

A novel recombinant immuno-tBid with a furin site effectively suppresses the growth of HER2-positive osteosarcoma cells *in vitro*

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Abstract. Immunotherapy is a promising strategy for the treatment of human epidermal growth factor receptor 2 (HER2)-positive tumors. Previously, we constructed an immuno-carboxy terminal fragment of Bid (immuno-tBid) and reported its specific and effective destruction of HER2-positive tumor cells. In this study, in order to further reduce the immunogenicity of the previous immuno-proapoptotic protein, we constructed a novel immuno-tBid by replacing domain II of *Pseudomonas* exotoxin A with a short furin cleavage sequence from the diphtheria toxin. In order to explore the possible application of this novel immuno-tBid in the treatment of osteosarcoma, we first examined the expression of the HER2 protein in a subclone of a human osteosarcoma cell line with relatively high metastatic potential (SOSP-9607-E10), as well as in clinical specimens of osteosarcoma. Quantitative real-time PCR and Western blot analysis revealed that the expression of HER2 was up-regulated in the SOSP-9607-E10 cells, while immunohistochemical analysis revealed that HER2 was overexpressed in

37% of the tissue specimens examined. Both HER2-positive SOSP-9607-E10 and SKBR-3 cells, as well as HER2-negative HeLa cells were transiently transfected with the novel immuno-tBid in order to study its specific pro-apoptotic effect. We demonstrate here that this novel immuno-tBid induces the specific destruction of HER2-overexpressing SOSP-9607-E10 cells through the release of cytochrome C. These results suggest that the novel immuno-tBid with a minimized exogenous fragment could represent a competitive approach for the treatment of HER2-positive osteosarcoma.

Introduction

Immunotoxins, which are hybrid proteins composed of plant or bacterial toxins linked with a targeting molecule, such as a monoclonal antibody (MAb), an antibody fragment, a growth factor, or a cytokine (1-4), have been proven to be effective in destroying tumor cells specifically. Similar to certain other well-demonstrated anti-tumor immunotoxins (5-7), the *Pseudomonas* exotoxin A (PEA)-based human epidermal growth factor receptor 2 (HER2)-targeted immunotoxin, e23sFv-PEA40, has shown potent and selective cytotoxicity to HER2-positive tumors both *in vitro* and *in vivo* (8). However, most of the clinical trials of the immunotoxin encountered the same obstacle, in that the foreign toxin fragments in immunotoxins induced strong humoral immune responses (9-14). In addition to the development of neutralizing antibodies against the plant or bacteria-derived toxins (9,10) which profoundly diminished the anti-tumor efficiency of the therapy, other side-effects including vascular leakage, and non-specific toxicity to vascular endothelial, liver, or renal cells (7,10-14) were also induced by immunotoxins. Therefore, studies are now focusing on immunotoxins containing human-derived effector proteins, such as human pro-apoptotic proteins, in order to minimize non-specific toxicity and immunogenicity (15).

Previously, we reported the potent and specific suppressing effect of the partially humanized fusion protein, immuno-carboxy terminal fragment of Bid (immuno-tBID), composed

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Abbreviations: HER-2, human epidermal growth factor receptor-2; PEA, *Pseudomonas* exotoxin A; FBS, fetal bovine serum; EGFR, epidermal growth factor receptor; PBS, phosphate-buffered saline; AIF, apoptosis inducing factor; MAb, monoclonal antibody

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of a HER2-specific single-chain antibody, the PEII domain and tBid, on HER2-overexpressing human breast cancer cells (SK-BR-3) and human ovarian cancer cells (SK-OV-3) (16,17). In order to further reduce the exogenous fragment and thus minimize immunogenicity, we constructed a novel class of immuno-proapoptotic proteins by replacing the PEII domain of previous immuno-proapoptotic proteins by a short furin cleavage sequence from the diphtheria toxin ($A_{187}GNRVRRSVG_{196}$, Fdt), and confirmed their efficiency in inducing cell death *in vitro* and reducing tumor size *in vivo* (18). Even though these novel immuno-proapoptotic proteins have been proven to be effective in inducing apoptosis in the HER2-overexpressing human breast tumor cell line, SKBR-3, and the human gastric cancer cell lines, SGC-7901 and AGS, their effect on human osteosarcoma has not yet been investigated. Thus the purpose of the present study, was to modify our previous immuno-tBid by introducing the short furin cleavage sequence, Fdt, and to study the possible proapoptotic effect of this novel immuno-tBid on HER2-overexpressing SOSP-9607-E10 cells *in vitro*.

