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Reduction of non-specific toxicity of immunotoxin by intein mediated reconstitution on target cells

Jing Wang[a], Lei Han[a], Junsheng Chen[a], Yueqing Xie[b], Hua Jiang[b], Jianwei Zhu[a,b,*]

[a] Engineering Research Center of Cell and Therapeutic Antibody, Ministry of Education, School of Pharmacy, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, People's Republic of China
[b] Jecho Laboratories, Inc., 7320 Executive Way, Frederick MD21704, USA

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ABSTRACT

Recombinant immunotoxins are chimeric proteins composed of a targeting peptide that binds to a specific tumor antigen and a toxin protein killing target cells. Recombinant immunotoxin exhibits potent cancer inhibiting effects both *in vivo* and *in vitro*. However, the non-specific toxicity causes severe syndromes limiting their clinical application. To reduce toxicity caused by recombinant immunotoxins in general, we divided an immunotoxin into two nontoxic segments that may restore toxic bioactivity on tumor cell surface based on the intein mediated trans-splicing reaction. Both split and reconstituted immunotoxins were tested for their biological activities. We found that the reconstituted immunotoxin retained antigen specificity and affinity toward cancer cells over-expressing HER2/neu. After being internalized into HER2/neu positive cells, the reconstituted immunotoxin showed comparable cytotoxicity as the original immunotoxin, while the split immunotoxin fragments showed no toxic activity to cells with or without HER2/neu expression. This approach can potentially be used under clinical settings to reduce non-specific toxicity by administering patients with inactive immunotoxin fragments. Cytotoxic effect only occurs at tumor sites where the inactive fragments bind, trans-splice and become active toxin.

1. Introduction

Immunotoxins are a category of immunoconjugate in which targeting parts are joined to protein toxins application of recombinant immunotoxins has been one of the hot spots in cancer immuno-therapy since they were first constructed thirty years ago [1–3]. Antibodies and growth factors were most commonly used cell-targeting motifs while toxic proteins from bacteria or plants were extremely potent cell-killing agents [4]. Immunotoxins recognize specific tumor cells through the ligand-receptor or antibody-antigen interaction, and then induce cell death after being internalized to the cytoplasm of the target cells [5]. Compared to the conventional chemo-therapy and radiation therapy, the targeting therapies of immunotoxins demonstrated higher efficacy and better safety [6]. However, due to expression of the specific antigens on normal cells, there still remained some unwanted toxicities of immunotoxins [7,8]. Although the targeting molecules dramatically reduced the side effects caused by the cytotoxic heterologous protein, non-specific toxicities frequently occurred during the tumor treatment, such as vascular leak syndrome caused by recombinant immunotoxins targeting the HER2/neu oncogene [9].

Inteins are auto-processing domains found in archaeal, eubacterial, and eukaryotic genes, which conduct protein-splicing naturally [10,11]. Protein splicing is an autocatalytic process in which intein excises itself from a precursor protein with ligation of the flanking protein (extein) [12,13]. The cis-splicing and trans-splicing are separately mediated by the continuous intein and the split intein, respectively. The split intein can fuse with two different external proteins (extein) and conjugate to form an active intein, which then excises itself out from the exteins and form a new peptide bond between the two exteins [14]. Intein-based methods were employed to facilitate protein purification and protein modifications [15,16]. Previously, our lab successfully utilized inteins in protein soluble expression and purification, and in generating bispecific antibodies [17,18].

To improve the safety profile of the immunotoxin therapy, we created a new approach that is to divide a toxic immunotoxin into two nontoxic segments that can be re-connected through protein ligation mediated by intein trans-splicing reaction. The cytotoxicity is resumed after the immunotoxin is reconstituted. In this report, we demonstrated

* Corresponding author at: Engineering Research Center of Cell and Therapeutic Antibody, Ministry of Education, School of Pharmacy, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, People's Republic of China.
E-mail address: jianweiz@sjtu.edu.cn (J. Zhu).

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the feasibility of this approach using *Npu DnaE*, a naturally occurring split intein from *Nostoc punctiforme* PCC73102, and PE38, a truncated form of Pseudomonas exotoxin A [19]. PE38 sequence was split into two segments and each were fused to N-intein and C-intein of *Npu DnaE*. The separated two segments of PE38 lost activity until they were reconstituted on the target cell surface through *Npu DnaE* mediated trans-splicing. Therefore, recombinant immunotoxins scFvPE38, composed of the scFv of Trasuzumab and PE38, kept inactive when delivered as two segments and restored activity after protein trans-splicing reaction.

2. Materials and methods

2.1. Cell lines and cell culture

The human ovarian adenocarcinoma cell line SK-OV-3 was a kind gift from Dr. Wei Han, Shanghai Jiao Tong University. The cell line was maintained in McCoy's 5A medium supplemented with 10% Fetal Bovine Serum (FBS). The human breast adenocarcinoma cell line MCF-7 was a kind gift from Dr. Yong Gan, Shanghai Institute of Materia Medica. The cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 0.01 mg/ml human recombinant insulin (Yeasen Biotech, China). The Chinese hamster ovary (CHO) cells (CHO-S; Invitrogen, Carlsbad, CA, USA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 0.1 mM MEM Non-Essential Amino Acids Solution. Culture was performed in a humidified atmosphere at 37 °C and 5% CO2. All the media and FBS were purchased from Gibco (USA).

