Gene therapy using plasmid DNA-encoded anti-HER2 antibody for cancers that overexpress HER2

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Plasmid DNA-encoded antibodies, or DNA-based monoclonal antibodies (dMABS), are delivered by intramuscular injection and in vivo electroporation (EP) and are effective in virus neutralization, although they have not been evaluated for tumor gene therapy. Here we investigated whether a dMAb was appropriate for tumor gene therapy. We constructed the expression plasmids coding for the heavy or light chain of a parental murine antibody of Herceptin with the antibody genes codon- and RNA-optimized and fused to the Kozak-IgE leader sequence in pVax1. Transfection of the plasmids into human muscle RD cells resulted in functional expression of the antibody, and this exhibited the same in vitro antiproliferative activity as Herceptin. A single intramuscular injection and in vivo EP of the plasmids (100 μg per head) resulted in high and sustained antibody expression in the sera of normal mice and in effective inhibition of tumor growth in nude mice bearing HER2-positive human breast carcinoma BT474 xenografts. The antitumor efficacy of the anti-HER2 dMAb was similar to that of four doses of intravenously injected 10 mg kg−1 Herceptin. The results demonstrate that the dMAb is effective in the treatment of HER2-positive breast cancer, suggesting that this dMAb may be applicable for tumor gene therapy.

INTRODUCTION

Therapeutic antibodies represent the most successful biological drugs. In the past 30 years, >40 therapeutic antibodies have been approved for clinical use in various indications, including cancers, autoimmune diseases, infectious disease and neovascular disorders.1 Thus therapeutic antibodies represent one of the fastest growing areas of the pharmaceutical industry. The global market for antibody therapeutics in 2014 was $75 billion.1 The remarkable success was driven by technological evolution from murine monoclonal antibodies (mAbs) to chimeric and humanized antibodies, human mAbs and antibody–drug conjugates.2 In oncology, therapeutic antibodies targeting growth factor receptors such as HER2/neu or epidermal growth factor receptor have been proven effective for the treatment of metastatic breast cancer or colorectal and head and neck cancers, respectively.3,6

The clinical impact of therapeutic antibodies is remarkable. However, production of the mAbs requires high-cost manufacturing and complicated technologies, especially those associated with large-scale mammalian cell culture and antibody purification.7–9 These requirements have limited their application to broader populations, particularly in developing countries. In this regard, the manufacturing for in vivo gene therapy is greatly simplified and the production costs are much lower, which will likely facilitate the application of therapeutic antibodies to a greater population. There are two general approaches to deliver genes into a cell: viral and non-viral. Viral vectors are highly efficient in introducing genes but can create some safety risks, whereas non-viral vectors are much safer than viral vectors but may have inefficient gene delivery.10–13 In the past several years, there has been a great advance in the plasmid DNA delivery technology that is utilized for in vivo production of proteins. This included codon optimization for expression in human cells, RNA optimization to improve mRNA stability as well as more efficient translation at the ribosomal level, the addition of specific leader sequences to enhance translation efficiency, the creation of synthetic inserts to further enhance production in vivo and the use of improved adaptive electroporation (EP) delivery protocols to improve in vivo delivery.14–25

The synthetic DNA-encoded antibody approach, or DNA-based monoclonal antibody (dMAb) technique, has not been applied to tumor gene therapy. In the present study, we evaluated a model antibody for tumor gene therapy. We constructed expression plasmids that can express the heavy or light chain of a parental murine mAb (mumAb4D5)31 of trastuzumab (Herceptin), which has been clinically used to treat metastatic breast cancers overexpressing HER2, and evaluated in vivo expression and antitumor efficacy after a single intramuscular injection and in vivo EP. The anti-HER2 dMAb resulted in high and sustained expression of the murine mAb in mouse sera and effective inhibition of tumor growth in nude mice bearing HER2-positive human breast carcinoma xenografts.
MATERIALS AND METHODS
Cells and reagents
HEK293T and human muscle RD cells (ATCC CCL-136, Manassas, VA, USA) were cultured in Dulbecco’s modified Eagle’s medium (Welgene, Gyeonsan-si, Korea) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT, USA). Human breast carcinoma BT474 cells were obtained from ATCC (lot number, 59758899) and cultured in Dulbecco’s modified Eagle’s medium/F12 (Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum. All cells were cultured in 5% CO2 in a 37 °C humidified incubator.

