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Anticancer Activity of the Antimicrobial Peptide Scolopendrasin VII Derived from the Centipede, *Scolopendra subspinipes mutilans*

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Previously, we performed *de novo* RNA sequencing of *Scolopendra subspinipes mutilans* using high-throughput sequencing technology and identified several antimicrobial peptide candidates. Among them, a cationic antimicrobial peptide, scolopendrasin VII, was selected based on its physicochemical properties, such as length, charge, and isoelectric point. Here, we assessed the anticancer activities of scolopendrasin VII against U937 and Jurkat leukemia cell lines. The results showed that scolopendrasin VII decreased the viability of the leukemia cells in MTS assays. Furthermore, flow cytometric analysis and acridine orange/ethidium bromide staining revealed that scolopendrasin VII induced necrosis in the leukemia cells. Scolopendrasin VII-induced necrosis was mediated by specific interaction with phosphatidylserine, which is enriched in the membrane of cancer cells. Taken together, these data indicated that scolopendrasin VII induced necrotic cell death in leukemia cells, probably through interaction with phosphatidylserine. The results provide a useful anticancer peptide candidate and an efficient strategy for new anticancer peptide development.

Keywords: Antimicrobial peptide, anticancer activity, necrosis, phosphatidylserine, scolopendrasin VII

Introduction

Scolopendra subspinipes mutilans, a species of centipede, has been used in folk and oriental medicine for its pharmaceutical efficacy in treating various diseases [10]. However, the pharmacological effect and useful medicinal ingredients of this centipede have not been fully determined. Antimicrobial peptides (AMPs) that have been identified and isolated from living organisms play a pivotal role in innate immunity [15]. Numerous AMPs have been investigated from vertebrates and invertebrates, including arthropods; however, only a few AMPs of the centipede were reported [11, 18].

AMPs have common characteristics such as low molecular weight, amphipathic structure, and net positive charge [12]. AMPs could be classified into five functional groups according to their activities: antibacterial activity, anticancer activity, antifungal activity, antiviral activity, and antiparasite activity [8]. The anticancer activities of AMPs have been investigated against various cancer cells, and AMPs have emerged as potential anticancer therapeutic alternatives with reduced side effects compared with conventional chemotherapeutics [4]. The proposed mechanisms of anticancer activity for AMPs are necrosis, apoptosis, and cell cycle arrest [9, 14].

Cancer is the leading causes of death worldwide [6]. The incidence and mortality rate of leukemia are relatively low compared with other primary cancers [6]. However, leukemia is the leading cause of cancer mortality in the population under age of 20 years in the United States [16] and is the most common cancer in children and adolescents aged 0–14 years in Korea [7]. Leukemia represents age-specific

incidence and mortality. The age-specific pattern of leukemia incidence is similar in Western countries. Here, we demonstrate the anticancer activity of scolopendrasin VII against leukemia cells. We showed that the anticancer effect of scolopendrasin VII is primarily due to its interaction with phosphatidylserine on the cell surface, which initiates necrosis in the cancer cells.

Materials and Methods

Peptide

The synthetic peptide scolopendrasin VII was synthesized using the solid-phase peptide synthesis method by Anygen Co., Ltd. (Gwangju, Korea). The peptide was dissolved in acidified distilled water (0.01% acetic acid) and stored at −20°C until use.

Cell Culture

U937 and Jurkat leukemia cells were maintained in RPMI-1640, and Raw 264.7 cells were maintained in DMEM supplemented with 10% FBS, penicillin G (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen, USA). Cells were cultured at 37°C in a humidified incubator with 5% CO_2.

MTS Assay

U937, Jurkat, and Raw264.7 cells plated in 96-well plates (2×10^4 cells/well) were treated with scolopendrasin VII at varying concentrations (50, 100, 200, and 300 μg/ml). After incubation for 24 h, the viability of the cells was assessed by the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay according to the manufacturer's protocol (Promega, USA). The optical density at 490 nm was measured with a microplate reader (Beckman DTX 8800 multi detector).

LDH Release Assay

Cells were seeded at 1×10^4 cells/well in a 96-well culture plate in assay medium (DMEM or RPMI-1640) and were treated with different doses of scolopendrasin VII for 24 h. For high control samples, 5 μl of lysis solution was added to positive control wells, and the plate was incubated for 15 min. Then, 100 μl of the reaction mixture was added to each well, including the high controls, and incubated for 30 min. Finally, 50 μl of stop solution was added to each well, and the absorbance was measured using a microplate reader at 490 nm. The percent cytotoxicity was calculated with the following equation: Cytotoxicity (%) = (sample value - low control) / (high control - low control) × 100.

