Carcinoembryonic Antigen—Targeted Selective Gene Therapy for Gastric Cancer through FZ33 Fiber-Modified Adenovirus Vectors

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Abstract

Purpose: A major problem when using the adenoaviral vectors for gene therapy applications is thought to be related to low transduction efficiency in cancer cells or to side effects in normal cells. There is an urgent requirement to improve the specificity of gene delivery in the context of cancer gene therapy.

Experimental Design: We constructed a genetically modified adenovirus incorporating an IgG Fc-binding motif from the Staphylococcus protein A, Z33, within the HI loop (Adv-FZ33). A remarkable degree of targeted gene delivery to gastric cancer cells was obtained with Adv-FZ33 with the fully human anti–carcinoembryonic antigen (CEA) monoclonal antibody, C2-45.

Results: In vitro LacZ or EGFP gene expression after Adv-FZ33 infection via C2-45 was 20 times higher than control monoclonal antibody in MKN-45 at 1,000 viral particles/cell. We generated Ax3CAUP-FZ33 (UP-FZ33), which is an Adv-FZ33 derivative vector expressing a therapeutic gene (i.e., Escherichia coli uracil phosphoribosyltransferase), which converts 5-fluorouracil (5-FU) directly to 5-fluoro-UMP. UP-FZ33 with C2-45 enhanced the cytotoxicity of 5-FU by 10.5-fold in terms of IC50 against MKN-45 compared with control IgG4. In a nude mouse peritoneal dissemination model, tumor growth in mice treated with UP-FZ33/C2-45/5-FU was significantly suppressed, and tumor volumes were less than one-fourth of those of the control IgG4 group (P < 0.05). The median survival time of the UP-FZ33/C2-45/5-FU group was significantly longer than those treated with PBS or 5-FU only (P < 0.01).

Conclusions: These data suggest that CEA-targeted FZ33 mutant adenovirus-mediated gene delivery offers a strong and selective therapeutic modality against CEA-producing cancers.

Disseminated peritoneal gastric cancer does not generally respond to current treatments, such as surgery, chemotherapy, and radiotherapy (1, 2). There is, therefore, considerable interest in the development of new therapeutic approaches. In this context, gene therapy offers a potentially valuable, alternative means of treating advanced gastric cancer.

At present, adenoviral vectors have been widely used for gene transfer into tumor cells primarily because they can be produced in high titers and can infect many different cell types (3). Adenovirus serotype 5 infection is mediated by the high-affinity binding of the fiber knob to the coxsackie-adenovirus receptor (CAR; refs. 4–6) followed by internalization mediated by the binding of RGD motifs in the penton base to integrins αvβ3 and α5β3 on the cell surface (4, 7, 8). However, widespread application of adenoviral vectors for cancer gene therapy is hampered by a lack of specificity for malignant cells, as the CAR is expressed on both normal and malignant cells (4). Therefore, the development of tropism-modified, tumor-targeted adenoviral vectors is a critical issue in further developing the cancer gene therapy approach. In recent years, adenoviral tropism can be modified to retarget adenovirus serotype 5 to alternate receptors, thereby rendering viral infection CAR independent. We noticed a strategy that IgG-binding domain (Z33), derived from staphylococcal protein A, was inserted into the adenoviral fiber protein (9, 10). The fiber retained the ability to assemble into trimers, bound IgG with high affinity, and was incorporated into vector particles.

The human carcinoembryonic antigen (CEA) gene family is a group of highly glycosylated homotypic/heterotypic cell surface and intercellular adhesion molecules that belong to the immunoglobulin gene superfamily (11). CEA is expressed on the surface of ~95% of human cancer cells as well as on the epithelial cells of normal gastrointestinal tissue and the fetal intestine (12, 13). However, in normal adult tissues, CEA is localized on the luminal side of the columnar epithelial cells (14), so that the antigen does not directly face blood or tissue fluid (13, 15). Tumor CEA may therefore be a viable target for adenoviral-mediated gene therapy. However, major problems

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occurred when a mouse anti-CEA antibody was used as an anticaner agent in clinical trials due to cross-reactivity of the monoclonal antibody (mAb) with normal tissues and also because of an immunogenic response against the mouse mAb (16, 17). The CEA family consists of seven expressed genes (13, 18). Among these, CEACAM1, CEACAM6, and CEACAM8 are quite impedimental in terms of targeting because they are also often expressed in various normal tissues (18–22). To circumvent this problem, we established novel fully human-specific CEA mAbs against tumor CEA (19–23).

In this study, we evaluated on both in vitro and in vivo levels the extent of retargeting toward and therapeutic effectiveness against CEA-positive gastric cancers when using the fully human CEA antibody complex with this modified vector.

