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Full Length Research Paper

Anti-proliferative activity of recombinant melittin expressed in *Escherichia coli* toward U937 cells

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Melittin, a toxic peptide found in bee venom, has been widely reported to have antimicrobial and anti-proliferative activity in many cell lines. While high-purity natural melittin is rare and expensive, the demand for melittin has increased, making it urgent to find a cheap, suitable method of mass production. In this study, the melittin gene of the Chinese bee (honeybee) was synthesized according to the sequence published in GenBank and cloned into the prokaryotic expression vector pGEX-6P-1. Recombinant melittin was then successfully expressed in *Escherichia coli.* The activity of affinity-purified recombinant melittin was determined in human leukemic U937 cells. Results show that the recombinant melittin had the same anti-proliferative activity in human leukemic U937 cells *in vitro* as natural one. This shows the promise of recombinant melittin as a replacement for the natural bee toxins used in drug development and other applications.

Key words: *Escherichia coli*, melittin, expression, anti-proliferative activity.

INTRODUCTION

Melittin is the major active ingredient from bee venom, making up 50% of its dry weight (Habermann, 1972). Mature melittin is a small, amphiphilic peptide containing 26 amino acid residues. Naturally, melittin is firstly expressed as a 70 amino acid promelittin without biology activity, and then matured through deletion of N-terminal 44 amino acid residues mediated by dipeptidylpeptidase IV (Vlasak et al., 1983). It has been reported that the melittin exhibits antimicrobial activity and pro-inflammatory effects (Sumikura et al., 2003). In addition, it can induce perturbations in the cell membrane and cause damage to enzyme systems (Habermann, 1972; Wade et al., 1990). It also has strong anti-cancer activity. Several types of cancer cells, including human leukemia and human renal, lung, liver, prostate, bladder and mammary cancer cells have been identified as targets of melittin (Katoh, 1995; Son et al., 2007). Moreover, melittin is capable of binding calmodulin, which has a role in cellular proliferation (Lee and Hait, 1985). Hait et al. (1985) also showed that melittin is one of the most powerful inhibitors of calmodulin activity and, as such, is an inhibitor of cell growth and clonogenicity of human and murine leukemic cells. Melittin also inhibits the melanotropin receptor in M2R melanoma cell membranes (Gerst and Salomon, 1987; Gerst et al., 1987). Additionally, melittin was found to induce apoptosis in vascular smooth muscle cells and in human leukemic cells (Son et al., 2006; Moon et al., 2008) as well as direct cytolysis in erythrocytes (Raghuraman and Chattopadhyay, 2005), mast cells (Nishikawa and Kitani, 2011) and thymocytes (Shaposhnikova et al., 1997). Currently, purification from bee venom and chemical synthesis are the research community's preferred approaches to obtain melittin. However, the cost factor of these methods limits the use of melittin in clinical and research applications. A new method of producing melittin, which is suitable for wider use, is necessary. In this project, we expressed melittin in *Escherichia coli* and evaluated its anti-proliferative activity

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Abbreviations: **IPTG**, Isopropyl-β-D-thiogalactopyranoside; **p-Akt**, phospho(p)-Akt; **SDS-PAGE**, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **HRP**, horseradish peroxidase; **GST**, glutathione S-transferase; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

in human leukemic U937 cells.

MATERIALS AND METHODS

Prokaryotic expression vector pGEX-6P-1, *E. coli* BL21 and PreScission protease were purchased from GE Healthcare Life Sciences (PA, U.S.). *Taq* DNA polymerase, dNTP, restriction endonuclease, X-gal, isopropyl-β-D-thiogalactopyranoside (IPTG), 100 bp DNA Ladder, PageRular™ Prestained Protein Ladder and Spectra™ Multicolor Low Range Protein Ladder were purchased from Fermentas (Shenzhen, China). Standard natural melittin (high performance liquid chromatography (HPLC) ≥ 85%) was purchased from Sigma (St. Louis, MO, U.S.). Cell culture plates (96 and 24 well) were purchased from Corning Ltd. (Shanghai, China). Antibodies against Akt and phospho(p)-Akt (p-Akt) were purchased from Cell Signaling Technology, Inc. (MA, U.S.). Antibody against β-actin was purchased from GenScript Corp. (Nanjing, China). Peroxidase-labeled goat anti-mouse immunoglobulin G was purchased from Thermo Fisher Scientific, Inc. (IL, U.S.).

Cells

Human leukemic U937 cells (ATCC, U.S.) were cultured at 37°C in a 5% (v/v) CO_2 humidified atmosphere and maintained in RPMI-1640 medium (Gibco) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco).