Recombinant HER2 (ERBB2-His) was obtained from A&R Therapeutics (Daejeon, Korea). Anti-mouse IgG F(ab’)2 antibody and peroxidase-conjugated anti-mouse IgG (Fc specific), anti-mouse IgG (H+L), anti-mouse IgG1 and anti-mouse IgG2a antibodies were obtained from Pierce Biotechnology (Thermo Fisher Scientific, Rockford, IL, USA).

Construction of 4DS-mIgG antibody expression plasmids
The light chain variable region (VL) sequence of mumAb4DS was modified by replacing asparagine at amino acid 65 with serine to remove potential N-glycosylation site. The modified VL was fused to mouse Cγ1 or Cγ2a to express mouse IgG1 or IgG2a isotype of mumAb4DS, respectively. The genes encoding the heavy or light chain were flanked with a Kozak-IgE leader sequence and cleavage sites for EcoRI and NotI. The resulting modified genes were codon- and RNA-optimized and synthesized by DNA synthesis service (GeneArt, Thermo Fisher Scientific, Washington, NC, USA), digested with EcoRI and NotI and individually subcloned into the EcoRI-NotI site of pVax1 (Invitrogen) and transfection reagent (Invitrogen) according to the supplier’s instruction.

Construction of 4D5-mIgG antibody expression plasmids
The heavy chain variable region (VH) of mumAb4DS was fused to mouse Cγ1 or Cγ2a to express mouse IgG1 or IgG2a isotype of mumAb4D5, respectively. The genes encoding the heavy or light chain were flanked with a Kozak-IgE leader sequence, the heavy chain gene encoding the heavy chain variable region (VH) of mumAb4D5 and mCk and subcloned into pVax1 to construct pVax1-4D5-mlgG-L.

Expression of 4DS-mIgG in RD and HEK293T cells
The heavy and light chain expression plasmids were co-transfected into the RD cells or HEK293T cells using the Lipofectamine 2000 transfection reagent (Invitrogen), according to the manufacturer’s instruction. Briefly, the cells (1 x 10⁶) were seeded in T75 flask at 24 h before transfection, and 10 μg of the pVax1 control or mixture of 5 μg each of the heavy and light chain expression plasmids was transfected. After transfection, the cells were cultured in serum-free medium for 48 h, and the culture supernatants were subjected to western blotting analysis and quantitative enzyme-linked immunosorbent assay (ELISA) to assess the expression levels of the antibody. Supernatants from pVax1-transfected cells were used as a negative control. The culture supernatants of transfected HEK293T cells were subjected to an affinity chromatography on Protein G-Sepharose 4B column (GE Lifesciences, Buckinghamshire, England) for antibody purification, according to the supplier’s instruction.

Western blotting analysis
Supernatants from transfected cells were subjected to 4–12% Bis-Tris sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the protein bands in the gel were transferred to polyvinylidene difluoride membranes (Invitrogen). The membranes were incubated with HRP-conjugated goat anti-mouse IgG (H+L) (Pierce), and immunoreactive bands were visualized using a HRP chromogenic substrate (Invitrogen).

Quantitative ELISA
Microtiter wells were coated with the purified anti-mouse IgG F(ab’)2 (100 ng) diluted in 100 μl phosphate buffered saline (PBS) at 4 °C overnight and blocked with skim milk (2%) in PBS for 1 h at 37 °C. Subsequently, diluted culture supernatant or normal mouse IgG (Pierce) as standard was added to each well and then incubated at 37 °C for 1 h. The wells were washed three times with PBS containing 0.05% Tween 20 (PBST), and then goat anti-mouse IgG (Fc specific)-HRP (1:10 000) was added and incubated at 37 °C for 1 h. The wells were washed with PBST and developed with O-phtalaldehyde (OPA) substrate (BD, San Diego, CA, USA). The absorbance was measured at 450 nm in a microtiter plate reader (Versa max, Molecular Devices, Sunnyvale, CA, USA).