**Materials and Methods**

**Cell culture.** We used two cancer cell lines (MKN-45 and MKN-74) derived from human gastric adenocarcinoma. A normal human hepatocyte cell line, Chang liver, was kindly provided by Dr. Niitsu (Sapporo Medical University, Sapporo, Japan). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum. We cultured Chinese hamster ovary (CHO) cell transfectants expressing CEACAM proteins in α-MEM (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum at 37°C in 5% CO2.

**pAx3-FZ33 plasmid construction.** Briefly, the FZ33 vectors were generated as follows (details are available on request). The cosmid pAx357-F/GRG (also designated simply as pAx3), derived from pAdex1cw (24), had FRGD mutant (25) with both the left side end and the right side end of the adenovirus serotype 5 genome (Fig. 1A). The DNA fragment with the Z33 peptide motif was synthesized by PCR with the 5′-primer GAAACCGGTCTCATCAAGTTTAACATGCA-GCAGCAGCGCCGCTTTTAC and the 3′-primer GTGCGTACGACATGATGCAGCTTTTAATCTTGCGCCGGCG using the 86-base oligonucleotide ATGCAGCAGCCGCGCCGCTTTTACGAGGCAAGGACCCAGACAGGAGAATAGACGAGCATTCG as a template. The amino acid sequence encoded by the PCR fragment was ETGLIKFNQQRFFYALEHDPNLNEEQRNANKSKIDDDASD. The resultant PCR product was inserted into psKIi-hiAN encoding the adenovirus serotype 5 fiber, resulting in the plasmid pSKII+Z33. The BstXI/BamHI fragment from the plasmid pSKII+Z33 was inserted into the pWE6.7R-F/wt-2 (25), resulting in pWE6.7R-FZ33. The EcoRI/RsrII fragment from pWE6.7R-FZ33 was ligated into pAx3, resulting in pAx3-FZ33. A EcoRI/BglII (blunted) fragment from pEGFP-N1 (Clontech) was inserted into the EcoRI/BglII (blunted) sites of the pCAcc (26), resulting in pCAEGFP. The ClaI fragment from
pCAGFP was ligated into the ClaI site of pAX3-FZ33, resulting in pAX3CAGFP-FZ33. The β-galactosidase (β-gal) expression cassette (CAZ3, 5,153 bp) was cut out from the pCAZ3 (25) by BamHI/BglII digestion, blunted by T4 DNA polymerase, and ligated into the ScaI site of pAX3-FZ33, resulting in pAX3CAZ3-FZ33. The Escherichia coli uracil phosphoribosyltransferase (UPRTase) gene expression cassette (27) was cut out from pCAUPI by SalI/HinIII (blunted) and ligated into the ScaI site of pAX3-FZ33, resulting in pAX3CAUIF-PFZ33.

**Generation of recombinant adenoviruses.** For the generation of recombinant adenovirus, each cosmid was transfected into 293 cells by lipofection using LipofectAMINE2000 reagent (Invitrogen). Plaques arising from the transfected 293 cells were isolated and evaluated by restriction enzyme digestion of the viral genome and sequencing of the expression units. The resultant adenoviral vectors were expanded in 293 cells and purified by cesium chloride ultracentrifugation. Purified viruses were dialyzed in PBS with 10% glycerol and stored at 4°C. Cells and purified recombinant adenovirus were used in experiments after restriction enzyme digestion and sequencing of the viral genome. To exclude the free antibody molecules in pCAGFP-FZ33, we ligated the β-galactosidase (β-gal) expression cassette (CAZ3, 5,153 bp) was cut out from the pCAZ3 (25) by BamHI/BglII digestion, blunted by T4 DNA polymerase, and ligated into the ScaI site of pAX3-FZ33, resulting in pAX3CAZ3-FZ33.

**Antibodies and reagents.** In the present study, we used the C2-45 clone (IgG, κ) as the fully human anti-CEA mAb. Preparation of the antibody has been described previously in detail (23). Mouse mAb clones F33-104 (IgG1, κ), F33-60 (IgG2a, κ), F82-81 (IgG2b, κ), and F11-12 (IgG3, κ) against human CEA (31) and mouse-human chimeric mAb ChF11-39 (IgG1; ref. 32) have been described previously. The 1B2 (IgG2a, κ) was purchased from IBL (Takasaki, Japan). A mouse anti-human CAR mAb (Rmcb), prepared as ascites fluid, was kindly provided by Dr. Jeffrey Bergelson (Children's Hospital of Philadelphia, Philadelphia. PA). The anti-α1,3β2 mAb MAB1961 and the anti-α1,6β2 MAB1976 were purchased from Chemicon International (Temecula, CA). Control human IgG4 was purchased from The Binding Site Ltd. (Birmingham, United Kingdom). Control mouse IgG and FITC-conjugated rabbit anti-mouse immunoglobulins were purchased from Sigma and Dako Japan (Kyoto, Japan), respectively. FITC-conjugated goat anti-human IgG (Fc) was purchased from Chemicon. CEA protein was purified from a liver metastasis derived from a colon adenocarcinoma (33).

