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The Suppression of Anti-Microbial Peptides on Leukemic THP-1 Monocytes

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Abstract: *Leukemia is the most common hematological malignancy. Chemotherapy, differentiation induction, and stem cell transplantation are available options for usual clinical therapies inleukemia. Antimicrobial peptides (AMPs) exert antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, and viruses by various mechanisms. In this study, we found that two AMPs (LPSBD-0 and LPSBD-2) showed lip opolysaccharides (LPS) binding capacity could suppress leukemia. Moreover, these two AMPs could result in oxidative stress and apoptosis in THP-1 leukemic monocytes in a dosage-dependent manner, not depending on autophage. These results suggest that these AMPs may havethe potential to be developed as anti-cancer peptides forhuman leukemia.*

Keywords: *Leukemia, Antimicrobial peptides (AMPs), oxidative stress, apoptosis, THP-1 leukemic monocytes*

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I. Introduction

Antimicrobial peptides (AMPs) belong to a large family of peptide molecules that typically contain <100 amino acids. They exist in various types of cells in both vertebrates and invertebrates. Previous studies have reported that AMPs facilitate promoting human health and reduce the cancer risk [1]. The AMPs play a crucial role in regulating the innate system, angiogenesis, and anticancer processes [2–4]. In addition, AMPs can specifically target certain proteins on the cancer cell membrane and induce cancer cell death, thus exhibiting potent toxicity in targeted cancer cells, suggesting that AMPs have potentials for application in antitumor therapy [5–8]. For example, pardaxin has been reported for application in anti-oral tumor [9].

Recently, the mechanism of AMPs against cancer cells has been described. AMPs arepredominantly composed of basic and hydrophobic aminoacids. AMPs are classified as α-helical, β-sheet, or extendedpeptides based on the secondary structure they adopt when incontact with cell membranes [10]. The outer leaflet of the cancer cellmembranes typically carries a net negative charge because ofits greater abundance ofO-sialoglycoproteins, heparan sulfateproteoglycans, and phosphatidylserine than compared withnormal cells, which have zwitterionic lipids in their membranesand are neutral in charge.AMPs mayhave greater binding affinity for neoplastic cells than normalcells, making AMPs exhibit selectively cytotoxicity for cancercells [11]. Several mechanisms have been reported for the anticanceractivities of AMPs mediated by AMPs-membrane interactions.The net positive charge and amphipathic nature ofAMPs provide them a selective ability to interact directly withthe plasma membrane of cancer cells to result in cell death [12]. This mechanism couldprevent the development of multiple drug resistance, such asthe overexpression of multidrug-efflux pump proteins [13], and combining AMPs with conventional anti-cancer drugs could provide additiveor synergistic effects in chemotherapies [14].

Anti-lipopolysaccharide factors (ALFs) were first characterizedfromTachypleustridentatus and describedthe LPS-binding domain of the MrALF from freshwaterprawnMacrobrachiumrosenbergii[15].The preliminary results of the viablecount showed that lipopolysaccharide binding-domain (LPSBD)-0 and LPSBD-2 peptides were not able to inhibitthe growth of human breast cancer cell line MDA-MB-231and human colon cancer cell line HCT116 [16].

Leukemia is the most common hematological malignancy.Chemotherapy, differentiation induction, and stem cell transplantationare available options for usual clinical therapies inleukemia. Recent studies have found that the proliferation of THP-1 leukemic cells could be limited through differentiation progress [17,18]. However,

the suppression of LPSBD0 (CQYSVNPKIKRFELYFKGRMW; MW = 2694.23) and LPSBD2 (CHYRVKPKIKRFEKYKGRMW; MW = 2652.15) on limitation of THP-1 leukemic cells is unclear. The aim of this study is to evaluate the anti-leukemic activity of LPSBD-0 and LPSBD-2 AMPs.