Indirect ELISA
Microtiter wells were coated with 100 ng of HER2 (ERBB2-His, A&R Therapeutics, Daejeon, South Korea) diluted in 100 μl PBS for 4 °C overnight and blocked with skim milk (2%) in PBS for 1 h at 37 °C. After
incubation, diluted culture supernatant or serum was added to each well and incubated for 1 h at 37 °C. The wells were washed three times with PBST, and then goat anti-mouse IgG-HRP was added and incubated for 1 h at 37 °C. The wells were washed and developed as described above. For quantitation of a murine MAb in the mouse sera, purified 4D5-mIgG1 or 4D5-mIgG2a was used as a standard.

In vivo assessment of 4D5-mIgG expression
Each 50 μg of the heavy and light chain plasmids in a 50 μl volume was injected intramuscularly into the female BALB/c mice (8 weeks of age), followed by EP using CELLECTRA adaptive constant current EP device (Inovio Pharmaceuticals Inc., Plymouth Meeting, PA, USA). Pulsing parameters for delivery were three pulses of 0.2 Amp constant current, 1 s apart, 52 ms in length. Each animal received a single administration of either experimental or control plasmid formulations. The mice were bled and sera were collected at each time point. Mouse sera were diluted to 1:50 and then analyzed by ELISAs.

In vitro tumor cell proliferation assay
BT474 cells (10^4 cells per well) were seeded in 96-well plates (SPL Life Sciences, Pocheon-si, Korea) and incubated at 37 °C in 5% CO₂ incubator for 24 h. Different concentrations of antibody were added to each well, and incubation was continued for 72 h. Water-soluble tetrazolium-1 (WST-1) reagent (10 μl, Roche Diagnostics GmbH, Mannheim, Germany) was added to each well and the plate was incubated in 5% CO₂ incubator for 2 h. The absorbance was measured at 450 nm with a reference at 650 nm in a microplate reader (Versa max, Molecular Devices).

In vivo tumor growth inhibition study
Female BALB/c nude (nu/nu) mice (4 weeks of age), obtained from Japan SLC, Inc. (Hamamatsu, Japan), were maintained for 2 weeks in a pathogen-free environment, and all animal experiments were performed in accordance with the Biototex Co., Ltd. (Cheongwon-gun, Korea) guidelines established for animal care and use. Estrogen (72 ng) was injected into the left flank of each mouse, and 2 days later, BT-474 cells (5 x 10^5 cells in 0.1 ml PBS) were subcutaneously injected into the right flank of each mouse. To test the antitumor activity of Herceptin, when tumors reached to 108–162 mm^3 in size, tumor-bearing mice were randomized into two groups (n = 8 per group), and Herceptin (8 mg kg⁻¹) or human Fc (hFc, 2.7 mg kg⁻¹) was intravenously injected into each mouse twice a week for 4 weeks. To test the antitumor activity of anti-HER2 dMAb, when tumors reached to 40–60 mm³ in size, 100 μg of plasmid DNA (4D5-mIgG2a expression plasmids or pVax1) or water as a control was injected intramuscularly into each animal followed by EP-mediated enhanced delivery by the CELLECTRA adaptive constant current EP device (Inovio Pharmaceuticals Inc.). At day 7, Herceptin (10 mg kg⁻¹) was intravenously injected into each mouse twice a week for 14 days. Body weight and tumor volume were measured twice a week for 22 days. Tumor volume was measured as the mean ± s.d. from the triplicate experiments.