**Flow cytometry.** The reactivities of anti-CEA mAbs with various cell lines and the expression levels of CEA, CAR, and integrins were analyzed using flow cytometry. Cultured cells were harvested by incubation with trypsin-EDTA solution (0.5%; Sigma) for 5 minutes. Detached cells were centrifuged and resuspended in PBS containing 2% bovine serum albumin at a concentration of 10^5/ml. The cells were first incubated with 10 μg/ml antibody for 1 hour on ice. Subsequently, the cells were washed and incubated with FITC-conjugated rabbit anti-mouse immunoglobulins for 30 minutes on ice and in the dark. After washing in 2% bovine serum albumin/PBS, the cells were resuspended in 500 μl of 2% bovine serum albumin/PBS. Analysis was done on a FACS-Calibur (Becton Dickinson, San Jose, CA).

**Transduction efficiency in vitro.** ECFP gene transduction was carried out by first plating cells in six-well plates at 2 × 10^5 per well 1 day before adenoviral vector infection. Cells were treated with serum-free medium containing of 3 μg/ml antibody (C2-45 or control isotype IgG) or without antibody at 37°C in 5% CO2 for 1 hour. After incubation, the cells were washed with serum-free medium and then cultured in 1,000 μL serum-free medium containing Ax3CAEGFP-FZ33 (1×10^9 VP/cell) at 37°C in 5% CO2 for 1 hour. After incubation, the cells were washed twice with serum-free medium and then cultured in medium with 10% fetal bovine serum for 24 hours. Unincorporated free antibody molecules would act as competitors of Adv-FZ33-mediated gene delivery to CEA-expressing cells. To exclude the free antibodies, we rinsed the target cells after the antibody treatment. Therefore, we added the viral vector in in vitro gene transfer assay. The transduction efficiency was measured for CEA-positive or CEA-negative cell lines. Green fluorescent protein (GFP) activity was measured by fluorescence-activated cell sorting analysis.

**Transgene expression in vitro.** The LacZ gene transduction was done according to the procedures of in vitro gene transfer assay. Cells were plated into 96-well plates at a density of 1 × 10^4 per well. Cells were treated with antibody (C2-45 or control isotype IgG) or without antibody and then infected with Ax3CAZ3-FZ33 at 100, 300, and 1,000 VP. Infected cells were cultured for 24 hours. Transgene expression was compared by infecting CEA-positive and CEA-negative cell lines. LacZ activity was measured by a β-gal reporter gene assay (Roche Molecular Biochemicals, Indianapolis, IN).

**Competition assay.** The specificity of targeting of the anti-CEA mAb was evaluated in CEA-CHO cells cultured in medium containing C2-45 and different concentrations of purified CEA. CEA-CHO cells were plated in 96-well plates at 1 × 10^5 per well. Cells were incubated in medium with purified CEA protein (0-10^8 ng/mL) and 3 μg/mL antibody (C2-45 or control human IgG4) for 1 hour. Thereafter, cells were infected with Ax3CAZ3-FZ33 at 1,000 VP for 1 hour. Transgene expression was measured by a β-gal reporter gene assay after 24 hours.

**5-Fluorouracil sensitivity in vitro.** E. coli UPRT gene transduction was done according to the procedures of in vitro gene transfer assay. Cells were plated into 24-well plates at 5 × 10^4 per well. These cells were treated with antibody (C2-45 or control human IgG4) or without antibody and infected with Ax3CAUIF-PFZ33 (UP-FZ33) at 100 VP for 1 hour. Infected cells were cultured in medium containing 5-fluorouracil (5-FU; 0-100 μmol/L) for 4 days. The relative sensitivities to 5-FU were compared among CEA-positive and CEA-negative cell lines. Cell survival was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

**LacZ expression in vivo mediated by Adv-FZ33 premixed with anti-CEA antibody.** Female athymic nude mice (BALB/c nu/nu) were obtained from Japan Charles River (Yokohama, Japan) and used to evaluate the ability and selectivity of Adv-FZ33 premixed with anti-CEA mAb to mediate gene transduction in disseminated gastric cancer in vivo. The mice were 4 weeks old at the beginning of each experiment. Freshly harvested MKN-45 (1 × 10^7 cells) that had been resuspended in PBS was injected into the peritoneal cavity. The Ax3CAZ3-FZ33 incubated with either 3 μg/mL C2-45 or IgG4 in serum-free medium. Vector-antibody premixture or vector alone was injected i.p. (5 × 10^5 VP/animal) into the mice 10 days after they received the MKN-45. Four days after vector administration, the mice were sacrificed and tissues were fixed by systemic perfusion with 4% paraformaldehyde for 15 minutes. To detect expression of β-gal, the peritoneal cavities were stained with X-gal solution [Sigma, 1 mg/mL X-gal in PBS (pH 7.2), 2 mmol/L MgCl2, 4 mmol/L potassium ferriyamde] for 4 hours.

