Cancer Gene Therapy with Plasmid Expressing Anti-HER2/Neu Toxin

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***Abstract*—**The HER2/neu receptor is over-expressed on the surface of many types of cancer cells, and is widely used for targeted delivery of anticancer drugs. We have created genetically engineered construct expressing the PE40 fragment of Pseudomonas toxin bound with the DARPin molecule which recognizes the HER2/neu receptor with high specificity. Intra-tumor injections of the construct complexed with polyethyleneimine led to growth retardation of D2F2/E2 tumors in mice. These results suggest a possibility of using this approach to develop new anticancer drugs.

***Keywords*—** gene therapy, DARPin, Pseudomonas exotoxin A, receptor HER2/neu, targeted therapy.

I. Introduction

The HER2\neu receptor is a member of the growth factors receptors family. Over-expression of this receptor is found in 20-30% carcinomas of the mammary gland, ovary and kidney, and is correlated with rapid growth of the tumor, resistance to chemotherapy and a bad prognosis of the disease outcome (1). Owing to its tumor-specificity the HER2\neu receptor is often employed in development of targeted anti-tumor drugs. Thus, the widely used Herceptin preparation is a monoclonal antibody binding to Her2/neu. The use of Herceptin increases the 5-year patient survival probability from 13 to 24 percent (2). The efficiency would likely be higher if 70% of the patients did not develop resistance to the drug. One way to overcome resistance to therapy and enhance its effectiveness is the conjugation of targeting molecules, e.g. antigens, with toxins. The most often used toxins for this purpose include the diphtheria toxin, pseudomonas toxin, and ricin. After getting into the cytoplasm, these toxins inactivate elongation factor 2, leading to cessation of protein synthesis. Their efficiency is very high, thus even one molecule of diphtheria toxin may kill the cell (3). A number of such toxins have been created and studied to date. However, their clinical efficiency as a rule is low. One of the main reasons for the failures is the high unspecific activity of the immunotoxins which prevents using them at concentrations that are optimal for destroying cancer cells (4).

A promising approach to reduce the toxic side effects of immunotoxins may be their local expression within tumor by using gene constructs which code for the required toxins. In this work we investigated feasibility of this approach for therapy of HER2/neu-positive mammary gland tumors. We created a construct CAG-ETA which expresses a fragment of pseudomonas toxin linked with the DARPin molecule that is capable of highly specific recognition of the HER2/neu receptor. We demonstrated that CAG-ETA construct, complexed with linear polyethyleneimine, can retard tumor growth following intra-tumor injections in mice.

II. Material and Methods

A. Plasmid construction

The coding sequence of the targeting module DARPin_9-29 (5) was amplified from plasmid pCG-Hnse-DARPin-d18-9-29 using the primers 5'–tattccatatggacctgggtaagaaactg (forward) and 5'-cgccaagcttttgcaggatttcagc (reverse). The PCR product was then treated with restriction endonucleases NdeI and HindIII and ligated with vector pET22b pretreated with the same endonucleases. The coding sequence of PE40 (a fragment of natural exotoxin A from Pseudomonas aeruginosa, amino acids 252-613) was amplified from plasmid pSD-4D5-ETA (6) together with the 16-amino-acid linker EFPKPSTPPGSSGGAP from the hinge region of murine IgG using the primers 5'-ccgttaagcttgggagttcccgaaaccgtccac (forward) and 5'-gcggaattcctacagttcgtctttatggtg (reverse). The resulting PCR product was treated with HindIII and EcoRI and cloned into pET22b in the same reading frame with the coding sequence of DARPin_9-29. The resulting plasmid was digested with NdeI and NotI to isolate the DARPin_9-29-PE40 fragment, which was ligated with the linker coding for the signal peptide of human albumin (primers 5`-aattcatgaagtgggtaacctttatttccctttttctctttagctcggcttattc-3` and 5`-agaataagccgagctaaagagaaaaagaagggaaataaaggttacccacttcatg-3`) together with plasmid PB-CAG-RL (7) digested with EcoRI and NotI. As a result of this three-component ligation the required plasmid CAG-ETA was obtained.

