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Rational design of an EGF-IL18 fusion protein: Implication for developing tumor therapeutics

Jian-Xin Lu [a,b], Ying Peng [a], Zhe-Feng Meng [a], Li-Qin Jin [a], Yong-Sui Lu [a], Min-Xin Guan [a,b,c,*]

[a] *Institute of Cellular and Molecular Medicine, School of Laboratory Medicine, Wenzhou Medical College, Wenzhou, Zhejiang 325035, China*
[b] *Zhejiang Provincial Key Laboratory of Medical Genetics, School of Life Sciences, Wenzhou Medical College, Wenzhou, Zhejiang 325035, China*
[c] *Division and Program in Human Genetics, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA*

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Abstract

Interleukin-18 (IL-18) is a proinflammatory cytokine. This protein has a role in regulating immune responses and exhibits significant anti-tumor activities. Epidermal growth factor (EGF) is an important growth factor that plays a central role in the regulation of cell cycle and differentiation. It was proposed that a targeted delivery of IL-18 by generation of IL-18-EGF fusion protein might decrease adverse effects and result in enhancing cytotoxic and antitumor activities. In the present study, a fusion protein, consisting of EGFR binding domain fused to human IL-18 mature peptide via a linker peptide of (Gly_4Ser) 3, was constructed and expressed in the insect cell line Sf9 using Bac-to-Bac baculovirus expression system. We showed that the purified recombinant fusion protein induced similar levels of IFN-γ to that of native IL-18 protein in human PBMC in the presence of ConA. Furthermore, EGF receptor competitive test in human epithelial cancer A431 cell line showed that EGF-IL18 fusion protein can specifically bind with EGFR by competing with native EGF protein. These suggest that this rationally designed protein can be further developed as novel tumor therapeutics.

Keywords: EGF-IL18 fusion protein; Tumor therapeutics; Cytokine; Expression; Targeting; Anti-tumor; IL-18; EGFR binding domain; Fusion proteins; Baculovirus expression system; Targeting tumor cells

Interleukin-18 (IL-18) or interferon-γ (IFN-γ) inducing factor (IGIF) is a proinflammatory cytokine, produced mainly by macrophage [1,2]. IL-18 precursor protein has a limited biological activity [3], while the mature form with 18.3 kDa, processed by the cystein proteases belonging to a caspase family [4], has many aspects of biological activities [5]. In particular, IL-18 augments cytotoxicity of NK cells and enhances proliferation of T cells [6,7]. It also promotes NK and T cells to secrete IFN-γ as well as granulocyte–macrophage CSF [8]. IL-18 folds into a β-trefoil structure that resembles that of IL-1 and its activity is via an IL-18 receptor (IL-18R) complex expressed on T and NK cells [9]. It has been shown that IL-18 has antitumor effects in multiple murine tumor models and human tumor cell lines in vitro [10–12]. Recent studies revealed that IL-18 has been involved in pathogenesis of several diseases, thereby having adverse effects on the tumor treatment [13–16].

Epidermal growth factor (EGF) is an important growth factor that plays a central role in the regulation of cell cycle and differentiation [17,18]. The EGF receptor (EGFR) is expressed at high levels in many types of tumor cells, such as squamous carcinoma, breast cancer, and endothelial cells. Structurally, EGF, harboring six cysteines, consists of three loops of domains, and the third loop of EGF, consisting of 15 amino acids,

* Corresponding author. Fax: +1 513 636 2261.
E-mail address: min-xin.guan@chmcc.org (M.-X. Guan).

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appears to be the binding site of EGFR, which has a potential to be used in targeted therapy for tumor [19–21].

It was proposed that a targeted delivery of IL-18 by generation of IL-18-EGF fusion protein might decrease adverse effects, but increase cytotoxic and antitumor activities [19,22]. In the present study, a fusion protein, consisting of EGFR binding domain fused to human IL-18 mature peptide via a linker peptide of (Gly_4Ser) 3, was constructed and expressed in the insect cell line Sf9 using Bac-to-Bac baculovirus expression system. The IFN-γ induction assay of fusion protein and EGF receptor competitive test were carried out.

Materials and methods

Chemicals, reagents, and cell lines. Restriction enzymes and pmd18-T plasmid as well as other molecular reagents were purchased from Sangon Biotec. (Shanghai, China). Other chemicals were purchased from Sigma (St. Louis, MO, USA). *Spodoptera frugiperda* Sf9 cell line and human epithelial cancer A431 cell line were purchased from (CCTCC, Wuhan, China). The Sf9 cell line was maintained in Grace's medium supplemented with 10% FBS (GibcoBRL, Gaithersburg, MD, USA). The A431 cell line was grown in F12 medium (Gibco) with 10% FCS. Bac-to-Bac (TM) baculovirus expression system was purchased from Gibco.