**Gene therapy using Adv-FZ33 premixed with anti-CEA mAb in mice with disseminated peritoneal gastric cancer.** Female mice (BALB/c nu/nu) were injected i.p. with MKN-45 (1 × 10^7 cells per mouse) in PBS (300 μL) to evaluate the ability of Adv-FZ33 premixed with anti-CEA mAb to mediate tumor-specific gene therapy against established tumors in vivo. The mice were then randomly assigned into five groups that received the following treatments: (a) UP-FZ33 containing 30 μg/mL C2-45 followed by 10 mg/kg 5-FU treatment, (b) UP-FZ33 containing 30 μg/mL control IgG4 followed by 10 mg/kg 5-FU treatment, (c) UP-FZ33 without antibody followed by 10 mg/kg 5-FU treatment, (d) PBS followed by 10 mg/kg 5-FU treatment, and (e) no treatment (PBS only). Four days after the MKN-45 injections, adenoviral vectors (5 × 10^9 VP) in a total volume of 300 μL were injected into the peritoneal cavity of the mice. Starting on the following day, 5-FU or PBS was given i.p. daily for 5 days. This treatment schedule was then repeated. Animals were sacrificed at 17 days after tumor inoculation and the disseminated peritoneal mass was collected and weighed from each animal (n = 3 in each group). To determine whether therapeutic reductions in tumor burden were also reflected by prolongation of the survival of the animals, an additional set of animals was inoculated and treated with the same protocols as above (n = 6 in each group) and survival times were evaluated. All of
the animal experiments were done under the guidelines established by Sapporo Medical University.

Statistical evaluation. Statistical values are presented as mean ± SD. The significance of differences between groups was assessed by a Student’s t test. Statistical significance was defined as P < 0.05. Survival was analyzed by the Kaplan-Meier method and differences were analyzed by a log-rank test.

Results

Construction of Adv-FZ33. The schema for construction of Adv-FZ33 is shown in Fig. 1B. A synthetic 33–amino acid IgG-binding domain (Z33), derived from staphylococcal protein A, was inserted into the HI loop of the knob protein. This modified fiber retained the ability to assemble into trimers, bound IgG with high affinity, and was incorporated into viral particles. Adv-FZ33 binds immunoglobulins and allows an antibody to redirect the vector to a new target molecule on the cell surface. Our Adv-FZ33 had intact CAR-binding structure (34) and retained CAR-binding ability (data not shown), which was in accord with the report by Volpers et al.

Particle numbers (VP/mL) to infectious titers (plaque-forming units/mL) ratios (VP/plaque-forming units) for the Adv-FZ33 used in those experiments were 35.6 (Ax3CAZ-FZ33), 65.6 (Ax3CAEGFP-FZ33), and 22.8 (Ax3CAUP-FZ33).

Specificity of anti-CEA mAb-mediated targeting. Firstly, we determined the specificity of the C2-45 antibody for CEA by flow cytometric analysis. We confirmed that C2-45 bound only to CEA-CHO (encoding CEACAM5 gene) cells and not to CEACAM8-CHO cells (Fig. 2A). Next, we determined whether Adv-FZ33 could infect CEA-expressing cells via an anti-CEA mAb. As shown in Fig. 2B, Adv-FZ33 alone or with a control mAb showed poor transduction of CEA-CHO cells. However, Adv-FZ33 with C2-45 showed a strongly enhanced rate of transduction. In contrast, no significant effect on transduction efficiency was observed when CEACAM8-CHO cells were exposed to Ax3CAEGFP-FZ33 with or without the antibody. We used Ax3CAZ3-FZ33 to carry out a competitive inhibition assay to confirm that the anti-CEA mAb-mediated transduction of CEA-CHO cells was the result of specific binding of the mAb to the vector and occurred via a CAR-independent cell entry pathway. In this experiment, the vector was pretreated with

![Fig. 2](image-url)