Figure 2. In vitro analyses of the expression and antitumor activity of 4D5-mIgG1 and 4D5-mIgG2a. (a) The human RD cells transfected with pVax1 as a control or the expression plasmids for mIgG1 or mIgG2a were cultured in serum-free medium, and the culture supernatants (19.5 μl per well) were subjected to western blotting analysis under non-reducing conditions using anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) conjugate (left panel). In addition, the 4D5-mIgG1 and 4D5-mIgG2a antibodies purified from the culture supernatant of transfected HEK293T cells were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions (right panel). (b) The amount of the expressed 4D5-mIgG1 or 4D5-mIgG2a in the culture supernatants of the transfected RD cells was measured by quantitative enzyme-linked immunosorbent assay (ELISA) using anti-mouse IgG (Fc-specific) antibody. (c) The antigen-binding activity of the expressed antibody in the culture supernatants of the transfected RD cells was analyzed by indirect ELISA. (d) Human breast cancer cells (BT474) were incubated with increasing concentrations of human Fc (hFc) as a negative control, Herceptin as a positive control or the purified 4D5-mIgG2a for 72 h and evaluated by a water-soluble tetrazolium-1 assay. Data shown are the mean ± s.d. from the triplicate experiments. OD, optical density.
calculated as follows: length × width² × 0.5. At the end of the study, tumor tissues were taken out and weighed. The tumor growth inhibition rate was calculated using the formula inhibition rate (%) = (1 − T/C) × 100, where T and C are the mean tumor volume or weight of the treated and control groups, respectively.

Statistical analysis
Data are presented as mean ± s.d., and statistical comparisons between groups were performed using one-way analysis of variance followed by Dunnett's test. A value of P < 0.05 was considered significant.

RESULTS
Construction of 4D5-mIgG antibody expression plasmids
To construct the expression plasmids for the anti-HER2 murine mAb, the VL sequence of mumAb4D5 was modified by replacing asparaginase at amino acid 65 with serine to remove a potential N-glycosylation site (Asn65-Arg66-Ser67), similar to that of Herceptin. The modified VL of mumAb4D5 was fused to mouse Ck to construct the light chain of mumAb4D5 while the VH of mumAb4D5 was fused to mouse Cγ1 or Cγ2a to construct the γ1 or γ2a heavy chain of mumAb4D5, respectively. The genes encoding the heavy or light chain were codon- and RNA-optimized and fused to the Kozak and IgE leader sequences. These optimized heavy and light chain genes were synthesized and separately subcloned into the EcoRI-Nool sites of pVax1 to construct heavy (pVax1-4D5-mIgG1-H or pVax1-4D5-mIgG2a-H) and light (pVax1-4D5-mIgG-L) chain expression plasmids, respectively (Figure 1). Thus the heavy and light chain genes were placed under the control of the strong human cytomegalovirus promoter for efficient expression in mammalian cells and tissues. The modified mumAb4D5 containing γ1 or γ2a was named 4D5-mIgG1 or 4D5-mIgG2a, respectively.

In vitro expression of 4D5-mIgG in human muscle RD cells
The heavy and light chain expression plasmids for 4D5-mIgG1 or 4D5-mIgG2a were co-transfected into human muscle RD cells using lipofectamine, and the culture supernatants were analyzed by western blotting analysis and ELISA while pVax1 was transfected into the cells as a negative control. The results showed that the 4D5-mIgG1 and 4D5-mIgG2a were expressed at similar levels (Figures 2a and b) and exhibited the same HER2-binding activity (Figure 2c). The antibody concentration in the supernatant was estimated to be 3.5 μg ml⁻¹ for 4D5-mIgG1 and 2.6 μg ml⁻¹ for 4D5-mIgG2a, as assessed by quantitative ELISA.

In vivo expression analysis of 4D5-mIgG1 and mIgG2a in mice after intramuscular injection and electroporation. (a) A total of 100 μg of the 4D5-mIgG1 or mIgG2a expression plasmids was injected into each BALB/c mouse (n = 5/group) and sera were taken at each time point. Sera were diluted 1:50 and then incubated in triplicate with HER2 antigen for indirect enzyme-linked immunosorbent assay (ELISA). Bound antibody was detected using goat anti-immunoglobulin G (IgG) (H+L)-horseradish peroxidase (HRP). (b and c) Indirect ELISA of the mouse sera using isotype-specific secondary antibody anti-IgG1-HRP (b) or anti-IgG2a-HRP (c). (d) The HER2-binding antibodies in the pooled sera were quantified by indirect ELISA using the purified 4D5-mIgG1 or mIgG2a antibody as standards. The values and bars represent the mean optical density (OD) and s.d.

