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ISSN 1479-2362
Chinese Journal of Agricultural Biotechnology **6**(1); 35–39
doi:10.1017/S1479236208002362

Construction of a membrane-lytic immunotoxin using melittin and epidermal growth factor

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Received 20 September 2007; Accepted 21 December 2007

First published in *Journal of Agricultural Biotechnology* 2008,16(3): 390–394

Abstract

Epidermal growth factor receptor (EGFR) is becoming a perfect target for killing carcinoma cells, especially because of its overexpression on the surface of these cells. Cationic antimicrobial peptides (CAP) have their own special mechanism of membrane-lytic cytotoxicity. In this study, a membrane-lytic immunotoxin (IT), chimeric protein MEGFMEL, was constructed to kill carcinoma cells with EGFR overexpression. This protein is composed of mouse (*Mus musculus*) epidermal growth factor (MEGF), as the target part, and melittin (MEL), as the cytotoxic part. Using *Escherichia coli* BL21 and pET30a as expression strain and vector, respectively, 63.45 μg/ml of MEGFMEL (68% purity) was obtained through low-temperature induction of expression and a thawing-freezing purification procedure (without cytolysis). *In vitro* activity measurement showed that this MEGFMEL significantly induced a lethal effect on A_{431} carcinoma cells overexpressing EGFR on the surface, with an LD_{50} value 52.6 μg/ml. The results suggest that the use of CAP as the toxin in the construction of unique membrane-lytic ITs aimed at EGFR is feasible.

Keywords: membrane-lytic immunotoxin; melittin; epidermal growth factor receptor

Introduction

Cationic antimicrobial peptides (CAP) are active peptides capable of killing pathogenic microorganisms in body fluid, an important component of the innate immune system. Studies on CAP application are becoming hot spots in veterinary medicine, pharmacology, physiology, etc. Wang *et al.* (2004b) reported the differences of expression of CAP *pr*-39 in various developmental stages of pigs. Zhang *et al.* (2005) cloned the chicken beta-defensin gene and analysed its sequence. Li *et al.* (2001) analysed the cow lectoferritin gene. Wang *et al.* (2004a) analysed the mouse lectoferritin gene. Huang and Wang (2005) expressed mouse lectoferritin heterologously in *Escherichia coli*. Shi *et al.* (2006) expressed heterologously the fusion protein composed of melittin and green fluorescence protein in insect cells. Zhao *et al.* (2006) expressed heterologously human lectoferritin in tomoto. As yet there are few reports about using CAP to create a target-sensitive toxin to kill target cells.

Immunotoxin (IT) kills specifically carcinoma cells. IT is composed of a target part – antibody and cytokine – which can specially bind to antigen or receptors over-expressed on carcinoma cells, and a cytotoxic part, which can kill carcinoma cells when the target part co-exists covalently (Pennell and Erickson, 2002). In normal conditions, epidermal growth factor receptor (EGFR) is widespread, but at low density (5000–10 000/molecules/cell) on mammal cell surfaces; it is blocked and unable to bind to its ligand. When the body is hurt, EGFRs become exposed and bind to their ligands to start healing, before

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https://doi.org/10.1017/S1479236208002362 Published online by Cambridge University Press

being blocked again. Due to out of control gene expression, EGFRs can become abnormally exposed or overexpressed, resulting in abnormal cell proliferation and carcinomas (Gullick, 1991). On the surface of many kinds of carcinomas, the EGFRs are overexpressed with normal form and steady phenotype, tightly binding to ligands such as epidermal growth factor (EGF) and transforming growth factor-α (TGFα). Davies and Chamberlin (1996) reported that EGFRs of over 40% of mastocarcinomas, 75% of ovary carcinomas, 81% of cervix carcinomas, 71% of oesophageal carcinomas and 50% of liver carcinomas overexpress 100 times more than normal and are exposed on the cell surface. Many kinds of carcinoma EGFR overexpression imply high metastasis rates and bad pronosis (Harris *et al.*, 1999; Kersemaekers *et al.*, 1999). EGFR is expected to be a target site for anticarcinoma ITs (Modi and Seidman, 2002).

Up to now, the cytotoxic part of ITs has been mainly from plant and microorganisms, such as ricin, diphtheria toxin (DT) and pseudomonas exotoxin (PE), the cytotoxic effect of which is to inhibit protein synthesis in target cells (Lambert *et al.*, 1991; Choe *et al.*, 1992; Kreitman, 2001). An internalization step is necessary for establishing the toxin's active efficiency. Melittin (MEL) is from bee venom, a typical membrane-lytic CAP which destroys target cell membranes without an internalization step. Using MEL as the cytotoxic part of IT, without an internalization step, will significantly raise cytotoxic activity.