2.2. Production and purification of recombinant immunotoxin

Objective genes were amplified with PCR using the synthetic primers and then overlapped using the following thermocycling protocols: 30 cycles at 98 °C for 10 s, 55 °C for 5 s, 72 °C for 10 s; 1 cycle at 72 °C for 5 min. The PCR fragments were inserted into pET30a between the *Nde*I and *Hind*III sites to generate vectors for expression. The vectors were named as pET-scFvPE38 (scFv: single-chain variable fragment), pET-scFvPMn (PMn: mutated PE38, $n = 1, 2, 3$, correspond to three different mutation sites), pET-scFvP_NI_N (P_N: N terminal of PE38; I_N: N terminal of split intein) and pET-I_CP_C (I_C: C terminal of split intein; P_C: C terminal of PE38), respectively.

The plasmids used in this reports were individually transformed into the *Escherichia coli* (*E.coli*) strain BL21 (DE3) for protein expression. The protein expression and purification was performed as previously described [20]. Briefly, protein expression was induced at 37 °C by addition of 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h. The inclusion bodies of target protein were isolated by centrifugation and then dissolved in denaturing buffer (100 mM Tris-HCl, 6 M guanidine hydrochloride, 1 mM EDTA, pH 7.4). After sonication with a probe, the thoroughly dissolved inclusion bodies were span at 12,000 rpm for 30 min. The supernatant was then rapidly diluted into 50 volume of refolding buffer (100 m M Tris-HCl, 0.5 M arginine, 1 mM reduced glutathione and 0.1 mM oxidized glutathione, pH 9.0), stirring at 4 °C for 12 h. For protein purification, the folded scFvPE38 and scFvP_NI_N were then applied onto Capto L columns. Solubilized I_CP_C appeared clean enough for trans-splicing reaction without further purification.

2.3. In vitro intein mediated trans-splicing reaction

The purified protein was finally diluted into reaction buffer (20 mM Tris-HCl, 0.1 mM NaCl, 1 mM DTT, 1 mM EDTA, and pH 8.0) at 37 °C for *in vitro* intein mediated trans-splicing reaction [21]. At different time points the reaction was stopped by adding SDS-PAGE loading buffer and boiled at 95 °C for 5 min. The mixture of trans-splicing reaction was analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and detected by Coomassie blue staining. For the reaction occurred in McCoy's 5A medium with FBS, the products were detected by Western blotting to monitor the trans-splicing reaction. A rabbit anti-ETA (Pseudomonas Exotoxin A) polyclonal antibody (Sigma-Aldrich, USA) and the HRP labeled goat anti-rabbit antibody (Singon Biotech, China) were used for the Western blotting analysis.

2.4. Binding assay and internalization analysis

The receptor binding affinity of the split and the original immunotoxins were evaluated by flow cytometry. One hundred microliter of cell suspension (1E6/ml) were incubated with various immunotoxins at 25 nM for 1 h on ice and then washed with phosphate buffered saline (PBS) before incubated with rabbit anti-ETA polyclonal antibody (1:100) for 1 h on ice. Herceptin was used as a positive HER2/neu-binding control while PBS group served as negative control. After PBS washing, alexa-488 conjugated anti-rabbit and anti-human secondary antibody (Singon Biotech, China) were added and incubated for half an hour on ice respectively. Fluorescence intensity of the cells were analyzed using flow cytometry (BD LSRFortessa, San Jose, CA, USA).

Internalization was evaluated as described by Cao et al. [22]. Briefly, cell slides were prepared for immunofluorescence detection. After separately treated with 50 nM original immunotoxins or 1 μM split immunotoxins (added 1 mM DTT in advance) for 4 h, the cells were fixed with 4% Paraformaldehyde and then permeabilized with 0.2% Triton X-100. Three percent of bovine serum albumin (BSA) was used to block non-specific binding before the addition of anti-ETA antibody and fluorescein isothiocyanate (FITC) conjugated secondary body. Cell nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). Immunofluorescence was visualized with a confocal laser-scanning microscope (LeicaTCS SP8, German).

2.5. In vitro cytotoxicity assay

About 5000 cells were plated in each well of a 96-well plate and cultivated for overnight at 37 °C before adding immunotoxins at various concentrations. After incubation for 72 h, the cytotoxicity of immunotoxins on different cells was measured by CCK-8 kit (Dojindo, Japan). The water-soluble tetrazolium salt WST-8 in CCK-8 kit was reduced by dehydrogenases in cells to give an orange colored product (formazan), which was soluble in the tissue culture medium. The absorbance monitored by a microtiter plate reader (Tecan infinite M200 Pro, Switzerland) at 450 nm was measured, which was correspond to the cell viability.

