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Computational modeling and functional analysis of Herpes simplex virus type-1 thymidine kinase and *Escherichia coli* cytosine deaminase fusion protein

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Abstract

Herpes simplex virus type-1 thymidine kinase (HSV-1TK) and *Escherichia coli* cytosine deaminase (CD) fusion protein was designed using InsightII software. The structural rationality of the fusion proteins incorporating a series of flexible linker peptide was analyzed, and a suitable linker peptide was chosen for further investigated. The recombinant plasmid containing the coding regions of HSV-1TK and CD cDNA connected by this linker peptide coding sequence was generated and subsequently transfected into the human embryonic kidney 293 cells (HEK293). The Western blotting indicated that the recombinant fusion protein existed as a dimer with a molecular weight of approximately 90 kDa. The toxicity of the prodrug on the recombinant plasmid-transfected human lung cancer cell line NCIH460 was evaluated, which showed that TKglyCD-expressing cells conferred upon cells prodrug sensitivities equivalent to that observed for each enzyme independently. Most noteworthy, cytotoxicity could be enhanced by concurrently treating TKglyCD-expressing cells with prodrugs GCV and 5-FC. The results indicate that we have successfully constructed a HSV-1TKglyCD fusion gene which might have a potential application for cancer gene therapy.

Keywords: Molecular modeling; Fusion peptide; Cytosine deaminase; Herpes simplex virus type-1 thymidine kinase

Suicide gene therapy which involves expression of genes encoding enzymes that convert nontoxic prodrugs into cytotoxic metabolites is a highlight in tumor gene therapy at present. The most widely studied suicide genes are the Herpes simplex virus type-1 thymidine kinase (HSV-1TK) gene and *Escherichia coli* cytosine deaminase (CD) gene [1–5]. HSV-TK catalyzes gancyclovir (GCV) into monophosphorylated gancyclovir that can then be converted into toxic gancyclovir triphosphate (GCV-TP) by other cellular kinases, which can cause cell growth inhibition and initiates cell death. CD can convert relatively nontoxic 5-fluorocytosine (5-FC) into a toxic metabolite 5-fluorouracil (5-FU). Both HSV-1TK and CD suicide gene therapy have been employed independently to eliminate tumor cells both in vitro and in vivo. Recently, some studies show that HSV-1TK and CD act in a synergistic way to kill tumors [6–10].

Hence, it was reasoned that the co-expression of HSV-1TK and CD, in conjunction with dual prodrug treatment, would be more effective in killing tumor cells than the employment of each enzyme/prodrug system independently. The purpose of choosing a linker that connects the HSV-1TK and CD genes is to avoid the function deficiency as a result of the interaction between those two proteins. Computational modeling of the HSV-1TKglyCD fusion peptide is an approach to finding an optimal linker.

In the present study, Herpes simplex virus type-1 thymidine kinase (HSV-1TK) and *E. coli* cytosine deaminase (CD) fusion proteins were designed using InsightII soft-

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ware, and a suitable linker peptide was chosen. We further constructed the recombinant fusion protein using this linker peptide. The function of that recombinant fusion protein was analyzed *in vitro*. The results of the present study demonstrate that the fusion of Herpes simplex virus type-1 thymidine kinase and the *E. coli* cytosine deaminase via a designed linker leads to the generation of a bioactive fusion protein.

Materials and methods

Molecular modeling. All modeling and simulations were conducted using the InsightII software (Biosym Technologies Inc. San Diego, CA). The crystal structures of HSV-TK (PDB code: 1K6W) and the CD (PDB code: 1OF1) were the starting point for the simulations, and the fusion protein models were combined as head–tail form of HSV-TK and CD using a series of linked peptide of different length. The consistent valence forcefield (CVFF) was selected prior to perform minimization and dynamics calculations. To account the solvent effect, the centered fusion protein is solvated in a sphere of TIP3P water molecules with radius 10 Å. The energy minimization procedure consisted of 1000 steps of steepest descent, and then a conjugate gradient minimization was taken until the root mean square (rms) gradient of the potential energy was less than 0.001 kcal/molA. The minimized coordinates of the protein were used as starting point for dynamics. The fusion protein was thermalized to 300 K with a gradient of 5 K per 6000 steps by randomly assigning individual velocities from the Gaussian distribution. After heating, the systems were allowed to equilibrate until the potential energy vs. time was approximately stable. Time-averaged structures were then determined over the last 100 ps of each simulation for the fusion protein. Data were collected every 0.5 ps. The most suitable link-peptide was picked out by analyzing the intermolecular energy between HSV-TK and CD. The energies of intermolecular interaction were calculated under the Docking module of InsightII.

