Construction of the plasmid coding for the expression of the EGFP-\textit{M-IL-2}^{88\text{Arg}, 125\text{Ala}} fusion protein and the anti-tumor effects exerted by the fusion protein in HeLa-60 cells

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Abstract. Gene therapy is a promising therapeutic option for the treatment of various cancers, and tumor-targeted plasmids encoding toxic protein genes are potential tools for gene therapy. In the present study, a recombinant plasmid containing the genes for the toxic protein melittin and interleukin-2 (IL-2) was constructed. Melittin and IL-2 are known to play key roles in immunoregulation and cancer therapy, but they each possess defects that limit the clinical application of these proteins. The present study aimed to construct a novel recombinant expression plasmid, pLEGFP-C1-M-IL-2^{88\text{Arg}, 125\text{Ala}}, and to improve the biological activity of IL-2 and melittin. The M-IL-2^{88\text{Arg}, 125\text{Ala}} gene was excised from the pPICZαA-M-IL-2^{88\text{Arg}, 125\text{Ala}} plasmid by polymerase chain reaction (PCR). The pLEGFP-C1 plasmid carrying the enhanced green fluorescent protein (EGFP) gene was used as a shuttle plasmid. Subsequent to digestion, the M-IL-2^{88\text{Arg}, 125\text{Ala}} gene was subcloned into the pLEGFP-C1 vector to build the pLEGFP-C1-M-IL-2^{88\text{Arg}, 125\text{Ala}} eukaryotic expression plasmid, which was identified by restriction enzyme digestion and gene sequencing. Confocal microscopy was used to determine the transfection efficiency subsequent to the plasmid being transfected into the cervical cancer HeLa cell line. The cells transfected with the pLEGFP-C1-M-IL-2^{88\text{Arg}, 125\text{Ala}} plasmid demonstrated a decreased transfection efficiency compared with the cells transfected with the pLEGFP-C1 plasmid. The cellular expression of M-IL-2^{88\text{Arg}, 125\text{Ala}} was detected by reverse transcription PCR and western blot analysis. Finally, cell counting kit-8 and apoptosis assays were performed to investigate the effects of the expression of the M-IL-2^{88\text{Arg}, 125\text{Ala}} fusion protein on HeLa cells and to analyze the antitumor activity of the protein. In conclusion, a recombinant eukaryotic pLEGFP-C1-M-IL-2^{88\text{Arg}, 125\text{Ala}} expression plasmid containing the M-IL-2^{88\text{Arg}, 125\text{Ala}} fusion gene was constructed and the M-IL-2^{88\text{Arg}, 125\text{Ala}} fusion protein was successfully expressed in HeLa cells. Furthermore, the M-IL-2^{88\text{Arg}, 125\text{Ala}} fusion protein was able to inhibit HeLa cell proliferation and induce apoptosis in the tumor cells. These findings may offer an alternative method for anticancer therapy. The present study has provided a basis for future studies into the M-IL-2^{88\text{Arg}, 125\text{Ala}} fusion gene.

Introduction

Melittin is a naturally-occurring cytolytic antimicrobial peptide that consists of 26 amino acids, with a molecular weight of 2846.46, and constitutes 40-50% of dry whole honeybee venom (1-4). Melittin has been proposed as a possible anticancer drugs with high potential and the antibacterial, antiviral, anti-inflammatory and anticancer properties demonstrated by the protein have been previously described (1-4). Numerous studies have reported several mechanisms of melittin cytotoxicity in various types of cancer cells, with mechanisms including growth inhibition, induction of apoptosis, cell cycle alterations and an effect on proliferation (5,6). The melittin peptide possesses numerous biological properties, but the hemolytic action of the melittin peptide limits the clinical applications of the protein (2).