Construction of fusion gene. Two step Over-Lap Extension PCR was used to construct fusion gene as shown in Fig. 1. The oligo primers for PCR amplification are as follows: F1 containing a linker peptide of (Gly_4Ser) 3 (underlined sequence) and 13 base of IL-18 cDNA (GenBank AF380360)—5′GTGGCGGTGGTTCCGGCGGTGGTGGCTCTGGTGGCGGCGGATCTTACTTTGGCAAGC 3′; F2, spanning the sequence encoding the EGF binding domain (underlined sequence) and 15 base of F1 5′ terminal—5′ATGCGCTGCTCCCATGGCTACACTGGTATTCGTTGCCAAGCAGTAGTTCTCGGTGGCGGTGGTTCC3′; F3, carrying 21 base of IL-18 cDNA 3′ terminal—5′GCTAGTCTTCGTTTTGAACAG3′.

For the first PCR, pucmT18 plasmid containing coding region of human IL-18 cDNA (a generous gift from Dr. Peng Ying) was used as template, F1 was used as forward primer, and F3 as reverse primer. In the second PCR, the product of the first PCR amplification was used as template, F2 was used as forward primer, and F3 as reverse primer. The resulting plasmid pFUS was generated by purifying second PCR product and cloning into pmd18-T vector. All resulting plasmids were verified for the nucleotide sequences. The fused gene was deposited in the database (GenBank Accession No.: AF454397).

Expression and purification of recombinant fusion protein. Recombinant bacmid DNA was lipofected into monolayer cultures of Sf9 cells. After 72 h of incubation at 27 °C in Grace's medium supplemented with 10% FBS, recombinant baculovirus was harvested and used for next infection. Log-phase Sf9 cells (viability >98%) were infected at an MOI of 5 by recombinant baculovirus. Infected Sf9 cells were grown in suspension at 27 °C for 48–72 h until the proportion of nonviable cells reached 20–40%. Cells were harvested by centrifugation and then resuspended in lysis buffer. Supernatants containing cytosolic proteins were recovered after removal of membrane fragments by centrifugation at 10,000*g* for 1 h at 4 °C. Purification of fusion protein was performed as detailed elsewhere [23]. IL-18 ELISA kit and Western blot analysis were used to determine the presence of fusion protein as detailed previously [24]. Protein purities were assessed by SDS–PAGE and subsequently Coomassie brilliant blue staining.

IFN-γ induction assay of fusion protein. To examine the biological activity of purified recombinant EGF-IL18 fusion protein, human IL-18 (from Sigma) and cytosolic preparations from Sf9 cells prior to infection by recombinant baculovirus along with recombinant control baculovirus were subjected to an IFN-γ induction assay in the presence of ConA. Briefly, human peripheral blood mononuclear cells (PBMC) were separated with Ficoll–Hypaque density gradient separation. After washing once with PBS, PBMC were suspended to 1×10^6 cells/ml in RPMI 1640 with 10% FCS and seeded in 96-well culture plate at 200 μl per well together with 10 μl cytosolic preparations or a series diluted concentration of fusion protein and human IL-18. The cultures were incubated for 48 h at 37 °C and the supernatants were assayed for human IFN-γ, by specific ELISA kit.

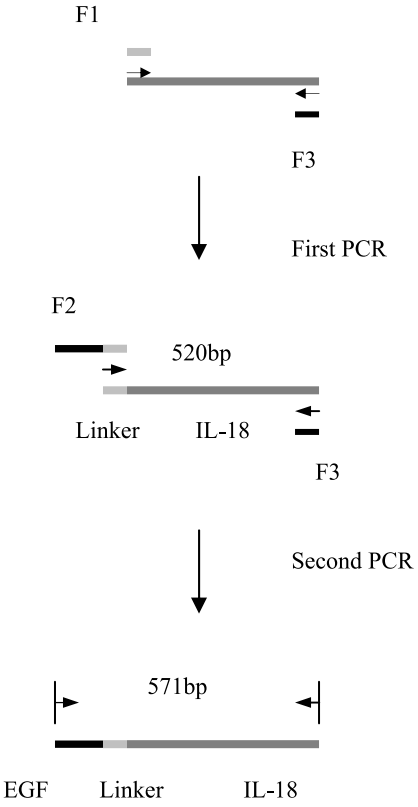

Fig. 1. Schematic diagram for the construction of EGF-IL-18 fusion gene. F1, F2, and F3 are primers for PCR amplification as detailed under Materials and methods.