Construction of recombinant expression vector

A pair of complementary oligonucleotides named Mel-1 (5′-GAT CCG GAA TTG GAG CAG TTC TGA AGG TAT TAA CCA CAG GAT TGC CCG CCC TCA TAA GTT GGA TTA AAC GTA AGA GGC AAC AGG GTT AGC-3′) and Mel-2 (5′-TCG AGC TAA CCC TGT TGC CTC TTA CGT TTA ATC CAA CTT ATG AGG GCG GGC AAT CCT GTG GTT AAT ACC TTC AGA ACT GCT CCA ATT CCG-3′) was designed according to the melittin sequence published in GenBank (Accession No. AF487907) and synthesized by GenScript (Nanjing, China). Both oligonucleotides were resuspended using annealing buffer [10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA] and annealed as follows: oligonucleotides were mixed together at a 1:1 molar ratio in a microcentrifuge tube at a concentration of 1 μM each, and incubated at 95°C for 5 min. Then, the oligos were allowed to gradually return to room temperature. The annealed dsDNA contained the mature melittin peptide coding sequence with an adhesive end corresponding to the recognition sequence of restriction endonuclease *Bam*H I at the 5′-end and an adhesive end corresponding to *Xho* I at the 3′-end. The annealed dsDNAs were then cloned into prokaryotic expression vector pGEX-6P-1 and double-cut by restriction endonucleases *Bam*H I and *Xho* I. The recombinant plasmid was identified by sequencing and is hereafter referred to as pGEX-6P1-melittin.

Expression of melittin in *E. coli*

Recombinant plasmid pGEX-6P-1-melittin was transformed into competent *E. coli* BL21 cells. Protein expression was induced as follows: A single colony was inoculated into 10 ml of Lysogeny broth (LB) medium supplemented with 100 μg/ml ampicillin and incubated with shaking at 37°C until OD_{600} reached 0.6. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM and incubation continued at 15°C overnight. The flask was placed on ice for 5 min and then the cells were harvested by centrifugation at 5,000 ×g for 5 min at 4°C. The pellet was collected and resuspended in 0.25 culture volume of cold 20 mM Tris-HCl (pH 8.0). The cells were ruptured by brief pulses of sonication on ice until the sample was no longer viscous. Supernatant and sediment were collected separately after centrifugation at 12,000 ×g for 10 min at 4°C and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Uninduced cells were prepared by the same method for use as negative controls.

Immunoblot analysis

Extracted protein samples prepared as the aforementioned were separated with SDS-PAGE and electrotransferred to nitrocellulose membrane for 1 h at 0.2 A in a Bio-Rad transblot apparatus. The membrane was incubated with mouse anti-glutathione S-transferase (GST) monoclonal antibody and the antibody-antigen complex was detected with secondary antibodies, goat anti-mouse IgG conjugated with horseradish peroxidase (HRP). All purchased products were used in accordance with the manufacturer's instructions.

Purification of GST-melittin expressed by *E. coli* and its cleavage

The recombinant GST-melittin fusion protein was purified by GST resin and cleaved by PreScission protease in 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT) at 4°C for 16 h according to the standard protocol. The melittin was eluted directly from the GST resin and 2 × Tris-tricine loading buffer was added to the purified protein and boiled at 100°C for 3 to 5 min. The protein was analyzed by Tris-tricine gel electrophoresis.

Assay of melittin activity

Effects on growth and proliferation of leukemic U937 cells

The effect of melittin on the viability of cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, leukemic U937 cells were plated at 1×10^4 cells per well in 96-well microtiter plates. After 24 h, cells were treated with 100 μl of culture medium containing either 1, 2, 4 or 8 μg/ml of melittin or a negative control. Melittin stock solutions were prepared at 2 mg/ml and mixed with fresh serum-free RPMI-1640 medium to the desired final concentrations. Each concentration of melittin was repeated in eight wells. Cell viability was determined after 4, 8, 24, 48 and 72 h of incubation. Twenty microliters of MTT (5 mg/ml in phosphate-buffered saline stock) was added to each well and were incubated for 3 h at 37°C to allow the color to develop. After careful removal of the medium, 100 μl of dimethyl sulfoxide (DMSO) was added to each well and was shaken carefully for 15 min at room temperature. The absorption was recorded on a microplate reader (BIO-RAD) at a wavelength of 570 nm (Bi et al., 2010).

