conducted a calcein leakage assay to elucidate the interaction of CopA3 with cell membrane molecules using artificial liposomes (Fig. 3B). Two types of calcein-entrapped liposomes consisting of PS, and sphingomyelin were prepared and the amount of calcein release was determined from the liposomes at different peptide concentrations. As a result, CopA3 induced calcein leakage from the PS liposomes. The result was consistent with the anti-E. faecalis activity of CopA3 in the presence of membrane components in radial diffusion assay (Fig. 3A). While polymixin B slightly induced calcein leakage from PS liposome, CopA3 well induced leakage of calcein dose-dependently. In contrast, when sphingomyelin liposome was treated with peptides, little or no calcein leakage was detected. These findings suggest that CopA3 interacts with heparin and PS, which could result in CopA3-induced cytotoxicity in cancer cells as well as in bacteria.

CopA3 induced apoptosis and necrosis in the gastric cancer cells
We assessed the nature of the cytotoxic effect of CopA3 to characterize the mechanism of reduced viability. Apoptosis of the cancer cells was examined by Annexin V/PI staining of the CopA3-treated gastric cancer cells (Fig. 4A). Annexin V binding to the CopA3-treated gastric cancer cells increased at a peptide concentration of 25 μM and 50 μM. The Annexin V-positive/PI-negative population reached maximum at 50 μM CopA3, while the Annexin V-negative/PI-positive population gradually increased as the peptide concentration increased and reached a maximum at the highest concentration of CopA3 (100 μM). These data indicate that CopA3 induced both apoptosis and necrosis in the gastric cancer cells, although necrotic cell death was dominant at higher concentrations of CopA3.

Acridine orange/ethidium bromide staining
Membrane integrity of the SNU-668 cells upon CopA3 treatment was analyzed by acridine orange/ethidium bromide staining. After CopA3 treatment for 1 h, the number of orange-colored nuclei increased in CopA3-treated cells with increasing CopA3 concentration, while the majority of control cells exhibited a green fluorescence (Fig. 4B). The cells treated with CopA3 exhibited an orange and orange-red fluorescence, indicating severe membrane disruption at high concentrations of CopA3.

DISCUSSION
We have described the anticancer activity of CopA3 against
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Fig. 4. CopA3 induces apoptosis and necrosis in SNU-668 human gastric cancer cells. (A) Flow cytometric analyses of the gastric cancer cells treated with various concentrations of CopA3 for 1 h. The cells were stained with annexin V-FITC and PI and subjected to flow cytometry. The distribution of the cells is presented as the percentage of total cells counted (10,000). (B) Gastric cancer cells were stained with acridine orange/ethidium bromide after incubation with CopA3. Cells were observed under a fluorescence microscope (200×). Live cells show green fluorescence, and necrotic cells show orange fluorescence.

the human gastric cancer cell lines. CopA3 selectively reduced the viability of the gastric cancer cells, while CopA3 did not exert a significant cytotoxic effect on Raw264.7 cells (Fig. 1A). Since PS and heparin inhibited CopA3-induced reduced viability of the cancer cells, the anticancer activity might be ascribed to the interaction between cationic AMPs and anionic surface molecules of cancer cells.

To investigate the anticancer mechanism of CopA3, we assessed the cytotoxicity of the peptide using the LDH release assay. CopA3 was found to disrupt the membrane integrity of the gastric cancer cells and thereby increase LDH release in a dose-dependent manner with increasing peptide concentrations (Fig. 1B). CopA3-treated Raw264.7 cells showed cytotoxic activity; however, the lytic activity was significantly lower compared to that in the gastric cancer cells. A lower percentage of FBS in the LDH release assay (1% FBS) compared to the MTS assay (10% FBS) might enhance cell death, resulting in higher LDH release at high CopA3 concentrations, which suggests that the lytic activity of CopA3 is influenced by serum levels. We examined the viability of Raw264.7 cells in serum-free conditions after CopA3 treatment and found that the viability of the cells remained > 70% at 50 µM CopA3, while the viability dropped to < 10% at 100 µM CopA3 (data not shown). These data indicate that an effective dose of CopA3 is ≤ 50 µM.