2.6. Detection of apoptosis

AnnexinV selectively binding to phosphatidylserine on the surface of apoptotic cells, a $Ca2^+$ dependent process, was used to confirm the cell apoptosis [23]. Briefly, 2E5 cells were seeded in each well of a 12-well plate and incubated overnight for confluence. The immunotoxins were added to induce cell apoptosis. After incubation for 48 h, cell suspensions were obtained through tyrosine digestion. The cells were incubated with AnnexinV-FITC and propidium iodide (PI) for 10 min at room temperature. The cells were washed and resuspended with AnnexinV binding buffer, and analyzed on the flow cytometer).

2.7. Data analysis

All results were generated through at least triplicate experiments with means ± standard error. The statistical differences between groups of data were analyzed by Students' test. A level of $P < 0.05$ was considered to be significant (*), and $P < 0.01$ was considered to be very significant (**).

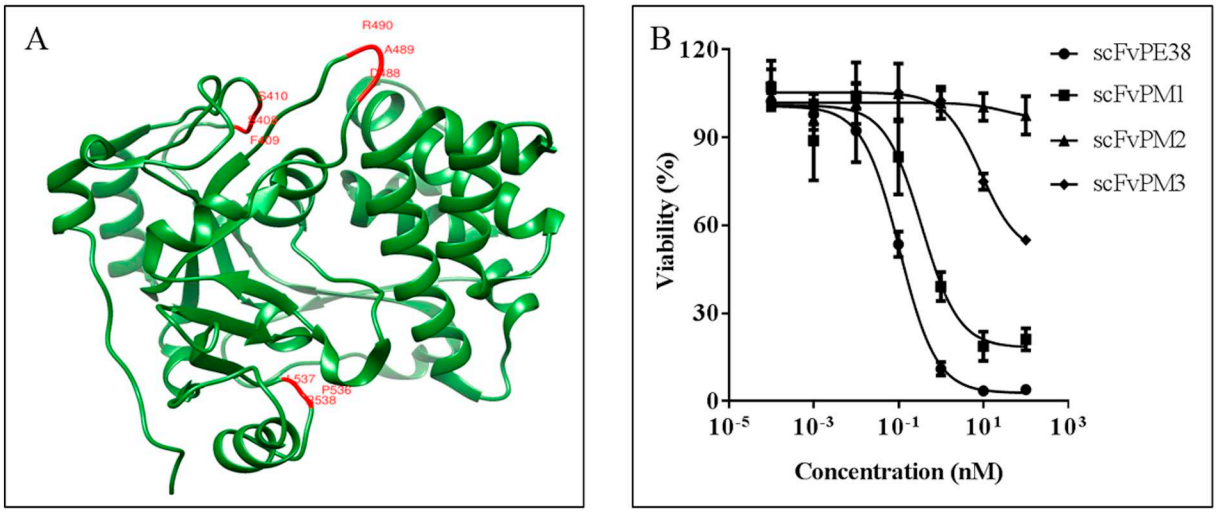

Fig. 1. Determination of the intein insertion site. A, The mutation sites in the three dimensional structure of PE38. B, Cytotoxicity of the wild-type and mutated scFvPE38 on SK-OV3 cells.

3. Results

3.1. Determination of the intein insertion site

PE38 is a 283-residue protein containing domains II, Ib and III of Psuedomonas Exotoxin A. Domain III is responsible for ADP-ribosyl transferase activities [24]. The ideal dividing point should ensure that the split segments are inactive, and after trans-splicing the toxic activity can be restored. Three locations (Ser408-Ser410, Asp488-Arg490 and Pro536-Arg538, Residue numbering is based on the amino acid sequence of native PE) were chosen as possible intein insertion site. All three sites are located on the loops of domain III and surface-exposed (Fig. 1A). A "CFN" sequence was followed the downstream of *Npu* C terminal to ensure sufficient splicing efficiency [25]. Some amino acid residues, such as His426, Tyr481, and Glu553, participate in conformational changes during the substrate-binding, therefore are essential for the full ADP-ribosylation activity of the PE [24]. In order to maintain their spatial positions, the "CFN" sequence flanking C terminal of *Npu DnaE* was introduced through site-specific mutagenesis rather than addition to the original sequence. The approach was adopted to avoid a "CFN" scar after splicing that might cause conformational changes and reduce the enzyme activity.

To examine the impact of the "CFN" mutagenesis on PE38's function, we constructed Trasuzumab scFv fusion with the wild-type PE38 (scFvPE38) or PE38/Ser408-Ser410 (scFvPM1), PE38/Asp488-Arg490 (scFvPM2) and PE38/Pro536-Arg538 (scFvPM3). The original and mutated immunotoxins were evaluated for HER2/neu targeting ability and their cytotoxicity on HER2/neu overexpressed SK-OV3 cells. Results of the CCK8 cytotoxicity assay showed that the mutations caused reduced cytotoxicity (Table 1), while IC_{50} of 0.11 nM of scFvPE38 was similar to the previously reported value [26]. Fig. 1B shows the dose response curves of the original and mutated PE38 immunotoxins. scFvPM1 (Ser408-Ser410) retained most of the cytotoxicity and was selected as the intein insertion site in subsequent studies.

Table 1
The IC_{50} of the wild-type and the mutated scFvPE38.

