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A Caspase-6 and Anti-Human Epidermal Growth Factor Receptor-2 (HER2) Antibody Chimeric Molecule Suppresses the Growth of HER2-Overexpressing Tumors

Yan-Ming Xu,* Li-Feng Wang,* Lin-Tao Jia,* Xiu-Chun Qiu,* Jing Zhao,* Cui-Juan Yu,† Rui Zhang,* Feng Zhu,* Cheng-Ji Wang,* Bo-Quan Jin,† Si-Yi Chen,‡ and An-Gang Yang 2*

Clinical studies have suggested that human epidermal growth factor receptor-2 (HER2) provide a useful target for antitumor therapy. We previously described the generation of a chimeric HER2-targeted immunocasp-3 protein. In this study, we extend the repertoire of chimeric proapoptotic proteins with immunocasp-6, a construct that comprises a HER2-specific single-chain Ab, a single-chain Pseudomonas exotoxin A, and an active caspase-6, which can directly cleave lamin A leading to nucleus damage and inducing programmed cell death. We demonstrate that the secreted immunocasp-6 molecule selectively recognizes and induces apoptosis in HER2-overexpressing tumor cells in vitro, but not in cells with undetectable HER2. The immunocasp-6 gene was next transferred into BALB/c athymic mice bearing human breast SK-BR-3 tumors by i.a. injection of liposome-encapsulated vectors, by intratumor injection of adenoviral vectors, or by i.v. injection of PBMC modified by retroviral infection. Regardless of the method used, expression of immunocasp-6 suppressed tumor growth and prolonged animal survival significantly. Our data show that the chimeric immunocasp-6 molecule can recognize HER2-positive tumor cells, promptly attack their nucleus, and induce their apoptotic death, suggesting the potential of this strategy for the treatment of human cancers that overexpress HER2. The Journal of Immunology, 2004, 173: 61–67.

Over the past decade, promising advances have been made in specific gene therapy for human tumors (1–5), including lead-in targeting, transcriptional targeting, and recognition targeting. Yet the need for more sensitive, effective, and specific treatments continues to drive this field of research. Human epidermal growth factor receptor-2 (HER2), a 185-kDa protein that transduces cell signaling, is critical in cell proliferation, differentiation, adhesion, and motility (6–8). Its overexpression as a result of gene amplification is detected in 20–40% of patients with breast, ovarian, endometrial, gastric, bladder, prostate, or lung cancer (9, 10). Studies indicate that HER2 plays a direct role in the pathogenesis and aggressiveness of tumors and is associated with a poor clinical outcome (11). Importantly, HER2, which is undetectable in normal adult tissues, could provide a useful target for specific gene therapy (12–14).

Caspases are vital elements in transducing apoptotic signals and executing apoptosis in mammalian cells. The apoptotic program requires the participation of at least two classes of caspases: initiators such as caspase-2, caspase-8, caspase-9, and caspase-10, and effectors such as caspase-3, caspase-6, and caspase-7 (15–20). Activation of caspase-6 induces apoptosis by cleaving lamin A and other substrates (21). Unlike its wild-type zymogen counterpart, active caspase-6 constructed in a reverse order of the subunits (22) is capable of autocatalytic processing in vitro, independent of apoptotic signals, and can induce apoptosis of tumor cells, making it an attractive candidate molecule for gene therapy.

To devise a more sensitive, effective, and specific strategy of antitumor therapy, we generated a novel fusion gene, immunocasp-6, consisting of NH2-terminal leader sequence to promote secretion of the recombinant immunocasp-6 protein fused with an anti-HER2 single-chain Ab, the translocation domain (domain II) of Pseudomonas exotoxin A (PEA), and an active caspase-6. As an accredited Ab, e23sFv, which was derived from a mouse mAb against human HER2, has been confirmed to bind the extracellular domain of HER2 protein with high affinity and to be internalized by endocytosis (23, 24). PEA is a single-chain toxin consisting of three major domains (I, II, and III) responsible for binding of the molecule to target cells, translocation of the molecule to the cytosol, and the induction of cell death, respectively (25). Indeed domain II of PEA has been reported to efficiently transfer the cellular toxicity domain to the cytoplasm (26–28). By replacing the cellular toxicity domain of PEA with active caspase-6, we sought to translocate the caspase into tumor cells in which it would induce apoptosis. An earlier study by our group has demonstrated that certain kinds of similar immuno-molecules effectively killed HER2-positive tumor cells (29). In this study, this concept was tested both in vitro and in a mouse model of HER2-positive tumors, using different methods of gene transfer.

