Single-chain antibody/activated BID chimeric protein effectively suppresses HER2-positive tumor growth

Xiu-Chun Qiu,1,3 Yan-Ming Xu,1 Fang Wang,1 Qing-Yu Fan,3 Li-Feng Wang,1 Bao-An Ma,3 Lin-Tao Jia,1 Jing Zhao,1 Yan-Ling Meng,2 Li-Bo Yao,1 Si-Yi Chen,4 and An-Gang Yang2

1State Key Laboratory of Cancer Biology, Department of Biochemistry and Molecular Biology, and 2Department of Immunology, Fourth Military Medical University; 3Department of Orthopaedics, Tangdu Hospital, Xi’an, People’s Republic of China and 4Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas

Abstract

BH3-interacting domain death agonist (BID) is a crucial element in death signaling pathways and is recognized as an intracellular link connecting the intrinsic mitochondrial apoptotic and extrinsic death receptor–mediated apoptotic pathways. Herein, we describe experiments conducted with a fusion protein, which was generated by fusing a human epidermal growth factor receptor-2 (HER2)–specific single-chain antibody with domain II of Pseudomonas exotoxin A and the truncated active BID (tBID). These experiments extend our previous work on several other immuno-proapoptotic proteins. Specifically, by excluding cells with undetectable HER2, we showed that the secreted immuno-tBID molecule selectively recognized and killed HER2-overexpressing tumor cells in vitro by attacking their mitochondria and inducing their apoptotic death. This apoptosis could only be inhibited partially by caspase pan-inhibitor zVAD and mitochondrial protease TAT-BH4. Subsequently, we transferred the immuno-tbid gene into BALB/c athymic mice bearing HER2-positive tumors together with other immuno-proapoptotic proteins using i.m. injections of liposome-encapsulated vectors. The expression of the immuno-tbid gene suppressed tumor growth and prolonged animal survival significantly. We also shortened the translocation domain of Pseudomonas exotoxin A II to only 10–amino acid sequence, which were crucial for furin cleavage. The new recombinant molecule retained the translocation efficiency and the ability of specific killing HER2-positive tumor cells. Our data showed that, compared with the toxins employed before, the chimeric immuno-tBID molecule can not only specifically recognize HER2-positive tumor cells but also certainly induce apoptosis even in the presence of zVAD and TAT-BH4, thereby suggesting an alternative approach to treating HER2/neu-positive tumors. [Mol Cancer Ther 2008;7(7):1890–9]

Introduction

Tumors are thought to arise through a series of mutations that alter the appropriate functions of oncoproteins. A large number of these oncoproteins are transmembrane molecules that participate in signal transduction. The human epidermal growth factor receptor-2 (HER2), a member of the epithelial growth factor receptor family, transduces cell signaling and plays key roles in cell differentiation, adhesion, and motility (1). It is expressed highly in many cancer types (2–6) but lowly in most normal adult tissues. From experimental and clinical viewpoints, HER2 is a key player in cell communication, whose overexpression may result in poor cancer prognoses, and was therefore chosen as a target for current clinical trials.

Two basic pathways, extrinsic and intrinsic, can direct a cell to undergo apoptosis. The extrinsic apoptotic pathway is mediated by the binding of apoptosis-inducing ligands, including the tumor necrosis factor, tumor necrosis factor–related apoptosis-inducing ligand, and the Fas ligand to their receptors (tumor necrosis factor receptor and Fas) on the cell surface. Alternatively, the intrinsic pathway is regulated by cytochrome c release (7) and is induced typically by stress, radiation, and chemotherapeutic drugs. Each of these pathways converges in the activation of cytosolic aspartate-specific proteases (caspases) that degrade cellular proteins and commit the cell to self-destruction.

