Enhancement of enzyme cytotoxicity mediated by HIV-1 TAT protein with Gly4 linker *in vitro*: a study with TAT-TK fusion construct☆

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Abstract

Background. Suicide gene therapy using herpes simplex virus type-1 (HSV-1) thymidine kinase (TK) is a widely exploited approach for gene therapy of cancer and other hyperproliferative disorders. Despite its popularity, clinical success has been so far hampered mostly by the relative inefficiency of TK gene transfer and its limited bystander effect. *Materials and Methods*. Here we report that fusion of TK to HIV-1 Tat protein with different Gly linker imparts cell membrane translocating ability to the enzyme and significantly increases its cytotoxic efficacy, and Gly_4 linker between fusion protein show a major impact to enhancement of TK tumor cells killing compared to Gly_0, Gly_2 and Gly_6. Experiments were performed by incubated HepG2 cells with Dulbecco's modified Eagle's medium (DMEM) containing 10 μg/ml different Gly linker fusion proteins. *Results*. The clearance of TK-expressing cells is confirmed by Immunofluorescence assay and results show that transcellular transfer of active HSV-1 thymidine kinase could be mediated by HIV-1 Tat. Meanwhile, the proportion of apoptosis cells detected by cell flow cytometry and survival cell populations by trypan blue suggested that the remarkable enhancement of fusion protein with Gly_4 linker cytotoxicity can be detected in different group *in vitro*. *Conclusions*. This modification of TK might constitute an important step in the optimization of TK suicide gene strategy for gene therapy of cellular proliferation. [Life Science Journal. 2009; 6(2): 55 – 60] (ISSN: 1097 – 8135).

Keywords: thymidine kinase; Tat; glycine; internalization effects

1 Introduction

Thymidine kinase (TK) from human herpes simplex virus type 1 (HSV-1) is the most extensively exploited gene for the control of cellular proliferation in gene therapy[1]. Cells expressing TK convert the nucleoside analogues acyclovir (ACV) and ganciclovir (GCV) into their phosphorylated forms, which are in turn incorporated into replicating DNA where they block further chain elongation and consequently induce cell death[2]. Prodrug gene therapy using TK has found application in several instances. However, the results so far obtained have shown only marginal clinical benefit, mainly due to the poor rate of delivery of the HSV-TK gene to tumor cells[3,4]. In the last few years, some proteins, which present the unusual characteristic of crossing the cell membranes through noncanonical processes of secretion or internalization, have been described. In particular, chemical cross linking of Tat peptides with heterologous proteins[5] or, more efficiently, production of recombinant proteins facilitates the intracellular delivery of these proteins. Monica and her colleagues have recently observed that recombinant proteins fused to full-length Tat (86 amino acids) efficiently enter the cells when present in the extracellular medium and are readily transported to the nucleus in an active form[6–8].

Given this peculiar characteristic of fusion protein, the

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success of this approach depends on the possibility either of expressing the suicide gene in the majority of cancer cells (a still unrealistic possibility) or of extending its effect to a proportion of cells sufficiently large to achieve complete cell killing after prodrug treatment. As far as the latter possibility is concerned, further improvements in the TK gene approach might also benefit from prolonged persistence of the K-producing cells in the context of the tumor mass for the whole duration of GCV treatment. Meanwhile, how to construct the fusion protein that confers TK a remarkably therapeutic efficacy is not still defined. To address this question well, we constructed a series of HIV-1-tat-TK fusion protein that contained Gly0, Gly2, Gly4 or Gly6 different linker between them. Our results show that fusion of Tat to TK permits transcellullar transfer of the enzyme in HepG2 cells, and the remarkable enhancement of fusion protein with Gly4 linker cytotoxicity can be detected in different group *in vitro*.

2 Materials and Methods

2.1 Materials

Plasmid pcDNA3-TK containing TK gene, pcDNA3-tat carrying truncated HIV-tat (1 – 200 nt) gene and *E. coli* DH5α were maintained in our laboratory. Eukaryotic expression vectors pcDNA3 and prokaryotic expression vector-PBK were from Invitrogen Co., Netherlands. Purification kit for PCR product was obtained from QIAGEN Company (Germany). Restriction endonucleases such as *Eco*RI, *Bam*HI, and T4 DNA ligase were from Huamei Bioengineering Company (Luoyang, China). All the PCR primers used in the study were designed by Genebank and synthesized by Takara Ltd. (Dalian, China). Sephadex G-100 and Sepharose CL-4B were from Pharmacia Ltd. Monoclonal antibodies of HIV-Tat and TK proteins were gently gift from MD. Zqi Han, Medical College North-western University, USA.