II. Materials and Methods

2.1. Cell culture

THP-1 cell line was obtained from the Bioresource Collectionand Research Center (BCRC) (Hsinchu, Taiwan). THP-1 cellswere incubated in RPMI-1640 medium(GIBCO BRL; Thermo,Carlsbad, CA, USA) supplemented with 10%FBS(GIBCO BRL), 2 mML-glutamine, and 1 mM sodium pyruvateat 37 ^{o}C in a 5% CO_2 and 95%humidity atmosphere.Anti-Bax, anti-Bcl2, anti-LC-3 and anti-GAPDH antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). LPSBD-0 and LPSBD-2 weresynthesized and purchased from LTKBioLaboratoriesInc.

2.2. Sample treatment

The THP-1 cells (1.5×10^5/mL) weretreated with LPSBD-0 or LPSBD-2 (20, 60, 100, 150, and 200 μg/mL) dissolvedin RPMI-1640 medium for 24 h. The numbers ofviable cells were counted by trypan blue dye exclusion aftertreatment.

2.3. Apoptosis/necrosis measurement

For apoptosis detection, cells were collected after treatment with LPSBD-0 or LPSBD-2 (20, 60, 100, 150, and 200 μg/mL) for 12 h. Cells were harvested, washed in ice-cold PBS and resuspended in 200 μL of binding buffer before being incubated in 5 μL of Annexin V-fluorescein isothiocyanate (FITC) (BD Biosciences) solution and 5 μL of propidium iodide (PI)at room temperature for 15 min in the dark. Then 300 μL of binding buffer was added for optimal cell amounts. Cells were analyzed by flow cytometry. Untreated cells were used as the control for double staining [19].

2.4. Measurements ofoxidative stress

The MitoSOX™ Red mitochondrial superoxide indicator is anovel fluorogenicdye for highly selective detection of superoxidein the mitochondria of live cells. The MitoSOX™Red reagent is a live-cell permeant and rapidly and selectivelytargets the mitochondria. Once in the mitochondria, the MitoSOX™ Red reagent is oxidized by superoxide and exhibitsred fluorescence. The MitoSOX™ Red reagent is readily oxidized bysuperoxide but not by other ROS- or reactive nitrogen species (RNS)-generating systems, and the oxidation of the probe is preventedby superoxide dismutase. The oxidation product becomeshighly fluorescent upon binding to nucleic acids. Mitochondrial superoxide is generated as a byproduct of oxidativephosphorylation. The assumption that mitochondria serve as the major intracellularsource of ROS has been largely based on experiments withisolated mitochondria rather than on direct measurements in livingcells. MitoSOX™ Red mitochondrial superoxide indicator is anovel fluorogenic dye for highly selective detection of superoxide in the mitochondria of live cells by using a fluorescence microscope. Cells were collected after treatment with LPSBD-0 or LPSBD-2 (200 μg/mL) for 12 h; and the oxidative stress was measured by MitoSOX stain.

2.5. Cell morphology

THP-1 cells were collected after treatment with LPSBD-0 or LPSBD-2 (20, 60, 100, 150, and 200 μg/mL) for 24 h. The cell morphologywas observed under an inverted microscope with a magnificationof 1000×.

2.6. Western Blot

Cells were rinsed with ice-cold PBS and lysed by RIPA lysis buffer with protease and phosphatase inhibitors for 20 min on ice. Then the cells were centrifuged at 12,000 xg for 10 min at 4 ^{o}C. Protein extracts (20 μg) were resolved using SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 200 V, 45 min). The protein bands were electrotransferred to nitrocellulose membranes (80 V, 120 min). Membranes were then treated with a 5% enhanced chemiluminescence (ECL) blocking agent (GE Healthcare Bio-Sciences) in saline buffer (T-TBS) containing 0.1% Tween-20, 10 mMTris-HCl, 150 mMNaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ at a pH of 7.4 for 1 h, and then incubated with the primary antibody overnight at 4 ^{o}C. Subsequently, membranes were washed three times in T-TBS and bound primary antibodies were detected using appropriate horseradish peroxidase-conjugated secondary antibodies, followed by analysis in an ECL plus Western blotting detection system (GE Healthcare Bio-Science).