	Mutation site	Original sequences	Mutated sequences	IC_{50} (nM)
scFvPE38	–	–	–	0.11
scFvPM1	408–410	SFS	CFN	0.35
scFvPM2	488–490	DAR	CFN	33.13
scFvPM3	536–538	PLR	CFN	8.85

3.2. Expression and purification of immunotoxin fragments fused with Npu DnaE intein

PE38 was split at residue 407–408 with "SFS (408–410)" mutated to "CFN". The resulted two fragments were named P_N and P_C. *Npu DnaE* intein is a naturally split intein composed of N-intein (I_N) and C-intein (Ic). P_N and P_C were separately fused to I_N and I_C (P_NI_N and IcPc). The scFv of Trastuzumab was composed of VL and VH, which was joined by a flexible $(GGGGS)_3$ peptide linker. scFv was fused to the N terminus of P_NI_N using another $(GGGGS)_3$ linker to form $scFvP_NI_N$ (Fig. 2A). Coding sequences of $scFvP_NI_N$ and the I_CP_C were separately inserted into vector pET30a. $ScFvP_NI_N$ and IcPc proteins were expressed in *E.coli* BL21 (DE3) as inclusion bodies under the induction conditions described in "Materials and methods". The inclusion bodies were isolated and proteins were purified as described in "Materials and Methods" to the purity about 90% (Fig. 2B).

3.3. Reconstituted immunotoxin through trans-splicing reaction in vitro

The purified $scFvP_NI_N$ and I_CP_C were mixed in reaction buffer (described in "Materials and Methods") to a final concentration of 5 μM for each component. The splicing reaction was carried out at 37 °C with the presence of 1 mM DTT to suppress formation of intermolecular disulfide bond. The reaction mixture was analyzed by SDS-PAGE. About 10% reaction products could be seen on the gel (Fig. 3A). Likewise the trans-splicing reaction was also carried out in McCoy's 5A medium supplemented with 10% FBS and the reaction mixture was analyzed by Western blotting. The reaction products with similar molecular weight to scFvPE38 could also be seen on the membrane (Fig. 3B). Some other cell medium such as DMEM and RPMI 1640 were also validated for the protein trans-splicing reaction (Dates not shown).

3.4. Comparison of the antigen-binding abilities of scFvPM1 and $scFvP_NI_N$

Three different cell lines (SK-OV3, MCF7 and CHO) with different HER2/neu expression levels were employed to determine whether the immunotoxins could bind antigen positive cells. Binding of the immunotoxins on cell surfaces was determined by flow cytometry. Herceptin, a HER2/neu specific monoclonal antibody, was used as positive control. Both $scFvP_NI_N$ and scFvPM1 bound to SK-OV3 and MCF7 but not CHO cells, as did Herceptin, according to the flow cytometry measurements (Fig. 4). SK-OV3 has a higher expression level of HER2/neu on cell surface than MCF7, while CHO has no expression [22,27]. Our results showed that scFvPM1 and $scFvP_NI_N$ bound to the

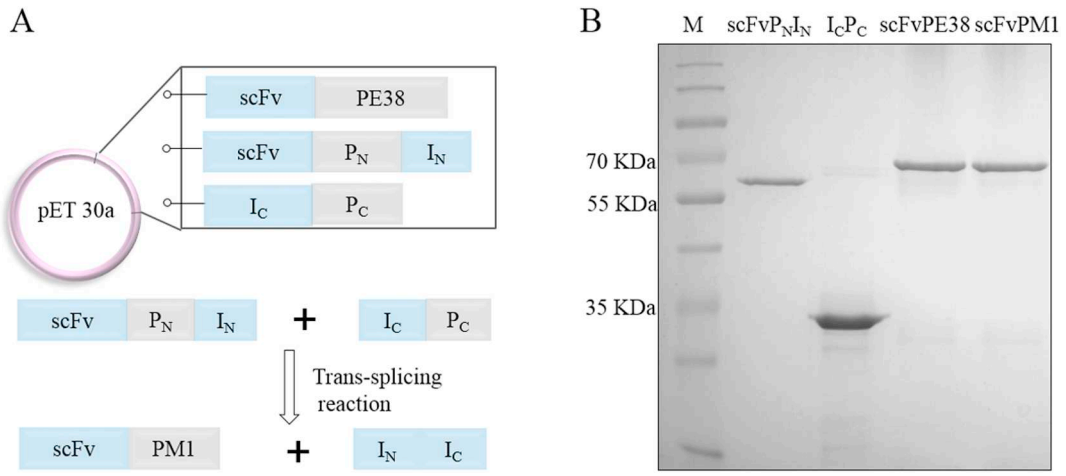

Fig. 2. Preparation of the original and split immunotoxins. A, schematic diagram of plasmid constructs and intein mediated trans-splicing reaction. B, SDS-PAGE analysis of purified immunotoxins.

