Construction of a novel fusion protein harboring mouse interferon γ and epidermal growth factor receptor binding domain and enhancement of its antitumor activity

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Abstract A novel fusion protein harboring mouse interferon γ and epidermal growth factor receptor binding domain was constructed with the method of genetic and protein engineering. The fusion protein kept complete antiviral activity with the titer of 10^8 IU per liter of culture. The EGF-RBD of the fusion protein exhibited competitive binding activity against ^{125}I-mEGF for mEGF receptors on A431 cells. The fusion protein was shown to be more potent in inhibiting the growth of cultured mouse breast carcinoma cells than interferon γ. Experimental data on mouse B16 malignant melanoma model indicated that the tumor weight of fusion protein-treated group was statistically significantly smaller than that of interferon γ-treated group. The work here provides a necessarily reliable clue for the upcoming clinical employment of a novel class of targeting interferons.

Keywords: interferon γ, epidermal growth factor, fusion protein, antitumor activity.

The conventional antitumor therapy at present usually involves cytotoxic drugs that have great side-effects on both tumor cells and normal tissues. A specific delivery system has been tried to mediate tumoricidal drugs to malignant cells by targeting killing, in order that they exert lesser or no side-effects on normal tissues when destroying tumors. Oncocytological data have provided the evidence that the epidermal growth factors are overexpressed to as many as 10^6 per cell on a variety of malignant cell surfaces such as glioma, squmous carcinoma, melanoma and breast carcinoma, which has provided a potential target for EGF receptor-specific targeting anti-tumor therapy. Interferon γ is a pleiotropic cytokine which possesses antiviral, antiproliferative, immune regulatory and antitumor effects. A fusion protein of human interferon γ and vaccinia virus growth factor fragment was constructed in our lab and was used to obtain partial effects we have expected. The antiproliferative activity of human-r-EGF3 fusion protein was higher than that of its parent IFN and this effect was closely related to their EGF recepter competition[1]. Unfortunately, interferon γ is born to be strictly species-specific and the above-mentioned fusion protein cannot be employed in animal experiments. To further verify the idea as well as the protein's *in vivo* antitumor efficacy, we have constructed a novel fusion protein harboring mouse interferon γ and epidermal growth factor receptor binding domain and proved both *in vitro* and *in vivo* enhancement of its antitumor activity compared with its parent IFN-γ counterpart.

1 Materials and methods

1.1 Materials

1.1.1 Chemicals and enzymes. Ristriction endonucleases, T4 DNA ligase, T4 DNA polymerase, T4 polynucleotide kinase and *E. coli* DNA polymerase I large fragment-Klenow enzyme were bought from Biolabs Inc., USA, and Boehringer Mannheim Company, Germany. Thermostable Taq DNA polymerase and dNTPs were from Sino-American Biotechnology Company. ^{125}I-mEGF was purchased from American DuPont, Inc. Standard mIFN-γ was a product from Gibco Company, USA.

1.1.2 Bacteria, plasmids and bacteriophages. *E. coli* JM 109 strain and Dh 5 α strain were supplied by our department. Plasmid pMifco-1 harboring mouse IFN-γ cDNA was a gift from Prof. Sidney Pestka, University of Dental Medicine, New Jersey. Cloning vectors pUC 19 was bought from American Bioloabs, Inc. Prokaryotic expression vector pBV 220 was provided by Prof. Zhang in our department. Bacteriophages M13mp18 and M13mp19 were purchased from Biolabs, Inc., USA.

1.1.3 Cell strains. Mouse L929 fibroblast was supplied by our department. Mouse Jyg-MC (A) breast carcinoma cell was bought from Riken Cell Bank, Japan. Human A 431 epidermoid carcinoma cell was a gift from Institute of Drugs and Bioproducts Verification, Chinese Academy of Medicine. Heat-inactivated fetal calf serum was purchased from Institute of Veterinary Medicine, Harbin, Heilongjiang Province. Cell culture mediums of Eagle's MEM, RPMI 1640, trypsine, Versene, Penicilline, streptomycin and glutamine were supplied by Department of Medium Supplement of our institute.