Effects on Akt signaling pathway

An immunoblotting assay was used to determine whether melittin induced apoptosis in leukemic U937 cells through the Akt signaling pathway. Total protein was prepared using a total protein kit (KeyGEN, China). The cells were lysed in 1 ml of ice-cold lysis buffer, 10 μl of phosphatase inhibitor, 1 μl of protease inhibitor and 10 μl of 100 mM phenylmethanesulfonyl fluoride (PMSF). After 15 min of slow shaking at 4°C, cells were centrifuged at 140,000 ×g for 30 min at 4°C, and the supernatants were collected. Protein

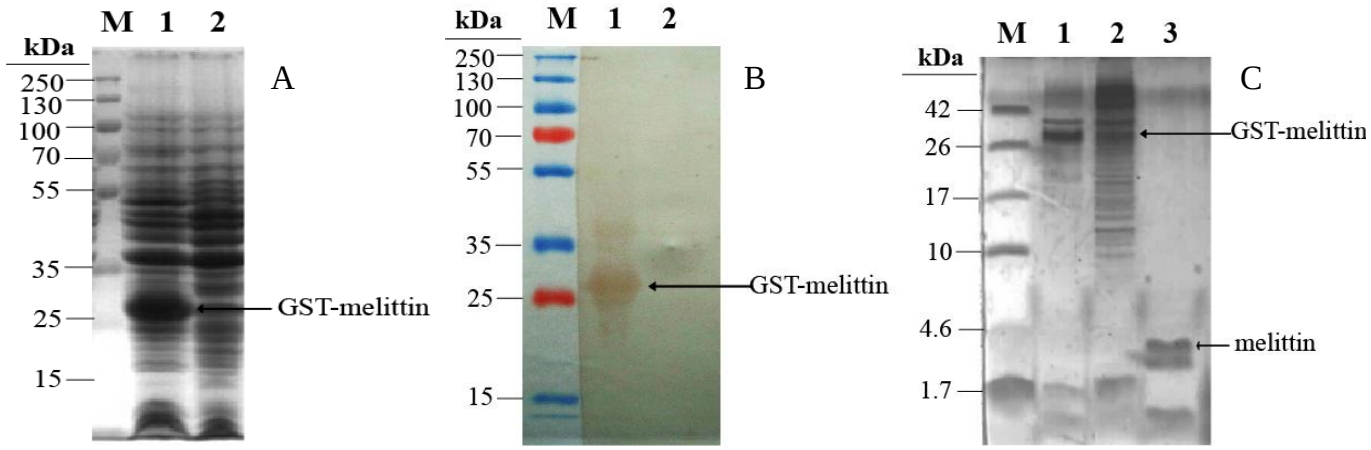

Figure 1. Expression and purification of the recombinant melittin. (A) SDS-PAGE analysis for expression of GST-melittin fusion protein in *E. coli*. (B) Western blot analysis for GST-melittin fusion protein. Lane M: Page Ruler^{TM} pre-stained protein Ladder; lane 1: Induced cells; lane 2: Uninduced cells. (C) Tris-tricine analysis for the proteolysis of purified GST-melittin protein. Lane M: Spectra^{TM} low range pre-stained protein ladder; lane 1: GST-melittin fusion proteins in inclusion body; lane 2: GST-melittin fusion proteins in supernatant; lane 3: Purified melittin.

concentration was determined using the bicinchoninic acid (BCA) method. For western blot analysis, equal amounts of total protein were loaded onto SDS-PAGE, and the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, U.S.), followed by incubation with specific primary antibodies overnight. After exposure to HRP-conjugated secondary antibody for 30 min, the protein bands were visualized using an enhanced chemiluminescence detection kit (ECL Kits, Pierce, U.S.) (Tu et al., 2008).

Statistical analysis

All data from MTT assays and western blot analyses are presented as mean ± standard deviation. Significant differences among the groups were determined using the student's *t*-test. A value of $P < 0.05$ was accepted as an indication of statistical significance.

RESULTS

Expression of recombinant melittin protein in *E. coli*

The pGEX-6P-1-melittin was transformed into *E. coli* BL21. The expression of GST-melittin was induced by 0.2 mM IPTG and 250 ×g shaking overnight at 15°C. Coomassie brilliant blue staining and western blot showed that the induced *E. coli* had one additional band at 28 to 29 kD than the uninduced *E. coli* (Figure 1A and B). The results show that GST-melittin was expressed in the induced *E. coli*. After *E. coli* underwent ultrasonic disruption, the inclusion bodies and supernatant were separated. Tris-tricine PAGE results showed that about half of the GST-melittin was in the inclusion body and about half was in the supernatant. The supernatant was purified using GST resin. Then, the PreScission protease was added to GST resin G and allowed to react at 4°C for 16 h. The eluted protein was collected and analyzed using Tris-tricine PAGE (Figure 1C). The results show that a band appeared at 1.6 to 4 kD in the gel, which was the recombinant melittin protein.