Materials and methods

Construction of expression plasmids. The gene encoding truncated Bid and the single-chain HER2 antibody (e23sFv), were generated in our previous study (16). The furin site sequence from the diphtheria toxin ($A_{187}GNRVRRSVG_{196}$, Fdt) was incorporated into tBid by PCR. The novel *immuno-tBid* gene was generated by the sequential fusion of the genes of a signal peptide (MKHLWFFLLLVAAPRWVLS), e23sFv, the furin cleavage sequence, Fdt, and tBid (bid Δ 1-60). This novel *immuno-tBid* gene was cloned into the expression vector, pCMV. Vector sequence was confirmed by DNA sequencing.

Cell culture and transfection. The HER2-positive human breast tumor cell line, SKBR-3, the HER2-negative human cervical carcinoma cell line, HeLa, and the subclone of a human osteosarcoma cell line with relatively high metastatic potential, SOSP-9607-E10, were maintained in DMEM or RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 4 mmol/l L-glutamine. At 24 h before transfection, cells were seeded in 12-well plates at a density of 1×10^5 cells per well. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the standard procedure. For transient expression, cells were harvested 72 h after transfection. For stable transfection, cells were selected in the medium containing 800 μ g/ml G418 (Invitrogen) for 2 to 3 weeks.

Immunohistochemical analysis. Paraffin-embedded, formalin-fixed resection specimens from patients were used for the immunohistochemical analyses. The study included 48 specimens from patients with osteosarcoma who presented to the Department of Orthopaedic Surgery in Tangdu Hospital from 2003 to 2004. The inclusion criteria were: Primary osteosarcoma without lung metastasis at the time of diagnosis, pre-operative chemotherapy with paclitaxel, with a post-operative follow-up of 24 months. For immunohistochemical staining, paraffin-embedded tissue sections were dewaxed,

hydrated, and incubated in 0.3% methanol- H_2O_2 for 20 min to remove endogenous peroxidase. After the sections were washed with phosphate-buffered saline (PBS) for three times and blocked with normal goat serum, they were incubated overnight at 4°C with mouse anti-HER2 polyclonal antibody (1:200, M0196, Changdao Corp., China). The sections were then incubated with goat anti-mouse IgG as the secondary antibody for 30 min and then processed with 3,3'-diaminobenzidine staining.

Quantitative real-time PCR (RT-PCR) for HER2 mRNA in SKBR-3, HeLa and SOSP-9607-E10 cells. Transcriptional levels of HER2 in SKBR-3, SOSP-9607-E10 and HeLa cells, were measured by quantitative RT-PCR using the SYBR Premix Ex Taq kit (Takara Bio, Madison, WI, USA). Total RNA was extracted from cultured cells by using TRIzol® Reagent (Invitrogen). cDNA was synthesized using 2 μ g of total RNA, oligo(dT)₁₈ primer and Superscript RT (from Invitrogen). Quantitative RT-PCR was performed using 1 μ l of a 1:5 dilution of first-strand cDNA and primers specific to HER2 and β -actin on a Prism 7500 cyler (Applied Biosystems). HER2 was amplified using the forward primer, 5'-TCA CCT ACA ACA CAG ACA CGT TTG-3', and the reverse primer, 5'-ATC CCA CGT CCG TAG AAG GTA-3'. PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide and visualized under UV light. All the experiments were repeated for at least three times and similar results were obtained.

Western blot analysis. Western blot analysis was performed to study the expression of the HER2 protein in SKBR-3, SOSP-9607-E10 and HeLa cells. Cells were washed with ice-cold PBS, scraped into ice-cold PBS, and thereafter lysed. The protein content was measured by the Bradford assay using bovine serum albumin as the standard. Protein from cell lysates was separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham Biosciences). The blotted membranes were then incubated with primary antibody that recognizes HER2 (1:500; NeoMarkers) overnight at 4°C in PBS Tween-20. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1:2000; Zhongshan) for 2 h at room temperature. Western blots were visualized using an enhanced chemiluminescence kit (Pierce).