1.2 Methods

1.2.1 Chemical synthesis and process of oligonucleotide fragments. Altogether four oligonucleotide fragments were synthesized in our experiments. Primers A and B were upstream and downstream primers for PCR reaction. Primers C and D were fragments for the synthesis of mEGF-RBS gene. The primers were synthesized on an automatic DNA synthesizer from American ABI Biosystems, Inc. and purified on an FPLC Mono Q column from Pharmacia, Sweden. The primers were assayed for their OD_{260} and OD_{280} values on a DU-70 Model Spectrophotometer from Bechman Company, USA, and the values were converted into primer concentration.

1.2.2 Polymerase chain reaction (PCR). The PCR reaction was performed as described in ref. [2] on Single BlockTM system purchased from American ERICOMP Company. A DNA fragment of 1.4 kb was cut from plasmid pMifco-1 as template. The PCR reaction involves deionized water 72 μL, 10×dNTPs 10 μL, 10×Taq enzyme buffer 10 μL, template DNA 40 ng, Primer A 2 μL (≈100 pmoles), Primer B 3.5 μL (≈100 pmoles) and Taq DNA polymerase 1 μL (2u). The cycle parameters are as follows: denaturing at 94℃ for 30 s, annealing at 55℃ for 30 s, elongating at 72℃ for 90 s, altogether 30 cycles with elongation at 72℃ for 10 min when the reaction ended.

1.2.3 Enzymatic synthesis of partially annealed oligonucleotides encoding mEGF-RBD gene.

Primers C and D were annealed by 12 matching base pairs at their 3′ termini. The annealing reaction involved deionized water 13 μL, 10 × sequencing buffer 2 μL, 10 × dNTPs 2 μL, Primer C 1 μL (1 μg), Primer D 1 μL (1 μg) and Klenow enzyme 1 μL (1u). The reaction was first boiled for 5 min without addition of Klenow enzyme, annealed at 45℃ for 2 h. 1μL Klenow enzyme (1 u) was then added and incubated at 37℃ for half an hour.

1.2.4 DNA cloning, extraction of plasmid DNA and transformation of bacteria. These experiments were done according to reference [2].

1.2.5 Isolation of single stranded recombinant phage DNA. Also done with ref. [2] as guidance.

1.2.6 DNA sequencing. Nucleotide sequence was determined with Sanger's dideoxy termination method in M13 phage system constructed[2].

1.2.7 Prokaryotic expression of target gene and SDS-PAGE characterization. Single colony of recombinant DH 5 α strain was inoculated into 3 mL ALB and incubated overnight by vigorous shaking at 30℃. The culture was diluted 50-fold in 3 mL ALB and still incubated at 30℃ until OD_{600} reached approximately 0.4. The temperature was quickly shifted to 42℃ at which the culture was further incubated for 6 h. 1 μL sample was withdrawn for SDS-PAGE electrophoresis on PhastGel Gradient 10—15 or 8—25 on 'Phast SystemTm', Pharmacia Inc. The samples were stained by Commasie Briliant Blue R 350 and destained (10% iceacetic acid and 30% methanol) for characterization.

1.2.8 Antiviral activity assay. Cytopathic inhibition effect assay was used as previously described[3]. Mouse L929 fibroblasts were seeded into a 96-well plate. 50 μL samples was serially diluted 4-fold in wells of each row and challenged by vesicular stomatitis virus (≈104 pfu) after 24 h. Results were read when virus control was totally cytopathic and the endpoints were defined as 1 unit of titer in 50% cytopathic well.

1.2.9 Antiproliferative assay. Mouse Jyg-MC(A) breast carcinoma cells were passaged in RPMI 1640 with split ratio of 1∶4 as dealt with elsewhere[4]. The cells were seeded in small flasks at density of 2.3 × 105 and the samples were added simultaneously to the final concentration of 2 000 u/mL with guanidine hydrochloride as control (final concentration of 7 mmol/L). The cells were treated daily with samples and the cells were counted every other day.

