

Benefits of gene transduction of granulocyte macrophage colony-stimulating factor in cancer vaccine using genetically modified dendritic cells

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Abstract. Granulocyte macrophage colony-stimulating factor (GM-CSF) is a key cytokine for the generation and stimulation of dendritic cells (DCs), and it may also play a pivotal role in promoting the survival of DCs. In this study, the feasibility of creating a cancer vaccine using DCs adenovirally transduced with the carcinoembryonic antigen (CEA) gene and the GM-CSF gene was examined. In addition, the effect of the co-transduction of GM-CSF gene on the lifespan of these genetically modified DCs was determined. A cytotoxic assay using peripheral blood mononuclear cell (PBMC)-derived cytotoxic T lymphocytes (CTLs) was performed in a 4-h ⁵¹Cr release assay. The apoptosis of DCs was examined by TdT-mediated dUTP-FITC nick end labeling (TUNEL) assay. CEA-specific CTLs were generated from PBMCs stimulated with genetically modified DCs expressing CEA. The cytotoxicity of these CTLs was augmented by co-transduction of DCs with the GM-CSF gene. Co-transduction of the GM-CSF gene into DCs inhibited apoptosis of these DCs themselves via up-regulation of Bcl-x_L expression, leading to the extension of the lifespan of these DCs. Furthermore, the transduction of the GM-CSF gene into DCs also suppressed the incidence of apoptosis of DCs induced by transforming growth factor-β1 (TGFβ-1). Immunotherapy using these genetically modified DCs may therefore be useful with several advantages as follows: i) adenoviral toxicity to DCs can be reduced; ii) the lifespan of vaccinated DCs can be prolonged; and iii) GM-CSF may protect DCs from apoptosis

induced by tumor-derived TGFβ-1 in the regional lymph nodes.

Introduction

Dendritic cells (DCs) are the most potent specialized antigen-presenting cells for the initiation of antigen-specific immune responses. Therefore, antigen-loaded DCs have been regarded as promising vaccines in cancer immunotherapy (1). Carcinoembryonic antigen (CEA) is an oncofetal glycoprotein expressed in a high percentage of tumors of epithelial origin such as colon, rectum, pancreas, gastric, breast, and therefore, is an attractive target for immunotherapy (2). This laboratory performed a pilot study of peripheral blood mononuclear cell (PBMC)-derived DCs loaded with the CEA peptide (CEA652) and observed low frequencies of CEA-specific cytotoxic T lymphocytes (CTL) responses in a few patients. In that study, no objective antitumor responses were recognized (3). Therefore, we have employed a gene-based vaccination strategy using DCs adenovirally transduced with the whole tumor-associated antigen (TAA) gene. This approach exploits the fact that these genetically modified DCs will produce multiple endogenous antigenic epitopes independent of the major histocompatibility complex (MHC) alleles. These vaccinations, using DCs adenovirally transduced with the TAA gene, elicited more efficient therapeutic immune responses against tumors than vaccinations using DCs pulsed with immunodominant peptides in mouse models (4). Other groups observed that human DCs transduced with a recombinant adenovirus encoding the CEA gene could effectively induce CEA-specific CTLs *in vitro* (5,6), thus indicating that this approach could be useful for immunotherapy.

The use of cytokines is the most common method to activate DCs. For instance, granulocyte macrophage colony-stimulating factor (GM-CSF) is highly effective for the generation and stimulation of DCs (7). Our previous studies demonstrated that a vaccination containing DCs co-transduced with the TAA gene and the GM-CSF gene elicited potent therapeutic immunity in subcutaneous tumors in mouse models (8,9), and also showed that the co-transduction of

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the GM-CSF gene enhanced the migratory capacity of DCs for draining the lymph node by up-regulation of CC chemokine receptor 7 (CCR7) expression (4,8). In addition, GM-CSF has other important functions for DCs. This cytokine promotes the survival of PBMC-derived DCs (10). Moreover, GM-CSF inhibits the apoptosis of DCs by inducing an increased expression of Bcl-2 and Bcl-x_L (11). Research indicates that tumors will stimulate the apoptotic death of key immunocompetent cells, including DCs (12). Specifically, tumor-derived transforming growth factor- β -1 (TGF β -1) has been reported to induce DC apoptosis in the sentinel lymph node (13). Therefore, we hypothesized that genetically modified DCs expressing CEA may demonstrate the prolongation of the DCs lifespan via up-regulation of Bcl-x_L by co-transduction of the GM-CSF gene. Therefore, vaccination using those DCs will be possible to elicit potent CEA-specific antitumor immunity, because the lifespan of antigen-bearing DCs in lymphoid tissues may be critical in determining the outcome of CTL responses.

In this study, human DCs were adenovirally transduced simultaneously with CEA and GM-CSF genes to determine whether the *in vitro* stimulation with these genetically modified DCs could induce a potent CEA-specific CTL responses against CEA-expressing target cells. In addition, the possibility that a synergistic effect of this co-transduction could increase life span of these genetically modified DCs was investigated.