Binding of Scolopendrasin VII to Membrane Components, Determined by Radial Diffusion Assay and the MTS Assay

The binding of scolopendrasin VII to the surface of the cancer cells was examined by assessing the effect of the cell membrane components on the antibacterial activity of scolopendrasin VII, using a radial diffusion assay. One microgram of scolopendrasin VII was incubated with varying concentrations of ganglioside, heparin, and phosphatidylserine for 10 min at 37°C in 10 mM sodium phosphate buffer. Then, 5 μl aliquots of each mixture were loaded into wells (3 mm in diameter) that were punched into the underlying agar containing washed mid-logarithmic multidrug resistant *P. aeruginosa* (MDRPA) (4×10^6 CFU/ml). The underlay agar consisted of 9 mM sodium phosphate, 1 mM sodium citrate buffer, 1% (w/v) agarose (Sigma, A6013), and 0.3 mg of tryptic soy broth. After incubation at 37°C for 3 h, 10 ml of overlay agar containing 1% agarose and 6% TSB was poured onto the underlay agar. The MTS assay was also carried out with scolopendrasin VII preincubated with varying concentrations of ganglioside, heparin, and phosphatidylserine as described above.

Annexin V/Propidium Iodide Staining

U937 cells were plated into 6-well tissue culture plates (1×10^6 cells/well) and treated with various concentrations of scolopendrasin VII (100, 200, and 300 μg/ml) or left untreated. After incubation for 1 h, cells were harvested by tryptic digestion, washed twice with cold PBS, and resuspended in 1× binding buffer (0.01 M Hepes/NaOH (pH 7.4), 0.14 M NaCl, and 2.5 mM $CaCl_2$). Cells (1×10^5 cells) were then transferred to a 1.5 ml tube containing 100 μl of binding buffer, and 5 μl of FITC-conjugated Annexin V (BD Bioscience, USA) and 5 μl of propidium iodide (PI) were added. The cells were gently mixed in a vortexer, protected from light, and incubated for 15 min at room temperature. After incubation, 400 μl of 1× binding buffer was added to each tube. Stained cells were measured by flow cytometry with a BD FACS Calibur cytometer (BD Biosciences, USA) and results were analyzed using CellQuest software (BD Biosciences, USA).

Acridine Orange/Ethidium Bromide Staining

U937 cells were seeded in 6-well tissue culture plates (1×10^6 cells/well), treated with varying concentrations of scolopendrasin VII (100, 200, and 300 μg/ml) or left untreated for 4 h, and then 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 $P < 0.05$.

Results and Discussion

Peptide

Previously, we analyzed the transcriptome of *S. s. mutilans* and identified AMP candidates using bioinformatics tools [20]. Scolopendrasin VII (sequence: FCTCNVKGFNAKNKRGIIYP-

NH_2) was one of the identified candidates, and its antimicrobial activity with no hemolytic effect was confirmed [20]. Scolopendrasin VII is a cationic peptide with a net charge of +4 at neutral pH. Cationic antimicrobial peptides (CAPs) are known to be the effector molecules of innate immunity that exhibit both antimicrobial activity and anticancer activity [14, 15]. Thus CAPs are a promising new agent as anticancer drugs, and the cationicity of peptide is an important factor for cytotoxicity to cancer cells. For instance, antimicrobial peptides (AcrAP1 and AcrAP2) were isolated from the venom of the Arabian scorpion *Androctonus crassicauda*. These natural peptides have no anticancer activity against four human cancer cell lines, whereas cationicity-enhanced analogs displayed potent anticancer effects on all four cancer cell lines [3]. Here, we have described the anticancer activity of scolopendrasin VII against human leukemia cancer cells.

Scolopendrasin VII Reduces the Viability of Leukemia Cells

Recently, it has been reported that several antimicrobial peptides from insects showed anticancer activities against leukemia cells [1, 17, 21]. These peptides are mostly cationic α-helical amphiphilic peptides, and the α-helix conformation of peptides is important for anticancer activity [19]. Therefore, we investigated the effect of the synthetic peptide scolopendrasin VII on the viability of the U937 and Jurkat leukemia cell lines. Cancer cells were treated with various concentrations of scolopendrasin VII (50, 100, 200, and 300 μg/ml) for 24 h, and cell viability was measured by the MTS assay. Scolopendrasin VII decreased the viability of the leukemia cells in a dose-dependent manner (Fig. 1). In contrast, Raw 264.7 cells were relatively resistant to scolopendrasin VII treatment, which suggests some specificity of scolopendrasin VII against the leukemia cells.