Cell viability assay. Cell viability of the transiently transfected cells was tested by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells transfected with *e23sFv-Fdt-tBid* were cultured in 96-well plates for 24 to 96 h, and then incubated with 20 μ l of 1.5 mg/ml MTT for 4 h. After that, the cells were treated with 150 μ l DMSO. Values at A_{490} nm were determined using the Sunrise microplate reader (Tecan). Each assay was performed in triplicate on at least three independent occasions.

Cell-killing assay. The cell-killing ratio of the transiently transfected cells was determined by cell counting after trypan blue staining. After the cells were detached and re-suspended in DMEM containing 10% FBS, they were stained with trypan blue. Unstained cells were counted on a blood cell

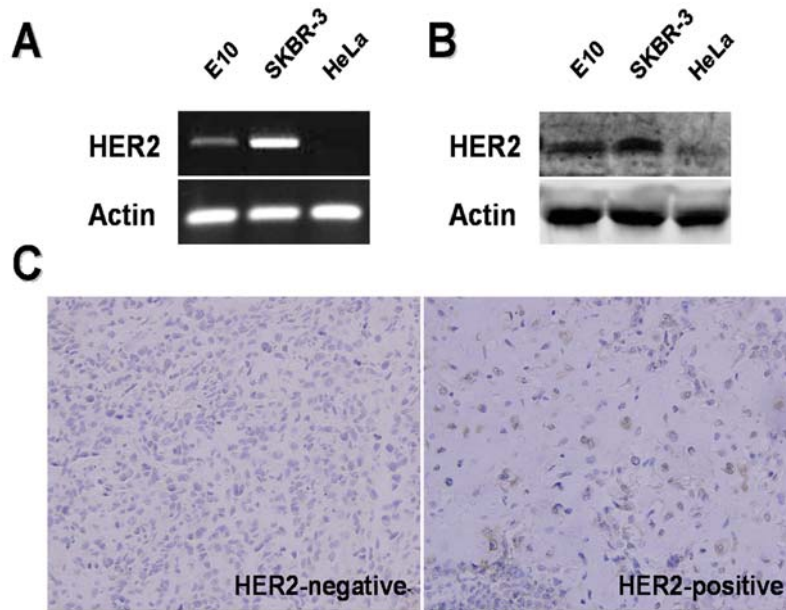


Figure 1. The expression of HER2 in the SOSP-9607-E10 cell line and clinical osteosarcoma specimens. (A) Transcriptional levels of HER2 in SKBR-3, SOSP-9607-E10 and HeLa cells. Overexpression of HER2 was detected in SOSP-9607-E10 cells by RT-PCR. (B) The expression of HER2 protein in SKBR-3, SOSP-9607-E10 and HeLa cells. As can be seen from the results of the Western blot analysis, HER2 was overexpressed in SOSP-9607-E10 cells. (C) Immunohistochemical analysis of the expression of HER2 in clinical osteosarcoma specimens (x400). The negative specimen showed no HER2-positive cells, while the positive specimen revealed numerous HER2-positive osteosarcoma cells.