Materials and Methods

Plasmids and DNA constructs

Human caspase-6 cDNA was obtained from Jurkat cells by RT-PCR. The active caspase-6 gene, in which the coding sequence of the small subunit was not replaced with the immunoglobulin gene, was inserted into an expression vector. The caspase-6 gene was then inserted into the human HER2 gene, resulting in the generation of the immunocasp-6 molecule. The immunocasp-6 gene was next transferred into BALB/c athymic mice bearing human breast SK-BR-3 tumors by i.a. injection of liposome-encapsulated vectors, by intratumor injection of adenoviral vectors, or by i.v. injection of PBMC modified by retroviral infection. Regardless of the method used, expression of immunocasp-6 suppressed tumor growth and prolonged animal survival significantly. Our data show that the chimeric immunocasp-6 molecule can recognize HER2-positive tumor cells, promptly attack their nucleus, and induce their apoptotic death, suggesting the potential of this strategy for the treatment of human cancers that overexpress HER2. The Journal of Immunology, 2004, 173: 61–67.
preceded that of the large subunit, was generated by PCR-based gene splicing using the overlap extension method (30). Recombinant immunocaspase-6 was generated by sequential fusion of the genes of a signal peptide (Met-Lys-His-Leu-Trp-Phe-Phe-Leu-Leu-Leu-Val-Ala-Ala-Pro-Arg-Trp-Val-Leu-Ser), a single-chain HER2 Ab (ε23Fv), a PEA translocation domain (from aa 253 to 364 or from 253 to 412) (31–33), and an active caspase-6 gene with or without NH₂-terminal fusing of a Val-Glu-Ile-Asp (VEID) peptide (Fig. 1). The immunocaspase-6 gene was cloned into a pCMV plasmid. To test the effect of the residual PEA translocation domain on active caspase-6, we used PCR to acquire a fusion gene that encodes a truncated PEA translocation domain (from aa 280 to 412) combined with a reverse caspase-6 gene (tpc-6). This construct was cloned into a pCDNA3 vector and further into a pLND vector, representing a commercial and inducible mammalian expression system (Complete Control; Stratagene, La Jolla, CA), in which ecdysone analogue (ponasterone A) was used to induce gene expression in HeLa cells. All constructs were verified by DNA sequencing.

Cells

Our human breast cancer (SK-BR-3) and human ovarian cancer (SKOV-3) cell lines are both HER2-positive, whereas the human uterine cervical carcinoma (HeLa), human glioma (BT235), mouse embryo fibroblasts (PA317 and NIH 3T3), and human embryo kidney epithelial (HEK 293) cell lines are all HER2-negative, as demonstrated by immunofluorescence staining (data not shown). The cells were all cultured in DMEM (Invitrogen, Carlsbad, CA) with 4 mM l-glutamine adjusted to contain 1.5 mM of sodium bicarbonate and 10% FCS at 37°C and 5% CO2. Human normal PBMCs were separated from blood and cultured in DMEM in the presence of 10% FCS and PHA (Sigma-Aldrich, St. Louis, MO).

Western blot analysis

The lysates of tpc-6-transfected cells and the serum-free supernatants of cells permanently transfected with immunocaspase-6 were analyzed by SDS-PAGE. Proteins were blotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) and detected with primary Abs that recognize caspase-6 (1:200; BD PharMingen, San Diego, CA), lamin A (1:50; Chemicon International, Temecula, CA), or lamin C (1:50; Chemicon International, Temecula, CA). Alkaline phosphatase-linked anti-mouse or anti-rabbit IgG (1:100; Sigma-Aldrich) were used as secondary Abs and detected with NBT-5-bromo-4-chloro-3-indolyl phosphate (Pierce, New York, NY).