EGF receptor competitive test on target cells. A431 cell line was cultured in 10 cm plate for 48–72 h until forming confluent monolayer culture. Confluent monolayer culture of A431 cells was dispensed at a concentration of 2×10^5 cells per well in 24-well plate. EGF-IL18 fusion protein was added to each well in the presence of ^{125}I-labeled human EGF. The assay was performed as described elsewhere [21]. The ^{125}I-labeled-EGF binding rate was calculated as follows:

$$\text{binding rate } (\%) = \frac{\text{experimental group cpm} - \text{nonspecific cpm}}{\text{maximal cpm} - \text{nonspecific cpm}}.$$

Results and discussion

To test if a targeted delivery of IL-18 by generation of the IL-18-EGF fusion protein might decrease adverse effects and increase cytotoxic and antitumor activities, IL-18-EGF fusion gene was constructed as shown in Fig. 1. In this system, EGF was mitogenic which can promote tumor cell growth, and the receptor binding domain of

EGF was responsible for the receptor binding but not mitogenic. Thus, the receptor binding domain of EGF was fused with the functional domains of IL-18. This construction would ensure that the fusion protein did not participate in intracellular signal transduction by intact EGF molecules, which could block EGF-γ-induced cell proliferation. IL-18 folds into a β-trefoil structure and EGFR binding domain harbors a α-helixes loop. In order to avoid interference of these polypeptides, we introduced a flexible peptide to link them, in this way, these two polypeptides can fully exert their biological activities. The structure analysis of IL-18 and its receptor shows that IL-18's monomer protein exhibits full effect by its receptor activation mechanism [25]. That would be very easy for the construction and expression of a multiple functional fusion protein with the very simple structure. Furthermore, the secondary structure of recombinant fusion proteins was predicated to have similar secondary structure to that of native IL-18. Thus, it was reasonable to expect that EGF-IL18 fusion protein has similar function to that of native IL-18 protein.

The resultant plasmid pFUS was first transfected in the *S. frugiperda* Sf9 cell line. The infected cells revealed a prominent cytopathic effect (CPE) as shown in Fig. 2. To test if the fusion protein was expressed in the Sf9 cells, the infected cells were collected and lysed. The resulting cytosolic preparations were separated by SDS–PAGE. Fig. 3A showed the electrophoretic pattern of cytosolic preparations from Sf9 cells. An ~20 kDa band, consistent with the anticipated molecular weight of recombinant EGF-IL18, appeared in the transfected Sf9 cells, implying the expression of this fusion protein. The recombinant EGF-IL18 protein was further purified form cytosolic preparations from Sf9 cells after infection. As shown in Fig. 3B, ~85% purity of the purified recombinant EGF-IL18 was achieved after purification. Subsequently, Western blot analysis using a mouse-anti-human IL-18 monoclonal antibody detected a band with the size of 20 kDa, corresponding to the predicted size of fusion protein, confirming that this fusion protein indeed was expressed in the transfected Sf9 cells.

The fusion protein was then examined for its biological activities. Fig. 4 showed that by IFN-γ induction

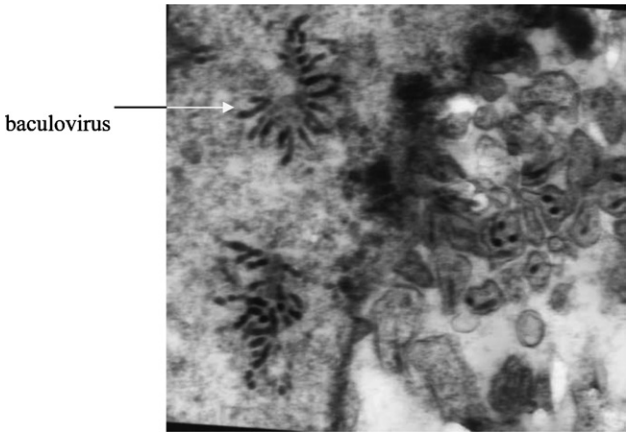

Fig. 2. Electron microscopic photograph of recombinant baculovirus in the infected Sf9 cells (3000×). The dense particles are virons indicated by arrow.

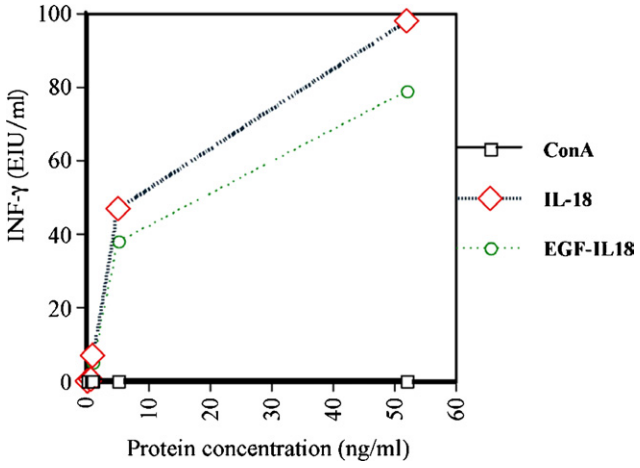

Fig. 4. IFN-γ induction assay. Various concentrations of native IL-18 and cytosolic preparations from Sf9 cells with pre-infection or infecting with recombinant baculovirus were subjected to an IFN-γ induction assay in the presence of ConA.