In this study we used MEL as the cytotoxic part and mouse (*Mus musculus*) epidermal growth factor (MEGF) as the target part, incorporating the two domains by a linker sequence into a single-molecule IT heterologously expressed in *E. coli*. The linker is flexible enough to make the two domains, MEGF and MEL, independently active, because the sequence of the linker is from the highly flexible immunoglobin hinge region. The positive charges of the cationic region of the MEL domain were neutralized by MEGF (the pI of MEGF is 4.6), which makes the MEGFMEL unable to bind to negatively charged normal cells. The MEGF domain could lead MEGFMEL binding to the carcinoma cells overexpressing EGFR, so that the amphipathic alpha helix of the MEL domain could then specifically destroy the carcinoma cell membrane.

Materials and methods

Plasmid and strains

Recombinant plasmids pGEMT*megf* and pUCT*mel* were developed in our laboratory (He *et al.*, 2002; Ruan *et al.*, 2005). Plasmid pET30a and *E. coli* BL21 were from our laboratory collection. T4 DNA ligase and restriction endonuclease enzymes (*Eco*RI, *Bam*HI, *Sac*I and *Hin*dIII) were purchased from Shanghai Sangon Co. Ltd (Shanghai, China). *E. coli* was grown in Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride) containing 50 μg/ml ampicillin. The linker sequence was synthesized by Shanghai Sangon Co. Ltd as follows:

linker$_1$: 5′-C CGT $(GGT)_4$ TCT $(GGT)_4$ TCT $(GGT)_3$ G-3′
linker$_2$: 3′-TC GAG GCA $(CCA)_4$ AGA $(CCA)_4$ AGA $(CCA)_3$ CCT AG-5′

Linker$_1$ and linker$_2$ became a double-strand linker with the cosmid sites *Sac*I at the 5′ end and *Bam*HI at 3′ end after renaturation.

Primers MEGF-P$_1$ (matching with the MEGF 5′ region) and MEL-P$_2$ (matching with the melittin 3′ region) were synthesized by Shanghai Sangon Co. Ltd, with the following sequences:

MEGF-P$_1$: 5′-CCCGAATTCAATAGTTACCCAGGATGCCC-AT-3′ (*Eco*RI site underlined)
MEL-P$_2$: 5′-CCCAAGCTTTTACTGTTGCCTCTTACGTTT-3′ (*Hin*dIII site underlined).

Construction of megfmel *chimeric DNA*

The *megf* sequence, with the sites *Eco*RI at the 5′ end and *Sac*I at the 3′ end, was generated by digesting the PCR product with *Eco*RI and *Sac*I restriction enzymes (*megf*/*Eco*RI + *Sac*I).

The *mel* sequence with the sites *Bam*HI at the 5′ end and *Hin*dIII at the 3′ end was generated by digesting the PCR product with *Bam*HI and *Hin*dIII restriction enzymes (*mel*/*Bam*HI + *Hin*dIII).

The *megf*/*Eco*RI + *Sac*I and *mel*/*Bam*HI + *Hin*dIII were ligated by linker (*Sac*I cosmid site at 5′ end and *Bam*HI cosmid site at 3′ end) with T4 DNA ligase. Then, the *megfmel* fusion gene was generated by PCR with primers MEGF-P$_1$ and MEL-P$_2$.

*Cloning of chimeric construct pET*megfmel

The *megfmel* PCR product was ligated to T-vector before digestion by *Eco*RI and *Sac*I restriction enzymes. The digested *megfmel* fragment was subcloned into the plasmid pET30a, which was previously digested with the same restriction endonuclease enzymes, to develop the chimeric construct pET*megfmel*. This construct was sequenced to confirm correct DNA sequence after PCR and digestion verification. The primer for sequencing was the universal primer of the pET vector: pET30-Pc (5′-TAATACGACT-CACTATAGGG-3′).

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5'-CCCGAATTC AAT AGT TAC CCA GGA TGC CCA TCC TCA TAT GAT GGT TAC TGC
CTC AAT GGT GGC GTG TGC ATG CAT ATT GAA TCA CAG GAC AGC TAC ACA TGC
AAC TGT GTT ATT GGC TAT TCT GGG GAT CGA TGT CAG AGT CGA GAC CTA CGA
TGG TGG GAG CTC CGT GGT GGT GGT GGT TCT GGT GGT GGT GGT TCT GGA GGA
GGA GGA TCC GGA ATT GGA GCA GTT CTG AAG GTA TTA ACC ACA GGA TTG CCC
GCC CTC ATA AGT TGG ATT AAA CGT AAG AGG CAA CAG TAA AAGCTTGGG-3'

Fig. 1. Sequence of the DNA insert in pET*megfmel*.