Construction of plasmids. The DNA fragment encoding HSV-1TK was obtained from the plasmid pNGVL-TK (National Gene Vector Laboratory, University of Michigan) by PCR performed with a sense primer (5′-CCG GAA TTC ACC ATG GCT TCG TAC CCC-3′) and an antisense primer (5′-CGC GGA TCC GTT AGC CTC CCC CAT CTC-3′). The first denaturation step was carried out at 94 °C for 2 min, followed by 30 cycles at 94 °C for 15 s, 52 °C for 30 s, 72 °C for 1 min and the final primer extension at 72 °C for 7 min. The PCR product was digested with EcoRI and BamHI, and then inserted into pcDNA3.1/HA-myc-His(−)Z (a modified pcDNA3.1 vector that contains a hemagglutinin epitope tag sequence immediately downstreaming the cloning site; gift of State Laboratory for Oncogene and Related Gene Research, Shanghai Jiaotong University) to yield pcDNA3.1/HA-myc-His(−)Z-TK. The DNA fragment encoding CD was obtained from CD-containing plasmid (reserved by our laboratory) by PCR performed under the similar conditions with a sense primer (5′-CCG GAA TTC ACC ATG GGG AGG CTA ACA GTG TC-3′) and an antisense primer (5′-CGC GGA TCC ACG TTT GTA ATC GAT GGC TTC-3′). After being digested with EcoRI and BamHI, the purified CD fragment was cloned into pcDNA3.1/HA-myc-His(−)Z to yield pcDNA3.1/HA-myc-His(−)Z-CD.

To construct the recombinant vector carrying the HSV-1TKglyCD fusion protein cDNA, PCR was performed to generate a linker and a modified CD cDNA, the primer 5′-CGC GGA TCC GGC GGG GGC GGT GGA GGA GGG GGT GGG AGG CTA ACA GTG TC-3′ was used as a sense primer which corresponds to the 5′-untranslated region (UTR) and the first five codons of the CD gene, all the nonglycine codons were changed into the glycine codons, and the vector was inserted with a BamHI site at 5′ end of both primers. The antisense primer 5′-CGC GGA TCC ACG TTT GTA ATC GAT GGC TTC-3′ is the same as the above antisense primer for the CD gene. The 1310-bp PCR product CD cDNA was digested with BamHI, and then was inserted into pcDNA3.1/HA-myc-His(−)Z-TK to yield pcDNA3.1/HA-myc-His(−)Z-TkglyCD. The orientation of inserted CD gene was validated by PstI cut, and the digested fragments of the positive clone were 442, 782, 2662, 4045 bp, respectively. Subsequently, the DNA fragment encoding HSV-1TKglyCD was verified by DNA sequencing.

Cell cultures. Human embryonic kidney 293 cell line (HEK293) was obtained from Invitrogen. Human lung cancer cell line (NCIH460) was obtained from Cells Bank of the Chinese Academy of Science (Shanghai, China). These cell lines were cultured in DMEM medium supplemented with 10% fetal bovine serum (Gibco) at 37 °C under 5% CO_2 in an incubator.

