Recombinant Immunoproapoptotic Proteins with Furin Site Can Translocate and Kill HER2-Positive Cancer Cells

Tao Wang,1 Jing Zhao,1 Jun-Lin Ren,2 Li Zhang,1 Wei-Hong Wen,2 Rui Zhang,1 Wei-Wei Qin,1 Lin-Tao Jia,1 Li-Bo Yao,1 Ying-Qi Zhang,3 Si-Yi Chen,1 and An-Gang Yang1

1State Key Laboratory of Cancer Biology, Departments of Biochemistry and Molecular Biology and Immunology and Biotechnology Center, Fourth Military Medical University, Xian, China and 2Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas

Abstract

We previously reported the selective killing of HER2-positive tumor cells by a class of immunoproapoptotic proteins containing single-chain antibody, translocation domain of Pseudomonas exotoxin A (domain II; PEA II), and constitutively active human apoptotic molecules. In this study, a novel class of antitumor immunoproapoptotic proteins was explored to mediate tumor-specific apoptosis both in vitro and in vivo. Three furin cleavage sequences, including a synthetic polyarginine tract, and two furin cleavable sequences from PEA and diphtheria toxin were respectively used to replace PEA II in the previously constructed immunoproapoptotic protein. When produced and secreted by the genetically modified Jurkat cells, the novel targeted proapoptotic proteins selectively bound to HER2, which is often overexpressed on tumor cell surface. Followed by receptor-mediated endocytosis and furin cleavage in the endosome, the recombinant proteins could translocate into the cytosol, leading to irreversible cell death. Moreover, delivery of these proteins by either i.m. plasmid injection or i.v. injection of plasmid-expressing Jurkat cells led to tumor regression and prolonged animal survival in a nude mouse xenograft tumor model, indicating in vivo antitumor activity of the recombinant proteins. We conclude that the new class of immunoproapoptotic proteins show comparable activity with PEA II-containing counterpart and provide an attractive therapeutic alternative as they contain much less exogenous fragments.

Introduction

Immunotoxins are hybrid proteins composed of plant or bacterial toxins linked with a targeting molecule, such as a monoclonal antibody, an antibody fragment, a growth factor, or a cytokine (1–4). Immunotoxins derive their toxic potency from the toxin and their specificity from the targeting moiety to which they are attached. Typically, the targeting moiety recognizes and delivers the whole molecule to specific receptors expressed on surface of target cells. After binding to the cell surface, conjugates are internalized via a receptor-mediated endocytosis, followed by cleavage-dependent release of active toxin from endosome to cytosol, wherein the toxin functions and eventually kills the cell.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: An-Gang Yang, Department of Immunology, Fourth Military Medical University, Xian, Shaanxi, 710032, China. Phone: 86-29-84774528; Fax: 86-29-83253816; E-mail: agyang@fmmu.edu.cn.

We have previously reported the generation of a Pseudomonas exotoxin A (PEA)-based immunotoxin, which contains an anti-HER2 single-chain antibody and PEA40 (e23sFv-PEA40; ref. 5). PEA40 is a cytotoxic composed of PEA domain II (PEA II), which is responsible for the enabling of translocation, and PEA III, which is for catalytic inactivation of protein synthesis. As some other well-demonstrated antitumor immunotoxin (6–8), e23sFv-PEA40 was shown to have potent and selective cytotoxicity to tumors in culture and nude mouse models. However, most of the immunotoxin clinical trials faced the same obstacle that immunotoxin would arouse strong humoral immune responses (9–14). In most cases, patients with normal immune systems develop neutralizing antibodies against the plant or bacterial-derived toxins (9, 10). Besides, vascular leakage, nonspecific toxicity to vascular endothelial, liver, or renal cells are other side effects of immunotoxin administration (8, 10–14). In short, clinical trial results suggest that it is the foreign toxin fragments that usually cause humoral immune response and therefore limit the clinical utility of immunotoxins.