Scolopendrasin VII Disrupts the Membrane Integrity of Leukemia Cells

We next determined the effect of scolopendrasin VII on the membrane integrity of the cancer cells by detecting the release of LDH. The amount of LDH released by the leukemia cells increased in a dose-dependent manner (Fig. 2). These data are consistent with the results of the cell viability assay, which suggests a specific cytotoxic effect of scolopendrasin VII on the leukemia cells. Recent studies have demonstrated that ABP-CM4 from the hemolymph of the Chinese silkworm *Bombyx mori* and polybia-MP1 from the venom of the social wasp *Polybia paulista* increased the release of LDH by leukemia cell lines in the presence of these peptides [1, 17]. These results indicate that the cationic peptides, including scolopendrasin VII, could affect the membrane integrity of cancer cells and induce cell membrane disruption.

Scolopendrasin VII Interacts with Phosphatidylserine, a Cancer Cell-Enriched Phospholipid

We determined the effect of pretreatment with cancer cell-specific membrane components (ganglioside, heparin, and phosphatidylserine) on the scolopendrasin VII-induced

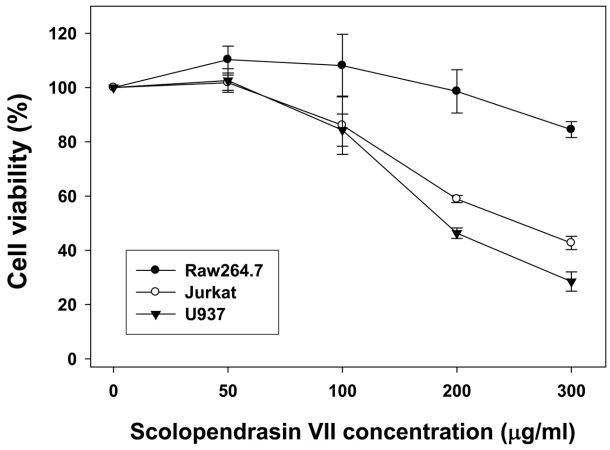

Fig. 1. Cell viability of the leukemia cells after scolopendrasin VII treatment.

Cell viability was measured by the MTS assay after 24 h incubation with the indicated concentrations of the peptides. Data shown are means ± standard deviations of triplicate experiments.

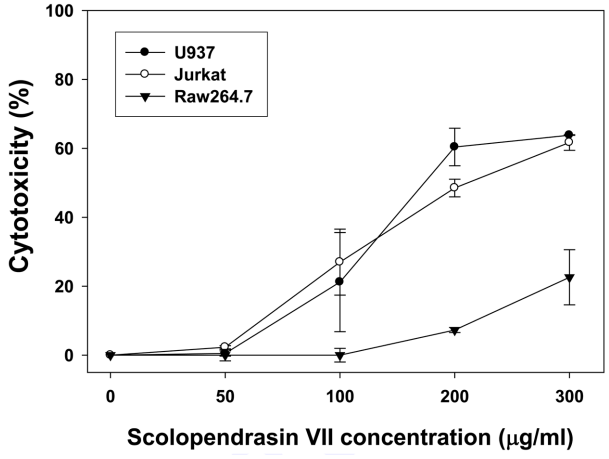

Fig. 2. Cytotoxic effect of scolopendrasin VII.

After 24 h incubation with the peptide, LDH released from each cell line was detected using the LDH release assay. Each symbol represents the mean value from triplicate experiments, and the error bars depict standard deviations.

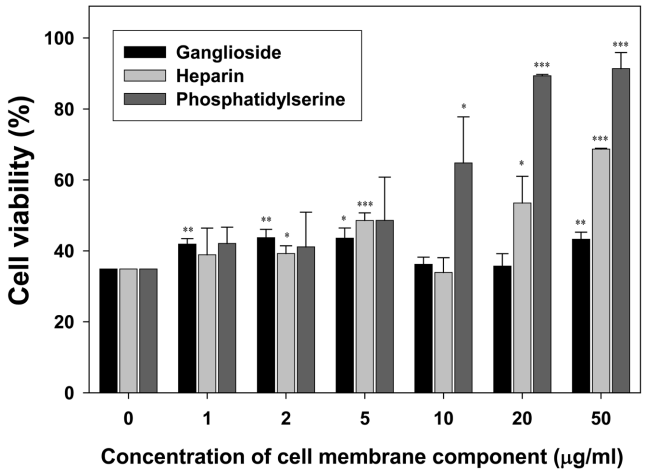

Fig. 3. Effect of anionic cancer cell-surface molecules on cell viability.