Antitumor efficacy of anti-HER2 dMAb against a HER2-positive human breast tumor
We examined the antitumor activity of 4D5-mIgG2a because it was produced at a higher level than 4D5-mIgG1 in mouse sera. The expression plasmids were transfected into HEK293T cells and the antibody was purified from the culture supernatant by affinity chromatography using a Protein A column. The purified 4D5-mIgG2a antibody, Herceptin (positive control) or human Fc (hFc, negative control) was added to HER2-positive BT474 cells and evaluated by a WST-1 assay. The 4D5-mIgG2a inhibited the proliferation of the cells as effectively as Herceptin (Figure 2d).

To evaluate the in vivo antitumor efficacy of anti-HER2 dMAb, a BT474 xenograft nude mouse model was first established and the antitumor efficacy of Herceptin was tested. Herceptin (8 mg kg⁻¹) or human Fc (2.7 mg kg⁻¹, control) was intravenously administered twice a week into the BT474 xenograft model for 28 days.
Herceptin moderately inhibited the tumor growth without affecting body weight (Figure 4a). Tumor volume was reduced by 54.6% in the Herceptin treatment group, compared with the isotype control. The tumor weight was decreased by 20.5% following the Herceptin treatment (Figure 4b).

Next the in vivo antitumor activity of anti-HER2 dMAb was evaluated in the same animal model. A single dose (100 μg per head) of the 4D5-mIgG2a expression plasmids, pVax1 or water was intramuscularly injected into nude mice (n=8 per group) bearing BT474 xenografts. At day 7 after DNA injection, Herceptin (10 mg kg\(^{-1}\)) was intravenously injected twice a week for 2 weeks. Tumor volume (c) and body weight (d) were measured twice a week while tumor weight (e) was measured at day 22. Results are represented as the average±s.e. (f) HER2-binding antibodies in the mouse sera were quantified by an indirect enzyme-linked immunosorbent assay using the purified 4D5-mIgG2a antibody as a standard. *\(P<0.05\), significant difference from the negative control group by Dunnett’s \(t\)-test. **\(P<0.01\), Significant differences from the negative control group by Dunnett’s \(t\)-test.

**Figure 4.** Antitumor activity mediated by pVax1-4D5-mIgG2a (H+L) in nude mice bearing BT474 xenografts. (a and b) Antitumor efficacy of Herceptin in a BT474 xenograft nude mouse model: Herceptin (8 mg kg\(^{-1}\)) or hFc (2.7 mg kg\(^{-1}\)) as a control was intravenously administered into each mouse twice a week for 4 weeks. Tumor volume was measured biweekly (a), and tumor weight was measured at the end of the experiment (b). Results are represented as the average±s.e. (c-e) Plasmid DNA or water was intramuscularly injected into nude mice (n=8 per group) bearing BT474 xenografts. At day 7 after DNA injection, Herceptin (10 mg kg\(^{-1}\)) was intravenously injected twice a week for 2 weeks. Tumor volume (c) and body weight (d) were measured twice a week while tumor weight (e) was measured at day 22. Results are represented as the average±s.e. (f) HER2-binding antibodies in the mouse sera were quantified by an indirect enzyme-linked immunosorbent assay using the purified 4D5-mIgG2a antibody as a standard. *\(P<0.05\), significant difference from the negative control group by Dunnett’s \(t\)-test. **\(P<0.01\), Significant differences from the negative control group by Dunnett’s \(t\)-test.