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B. Transfection in vivo

Transfection was carried out using linear polyethyleneimine (25 kD, Polysciences, cat#23966). Dry polyethyleneimine was dissolved in 0.2 N HCl to 5 mg/ml, aliquoted and stored at -200 C. Plasmid DNA was isolated from bacterial cells using a GeneJET Plasmid Maxiprep Kit purchased from Fermentas (cat#K0492). For each intra-tumor injection 25 µg of plasmid DNA was dissolved in 50 µl of 5% glucose solution and mixed with 50 µl of 5% glucose solution containing 2 mg/ml polyethyleneimine. The mixture was thoroughly stirred and kept at room temperature for at least 15 min; it was used to inject mice within an hour after preparation.

C. Assessment of anti-tumor activity in vivo

D2F2/E2 cells were grown in Dulbecco-modified Eagle medium (DMEM) supplemented with inactivated 10% calf fetal serum, 2 mM L-glutamin to visual density of 90-100%, removed from plastic and resuspended in phosphate-buffered saline (PBS). Cell concentration was measured in a hemocytometer (Goryaev chamber) and brought to 4x106 cells/ml. The cell suspension was injected subcutaneously (100 µl) to BALB/c mice into the infrascapular region with an insulin syringe. Ten days later mice that had palpable tumors were selected and randomly distributed among experimental and control groups. Each group had 7 animals. The experimental or control plasmid complexed with polyethyleneimine were injected in 100 µl into the tumor area with an insulin syringe. The length and width of the tumors were measured with a slide caliper. Tumor volumes were calculated using the formula V(mm3) = a (mm) × b2(mm2)/2, where a and b are length and width, respectively. All experiments with animals were approved by Institutional Animal Care and Use Committee. The maximum allowable tumor size was 2 cm3, at which point the mouse was sacrificed by cervical dislocation.

D. Statistical analysis

Statistical analysis of tumor size distribution in the experimental and control groups was done using the Mann-Whitney test (http://www.socscistatistics.com/tests/mannwhitney/default2.aspx)..

III. Results and Discussion

Perhaps the main difficulty of using gene constructs for local expression of strong targeted toxins is the low level of their expression. As a rule, onset of synthesis of the toxin in a transfected cells leads to its death, and the cell has enough time to synthesize the toxin only in very small quantities. This explains the fact that most studies that employ expression of potent toxins for gene therapy employ non-secreted toxins which are designed to destroy only the transfected cells (e.g. 8, 9). More recently works on gene therapy with constructs encoding secreted toxins have been published (10, 11). These studies report the so-called bystander effect which means that in spite of a low level of expression, in some cases this approach can offers not only destruction of transfected cells but also intact cells. In our work we decided to create a gene construct based on the toxin DARPin_9-29-ETA and examine the presence of tumor-suppressive effect on the breast cancer model. The DARPin_9-29-ETA toxin consists of pseudomonas toxin fragment (the translocational domain II and domains Ib and III of the natural toxin that are responsible for ADP-ribosylation of eukaryotic elongation factor 2) linked to the DARPin molecule which has high affinity to the HER2/neu receptor. The toxin DARPin_9-29-ETA was selected for gene therapy as most efficient at very low concentrations. We believe that since the cell will synthesize the protein for a very short time, to achieve the bystander effect it is necessary to use the most powerful toxins. The design of such a targeted toxins is well reviewed in a recent paper (12). The DARPin_9-29-ETA toxin was derived from 4D5-ETA (6) by way of replacing the targeting portion of antibody 4D5 with the ankyrin repeats which specifically recognize the HER2/neu receptor (5). According to our information, the change increases cytotoxicity of 4D5-ETA (IC50 = 22 nM) by more than 4 orders of magnitude. The CAG-ETA plasmid was created to express DARPin_9-29-ETA in mammalian cells. It consists of a strong synthetic CAG promoter which controls expression of the DARPin_9-29-ETA, linked to coding sequence of signal peptide of human albumin. We have chosen to use linear polyethyleneimine for delivery of our construct into the murine cells. It offers lower efficiency of transfection than viruses but it is much cheaper, easier to use, and less immunogenic, which facilitates using multiple injections. We induced tumors in mice with D2F2/E2 cells. This line was isolated from mammary gland tumors of BALB/c mice. A distinguishing feature of these cells is expression of the human HER2/neu receptor, thus making them suitable for testing compounds that bind to this receptor. Despite the presence of the human HER2/neu receptor at their surface, D2F2/E2 cells are capable of inducing tumors when injected to BALB/c mice. D2F2/E2 cells have shown low transfection efficiency in our in vitro experiments regardless of transfection agent employed. For instance, with DNA-polyethyleneimine complexes the efficiency was about 10%. Despite the low transfection efficiency, we decided to study the possibility of inhibiting D2F2/E2-induced tumors by intra-tumor injections with CAG-ETA-polyethyleneimine complexes. In initial experiments we injected mice only once on day 16 after tumor induction (Fig 1a). It can be seen that a single injection did not affect tumor growth to any significant extent. In the next series of experiments we injected mice on days 10, 14 and 20 after induction (Fig.1b). The difference between experimental and control groups was noticeable starting from day 24, and on day 42 the average difference in tumor volume reached 1.8-fold. This difference is statistically significant at P=0.045. Thus, we can conclude that three consecutive injections of the CAG-ETA-polyethyleneimine complexes result in retardation of D2F2/E2 tumor growth in mice.