It has been reported that enantiomeric 9-mer peptide derived beetle defensins showed anticancer activity against several cancer cell lines, and the peptides selectively targeted PS on the surface of cancer cell membranes (15). In addition, diastereomeric lytic peptides also interacted with PS in PS-containing model membranes (16). Therefore, we examined whether the viability of gastric cancer cells was affected by CopA3 in the presence of anionic cell surface molecules (Fig. 2). Results from these experiments strongly suggested that cell-surface PS was a target molecule for CopA3 binding. To support this hypothesis, we further analyzed the antimicrobial activity of CopA3 in the presence of cell membrane molecules by using a radial diffusion assay (Fig. 3A) and induction of calcine leakage from liposome by CopA3 treatment in calcine leakage assay (Fig. 3B). The results of the present study revealed that the binding affinity of CopA3 for PS was shown to be critical for the anticancer activity. Thus, the results from these experiments indicated that PS might be a potential target for CopA3 binding, in addition to heparin. Buforin IIb is a cell-penetrating anticancer peptide that causes apoptosis and targets cancer cells through interactions with gangliosides (17). However, CopA3 did not bind to gangliosides and retained its antibacterial activity in the presence of gangliosides. Thus, it is likely that the target molecules on the surface of cancer cell are dependent on the peptide’s mechanism of action.

The mechanism of CopA3-induced cell death was further assessed by flow cytometry analysis with annexin V and PI staining (Fig. 4A). The results revealed that cell death of gastric cancer cells depend on CopA3 concentration and incubation time. Induction of apoptotic or necrotic cell death by a lactoferrin-derived peptide (Pep1) was also shown to be concentration-dependent (18). CopA3-induced necrosis was more prevalent than CopA3-induced apoptosis at a high peptide concentration. Flow cytometric analysis of Annexin V/PI binding showed CopA3-induced apoptosis predominated with a
short incubation time (1 h) (Fig. 4A) at a low CopA3 concent-
tration, while necrotic cell death was more prevalent over an
apoptosis longer incubation time (4 h) at both low and high
CopA3 concentrations (data not shown). These data suggest
that CopA3-induced apoptosis is transient and was only ini-
tiated at low CopA3 concentrations. In addition, we stained
CopA3-treated SNU-668 cells with acridine orange/ethidium
bromide to verify the annexin V/PI staining results (Fig. 4B).
These results indicated that CopA3-treated cells were not un-
dergoing apoptosis; strong staining with EtBr indicated that
the cells were primarily necrotic.

In conclusion, our findings demonstrate that CopA3 displays
selective anticancer activity in human gastric cancer cells. In
vitro analysis by the LDH release assay, flow cytometric anal-
ysis, and acridine orange/ethidium bromide staining indicated
that CopA3 primarily induced necrosis in the cancer cells. The
anticancer and antimicrobial activities of CopA3 were reversed
by incubation with heparin and PS, which suggests that these
activities are dependent on an interaction between CopA3 and
heparin/PS. Collectively, these results suggest the potential util-
ity of CopA3 as a therapeutic for gastric cancer.

MATERIALS AND METHODS

Peptide

The homodimeric peptide CopA3 was synthesized using the
solid-phase peptide synthesis method by Anygen Co., Ltd.
(Gwangju, Korea). The peptide was dissolved in acidified dis-
tilled water (0.01% acetic acid) and stored at −20°C until use.
CopA3 consists of a homodimer of two subunits that contain
nine residues (LLCIALRKK-NH2) each. The two monomers are
linked by a single disulfide bond between the third Cys of
each monomer. The total net charge of CopA3 is +6.

Cell culture

Raw 264.7, Caki, and HeLa cells were maintained in DMEM,
and SNU-668 cells were maintained in RPMI-1640 medium
supplemented with 10% FBS, penicillin G (100 U/ml), and
streptomycin (100 μg/ml) (Invitrogen, USA). Cells were cul-
tured at 37°C in a humidified incubator with 5% CO2.

MTS assay for cell viability

Cells plated into 96-well tissue culture plates (2 × 10^4 cells
per well) on the previous day were treated with various con-
centrations (10, 25, 50, and 100 μM) of CopA3 or without
CopA3. After incubation for 24 h, the viability of the cancer
cells was assessed by the Cell Titer 96 AQueous One Solution
Cell Proliferation Assay according to the manufacturer’s proto-
col (Promega, USA). The optical density at 490 nm was meas-
ured with a microplate reader (Beckman DTX 8800 multi de-
tector). To investigate the interaction of CopA3 with cell mem-
brane components, cells were treated with ganglioside, hepa-
in, or PS at the indicated concentration in the presence of
CopA3. Then, cell viability was measured as described above.