1.2.10 mEGF receptor binding activity assay. Human A 431 epidermoid carcinoma cells were seeded in a 24-well plate until they reached confluence as previously described[5]. Wells were washed three times with prewarmed Hank's solution and 1 mL 0.1% BSA plus RPMI 1640 was filled into each well. Final concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} mol/L were added to the samples respectively and the samples were incubated at 25℃ for 30 min followed by addition of 10 μL ^{125}I-mEGF (≈20 000 cpm) for further 30 min. The contents in each well were washed four times and lysed with 200 μL of 0.5 mol/L NaOH and 0.1% SDS at 37℃ for 1 h. The solution in each well was transferred to a vial for γ ray counting on 'GAMMA 5500 COUNTING SYSTEM', Bechman Inc.

1.2.11 Large-scale preparation and partial purification of samples. DH 5 α bacteria strains

harboring IFN-γ and fusion gene were amplified in 1 000 mL of culture as mentioned above and purified by two methods. (ⅰ) The bacteria containing fusion protein were disintegrated by ultrasound treatment. The pellet was washed with water and 4 mol/L urea and dissolved in 50 mL 7 mol/L GuCl. 50 mmol/L PB to final volume of 175 mL with GuCl concentration of 2 mol/L was added to the supernatant. The solution was stored at 4℃ overnight and diluted 10-fold the next day, with GuCl concentration of 0.2 mol/L. The solution was still placed at 4℃ and on the following day dialyzed against 20 mmol/L pB at 4℃ overnight. The samples were eventually covered with PEG-6000 powder at 4℃ for 12 h and centrifuged. The supernatant was sterilized by passage through a 0.22 μmol/L filter and stored at −70℃. (ⅱ) The bacteria containing interferon γ were treated with 7 mol/L guanidine chloride and diluted and renatured similarly as above mentioned except that the GuCl concentrations were 2 and 1 mol/L respectively.

1.2.12 Animal model establishment. 50 female C57 mice aged 7—8 weeks with average weight of 20 g were bought from Animal Center, Beijing Medical University and were divided into 5 groups. Mouse B16 melanomas were passaged in mice and excised on the 13th day. Tumors were ground in normal saline and the suspension was inoculated subcutaneously at the right axilla of each mouse. Samples were given daily from the following day on and the mice were sacrificed on the 12th day. The tumors were excised, weighed and the data were statistically processed.

2 Results

2.1 Reconstruction of rMuIFN-γ cDNA with PCR

The plasmid of pMifco-1 obtained from Prof. Pestka contained the following elements as 5′ non-translating region, signal peptide sequence, mature peptide coding region and 3′ untranslating region, which was suited for eukaryotic expression. In order to fuse the gene with mEGF-RBS and express it in *E. coli*, we reconstructed the cDNA gene with PCR method. The sequence of the primers were as follows:

Primer A 5′ CCGAATTCTTATGCACGGCACAGTCATTGAAAG 3′ (33 mer),

Primer B 5′ AAGGATCCTTACCCGGGGCGACTCCTTTTCCGCTTCC 3′ (37 mer).

A 0.4-kb fragment of PCR product was obtained with an EcoR I fragment of 1.4 kb digested from pMifco-1 as template and subcloned into pUC 19. Restriction map of the PCR product was proved to be correct by agarose gel electrophoresis. DNA sequencing also demonstrated that the nucleotide sequence of the amplified gene was in accordance with previous design. The first amino acid at 5′ terminus of the peptide was histidine and at 3′ was arginine at position 132 followed by proline and glycine, whose codons were CCCGGG.

2.2 Enzymatic synthesis of partially annealed oligonucleotides encoding mEGF-RBS

The sequences of the two oligonucleotides were illustrated as follows:

Primer C 5′ AAGAATTCCATGCCCGGGGGTTGCGTTATCGGTTACTCTGGTGATCGA 3′ (48 mer),

Primer D 5′ ATGGATCCTTAAAGATCTCGAGTTTGACATCGATCACCAGAGTAA 3′ (45

mer).