Transient transfection and cytotoxicity assays. The plasmid pcDNA3.1/HA-myc-His(−)Z and the recombinant transfer vectors pcDNA3.1/HA-myc-His(−)Z-TK, pcDNA3.1/HA-myc-His(−)Z-CD, and pcDNA3.1/HA-myc-His(−)Z-TKglyCD were transfected into NCIH460 cells using the Lipofectamine 2000 (Invitrogen). After 24 h incubation, transduced NCIH460 cells were detached by trypsinization and were immediately seeded into 96-well plates (5×10^3 cells per well) in 200 μl medium containing various concentrations of 5-FC {(0, 20, 40, 60, 80 μg/ml) Sigma} or GCV{(0, 0.2, 0.4, 0.6, 0.8 μg/ml) Syntex} for 72 h. The sensitivities of the transduced NCIH460 cells in 96-well plates to GCV and 5-FC were quantified using Cell Counting Kit-8 (Dojindo). Briefly, at the time of the assay, the cells were stained with 10 μl CCK-8 solution at 37 °C for 4 h. Absorbance was measured at 450 nm using a microplate reader (Wako, Osaka, Japan). Each group set consisted of three repeated wells. The results were calculated as mean values of three wells per treatment group. The tests were repeated three times.

Western blotting analysis. To determine the HSV-1TK, CD, and HSV-1TKglyCD-expression, each recombinant plasmid-transduced HEK293 cells were lysed in the SDS sample buffer (containing 10 mM β-mercaptoethanol, 100 mM Tris–Cl pH 6.8, 2% SDS, and 0.1% bromo phenol blue). Boiled samples were separated by SDS–PAGE in 10% gels and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was probed with Anti-HA monoclonal antibody (Sigma) followed by a horseradish peroxidase-conjugated secondary antibody (Sigma). Bands were visualized using the ECL chemiluminescence detection system (Amersham).

Statistical analysis. The cell viability data from the different groups were compared by analysis of variance (ANOVA) using SPSS 12.0 statistical software. A value of $P < 0.05$ was considered statistically significant.

Results and discussion

Modeling of HSV-1TKglyCD fusion protein

Molecular modeling is one of the most reliable methods to predict models of protein [11–16]. According to the crystal structures of the HSV-TK (PDB code: 1K6W) and the CD (PDB code: 1OF1), the structural model of the recombinant HSV-TKglyCD fusion proteins was built by using InsightII software. The model was optimized with energy minimization using steepest descent and conjugated gradient method. Subsequently, the conformation of the fusion protein was subject to molecular dynamic run. The average structures obtained and computed on the last 100 ps were further minimized. The minimization stage was judged on the basis of the root mean square (rms) deviation from the crystal structure and computational efficiency. The optimized structures of the fusion protein with the three kinds of linkers were obtained. The system energy and intermolecular energy between HSV-TK and CD were determined. The length of the linker peptides has effect on the function of the fusion proteins.

Choice of suitable link-peptide

Construction of functional fusion proteins often requires a linker sequence that adopts an extended conformation to allow for maximal flexibility. In this study, linker sequences were selected based on the molecular modeling methods. We designed three linker sequences which had various sequence lengths. The complete model of fusion protein of HSV-TK and CD was built using a series of linked peptides. Then they were subject to minimization and dynamic runs. The energy of the interaction between two proteins in fusion protein was determined. The maximal partial intermolecular actions between HSV-TK in fusion protein with different linker peptides and CD are listed in Table 1. Results indicated that HSV-TK with 11aa connecting peptide produced the lowest energy with CD. So the peptide GSG GGG GGG GG was used as linker peptide to connect the two moieties of the fusion protein. The module of interaction between HSV-TK and CD (11aa link-peptide) was also shown in Fig. 1. It was obviously shown that the HSV-TK could not interact with CD, and the fused enzymes connected by GSG GGG GGG GG linker do not greatly alter either the catalytic sites or the structures. The binding sites of both proteins were not affected and this fused protein would most likely bind substrates. The results suggested that it should be possible to produce a functional fusion protein with a GSG GGG GGG GG linker between HSV-TK and CD.