Viability assay of leukemia cancer cells was performed by mixing with varying amounts of ganglioside, heparin, or phosphatidylserine with 200 μg/ml scolopendrasin VII. Data are expressed as means ± standard deviation from three individual measurements. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, compared with non-treated control.

viability reduction of the cancer cells. The viability of scolopendrasin VII-treated cancer cells increased after treatment with increasing concentrations of phosphatidylserine in a dose-dependent manner (Fig. 3). The effect of the cancer cell-specific membrane components on the antibacterial activity of scolopendrasin VII was also examined. One microgram of scolopendrasin VII was incubated with different concentrations of ganglioside, heparin, or phosphatidylserine, and each mixture was tested in the radial diffusion assay. The antibacterial activity of scolopendrasin VII was significantly reduced with increasing concentrations of phosphatidylserine (Fig. 4). These findings suggest that scolopendrasin VII interacts with phosphatidylserine, which could result in scolopendrasin VII-induced cytotoxicity in the cancer cells.

Previously, Wang *et al.* [17] reported that the relative amount of cell-surface phosphatidylserine was determined by annexin V-binding to nontumorigenic and tumorigenic cells. The result showed that human myelogenous leukemia K562 cells express high levels of phosphatidylserine. It is known that tumor cell membranes have a higher phosphatidylserine composition than normal cells [2]. In addition, sphingomyelin and phosphatidylcholine are the major phospholipids in the outer monolayer of the mouse erythrocyte membranes without phosphatidylserine [13]. In our previous study, scolopendrasin VII had no hemolytic activity against mouse erythrocytes [20]. Therefore, the results suggest that cell-surface phosphatidylserine is a target molecule for scolopendrasin VII binding. The interaction of scolopendrasin VII with phosphatidylserine could be an initial step for the cytotoxic effect of scolopendrasin VII against the leukemia cells.

Flow Cytometry and Acridine Orange/Ethidium Bromide Staining

We assessed the nature of the cytotoxic effect of

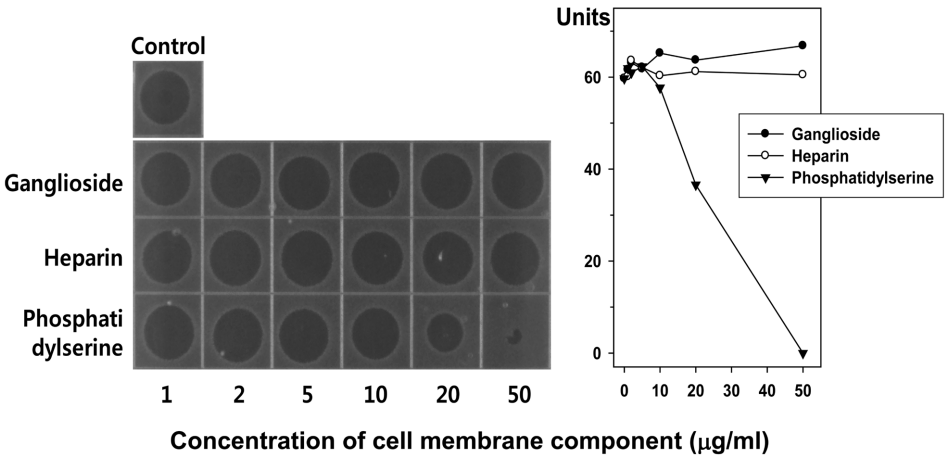

Fig. 4. Specific binding of scolopendrasin VII to phosphatidylserine.

The radial diffusion assay was conducted by mixing varying amounts of ganglioside, heparin, or phosphatidylserine with 1 μg of scolopendrasin VII. The mixture of peptides with the cell surface molecules were loaded into wells of the assay plate seeded with MDRPA, which has been confirmed to be highly susceptible to scolopendrasin VII. The left panel shows a photo of the gel from the radial diffusion assay. The right panel shows the antibacterial activity of scolopendrasin VII in the mixture, plotted against the concentration of the cell surface molecules. The diameters of the clearing zone are expressed in units (1 mm = 10 units). The results shown are representative of three independent experiments.