Human interleukin-2 (hIL-2) acts as a T cell growth factor and plays a key role in the generation of antitumor immunity mediated by cytotoxic T lymphocytes. IL-2 is able to influence several types of cancer effectively, including renal cell carcinoma, melanoma and liver cancer (7-9). In addition, recombination of the IL-2 gene has been clinically used as a type of drug to treat a variety of tumors (10,11). Despite the functions of IL-2, the toxicity and short half-life of IL-2 also limit the clinical application of this protein (7). Therefore, numerous studies worldwide have investigated mutant recombinant hIL-2 to increase the biological activity of the protein and to minimize the corresponding systemic toxicity (12-14). In the present study, the IL-2 gene was modified by site-specific mutagenesis procedures into IL-2^{88\text{Arg}, 125\text{Ala}}. An associated study has indicated that mutant hIL-2, which possesses an
asparagine to arginine substitution at residue 88, is more selective for T cells than NK cells compared with the wild-type hIL-2 \textit{in vitro} (12). The free cysteine at 125 is not recognized by the IL-2 receptor and contributes to the coagulation of protein. Substituting cysteine with alanine at residue 125 improves this defect and enhances the function of IL-2 (15,16).

\textit{M-IL-2}(88Arg, 125Ala) is a fusion protein consisting of melittin and IL-2 (88Arg, 125Ala) and the two proteins are connected to each other by the (Gly,Ser) linker peptide. The fusion protein is expected to combine the function of mutant IL-2 and melittin. The lethal effect of \textit{M-IL-2}(88Arg, 125Ala) on host bacteria has been reported (17).

In the present study, a recombinant eukaryotic expression plasmid containing the \textit{M-IL-2}(88Arg, 125Ala) fusion gene linked with the enhanced green fluorescent protein (EGFP) gene was constructed and the protein was successfully expressed, with the antitumor bioactivity of the protein being identified. The result indicated that this fusion protein was able to inhibit the proliferation and induce the apoptosis of HeLa cells. The \textit{M-IL-2}(88Arg, 125Ala) fusion protein may be a potential agent for cancer therapy, and is therefore important for future studies.

Materials and methods

Materials. The pLEGFP-C1 vector, \textit{Escherichia coli} strain DH5α and \textit{E. coli} DH5α cells containing the plasmid that coded for \textit{pPICZαA/M-IL-2}(88Arg, 125Ala) were provided by the Department of Microbiology of Qindao University (Qingdao, Shandong, China). The strains were propagated in Luria-Bertani broth, consisting of 10.0 g/l tryptone, 10.0 g/l NaCl and 5.0 g/l yeast extract (pH 7.2-7.5), supplemented with 25 µg/ml Zeocin or 100 µg/ml ampicillin (Takara Biotechnology, Co., Ltd., Dalian, Liaoning, China). The HeLa-60 cell line was purchased from the Shanghai Cell Resource Center of the Chinese Academy of Sciences (Shanghai, China). The HeLa-60 cells were propagated in HyClone RPMI-1640 medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and maintained at 37°C in a humidified atmosphere containing 5% (v/v) CO₂. The gel and plasmid extraction kits, \textit{BamHI}, HindIII, EcoRI and KpnI restriction enzymes, and T4 DNA ligase were purchased from Takara Biotechnology, Co., Ltd. The 2X Dream Taq Green PCR Master Mix and RevertAid First Strand cDNA Synthesis kit were obtained from Fermentas (Waltham, MA, USA). TRIZol reagent and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA). Guava Nexin reagent was obtained from EMD Millipore (Billerica, MA, USA). Rabbit anti-hIL-2 and goat anti-rabbit immunoglobulin G were obtained from Bioss (Woburn, MA, USA). Cell counting kit-8 (CCK-8) and dimethyl sulfoxide were obtained from Signalway Antibody LLC (Shanghai, China).

Construction of the pLEGFP-C1-M-IL-2(88Arg, 125Ala) plasmid (Fig. 1)

\textit{M-IL-2}(88Arg, 125Ala) gene amplification and sequencing. The \textit{M-IL-2}(88Arg, 125Ala) gene was amplified from the \textit{pPICZαA/M-IL-2}(88Arg, 125Ala) plasmid by polymerase chain reaction (PCR) using the p1 and p2 primers (Table I), which added HindIII and \textit{BamHI} restriction sites to the 5’ and 3’ ends of the gene, respectively. The parameters for PCR were as follows: Pre-denaturation at 94°C for 4 min; denaturation at 94°C for 45 sec; annealing at 56°C for 30 sec; extension at 72°C for 30 sec; and further extension at 72°C for 10 min. This protocol was performed for 30 cycles. The \textit{pPICZαA/M-IL-2}(88Arg, 125Ala) plasmid was identified by restriction analysis, which was performed using the \textit{EcoRI} and \textit{KpnI} enzymes, and the plasmid was sequenced using p3 and p4 primers (Table I) at Sangon Biotech Co., Ltd. (Shanghai, China).