Table 1
List of linker sequences and intermolecular energy

Link-peptide sequence	Intermolecular energy (kcal/mol)		
	Vdw	Elect	Total
GSGGGGGGGGG	−0.859	−1.539	−2.389
GSGGGGGGG	−2.554	−2.580	−5.134
GSGGGGG	−7.772	−1.610	−9.382

Construction of HSV-1TK gene, CD gene, and HSV-1TKglyCD fusion gene

First, construction of the HSV-1TK and CD gene expression vector required modification of both the CD and HSV-1TK genes. Using PCR, the TGA stop codons of HSV-1TK and CD were changed to allow translational read through into HAtag sequence. So it is convenient to detect the expression of target gene by anti-HA antibody. The sequence of the inserted gene was firstly identified by EcoRI and BamHI digestion. Two segments of approximate 5.5 and 1.1 kb (or 1.3 kb) were extracted from the 6.6 kb (or 6.8 kb) recombinant plasmid, which were equal to the size of the pcDNA3.1/HA-myc-His(−)Z plasmid and HSV-1TK (or CD) gene, respectively. Sequencing results showed that the nucleotides sequences of the inserted genes were completely consistent with the nucleotides sequences of the HSV-1TK gene (GenBank: 59889) and the CD gene (GenBank: 45356868).

In order to construct the DNA fragment encoding the HSV-1TKglyCD fusion protein, PCR was also employed to change the TGA stop codon and convert the 5′-UTR of the CD gene into a polyglycine tract. Upon ligation, the CD gene was fused in-frame with the upstream HSV-1TK gene and the downstream HAtag sequence, joining the two enzymatic moieties by a linker. The linker consists of 33 nucleotides encoding GSG GGG GGG GG, which is rich in flexibility.

The fragments of the recombinant plasmid cut by single BamHI were 6.6 and 1.3 kb, which were equal to the sizes of the pcDNA3.1/HA-myc-His(−)Z-TK plasmid and CD gene, respectively. Subsequently, the fragments of the posi-

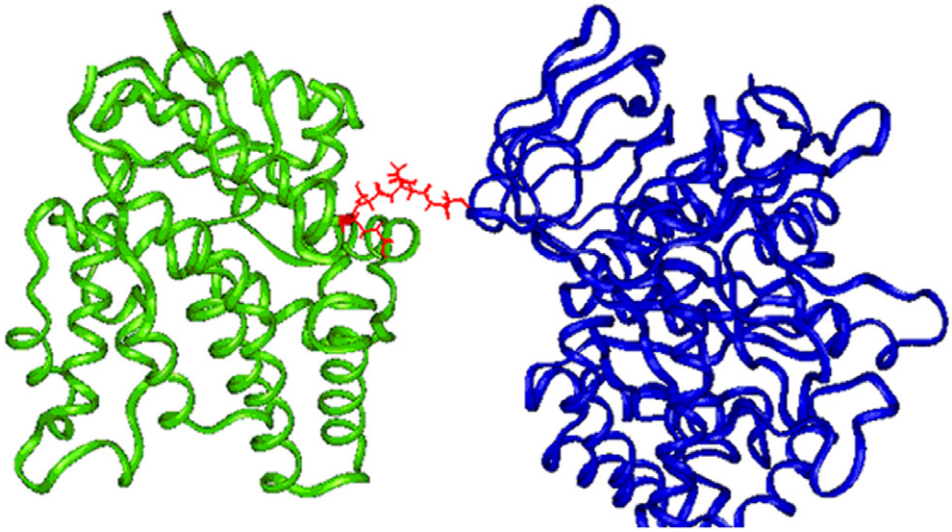

Fig. 1. A 3D structure of HSV-1TK and CD fusion protein obtained by molecular modeling method. HSV-1TK is represented by green color, CD is represented by blue color, and the linker peptide GSG GGG GGG GG is represented by red color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tive recombinant plasmid in which the inserted CD gene was forward orientated were cut by PstI were 442, 782, 2662, and 4045 bp. The derived plasmid was verified by DNA sequencing and named as pcDNA3.1/HA-myc-His(−)Z-TKglyCD.

This linker was designed to provide the flexibility necessary for proper protein folding and independent enzymatic functions. The fusion protein may thus guarantee stoichiometric expression of the CD and TK protein moieties.