**Fig. 2.** Specificity of anti-CEA mAb-mediated targeting. A, analysis of the expression of CEA on CEACAM-transfected CHO cells by flow cytometry. Open histograms, staining with an isotype control antibody; shaded histograms, staining with C2-45. B, transduction efficiency of CEACAM-transfected CHO cells evaluated by flow cytometry. Cells were infected with Ax3CAEGFP-FZ33 at 1,000 VP/cell after incubation with C2-45 or isotype control antibodies (3 μg/mL). Open histograms, staining with an isotype control antibody; shaded histograms, staining with C2-45. Numbers, percentages of GFP-positive cells. C, competitive inhibition assay. CEA-CHO cells were transduced with Ax3CAZ3-FZ33 at 1,000 VP/cell after preincubation with C2-45 (●) or isotype control antibodies (○, 1 μg/mL) in the presence of purified CEA protein. Points, mean; bars, SD. RLU, relative light units.
either C2-45 (100 ng) or control IgG4 (100 ng) before transduction of CEA-CHO cells in the presence of CEA protein (0-10^4 ng/mL). As shown in Fig. 2C, the high level concentration of CEA protein reduced β-gal activity in cells transduced by vector pretreated with C2-45 but not in cells transduced with vector pretreated with the control antibody. In the presence of 10^4 ng/mL CEA protein, β-gal activity was decreased to 41% following infection of vector pretreated with C2-45 compared with control (no addition of CEA protein). However, the absolute value of β-gal activity was ~10 times higher in cells transduced by the C2-45-treated vector than in cells exposed to the vector pretreated with the control antibody.

We also screened other mAbs against human CEA to determine whether they could enhance the transduction efficiency of CEA-positive cells with Adv-FZ33 (Table 1). Although transduction efficiency was increased when Adv-FZ33 was bound to various mouse anti-CEA mAbs, none showed the same enhancement of gene expression in CEA-positive cells as C2-45.

Expression of CAR and integrins in CEA-positive and CEA-negative cell lines. We screened two cancer cell lines, MKN-45 and MKN-74, and Chang liver with antibodies against various cell surface markers (Fig. 3A). The CEA-positive cell line MKN-45, but not the CEA-negative MKN-74 cell line or Chang liver cells, showed a strong reaction with C2-45. All three cell lines showed evident expression of the primary adenovirus receptor, CAR, and the secondary adenovirus receptors, integrins αvβ3 and αvβ5. These observations indicate that CAR or integrin expression in human cell lines cannot be used as specific targets in cancer gene therapy.

Improvement of transduction efficiency of CEA-positive cells in vitro. As shown in Fig. 3B, MKN-74 and Chang liver gave the same levels of gene transfer following application of either Ax3CAEGFP-FZ33 pretreated with C2-45 or control antibody. In contrast, gene transfer efficiency was significantly increased in the MKN-45 using vector treated with C2-45 compared with the control antibody. MKN-45 infected with 10 VP/cell showed a 7.82-fold enhancement in gene transfer. At a concentration of 1,000 VP/cell, C2-45-mediated adenoviral infection induced 92.29% transduction.

Improvement of transgene expression in CEA-positive cells in vitro. We next sought to determine whether transgene expression was increased in a similar fashion to transduction efficiency using the modified adenovirus (Fig. 3C). In Chang liver and MKN-74, application of Ax3CAZ3-FZ33 that had been pretreated with C2-45 gave transgene expression equal to that for the adenovirus treated with the control antibody or adenovirus alone. In contrast, β-gal activity in MKN-45 infected with Ax3CAZ3-FZ33 pretreated with C2-45 was significantly increased compared with cells infected with the combination of adenovirus-control antibody or adenovirus alone. In MKN-45, C2-45-mediated vector infection at a 100, 300, or 1,000 VP/cell showed 4.5-, 9.4-, or 21.8-fold enhancement, respectively, in β-gal activity compared with vector alone.

UPRT gene transduction sensitizes CEA-positive cells to 5-FU in vitro. E. coli UPRT is a pyrimidine salvage enzyme that catalyzes the synthesis of UMP, a precursor of the pyrimidine nucleotide, from uracil and phosphoribosylpyrophosphate. The sensitivity of human CEA-positive or CEA-negative gastric cancer cell lines to 5-FU was evaluated using UP-FZ33. Transduction of the UPRT gene restores drug sensitivity to cancer cells that show resistance to 5-FU (32). As shown in Fig. 4, when the cells were infected with UP-FZ33 alone, the IC_{50} of 5-FU decreased from 24 to 15 μmol/L for MKN-74 and from 1.8 to 0.22 μmol/L for Chang liver cells. With respect to MKN-45, IC_{50} did not change as 3.1 μmol/L. When antibody-pretreated cells were infected with UP-FZ33, 5-FU sensitivity of IgG4-treated cells increased compared with C2-45, although there was no significant difference in the IC_{50} with respect to MKN-74. For Chang liver, there was no difference in sensitivity between cells treated with vector alone or with vector treated with either C2-45 or the control antibody. In terms of the MKN-45, UP-FZ33 with C2-45 significantly increased the sensitivity of these cells to 5-FU, with a 10.5-fold difference in IC_{50} (0.081 μmol/L with C2-45 versus 0.85 μmol/L with the IgG4 antibody).