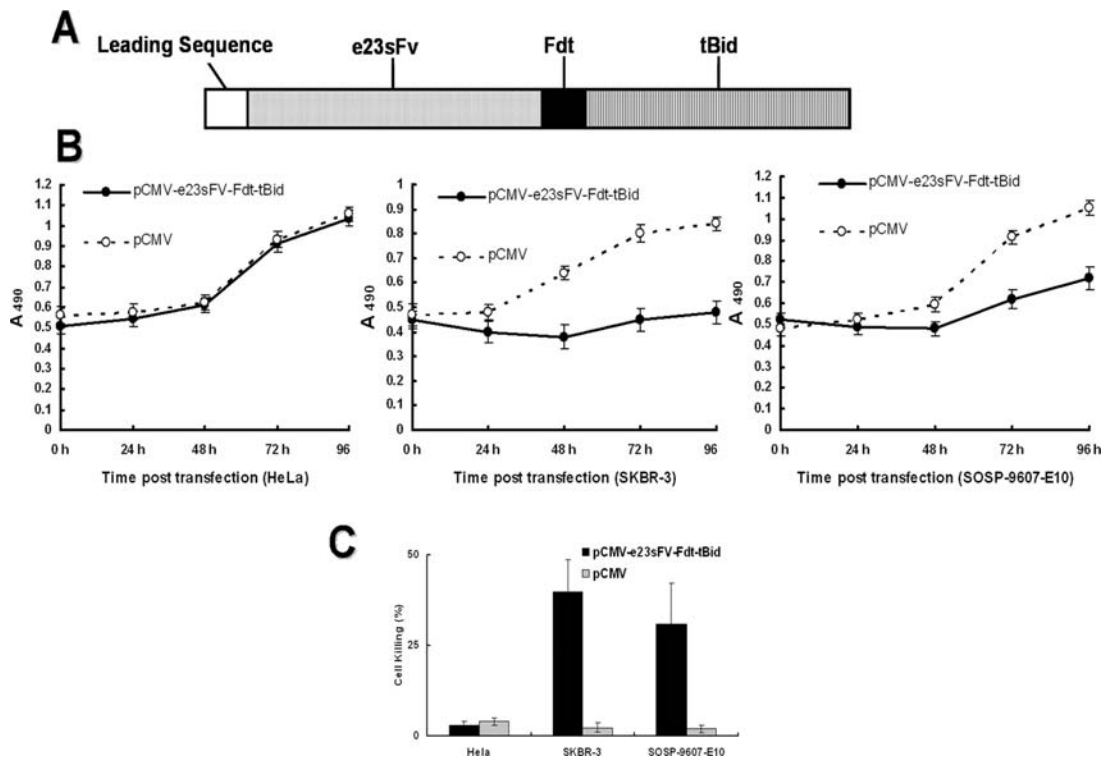


Figure 2. The novel immuno-tBid induced cell death in transiently transfected SOSP-9607-E10 cells. (A) The novel recombinant gene consists of a signal peptide, the HER2 antibody, e23sFv, the furin cleavage sequence, Fdt, and tBID (bidΔ1-60). (B) MTT assay of SKBR-3, SOSP-9607-E10 and HeLa cells transfected with the novel immuno-tBid protein expression constructs. (C) SKBR-3, SOSP-9607-E10 and HeLa cells were transiently transfected with the novel immuno-tBid protein expression constructs and the suppression percentages were calculated.

counting plate, and the cell-killing ratio was determined as follows: $(N_c - N_t)/N_c \times 100\%$. In this formula N_t and N_c represent the number of cells in the treatment and control groups, respectively.

Flow cytometry assay and TUNEL staining for detection of apoptosis. SOSP-9607-E10 cells were seeded at a density of 1×10^5 cells per well on slides in 12-well Costar transwell plates. HeLa cells stably transfected with *e23sFv-Fdt-tBid* or