To minimize nonspecific toxicity and immunogenicity, investigators have set about to explore immunotoxins containing human-derived effector proteins, such as human RNase (15). Similarly, we previously reported the use of human proapoptotic proteins, such as active caspase-3 (16), caspase-6 (17), granzyme B (18), and truncated apoptosis-inducing factor (19). Those human toxic moieties were substituted for the catalytic domain of PEA40, which was conjugated to an anti-HER2 antibody (5). The resulting partially humanized immunotoxins were composed of an anti-HER2 single-chain antibody (20), a PEA translocation domain, and one of the above-mentioned human proapoptotic proteins. Such immunoproapoptotic proteins retained potency to kill HER2-positive tumor cells both in vitro and in vivo (16–19). To further reduce exogenous fragment, we generated truncated variants of PEA II to identify the minimal sequence required for translocation. Our results showed that consistent with previous reports (21), deletion of either the first or last α-helix of PEA II led to translocation activity comparable with the native PEA translocation domain. Strikingly, we observed that when e23sFv and granzyme B were coupled with a rather small PEA II-derived sequence containing only six amino acid residues around furin cleavage site, this recombinant protein also showed antitumor activity, although the cytotoxicity comparatively decreased compared with the full-length PEA II conjugate. This unexpected finding indicates a crucial role of furin cleavage in the proapoptotic activity of immunotoxins.

Furin is a cellular endoprotease and has been implicated in proteolytic activating large numbers of secreted proteins. Mature furin localizes principally to the trans-Golgi network, the cell surface, and early endosomes (22–24). Furin-mediated cleavage of PEA and PEA-derived immunotoxins is both an obligatory and
rate-limiting step for their cytotoxic activity (25, 26). In fact, only 5% to 10% of cell-associated PEA can be cleaved by furin within cells (27). In this study, we report the generation of a new class of conjugates, called novel immunoproapoptotic proteins, which contain furin-sensitive sequences. These furin sensitive sequences include sites from PEA (T_{273}RHRQPRGWE_{282}, Fpe), diphtheria toxin (A_{187}GNRVRRSRSV_{196}, Fdt), and a synthetic polyarginine tract (RRRRRRRRRR, R_9). We, here, describe the construction and characterization of a novel class of immunoproapoptotic proteins containing these furin cleavage sequences. We present evidence that these novel immunoproapoptotic proteins efficiently and selectively bind to HER2-overexpressing tumor cells, followed by endocytosis, furin-mediated cleavage, and translocation, resulting in induced cell death and reduction of tumor size in nude mice.

Materials and Methods

Plasmids construction. The genes encoding e23sFv, PEA II, and casp3/GrB were generated in our previous study (16, 18). Truncated PEA II fragments were amplified by PCR using a casp3-encoding plasmid as template (16). Furin site sequences were incorporated into casp3, GrB, or enhanced green fluorescent protein (EGFP) by PCR. Generation of the recombinant genes involved a sequential fusion of the genes of a signal peptide. Fragments encoding e23sFv, casp3/GrB, truncated PEA II, or the furin sites were cloned downstream and in frame with DNA encoding a signal sequence (MKHLWFLLLLAVPRWLS; Figs. 1A and 2A) in the expression vector pCMV. Vector sequences were confirmed by DNA sequencing.

Cell culture and transfection. The human breast tumor cell line SKBR-3, the human gastric cancer cell lines SGC-7901 and AGS, the human hepatocellular carcinoma cell line Hep G2, the human cervical cancer cell line HeLa, and the human epidermoid carcinoma cell line A431 were cultured in RPMI 1640 medium containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA). The HeLa cells were transiently transfected with pCMV or vectors encoding variant constructs described in A. C, time course of cell number in SKBR-3 transfected as described. Columns, mean; bars, SE. D, immunocytochemical staining of GrB in SKBR-3 cells 72 h after transfection with pCMV or variant constructs described in A. Magnification, 400×.

