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# Expression of the fusion protein recombinant human granulocyte-macrophage colony stimulating factor and leukemia inhibitory factor in a baculovirus vector system

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**A fusion gene coding human granulocyte-macrophage colony stimulating factor (GM-CSF) and leukemia inhibitory factor (LIF) cDNAs was inserted into the transfer vector pSXIVVI$^+$ X3 with the control of Syn and XIV promoters. The Sf9 cells (*Spodoptera frugiperda*) were co-transfected with the recombinant plasmid and TnNPV DNA (Trichoplusia ni nuclear polyhedrosis virus DNA). The fusion protein recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF) and leukemia inhibitory factor (LIF) could be synthesized in cells infected with recombinant virus at a level of about 23% of their total cellular protein. Activity analysis of the fusion protein in infected cells revealed that it exhibited the dual activities of GM-CSF and LIF. Western blot analysis of the expressed fusion protein in infected larvae showed that the virus-mediated fusion protein, with a molecular weight of ~35 kDa, is confirmed with immunoreactivity.**

**Keywords:** GM-CSF; LIF; baculovirus system; transfer vector; gene expression

## Introduction

Granulocyte-macrophage colony stimulating factor (GM-CSF) and leukemia inhibitory factor (LIF) are two kinds of related and overlapped hematopoietic stimulating factors [8,9,16]. Investigations of cytokine receptors revealed that the two cytokines can display different or the same biological activities by activating different or the same signal transduction systems, respectively [1,3]. GM-CSF has an increasing clinical application in the treatment of neutropenia and aplastic anemia, and has greatly reduced the infection risk associated with bone marrow transplantation by accelerating neutrophil production [2]. LIF is a cytokine with a wide bioactivity spectrum, which can induce differentiation and inhibit growth of leukemia cells, regulate growth and development of embryos, and stimulate hematopoiesis of bone marrow megakaryocyte cells [10].

The baculovirus expression vector system has been widely used to express a variety of heterologous genes in insect cells and caterpillars [7,11,12]. Insect cells provide a suitable environment for post-translational modifications and folding of the protein product such that the foreign proteins synthesized resemble their authentic counterparts in almost all respects [7], and the insect larval system offers an exciting alternative because of lesser equipment requirements and simple operations involved in the mass-scale production (rearing and maintenance of larvae) compared to tissue-cultured cells. Hence, the insect system remains the system of choice for expressing genes for therapeutic or vaccine purposes.

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The objective of this study is to construct a fusion protein with the dual activities of GM-CSF and LIF, expressed in a baculovirus vector system. It is expected to provide a new approach to searching for new and efficient cytokines.

## Materials and methods

### *Cell line, plasmid and virus*

p18GMCSF containing GM-CSF cDNA and p18LIF containing LIF cDNA were from Beijing Institute of Radiation Medicine, Beijing, China. Baculovirus transfer vector pSXIVVI$^+$ X3 [17] was used for expression of the plasmid. *E. coli* DH5$\alpha$ was used for transformation. Trichoplusia ni nuclear polyhedrosis virus (TnNPV) (NERC, Institute of Virology, Oxford, UK) and its recombinant parent virus TnNPV-SVI$^-$ G DNA(gal$^+$ OCC$^-$) containing Syn and XIV promoters [17], and Sf9 cell were used. The medium was TNM-FH complete medium supplemented with 10% fetal bovine serum [4].

### *DNA manipulation*

Plasmid DNA manipulation was carried out as described [13]. The clones were sequenced by dideoxynucleotide chain termination [14] with the Circumvent sequencing kit (New England Biolabs, Beverly, MA, USA) following the manufacturer's instructions.

### *Construction of transfer vector carrying GM-CSF and LIF cDNAs*

The *Bam*HI + *Pst*I fragment carrying cDNA encoding LIF from pUC18LIF was subcloned downstream of the GM-CSF gene in pUC18GM1 and the cloned plasmid pUC18GM-CSF-LIF carrying the fusion gene GM-CSF-LIF was obtained. Finally, the *Eco*RI + *Pst*I fragment of the fusion gene was cloned into transfer vector plasmid

pSXIVVI$^+$ X3 (5.8 kb), the ligation mix was transformed (*E. coli* DH5$\alpha$), thus recombinant plasmid pSXIVVI$^+$ X3-GM-CSF-LIF (6.8 kb) was generated.

### *Construction of recombinant baculovirus containing GM-CSF-LIF gene*

A partial physical map of the recombinant plasmid, pSXIVVI$^+$ X3-GM-CSF-LIF, used for generating recombinant virus, is illustrated in Figure 1. For constructing the recombinant baculovirus, Sf9 insect cells were co-transfected [15] with the recombinant plasmid and TnNPV-SVI$^-$G DNA. The recombinant baculovirus, designated TnNPV-GM-CSFLIF-OCC$^+$, was obtained by plaque purification [12,15].