Caspase-6 activity assay

To assay for caspase-6 activity in vitro (Active Caspase-6 set; BD PharMingen), we added 10 μl of Acetyl (Ac)-VEID-7-amino-4-trifluoromethylcoumarin (AFC) into 1 ml of caspase-6 activity assay buffer: 20 mM Pipes (pH 7.2), 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% (v/v) CHAPS, 10% sucrose. In a separate tube, 10 μl of Ac-VEID-CHO and 10 μl of Ac-VEID-CHO, which was an inhibitor of caspase-6, were added into 1 ml of caspase-6 activity assay buffer. Then lysate of tpc-6-transfected cells induced by ponasterone A were added for various time points to each tube. After incubation for 1 h at 37°C, the AFC liberated from the Ac-VEID-AFC was measured with a spectrofluorometer with an excitation wavelength of 400 nm and an emission wavelength of 480–520 nm (peak, 505 nm).

DNA fragmentation assay

Genomic DNA was isolated with the Apoptosis DNA Ladder kit (Boehringer Mannheim, Mannheim, Germany) from pIND/tpc-6-transfected cells that had been induced by 20 μmol/L ponasterone A for 24 h. Agarose gel electrophoresis was performed with 50 V for 2 h.

PCR amplification assay

Jurkat cells were transfected with constructed pCMV-immunocaspase-6 using pCMV as a control, and were selected with 800 μg/ml G418 (Invitrogen) for over 2 wk. The genomic DNA was extracted from the cells. Using 5′-GTCTCAAGGGCAAGGCCAC-3′ as the upstream primer and 5′-CTGCAGCGGATARCCGCA-3′ as a downstream primer, genomic PCR amplification was performed with 96°C denaturing 30 s, 55°C annealing 1 min and 72°C extending 1 min. The PCR products were analyzed through agarose gel electrophoresis.

Dot blot analysis

The total RNAs were isolated from the Jurkat cells transfected and were dotted into a nitrocellulose membrane with PCR products of caspase-6 large subunit as a positive control and PCR products of PEA translocation domain as a negative control. The cDNA of caspase-6 large subunit was labeled by digoxigenin to be a probe, and the dots were detected following the user manual of digoxigenin DNA Labeling and Detection kit (Boehringer Mannheim).

Retrovirus production

Immunocaspase-6 gene was cloned into a retroviral vector: pLNCX, then pLNCX/immunocaspase-6 was transfected into PA317 packaging cells with pLNCX only serving as a control. The virus-containing supernatants, Re-immunocaspase-6 and Re alone, which were collected respectively from the culture medium of PA317 cells transfected with pLNCX/immunocaspase-6 and pLNCX alone, were frozen in aliquots at −80°C. The viral titers were determined by infecting NIH 3T3 cells with serially diluted supernatant fluids in the presence of 10 μg/ml polybrene (Sigma-Aldrich); the titers of both Re-immunocaspase-6 and Re were 4 × 10⁵ PFU/ml.

Adenovirus production

Immunocaspase-6 was cloned into an adenoviral pShuttleCMV vector (Stratagene). The resultant pShuttleCMV/immunocaspase-6 and pShuttleCMV were cotransfected separately with an adenoviral backbone plasmid, and were selected with 800 μg/ml G418 (In-vitrogen) for over 2 wk. The genomic DNA was extracted from the cells.

FIGURE 1. Schematic diagram of caspase-6-based gene constructs.
pAdeasy-1, into BJ5183 cells, in which efficient homologous recombination yielded adenoviral DNA harboring the immunocasp-6 gene. The recombinant DNA was identified by restriction endonuclease digestion and then transfected into HEK 293 cells after linearization. The cells were collected 1–10 days after transfection, when a cytopathic effect appeared, and the generated recombinant adenoviruses were isolated. Recombinant adenoviruses, Ad-immunocasp-6 and Ad alone, were obtained, and titers were determined by plaque assay of the infected HEK 293 cells with serially diluted supernatant. The titers of the primary viral stocks produced are generally in the range from 10^7 to 10^8 PFU/ml. Virus stocks were amplified by culturing HEK 293 with low-passage virus stocks. One round of amplification generally produced a 10-fold increase in titer. Amplification continued until the titer reached 10^12 PFU/ml.