Inhibition of viability and proliferation of leukemic U937 cells by recombinant melittin

Leukemic U937 cells were seeded at 1×10^4 cells/well and were treated with different concentrations (1, 2, 4 and 8 µg/ml) of recombinant melittin for different duration of incubation (4, 8, 24, 48 and 72 h). Cell viability was determined by MTT assay. The OD_{570} values are presented as mean ± SD. Significance was determined using the student's *t*-test (*$P < 0.05$ versus control, **$P < 0.01$ versus control) (Figure 2). The results show that the recombinant melittin at a concentration of 1 µg/ml had no obvious inhibitory effect on the human leukemic U937 proliferation. However, the recombinant melittin at a concentration of 2 µg/ml could inhibit the leukemic U937 proliferation. The inhibitory effect was particularly obvious after 24 h of exposure. The recombinant melittin at concentrations of 4 and 8 µg/ml showed a strong inhibitory activity of proliferation that was sustained over time.

Interference with expression of p-Akt in leukemic U937 cells by recombinant melittin

The Akt signaling pathway is known to be involved in cell growth, differentiation and apoptosis. In this experiment, we investigated the expression and phosphorylation levels of Akt after treatment with various concentrations of melittin for 4 h. As shown in Figure 3, Akt phosphorylation decreased after exposure to 2 and 4 µg/ml melittin, while the total Akt protein levels remained constant under each

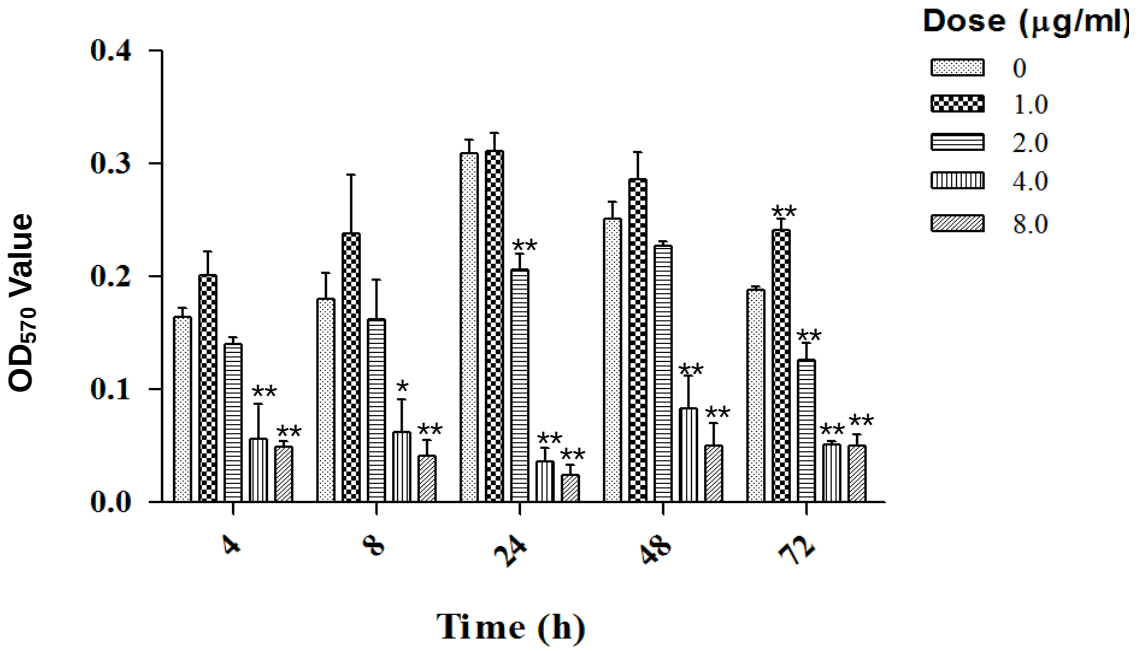

Figure 2. Anti-proliferative effects of melittin on human leukemic U937 cells. Melittin was added to human leukemic U937 cells (1×10^4 cells/well in 96-well plates) at final concentrations of 0, 1, 2, 4 and 8 μg/ml for 4, 8, 24, 48 and 72 h, respectively. The cells were then subjected to MTT assay. *P < 0.05, **P < 0.01.