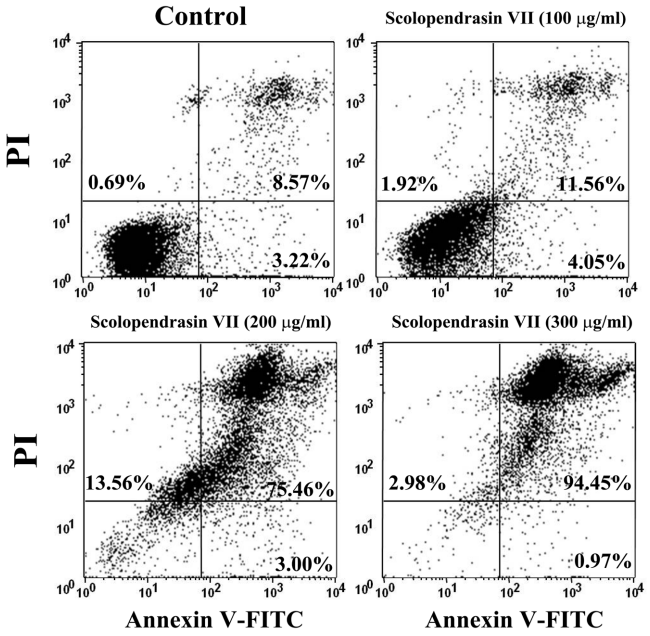

Fig. 5. Scolopendrasin VII induces necrosis in U937 human leukemia cells.

Flow cytometric analyses of the leukemia cells treated with various concentrations of scolopendrasin VII for 4 h were performed. 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).

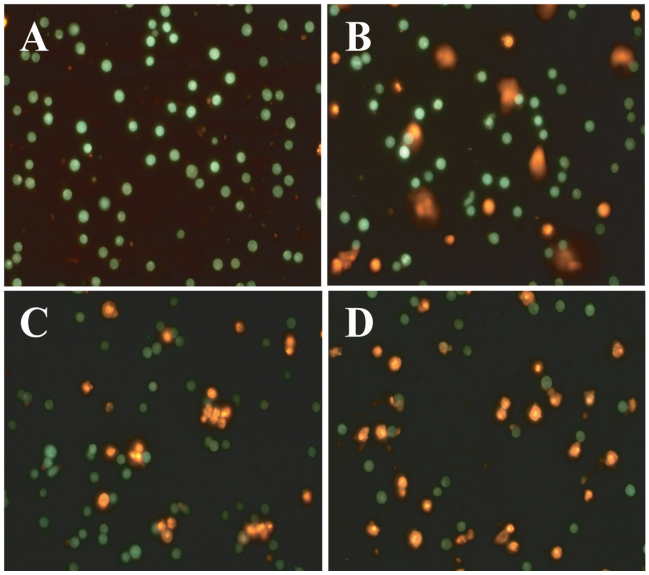

Fig. 6. Scolopendrasin VII-treated U937 leukemia cells stained with acridine orange/ethidium bromide.

Cells were observed under a fluorescence microscope (×400). Live cells show green fluorescence, whereas necrotic cells show orange fluorescence. (**A**) Control (no treatment); (**B**) treatment with scolopendrasin VII (100 μg/ml); (**C**) treatment with scolopendrasin VII (200 μg/ml); (D) treatment with scolopendrasin VII (300 μg/ml).

scolopendrasin VII to characterize the mechanism of the reduced viability. Cancer cell apoptosis was examined by Annexin V/PI staining of the scolopendrasin VII-treated leukemia cancer cells (Fig. 5). The Annexin V-positive/PI-positive population increased at peptide concentrations of 200 and 300 μg/ml. The Annexin V-positive/PI-positive population reached a maximum at 300 μg/ml scolopendrasin VII. A similar shift in the cell population following scolopendrasin VII treatment was also observed in Jurkat cells (data not shown). These data indicate that scolopendrasin VII induces necrosis in the leukemia cancer cells.

The membrane integrity of the U937 cells following scolopendrasin VII treatment was analyzed by acridine orange/ethidium bromide staining (Fig. 6). After 4 h of scolopendrasin VII treatment, the number of orange-colored nuclei increased in scolopendrasin VII-treated cells in a concentration-dependent manner. The cells treated with scolopendrasin VII exhibited an orange and orange-red fluorescence, indicating increased membrane disruption with increased concentrations of scolopendrasin VII. A similar phenomenon was also observed in Jurkat cells (data not shown). The cells treated with scolopendrasin VII exhibited an orange fluorescence, indicating that scolopendrasin VII-treated cells were primarily necrotic. Taken together, these results indicate that scolopendrasin VII-treated leukemia cells were primarily undergoing necrotic cell death.

In conclusion, scolopendrasin VII shows selective anticancer activity against leukemia cells such as U937 and Jurkat cells. LDH release assay, flow cytometric analysis, and acridine orange/ethidium bromide staining indicated that scolopendrasin VII induces necrosis in the cancer cells. The anticancer activities of scolopendrasin VII are dependent on its interaction with phosphatidylserine. Collectively, these results would be directly applicable in the development of novel cancer therapeutics from *S. s. mutilans* AMPs.

Acknowledgments

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