Construction and identification of the recombinant eukaryotic expression plasmid pLEGFP-C1-M-IL-2(88Arg, 125Ala). The purified PCR products of \textit{M-IL-2}(88Arg, 125Ala) and the backbone plasmid pLEGFP-C1 were digested by the \textit{BamHI} and HindIII restriction enzymes, respectively. The digestion products were purified and isolated, and the target gene \textit{M-IL-2}(88Arg, 125Ala) was then subcloned into the \textit{pLEGFP-C1} plasmid using T4 DNA ligase, forming the recombinant plasmid pLEGFP-C1-M-IL-2(88Arg, 125Ala). This plasmid was transformed into competent \textit{E. coli} DH5α cells and the cells were selected on solid Luria-Bertani broth plates containing 10.0 g/l tryptone, 10.0 g/l NaCl, 5.0 g/l yeast extract and 15.0 g/l agar (pH 7.2-7.5), in addition to 100 µg/ml ampicillin. The recombinant plasmid was amplified in positive transformants and extracted using the plasmid extraction kit. The plasmid was then identified by restriction analysis using \textit{BamHI} and HindIII, PCR and sequencing with the p5 and p6 primers (Table I) at Sangon Biotech Co., Ltd.

Expression of the recombinant eukaryotic expression plasmid pLEGFP-C1-M-IL-2(88Arg, 125Ala) in HeLa cells

Cell culture and transient transfection. Human cervical cancer cells (HeLa cells) were propagated in RPMI-1640 medium supplemented with 10% fetal bovine serum and maintained at 37°C in a humidified atmosphere containing 5% (v/v) CO₂. The cells were plated 24 h prior to transfection onto six-well plates for the mRNA assay, western blotting and apoptosis assay, and onto 96-well plates for the growth curve assay. The cells were then divided into the non-transfection, Lipofectamine 2000, Lipofectamine 2000 and empty pLEGFP-C1 plasmid, and Lipofectamine 2000 and recombinant pLEGFP-C1-M-IL-2(88Arg, 125Ala) plasmid groups. The transfection was performed according to the instructions provided by the manufacturer of the Lipofectamine 2000 when the cell confluence reached 90%. Transfection efficiency was measured 24 h subsequent to transfection using an Olympus confocal microscope (Olympus, Tokyo, Japan), and the cells were subjected to the subsequent assays.

Detection of \textit{M-IL-2}(88Arg, 125Ala) mRNA expression by \textit{RT-PCR}. Total RNA was extracted from HeLa cells using TRIZol reagent. First-strand cDNA was synthesized using the RevertAid First Strand cDNA Synthesis kit. \textit{RT-PCR} was then conducted according to the manufacturer's instructions, using the first-strand cDNA as a template and the primers p1, p2, p7 and p8 (Table I). In each reaction, GAPDH was used as an internal control. The parameters for PCR were as follows: Pre-denaturation at 94°C for 4 min; denaturation at 94°C for 45 sec; annealing at 56°C for 30 sec; extension at 72°C for 30 sec; and further extension at 72°C for 10 min.
This PCR protocol was performed for 30 cycles. The resulting PCR products were analyzed with a Vilber Lourmat imaging system (Quantum ST4, Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany).

**Western blot analysis.** The cells on the bottom surface of the culture plate and in the medium were harvested at 48 h subsequent to transfection. Total protein was extracted using radio immunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) and the concentration of the protein was identified using the bicinchoninic acid protein assay kit (Beijing ComWin Biotech Co., Ltd., Beijing, China). Various samples containing the same amount of protein were loaded on a 12% SDS-PAGE gel and the gel was wet-transferred onto polyvinylidene fluoride membranes (Virostat, Inc., Westbrook, ME, USA). The membranes were blocked using Tris-buffered saline with Tween 20 (Cell Signaling Technology, Inc., Danvers, MA, USA) containing 5% skim milk for 2 h and then incubated with the secondary horseradish peroxidase-conjugated polyclonal goat anti-rabbit antibody (1:10,000; cat. no. ASS1009, Bioss, Inc.) at room temperature for 2 h. The results were detected using the Vilber Lourmat imaging system.