Materials and methods

Cell lines. Autologous Epstein-Barr virus-transformed B-lymphoblastoid cell lines (EBV-LCLs) were generated using standard methods (14) from healthy donor PBMCs transformed by EBV using the B95-8 Kit (Immuno-Biological-Laboratories, Gunma, Japan) and maintained in TIL Media (Immuno-Biological-Laboratories). Human embryonic kidney cell line 293 (American Type Culture Collection, Rockville, MD) was grown in DMEM (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Co., Carlsbad, CA), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen).

Generation of DCs. For the generation of DCs and the induction of CTLs, RPMI-1640 medium (Bio Whittaker, Walkersville, MD) supplemented with 5% heat-inactivated human AB serum (ICN Biomedicals, Glen Cove, NY), 100 U/ml penicillin, 100 μ g/ml streptomycin and 1 mM MEN sodium pyruvate, 0.1 mM MEN non-essential amino acids and 2 mM L-Glutamine (Bio Whittaker) were used (complete medium). DCs were prepared from PBMCs as previously described (3,15). Briefly, PBMCs were isolated from healthy donors by density centrifugation on Ficoll-Hypaque gradients (Amersham Biosciences, Piscataway, NJ) for 20 min at 1800 rpm at room temperature. After three washes, the cells were resuspended at 4x10⁶/ml in complete medium and incubated for 1.5 h in 75-cm² tissue culture flasks at a total volume of 10 ml/flask. The non-adherent cells were gently washed out with complete medium. The remaining plastic-adherent cells were cultured

in complete medium supplemented with 1000 U/ml recombinant human (rh) GM-CSF (Kirin Brewery Co., Tokyo, Japan) and 1000 U/ml rh interleukin (IL)-4 (kindly provided by Ono Pharmaceutical Co., Tokyo, Japan). After 7 days, the non-adherent cells were removed and were used as immature DCs. These DC preparations were stained with FITC or PE-conjugated monoclonal antibodies against human cell surface molecules, and examined by flow cytometry. Approximately 99% of the cells showed high levels of CD11b and CD11c, >99% of the cells showed high levels of HLA class I and class II, and around 5% of the cells showed low levels of CD14, CD80 and CD83 (data not shown).

Generation of recombinant adenoviral vectors. The GM-CSF cDNA fragment was excised from the plasmid pORF-hGM-CSF containing the complete human GM-CSF cDNA (InvivoGen, San Diego, CA) by *SgrAI* and *NheI*, blunt-ended, and ligated into the *SwaI* site of cosmid vector pAxCawt (Takara, Shiga, Japan) to yield pAxCahGM-CSF. The CEA cDNA fragment was excised from the plasmid pL107.1 containing the complete human CEA cDNA, and pAxCACEA was generated as previously described (9). The recombinant adenoviral vectors, AxCahGM-CSF and AxCACEA were generated by the COS-TPC method as previously described (16). The recombinant AxCALacZ expressing a LacZ reporter gene was also generated by the COS-TPC method (16).

Gene transduction of DCs. DCs were transfected with recombinant adenoviral (Ad) vectors using the centrifugal method (17). Briefly, DCs were mixed with recombinant Ad vectors at various multiplicities of infections (MOIs) in a final volume of 1 ml of phosphate-buffered saline (PBS) containing 1% human AB serum, 10 U/ml penicillin and 10 μ g/ml streptomycin, and they were centrifugated at 2000 x g at 37° for 2 h. The DCs were then washed twice with PBS, counted, and placed in 6-well plates at a density of 1.0x10⁶ cells/well in 3 ml of complete medium supplemented with 0.1 KE/ml of OK-432 (Chugai Pharmaceutical Co., Tokyo, Japan) (18). After 48 h, these DCs were used for the experiments.

Induction of CEA-specific CTLs and cytotoxicity assay. Immature DCs (HLA02/24) were transfected with AxCACEA at a MOI of 100, AxCALacZ at a MOI of 100, or AxCACEA at a MOI of 100 plus AxCahGM-CSF at various MOIs and then were cultured in the presence of 0.1 KE/ml of OK-432. After 48 h, these genetically modified DCs were used as stimulator cells. Autologous PBMCs (HLA02/24) were used as responder cells. On day 0, 4x10⁶ responder cells and 2x10⁵ stimulator cells were mixed in complete medium containing 10 ng/ml of rh IL-7 (Genzyme Techne Co., Cambridge, MA), and cultured in a 24-well plate at a total volume of 1 ml/well. On day 2, complete medium containing 20 U/ml of rh IL-2 (Shionogi Pharmaceutical Co., Osaka, Japan) was added at a total volume of 2 ml/well. On days 7 and 14, the cultures were restimulated with gene-transduced DCs at a ratio of 20:1. Complete medium containing 20 U/ml of rh IL-2 was added every 2 to 3 days. On day 21, after 3 cycles of stimulation by genetically

modified DCs, a cytotoxic assay was performed using a 4-h ^{51}Cr release assay. CEA cDNA-transduced autologous EBV-LCLs and LacZ cDNA-transduced LCLs were used as target cells. The abbreviations for the vectors used are: DC-AxCACEA, DCs transfected with AxCACEA; DC-AxCACEA/GM-CSF, DCs transfected with AxCACEA and AxCAhGM-CSF; and LCL-AxCACEA, EBV-LCLs transfected with AxCACEA.