BH3-interacting domain death agonist (BID) is a proapoptotic BH3 domain–only member of the Bcl-2 family thought to exert its death-inducing effect by stimulating the release of proapoptotic factors [cytochrome c, apoptosis-inducing factor (AIF), and procaspase-9] from the mitochondria. Full-length BID, normally present in the cytosol, is cleaved by activated caspase-8 into a 15.5-kDa COOH-terminal fragment (tBID), which translocates to the mitochondria and causes cytochrome c release (8, 9). Because BID is recognized as an intracellular link connecting the extrinsic and intrinsic apoptotic pathways, the application of BID to kill tumor cells may be an efficient strategy for treating malignant disease.
During the past decade, we were devoted to studying sensitive, efficient, and specific cancer gene therapies, especially with regard to cancers overexpressing HER2. Several immuno-proapoptotic proteins (immuno-caspase-3, immuno-caspase-6, and immuno-granzyme B) that comprise a HER2-specific single-chain antibody (ε23sFv; ref. 10), translocation domain of Pseudomonas exotoxin A (PEA) (ref. 11, 12), which can introduce COOH-terminal domain transferring from endosome to cytosol, and proapoptotic proteins were shown to have potent selective antitumor effects both in vitro and in vivo (13–17). However, the immuno-proapoptotic proteins could be inhibited or even blocked by some intracellular caspase inhibitors and/or mitochondrial protectors, and as a heterogeneous protein, PEA II employed could also induce the failed treatment.

To develop a more sensitive strategy for applying antitumor therapy, we generated a chimeric molecule, which replaced the domain III in immuno-proapoptotic proteins with tBid to generate immuno-tBid (ε23sFv-PEA II-BIDΔ1-60/75). The specific cell-killing efficiency of ε23sFv-PEA II-BIDΔ1-60/75 was conducted with and without the presence of caspase pan-inhibitor zVAD and mitochondrial protector TAT-BH4. To generate a recombinant therapeutic gene without the presence of any heterogeneous genes, we designed immuno-tbid molecules expressing a 10-peptide region (TRHRQPRGWE) derived from amino acids 273 to 282 of PEA and was cloned into pCMV plasmid. All constructs were verified by DNA sequencing.

**Materials and Methods**

**Construction of Expression Plasmids**

The RNA samples were extracted from Jurkat cells with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription-PCR was done to amplify the human bid cDNA. The truncated bid genes, bidΔ1-60 and bidΔ1-75, were obtained by PCR. Using PCR-based overlap extension gene splicing method as described previously (14–16), recombinant immuno-tbid was generated by sequential fusion of the genes into a signal peptide (MKHLWFLLLVLAAPRWVLS), a single-chain HER2 antibody (ε23sFv, comprising the linked heavy and light chain variable regions derived from a mouse monoclonal antibody (mAb) ε23 with high-affinity binding to the extracellular domain of HER2 protein), a PEA translocation domain (amino acids 253-358 or 253-364), and a truncated bid gene (bidΔ1-60 or bidΔ1-75). The immuno-tbid gene was cloned into a pCMV plasmid. We also cloned the genes of a partial PEA translocation domain (amino acids 280-358 and 280-364) and fused them to the 5’ end of a bid gene (tbidΔ1-60 or tbidΔ1-75). The resulting fusion genes were cloned into pcDNA3 and pIRE2-EGFP (BD Biosciences Clontech). Through PCR, immuno-tbid gene was also constructed with a 10-peptide (TRHRQPRGWE) translocation domain, which was derived from amino acids 273 to 282 of PEA, and was cloned into pCMV plasmid. All constructs were verified by DNA sequencing.

**Cell Cultures**

As reported previously, our human breast cancer (SK-BR-3) and human ovarian cancer (SK-OV-3) are both HER2/neu-overexpressing cells, whereas the human cervical carcinoma (HeLa), African green monkey kidney (COS-7), human umbilical vein endothelial (ECV-304), and human laryngeal cancer (Hep2) are all HER2/neu low-expressing cells (16, 17). The cells were cultured with RPMI 1640 or DMEM (Invitrogen) with 4 mmol/L l-glutamine supplemented with 10% fetal bovine serum and antibiotics. All cells were cultured at 37°C in 95% air-5% CO2.