Figure 1. Selective cytotoxicity of immuno-GrB with truncated PEA II variants. A, schematic diagram of immuno-GrBs containing signal sequence, e23sFv, portions of the PEA translocation domain, and GrB. B, immunofluorescent staining of GrB in HeLa cells 72 h after transient transfection with pCMV or vectors encoding variant constructs described in A. C, time course of cell number in SKBR-3 transfected as described. Columns, mean; bars, SE. D, immunocytochemical staining of GrB in SKBR-3 cells 72 h after transfection with pCMV or variant constructs described in A. Magnification, 400×.
Carcinoma cell line HeLa, and the human lymphoma cell line Jurkat were maintained in DMEM or RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in 5% CO₂. At 24 h before transfection, cells were seeded in 12-well plates at a density of 1 x 10⁵ cells per well. Transfection was performed with Lipofectamine 2000 (Invitrogen) following the standard procedure. For transient expression, cells were harvested 72 h after transfection, unless stated otherwise. For stable transfection, cells were selected in medium containing 800 μg/mL G418 (Invitrogen) for 2 to 3 weeks.

Cell viability assay. For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cells transfected with novel immunopro-apoptotic protein genes were cultured in 96-well plates for 24 to 96 h. Cells were then incubated with 20 μL of 1.5 mg/mL MTT per well for 4 h, followed by the addition of 150 μL DMSO. A₄₉₀ nm values were determined using a Sunrise microplate reader (Tecan). Each assay was performed in triplicate on at least three independent occasions.

Immunocytochemical and histologic analyses. Cells on coverslips were transfected with DNA and stained 72 h after transfection. The samples were fixed in 4% paraformaldehyde (Invitrogen) following the standard procedure. For transient expression, cells were harvested 72 h after transfection, unless stated otherwise. For stable transfection, cells were selected in medium containing 800 μg/mL G418 (Invitrogen) for 2 to 3 weeks.

Figure 2. Expression and secretion of novel immunopro-apoptotic proteins containing optimal furin-cleavable peptides. A, schematic diagram of novel immunopro-apoptotic proteins comprising signal sequence, e23sFv, furin-cleavable peptides, and proapoptotic proteins or EGFP. B, Western blot analysis of concentrated cell culture medium from Jurkat cells stably transfected with novel immunopro-apoptotic protein genes after 72-h culture. Blots were probed with anti-casp3, anti-GrB, and anti-EGFP antibody, as indicated. C, Western blot analysis of in vitro furin cleavage of concentrated culture supernatants from transfected Jurkat cells. Culture supernatants were concentrated and incubated with recombinant furin at room temperature for 16 h at pH 5.4 or pH 7.2. Cleavage rates were calculated from densitometric estimates.
Sections were then stained with anti-GrB or rabbit anti-human active caspase-3 (1:500; BD PharMingen) as described above.

**TUNEL assay.** Paraffin-embedded tissue sections were dewaxed and hydrated following standard procedures. Specimens were covered with 100 μL of 20 μg/mL proteinase K at room temperature for 20 min. After washing with TBS, specimens were rinsed with 100 μL of 1× TdT equilibration buffer, and incubated at room temperature for 10 to 30 min. Next, specimens were treated with 60 μL of TdT labeling reaction mixture. Finally, slides were incubated in a humidified chamber at 37°C for 1.5 h before detection using a fluorescence microscope.

**Furin cleavage assay.** Stably transfected Jurkat cells were cultured in T-75 flasks (Corning) with serum-free medium for 72 h. Supernatants were concentrated by centrifugation for 30 min at 4°C at 3,000×g through Amicon Ultra concentrators (30,000 MWCO, Millipore). Proper buffers were added to the device, and centrifugation was performed again as above to concentrate and exchange buffers to what were applied in the later furin cleavage assay. All the reaction buffer solutions contained final concentrations of 3 mmol/L CaCl2 and 0.1% Triton X-100. For pH 5.4, 50 mmol/L sodium acetate buffer were used, and for pH 7.2, 10 mmol/L HEPES buffer were used. Recombinant human furin (200 ng R&D Systems) was used in each reaction, and the final total reaction mixtures were 100 μL per reaction. Reaction mixtures were incubated for 16 h at room temperature on a rocking platform. SDS loading buffer was added to quench the reaction, and proteins were analyzed by SDS-PAGE and autoradiography. The percentage of furin cleavage rate was calculated using Glyko Bandscan software (Version 5.0) from the formula: cleavage rate = value of gray scale of cleaved protein / value of gray scale of total protein.