Blocking of the CTL activity. Blocking of the CTL activity against LCL-AxCACEA was achieved by incubating either effector cells or target cells with anti-human CD4 (RPA-T4), anti-human CD8 (RPA-T8), anti-human HLA-A,B,C (G46-2.6) (BD PharMingen, San Diego, CA) or anti-mouse IgG (control antibody, Dako, Glostrup, Denmark) antibodies at a dose 10 μl /well for 30 min at 4°C before a 4-h ^{51}Cr -release assay.

Flow cytometric analysis. The cell surface antigens were determined using a FACSCalibur (Becton-Dickinson, Mountain View, CA) with CellQuest software. The cells (2×10^5) were incubated with specific antibodies in PBS for 30 min at 4°C, rinsed twice, and the cells were analyzed. To assay for intracellular expression of GM-CSF and Bcl-x_L, IntraPrep permeabilization reagents were used for cytoplasmic immunophenotyping (Coulter Co., Marseille, France). The following antibodies were used for flow cytometry: PE-conjugated anti-human CD80 monoclonal antibody (mAb) (L307.4), PE-conjugated anti-human CD83 mAb (HB15e), PE-conjugated anti-human CD86 mAb (2331), PE-conjugated anti-human CCR7 mAb (3D12) (BD PharMingen), mouse anti-human CEA mAb (NCL-CEA-2) (Novocastra, Newcastle, UK), rat anti-human GM-CSF (BVD2-21C11) (Beckman Coulter, Carlsbad, CA) and mouse anti-human Bcl-x_L mAb (H-5) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). FITC-conjugated anti-mouse IgG (Dako) and PE-conjugated anti-rat IgG (SouthernBiotech, Birmingham, AL) was used as a secondary antibody for the unconjugated mAb, at 4°C for 30 min.

Enzyme-linked immunosorbent assay (ELISA). Genetically modified DCs were seeded at a concentration of 1×10^6 cells/well on a 48-well plate for 48 h in a complete medium. Thereafter, the supernatants were harvested and GM-CSF level was measured using hGM-CSF ELISA kit (Endogen, Inc., Woburn, MA). Each assay was performed on duplicate samples.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from genetically modified DCs and RT-PCR was performed to examine the Bcl-x_L mRNA expression using a pair of specific primers (sense: 5'-TTGGACAATGGACTGGTTGA-3'; antisense: 5'-GTAGAGTGGATGGTCAGTG-3'). To ensure the quality of the procedure, RT-PCR was performed on the samples using specific primers for β -actin.

Evaluation of the DCs lifespan. Genetically modified DCs were seeded in 96-well plates at a density of 2×10^5 cells/well under cytokine-free conditions. DC survival was quantified

by measurement the cell viability by trypan blue exclusive method and it was displayed as a percentage of the viable cells.

Evaluation of apoptosis in DCs. Apoptosis of DCs was evaluated by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-FITC nick end labeling (TUNEL) assay using Mebstain Apoptosis Kit Direct (Coulter Co.). Genetically modified DCs were seeded under cytokine-free conditions. After 1 or 4 days the DCs were collected and washed twice with PBS containing 2 % bovine serum albumin (BSA-PBS), and gently suspended in ice-cold 4% paraformaldehyde for 30 min. The fixed cells were then washed with 70% ethanol and the dehydrated cells were incubated for 30 min at -20°C. Thereafter, the cells were incubated for 1 h at 37°C in the dark in reaction buffer, with dUTP-FITC in order to label the 3'-OH ends of the fragmented DNA. After washing with BSA-PBS, the nuclei stained with TdT-mediated dUTP-FITC of the cells were analyzed by flow cytometry.

Apoptosis of the DCs induced by TGF β -1 was also examined. Genetically modified DCs were incubated for 48 h in the presence of rh TGF β -1 (40 ng/ml) (Sigma, St. Louis, MO). Next, the apoptotic cells were quantified by TUNEL assay as described above.

The experimental procedure. This experiment was approved by the committee of recombinant DNA experiment in Wakayama Medical University. All experiments were performed in accordance with the guidelines of this committee. We obtained informed written consents from 3 healthy donors before experiments.

Results

The adenoviral transduction efficiency of DCs. The ability of recombinant adenoviral vector AxCACEA to transfer and express CEA gene in DCs was evaluated. The DCs generated from PBMCs showed only background levels of CEA activity. The expression of CEA was readily detected using the centrifugal method in the majority (47%) of DCs transfected with AxCACEA at a MOI of 100 (data not shown), and was further increased at a MOI of >100 in a dose-dependent manner (MOI 200, 55%; MOI 300, 63%) (data not shown). Next, the viability of DCs transfected with AxCACEA at various MOIs was determined. At a MOI ≤ 100 , the viability of transduced DCs was similar to that of non-transduced DCs, whereas at a MOI ≥ 200 , the viability of transduced DCs decreased in a dose-dependent manner (data not shown). Based on these observations, all subsequent studies were carried out with AxCACEA at a MOI of 100. In addition, the expression of CEA in target cells was measured. CEA expression of AxCACEA-infected autologous LCLs at a MOI of 100 demonstrated a 95% frequency, although mean fluorescence intensity in LCL-AxCALacZ was not detected (data not shown).