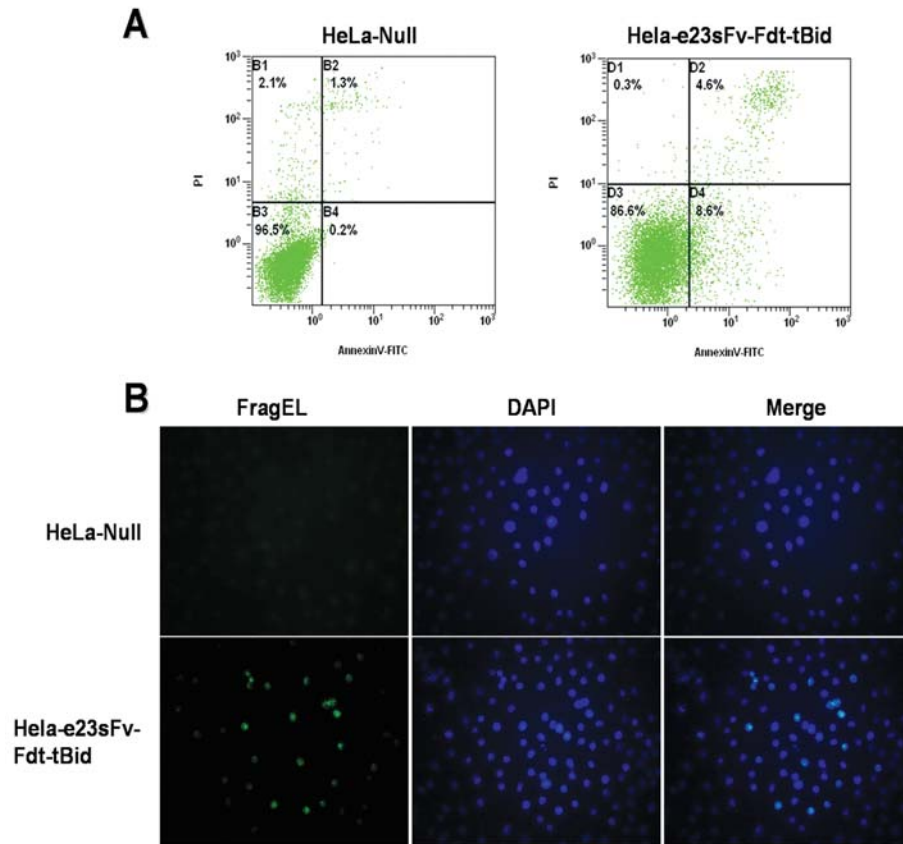


Figure 3. SOS-9607-E10 cells were co-cultured with immuno-tBid protein-expressing HeLa cells, and apoptosis was observed by flow cytometry (A) and TUNEL staining (B).

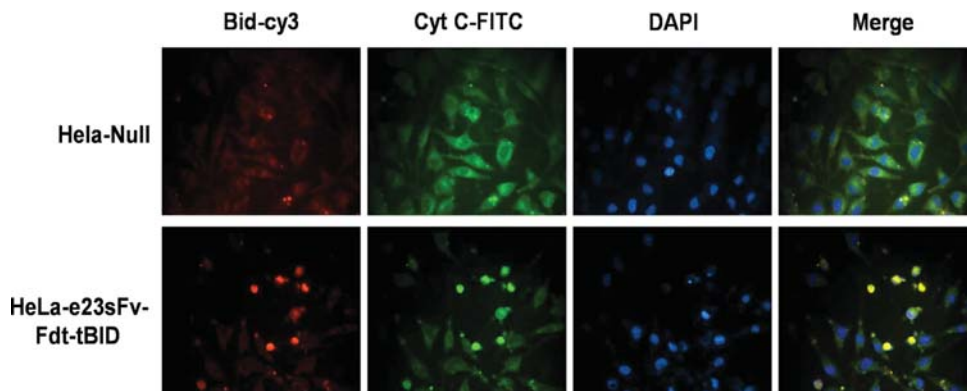


Figure 4. Secreted chimeric protein induced apoptosis through Bid and cytochrome C. SOS-9607-E10 cells co-cultured with immuno-tBid protein-expressing HeLa cells exhibited intensive cytoplasmic fluorescence of both Bid and cytochrome C, suggesting that apoptosis was induced by the expression of Bid and, subsequently, the secretion of cytochrome C.

control cells transfected with pCMV, were placed in the top chamber at a density of 3×10^5 cells per well. Cells were co-cultured at E/T ratios of 3:1 for 8 h. The apoptosis of the cells in the lower chamber was then detected by flow cytometry assay and TUNEL staining. As for flow cytometry assay, cells in the lower chamber were collected, stained with Annexin V-FITC/PI following standard procedures, and finally analyzed. As for TUNEL staining, cells were fixed, stained using the TdT-FragEL™ DNA Fragmentation

Detection kit (Calbiochem) and observed thereafter using a fluorescence microscope.

Immunofluorescence. Immunofluorescence assay was performed in order to study whether the apoptosis of the HER2-positive tumor cells was induced by tBid and subsequent release of cytochrome C from mitochondria. SOS-9607-E10 cells were co-cultured with HeLa-e23sFv-Fdt-tBID or HeLa-Null cells as mentioned above. After that,

SOSP-9607-E10 cells in the lower chamber were fixed in 4% paraformaldehyde, permeabilized with PBS containing 0.1% Triton X-100, and blocked with 2% normal rabbit serum. Then the cells were stained with antibodies recognizing Bid (C20, 1:200; Santa Cruz Biotechnologies) and cytochrome C (H-104, 1:200; Santa Cruz Biotechnologies) as the primary antibodies, with biotin-linked anti-goat IgG (1:100; Santa Cruz Biotechnologies) and FITC-linked anti-rabbit IgG (1:100; Sigma) as the secondary antibodies and with Cy3-linked anti-biotin antibodies (1:100; Sigma) as the tertiary antibody. Nucleus staining was performed with 4,6-diamidino-2-phenylindole.