2.2 Chimer genes preparation

Primers for polymerase chain reaction(PCR): Recombinant protein of HIV tat and TK gene containing 2 glycines. HIV Tat left primer: 5'-GTGGATCCATGGAGCCAGTAGATCCTA-3', HIV Tat: 5'-ATCGAAGCATACCTCCCTTTTCCTTCGGGCCAG-3', TK left primer: 5'-GGAGGTATGCTTCGTAC CCCTGCCATC-3', TK right primer: 5'-CAGGATCCAGTTAGCCTCCCCCATCTC-3'.

Recombinant protein of HIV tat and TK gene containing 4 glycines HIV Tat left primer: 5'-GTGGATCCATGGAGCCAGTAGATCCTA-3', HIV Tat right primer: 5'-GCATACCTCCACCTCCCTTTTCCTTCGGGCCAG-3', TK left primer: 5'-GGAGGTGGAGGTATGCTTCGTACCCCTGCC-3', TK right primer: 5'-CAGGATCCAGTTAGCCTCCCCCATCTC-3'.

Recombinant protein of HIV tat and TK gene containing 6 glycines HIV Tat left primer: 5'-GTGGATCCATGGAGCCAGTAGATCCTA-3', HIV Tat right primer: 5'-ACCTCCACCTCCACCTCCCTTTTCCTTCGGGC-3', TK left primer: 5'-GGAGGTGGAGGTGGAGGTATGCTTCGTAC-3', TK right primer: 5'-CAGGATCCAGTTAGCCTCCCCCA TCTC-3'.

2.3 Ligation of objected genes and clone

2.3.1 The 1st ligation of Tat and TK (Recombinant protein containing 0 or 2 glycines). Extracted the pcDNA3-tat and pcDNA3-TK plasmid as routine methods, HIV Tat left primer: 5'-GTGAATTCATGGAGCCAGTAGATCCTA-3' (containing *Eco*RI site, underline showed), HIV Tat right primer: 5'-ATCGAAGCATACCTCCCTTTTCCTT CGGGCCAG-3', TK left primer: 5'-GGAGGTATGCTTCGTACCCCTGCCATC-3', TK right primer: 5'-CAGGATC CAGTTAGCCTCCCCCATCTC-3' (containing *Bam*HI site, underline showed), amplified Tat and TK gene, the PCR profiles were denaturation at 92 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s for 30 cycles, at last extension at 72 °C for 10 min. Amplification products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and quantified until use.

The ligation of antisense fragment of TK gene with HIV tat gene segment was performed by gene SOEing PCR[11] with Puf enzyme. Briefly, there were a sense and antisene sequence between the primers of Tat and TK gene (underline showed): HIV Tat right primer: 5'-ATCGAAGCATACCTCCCTTTTCCTTCGGGCCAG-3', TK left primer: 5'-GGAGGTATGCTTCGTACC CCTGCCATC-3' (Slanting base pairs are codon of glycine). The above products of Tat and TK were mixed as a certain proportion. The fragment of HIV tat-Gly(2)-TK was further amplified by PCR with the major round to be 92 ºC for 30 s, 50 ºC for 45 s and 72 ºC for 60 s for 30 cycles, and the final extension step being 10 min at 72 ºC, in which the HIV Tat left primer: 5'-GTGAATTCATGGAGCCAGTAGATCCTA-3', and the TK right primer: 5'-CAGGATCCAGTTAG CCTCCCCCATCTC-3', were employed as primers. Amplification products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and quantified for the next PCR ligations or cloned.