An ELISA analysis demonstrated that the DCs transfected with AxCAhGM-CSF at various MOIs produced high concentrations of GM-CSF in a dose-dependent manner. In contrast, the DCs transfected with AxCALacZ showed no secretion of GM-CSF. The GM-CSF production from the

Table I. Human GM-CSF production from DCs transfected with AxCAhGM-CSF at various MOIs.

	MOI	ng/1x10 ⁶ DCs	
		DC	DC-AxCACEA
AxCAhGM-CSF			
	0	0	0
	2	0.8	1.1
	5	1.2	2.3
	15	3.7	5.2
	30	7.5	7.9
	50	20.4	17.5
	100	42.8	22.1
	200	126.4	165.9
	300	188.0	226.5
AxCALacZ			
	50	0	0
	100	0	0

DCs or DCs-AxCACEA (DCs were transfected with AxCACEA at a MOI of 100) were transfected with AxCAhGM-CSF or AxCALacZ at various MOIs and cultured at a concentration of 1x10⁶ cells per well for 48 h. Then supernatants were harvested and assayed by ELISA.

DCs transfected with AxCAhGM-CSF was not affected by the co-transfection of 100 MOI of AxCACEA (Table I).

We also analyzed the frequency of DCs simultaneously expressing CEA and GM-CSF in DCs co-transfected with AxCACEA and AxCAGM-CSF. As shown in Fig. 1, 21% of DC-AxCACEA/GM-CSF was expressed both of CEA and GM-CSF.

The effect of GM-CSF gene transduction on the expression of costimulatory molecules of DCs. To examine the effect of co-transduction with the GM-CSF gene on the expression of costimulatory molecules and CCR7, DCs were transfected with various recombinant Ad vectors, and the expression of CD80, CD83, CD86 and CCR7 on DCs were determined by flow cytometry. As shown in Fig. 2, mature DCs, stimulated with OK-432, showed higher expression of all those molecules than the immature DCs. CCR7 expression on DCs was obviously enhanced by adenoviral transduction with the GM-CSF gene, while the expressions of CD80, CD83 and CD86 were unchanged (Fig. 2).

CEA-specific cytotoxic responses induced by CEA gene-transduced DCs. The ability of genetically modified DCs expressing CEA to induce CEA-specific cytotoxic responses was evaluated. The lymphocytes stimulated by DC-AxCACEA showed cytotoxic activity against LCL-AxCACEA, while they showed no cytotoxicity against LCL-AxCALacZ. Furthermore, the lymphocytes stimulated by DC-AxCALacZ showed no cytotoxic activity against either the LCLs expressing CEA, or the LCL-AxCALacZ (Fig. 3). An anti-

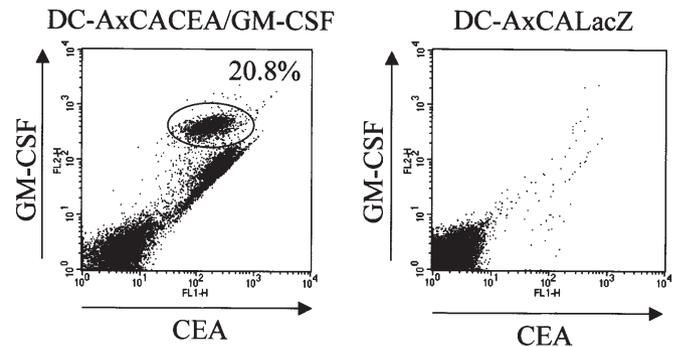


Figure 1. The frequency of genetically modified DCs expressing both CEA and GM-CSF. DCs-AxCACEA/GM-CSF (MOI 100/30) or DCs-AxCALacZ (MOI 100) were isolated and dual-stained by incubation with PE-labeled anti GM-CSF mAb and FITC-labeled anti-CEA mAb, and then, cells were analyzed by flow cytometry. The values show the percentage of doubly stained cells in 10,000 cells.

body blocking assay was used to assess the functional phenotypes of these PBMC-derived CTLs induced by DC-AxCACEA. The cytotoxic activity of CTLs against LCL-AxCACEA induced by the stimulation of DC-AxCACEA was blocked by anti-CD8 Ab and anti-HLA class I Ab, but not by anti-CD4 Ab or control Ab (data not shown). These results suggest that genetically modified DCs expressing CEA can induce an HLA class I restricted CEA-specific CD8⁺ CTL responses, without initiating the adenovirus vector-specific CTL responses.