**Recombinant immunoproapoptotic protein transduction.** SGC-7901 cells were seeded at a density of 1×10⁶ cells per well on slides in 12-well Costar transwell plates. Jurkat-e23sFv-Fdt-EGFP or Jurkat-e23sFv-Null-EGFP cells at a density of 5×10⁵ cells per well were placed in the top chamber. Cells were cotransfected at E/T ratios of 3:1 for 8 h. Cells in the lower chamber were then fixed, permeabilized, and blocked as described above. After overnight incubation at 4°C in rabbit anti-EEA1 (1:200, Abcam) and mouse anti-GFP (1:500, Clontech), cells were washed in PBS and incubated in biotin-labeled anti-rabbit (1:100; Sigma) for 2 h at room temperature. Cells were then washed as before and incubated in a Cy3-conjugated avidin-biotin (Sigma) and FITC-conjugated antimouse IgG (1:1,000; Sigma) for 1 h at room temperature. Finally, cells were washed and mounted on slides. Cells were imaged by laser scanning confocal microscopy (FluoView FV1000, Olympus).

**Western blot analysis.** Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham Biosciences). Membranes were incubated with primary antibodies that recognize caspase-3 (1:500; BD PharMingen), granzyme B (1:200; Santa Cruz Biotechnology), GFP (1:1,000; Clontech), HER2 (1:500; NeoMarkers), or poly(ADP-ribose) polymerase (PARP; 1:200; Santa Cruz Biotechnology) overnight at 4°C in PBST. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000; ZhongShan) for 2 h at room temperature. Western blots were visualized using an enhanced chemiluminescence kit (Pierce).

**In vitro cytotoxicity assay.** Tumor cells were cocultured with transfected Jurkat cells at a ratio of 1:3 in a 12-well Costar transwell, separated by a filter with 0.4 μm pores. Viable tumor cells were counted by trypan blue exclusion at each indicated time point. The percentages of cell killing were determined as follows: 1 – (the number of cells cocultured with stably transfected Jurkat / the number of cells cocultured with Jurkat controls) × 100%.

**Antitumor activity of Fdt linked immunocasp3 in vitro.** An SGC-7901 xenograft mouse model was established by s.c. injection of 5×10⁶ cells in the right hind flank of nude mice (4-6 weeks old; 18-22 g, weight). Tumors grew to 5 to 7 mm in diameter within 2 weeks, upon which time mice were randomly assigned to groups of five mice each. One group of mice received six doses of 10 μg of pCMV-e23sFv-Fdt-Rcasp3 or pCMV-e23sFv-PEA II-casp3 mixed with Lipofectamine every 3 days i.m. in the right posterior limb. Another group of five mice received three weekly i.v. injections of 2×10⁶ Jurkat-e23sFv-Fdt-Rcasp3 or Jurkat-e23sFv-PEA II-Rcasp3 cells.

Control mice were injected with either liposome-mixed empty pCMV vector or unmodified Jurkat cells. Tumor growth was monitored by caliper, measuring two perpendicular tumor diameters every 3 days, and the volume of the tumor was calculated from the formula: tumor volume = (width)² × length / 2 (28). Mouse survival was also recorded. Mice were then sacrificed by cervical dislocation and dissected, and tissues were embedded in paraffin for histologic analysis.