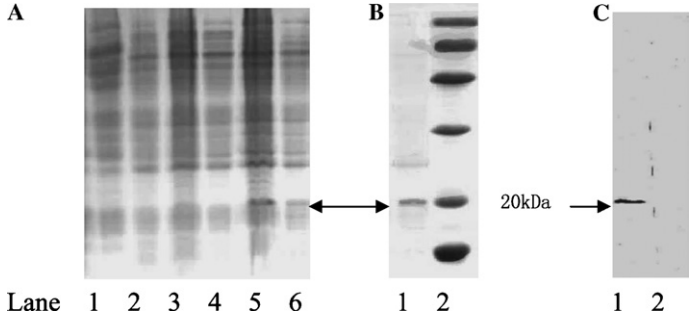

Fig. 3. SDS–PAGE analysis of fusion protein and Western blot analysis. (A) Lane 1, cytosolic preparations from the Sf9 cell prior to infection; lanes 2–4, cytosolic preparations after infection by recombinant baculovirus GUS; lanes 5 and 6, cytosolic preparations after infection by recombinant baculovirus EGF-IL18. (B) Lane 1, purified EGF-IL18 protein; lane 2, low molecular weight protein marker. (C) Western blot analysis. The blot was hybridized with a mouse anti-human IL-18 monoclonal antibody.

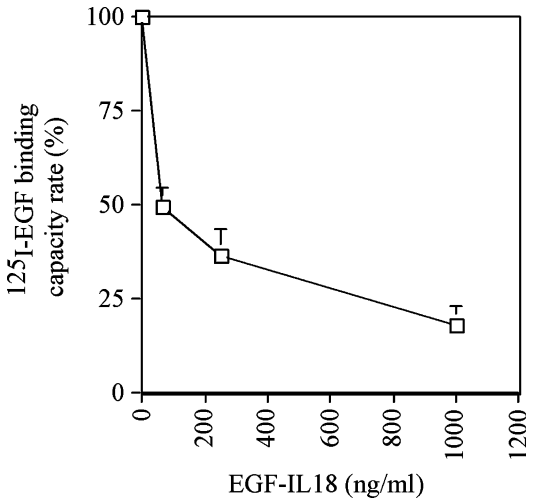

Fig. 5. EGF receptor competitive test. Human A431 cells were cultured and dispensed at a concentration of 2×10^{-5} cells per well. Equal amount of ^{125}I-labeled EGF was added to each well. Various concentrations of EGF-IL18 fusion protein were added to each well. The ^{125}I-EGF binding rate was calculated as detailed under Materials and methods.

assay, the purified recombinant fusion protein, similar to the native IL-18 protein, appeared to induce IFN-γ in human PBMC in a dose-dependent manner in the presence of ConA. In particular, there was the same tendency to induce IFN-γ between native IL-18 protein and EGF-IL18 fusion protein. Likely, the purity of EGF-IL18 fusion protein may contribute to the slight difference of activity between two proteins. The result indicates that the fusion protein has similar activities as IL-18, thereby initiating IL-18's receptor activation path to induce IFN-γ human PBMC. Thus, the fusion protein may have a potential for the antitumor treatment.

The capacity of recombinant EGF-IL18 binding with EGFR was then assessed by EGF receptor competitive test in the human epithelial cancer A431 cell line. As shown in Fig. 5, ^{125}I-labeled EGF shows 100% capacity to bind with EGFR in A431 cells in the absence of EGF-IL18. With increasing concentration of EGF-IL18 fusion protein, the activity of ^{125}I-labeled EGF was decreased, suggesting that EGF-IL18 can specifically bind with EGFR by competing with native EGF protein. The results imply that EGF-IL18 fusion protein can specifically target at tumor cells expressing EGFR.

It was suggested that Fas–Fas ligand and perforin-induced tumor apoptosis play a critical role in the antitumor or effects mediated by human IL-18 [26]. The fusion protein was able to bind with tumor cells and T or NK cell simultaneously, which appears to guide NK cells close together with tumor cells. By increasing Fas-L on NK cells, the fusion protein may increase NK cell-mediated cytotoxic and Fas–Fas ligand-induced tumor apoptosis. Fusing a tumor specific (EGFR) binding domain with IL-18 appears to block cell proliferation stimulated by EGF and cause little adverse effects in treatment by targeting tumor cells. Moreover, due to targeting tumor cells more directly, the fusion protein is expected to initiate more balanced Th1 cell-mediated antitumor immune response and IFN-γ-mediated antitumor effect. These suggest that this rationally designed protein can be further developed as novel tumor therapeutics.

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