The boosting effect of transduction of the GM-CSF gene on cytotoxic activity. The optimal MOI of Ad vector AxCAhGM-CSF for enhancing the cytotoxic activity of the PBMC-derived CTLs was examined. The cytotoxic activity of the lymphocytes against LCL-AxCACEA induced by *in vitro* stimulation with DC-AxCACEA was significantly enhanced by co-transduction of the GM-CSF gene (Fig. 4A and B), and the optimal dose of AxCAhGM-CSF was suggested to be a MOI of 30 (Fig. 4A). In this study, the dose of AxCAhGM-CSF was fixed at that dose. On the other hand, the lymphocytes stimulated by DC-AxCACEA/GM-CSF showed no cytotoxic activity against LCL-AxCACEA (Fig. 4C). These results suggest that co-transduction of the GM-CSF gene into DCs expressing CEA can induce the capacity of DCs to prime CEA-specific CTL responses.

Effect of transduction of the GM-CSF gene on the apoptosis and the lifespan of genetically modified DCs. The survival of DCs in cultures was determined using the trypan blue exclusion method in order to examine the effect of the GM-CSF gene transduction on the lifespan of DCs. The percentage of viable cells among DC-AxCACEA/GM-CSF was significantly elevated compared with the other groups. Interestingly, approximately 20% of DCs simultaneously expressing CEA and GM-CSF were still alive on day 6, while all DCs expressing CEA alone were dead (Fig. 5A). Next, the expression of anti-apoptotic protein, Bcl-x_L, in genetically modified DCs was observed by flow cytometry. The expression of Bcl-x_L in genetically modified DCs was markedly augmented by the co-transduction of the GM-CSF

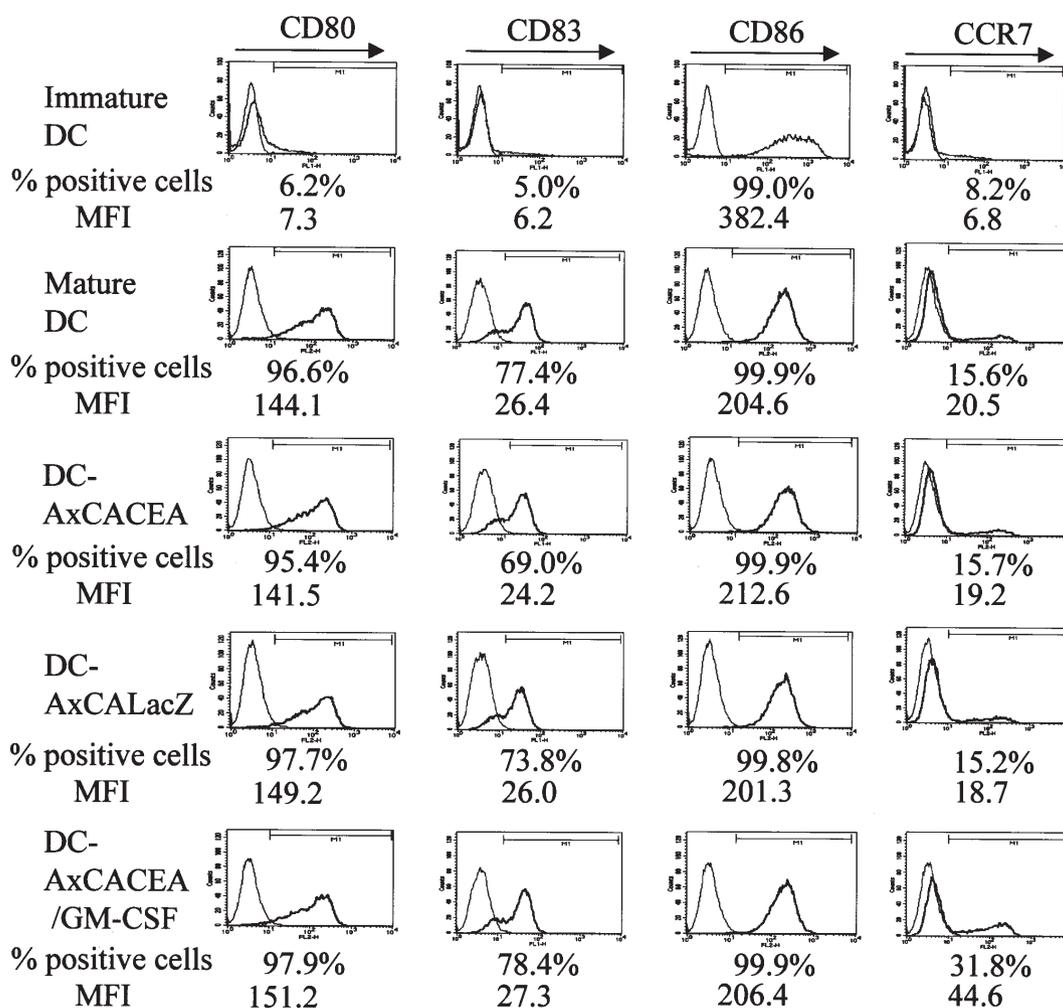


Figure 2. The expression of costimulatory molecules and CC chemokine receptor 7 on genetically modified DCs. The expression of cell surface antigens was analyzed by flow cytometry. The values of % positive cells show the percentage of positively stained cells. The values of MFI show the mean fluorescence intensity of activation markers on genetically modified DCs. Experiments were performed from 3 different donors to confirm the reproducibility of the results, and similar results were obtained.