LDH release assay

Cell membrane integrity was analyzed by measuring LDH
activity. LDH activity was monitored using a Cytotoxicity De-
tection Kit (Roche Applied Science, Germany). In brief, cells
were seeded at 1 × 10^5 cells per well in a 96-well culture
plate in assay medium (DMEM or RPMI-1640 containing 1% FBS)
and were treated with different doses of CopA3. After 24 h
of incubation, 5 μl of lysis solution was added to high con-
trol samples as a positive control, and the plate was incubated
for an additional 15 min. Then, 100 μl of the reaction mixture
was added to each well, followed by a 15-min incubation.
Finally, 50 μl stop solution was added to each well, and the
absorbance was measured using a microplate reader at 490
nm. The percent cytotoxicity was calculated by the following
equation: Cytotoxicity (%) = (exp. value - low control) / (high
control − low control) × 100

Binding of CopA3 to cancer cell-specific membrane
components using the radial diffusion assay

The ability of CopA3 to bind to the surface of SNU-668 cells
was examined by assessing the effect of cancer cell membrane
components on the anti-E. faecalis activity of CopA3 in a radial
diffusion assay (19). One microgram of CopA3 was incubated
with different amounts of ganglioside, heparin, PS, phosphati-
dylcholine (PC), phosphatidylethanolamine (PE), or sphingo-
myelin for 10 min at 37°C in 10 mM sodium phosphate buffer
(pH 7.4). Then, 5-μl aliquots of each mixture were loaded into
wells (3-mm diameter) that had been punched in the underlay
agar containing washed mid-logarithmic E. faecalis (4 × 10^5
colony forming units). The underlay agar consisted of 9 mM
sodium phosphate, 1 mM sodium citrate buffer, 1% (w/v) agar-
ose (Sigma, A6013), and 0.3 mg of tryptic soy broth (TSB;
Difco). After incubation at 37°C for 3 h, a 10-ml overlay agar of
1% agarose and 6% TSB was poured onto the underlay
agar. The diameters of the clearing zones were used to in-
dicate the antibacterial activity, which were plotted against the
cell membrane component concentrations.

Binding of CopA3 to cancer cell-specific membrane
components using calcein leakage assay

The lipid vesicles were prepared and the calcein leakage assay
was performed according to the procedure described previ-
ously (20). Calcein-entrapped vesicles were obtained by elution
with 10 mM Tris buffer (pH 7.4) containing 130 mM NaCl on
a Sephadex G-50 column. In the calcein leakage assay, the flu-
orescence intensity was measured using a fluorometer (VARIAN,
USA) with excitation and emission values of 490 and
520 nm, respectively. The percent dye-release was eval-
uated by the following equation: leakage (%) = (F − F0) / (Fmax
− F0) × 100, where F0 was the fluorescence intensity of the
control vesicle, and F and Fmax were the fluorescence intens-
ities achieved by peptides and 1% (w/v) Triton X-100, respec-
atively.
Annexin V/Propidium iodide (PI) staining
SNU-668 cells were plated into 6-well tissue culture plates (1 × 10⁵ cells per well) and treated with various concentrations of CopA3 (25, 50, and 100 μM) or without CopA3. After incubation for 1 h, cells were harvested and washed twice with cold PBS and resuspended in 1× Binding buffer (0.01 M Hepes/NaOH (pH 7.4), 0.14 M NaCl, 2.5 mM CaCl₂). Cells (1 × 10⁵ cells) were then transferred to a 1.5-ml tube containing 100 μl of binding buffer, and 5 μl of FITC-conjugated Annexin V and propidium iodide (PI) was added. The cells were vortexed gently and incubated for 15 min at room temperature in the dark. After incubation, 400 μl of 1× binding buffer was added to each tube. Stained cells were measured by flow cytometry with a BD FACSCalibur cytometer (BD Biosciences, USA) and CellQuest software (BD Biosciences, USA) was used for analysis of the results.

Acridine orange/ethidium bromide staining
Cells were seeded in 6-well tissue culture plates (1 × 10⁶ cells per well), treated with CopA3 (25, 50, and 100 μM) or without CopA3 for 1 h, and washed with PBS. Cells were then stained with acridine orange (3 μg/ml) and ethidium bromide (10 μg/ml) and observed immediately under a fluorescence microscope (Carl Zeiss, Germany).

Statistical analysis
Data are presented as an average with standard deviation (mean ± SD) of at least three independent experiments. Differences among groups were evaluated by Duncan post-hoc ANOVA analysis and considered statistically significant at P < 0.05.

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