Primer C was anealed with Primer D *in vitro* and catalyzed by Klenow enzyme. The newly synthesized double stranded DNA was cloned into pUC 19 for restriction map determination and into M13 for sequencing. The gene coded for a 15 amino acid peptide from cysteine at position 33 to leucine at position 47. Two unique restriction sites of Bgl Ⅱ and Xho Ⅰ were included for recombinant characterization. The linker peptide of proline-glycine-glycine was encoded by the sequence of CCCGGGGGT just before the cysteine which was an Sma I site for gene fusion.

2.3 Construction of mIFN-γ/mEGF-RBD fusion gene

A fusion gene was obtained by inserting a small fragment digested from PCR reconstructed gene with Sma Ⅰ and EcoR Ⅰ at Sma Ⅰ site of mEGF-RBS and sequenced to be correct compared with the designed sequence (figure 1).

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                ATG CAC GGC ACA GTC ATT GAA AGC CTA GAA AGT CTG AAT
                Met His Gly Thr Val Ile Glu Ser Leu Glu Ser Leu Asn
AAC TAT TTT AAC TCA AGT GGC ATA GAT GTG GAA GAA AAG AGT CTC TTC
Asn Tyr Phe Asn Ser Ser Gly Ile Asp Val Glu Glu Lys Ser Leu Phe
TTG GAT ATC TGG AGG AAC TGG CAA AAG GAT GGT GAC ATG AAA ATC CTG
Leu Asp Ile Trp Arg Asn Trp Gln Lys Asp Gly Asp Met Lys Ile Leu
CAG AGC CAG ATT ATC TCT TTC TAC CTC AGA CTC TTT GAA GTC TTG AAA
Gln Ser Gln Ile Ile Ser Phe Tyr Leu Arg Leu Phe Glu Val Leu Lys
GAC AAT CAG GCC ATC AGC AAC AAC ATA AGC GTC ATT GAA TCA CAC CTG
Asp Asn Gln Ala Ile Ser Asn Asn Ile Ser Val Ile Glu Ser His Leu
ATT ACT ACC TTC TTC AGC AAC AGC AAG GCG AAA AAG GAT GCA TTC ATG
Ile Thr Thr Phe Phe Ser Asn Ser Lys Ala Lys Lys Asp Ala Phe Met
AGT ATT GCC AAG TTT GAG GTC AAC AAC CCA CAG GTC CAG CGC CAA GCA
Ser Ile Ala Lys Phe Glu Val Asn Asn Pro Gln Val Gln Arg Gln Ala
TTC AAT GAG CTC ATC CGA GTG GTC CAC CAC CTG TTG CCG GAA TCC AGC
Phe Asn Glu Leu Ile Arg Val Val His Gln Leu Leu Pro Glu Ser Ser
CTC AGG AAG CGG AAA AGG AGT CGC CCC GGG GGT TGC GTT ATC GGT TAC
Leu Arg Lys Arg Lys Arg Ser Arg Pro Gly Gly Cys Val Ile Gly Tyr
TCT GGT GAT CGA TGT CAA ACT CGA GAT CTT TAA
Ser Gly Asp Arg Cys Gln Thr Arg Asp Leu **
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Fig. 1. Complete nucleotide sequence of mEGF-RBS.

2.4 Prokaryotic expression of the fusion gene

The prokaryotic expression vector pBV220 was constructed by Prof. Zhang *et al*. and was employed for our expression of γ interferon and the fusion gene, whose level was as high as 30% of the total bacterial protein. Two bands of 14.9 and 16.7 ku in 42℃ -induced proteins were identified by SDS-PAGE electrophoresis compared with 0℃ non-induced samples, completely complied with the expected molecular weight deduced from DNA sequence (figure 2).