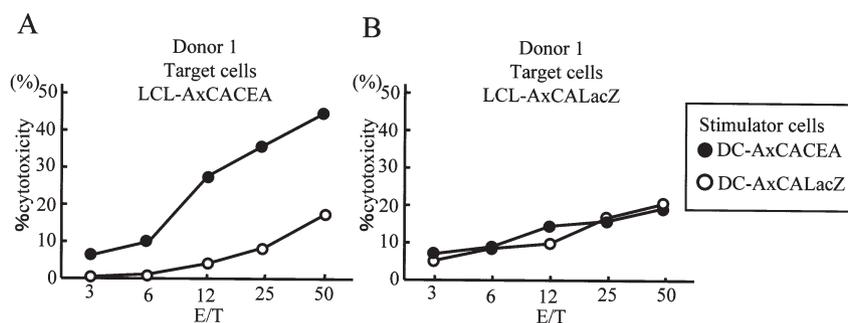


Figure 3. Cytotoxic activity of CTLs against CEA-expressing target cells induced by CEA cDNA-transduced DCs. CEA cDNA-transduced DCs (●) or LacZ cDNA-transduced DCs (○) were used as stimulator cells. Autologous PBMCs were used as responder cells. The CTL culture after 3 cycles of restimulations *in vitro* was used as effector to test the lysis of the following targets: LCL-AxCACEA (A) and LCL-AxCALacZ (B). Experiments were performed from 3 different donors to confirm the reproducibility of the results, and similar results were obtained.

gene (Fig. 5B). In addition, mRNA expression of Bcl-x_L in genetically modified DCs was examined by RT-PCR. Bcl-x_L mRNA-expression in genetically modified DCs expressing CEA was markedly augmented by the co-transduction with the GM-CSF gene (Fig. 5B). To verify whether the co-transduction with the GM-CSF gene into DCs expressing CEA

inhibited apoptosis, a TUNEL assay was performed. As shown in Fig. 5C, GM-CSF gene transduction inhibited the apoptosis of genetically modified DCs. When compared with DC-AxCACEA and DC-AxCALacZ, the apoptotic cell-population among DC-AxCACEA/GM-CSF significantly reduced after 4 days. These results suggest that although

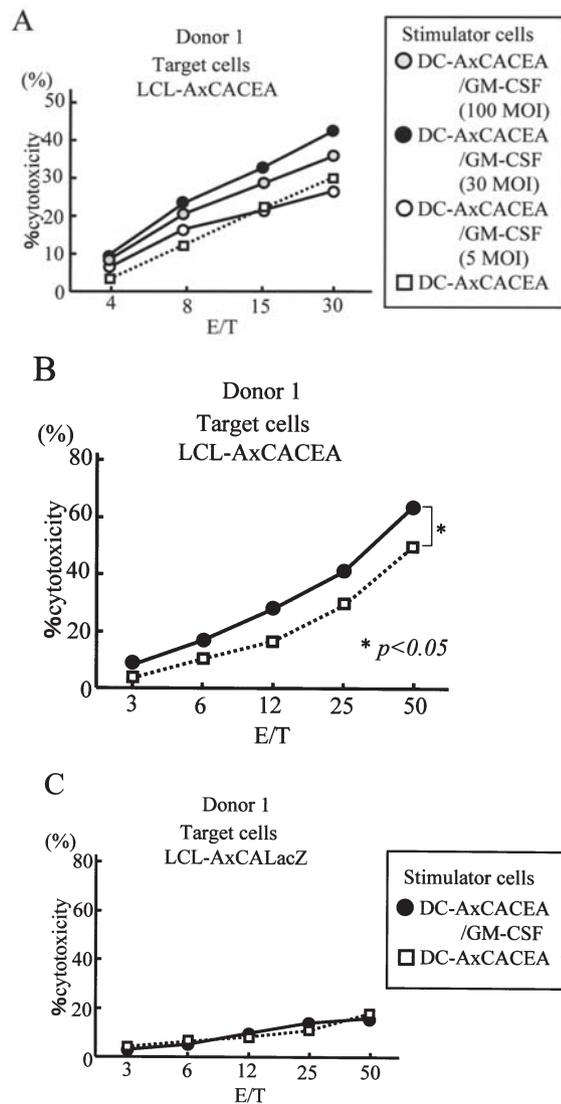


Figure 4. The boosting effect of transduction of the GM-CSF gene on cytotoxic activity. (A) The optimal MOI of AxCaGM-CSF for enhancing the cytotoxic activity of the CTLs. DCs were transfected with AxCACEA (\square) at a MOI of 100. Furthermore, DCs were transfected simultaneously with AxCACEA (MOI 100) and AxCaGM-CSF: MOI of 100 (\circ), 30 (\bullet), 5 (\circ). These genetically modified DCs were used as stimulator cells. After 3 cycles of restimulations *in vitro*, CTLs were used as effector to test the lysis of the target, LCL-AxCACEA. Experiments were performed from 3 different donors to confirm the reproducibility of the results, and similar results were obtained. (B and C) The cytotoxic activity of the CTLs against LCL-AxCACEA (B) and LCL-AxCALacZ (C) induced by *in vitro* stimulation with DC-AxCACEA (MOI 100) (\square) or DC-AxCACEA/GM-CSF (MOI 100/30) (\bullet). *The difference between DC-AxCACEA/GM-CSF and DC-AxCACEA was significant (E/T=50, $p < 0.05$). Statistical analysis was performed from 3 different donors by ANOVA and the Fisher's test. A p -value of < 0.05 was considered to be significant.