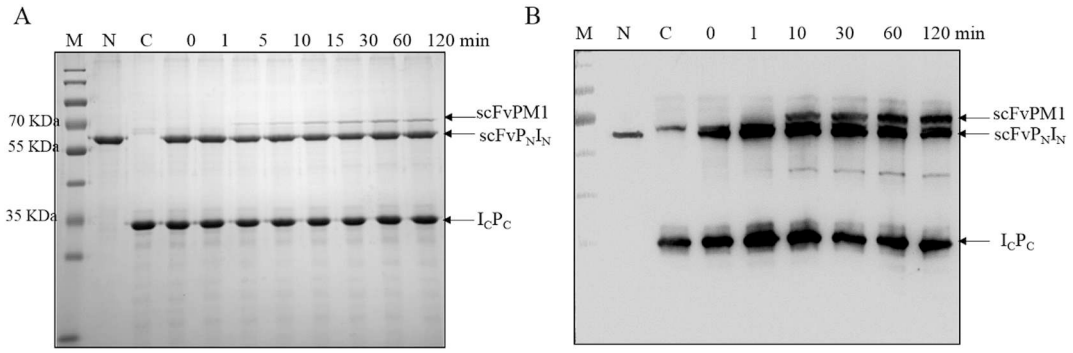

Fig. 3. Reconstituting scFvPM1 by split intein–mediated protein trans-splicing. A, SDS-PAGE analysis of trans-splicing reaction between scFvP_NI_N (N) and IcPc (C) at different time points in reaction buffer (20 mM Tris-HCl, 0.1 mM NaCl, 1 mM DTT, 1 mM EDTA, and pH 8.0). B, Western blotting analysis of successful reconstitution of covalently linked full-length scFvPM1 in FBS supplemented cell medium.

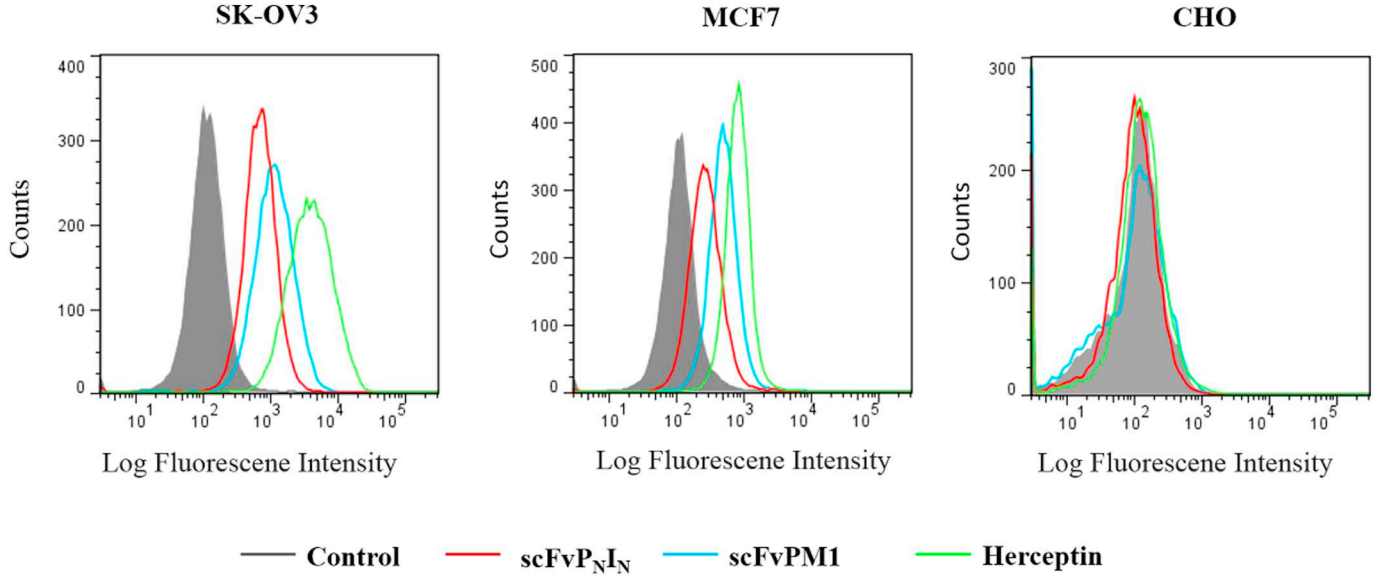

Fig. 4. Specific binding activity of scFvP_NI_N and scFvPM1. The binding behavior of the constructs were determined by flow cytometry on three cell lines: HER2/neu positive (SK-OV3 and MCF7) and negative cells (CHO cells). Antigen expression was determined by the use of monoclonal antibody Herceptin directed against HER2/neu.