**Immunoblotting**

The immuno-tbid transfected cells were cultured with serum-free medium for 3 days and the medium was collected and concentrated for 20 times and then subjected to immunoblotting, which was done following the manufacturer’s instructions (Amersham Pharmacia Biotech). Proteins with 15 μg were separated by 10% SDS-PAGE, blotted onto polyvinylidene difluoride membranes, and analyzed with a 1:1,000 dilution of a BID polyclonal goat antibody (C20; Santa Cruz Biotechnologies). Immunoreactive bands were developed with 3,3-diaminobenzidine staining.

**Immunofluorescence**

The cells were cultured on coverslips in DMEM containing 10% fetal bovine serum, fixed with a freshly prepared paraformaldehyde solution [4% in PBS (pH 7.4)] for 10 min at room temperature, and permeated with 0.1% Triton X-100 for 15 min. The cells were stained with antibodies recognizing BID (C20, 1:200; Santa Cruz Biotechnologies), AIF (H-300, 1:100; 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 Cy3-linked anti-biotin antibodies (1:100; Sigma) as the tertiary antibody. 4’,6-Diamidino-2-phenylindole was also used for nucleus staining.

**Immunohistologic Staining**

The muscle tissue near the injection spot and tumors were collected and fixed in formalin and were embedded in paraffin after treatment, and the 5 μm sections were cut onto 3-amino-propyltriethoxysilane-coated slides. The slides were dewaxed in xylene and washed in 95% (v/v) ethanol. For immunohistochemistry staining, the muscle tissues were incubated for 30 min in 0.3% hydrogen peroxide solution, washed in PBS, placed in citrate buffer, and microwaved at 900 W for 20 min. All the slides were then dried and blocked for 1 h with the appropriate serum in a humidified chamber. Primary antibody was added overnight at 4°C. BID was analyzed by using a 1:200 dilution of a polyclonal goat antibody, and AIF was analyzed by using a 1:100 dilution of rabbit antibody. The sections were incubated with secondary antibodies for 30 min and then processed with 3,3-diaminobenzidine staining.
Annexin V-FITC Staining

The transfected HeLa cells were collected, and Annexin V-FITC/PI staining was done according to the manufacturer’s instructions (BD PharMingen). Cells were then analyzed by flow cytometry.

APO2.7 Antibody Staining

The cells were washed twice with PBS containing 5% fetal bovine serum, and 1 × 10⁵ cells were suspended in 0.5 mL PBS with 5% fetal bovine serum and 0.02% NaN₃ and incubated with mAbs for 30 min at 4°C. For apoptosis detection, we used the mouse phycoerythrin-conjugated mAb APO2.7 (clone 2.7 A6A3; Beckman Coulter) for detecting 7A6 antigen expressed by cells undergoing apoptosis. Flow cytometric analysis was done on a flow cytometer equipped with a 15 mW argon-ion laser with excitation capabilities at 488 nm.

Measurements of Mitochondrial Membrane Potential

The mitochondrial membrane potential of intact cells was measured by flow cytometry with a fluorescent dye that accumulates in mitochondria as a function of membrane potential (3,3'-dihexyloxa-carbocyanine iodide) staining. HeLa cells after transfection were subjected to trypsinization and treated with 3,3'-dihexyloxa-carbocyanine iodide (Invitrogen) in PBS for 15 min at 37°C and subsequently analyzed by fluorescence-activated cell sorting.

Cell-Killing Assay

Cell-killing ratio was determined by cell counting after trypsin blue staining, which was used to tell the living cells from the dead ones. The assay was done as follows: (a) detaching the adherent cells with 0.05% (m/v) trypsin (1:250) at 37°C; (b) resuspending the cells in DMEM containing 10% fetal bovine serum in a certain volume; (c) staining the cells with trypan blue to the final concentration of 0.04%; (d) counting respectively the unstained cells in the four squares, each containing 16 smaller squares; (e) calculating the cell concentration to be [([N1 + N2 + N3 + N4]) / 4] × 10²/mL; and (f) the cell number is that the result of the cell concentration times the total volume, and the cell-killing ratio is that the result of (Nc - Nh) / Nc% in which Nt and Nc are the cell number of treatment and control groups, respectively.