the apoptosis of DCs was induced by adenoviral transduction, co-transduction of DCs with the GM-CSF gene led to the inhibition of apoptosis of these DCs through the enhancement of anti-apoptotic protein Bcl- x_L expression.

Effect of transduction of the GM-CSF gene on the apoptosis of genetically modified DCs induced by TGF β -1. In order to first reconfirm the *in vitro* effects of TGF β -1 on the cultured DCs, DCs were incubated with TGF β -1 (40 ng/ml) for 48 h

and then stained with TUNEL to evaluate apoptosis. TGF β -1 was found to induce significant increases in the incidence of apoptosis on the cultured DCs (Fig. 6). To determine whether the co-transduction with the GM-CSF gene into genetically modified DCs expressing CEA might reduce the apoptosis caused by TGF β -1, the difference in the incidence of apoptosis between DC-AxCACEA and DC-AxCACEA/GM-CSF was observed in the presence of TGF β -1. The incidence of apoptosis among genetically modified DCs expressing CEA caused by TGF β -1 was strongly inhibited by co-transduction with the GM-CSF gene (Fig. 6).

Discussion

We have examined the potential cancer vaccine therapy provided by DCs adenovirally transduced with the TAA gene and cytokine gene in mouse models (8,9,19). In this study, the potency of DCs adenovirally transduced with the CEA gene and the GM-CSF gene as the cancer vaccine in human was examined *in vitro*, in order to assess the feasibility of this strategy. The results showed that CEA-specific CTLs were generated from PBMCs stimulated with adenovirally modified DCs expressing CEA. Moreover, the cytotoxicity of CTLs against CEA-expressing target cells was augmented by co-transduction of DCs with the GM-CSF gene. These experiments also demonstrated that the co-transduction with the GM-CSF gene into DCs expressing CEA inhibited apoptosis of these cells via up-regulation of Bcl- x_L expression, thus resulting in extending the lifespan of the DCs.

The present study showed that *ex vivo* adenoviral gene transduction using a centrifugal method is a highly efficient method of gene transfer into human DCs (17), and the viability of DCs, which were adenovirally transduced with the CEA gene at a MOI of 100, was similar to that of naive DCs immediately after gene transduction. However, the viability of those DCs decreased day by day during *in vitro* culture under cytokine-free conditions. The current focus on DC-based gene immunotherapy using adenovirus vectors has shed light on the damage to DCs caused by adenoviral toxicity (20). Considering the clinical application of this strategy, it is important to develop an effective protocol resulting in extending the lifespan of genetically modified DCs used for the vaccine.

GM-CSF is a cytokine involved in proliferation and differentiation of hemopoietic cells (10). It has been recognized in an anti-apoptotic cytokine in DCs (11), and it has been shown to prolong the lifespan of DCs in an *in vitro* culture (10,11,21). There is convincing evidence that the increased survival of DCs in the presence of GM-CSF correlates with the increased expression of anti-apoptotic protein Bcl-2 (11,22,23). Bcl- x_L , a member of the Bcl-2 family, is capable of suppressing apoptosis in various cells (12,24). It also plays an important role in the regulation of hemopoiesis and the survival of immunocompetent cells (22,25,26). It has been demonstrated that Bcl- x_L is required for the promotion of DC survival by Toll-like receptor (TLR) ligands and T cell costimulatory molecules (26,27). In this study, the transduction of the GM-CSF gene into DCs inhibited apoptosis in these genetically modified DCs through the up-regulation of Bcl- x_L , thus resulting in