**The anti-tumor effects of the M-IL-2(88Arg, 125Ala) fusion protein.** The CCK-8 and apoptosis assays were performed to analyze the effects of M-IL-2(88Arg, 125Ala) expression on HeLa cells. Four groups were designed as follows: Experimental group consisting of cells transfected with pLEGFP-C1-M-IL-2(88Arg, 125Ala); control group consisting of cells transfected with pLEGFP-C1; control group consisting of cells transfected with only Lipofectamine 2000; and normal group consisting of non-transfected cells. The cells were seeded into 96-well plates for the CCK-8 assay, and each well was administered with 10 µl CCK-8 solution at 24, 48 and 72 h subsequent to transfection. The cells were then incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 4 h. The optical density (OD) of the cells was measured at 450 nm using a plate reader (Sunrise; Tecan Schweiz AG, Männedorf, Switzerland). The cells were seeded into six-well plates for the apoptosis assay. The cells that attached and detached were harvested at 72 h subsequent to transfection and incubated for 20 min in the dark following the addition of Guava Nexin.
Reagent. The experimental data were acquired using a Guava Mini flow cytometer (EMD Millipore, Billerica, MA, USA).

Statistical analysis. All data are expressed as the mean ± standard deviation. Statistical comparisons between groups were analyzed using one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of the pPICzA/M-IL-2(A/Arg, 125Ala) plasmid. The pPICzA/M-IL-2(A/Arg, 125Ala) plasmid was identified by EcoRI and KpnI digestion, as shown in Fig. 2A. The digestion and sequencing results confirmed the identity of the plasmid within the cells to be the pPICzA/M-IL-2(A/Arg, 125Ala) plasmid.

Construction of the pLEGFP-C1-M-IL-2(A/Arg, 125Ala) plasmid. The purified PCR products of M-IL-2(A/Arg, 125Ala) were inserted into the backbone of the pLEGFP-C1 plasmid subsequent to digestions by BamHI and HindIII (Fig. 2B and C). Subsequent to transformation, the positive transformants were selected on SLB plates containing 100 µg/ml ampicillin. The 726-bp products of PCR performed on pLEGFP-C1-M-IL-2(A/Arg, 125Ala) with primers p5 and p6 and restriction analysis with BamHI and HindIII (Fig. 2D and E) all demonstrated that the recombinant plasmid pLEGFP-C1-M-IL-2(A/Arg, 125Ala) was constructed successfully. The results of the sequencing analysis were also consistent with the expected findings.

Expression of the recombinant eukaryotic expression plasmid pLEGFP-C1-M-IL-2(A/Arg, 125Ala) in HeLa cells. To investigate the ability of pLEGFP-C1-M-IL-2(A/Arg, 125Ala) to express the M-IL-2(A/Arg, 125Ala) fusion protein, transfection, RT-PCR and western blot analysis were performed. The results demonstrated that cells transfected with pLEGFP-C1-M-IL-2(A/Arg, 125Ala) expressed the M-IL-2(A/Arg, 125Ala) fusion protein compared with the cells transfected with the empty pLEGFP-C1 plasmid and the non-transfected cells (Fig. 3A-C).

The effects of the M-IL-2(A/Arg, 125Ala) fusion protein on HeLa cells. The effect of the M-IL-2(A/Arg, 125Ala) fusion...
protein on HeLa cells was detected by the CCK-8 and apoptosis assays. The morphology of the cells was observed at 24, 48 and 72 h (Fig. 4) and the CCK-8 assay was performed at 24, 48 and 72 h subsequent to transfection (Fig. 5). The apoptosis assay was performed at 72 h post-transfection (Fig. 6). The results indicted that the cells in the group transfected with the pLEGFP-C1-M-IL-2(88Arg, 125Ala) plasmid grew at a slower rate compared with the control groups. It was therefore indicated that the overexpression of the fusion protein inhibited the proliferation of HeLa cells and induced apoptosis.

### Discussion

Tumor-targeted plasmids encoding toxic protein genes are reliable tools in gene therapy that are able to be used in various tumors (18,19). In the present study, a novel recombinant eukaryotic expression plasmid, which contained the genes for a toxic fusion protein that was comprised of melittin and IL-2(88Arg, 125Ala), was constructed initially and the plasmid was then successfully transfected into HeLa cells.