Assessment of In vivo Antitumor Activity of Immuno-tBID

BALB/c athymic mice (6-8 weeks old) were inoculated s.c. with 2 × 10⁶ human breast cancer SK-BR-3 cells. The tumors were allowed to grow and establish until they reached a diameter of 5 to 7 mm (designated day 0). The mice were then divided randomly into groups for the various treatments. The mice bearing SK-BR-3 tumors were subjected to liposome-mediated immuno-tBid gene treatments. The mice were administered i.m. with 10 μg pCMV/immuno-tBid or 10 μg control vector, pCMV, both encapsulated by 20 μL liposomes. Each treatment used 10 mice and was done every 3 days, five times total.

Statistical Analysis

Statistical analyses were done with the SPSS10.0 software package for Windows (SPSS). The survival rates were analyzed using the Kaplan-Meier method, and comparisons among treatment groups were obtained with the log-rank test. Tumor volumes were analyzed with the analysis of covariance, with treatment group comparisons made by covariance testing after eliminating any initial group differences. Statistical significance was based on P < 0.05.

Results

Expression of tBID Contributes to Effective Cell Death

Most of the death agonists in the Bcl2 family, such as BID, contain a BH3 domain that is essential for binding to Bcl2 and capable of inducing apoptosis (18–21). Once BID is cleaved, the truncated portion containing the BH3 domain becomes lethal to cells. Two potential cleavage sites reside in the NH²-terminal portion of human BID, S²⁶⁷LQTG²⁶⁸ and R²⁷⁶EADS²⁹⁶, which match perfectly with the preferred cleavage sites for caspase-8 and granzyme B, respectively.

To investigate whether the same cytotoxic effect could be achieved in mammalian cells by the expressed truncated BID protein in the cytoplasm, we generated two truncated bid gene (bidΔ1-60 or bidΔ1-75; Fig. 1A) expression plasmids and transiently transfected them into COS-7 and HeLa cells. As shown in Fig. 1B, in contrast to the mock transfectants, the transient expression of the truncated BID (BIDΔ1-60 or BIDΔ1-75) gene led to an apparent delay in cell growth after transfection.

PEA (280-358) Fused tBID-Induced Apoptosis by Releasing Cytochrome c and the AIF

PEA is a single-chain toxin consisting of three major domains responsible for binding of the molecule to target cells, its translocation to the cytosol, and the induction of cell death, respectively (11). Indeed the domain II of PEA has been reported to efficiently transfer the cellular toxicity into the mitochondria (12). Because endosomal cleavage always occurs between Arg⁷⁷⁹ and Gly⁷⁸⁰ residues in the PEA translocation domain, it is necessary to explore whether fusing a portion of the PEA translocation domain (amino acids 280-358) would affect the proapoptotic activity of BIDΔ1-60 and BIDΔ1-75. PEA (280-358)-BIDΔ1-60 or PEA (280-358)-BIDΔ1-75 genes were constructed by adding the encoding sequence of NH²-terminal domain with PEA (280-358) to the BIDΔ1-60 or BIDΔ1-75 genes. The terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining, which is the most commonly used method for detecting DNA degradation in apoptotic cells, was done followed with single blind counting after 24 h (Fig. 1C). These results indicated that all constructs induced cellular apoptosis despite the use of the PEA (280-358) fusion. Moreover, the Annexin V-FITC staining done 12 h after the transfection also revealed that, compared with the control cells, apoptotic cells were present in 11.1%, 10.9%, 11.9%, and 9.2% of the cells transfected with BIDΔ1-60, BIDΔ1-75,
PEA (280-358)-BIDΔ1-60, or PEA (280-358)-BIDΔ1-75, respectively (Fig. 1D).

To further show the damage of mitochondria by PEA (280-358)-BIDΔ1-60 and PEA (280-358)-BIDΔ1-75, we studied the expression level of the APO2.7 antigen, which is detectable after mitochondrial pathway apoptosis induction. The APO2.7 antigen (also named 7A6), recognized by clone 2.7A6A3, is a 38-kDa protein localized on the mitochondrial membrane with an expression profile that is restricted to cells undergoing apoptosis. Specifically, APO2.7 expression induction appears as an early event during apoptosis, whereas the normal viable cells are negative or weakly positive for this gene. After transfection with the BIDΔ1-60, BIDΔ1-75, PEA (280-358)-BIDΔ1-60, or PEA (280-358)-BIDΔ1-75 genes, the APO2.7 antigen was presented on the mitochondrial surface of HeLa cell (Fig. 2A).