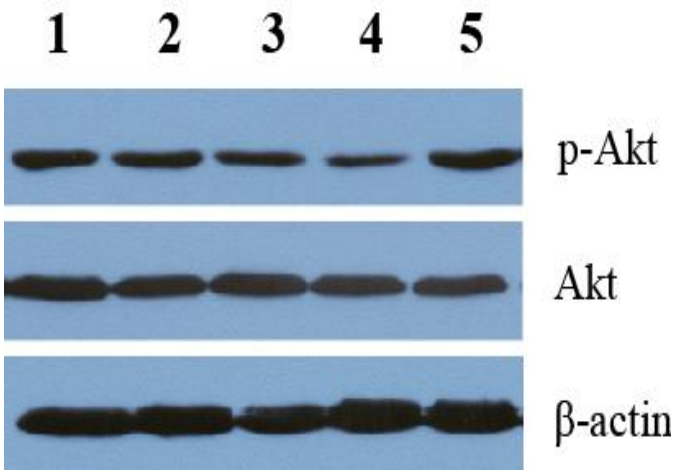

Figure 3. Melittin induced apoptosis in human leukemic U937 cells through the Akt signaling pathway. The cells were treated with 1, 2 or 4 μg/ml melittin for 4 h and then lysed. The total protein, found to be 25 μg by SDS-PAGE, was transferred to nitrocellulose and was probed with specific antibodies, respectively against p-Akt and Akt. β-Actin was used as an internal loading control. Lane 1 shows the effects of 1 μg/ml of natural melittin on the U937 cells; lanes 2, 3 and 4 show the effects of 1, 2 and 4 μg/ml recombinant melittin, respectively on the U937 cells; lane 5, shows the effects of the phosphate buffer used as a control.

treatment condition. These results show that recombinant melittin may act through the Akt signaling pathway to inhibit the proliferation of human leukemic U937 cells.

DISCUSSION

In this report, a melittin gene from the Chinese honeybee was expressed in *E. coli* using the pGEX expression system. The expression conditions of the fusion protein for the *E. coli* BL21 transformant were determined by optimizing IPTG concentration, temperature and duration of induction. With full nutrition, the optimum expression level was reached by the addition of IPTG at a final concentration of 0.2 mM followed by culture at 15°C overnight. SDS-PAGE proved that about 50% of the fusion protein appeared in the inclusion bodies and about 50% were soluble. We obtained the desired protein from the GST-fusion protein by using PreScission protease, as evidenced by Tris-tricine PAGE, which further verified the feasibility to produce active melittin by genetic engineering methods. Shi et al. (2004) successfully expressed the melittin of *Apis cerana cerana* in *E. coli* and found that the recombinant melittin had agglutination activity toward rabbit platelets .

Melittin has been reported to induce apoptosis in several cancer cells (Wang et al., 2009) and rheumatoid arthritis synovial fibroblasts *in vitro* and *in vivo* (Nah et al., 2008). In order to determine whether the recombinant melittin could act as natural one, we observed its effects on the viability and proliferation of human leukemic U937 cells. We found that the low-concentration recombinant melittin had no obvious inhibitory activity in the U937 cells, but its inhibitory effects on the proliferation of U937 cells were observed especially at 24 h of incubation when

the concentration was increased to 2 µg/ml. The recombinant melittin showed a strong ability to inhibit cellular proliferation at concentrations of 4 or 8 µg/ml, and this inhibitory activity was maintained over time. The enhancement of inhibitory activity with the increasing melittin concentration was also demonstrated by Moon et al. (2008), who used the commercial melittin to inhibit the proliferation of U937 cells and normal cells.

The PI3K/Akt signal pathway plays critical roles in regulating cell survival and death in many settings. The PI3K/Akt pathways are more often associated with cell survival through activation of anti-apoptotic downstream effectors (Xia et al., 1995; Cross et al., 2000). The PI3K/Akt pathway was also found to be important for regulating natural melittin-induced apoptosis in leukemic U937 cells (Moon et al., 2006, 2008). Son et al. (2006, 2007) also indicated that the natural melittin inhibited vascular smooth muscle cell (VSMC) proliferation through suppression of Akt. To determine whether the same mechanism is involved in the action of the recombinant melittin, we detected the phosphorylation levels of Akt protein in the U937 cells after treatment with the recombinant melittin. The results show that the levels of p-AKT were decreased after the cells were treated with the recombinant melittin at a concentration more than 2 µg/ml. This phenomenon suggests that the recombinant melittin may have the same bioactivity as the natural one.

In summary, we have demonstrated that the recombinant melittin can be abundantly acquired through genetic engineering methods, and the recombinant melittin can inhibit the U937 cells proliferation as the natural one probably through the PI3K/Akt signal pathway. In view of accumulating evidence that the recombinant melittin may be an important determinant of a research subject and a tool in clinical therapy and could replace the natural one for development of antitumor drugs.

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