### Tumor growth model

Six- to eight-week-old BALB/c athymic mice were purchased from National Rodent Laboratory Animal Resources, Shanghai Branch (Shanghai, China), and were cared and used in compliance with institutional guidelines. The mice were inoculated s.c. with 2 × 10^6 human breast cancer SK-BR-3 cells. Tumors were allowed to grow until they reached a diameter of 5–7 mm (day 0). The mice were then randomly divided into different treatment groups.

### Antitumor activity of immunocasp-6 in vivo

Mice bearing SK-BR-3 tumors were treated with the immunocasp-6 delivered by the following vehicles. In one set of experiments, mice received 10 μg of pCMV/immunocasp-6 or 10 μg of pCMV control vector, both encapsulated by 20 μl of liposome every 3 days. In the second set of experiments, mice were injected i.v. each week with human PBMCs 2 × 10^8 PFU adenovirus Ad-immunocasp-6, 1 × 10^8 PFU Ad (control adenoviral construct), or PBS (control treatment) were given to the groups.

### Statistical analysis

Statistical analysis was performed with the SPSS10.0 software package for Windows (SPSS, Chicago, IL). 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 (ANCOVA) method, with comparisons between treatment groups made by covariance test with the beginning differences occurring by grouping eliminated. Statistical significance was based on a value of p ≤ 0.05.

### Results

The underlying hypothesis of this study was that, an immunocasp-6 gene would be expressed and secreted from the transfected cells, analogous to previous results with immunotoxins (5, 23, 29, and 34). The protein would then bind to HER2-positive tumor cells, be internalized, undergo autoprocessing between PEA Arg^{270} and Gly^{280}, and induce apoptotic cell death (35).

**FIGURE 3.** Proapoptotic effect of tpc-6 gene transfection. A, Survival curves for HeLa cells tested at 1–6 days after treatment with ponasterone A or mock treatment. B, Genomic DNA was isolated from tpc-6 gene-modified HeLa cells at 24 or 48 h after addition of 20 μmol/L ponasterone A and separated on 0.7% agarose gel: DNA marker (M1) 2000, 1000, 750, 500, 250, and 100 bp; DNA ladder marker (M2); aa 364, 48 h (lane 1); aa 364, 48 h, VEID (lane 2); aa 412, 48 h (lane 3); aa 412, 48 h, VEID (lane 4); aa 412, 24 h, VEID (lane 5); aa 412, 12 h, VEID (lane 6); vector, 48 h (lane 7).

**FIGURE 4.** Detection of immunocasp-6 protein secreted by genetically modified Jurkat cells. The clones were established by transfection of Jurkat cells with pCMV/immunocasp-6 (with pCMV as a control) by selection with G418 (800 μg/ml). A, Genomic DNA was isolated from the Jurkat cell clones and analyzed by PCR: DNA marker (M) 2000, 1000, 750, 500, 250, and 100 bp; aa 412, VEID (lane 1); aa 412 (lane 2); aa 364, VEID (lane 3); aa 364 (lane 4), vector (lane 5). B, The Jurkat cell clones were collected and isolated for dot blot analysis to demonstrate the transcription of immunocasp-6 genes: positive control (dot blot 1), PCR products of caspase-6 large subunit; negative control (dot blot 2), PCR products of PEA translocation domain 253–412; Jurkat pCMV (dot blot 3); Jurkat pCMV/immunocasp-6 (dot blot 4) aa 412, VEID; Jurkat pCMV/immunocasp-6 (dot blot 5) aa 412, Jurkat pCMV/immunocasp-6 (dot blot 6), aa 364, VEID; Jurkat pCMV/immunocasp-6 (dot blot 7) aa 364. C, Serum-free medium of immunocasp-6 gene modified Jurkat cells was condensed 3 day after its renewal. Western blot analysis was performed to examine the secretion of immunocasp-6 proteins in the medium: Medium pCMV (lane 1); medium pCMV/immunocasp-6 (lane 2) aa 412, VEID (84 kDa); medium pCMV/immunocasp-6 (lane 3) aa 412 (84 kDa); medium pCMV/immunocasp-6 (lane 4) aa 364, VEID (78 kDa); medium pCMV/immunocasp-6 (lane 5) aa 364 (78 kDa).
analyzed their expression by Western blot analysis and assessed the cleavage of the caspase-6 to caspase-8 and to lamin A (Fig. 2A), a structural protein present in the nuclear envelope of cells that is typically cleaved during apoptosis (36). When evaluated by substrate cleavage assays (Active Caspase-6 set; BD PharMingen), all four types of tpc-6 showed the ability to cleave the test substrate (Fig. 2B); the highest efficiency of cleavage (Fig. 2C) was achieved with tpc-6 (aa 280–412; VEID).