2.7.Statistical Analysis

Experimental results were averaged triplicateanalysis. The data were recorded as the mean ± SD (n =3).Single-factor analysis of variance (ANOVA) was computedusing the SPSS software.

III. Results and Discussion

3.1. The effects of LPSBD-0 or LPSBD-2 on cell viability and morphology in leukemic THP-1 cells

The primary amino acid sequence of the synthetic LPSBD-0 and LPSBD-2 are CQYSVNPKIKRFELYFKGRMW (MW = 2694.23)and CHYRVKPKIKRFEKYKGRMW(MW = 2652.15), respectively.We found that the cell viability of THP-1 leukemic cells was suppressed by LPSBD-0 or LPSBD-2 treatment after 24 h as shown in **Figure 1**. Moreover, the suppression of THP-1 leukemic cells by LPSBD-2 was greater than LPSBD-0 at 100, 150, and 200 μg/mL.

In addition, the cell debris was observed in the THP-1 leukemic cells treated by LPSBD-0 and LPSBD-2 in a dosage-dependent manner after 24 h as shown in **Figures 2 and 3**.

To confirm the potential of LPSBD-0 and LPSBD-2induced THP-1 leukemic cells death, we investigated the apoptosis and necrosis of THP-1 leukemic cells using the PI/Annexin-V double stain. This staining method combined with flow cytometry enables quantitatively assessing living (Annexin-V-FITC negative/PI negative), early apoptotic (Annexin-V-FITC positive/PI negative), late apoptotic/necrotic (Annexin-V-FITC positive/PI positive), and dead (Annexin-V-FITC negative/PI positive) cells. As shown in **Figure4**, both LPSBD-0 and LPSBD-2 clearly resulted in cell dead in THP-1 leukemic cellsafter 12 h treatment.

3.2. The effects of LPSBD-0 or LPSBD-2 on mitochondrial function in leukemic THP-1 cells

Most antibacterial and anticancer peptides employ cell membrane disruption by lytic activity, or some peptides employ apoptosis in cancer cells through mitochondrial damage. It is believed that the mode of action originates from electrostatic interaction between cationic peptides and anionic cell wall components of bacterial and cancer cells.The assumption that mitochondria serve as the major intracellularsource of reactive oxygen species (ROS) has been largely based on experiments withisolated mitochondria rather than on direct measurements in livingcells.It clearly suggested that an elevation of oxidative stress in THP-1 leukemic cells treated by LPSBD-0 or LPSBD-2 at 200 μg/mL after 12 h according to the MitoSOX stain **(Figure 5)**.

The apoptotic protein marker, Bcl2 and Bax, and an autophagic protein marker, microtubule-associated protein-1 light chain-3 (LC3), were then determined in the total protein extracts of the cells. Results were shown as **Figure 6**, LPSBD-0 or LPSBD-2 at 200 μg/mL after 12 h treatment could result in alleviation in Bcl2 and elevation in Bax in THP-2 leukemic cells, but not affected LC-3. These results suggested that LPSBD-0 or LPSBD-2 suppressed THP-1 leukemic cells maybe depend on apoptosis.

IV. Discussion

Among the numerous candidates for novel anticanceragents, host defense peptides have emerged as highlypromising antitumor therapeutics given that the cellmembrane can be targeted and resistance may be less likelyto occur [20].Several host defensepeptides, including pardaxin [21], epinecidin-1 [22] and pleurocidin[23] from fishesand temporin-1CEa [24] and tryptophyllin[25] from frogshave beenidentified as exhibiting anti-cancer activities. Arthropodsare rich sources of anti-cancer peptides, such as the cecropinfamily from insects. These peptides can be classified as alpha-helical, beta-sheet,or extended peptides based on the secondary structurethat they adopt in action [20]. TheLPSBDs are derived from the lipopolysaccharide-bindingmotif of the host defence peptide ALF from the freshwaterprawn M. rosenbergii[15].