2.3.2 The 2nd ligation of Tat and TK (Recombinant protein containing 4 glycines). The above prod-

ucts of HIV tat-Gly(2)-TK were amplified, HIV Tat left primer: 5'-GTGAATTCATGGAGCCAGTAGATCCTA-3', HIV Tat right primer: 5'-GCATACCTCCACCTCCCTTTTCCTTCGGGCCAG-3' and TK left primer: 5'-GGAGGTGGAGGTATGCTTCGTACCCCTGCC-3', TK right primer: 5'-CAGGATCCAGTTAGCCTCCCCCATCTC-3' was employed as two pair primers to amplify respectively for the first step PCR. The two products of the first step PCR were mixed as a certain proportion, HIV Tat left primer: 5'-GTGAATTCATGGAGCCAGTAGATCCTA-3' and TK right primer: 5'-CAGGATCCAGTTAGCCTCCCCCATCTC-3' were employed for the 2nd step PCR. Amplification products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and quantified for the next PCR ligations or cloned.

2.3.3 The 3rd ligation of Tat and TK (Recombinant protein containing 6 glycines). The above products of HIV tat-Gly(4)-TK were amplified, HIV Tat left primer: 5'-GTGAATTCATGGAGCCAGTAGATCCTA-3', HIV Tat right primer: 5'-ACCTCCACCTCCACCTCCCTTTTCCTTCGGGC-3' and TK left primer: 5'-GGAGGT GGAGGTGGAGGTATGCTTCGTAC-3', TK right primer: 5'-CAGGATCCAGTTAG CCTCCCCCATCTC-3' were employed as two pair primers to amplify respectively for the first step PCR. The two products of the first step PCR were mixed as a certain proportion, HIV Tat left primer: 5'-GTGAATTCATGGAGCCAGTAGATCCTA-3' and TK right primer: 5'-CAGGATCCAGTTAGCCTCCCCCATCTC-3' were employed for the 2nd step PCR. Amplification products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and quantified for clone.

2.4 Clone and determination of ligation products

2.4.1 Determination of chimer Tat-Gly-TK gene. The above 3 kinds of PCR ligation products, pcDNA3-TK and PBK vector were digested by *Eco*RI and *Bam*HI, purified, ligated by T4 ligase. Ligation products were transferred into DH5α with routine principle, sifted and maintained at 37 ºC, poke out single clone of DH5α and cultured. Both of the newly-constructed vectors were confirmed by restriction endonuclease digestion, PCR with specific primers and finally by DNA sequencing (Baosheng Co., Dalian, China).

2.4.2 Expressed and extracted of the recombinant Tat-Gly-TK protein. The positive *E. coli* after determination was planted in LB medium with gentamicin, culture at 37 ºC. While OD value (A_{600}) of *E. coli* was coming to 0.4 – 0.6, isopropyl-1-thio-β-D-galactoside (IPTG, destined titer, 0.4 mM) was added and induced expressed, collected the *E. coli* after 6 h, destroyed the *E. coli* by ultrasonic in ice-cold and washed by 4 M urea buffer[12–14].

2.4.3 Recovery and purified of the recombinant Tat-Gly-TK protein. *E. coli* were resolved into the buffer containing 8M urea, passed Sephadex G-100 and acquired the crude protein. The crude protein were incubated in pH 8.0 buffer (containing 10 mM PBS, 5 mM reduction-glutathione, 2 mM oxidation-glutathione) at 16 ºC for 12 h, after purifying through Sepharose CL-4B which was incubated with HIV tat monoclonal antibody[9] or TK protein monoclonal antibody, and recombinant proteins were loaded on a 10% SDS-polyacrylamide gel and visualized by Coomassie Blue staining[12–14].

2.4.4 Determination on the biological property of Tat-Gly-TK. Cells: To study the biological property of recombinant Tat internalization, HepG2 cells were divided into 6 groups (HIV tat protein group, tat-TK protein group, Tat-$(Gly)_2$-TK protein group, tat-$(Gly)_4$-TK protein group, tat-$(gly)_6$-TK protein group and the positive control TK protein) and seeded in 8-well dishes at the density of $(1 – 2) \times 10^4$ cells/cm^2 in DMEM containing 10% fetal calf serum. After 24 h, cell cultures were washed twice and incubated for an additional 24 h in fresh medium containing 10% fetal calf serum, 100 μM chloroquine, and recombinant Tat protein. Incubation in the presence of chloroquine favors Tat uptake by modifying the pH of endolysosomal vesicles and preventing protein degradation[10,11]. After 24 h, the medium was changed to DMEM, 10% fetal calf serum, and cells were incubated for an additional 24 h. At the end of incubation, cells were fixed and determined by immuno-staining. Alternatively, cells were cultured for 3 days continued with DMEM containing 10 μg/ml gencilorvir, at last cells collected , stained by trypan blue and analyzed by FACS[12].