BID is believed to exert its proapoptotic effect by inducing the release of proapoptotic factors, such as cytochrome c and AIF, from the mitochondria. More recently, it has been shown that BID partially permeates the outer mitochondrial membrane and causes the release of several proteins from the intermembrane space. As assayed by staining with an apoptosis-dependent fluorescent dye (3,3′-dihexyloxycarbocyanine iodide), the mitochondrial membrane potential of the BIDΔ1-60, BIDΔ1-75, PEA (280-358)-BIDΔ1-60, or PEA (280-358)-BIDΔ1-75 gene cells declined, suggesting the possible leakage of internal molecules from mitochondria (Fig. 2B). To further determine cytochrome c and AIF release from the mitochondria, transfected HeLa cells were subjected to immunofluorescent analysis (Fig. 2C). Moreover, the leakage of nuclear materials, which was stained by 4′,6-diamidino-2-phenylindole into the cytoplasm, was observed in the cells expressing BIDΔ1-60, BIDΔ1-75, PEA (280-358)-BIDΔ1-60, or PEA (280-358)-BIDΔ1-75. However, the subsequent apoptosis was inhibited partially by a mitochondria protector TAT-BH4 but not by the caspase pan-inhibitor z-VAD (Fig. 2D), suggesting the activity of AIF-inducing caspase-independent apoptosis. In our study,
the expression of truncated BID caused irreversible cell death through apoptosis induction by AIF and cytochrome c release despite the fusion of PEA domain II fragment (amino acids 280-358) at its NH₂ terminus.

**Figure 2.** Cytochrome c and AIF leakage from bidΔ1-60/75- and PEA(280-358)-bidΔ1-60/75-transfected cells. **A,** HeLa cells were transiently transfected with pCDNA3, pCDNA3-bidΔ1-60/75, or pCDNA3-PEA(280-358)-bidΔ1-60/75. After 24 h, the cells were subjected to Apo2.7 antibody staining followed by flow cytometry analysis. **B,** transfected HeLa cells were subjected to trypsinization and analysis of the mitochondrial membrane potential by flow cytometry after 3,3'-dihexyloxa-carbocyanine iodide staining. **C,** meanwhile, HeLa cells after transfection were subjected to immunofluorescent staining with a mixture of anti-cytochrome c or anti-AIF antibody/FITC and anti-BID antibody/Cy3 together with 4',6-diamidino-2-phenylindole nucleus staining. Magnification, ×1,000. **D,** after 30-min exposure to 20 μmol/L zVAD or 20 μmol/L TAT-BH4, HeLa cells were transfected with pCDNA3, pCDNA3-bidΔ1-60/75, or pCDNA3-PEA(280-358)-bidΔ1-60/75. Then, the cells were subjected to trypan blue staining after 24 h and the negative cells were counted. Accordingly, the cell-killing percentages were calculated.

**Selective Killing of Tumor Cells Overexpressing HER2 by the Secreted e23sFv-PEA II (253-358)-BIDΔ1-60/75 Protein**

To induce specific tumoricidal effects, we fused the gene encoding an anti-HER2 single-chain antibody (e23sFv)
incubation periods (Fig. 4B), inhibited partially by zVAD, and (Fig. 4B). The cell-killing activity was enhanced by prolonged SK-OV-3) specifically but not the Hep-2 or the ECV-304 cells when the killing rate peaked. The cell-counting process wielded the more powerful death-inducing activity and showed that the HeLa/HER2/neu-overexpressing tumor cells, but none of the cell lines lacked the ability to express HER2 (including HeLa cells).