Statistical analysis. Statistical analysis was performed with the SPSS 12.0 software package for Windows (SPSS). Statistical significance was defined as a value of $p \leq 0.05$.

Results

Expression of HER2 in SOSP-9607-E10 cell line and clinical specimens. The HER2-positive human breast tumor cells, SKBR-3, the HER2-negative human cervical carcinoma cells, HeLa, and the subclone of a human osteosarcoma cell line with relatively high metastatic potential, SOSP-9607-E10, were subjected to RT-PCR and Western blot analysis in order to determine the expression of HER2 on both the mRNA and protein levels. As can be seen from the results of RT-PCR (Fig. 1A) and Western blot analysis (Fig. 1B), the SKBR-3 cells, in accordance with other studies, exhibited a significantly intensified expression of HER2. Though the intensity of HER2 expression in the SOSP-9607-E10 cells was not as strong as that of the SKBR-3 cells, it was, indeed, markedly stronger than that of the HeLa cells.

As for the immunohistochemical staining of the clinical specimens, 18 out of the 48 specimens (37%) exhibited moderate or strong immunostaining of HER2. Fig. 1C shows representative images of a positively- and negatively-stained specimen, respectively.

The novel immuno-tBid can induce cell death in SOSP-9607-E10 cells. As can be seen from Fig. 2A, the novel recombinant gene consisted of a signal peptide, the HER2 antibody, e23sFv, the furin cleavage sequence, Fdt, and tBid (bid Δ 1-60). In order to test the possible suppressing effect of this recombinant molecule on HER2-overexpressing cells, the viability of the cells transiently transfected with the recombinant molecule, was studied by MTT assay and trypan blue staining. Apparent cell death was observed in the HER2-positive SKBR-3 and SOSP-9607-E10 cells, but not in the HER2-negative HeLa cells (Fig. 2B and C).

Secreted e23sFv-Fdt-tBid protein can induce apoptosis in SOSP-9607-E10 cells. In order to investigate the pro-apoptotic effect of the secreted recombinant protein, HeLa cells stably transfected with the recombinant gene were co-cultured with SOSP-9607-E10 cells. As can be seen from the flow cytometry results (Fig. 3A), a large percentage (16.1%) of SOSP-9607-E10 cells underwent apoptosis after they were co-cultured with HeLa cells expressing the novel immuno-tBid. This pro-apoptotic effect was also confirmed by TUNEL

staining (Fig. 3B). A significant number of SOSP-9607-E10 cells in the HeLa-e23sFv-Fdt-tBid group exhibited green fluorescence, indicating that they are apoptotic. The above results suggest that the novel immuno-tBid secreted by transfected HeLa cells is very potent in inducing apoptosis in HER2-positive osteosarcoma cells.

Secreted fusion protein induced apoptosis through Bid and cytochrome C. Immunofluorescence staining was performed to further prove that the pro-apoptotic effect of the fusion protein was induced by the translocation of Bid to the cytoplasm and the consequent cytochrome C release from mitochondria. As shown in Fig. 4, SOSP-9607-E10 cells co-cultured with HeLa cells stably secreting the recombinant protein, exhibited intensive cytoplasmic fluorescence of both Bid and cytochrome C. These results suggest that, after the internalization of the recombinant protein, tBid translocates into the cytoplasm, thus inducing cytochrome C release and finally causing apoptosis in HER2-positive osteosarcoma cells.

Discussion

In the present study, we constructed the novel immuno-proapoptotic protein, e23sFv-Fdt-Bid Δ 1-60, and confirmed its efficient pro-apoptotic effect on HER2-overexpressing osteosarcoma cells.