**Statistical analysis.** Statistical analysis was performed with the SPSS12.0 software package for Windows (SPSS). Survival rates were analyzed by the Kaplan-Meier method, with comparisons between treatment groups made by the log-rank test. Tumor volumes were analyzed by the analysis of covariance method, with comparisons between treatment groups made by covariance test. Statistical significance was defined as P ≤ 0.05.

**Results**

**Immunoproapoptotic proteins containing truncated PEA II can induce HER2-positive tumor cell death.** PEA II consists of six α-helices (A–F), and when PEA is internalized into a cell, furin-dependent cleavage occurs between helices A and B (R279-G280).

To identify the minimal segment of PEA II required for translocation, we generated three deletion constructs bearing different helices: PEA253-358 (deletion of helix F), PEA275-358 (deletion of helix A and F), and PEA275-280 (deletion of all six helices). The deletion constructs were fused with anti-HER2 single-chain antibody (ε23sFv) and the proapoptotic granzyme B genes, producing a series of truncated immuno-GrB molecules (Fig. 1A). In addition, we generated a full-length PEA II fusion construct as the positive control and a PEA II–null fusion construct as the negative control.

Next, we transfected either HER2-positive human breast tumor SKBR-3 cells or HER2-negative human cervical carcinoma HeLa cells (29, 30) with the PEA II truncated constructs (Fig. 1B). Translocation was assessed indirectly by measurement of GrB-triggered cytotoxicity. The fusion proteins were secreted properly from both SKBR-3 and HeLa cells (data not shown). As expected, transfected HeLa cells proliferated at a rate that was similar to untransfected cells (data not shown), suggesting that expression of the chimeric protein was not toxic. In contrast, transfected SKBR-3 cells exhibited reduced cell number (Fig. 1C) and morphologic changes consistent with increased cell death (Fig. 1D). Interestingly, the smallest construct, containing PEA275-280, led to reduced but detectable cytotoxicity in SKBR-3 cells, indicating the furin site alone is necessary for GrB delivery to the cytosol. As this construct contains the least PEA fragment, it was thus chosen for further optimization of furin cleavage and cytotoxic activity.

**Expression and secretion of novel immunoproapoptotic proteins containing optimal furin cleavable sequences.** To increase furin-mediated cleavage and release of active effectors to the cytosol, we next attempted to alter the sequence of the amino acids, linking antibody and proapoptotic moieties in three different constructs. In the first construct, we added additional amino acid residues to the PEA275-280 sequence (T27>RHRQPRGW282, Fpe) to enhance the rate of furin-mediated cleavage (23, 24). In the second construct, we used the furin site from diphtheria toxin (A19-GNRRRSVG196, Fdt), which has been reported to facilitate furin-mediated cleavage (23, 24). In the third construct, we used the arginine-rich sequence (RRRRRRRRR, R₉), a furin cleavage sequence with multiple cleavage sites. These three putative furin-sensitive linkers were cloned between ε23sFv gene, with signal sequence, and apoptosis-inducing protein (casp3 or GrB) genes or the EGFP gene under the control of the CMV promoter (Fig. 2A).
Two other plasmids, in which e23sFv and apoptosis-inducing proteins were linked directly or by wild-type PEA II, were also generated as controls (Fig. 2A).

CD4+ Jurkat cells were then transfected with these expression plasmids and selected by G418 selection for stable expression. Expression of the transfected constructs was confirmed by reverse transcription–PCR (RT-PCR) using primers specific to a 750-bp e23sFv fragment (data not shown). Expression and secretion of fusion proteins was confirmed by Western blot analysis of cell culture supernatants (Fig. 2B). Stably transfected Jurkat cells showed similar proliferation rates compared with parental cells (Supplementary Fig. S1).