As shown in Table 2, it is to be noted that UP-FZ33 with a control IgG4 gave significant (3.6- to 3.3-fold) increase in 5-FU sensitivity in MKN-45 or MKN-74, respectively, compared with the vector alone. The mechanism underlying this increase is not clear. This effect was observed only when Adv-FZ33 and antibody were simultaneously applied to the target cell.

Ax3CAZ3-FZ33 premixed with C2-45 enhances selective gene transfer in CEA-positive cells in vivo. To evaluate selective gene transfer in vivo, mice with disseminated peritoneal tumors were given an i.p. injection of Ax3CAZ3-FZ33 with or without antibody. In in vivo gene transfer assay, we first mixed adenoviruses with antibodies and then injected into mice. This method seems to be more practical in terms of future clinical application. The peritoneal cavity of a mouse that received Ax3CAZ3-FZ33 with C2-45 showed selectively enhanced expression of β-gal in disseminated tumors (Fig. 5A). In contrast, a mouse receiving Ax3CAZ3-FZ33 with the control IgG4 showed little expression of β-gal. X-gal-positive staining was present only in cancer cells not in other organs, including

<table>
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NOTE: Representative values are mean fluorescence intensity of histograms.
Abbreviations: MFI, mean fluorescence intensity; hlgG, human IgG control antibody, mlgG, mouse IgG control antibody.
liver, spleen, and kidney. These observations show that Ax3CAZ3-FZ33 with C2-45 is able to selectively augment expression of a transgene in CEA-positive gastric cancer cells in vivo. Analyses of tissue sections showed that the LacZ gene expression was limited to superficial layers of the peritoneal tumors (Fig. 5B).

**Therapeutic effect of Ax3CAIP-FZ33 premixed with anti-CEA mAb plus 5-FU in vivo.** The macroscopic appearances of the abdominal cavities of treated mice is shown in Fig. 6A. A very large main tumor below the stomach and large multiple disseminated tumors were observed on the mesentery in the PBS-only group (total weight, 1.14 ± 0.02 g) and the 5-FU-only group (total weight, 0.99 ± 0.23 g). A very large main tumor and medium-sized multiple disseminated tumors were observed in the UP-FZ33/IgG4/5-FU group (total weight, 0.52 ± 0.19 g) and the UP-FZ33/5-FU group (total weight, 0.57 ± 0.10 g). On the other hand, tumor growth in mice treated with UP-FZ33/C2-45/5-FU was significantly suppressed, and tumor volumes were less than one-fourth of those of the control IgG4 group. As C2-45 increased 5-FU sensitivity much more remarkably (i.e., 6.4 in the 5-FU-only group, 27.7 in the UP-FZ33/C2-45/5-FU group, 10.7 in the UP-FZ33/5-FU group, and 25.7 in the UP-FZ33/IgG4/5-FU group), tumor volumes in UP-FZ33/C2-45/5-FU were 8.1 in the UP-FZ33/5-FU group, 32 days (UP-FZ33/IgG4/5-FU), and 42 days (UP-FZ33/C2-45/5-FU; Fig. 6C). The median survival times of various treatment groups were 22 days (PBS only), 23 days (5-FU only), 32 days (UP-FZ33/IgG4/5-FU), and 42 days (UP-FZ33/C2-45/5-FU; Fig. 6C). The median survival time of the UP-FZ33/C2-45/5-FU group was significantly longer than those of the PBS-only and 5-FU-only treatment groups (P < 0.01 versus PBS and 5-FU groups). On the other hand, no significant difference was present between the UP-FZ33/C2-45/5-FU or UP-FZ33/IgG4/5-FU groups and the PBS-only or 5-FU-only treatment groups. When we used the >25% body weight loss as survival end point, the survival curve showed essentially the same significance as the Fig. 6D (data not shown).

### Discussion

In this study, we found that the strategy of Adv-FZ33 with an anti-CEA mAb can result in improved effectiveness, both in vitro and in vivo, of antitumor treatments of gastric cancer cells expressing CEA.