In the past decade, we carried out a series of studies on antibody-directed and cell-mediated cancer immunotherapy by combining the specificity of antibodies and the potent cytotoxicity of pro-apoptotic proteins. Up to now, a number of pro-apoptotic effectors, including caspase-3 (19), caspase-6 (20), granzyme B (21), tBid (16,17) and apoptosis inducing factor (AIF) (22), have been used to construct our immuno-proapoptotic proteins and have been confirmed to be efficient in inducing targeted apoptosis both *in vitro* and *in vivo*. Among all these recombinant proteins, immuno-tBid has been proven to be a bright prospect for future therapeutic application, as Bid is an intracellular link connecting the receptor- and mitochondrial-mediated apoptotic pathways (23,24) and thus guarantees efficiency when either pathway is unavailable in tumor cells. However, one obstacle that hinders the clinical application of immuno-tBid is its immunogenicity. In order to reduce the immunogenicity of immuno-tBid, we further refined it by replacing the PEA translocation domain with a smaller exogenous fragment, a furin sensitive sequence derived from the diphtheria toxin (A₁₈₇G_{NRVRRS}V_G₁₉₆, Fdt). As this new immuno-proapoptotic protein consists mainly of humanized antibodies and human apoptotic effectors, it possesses putatively minor immunogenicity. With reduced possibility of inducing immune response and systemic toxicity, this novel immuno-tBid promises improved therapeutic utility for the long-term treatment of tumors that overexpress HER2.

The transmembrane glycoprotein, HER2, classified as a member of the epidermal growth factor receptor (EGFR) family, has been reported to be overexpressed in many adenocarcinomas, including breast, ovarian, lung and stomach tumors (25-28), and is thus recognized as an ideal molecular target for cancer gene therapy. Although HER2 is generally

recognized as an indicator of tumor migration and poor prognosis, its role in osteosarcoma is controversial (29-37). In this study, we investigated the expression of HER2 in SOSP-9607-E10 cells, a previously established subculture of osteosarcoma cells with relatively high metastatic potential (38). In accordance with our previous flow cytometry results (17), RT-PCR and Western blot analysis in this study demonstrated an increased HER2 expression in SOSP-9607-E10 cells. Further immunohistochemical analysis of the HER2 expression in clinical specimens also revealed overexpression in 37% of all the examined specimens. Taking all these results together, we hypothesized that HER2 could be an effective target for the immunotherapy of osteosarcoma, especially the type with high metastatic potential.

In order to verify the HER2-targeted suppressing effect of the novel immuno-tBid, we transfected the recombinant gene into SOSP-9607-E10 cells, HER2-positive SKBR-3 cells, as well as into HER2-negative HeLa cells, and compared the vitality of these cells after transient transfection. The effective destruction of SOSP-9607-E10 cells by the novel immuno-tBid was confirmed by both MTT assay and trypan blue staining. Significant cell death was observed in both SOSP-9607-E10 and SKBR-3 cells, but not in HeLa cells, at 48 h after transfection. The percentage of cell destruction was 39.7 and 31% for the SKBR-3 and SOSP-9607-E10 cells, respectively. These results confirm our hypothesis that the recombinant gene encoding the novel immuno-tBid can induce the targeted suppression of HER2-positive SOSP-9607-E10 cells.

As for future therapeutic application, our aim is to transfect the novel immuno-tBid gene into lymphocytes. These modified lymphocytes would secrete the immunotoxin after they 'home' to the tumor site. It is believed that the secreted novel immuno-tBid would bind to HER2-overexpressing osteosarcoma cells, and subsequently induce apoptosis in these tumor cells. In this preliminary study, we transfected the recombinant gene into HeLa cells and investigated the pro-apoptotic effect of the secreted immuno-toxin on SOSP-9607-E10 cells. As expected, secreted immunotoxin was detected in culture medium of transfected HeLa cells by Western blot analysis (data not shown). Moreover, TUNEL staining and flow cytometry revealed that a significant number of the SOSP-9607-E10 cells co-cultured with modified HeLa cells underwent a typical process of apoptosis. With the understanding that tBid exerts its pro-apoptotic effect by inducing the release of pro-apoptotic factors, such as cytochrome C, AIF, and procaspase 9, from mitochondria (22,23), we performed the immunofluorescence assay in order to further confirm the pro-apoptotic effect of the novel immuno-tBid on SOSP-9607-E10 cells. Again, the potent pro-apoptotic effect of the novel immuno-tBid was proven by the fluorescence of both Bid and its downstream pro-apoptotic factor, cytochrome C, in the cytoplasm.

Thus, it can be concluded that, the novel immuno-tBid with the minimized exogenous fragment can effectively and selectively destroy HER2-positive osteosarcoma cells. This novel immunotoxin could be a promising candidate for the future development of long-term immunotherapies with maximal cell-suppressing ability and minimal non-specific damage to normal tissues.

Acknowledgements

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