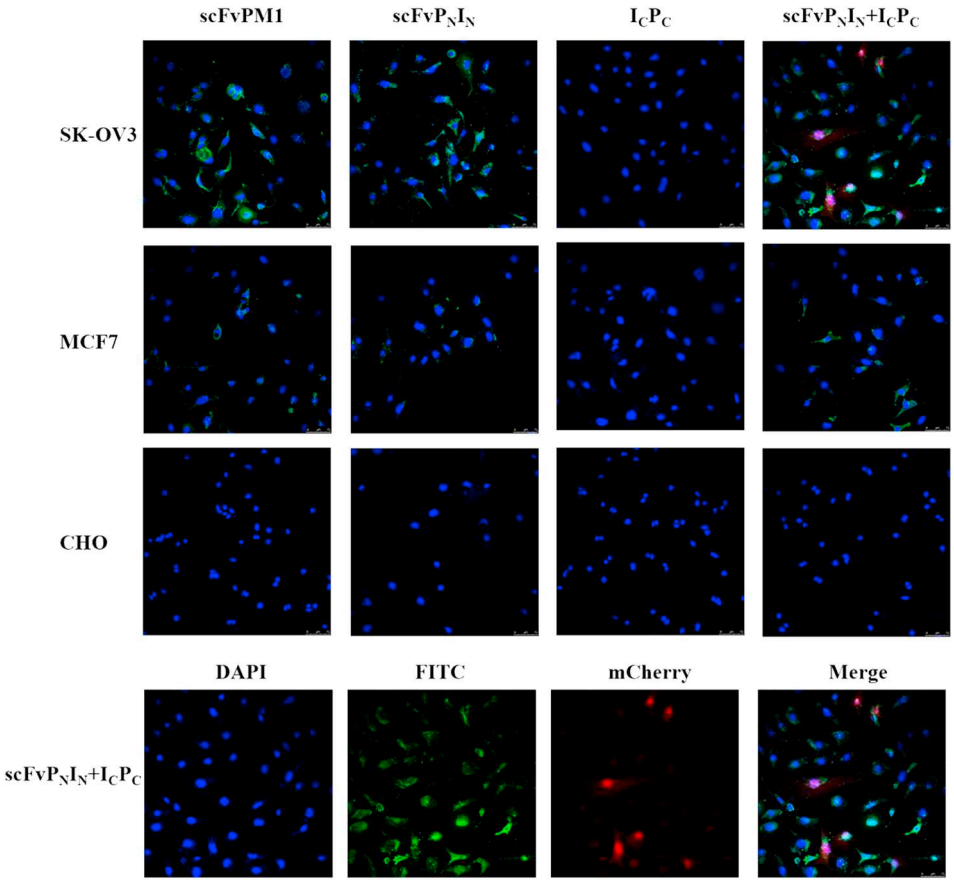

Fig. 5. Internalization analysis of HER2/neu -positive and -negative cells after 4 h treatment of immunotoxins. Cells were subjected to immunofluorescent staining with anti-ETA antibody and FITC conjugated secondary antibody, with DAPI nuclear counterstaining. The images were taken using 20 objective.

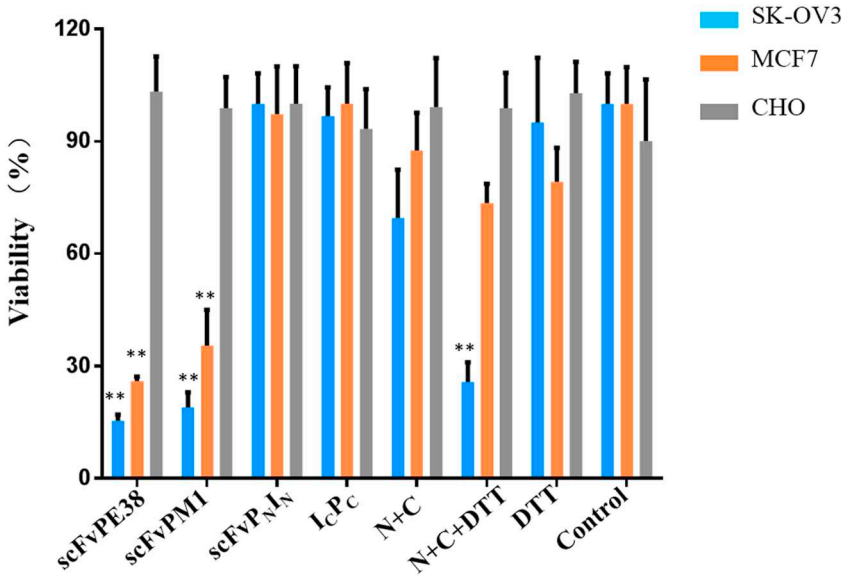

Fig. 6. Cell viability assay to determine the specific cytotoxicity of the original (50 nM) and the split immunotoxins (2.5 μM for N and C), SK-OV3, MCF7 and CHO cells were separately incubated for 72 h at 37 °C and viability was determined using an CCK8 kit ($n = 5$, *:$0.01 < p < 0.05$, **: $p < 0.01$).

cells differently, possibly due to the number of HER2/neu on the cell surfaces (Fig. 4).

3.5. Internalization of the original and reconstituted immunotoxin

A laser-scanning confocal microscopy was employed to analyze the internalization of the immunotoxins by SK-OV3, MCF7 and CHO cells. Cellular uptake of either $scFvP_NI_N$ or scFvPM1 could be reflected by the green fluorescence coming from the FITC labeled secondary antibody. To detect the spliced products of $scFvP_NI_N$ and I_CP_C, a mCherry tag was added to the C terminal of I_CP_C (I_CP_C-mC) to exhibit red color. Reconstituted scFvPM1 could be observed with both green and red