We have shown that Adv-FZ33 with C2-45 enhanced transduction efficiency and gene delivery in CEA-expressing CHO cells. As the CHO cells do not express CAR, the infection by Adv-FZ33 with C2-45 must have been via a CAR-independent pathway. CEA is a glycosphingolipidiniositol anchor-type protein that is linked to the cell surface (35, 36). If an antibody binds to a glycosphingolipidiniositol-type antigen, it is not necessarily the case that the signal will be internalized by the cell. Therefore, the infection pathway of Adv-FZ33 with C2-45 will involve an initial attachment to the cell surface CEA (instead of CAR) followed by internalization through interaction of the penton base of the viral capsid with αβ3 class integrins. Indeed, CHO cells have high expression of αvβ3 integrin. Next, we evaluated gene delivery to human gastric cancer cell lines that highly express CAR and showed that Adv-FZ33 with C2-45 enhanced gene transduction through binding to the CEA on target cells compared with the only CAR-dependent pathway. Thus, CEA seems to be an attractive target molecule for the attachment of adenovirus vectors in gastric cancer gene therapy. The levels of αvβ3 or αvβ5 detected on the MKN-45 were rather low. Whereas αvβ3 and αvβ5 integrins act as key molecule for cellular internalization of adenovirus, other integrin heterodimers, such as αvβ1 or αvβ3 (37–39), might play an important role for adenovirus internalization in the MKN-45.

We also examined whether other mouse mAbs against human CEA would likewise enhance transduction efficiency when combined with Adv-FZ33. Although these antibodies had comparatively high reactivity for the antigen, gene expression was weak compared with C2-45. Clearly, choice of antibody will be an important determinant for successful application of this strategy.

We showed that MKN-45 in vitro showed dramatically enhanced sensitivity to 5-FU in the presence of Adv-FZ33 with an anti-CEA antibody-mediated UPRT gene. MKN-45 infected with LacZ-FZ33 with C2-45 showed a 5-fold enhancement in β-gal activity, whereas the same amount of UP-FZ33 with C2-45 increased 5-FU sensitivity much more remarkably (i.e., 10-fold). One possible explanation for this observation is the “bystander effect” that can occur where there is cellular contact between infected and uninfected cells (27).

Two issues need to be considered with regard to targeting of cancer cells in vivo using Adv-FZ33-anti-CEA mAb combination: (a) the stability of antibody-adenovirus vector complexes and (b) whether circulating CEA derived from tumor cells could inhibit a vector-antibody complex. With respect to the first point, as far as use of cancer gene therapy in a clinical setting is concerned, it is important that the antibody-adenovirus vector complexes are stable in an environment containing circulating immunoglobulins (~10 mg/mL). Henning et al. reported that antibody-mediated gene transfer was completely abolished when adenovirus vector-heceptin complexes were incubated with 50% whole normal mouse or human serum (40–42). It is expected that antibody-Adv-FZ33 complexes might be unstable when they were given systemically into the blood. Therefore, covalent cross-linking of adenovirus with antibody would enhance the stability of the conjugate, leading to the more effective gene transduction to the target cells. However, in our experimental model, administration of a combination of Adv-FZ33 with
Fig. 3. Improvement of gene transduction in CEA-positive gastric cancer cells in vitro. 

A, expression of human CAR, integrins, and CEA in MKN-45, MKN-74, and Chang-liver cells. Open histograms, staining with an isotype control antibody; shaded histograms, staining with C2-45, anti-CAR, anti-αvβ3, and αvβ5, respectively. 

B, transduction efficiency in Ax3CAEGFP-FZ33-infected human cells was evaluated by flow cytometry. Cells were infected with Ax3CAEGFP-FZ33 at 0 to 1,000 VP/cell after incubation with C2-45 or control IgG4. Open histograms, isotype control antibody without Adv-FZ33; shaded histograms, EGFP gene expression by Adv-FZ33 with C2-45 or control IgG4. Numbers, percentages of GFP-positive cells.
anti-CEA mAb was able to augment expression of the transgene selectively in CEA-positive gastric cancer cells in vivo. The vector-antibody combination may be able to target cancer cells in the peritoneal cavity for three reasons: (a) the concentration of immunoglobulins in the peritoneal cavity is lower than found in serum, (b) vector-mAb complexes could come directly into contact with the tumor cells because MKN-45 do not produce ascites in the disseminated
peritoneal model, and (c) the peritoneal cavity is a closed space in which viral infection is comparatively easy despite the presence of physiologic ascites. With regard to the second point, free CEA-derived from MKN-45 did not inhibit the vector-anti-CEA mAb conjugation in the abdominal cavity. In vitro, at high levels of CEA protein (10,000 ng/mL), the expression level of the reporter gene was >10 times higher with C2-45 than that obtained with the control antibody. Our observations are consistent with those of Nolan et al. who described an immunization strategy involving direct genetic alteration of T cells to create chimeric immunoglobulin T-cell receptors. These receptors can bind to CEA through anti-CEA single-chain Fv (43). In their study, incubation in the presence of soluble CEA at 10,000 ng/mL did not interfere with complex formation. In cancer patients, serum CEA levels may reach 1,000 ng/mL compared with the normal level of <5 ng/mL. In our experimental model of disseminated abdominal cancer, the maximum value of CEA concentration in the blood was 200 ng/mL in the untreated group (data not shown).