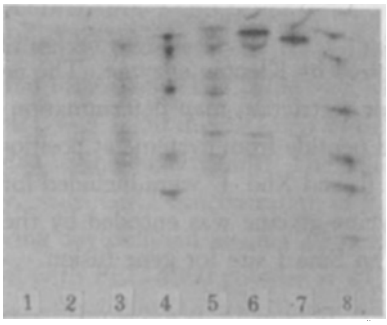

Fig. 2. SDS-PAGE analysis of prokaryotic expression products of target gene. 1, pBV220 amplified at 30℃; 2, rMuIFN-γ mutant amplified at 30℃; 3, fusion gene amplified at 30℃; 4, protein molecular weight standards: 14.4, 18.6, 24, 45 and 66ku; 5. pBV220 induced at 42℃; 6, rMuIFN-γ mutant induced at 42℃; 7, fusion gene induced at 42℃; 8, protein molecular weight standards: 14.4, 20.1, 30, 43, 67 and 94 ku.

2.5 Antiviral activity assay

The average antiviral titers of the expressed proteins reached 10^8 IU per liter of culture by three separate assays.

2.6 mEmEGF receptor binding activity assay

The result is shown in table 1.

2.7 *In vitro* antitumor activity assay.

The data are exhibited in table 2.

2.8 *In vivo* antitumor activity assay

See table 3 and figure 3.

Table 1 Radioactivity counting on A431 cells after competitive binding of different concentrations of samples with ^{125}I-mEGF against mEGF receptors

	Sample concentration (− log *M*)					Maximal binding
	10	9	8	7	6	
γ	(99%)	(91%)	(86%)	(66%)	(58%)	(100%)
	1 021	944	891	680	601	1 035
Fu	(97%)	(74%)	(48%)	(38%)	(17%)	(100%)
	1 000	765	459	392	176	1 030

Table 2 Antiproliferative activity of samples on mouse breast carcinoma cells

Sample	Cell number($\times 10^4$)			
	Day 1	Day 3	Day 5	Day 7
GuCl control	23	22	20	15
IFN-γ	23	21	18	10
Fu	23	20	15	5

Table 3 Tumor weight and statistical analysis

Treatment	Average tumor weight/g	Number of mice when sacrificed	Inhibition rate of tumor growth	*P* value	
				cf. with (1)	cf. with (4)
(1)	3.6±0.6	10	0	/	<0.01
(2)	1.04±0.4	10	72	<0.01	<0.01
(3)	1.6±0.7	10	55	<0.01	0.01<*p*<0.05
(4)	2.6±0.8	10	27	<0.01	/

(1) *E. coli* protein (−) control; (2) cyclophosphamide (+) control; (3) fusion protein at concentration of 5×105 IU; (4) IFN-γ at concentration of 5×105 IU.

3 Discussion

In this paper we have constructed a novel fusion protein harboring murine interferon γ and

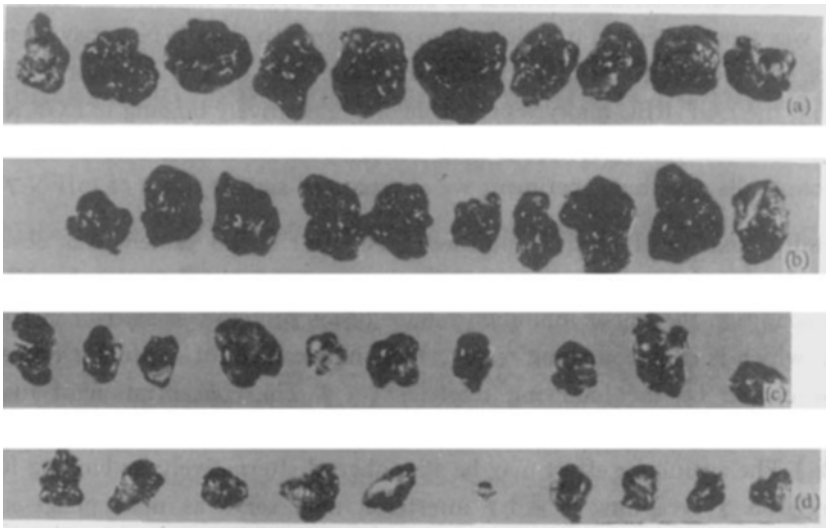

Fig. 3. Solid melanoma tumors excised from mice in different experiment groups. (a), (b), (c) and (d) denote *E. coli* protein control, IFN-γ (500 000 units), fusion protein (500 000 units) and cyclophosphamide, respectively.