Immunofluorescence assay and trypan blue exclusion test: Fixed cells were washed twice with 0.1% Triton X-100 in PBS for 10 min and incubated with monoclonal antibodies of HIV tat in PBS supplemented with 0.15% glycine and 0.5% bovine serum albumin. Images obtained by microscopy using an Olympus FV300 microscope. Green-fluorescence in cells showed recombinant Tat internalization. Meanwhile, the photograph recorded cell shapes and the survival cell ratio was assessed by the 0.1% trypan blue exclusion test on the 3rd day of post-transfection and the data determined were analyzed by statistics (*t*-test).

Flow cytometry. To analyze HIV Tat internalization by cell cytometry, cells were washed four times with PBS, stained by ethidium iodide, washed with PBS again and analyzed with a FACScan flow cytometer (Becton

Dickinson). A total of 10,000 events per sample were considered.

3 Results

According to the length of glys in the HIV Tat-$(Gly)_n$-TK, the diffreent primers were empolyed for the first PCR or the second PCR. We acquired a series of chimer genes of HIV Tat-$(Gly)_n$-TK inserted different numbers of glycines, and fragments were about 1200 bp as expected (Figure 1).

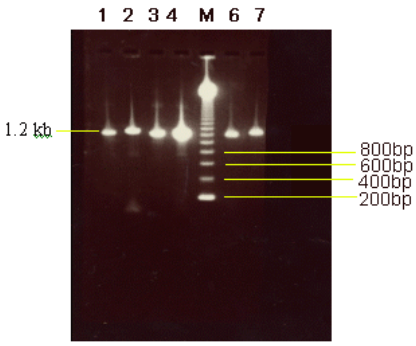

Figure 1. PCR of four fragments which contained 0, 2, 4 or 6 Glycines. M: Marker; Lane 1: HIV Tat-TK; Lane 2: HIV Tat-$(Gly)_2$-TK; Lane 3: HIV Tat-$(Gly)_4$-TK; Lane 4: HIV Tat-$(Gly)_6$-TK.

3.1 SDS-PAGE of recombinant protein

The chimer genes of HIV Tat-$(Gly)_n$-TK were expressed in *E. coli* and purified by Sepharose CL-4B decorated by Tat monoclonal antibody, SDS-PAGE (Figure 2). Recombinant proteins were loaded on a 10% SDS-polyacrylamide gel and visualized by Coomassie Blue staining. The weight was about 53 KD as expected (the protein of HIV Tat-$(Gly)_6$-TK gene expressed is the same as HIV Tat-$(Gly)_4$-TK, the data isn't shown).

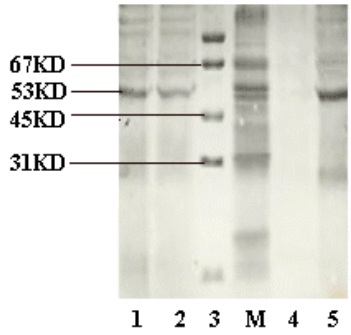

Figure 2. SDS-PAGE of recombined protein after purify. M: Marker; Lane 1: HIV Tat-TK; Lane 2: HIV Tat-$(Gly)_2$-TK; Lane 3: mixture protein of E coli after ultrasonic destroy; Lane 4:HIV Tat-$(Gly)_4$-TK; Lane 5: HIV Tat-$(Gly)_6$-TK.

3.2 Immunofluorescence assay

HepG2 were incubated with the fusion protein of HIV Tat-$(Gly)_n$-TK for 3 days, and the cells were determined by immunofluorescence assay. Excepted for TK group, the green-fluorescence was showed in the cells of the other 5 groups and the ratios of translocation to the nucleus in 5 group were the same extend. But in the TK group, there was no green-fluorescence in the cells. Images were obtained by microscopy (Figure 3).