In vitro cleavage of novel immunoproapoptotic proteins by furin. Secreted fusion proteins from stably-transfected Jurkat cells were collected and subjected to proteolysis with recombinant furin. At pH 7.2, PEA II was uncleavable as previously reported (25, 31, 32). In contrast, cleavage of Fpe (6.9% of total), Fdt (36.4%), and Rv (38.7%) was observed at pH 7.2. At pH 5.4, PEA II, Fpe, Fdt, and Rv were all cleaved more efficiently (39.2%, 49.4%, 68.2%, and 73.7%, respectively). However, the negative control recombinant protein, in which e23sFv directly fused with EGFP, was not cleaved at either pH (Fig. 2C). According to the results, Fdt and Rv were more efficiently cleaved among all of the constructs in both pH conditions. In addition, we observed that although an acidic environment is not obligatory for the cleavage of furin sensitive sites, lower pH, which is similar to the pH environment of endosome, greatly enhances the cleavage rate of these sequences.

Translocation of novel immunoproapoptotic proteins in SGC-7901 cells. Jurkat cells stably transfected with e23sFv-Fdt-EGFP or e23sFv-Null-EGFP were cocultured with HER2-positive SGC-7901 cells for 8 h at an E/T ratio of 3:1. Under fluorescence microscope, the majority of SGC-7901 cells were observed taking up the fusion proteins secreted by Jurkat cells (data not shown). To examine the translocation of these fusion proteins in HER2-positive cells, we performed immunofluorescent staining using both anti-EGFP and anti-EEA1 antibodies. EEA1 is a membrane-bound protein component specific to the early endosome (33, 34). The e23sFv-Null-EGFP fusion protein showed a punctuate distribution in SGC-7901 cells which colocalized predominantly with the early endosome marker (Fig. 3), suggesting a lack of translocation to the cytosol. In contrast, e23sFv-Fdt-EGFP exhibited both a punctate colocalization with EEA1, as well as a diffuse cytoplasmic fluorescence, indicating translocation of the fusion protein from early endosome to cytosol (Fig. 3). These observations strongly suggest that furin-mediated cleavage leads to cytosolic translocation of immunoproapoptotic proteins in gastric cancer SGC-7901 cells.

Novel immunoproapoptotic proteins specifically kill HER2-overexpressing tumor cells in vitro. Recombinant proteins were transiently expressed in HER2-positive SGC-7901 and HER2-negative HeLa cells. Apparent cell death was observed in SGC-7901 cells but not HeLa cells at 48 h after transfection (Fig. 4B), presumably due to the presence of HER2 on the surface of SGC-7901 cells. Indirect immunofluorescent staining showed cells expressing the targeted proteins underwent clear nuclear chromatin condensation 72 h after transfection (data not shown). Next, we investigated degree of apoptosis in transfected SGC-7901 cells by detecting the cleavage of PARP. As expected, PARP was cleaved 72 h after transfection with all constructs containing furin cleavage sites, but not in the null-negative control group (Fig. 4A).

Next, we compared in vitro cytotoxicity of the novel immunoproapoptotic proteins by coculturing transfected Jurkat cells with SGC-7901 cells at a ratio of 3:1. In the case of casp3-conjugated fusion proteins, we observed apparent cell death in the PEA II group 24 h after coculture and a killing rate as high as 80% 4 days after transfection (Fig. 4C, left). Similarly, furin cleavable sequences–linked groups, namely Fpe, Fdt, and Rv, showed time-dependent killing activity, but their cytotoxicity was weaker than that of PEA II (Fig. 4C, left). Fpe-containing fusion proteins contributed less to cell killing than Fdt-containing and Rv-containing
fusion proteins, possibly due to inefficient furin cleavage. Similar results were obtained with GrB-conjugated fusion proteins, although GrB-mediated killing was not as potent as casp3 (Fig. 4C, right).