In the presence of 20 µmol/L ponasterone A, cells transfected with pIND/tpc-6 clearly expressed tpc-6 proteins, which accounted for the induction of irreversible cell death. The cell death ratio correlated with the time after ponasterone A treatment (Fig. 3A). Definitive evidence of apoptosis was provided by agarose gel electrophoresis, showing the fragmentation of genomic DNA (i.e., DNA ladder) (Fig. 3B). Taken together, these results show that tpc-6 can produce strong proapoptotic activity in vitro, with little or no evidence of adverse effects from the NH2-terminal leader sequence present in the fusion product.

**Immunocasp-6 modified Jurkat cells specifically kill HER2-positive tumor cells in vitro**

To investigate the HER2 targeted proapoptotic activity of immunocasp-6 proteins, we transfected human lymphoma Jurkat cells with pCMV/immunocasp-6 vectors, obtaining cell clones that stably expressed the gene of interest. PCR analysis of genomic DNA from the transfected Jurkat cells showed integration of immunocasp-6 (Fig. 4A). RNA dot blots confirmed the presence of immunocasp-6 mRNA in the cells (Fig. 4B), whereas Western blotting of concentrated culture medium demonstrated the expression and the secretion of immunocasp-6 proteins (Fig. 4C).

The growth of Jurkat cells expressing immunocasp-6 appeared normal, suggesting that the chimeric protein does not adversely affect the Jurkat cells themselves (Fig. 5A). Human breast carcinoma SK-BR-3 cells and human ovarian cancer SKOV-3 cells that overexpress HER2, and human uterocervical carcinoma HeLa cells and human glioma cancer BT325 cells, which are HER2-negative, were then cultured in the growth medium of Jurkat-immunocasp-6 cells. Fig. 5C shows that the medium specifically killed HER2-positive tumor cells (SK-BR-3 and SKOV-3) in a time-dependent manner (Fig. 5C), but not HER2-negative tumor cells (HeLa and BT325) (Fig. 5D). Among the four types of immunocasp-6 molecules that were tested, immunocasp-6 (aa 412; VEID) was the most efficient cytotoxic protein (Fig. 5B) and was chosen for further study. In our experiment, SK-BR-3 cells were more sensitive to the apoptotic activity of immunocasp-6 proteins than were SKOV-3 cells, consistent with the relatively high level of HER2 expression on SK-BR-3 cells. Finally, cocultivation experiments showed that Jurkat-immunocasp-6 cells could specifically recognize and kill HER2-positive tumor cells in vitro (Fig. 5E).

**Liposome-mediated immunocasp-6 gene transduction suppresses the growth of HER2-positive tumor in vivo**

Murine xenograft models were randomly divided into two treatment groups that received i.m. injections of 10 µg of liposome-encapsulated pCMV/immunocasp-6 or pCMV alone. As shown in Fig. 6A, tumors treated with the liposome pCMV/immunocasp-6
complex grew more slowly than those treated with liposome-pCMV complex, suggesting that the immunocasp-6 protein can suppress HER2-positive tumors upon secretion by genetically modified muscle cells (*p* < 0.00002 by ANCOVA test). Similarly, mice treated with pCMV/immunocasp-6 survived longer than mice treated with a control vector (*p* = 0.0289 by the log-rank test). Immunohistochemical analysis confirmed the presence of caspase-6 activity in tumors treated with pCMV/immunocasp-6, but not in those treated with pCMV (Fig. 6B). The TUNEL assay identified apoptotic cells in tumor tissues treated with liposome pCMV/immunocasp-6 (Fig. 6C).