HER2/neu-Positive Tumor Growth Was Suppressed by e23sFv-PEA II-bid.1-60 Transduction In vivo

The 30 mice with xenografts were divided randomly into three groups to receive i.m. injections of liposome-encapsulated pCMV/e23sFv-PEA II-BID.1-60, pCMV vector alone, or PBS-liposome mixture, respectively. The tumor growth and survival rates were traced and analyzed. The tumors treated with the liposome-pCMV/e23sFv-PEA II-BID.1-60 complex grew more slowly than those treated with the liposome-pCMV complex (Fig. 5B), which suggested that the e23sFv-PEA II-BID.1-60 protein could strongly suppress the HER2/neu-positive tumor on secretion by genetically modifying the tumor cells themselves. Meanwhile, the mice treated with the pCMV/e23sFv-PEA II-BID.1-60 construct exhibited prolonged survival time compared with those treated with a control vector (Fig. 5B; \( P < 0.05 \)).

Immunohistochemical or immunofluorescence analysis confirmed BID-positive staining in muscles (Fig. 5A) and tumors (Fig. 5C) treated with pCMV-e23sFv-PEA II-BID.1-60 but not in those treated with pCMV vector alone. Apoptotic cells were discovered using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay in tumor tissues treated with liposome-pCMV/e23sFv-PEA II-BID.1-60 (Fig. 5D).

Figure 3. Immuno-tBID expressed secretively. A, immuno-tbid constructs were generated by fusing the e23sFv gene in-frame with the 5′ end of the fusion genes encoding the PEA domain II (amino acids 280–358) and bid.1-60/75. B, HeLa and SK-BR-3 cells were transiently transfected with e23sFv-PEA II-bid.1-60, respectively, and immunofluorescent staining was done with anti-BID antibody/Cy3. Magnification. \( \times 1000 \). C, HeLa cells were transfected with e23sFv-PEA II-bid.1-60 and e23sFv-PEA II-bid.1-75 followed by 800 \( \mu \)g/mL G418 selecting. The medium of 1 \( \times 10^6 \) e23sFv-PEA II-bid.1-60 gene-modified HeLa cells after 48 h of cultivation was concentrated and subjected to immunoblotting with a BID antibody. Lane 1, protein marker; lane 2, vector; lane 3, e23sFv-PEA II-bid.1-60; lane 4, e23sFv-PEA II-bid.1-75.
Selective Cell-Killing Effect Induced by e23sFv-TP-BIDΔ1-60 In vitro and In vivo

Our study shows that the chimeric protein including the PEAII(253-358) translocation domain has a potent selective antitumor effect both in vitro and in vivo. This result prompted us to shorten the PEA translocation domain and transfect the PEA-amended constructs into appropriate cell lines. We engineered these e23sFv-TP-bidΔ1-60 constructs by fusing the e23sFv gene in-frame with the 5’ end of the fusion genes encoding the PEA domain II (amino acids 273-282) and BIDΔ1-60. We evaluated its antitumor activity in vitro and then constructed and analyzed similarly a recombinant gene without a translocation domain as a negative control. This was constructed by fusing the antibody gene against HER2 with the 5’ end of the BIDΔ1-60 gene directly (Fig. 6A).

The pCMV/e23sFv-PEAII-BIDΔ1-60, e23sFv-TP-BIDΔ1-60, and e23sFv-BIDΔ1-60 were stably transfected into Jurkat and CHO cells, respectively, followed by G418 selection. The G418-resistant cells were subcloned, and the immunoblotting of the culture medium confirmed the stable secretion of the chimeric proteins. We showed through fluorescence microscopy that after transiently transfecting these genes into the HER2-positive breast cancer SK-BR-3 and gastric cancer SGC7901 cells under LipofectAMINE mediation, sub-G1 peaks were appeared nearly 48 h later (Fig. 6B) in e23sFv-PEA II-BIDΔ1-60 and e23sFv-TP-BIDΔ1-60 transfectant groups. Moreover, after being cocultivated with e23sFv-PEA II-BIDΔ1-60 or e23sFv-TP-BIDΔ1-60 modified Jurkat cells, the SK-BR-3 cells died in an apoptotic manner (Fig. 6C).