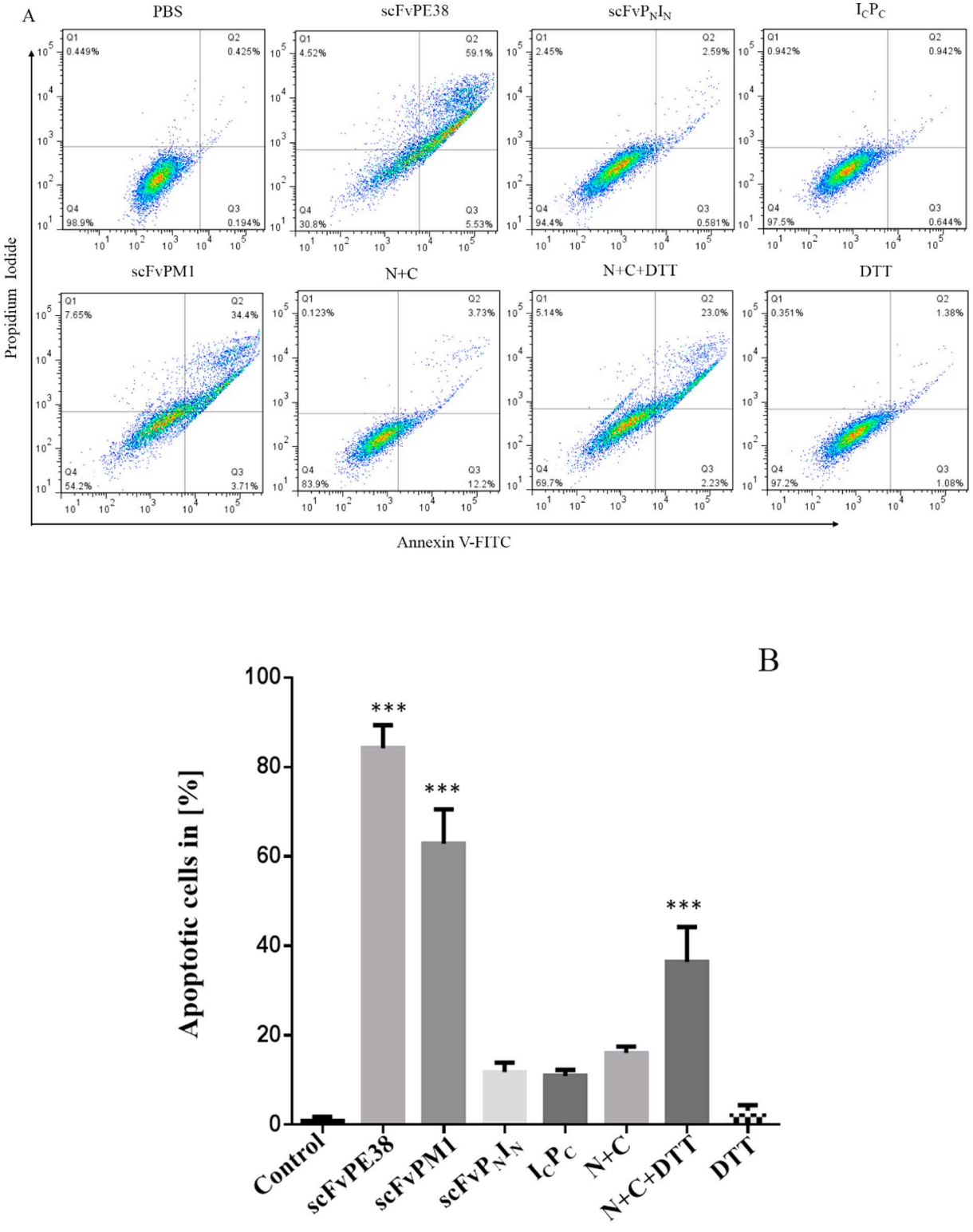

Fig. 7. Induction of apoptosis by the original (50 nM) and the split immunotoxins (2.5 μM for N and C). A. SK-OV3 were incubated with the immunotoxins for 48 h before analyzed with flow cytometry. B. the sum of early-apoptotic and late apoptotic cells of three independent experiments. Error bars represent the standard error of the means. Statistical analysis was carried out using a two-tailed unpaired Student's *t*-test (***$p < 0.01$).

fluorescence. After treated with immunotoxins for 4 h, both SK-OV3 and MCF7 showed internalization of scFvPM1 and scFvP$_N$I$_N$, while the green fluorescence on SK-OV3 was a little stronger than on MCF7. The co-localization of green and red fluorescence in SK-OV3 cells indicated the internalization of the reconstituted scFvPM1 (Fig. 5). In contrast, when incubated with HER2/neu negative CHO cells for 4 h, only the blue fluorescence of DAPI was detected indicating no internalization occurred on CHO cells (Fig. 5). Besides, when I$_C$P$_C$-mC was applied alone, no obvious intracellular red fluorescence could be seen on the three cell lines (Fig. 5). The results suggested that the internalization was receptor mediated endocytosis.