### *Activity assay of fusion protein in infected cells*

Purified recombinant virus was infected with Sf9 cells ($3 \times 10^6$ cell, MOI = 10 PFU cell$^{-1}$). After 1 h the virus solutions were dislodged and 5 ml of medium TNM-FH containing 10% FBS (fetal bovine serum) was added and cells were cultured at 28°C. The infected cells were washed twice with phosphate-buffered saline (PBS) and dissolved in 1 ml of PBS. Cells were lysed by the freeze-thaw method, and centrifuged for 5 min at 95 000 $\times$ *g*. The supernatant phase from infected-cell lysates was used for the assay. (1) The activity of GM-CSF was assayed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric analysis [6]. Briefly, 50 000 TF-1 cells ml$^{-1}$ (Chinese Academy of Military Medical Sciences, Beijing, China) in log phase were prepared in 96-well microtiter plates with a final volume of 0.1 ml per well. Ten-fold serial dilutions of GM-CSF (with final concentrations of 0.01, 0.1, 1, 10, 100, and 1000 $\mu$g L$^{-1}$) were then added into cells, three replications for every concentration. Plates were incubated at 37°C for 48 h. MTT solution (final concentration 0.5 g L$^{-1}$ diluted by PBS) was added to all wells, and plates were incubated at 37°C for 6 h. Acid-isopropanol (0.1 ml of 0.04 mol L$^{-1}$ HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the crystals. The A values were read on an enzyme-linked assay instrument (Bio-Rad, Beijing, China) with a test wavelength of 570 nm and a reference wavelength of 630 nm. (2) LIF activity was measured by semi-solid culture of HL60 (Beijing Institute of Radiation Medicine) *in vitro* [1,8]. Log phase HL60 cells (300 ml$^{-1}$) were suspended in solutions mixed with 2 ml of FBS, stimulating factor and 1640 medium, the solutions were heated at 37°C in a waterbath for 10 min and 0.2 ml of agar (30 g L$^{-1}$) was added, 1 ml of the solution was added to each petri dish, then transferred into an elastic box and cultured at 37°C under 5% carbon dioxide for 7 days. A colony was counted when it contained more than 50 cells. Three dilutions for rhGM-CSF-LIF were made: 0.01, 0.1 and 1 mg L$^{-1}$.

### *Western blot analysis of fusion protein in infected larvae*

Fourth instar *Plusia agnata* larvae were fed a diet mixed with recombinant virus polyhedrin, sixth instar larvae were harvested 4 days post-infection. Total cell extracts of virus-infected caterpillars were prepared and used for further analysis [4]. Western blot analysis was carried out as described [13]. Larval protein extracts separated by SDS-PAGE (using slab gels with 10% polyacrylamide separating gel and 4% stacking gel) were probed with mouse anti-human GM-CSF monoclonal antibody (Chinese Academy of Military Medical Sciences) with a dilution of 1:400, the membranes were incubated in goat anti-mouse IgG conjugated with horseradish peroxidase (BioRad Laboratories) with a dilution of 1:500 according to the manufacturer's instructions.

## Results and discussion

### *Identification of recombinant plasmid and virus*

The sequencing analysis of recombinant plasmid pSXIVVI$^+$ X3-GM-CSF-LIF (6.8 kb) revealed that the sequences of the fusion gene were as expected (data not shown). The partial sequences in the chimeric region are shown in Figure 1. Enzyme digestion identification of recombinant baculovirus TnNPV-GM-CSFLIF-OCC$^+$ revealed that *Eco*RI + *Pst*I-digested recombinant baculovirus had a 0.93-kb band (equivalent to the sum of GM-CSF fragment (about 500 kb) and LIF fragment (about 420 kb)), which was not visible in the two enzymes digestion map of parent virus (data not shown).