**Retrovirus-mediated transfer of the immunocasp-6 gene prolongs the survival of HER2-positive tumor-bearing mice**

Mouse SK-BR-3 tumor xenograft models were injected i.v. with 2 × 10⁵ human PBMCs that had been infected with recombinant retroviruses (Re) containing either pLNCX/immunocasp-6 or a control pLNCX vector. The human carcinomas in mice given the immunocasp-6-transduced PBMCs grew more slowly than those treated with mock-transduced PBMCs (Fig. 6A; *p* = 0.0102 by ANCOVA test). Immunohistochemical staining with a caspase-6 Ab indicated that tumors treated with Re-immunocasp-6 were caspase-6 positive, whereas those given Re alone were negative.

**FIGURE 6.** Antitumor effects of immunocasp-6 targeted to HER2 by a liposome-encapsulated, retrovirus-medi-ated, or adenovirus-mediated delivery system. An SK-BR-3 xenograft mouse model was established by s.c. injection of 1 × 10⁶ cells. The mice were injected i.m. with pCMV/immunocasp-6 or pCMV (eight mice each group), both encapsulated by 20 µl of liposome every 3 days five times or injected i.v. once a week (twice) with PBMCs expressing a modified immunocasp-6 gene from a retroviral vector (eight mice each group) or treated on 3 consecutive days by intratumorally injection of recombinant adenovirus (10 mice each group). A. Tumor sizes were measured throughout the experiment. B. Distributions of the killer proteins were detected in tumor tissues by immunohistochemistry. C. Tumor tissues from mouse model were studied by TUNEL staining to evaluate the proportion of apoptotic cells.

**FIGURE 7.** Comparison of survival rates of mice bearing SK-BR-3 xenografts in all groups. The mice received intratumoral injections of 10 µg of pCMV/immunocasp-6 (eight mice) or 10 µg pCMV (12 mice) (both encapsulated by 20 µl of liposome) once a week or weekly injections of PBMCs gene modified by retrovirus infection (10 mice each group), or were treated 3 consecutive days by intratumoral injection of recombinant adenovirus (10 mice each group). Mouse survival times were recorded and survival rates were analyzed by the Kaplan-Meier method; comparisons between treatment groups were made by the log-rank test.
(Fig. 6B). TUNEL staining revealed many apoptotic cells in tumor tissues treated with Re-immunocasp-6 (Fig. 6C). The survival rate of mice treated with Re-immunocasp-6 was significantly higher than the control rate (Fig. 7; \( p = 0.0090 \) by the log-rank test).

Injection of recombinant adenovirus containing the immunocasp-6 gene increases the survival rate of mice engrafted with HER2-positive tumors

Mice bearing SK-BR-3 human tumors were randomly divided into three groups (10 animals each) and given intratumor injections of \( 1 \times 10^6 \) PFU adenovirus/immunocasp-6, \( 1 \times 10^6 \) PFU adenovirus/void, or PBS on 3 consecutive days. Rapid uptake of viruses and viral particles was apparent by 24 h after each injection. Tumors treated with Ad-immunocasp-6 grew more slowly than those treated with Ad or PBS alone (Fig. 6A; \( p = 0.0042 \) by ANCOVA test). An immunohistochemical assay showed that tumors treated with Ad-immunocasp-6 were caspase-6-positive, whereas those treated with adenovirus/void or PBS were all negative (Fig. 6B). Apoptotic cells were clearly present by TUNEL staining in tumor tissues treated with Ad-immunocasp-6 (Fig. 6C). The survival rate of mice treated with Ad-immunocasp-6 was significantly higher than either of the controls (Fig. 7; \( p = 0.0198 \)), which were essentially identical.