To investigate the efficiency of the e23sFv-TP-BIDΔ1-60 fragment and the diversity of several immuno-proapoptotic proteins, the SGC7901 recipient (1 x 10^6) nude mice were injected i.m. with 10 μg LipofectAMINE-encapsulated pCMV/e23sFv-TP-BIDΔ1-60, e23sFv-PEA II-BIDΔ1-60, immuno-caspase-3, immuno-caspase-6, or immuno-granzyme B, and pCMV as a control. The result showed that e23sFv-TP-BIDΔ1-60 and e23sFv-PEA II-BIDΔ1-60 prolonged the mouse survival time remarkably. Moreover, this effect was higher than that observed for immuno-granzyme B and similar to that observed for immuno-caspase-3 and immuno-caspase-6 (Fig. 6D).

Discussion

The HER2 oncogene, also called ErbB-2/neu, is the human homologue of the neu oncogene identified in DNA from rat neuroblastosomas as induced by ethylnitrosourea (22). Located on chromosome 17q, HER2

Figure 4. Selective cytoxicity of immuno-TBID-secreting Jurkat cells toward tumor cells. A, HER2-positive tumor cells (SK-BR-3) were cultured with the conditioned medium of e23sFv-PEA II-bidΔ1-60 modified HeLa cells and counted. The cell-killing percentages were calculated over 5 d from the cell counts. B, SK-BR-3, SK-OV-3, Hep-2, and ECV-304 were counted after 1 to 6 d of culture in the conditioned medium of e23sFv-PEA II-bidΔ1-60 (C) and immuno-caspase-3, immuno-caspase-6, and immuno-granzyme B modified cells (D), respectively, and the killing percentages were calculated.
encodes a transmembrane glycoprotein (p185) that was classified as a member of the epidermal growth factor receptor family. The HER2 protein is reportedly overexpressed in several adenocarcinomas, including breast, ovarian, lung, and stomach (2–6). Sufficient evidence has suggested that patients with HER2-overexpressing tumors exhibit a reduced response to conventional treatments (23). Because it is overexpressed in tumor cells but is not detected in normal cells, HER2 is an ideal target molecular for cancer gene therapy to exploit differences at the molecular level between normal and malignant cells. Several new therapeutic approaches have been developed that are based on mAbs or on antisense technology targeting the cells overexpressing HER2. Humanized anti-HER2 mAbs alone or conjugated either to toxins or enzymes directed against the extracellular domain of HER2 have been shown to be active in metastatic cancers overexpressing HER2 in xenograft mice (10, 24–26). Many early studies noted the improvement in limiting tumor growth in HER2-positive cell lines using an immunotoxin constructed by single-chain Fv modified PEA to convert from a cytostatic to a cytotoxic effect both in vitro and in vivo. Unfortunately, the in vivo use of immunotoxins for cancer therapy is not entirely advantageous in its current form. Reportedly, administration of analogous immunotoxins can result in the development of allergic reactions, infusion reactions, and lung problems in clinical experiments (26, 27). This is likely due to the heterogeneous nature of these toxins.

The most promising strategy for treating HER2-positive tumors will likely combine mAbs targeting of cancer cells with a maximal cell-killing ability while minimizing the potential damage to the surrounding normal tissues. Our approach to this end is using the anti-HER2 single-chain Fv targeted to tumor cells and with the minimized translocation domain to introduce a human gene encoding a key proapoptotic protein into the cytoplasm. Previously, several proapoptotic molecules, including caspase-3, caspase-6, granzyme B, and AIF were constructed into our immuno-proapoptotic proteins and were determined to be efficient in vitro and in vivo. Herein, we introduced tBID into this strategy by engineering several immuno-tbid constructs.

BID is a proapoptotic, BH3 domain–only member of the Bcl-2 family. As a key player in the apoptosis pathway, BID is a cytosolic protein in nonapoptotic cells, presenting in a variety of tissues (28). On induction of certain types of apoptosis, BID is cleaved by caspase-8, and its COOH-terminal fragment translocates to the mitochondria, where it exerts its proapoptotic effect by inducing the release of proapoptotic factors, such as cytochrome c, AIF, and procaspase 9 (8, 9). The COOH-terminal fragment of BID (called tBID) is regarded as the active form, which is far more potent in inducing apoptosis than the full-length BID molecule.