The hIL-2 protein acts as a T cell growth factor and plays a key role in the generation of the antitumor immunity that is

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### Table I. Primers used in the present study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>DNA sequence (5’‑3’)</th>
<th>Restriction enzyme</th>
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<tbody>
<tr>
<td>p1</td>
<td>CCCAAGCTTCGGGAATTGGAGCAGTTCT</td>
<td>HindIII</td>
</tr>
<tr>
<td>p2</td>
<td>CCGGATCCAGTTAGTGTGGAGATGATG</td>
<td>BamHI</td>
</tr>
<tr>
<td>p3</td>
<td>GACGTGTTCAATTCTGACAAGC</td>
<td></td>
</tr>
<tr>
<td>p4</td>
<td>GCAATGGCATCTGACATCC</td>
<td></td>
</tr>
<tr>
<td>p5</td>
<td>CATGCTCTGCTGGAGTTCGTG</td>
<td></td>
</tr>
<tr>
<td>p6</td>
<td>ACCTACACGTGGGCTTTTCATT</td>
<td></td>
</tr>
<tr>
<td>p7</td>
<td>CAAGTGCACTCCATGACAAACTTTG</td>
<td></td>
</tr>
<tr>
<td>p8</td>
<td>GTCCACACCCTGTTGCTGTAG</td>
<td></td>
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</tbody>
</table>

Underlining indicates the restriction site sequences.
Figure 4. The morphology of various HeLa cell treatment groups were observed at 24, 48 and 72 h subsequent to transfection. (A) Cells transfected with pLEGFP-C1-M-IL-2(88Arg, 125Ala). (B) pLEGFP-C1-transfected cells. (C) Lipofectamine 2000-transfected cells. (D) non-transfected cells.

Figure 5. Evaluation of the effects of M-IL-2(88Arg, 125Ala) overexpression on the proliferation of HeLa cells by CCK-8 analysis at 24, 48 and 72 h subsequent to transfection. *P<0.05 Group R vs. Groups L, E and N at 48 and 72 h. R, cells transfected with pLEGFP-C1-M-IL-2(88Arg, 125Ala); E, pLEGFP-C1-transfected cells; L, Lipofectamine 2000-transfected cells; N, non-transfected cells.

Figure 6. Representative dot plots from flow cytometric analysis of apoptosis at 72 h after transfection. *P<0.05 Group R vs. Groups L, E and N at 72 h. R, cells transfected with pLEGFP-C1-M-IL-2(88Arg, 125Ala); E, pLEGFP-C1-transfected cells; L, Lipofectamine 2000-transfected cells; N, non-transfected cells; 7-AAD, 7-aminoactinomycin D; PE, phycoerythrin.
mediated by cytotoxic T lymphocytes, as hIL-2 stimulates the local accumulation of cluster of differentiation 8(+) T cells and NK cells (20). IL-2 was also one of the first cytokines used for gene therapy and was found to possess a wide range of target immune cells. The administration of IL-2 has been recognized as a useful tool in immunotherapy for various tumors, including melanoma, prostate cancer and neuroblastoma (1,21), but a short half-life and resulting systemic toxicity limit the application of this protein.

Melittin is a naturally-occurring cytolytic antimicrobial peptide that possesses numerous biological functions. The protein has been used as a traditional medicine to treat back pain, rheumatism and skin diseases due to antibacterial, antiviral and anti-inflammatory effects, and certain studies have demonstrated the antitumor activity of melittin (22,23). Despite these properties, melittin also demonstrates several defects.

In the present study, the IL-2 gene sequence was modified and the fusion gene M-IL-2(88Arg,125Ala) was constructed to overcome the respective defects of melittin and IL-2, in addition to improving the function of these proteins and offering a novel tool for anti-tumor therapy. The M-IL-2(88Arg,125Ala) fusion gene was first used in the HeLa tumor cells and the results demonstrated that the fusion protein was able to inhibit the proliferation and induce the apoptosis of HeLa cells. In conclusion, a recombinant eukaryotic expression plasmid, pLEGP-F1-M-IL-2(88Arg,125Ala), was constructed to contain the M-IL-2(88Arg,125Ala) fusion mutant gene, and the plasmid resulted in the successful expression of the M-IL-2(88Arg,125Ala) fusion protein. The present results demonstrated that the fusion protein was able to inhibit the proliferation and induce the apoptosis of HeLa cells. A strong basis for future studies into this fusion gene was therefore created in the present study.

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References