Discussion

Many strategies of gene therapy have been designed to kill cancer cells, including their transduction with suicide genes or tumor suppressor genes or activation of the immune system against the tumor cells (37–40). Although many problems have impeded the practical use of gene-based therapy for human cancers, the search for safe, effective, and tissue-specific therapeutic gene products continues. We therefore, generated a novel immunocasp-6 gene by fusing a leader sequence, single-chain HER2 (ε23sFv) Ab and the translocation domain of PEA to the active caspase-6. To select the construct with the greatest tumor killing potential, we evaluated two kinds of translocation domains (PEA; aa 253–364 and aa 253–412) and two versions of active caspase-6 (one with and one without the specific cleavage site of caspase, VEID, in the NH₂ terminus); hence, four types of immunocasp-6 genes were generated altogether.

After confirming that the single-chain HER2 (ε23sFv) Ab will induce immunocasp-6 protein to bind selectively to target cells and that active caspase-6 will kill the detected cells by inducing apoptosis, we showed that the residual PEA translocation domain lacks any undesirable effects on the ability of caspase-6 to kill cells transfected with the ptc-6 construct. Second, Jurkat human lymphoma cells that continuously deliver immunocasp-6 were generated by modifying the cells in vitro. When cocultivated with the modified cells or cultured in their medium, HER2-positive tumor cells (SK-BR-3 and SKOV-3) but not HER2-negative cells, were killed in significant numbers (killing rate up to 94.3%).

The immunocasp-6 gene was also introduced i.m. or intratumorally in SK-BR-3 xenograft mouse models by either liposome encapsulation or adenovirus-mediated infection. Alternatively, the mice were given i.v. injections of PBMCs modified in vitro with the human immunocasp-6 gene. All of the reported treatments strongly inhibited tumor growth and significantly prolonged mouse survival (Fig. 7), without damaging the normal tissues, as judged from the results of immunohistochemistry assay and H&E staining. Thus, our in vitro and in vivo data correlated well, indicating that immunocaspase-6 gene transduction led to strong antitumor activity regardless of the method of delivery used.

Cationic liposome was used as a nonviral in vivo gene transfer system in this study. Compared with liposome-pCMV group, the survival rate of mice treated with liposome pCMV/immunocasp-6 was remarkably increased (\( p = 0.0289 \)), and this comparison demonstrated the efficacy of immunocasp-6 gene to HER2-overexpressing tumors. However, using cationic liposome for in vivo gene transfer is usually less efficient, although it is the most frequently used nonviral gene transfer system in vitro. In our study, compared with adenovirus or retrovirus gene delivery system, liposome seemed adverse for immunocasp-6 to achieve the pro-apoptotic effect in vivo. The comparison between liposome pCMV/immunocasp-6 and other groups, in which immunocasp-6 gene was delivered by different methods, revealed that cationic liposome we used in this study might be somewhat toxic to nude mice. Moreover, among the control groups, log-rank test of survival analysis by SPSS 10.0 (Fig. 7) showed that the survival of liposome-pCMV group was obviously lower than that of any other control groups (\( p = 0.0067 \)), whereas there was little difference between the other three controls (\( p = 0.8149 \)). Considering this toxicity, we thought unlike PBS, adenoviruses, and retroviruses, liposome might be unfit for continuous treatment in vivo, although it showed low toxicity to the cells cultured in vitro.

In this study, two viral vectors that provided effective methods of in vivo gene delivery for therapeutic purposes (41, 42) were used: retrovirus and adenovirus. In the treatment of SK-BR-3 xenograft mouse models, we found that retrovirus-immunocasp-6 (\( p = 0.0090 \)) and adenovirus-immunocasp-6 (\( p = 0.00198 \)) were similarly effective in raising the survival rate of the nude mice. There was a marked difference in the survival rate between the control and the treatment groups. Meanwhile, there was no significant difference between the survival rate of both the control (\( p = 0.4766 \)) and the treatment (\( p = 0.7248 \)) groups. However, the above comparison between the different therapeutic approaches may have limitations as in our experiment we did not use the unitarian criteria on the activity and titer of different virus or the number of lymphocytes injected into the mice, which needs further study indeed. Whether the retrovirus or the adenovirus vector will be used, various aspects should be further probed into so as to improve this novel gene therapy strategy.

In summary, the results reported describe the novel immunocasp-6 gene of detecting and killing HER2-positive tumor cells efficiently and selectively, which may hold great promise for the generation of a novel therapy for HER2-overexpressing tumors.

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