Expression and purification of the chimeric protein

The plasmid pET*megfmel* was transformed into *E. coli* BL21 cells to generate the recombinant strain BL21-pET*megfmel*, while pET30a was transformed into *E. coli* BL21 cells to generate the strain BL21-pET30a as the negative control. The transformed bacteria were plated on LB plates. The bacterial cultures were induced with iso-propyl β-D-thiogalactopyranoside (IPTG; final concentration was 0.50 mmol/l) when the optical density of the BL21-pET*megfmel* and BL21-pET30a reached 0.5 OD_{600}. The expression product was the chimeric protein MEGFMEL with 50 amino acid residues of pET30a at its N-terminal end and at its C-terminal end. The chimeric protein MEGFMEL was purified using the thawing and freezing method (Ruan *et al*., 2005) and detected on a 15% SDS–PAGE gel (Wang and Fan, 2000).

Quantification of the purified protein

The concentration of the target protein was measured by the Folin-phenol method according to Zhou (1991).

Bioactivity assay of the recombinant expressed product

We evaluated the cytotoxic activity of the product through a [^{3}H]thymine deoxyribonucleoside (Tdr) incorporation assay, with A_{431} as the target cells. The whole procedure was as follows:

The concentration of A_{431} cells was adjusted to 5×10^4/ml with RPMI-1640 medium, then 10 μCi [^{3}H]Tdr were added to 1 ml target cell solution and the culture was shaken for 6 h at 37 °C to thoroughly incorporate [^{3}H]Tdr into the daughter cells' DNA. A_{431} cells were then washed three times with RPMI-1640 medium and their concentration adjusted to 5×10^4/ml. Fifty microlitres of the cell suspension were added to the 96-well culture plate, followed by the gradient dilution of recombinant chimeric protein MEGFMEL in each well. The BL21-pET30a expressed product was used as the negative control, while 1.0 mol/l NaOH served as positive control.

Cells in the 96-well plate were incubated for 12 h at 37 °C. A volume of 50 μl culture medium containing 1.0 mg/ml trypsinase and 10 μg/ml DNase was transferred to each well. The positive control received, in addition, 1.0 mol/l HCl to neutralize NaOH. The plate was finally incubated for 1 h at 37 °C to ensure the disintegration of dead cells and their DNA to release the [^{3}H]Tdr.

The counts per minute (cpm) in the supernatant and deposit in each well were determined and the cytotoxic activity was calculated. The cytotoxic activity was calculated using the formula: [cpm in supernatant/(cpm in supernatant + cpm in deposit)] × 100%. The concentration of the protein of interest when cytotoxic activity was 50% gave the LD_{50} (median lethal dose).

Statistical analysis

The data analyses were performed using SAS/Statistic Version 6.12 software program (SAS Institute Inc., Cary, North Carolina, USA). The *F*-test was used to determine the statistically significant differences between the two groups in each parameter.

Results

*Vector pET*megfmel *verification*

The vector pET*megfmel* was sequenced to verify the DNA sequence after PCR and digestion processes. The sequencing result confirmed the existence of the DNA insert with the sequence shown in Fig. 1.

Electrophoresis assay and concentration of the chimeric protein MEGFMEL

After the preliminary purification used in this assay, the recombinant expressed product of BL21-pET*megfmel* migrated with an apparent molecular size of 14.0 kDa, as expected (Fig. 2, lane 2), while nothing was generated in the negative control strain BL21-pET30a (Fig. 2, lane 1). This result confirmed the success of the recombinant expression of chimeric protein MEGFMEL. The purity quotient of the product was 68%, according to grey scale scanning (Fig. 2). The calculated concentration of the chimeric protein MEGFMEL was 63.45 μg/ml.

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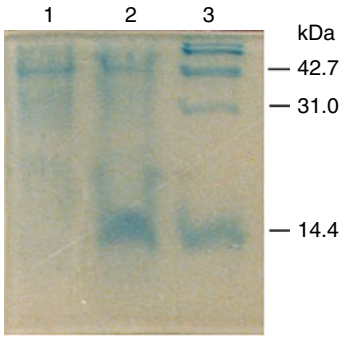


Fig. 2. Detection of the chimeric protein MEGFMEL by SDS-PAGE electrophoresis. Lanes: 1, BL21-pET30a; 2, BL21-pET*megfmel*; 3, marker. (See online for a colour version of this figure.)