To confirm the specificity of the cytotoxicity, e23sFv-Fdt-casp3/GrB-transfected Jurkat cells were cocultured in vitro with four different HER2-positive human tumor cell lines (SKBR-3, SGC-7901, AGS, and Hep G2) and control HeLa cells that express undetectable...
levels of HER2 (Fig. 5A). We observed significant killing of SKBR-3, SGC-7901, and AGS cells, moderate killing of Hep G2 cells, and no killing of HeLa cells (Fig. 5B). Consistent with our previous observations, stronger cytotoxicity was observed with casp3 conjugates than with GrB conjugates (Fig. 5B). Taken together, we conclude that immunoparaptotic proteins are capable of recognizing and destroying HER2-expressing cells, but not cells lacking detectable HER2 expression.

Secreted e23sFv-Fdt-casp3 effectively suppresses the growth of HER2-overexpressing tumor cells and prolongs survival in nude mice. The antitumor activity of immunoparaptotic proteins was evaluated in vivo in a nude mouse xenograft model containing HER2-overexpressing SGC-7901 cells. Because of its potent in vitro antitumor activity, we chose to use e23sFv-Fdt-casp3 in vivo. Nude mice were injected with SGC-7901 cells (see Materials and Methods).

One group of mice were given six i.m. doses of Lipofectamine-encapsulated pCMV-e23sFv-Fdt-casp3, pCMV-e23sFv-PEA II-casp3, or empty pCMV every 3 days over the course of the study. e23sFv-Fdt-casp3 expression in muscle cells, as evaluated with an anti-caspase-3 antibody, was found most obvious 24 h after i.m. injection and almost undetectable on day 4 (Fig. 5C). Another group of gastric cancer–bearing mice received three weekly i.v. injections of 2 x 10^7 Jurkat cells expressing e23sFv-Fdt-casp3, e23sFv-PEA II-casp3, or control Jurkat cells. Injected Jurkat cells were found to be localized to tumor tissues, liver, and spleen but hardly detectable in the heart, kidney, and lung (Fig. 5D; Supplementary Fig. S2). Remarkably, Jurkat cells were detected in tumor tissues 1 day after injection and were still evident 1 week later (Fig. 5D).

According to the statistical data, both the Jurkat secreting treated groups and the vector Lipofectamine treated groups led to...
a decrease in tumor volume and prolonged mouse survival time compared with controls (Fig. 6A and B). However, the Jurkat secreting groups were more efficient than the vector Lipofectamine groups in reducing tumor size \((P < 0.05)\), suggesting that the recombinant protein, which is continuously produced by the transduced cells inside tumors, accumulated locally to exert its antitumor effects. Interestingly, no obvious differences were observed between e23sFv-Fdt-casp3–injected and e23sFv-PEA II-casp3–injected groups, regardless of the method of delivery (Fig. 6A and B).

We next examined the localization of recombinant proteins in the mice that received genetically modified Jurkat cells. Immunohistochemical staining confirmed that secreted proteins were localized to tumor tissues, but not heart, liver, spleen, lung, kidney (Fig. 6C), or tumor tissues of control mice (data not shown). There was neither histologic abnormality nor apoptosis in liver, lung, kidney, or heart (Supplementary Fig. S3). In contrast, numerous apoptotic cells in tumor tissues were detected by TUNEL analysis (Fig. 6D), suggesting that immunoproapoptotic proteins secreted by the transfected cells \textit{in vivo} can effectively target and suppress cancer cells overexpressing HER2.