Expression and functional analysis of the HSV-1TKglyCD fusion gene

To test HSV-1TKglyCD fusion gene expression, we analyzed by Western blotting the proteins from the HEK293 cells transduced with plasmids pcDNA3.1/HA-myc-His(−)Z, pcDNA3.1/HA-myc-His(−)Z-TK, pcDNA3.1/HA-myc-His(−)Z-CD, and pcDNA3.1/HA-myc-His(−)Z-TKglyCD. In the pcDNA3.1/HA-myc-His(−)Z-TKglyCD-transduced cells, Western blotting analysis revealed a single band displaying an apparent molecular weight of 90 kDa, which is expected for the product of a TKglyCD fusion gene. In addition, we also detected the expressing products of TK and CD genes in the corresponding pcDNA3.1/HA-myc-His(−)Z-TK and pcDNA3.1/HA-myc-His(−)Z-CD-transduced cells, respectively. As expected, no gene products were detected in cells transduced with the pcDNA3.1/HA-myc-His(−)Z vector (Fig. 2).

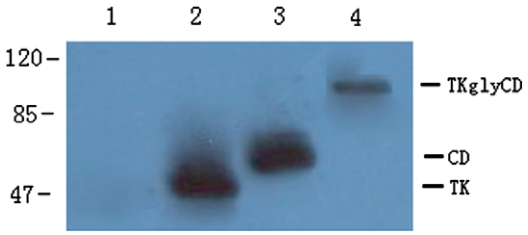

Fig. 2. Western blotting of HEK293 cells transduced with pcDNA3.1/HA-myc-His(−)Z-TK, pcDNA3.1/HA-*myc*-His(−)Z-CD, and pcDNA3.1/HA-myc-His(−)Z-TKglyCD. The blot was probed with anti-HA monoclonal antibody which reacted with HAtag fused into the HSV-1TK, CD, and TKglyCD. Line 1, cells transduced with the pcDNA3.1/HA-myc-His(−)Z. Line 2, cells transduced with the pcDNA3.1/HA-*myc*-His(−)Z-TK. Line 3, cells transduced with the pcDN A3.1 /HA-myc-His(−)Z-CD. Line 4, cells transduced with the pcDNA3.1/HA-myc-His(−)Z-TKglyCD.

To assess whether the HSV-1TKglyCD fusion protein is functional, we analyzed the HSV-1TKglyCD fusion protein rendered-NCIH460 cells susceptible to CD- or HSV-1TK-specific prodrugs by measuring the cytotoxicity after treatment with various concentrations of 5-FC and GCV. The results demonstrated that the control cells were resistant to the CD-specific prodrug 5-FC (Fig. 3A). NCIH460 cells expressing CD alone or TKglyCD, however, were highly sensitive to 5-FC. The IC_{50} value (concentration of prodrug required to inhibit colony formation by 50%) for TKglyCD (59 μg/ml 5-FC) and CD-expressing cells (57 μg/ml 5-FC) were not significantly different ($P > 0.05$). As illustrated in Fig. 3B, NCIH460 cells transduced with pcDNA3.1/HA-myc-His(−)Z were resistant to *GCV*. Markedly contrasted, NCIH460 cells expressing either HSV-1TK or TKglyCD were highly sensitive to GCV in a concentration-dependent manner. IC_{50} values for HSV-1TK (0.59 μg/ml GCV) and TKglyCD-expressing cells (0.60 μg/ml GCV) were nearly identical ($P > 0.05$). The cell survival studies indicated that the 90-kDa TKglyCD fusion protein should be bifunctional, possessing both CD and HSV-1TK activity and being able to confer the prodrug sensitivity to the transduced tumor cells.

Because HSV-1TK and CD act in a synergistic way to kill tumors, We further analyzed whether expression of TKglyCD, in conjunction with dual prodrug treatment, would be more effective in killing tumor cells than employing each enzyme/prodrug system independently. The extent to which the double suicide gene therapy could enhance cytotoxicity was investigated by treating transduced NCIH460 cells with a combination of 5-FC and GCV. As expected, co-treatment of TKglyCD-expressing cells with both 5-FC and GCV achieved synergistic cytotoxicity enhancement (Fig. 4).