### *Activity of fusion protein in infected cells*

MTT colorimetric analysis demonstrated that rhGM-CSF-LIF has the activity of GM-CSF and can support the growth of TF1 cell lines *in vitro* (Figure 2). The LIF activity assay shows that the fusion protein rhGM-CSF-LIF can suppress remarkably the clonal formation of HL60 *in vitro* ($P < 0.001$), and the suppression is strengthened as the dose increases ($P < 0.001$) (Figure 2). Densitometric scanning of SDS-PAGE gels revealed that ~23% of the total

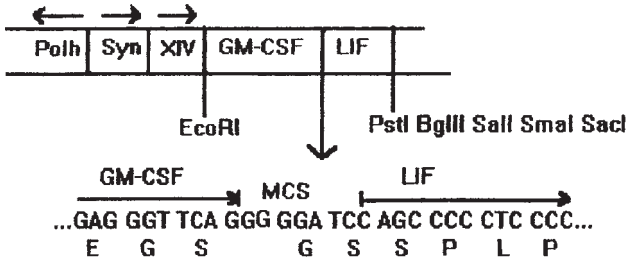


**Figure 1** Partial physical map of the recombinant plasmid pSXIVVI$^+$ X3-GM-CSF-LIF and sequences of GM-CSF and LIF junction. Polh, polyhedrin promoter; Syn, synthesis promoter; XIV, XIV promoter; MCS, multiple cloning sites.

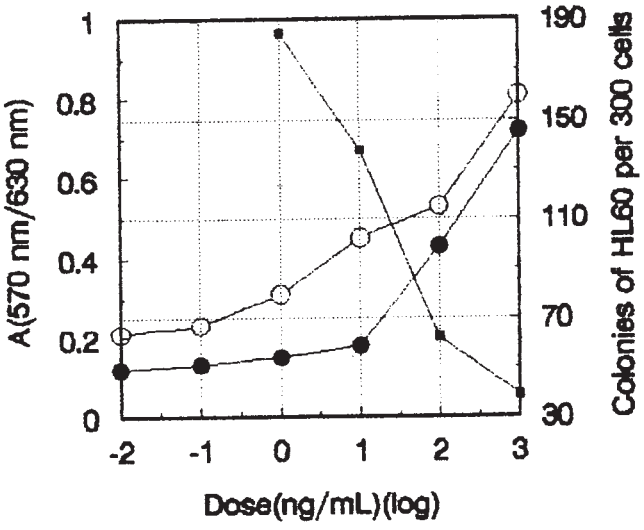


**Figure 2** Growth responses of TF1 cells to fusion protein expressed in Sf9 cells infected with recombinant baculovirus TnNPV-GM-CSF-LIF-OCC+. The zero-point of dose corresponds to the colony amount in the control. ○, A value for rhGM-CSF; ●, a value for rhGM-CSF-LIF; ■, colonies.

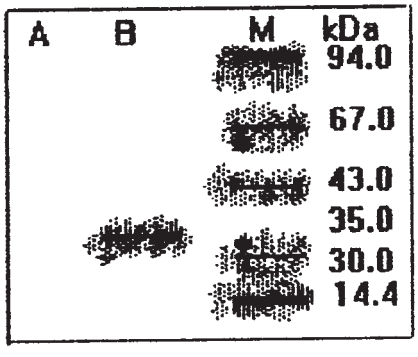


**Figure 3** Western blot of fusion protein expressed in larvae infected with recombinant baculovirus TnNPV-GM-CSF-LIF-OCC+. (A) Larvae infected with TnNPV for 72 h postinfection. (B) Larvae infected with recombinant virus TnNPV-GM-CSF-LIF-OCC+ 4 days postinfection. (M) Low molecular weight markers.

Coomassie brilliant blue-stained protein of the infected cells was represented by the fusion gene rhGM-CSF-LIF (data not shown).

### *Expression of fusion protein in infected larvae*

*P. agnata* larvae were infected with the recombinant baculovirus TnNPV-GM-CSF-LIF-OCC+ simply by mixing the recombinant baculovirus into the insect diet. Larval protein extracts were analyzed by SDS-PAGE and Western blot. The result shows that the fusion gene rhGM-CSF-LIF can also be expressed in the larvae, with a molecular weight of ~35 kDa, reacted with mouse anti-human GM-CSF monoclonal antibody (Figure 3).

It is likely that GM-CSF and LIF can be folded as the natural three-dimensional structure due to the existence of a protein linker consisted of glycine and serine, the protein linker occurs frequently in the hinge region and turning point and has broad mobility, such that GM-CSF and LIF can interact with their own receptor. Since the two functional regions determining the biological activity of human GM-CSF are amino-terminal-proximal and the functional region affecting the activity of LIF has not been reported, we placed GM-CSF in the amino-terminal portion of the fusion protein and LIF in the carboxyl-terminal portion of the fusion protein [5].

These results demonstrate that the cDNAs encoding human GM-CSF and LIF can be expressed in an occluded recombinant baculovirus expression system under the control of synthetic and XIV promoters. Since the expressed fusion protein exhibits the GM-CSF activity and suppresses growth of tumor cells, it may become an efficient anti-tumor factor. Since the recombinant fusion protein can be produced in caterpillars, it is valuable for mass-scale production of such a fusion protein with simple equipment.

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