Our recent study has found that the LPSBD-0 and LPSBD-2 peptidesexhibit high potential of forming beta-hairpin AMPs composed of two anti-parallel beta-sheets connected by oneloop. The beta-strands of LPSBD-0 are longer than beta-strandsof LPSBD-2 [16]. This difference may be associated with thedifference of anti-tumor activities between the two peptides.Several AMPs of similar structure, including tachyplesinwhich consists of 17 amino acid residues arranged intwo anti-parallel beta-sheets and induces apoptosis in HL-60cells [26]. Another exampleis protegrin a peptide with 18 amino acids thatexhibits membranolytic activity leading to human histiocyticlymphoma cell line U937 necrosis [27]. BothLPSBD-0 and LPSBD-2 exhibited significant anti-proliferativeactivities on the A549 and Hep3B cells [16].Reduction of cell viabilitywas observed in THP-1 leukemic cells treated with theLPSBD-0 and LPSBD-2**(Figure 1; Figure 4)**. Small pieces of debris were producedin the cultures of LPSBD-treated A549 and Hep3Bcells, and membrane permeability was increased in bothcell types [16]. Similar results were also found in THP-1 leukemic cells treated by LPSBD-0 and LPSBD-2 **(Figure 2; Figure 3)**.

Some AMPs inhibit DNA and/or RNA synthesis as well as apoptosis induction by reactive oxygen species (ROS) accumulation and mitochondrial dysfunction. Specifically, mitochondria play a major role in the apoptotic pathway. During apoptosis induced by AMPs, cells undergo cytochrome c release, caspase activation, phosphatidylserine externalization, plasma or mitochondrial membrane depolarization, DNA and nuclei damage, cell shrinkage, apoptotic body formation, and membrane blebbing [28]. We found that LPSBD-0 and LPSBD-2 could result in oxidative stress in THP-1 leukemic cells after 12 h treatment **(Figure 5)**.

During autophagy, LC3 wasup-regulated for the initiation and formation of autophagosomes [29].Some members of the Bcl2 family, such as Bcl2, is anapoptotic regulator to suppress cell death, while other homologues

including Bax and Bak exhibit powerful deathpromoting abilities. The Bcl2 family proteins play a centralrole in the process of cell apoptosis by interfering withcaspases. It is well established that the ratio between Bcl2 andBax is an important factor in the regulation of apoptosis [30]. We found that LPSBD-0 and LPSBD-2 could result in regulation on Bcl2 and Bax in THP-1 leukemic monocytes, but not affecting LC-3, suggesting that the suppression of cell viability in THP-1 leukemic monocytes treated by LPSBD-0 or LPSBD-2 maybe activate apoptotic pathway.

The ability of LPSBDs to change membrane permeabilitymay be remarkably useful for addressing the anticancerdrug resistance that occurs through the activation ofefflux pumps [31]. The cancer cell membrane leakage causedby the LPSBDs may be able to bypass the effect of multidrugresistance efflux pumps. A combination of the LPSBDpeptides and anti-cancer drugs may be a novel option forcancer chemotherapy.Results indicated that LPSBD0 and LPSBD2 could result in oxidative stress and apoptosis in THP-1 leukemic monocytes in a dosage-dependent manner. These results suggest that these AMPs may havethe potential to be developed as anti-cancer peptides forhuman leukemia **(Figure 6)**.

V. Conclusion

Antimicrobial peptides (AMPs) exert antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, and viruses by various mechanisms. LPSBD-0 and LPSBD-2 commonly possess particular characteristics by harboring cationic and amphipathic structures and binding to cell membranes, resulting in the leakage of essential cell contents by forming pores or disturbing lipid organization. These membrane disruptive mechanisms of LPSBD-0 and LPSBD-2 are possible to explain according to the various structure forming pores in the membrane.

Conflict of interest

The authors declare no competing financial interest.

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