The construction of a HSV-1TKglyCD fusion protein involves connecting the HSV-1TK with CD by a linker

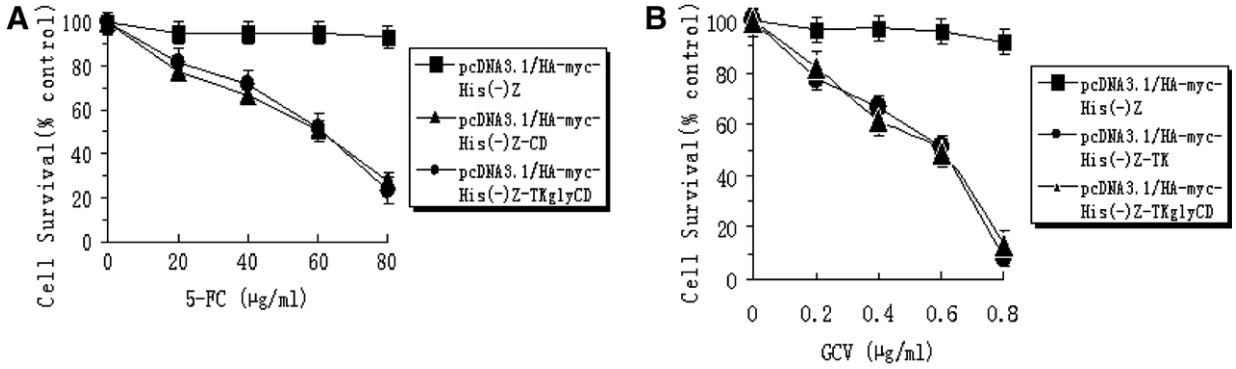

Fig. 3. Toxicity of prodrugs to NCIH460 cells transfected with pcDNA3.1/HA-myc-His(−)Z-CD, pcDNA3.1/HA-myc-His(−)Z-TK, pcDNA3.1/HA-myc-His(−)Z-TKglyCD, or control vector pcDNA3.l/HA-myc-His(−)Z. (A) Cells were treated with 5-FC at the indicated concentrations for 72 h. (B) Cells were treated with GCV at indicated concentrations for 72 h. Each group set consisted of three repeated wells. The tests were repeated three times.

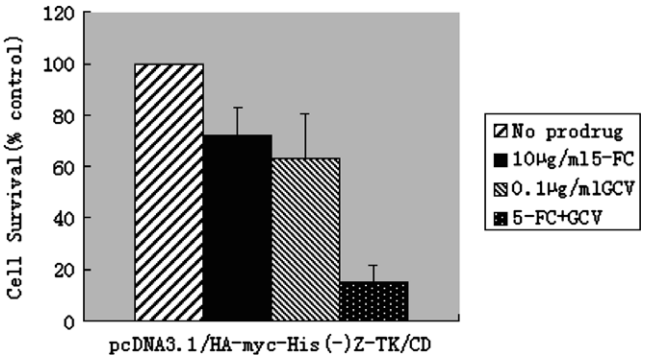

Fig. 4. TKglyCD-expressing cells were treated with 10 μg/ml 5-FC and 0.1 μg/ml GCV alone or in combination for 72 h. Levels of additive cytotoxicity are indicated. Each group set consisted of three repeated wells. The tests were repeated three times.

sequence which aims to allow the correct formation and functioning of their corresponding products. It is well known that the linker length and composition is particularly important in the construction of those functional fusion proteins. The linker sequence must be flexible and long enough to achieve this purpose. Glycine is generally used in the designed linkers because a glycine-rich linker will be more flexible than a linker of comparable length composed of non-glycine residues [17]. Therefore, we chose the glycine as the composition of linker. To asses the effects of changing the number of glycines in the linker, we have used InsightII software to built the structural model of HSV-TKglyCD fusion protein. This program automatically generates a refined homology model of a protein. Given only the sequence alignment to a known 3D protein structure, we expediently chose an appropriate linker for design fusion protein.

Together, the results from this study demonstrated that the addition of a flexible linker between the two subunits of such composite suicide genes allows the expression of a single chain fusion protein retaining functional activity. Most noteworthy, cytotoxicity could be enhanced by concurrently treating TKglyCD-expressing cells with prodrugs GCV and 5-FC. Conclusively, we have successfully constructed a HSV-1TKglyCD fusion gene which might have a potential clinical application.

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