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Next the in vivo antitumor activity of anti-HER2 dMAb was evaluated in the same animal model. A single dose (100 μg per head) of the 4D5-mIgG2a expression plasmids, pVax1 or water was intramuscularly injected into nude mice (n=8) bearing human breast carcinoma BT474 xenografts (40–60 mm\(^3\) in size), followed by EP. Seven days later, Herceptin (10 mg kg\(^{-1}\)) was intravenously administered twice a week into the animal model for 14 days, because in the in vivo expression analysis, antibody was generated in the mouse sera by 7 days after the plasmid DNA injection. Tumor volumes were measured for 3 weeks and tumor weights were measured at day 22 after injection. Administration of 4D5-mIgG2a expression plasmids inhibited the tumor growth compared with the pVax1 vector without affecting body weight (Figures 4c and d). Tumor volume was reduced by 43.0% in the 4D5-mIgG2a treatment group while tumor weight was decreased by 27.6% (Figure 4e). Herceptin inhibited the tumor growth by 45.4% compared with the water treatment group. The results indicate that the therapeutic efficacy of a single intramuscular injection of 100 μg of the 4D5-mIgG2a expression plasmids is similar to that of four intravenous injections of 10 mg kg\(^{-1}\) Herceptin. Finally, the serum antibody concentration at the end of the experiment was measured by an indirect ELISA. The average antibody concentration of 4D5-mIgG2a was estimated to be 3.8 μg ml\(^{-1}\) (Figure 4f).

The mouse having the highest serum antibody concentration had
the smallest tumor volume, but the antibody concentration was not correlated with antigen efficacy.

**DISCUSSION**

Intramuscular injection and *in vivo* EP of a dMAb, or plasmid DNA-encoded antibody, is effective in neutralizing viruses, such as the HIV and Dengue virus.29,30 However, dMAbs have not yet been evaluated for therapeutic purposes, such as tumor gene therapy. In this study, we constructed expression plasmids that expressed a parental murine antibody of Herceptin, as a model antibody, and evaluated the *in vivo* expression and antitumor efficacy. The antibody was functionally expressed in human RD cells and exhibited the same *in vitro* antitumor activity as Herceptin. A single intramuscular injection and *in vivo* expression of the plasmid (100 μg) resulted in high and sustained antibody expression in the sera of normal mice and in effective inhibition of tumor growth in nude mice bearing HER2-positive human breast carcinoma BT474 xenografts. The antitumor efficacy of the anti-HER2 dMAb (100 μg) was similar to that of four doses of intravenously injected Herceptin (10 mg kg⁻¹). The results suggest that the dMAb can be applied to tumor gene therapy. To our knowledge, our study is the first to show the effectiveness of the dMAb approach in tumor gene therapy.

Previous studies on tumor gene therapy showed that a single administration of adenovirus or adeno-associated virus carrying genes coding for an anti-HER2 mAb led to high expression levels of antibody in the serum with concomitant suppression of tumor growth in nude mice bearing HER2-positive carcinoma xenografts.14,31 However, there have been concerns that viral vectors can induce minor adverse effect such as fever, myalgia and disseminated infection as well as produce neutralizing antibody, thereby limiting multiple injections.32 Plasmid DNA is safe, stable, easily produced and well tolerated in humans.28,29 In addition, our study showed that the dMAb approach yields a high serum antibody concentration. Taken together, the dMAb strategy may have an advantage over viral vector-mediated gene delivery for tumor gene therapy.

Herceptin is administered either once a week or once every 3 weeks intravenously for 30–90 min to achieve a minimum serum level of > 10 μg ml⁻¹.33 The recommended duration of Herceptin treatment is currently 1 year or until the progression of the disease. In addition, as production of antibody requires high-cost manufacturing and complicated technologies,7,9 Herceptin therapy has been a burden on patients and the health-care system in terms of time and cost. In the present study, we observed that a single intramuscular injection of 100 μg of the expression plasmids exhibited almost the same therapeutic efficacy as that of four intravenous injections of 10 mg kg⁻¹ Herceptin. This result, taken together with the low production costs and simple manufacturing steps involved with plasmid DNA, suggests that dMAbs may be more simple and efficient than antibody protein therapy. Thus this approach may facilitate the application of therapeutic antibodies to a broader population and benefit more patients. In conclusion, we have demonstrated the therapeutic effectiveness of anti-HER2 dMAb in the treatment of HER2-positive breast cancer and found that dMAb is an attractive tumor gene therapy approach.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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