The tBID molecule was modified into a novel chimeric protein named immuno-tbid. This construction was achieved by fusing tBID to an anti-HER2 single-chain antibody and a NH2-terminal PEA translocation domain. In
theory, the e23sFv-PEA II-BIDΔ1-60/75 molecule can bind to and enter HER2-overexpressing tumor cells. To select the tBID construct with the greater tumor-killing potential, we evaluated two versions of active BID (BIDΔ1-60 and BIDΔ1-75).

Given that endocytosis of the e23sFv-PEA II-BIDΔ1-60/75 protein by HER2-overexpressing tumor cells would result in the release of PEA II-tBID to the cytosol, we investigated whether the PEA-derived peptide affected the tBID activity. Based on the well-characterized translocation mechanism of the PEA domain II in chimeric toxins (11, 12), autocleavage of the PEA translocation domain between Arg279 and Gly280 residues in the target cell endosomes would give rise to a COOH-terminal fragment, termed PEA (280-358)-BIDΔ1-60/75. The resulting fragment would consequently translocate to the cytosol and induce the cell to undergo apoptosis. The data from both transient and inducible expression systems showed that the PEA II-tBID form was active and caused cell death effectively in a manner similar to that seen with the tbid transfectants.

Furthermore, by cultivating in the medium of the genetically modified HeLa cells, our in vitro and in vivo data showed significant killing of the HER2-overexpressing tumor cells but not the HER2 low-expressing tumor cells or normal cells. In contrast to the immuno-apoptotic proteins that we generated previously (13–17), the e23sFv-PEA II-BIDΔ1-60/75 molecule exerts only minor immunogenicity potential and therefore should be applicable to long-term treatment of tumors that overexpress HER2. Moreover, unlike our immuno-caspase-3, immuno-caspase-6, or immuno-granzyme B constructs, e23sFv-PEA II-BIDΔ1-60/75 would be useful generally when either the receptor or mitochondrial-mediated apoptotic pathway is not available in tumor cells. Because of the uncertainty of whether the minimal fragment of PEA is necessary for its translocation activity, we constructed a short translocation peptide to link e23sFv and tBID with only 10 amino acids derived from the furin cleavage site (29) of PEA, called e23sFv-TP-BIDΔ1-60, and compared its antitumor effects with those of immuno-apoptotic proteins in vitro and in vitro. Importantly, the molecules inhibited cell proliferation similarly and killed tumor cells overexpressing HER2. We also compared the in vitro effects of the immuno-tBID and immuno-TP-tBID constructs with those of other immuno-proapoptotic proteins that we constructed previously. The result showed that immuno-tBID and immuno-TP-tBID could inhibit tumor growth more strongly and prolong longevity in mice. This advantage may reflect a smaller influence of the shorter NH2-terminal PEA-derived peptide on tBID activity and on the ability of the molecule to escape from endosomes.

A major concern in the development of HER2-targeted therapies is the expression of the target antigen on normal
tissues. Numerous studies with Herceptin (trastuzumab), although showing significant clinical benefits in patients with HER2-positive primary and metastatic breast cancers, have reported notable cardiotoxicity in a few patients presumably because of interference with normal HER2 signaling in the heart (30). By contrast, immuno-TP-tBID fusion proteins are expected to be less toxic over repeated treatments because of enhanced antitumor activity through the addition of an apoptotic effect molecule that permits decreased doses of the targeted killer protein to be used in vivo.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank Drs. Dai-ming Fan and Yong Zhou for useful suggestions and encouragement and Yun-xin Cao for assistance with the flow cytometric analysis.

References
Molecular Cancer Therapeutics

Single-chain antibody/activated BID chimeric protein effectively suppresses HER2-positive tumor growth

Xiu-Chun Qiu, Yan-Ming Xu, Fang Wang, et al.


Updated version
Access the most recent version of this article at:
http://mct.aacrjournals.org/content/7/7/1890

Cited articles
This article cites 30 articles, 13 of which you can access for free at:
http://mct.aacrjournals.org/content/7/7/1890.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/7/7/1890.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.