Chimeric protein MEGFMEL bioactivity

The recombinant chimeric protein MEGFMEL was diluted with distilled water to give the following concentrations: 60, 30, 15, 7.5, 3.75, 1.875 and 0.9375 μg/ml. Cytotoxic activity assays of the chimeric protein MEGFMEL were performed with A_{431} as target cells using [^{3}H]Tdr incorporation assay (Table 1).

The results in Table 1 show that the cytotoxic activity of the recombinant chimeric protein MEGFMEL to the A_{431} cells was significant. In addition, the cytotoxic activity was evaluated by further dilutions between 60 and 30 μg/ml. The LD_{50} of the protein was estimated as 52.6 μg/ml (cytotoxic activity 50.39 ± 5.45%). Because of the presence of some impure protein in the recombinant product, the LD_{50} of the recombinant MEGFMEL should actually be much lower. A higher protein purification could possibly increase the cytotoxic activity.

Discussion

The carcinoma cells A_{431} overexpress EGFR on the surface. They are perfect target cells for the assay of ITs targetting EGFR. However, up to now, the cytotoxic part of ITs has been from plants and microorganisms (PE, DT, ricin, etc.). The strong immunogenicity and essential internalization step affect the efficiency of these ITs (Lambert *et al.*, 1991; Choe *et al.*, 1992; Kreitman, 2001). CAP is becoming a potential toxin for IT construction due to its strong lethal effect and membrane-lytic cytotoxicity mechanism. In this study, a membrane-lytic IT, the chimeric protein MEGFMEL (composed of MEGF as the target part and MEL as the cytotoxic part), was constructed to neutralize carcinoma cells through EGFR overexpression. *In vitro* activity measurement showed that MEGFMEL had a strong lethal effect on A_{431} carcinoma cells.

The N-terminal end of the recombinant chimeric protein MEGFMEL is built up of 50 amino acid residues of pET30a. Previous studies have demonstrated that changes of the amino acid residues of the epidermal growth factor, except 4–48, have no influence on its activity (Burgess *et al.*, 1988). Our previous study showed that the activity of MEGF (57% purity) with an additional pET30a sequence at the N-terminal end was 30% of that of the standard MEGF (Ruan *et al.*, 2005). The present study has proved that MEGF with an additional pET30a sequence at the N-terminal end can ensure the affinity of the recombinant chimeric protein MEGFMEL to EGFR.

Some problems, however, should be considered to ensure clinical application of the IT. These problems include maintaining the half-life *in vivo*, keeping the immunogenicity as low as possible, improving safety testing, etc. The results in this study showed that using CAP as the cytotoxic part to construct an EGFR-targetting IT is possible. It is expected that a human CAP source (human beta defensins, hBDs) will be developed as the cytotoxic part and human epidermal growth factor (hEGF) as the target part to build more efficient ITs.

Table 1. Cytotoxic activity of the recombinant chimeric protein MEGFMEL to A_{431} cells

	CPM in supernatant	CPM in deposit	Cytotoxic activity (%)
Negative control	185 ± 66	4031 ± 335	4.39 ± 0.28
Positive control	4318 ± 419	312 ± 78	91.29 ± 9.41**
60.0000 μg/ml MEGFMEL	2493 ± 422	2027 ± 428	55.15 ± 12.55**
30.0000 μg/ml MEGFMEL	1395 ± 312	2621 ± 93	34.74 ± 3.06**
15.0000 μg/ml MEGFMEL	988 ± 182	3111 ± 226	24.10 ± 3.27**
7.5000 μg/ml MEGFMEL	774 ± 56	3488 ± 331	18.16 ± 2.16**
3.7500 μg/ml MEGFMEL	428 ± 105	3708 ± 112	10.35 ± 1.86**
1.8750 μg/ml MEGFMEL	369 ± 38	4005 ± 285	8.44 ± 0.72**
0.9375 μg/ml MEGFMEL	265 ± 88	3813 ± 128	6.50 ± 0.54**

CPM, counts per minute.
**, Significant difference compared to negative control at $P < 0.01$.

https://doi.org/10.1017/S1479236208002362 Published online by Cambridge University Press

Acknowledgements

This work was supported by the Zhejiang Natural Science Foundation and Hangzhou Science and Technology Plan.

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