3.3 Assessment by trypan blue exclusion test

The lethiferous effect of HIV-tat-$(Gly)_n$-TK on the HepG2 cells was continued to be aggravated on the 3rd day posttransfection, in which the majority of the transfected HepG2 cells were observed shrunken, rounded in shape and even dead. With the assessment by trypan blue exclusion test, the dead cell ratios of recombinant pro-

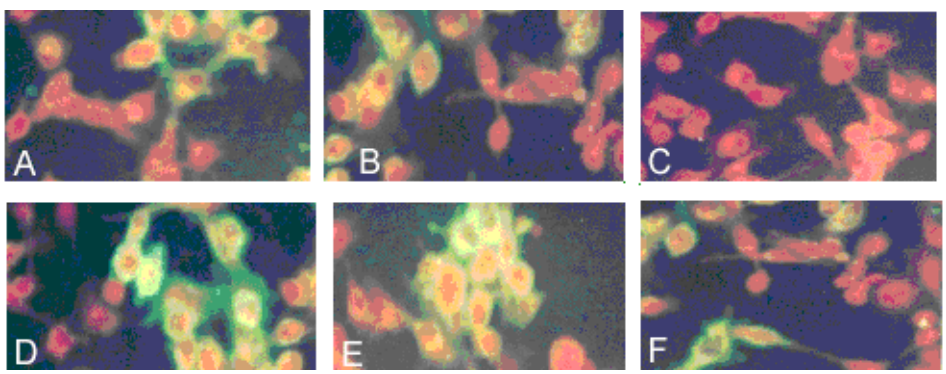

Figure 3. Immunofluorescence assay. A: HIV Tat; B: HIV Tat-$(Gly)_2$-TK; C: PBK-TK; D: HIV Tat-$(Gly)_4$-TK; E: HIV Tat-$(Gly)_6$-TK; F: HIV Tat-TK.

teins containing 0, 2, 4, 6 glycines or pcDNA3-TK (the positive on the 3rd day posttransfection) were 58.4%, 65.4%, 80.2% and 56.7% in order, in the negative control (HIV tat or TK protein group) only 9.1%. There were obviousdifference in the above groups (t-test, $P < 0.05$).

3.4 Flow cytometry

Cells were cultured for 3 days continued with DMEM containing 10 μg/ml gencilorvir. After scan of FACScan flow cytometer, the apoptosis cell ratios of recombinant proteins containing 0, 2, 4, or 6 glycines on the 3rd day posttransfection were 8.31%, 12.69%, 14.77% or 4.36% in order, and in the control (HIV tat or TK protein group) was only 1.00%. There were obviousdifference in the above groups (t-test, $P < 0.05$).

4 Conclusion

The efficacy of HSV-1 TK-GCV suicide gene therapy of cancer, as well as of other hyperproliferative conditions including arterial restenosis, has been widely attributed to the bystander effect[13,14]. This phenomenon is credited with providing complete tumor regression when only a small percentage of tumor cells express the enzyme. However, the marginal clinical benefit so far observed in patients clearly indicates that the natural bystander effect of TK does not sufficiently compensate a poor rate of delivery of the HSV-TK gene to tumor cells[15,16]. Thus, clinical effectiveness of suicide gene therapy is entirely dependent, on the one hand, on the development of novel strategies to increase bystander cell killing and, on the other and, on the improvement of the efficacy of gene transfer.

In this work, we show that tagging TK with HIV-1 Tat confers trafficking capacity to the enzyme and a major impact to enhancement of TK tumor cells killing was observed in the Gly_4 linker between fusion protein compared to Gly_0, Gly_2 and Gly_6. Different chimer genes were obtained by DNA recombinant technique and then clone into prokaryotic expression vector – PBK, fusion protein purified by monoclonal antibody of HIV tat, containing 0, 2, 4, or 6 glycines linker, cultured with HepG2 cells, proportion of apoptosis cell 8.31%, 12.69%, 14.77% or 4.36%, respectively, suggested that fusion protein inserted Gly_4 between them contributed a remarkable enhancement of TK activity to HepG2 killing, these results are agreement with trypan blue stain experiment.

Taken together, these results indicate that Tat may serve as a protein delivery tool by which candidate proteins can be administered in a functional form to HepG2 cells. It must be appreciated that a 1-fold to 2-fold amplification in TK efficacy might not be sufficient in itself to render this suicide gene therapy approach of cancer immediately effective in a clinical setting. However, such amplification in protein delivery might be additive to the other ameliorations to TK gene therapy that are currently being considered, including amino acid modification to increase enzymatic efficacy[17,18], increased canonical bystander effect[19,20], and, clearly, improved gene delivery. It is conceivable that clinical success might eventually be attained through a combination of improvements in all these different aspects of suicide gene therapy.

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