epidermal growth factor receptor binding domain, the sequence of which has been proved correct. Several aspects were taken into consideration when designing the experiments. First, EGF is mitogenic while γ interferon inhibits cell growth. If both proteins were introduced to cell surface without selection, their biological effects would be mutually sure to offset. Thus we deliberately introduced the C′ terminal domain of mEGF that was simply responsible for receptor binding but not mitogenic to make sure that the fusion protein did not participate in intracellular signal transduction conducted by contact EGF molecules, and the cell proliferation stimulated by EGF is blocked. IFN-γ can fully exert its antitumor effect. Second, the conformational structure of IFN-γ that was mainly composed of α helix[6] should not interact with that of EGF which mainly consist of β-sheet[7]. We have also deleted the C′ terminal cysteine of IFN-γ in order that there might not form disulfide bonds with the two cysteines in C loop of EGF. A peptide linker was necessary to minimize the possible impact of the strong cationic 'KRKRS' like structure at the C′ terminus of IFN-γ on EGF's receptor binding domain. Considering that proline is an α-helix blocker and glycine has the simplest structure without side chains, besides, both amino acids are devoid of strong polarity, we have chosen Pro-Gly-Gly as a peptide linker, whose codons coincide with Sma I restriction site CCCGGG. We selected *E. coli* expression as the characterization of fusion protein. In view of this, we have deleted both 5′ and 3′ untranslated regions of IFN-γ cDNA as well as peptide sequence containing Cys6, Cys20 and Cys22 that might form undesired -S-S- bridges by cysteine mispairing, though the signal peptide had been shown to be benefitial to protein purification[8], and introduced termination codon of TAA which was the most powerful one among TAA, TGA and TAG. When designing mEGF-RBS gene we also chose preferable codons of *E. coli* and give consideration to the restriction sites such as EcoR I, BamH I, Sma I, Xho I, Bgl II, etc.

The maintenance of high antiviral activity of IFN-γ after substitution of Pro and Gly for the C terminal Cys suggested that it is structurally unimportant. In antiproliferative assay we used 7

mol/L guanidine hydrochloride as control to exclude its cytoxic inhibition. The fact that the fusion protein was more potent in inhibiting tumor cell growth than IFN-γ may be mediated by the abundance of EGF receptors on breast carcinoma cells. The data of receptor binding experiment suggested that the EGF-RBD mioty was capable of competitively binding to EGF receptors against ^{125}I-mEGF. Eisenkraft *et al*. reported the down regulation effect of EGF receptors by IFN-α on renal carcinoma cells. In this experiment we also noticed similar effect of mIFN-γ.

No possible toxicity of partially purified IFN-γ nor fusion protein was observed in mouse model at the dosage of 5 × 105 IU per mouse after a consecutive injection for 10 d. Under this prerequisite condition the tumor inhibition rate of fusion protein was statistically higher than that of mIFN-γ, which is an encouraging result. The enhancement of antitumor effect of fusion protein may be caused by the following reasons: (ⅰ) The concentration of fusion protein on melanoma cell surfaces was greatly increased through specific targeting of EGF receptor binding domain. (ⅱ) The antitumor effect may be strenghened alternatively by binding fusion protein to cellular interferon γ receptors. (ⅲ) γ interferon may serve as macrophage-activating factor (MAF) to Ⅱ antigen on the surface of macrophage. (ⅳ) The activity of natural killer lymphocytes is raised. (ⅴ) Intracellular half life of fusion protein is prolonged due to C terminal fusion with EGF-RBD and insusceptibility to proteolysis in *E. coli*.

This paper established a more promising fusion protein for antitumor therapy, and provided necessarily reliable evidence for clinical employment of brand-new targeting interferons.

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