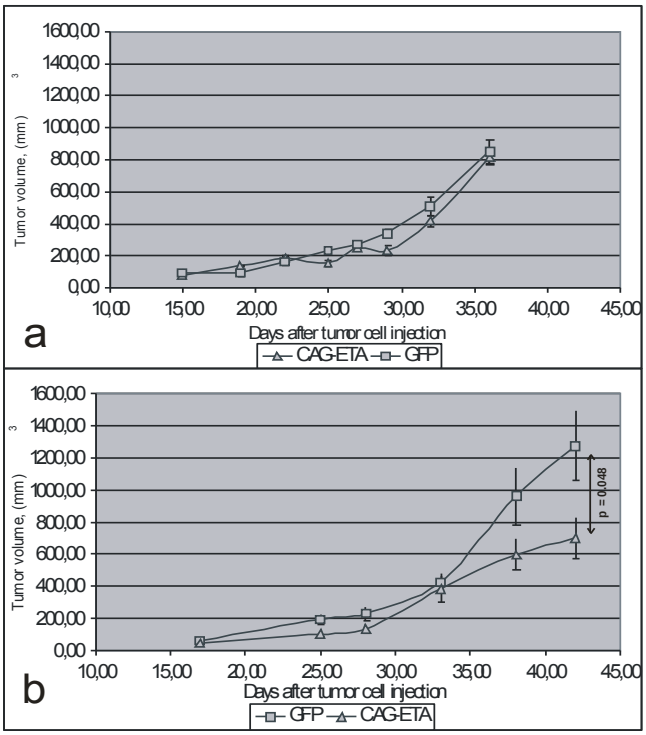

Fig. 1. Progression of D2F2/E2 tumors in BALB/c mice following injections of the CAG-ETA-polyethyleneimine complexes. Results are presented as mean values±SEM (error bars) (A) A single injection on day 16 of tumor progression. (B) Three injections on days 10, 14 and 20

IV. CONCLUSION

We have demonstrated statistically significant tumor-suppressive effect of injecting CAG-ETA-polyethyleneimine complexes into mouse tumors consisting of D2F2/E2 cells. The multiple injections of complexes are needed to achieve significant tumor retardation. After triple injection, the tumor volume in the experimental group was about 1.8 times less than in the control group. Thus, these data demonstrate the feasibility of using this approach to develop anti-cancer therapy.

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