Discussion

Obstacles facing immunotherapy are the limited accessibility of antibodies or antibody conjugates to solid tumors and the difficulty in obtaining tumor-specific killer cells by genetically modifying lymphocytes. In our previous work, we established an antibody-directed and cell-mediated cancer therapy strategy, which combines the specificity of antibodies, the potent cytotoxicity of proapoptotic proteins, and the intrinsic ability of lymphocytes to home and penetrate tissue (16–19). The potent \textit{in vivo} antitumor activity of such a strategy is probably a result of the migration of the lymphocytes to tumors as a targeted toxin carrier and production and accumulation of the targeted toxins inside tumors as a producer. Here, we optimized this strategy by reducing exogenous PEA fragment. We designed three novel, short furin sensitive sites, of \(\sim 10\) amino acids each, to be used as linkers in combination with an anti-HER2 single-chain antibody and the proapoptotic proteins caspase-3 and granzyme B. We predict that these furin cleavable sites might further attenuate nonspecific cytotoxicity, thus improving its utility as an anticancer treatment.
In this study, we have described the construction and functional characteristics of novel immunopropaptotic proteins, which included a poly-arginine tract (R₉) or a furin site sequence from PEA (amino acids 273-282, Fpe) or diphtheria toxin (amino acids 187-196, Fdt). Our in vitro results revealed that these novel immunopropaptotic proteins killed HER2-overexpressing tumor cells, but not HER2-negative cells. Moreover, these novel immunopropaptotic proteins were also functional in vivo, as they led to reduced tumor sizes in a murine xenograft model of HER2-overexpressing gastric cancer cells.

Unlike previously reported immunotoxins, which have been shown to cause immune systems to develop neutralizing antibodies and thus prevent retreatment, our newly generated novel immunopropaptotic proteins consist mainly of humanized antibodies and human apoptotic effectors. These novel immunopropaptotic proteins possess putatively minor immunogenicity and therefore reduce the possibility of inducing immune response and systematic toxicity, suggesting improved therapeutic utility for long-term treatment of tumors that overexpress HER2.

In this study, three short sequences (Fpe, Fdt, and R₉) were examined as potential furin-sensitive sites. Fpe, which lacked a basic residue in the P₂ position, exhibited relatively low cleavage efficiency, whereas Fdt and R₉ exhibited relatively high cleavage efficiency in vitro. Given that poly-arginine meets the most stringent furin specificity requirements (24) and that R-X-X-R is the minimal furin cleavage site (22–24), R₉ was predicted to be the suitable sequence for furin cleavage, with six possible cleavage sites. Because arginine-rich sequences have been shown to function as protein transduction domain (PTD; refs. 35–38), we suspected that after furin cleavage, cleaved R₉ might aid in translocating the proapoptotic molecule from endosome to cytosol, thus leading to enhanced proapoptotic activity. However, our results showed that the R₉-Casp3/Grb conjugates did not show enhanced cell killing compared with Fdt counterparts (Fig. 4B and C). One explanation for this observation is that with six possible cleavage sites, the remaining linker might be inadequate to aid translocation. We also noticed that our R₉ conjugates did not show nonspecific cellular uptake as PTD usually does when PTD is fused to either the N or COOH terminus of a protein (35–38). This may be due to the inaccessibility of R₉ to the surface of the cell membrane as a result of steric hindrance of the antibody and proapoptotic moiety.

Both active caspase-3 and granzyme B were used as proapoptotic molecules and both proved to be efficient at cell killing, suggesting the furin-sensitive linker may serve as a universally useful strategy by which to introduce effector proteins. Our results indicate that furin cleavage is a key step in the targeted killing process. However, the mechanism of translocation is not clear thus far. In our work, granzyme B was less cytotoxic than caspase-3. However, novel immunopropaptotic proteins containing granzyme B would still be useful in the treatment of tumors deficient in the caspase-dependent apoptotic pathway (39, 40). Given that almost any antigen can be targeted on the cell surface, this approach would be applicable to treatment of cancer, autoimmune diseases, and virus infection.

Acknowledgments


Grant support: National High Technology Research and Development Program of China grant 2006AA02A255 (A.G. Yang) and 2007AA021014 (A.G. Yang), Program for Changjiang Scholars and Innovative Research Team in University IRT0459 (A.G. Yang), National Basic Research Program of China 2004CB118805 (L.T. Jia), and National Natural Science Foundation of China 30701006 (T. Wang), 3030810 (A.G. Yang), and 30400403 (J. Zhao).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

Recombinant Immunoprapoptotic Proteins with Furin Site Can Translocate and Kill HER2-Positive Cancer Cells

Tao Wang, Jing Zhao, Jun-Lin Ren, et al.