Furthermore, we showed that the survival rate of mice given UP-FZ33/C2-45/5-FU was significantly higher than those that received either PBS or 5-FU only. However, injection of vector-anti-CEA mAb complexes could not abrogate tumor development completely. By staining the peritoneal cavity with X-gal, we found that the transgene was expressed in surface cell layers of the peritoneal tumors. It seems that it will be difficult to deliver a transgene into deeper layers of the tumors using i.p. injection of adenoviral vectors. To determine if an earlier application of the vectors could overcome this problem, we began treatment 4 days after i.p. injection of MKN-45 when the tumors were only macroscopic nodules. We found that tumor volumes in mice treated with UP-FZ33/C2-45/5-FU were significantly smaller than those in the control IgG4 group on day 17 after tumor cell inoculation. However, the number of surviving mice treated with UP-FZ33/C2-45/5-FU decreased rapidly from day 30 after implantation. Use of a replication-competent adenovirus or a killer gene with a potent bystander effect, such as thymidine kinase, may improve treatment outcomes. However, our UP-FZ33/C2-45/5-FU treatment exerted a powerful antitumor effect compared with previous investigations using adenoviral vectors. Lan et al., for example, studied an adenoviral vector carrying the CEA promoter to direct cytosine deaminase (AdCEA-CD) transgene expression and investigated its therapeutic value in mice with disseminated peritoneal tumors, the latter the result of i.p injection of MKN-45 (44). AdCEA-CD required 14 × 10^9 plaque-forming units (approximately equivalent to 70 × 10^10 VP) to obtain a statistical significant difference between groups treated with AdCEA-CD/5-fluorocytosine compared with 5-fluorocytosine only. In our animal experiments, statistical significance difference between the UP-FZ33/C2-45/

<table>
<thead>
<tr>
<th>IC50 (µmol/L) of 5-FU in Ax3CAUP-FZ33-infected cells</th>
<th>MKN-74</th>
<th>Chang liver</th>
<th>MKN-45</th>
</tr>
</thead>
<tbody>
<tr>
<td>No infection</td>
<td>24</td>
<td>1.8</td>
<td>3.1</td>
</tr>
<tr>
<td>Ax3CAUP-FZ33</td>
<td>15</td>
<td>0.22</td>
<td>3.1</td>
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<tr>
<td>Ax3CAUP-FZ33 with IgG4</td>
<td>4.6</td>
<td>0.20</td>
<td>0.85</td>
</tr>
<tr>
<td>Ax3CAUP-FZ33 with C2-45</td>
<td>13</td>
<td>0.25</td>
<td>0.081</td>
</tr>
</tbody>
</table>

NOTE: IC50 were determined by dose-response curve crossing the 50% lines.

Fig. 5. Distribution of the LacZ gene expression after i.p. injection of Adv-FZ33 anti-CEA antibody mixture in mice with disseminated abdominal cancer. Ten days after MKN-45 inoculation, mice were given an i.p. injection (5 × 10^9 VP) of Ax3CAZ3-FZ33 premixed with either C2-45 or isotype control antibodies. Four days later, the mice were sacrificed and X-gal staining was done. A, macroscopic appearance of peritoneal cavity after injection of Ax3CAZ3-FZ33 alone (left), Ax3CAZ3-FZ33/IgG4 (middle), and Ax3CAZ3-FZ33/C2-45 (right). Mice that received Ax3CAZ3-FZ33 or Ax3CAZ3-FZ33/IgG4 showed faint staining of the main tumor (arrowhead) and metastatic nodules (arrow). Mice that received Ax3CAZ3-FZ33/C2-45 showed clear staining of both the main tumor and metastatic nodules. In all groups, normal organs were not stained by X-gal. B, sections through the main tumors of mice that received Ax3CAZ3-FZ33 alone (left), Ax3CAZ3-FZ33/IgG4 (middle), or Ax3CAZ3-FZ33/C2-45 (right). LacZ expression is confined to the superficial layers of the tumor. Magnification, × 40.
5-FU and 5-FU groups was obtained only by UP-FZ33 infection with $10 \times 10^9$ VP. Therefore, our use of C2-45-binding Adv-FZ33 can result in effective targeting of CEA-positive cells.

Recently, several investigators have described other targeting strategies using adenovirus vectors (45–49). However, these approaches are time consuming and it is costly to construct recombinant vectors against candidate target molecules for
References

Carcinoembryonic Antigen–Targeted Selective Gene Therapy for Gastric Cancer through FZ33 Fiber-Modified Adenovirus Vectors

Toshihiro Tanaka, Jianhua Huang, Sachie Hirai, et al.


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