3.6. Effect of the immunotoxin on tumor cell lines

After confirming that the immunotoxins bound specifically to HER2/neu + cells and could be internalized, the next step was to determine their cytotoxic potential toward the HER2/neu ± tumor cell lines. scFvPE38, scfvPM1 and the reconstituted immunotoxin showed obvious toxicity toward SK-OV3 and MCF7 cells, while the mixture of $scFvP_NI_N$ and I_CP_C had no toxicity (Fig. 6). When DTT was simultaneously applied to the mixture of $scFvP_NI_N$ and I_CP_C, the cytotoxicity could be seen on both SK-OV3 and MCF7 cells which meant the reconstituted immunotoxins were produced by protein trans-splicing reaction. There was no significant difference between the blank control cells and the DTT-only treated cells (Fig. 6). Therefore, toxicity caused by DTT to the cells was negligible. Neither $scFvP_NI_N$ nor I_CP_C alone showed cytotoxicity to the cells even at high concentration (2.5 μM) indicating the non-toxic property of the split fragments. Comparing with SK-OV3 and MCF7 cells, viability of CHO cells was not influenced by any of the treatment (Fig. 6).

3.7. Apoptosis induced by the reconstituted immunotoxin in HER2/neu positive cells

PE38 inhibits protein synthesis in eukaryotic cells by catalyzing the ADP-ribosyl moiety transfer from oxidized NAD to elongation factor 2 (EF-2), which causes cell apoptosis [28]. To confirm the apoptosis induced by the reconstituted scFvPM1, we carried out an AnnexinV-PI assay described in “Materials and Methods” on the HER2/neu positive SK-OV3 cell line. The original scFvPE38, as a positive control, induced about 84% cell apoptosis when compared with untreated cells (Fig. 7B). After intein mediated protein splicing, the reconstituted scFvPM1 induced about 54% cell apoptosis (Fig. 7B). There was no apoptosis detected with the individual alone or the mixture of $scFvP_NI_N$ and I_CP_C (Fig. 7A), which was validated by the results from the cell viability assay (Fig. 6).

4. Discussion

Immunotoxins play an important role in cancer immunotherapy due to its high efficacy and low dosage [29]. However, the non-specific toxicity limits their further clinical application [30]. Strategies to reduce the non-specific toxicity of immunotoxin are highly desirable. Recombinant immunotoxin scFvPE38 reduces the non-specific toxicity of PE38 through specifically target HER2/neu positive cells. Nevertheless, normal tissues with some level of HER2/neu were still suffered from the undesired toxicity of scFvPE38 [31]. When an immunotoxin is divided to two nontoxic segments that can be reconnected to restore toxic activity on tumor cell surface based on the intein mediated trans-splicing reaction, the non-specific toxicity of an immunotoxin could potentially be greatly reduced. This new approach can be applied to reduce non-specific cytotoxicity when the target antigen expressed widely on in multiple organs and tissues.

It was the first time to use a split intein to reconstitute an immunotoxin on the target cell surface through trans-splicing reaction. Successful reconstitution of an immunotoxin may allow delivery of the two split parts separately in practice to eliminate toxicity till they meet on cell surface. When the targeting part of the immunotoxin is first attached to the cancer cell surface, a high concentration of the second part could be dosed. Both parts could be nontoxic during circulation until the complete immunotoxin was reconstituted on the target cell. The tumor microenvironment is highly reducing and hypoxic, which would facilitate the splicing reaction mediated by intein to reconstitute the two parts of immunotoxin [32,33].

The toxin peptides used in immunotoxin are highly toxic [2]. Most of the immunotoxins inhibited cell growth in the low picomolar range [31,34,35]. The high cytotoxicity of immunotoxin is double-edged and has brought severe side effects toward normal tissues even at low concentrations. In this study, the internalization analysis of the immunotoxins was conducted on three different tumor cell lines separately as additional fluorescence labeling would be needed to distinguish them [22,26] if HER2/Neu-negative cells co-cultured with HER2/Neu-positive cancer cells. The results of the confocal observation showed that the fluorescence signal of $scFvP_NI_N$ and I_CP_C was only visible in HER2/neu-positive cells but not in HER2/neu-negative cells. The fragment I_CP_C alone was't internalized into the tumor cells. After the trans-splicing reaction, the produced scFvPM1 could be taken up by the target cells through receptor mediated endocytosis. The split immunotoxin showed eliminated toxicity to the antigen positive and negative cells. Remarkably, the reconstituted immunotoxin was cytotoxic to the antigen positive cells, despite a reduced effect was observed, possibly due to the amino acid mutations in PE38.

The dramatic charge segregation was observed between N- and C-inteins in the DnaE intein family [36,37]. The C-intein engaged the N-intein through electrostatic interactions to form a compact binding intermediate. This intermediate then performed the canonical splicing mechanism to yield the native trans-splicing complex [38].The trans-splicing efficiency is affected by the extein conformation and the reaction condition. The reaction temperature and pH should be close to the physiological condition to facilitate *in vivo* application. Soluble expression of the split fragments might improve the splicing reaction efficiency.

In conclusion, we describe here a new approach to utilize immunotoxin for killing cancer cells by reconstituting an immunotoxin through intein mediated trans-splicing. Non-specific toxicity was greatly reduced because the split partners have no toxic activity until the trans-splicing reaction occurs at specific cell surface. There is no toxicity observed when the two parts of the split immunotoxin delivered separately even at very high concentrations. Considering the above, the intein mediated trans-splicing approach to reduce the non-specific toxicity of immunotoxin is promising for future clinical applications. Further animal and human trials would be necessary to demonstrate this unique innovative approach.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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