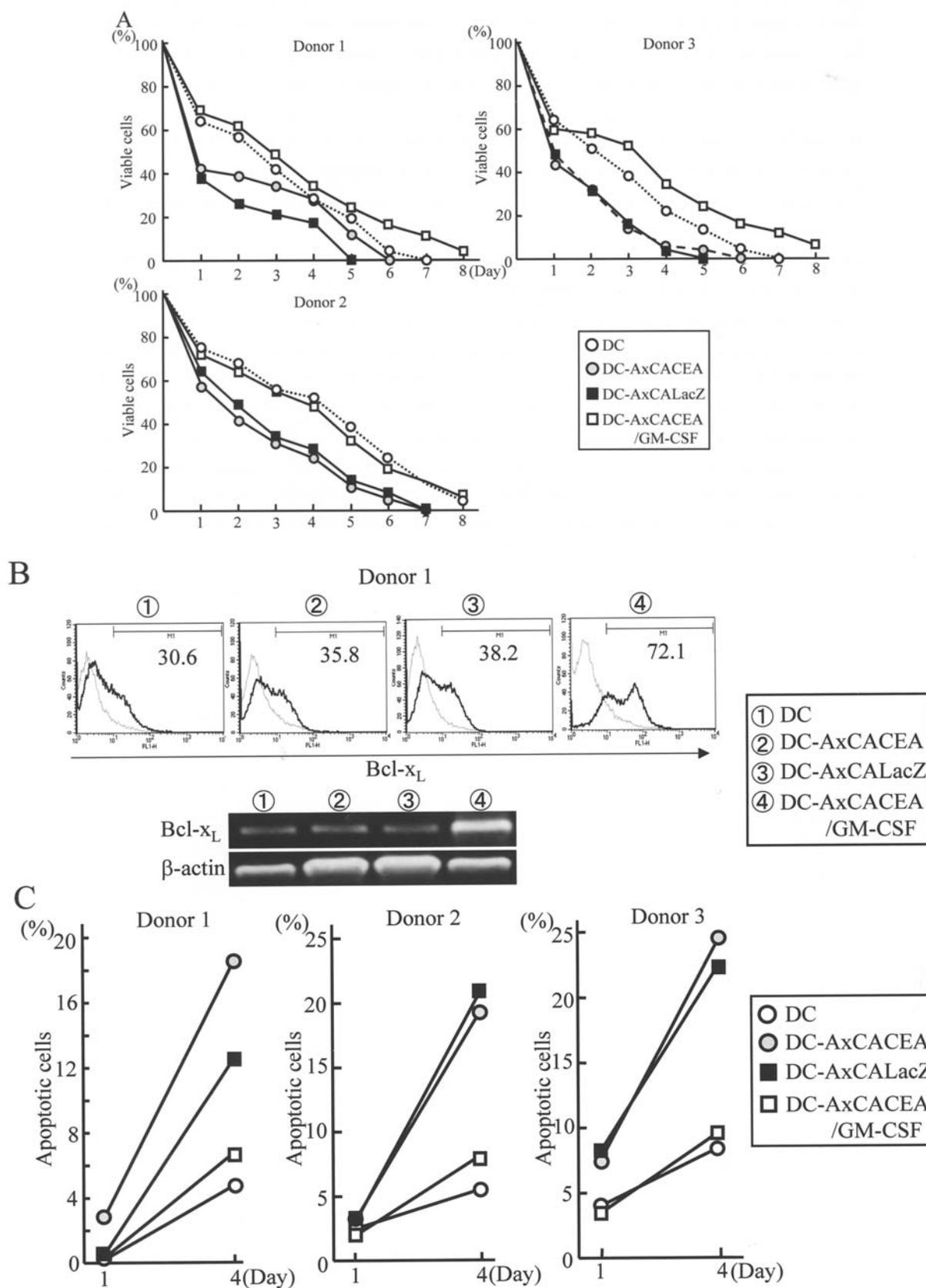


Figure 5. Effect of the transduction of the GM-CSF gene on the apoptosis and the lifespan of genetically modified DCs. (A) Evaluation of the lifespan of DCs. DCs or genetically modified DCs were seeded in a long-term culture under cytokine-free conditions. DC survival was quantified by the measurement of cell viability by trypan blue exclusive method and was displayed as a percentage of viable cells. Experiments were performed from 3 different donors. (B) The expression of Bcl- x_L in genetically modified DCs. The expression of Bcl- x_L protein in genetically modified DCs was analyzed by flow cytometry. The values show the percentage of positively stained cells. Total RNA was extracted from these genetically modified DCs, and expression of Bcl- x_L mRNA was assessed by RT-PCR using a pair of specific primers. (C) TUNEL assay. The nuclei stained with TdT-mediated dUTP-FITC of the genetically modified DCs were analyzed by flow cytometry. The values show the percentage of apoptotic cells in 10,000 cells. Experiments were performed from 3 different donors.

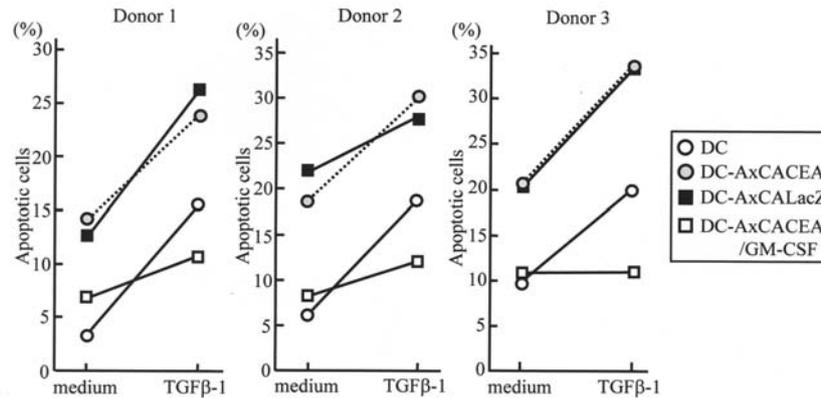


Figure 6. Effect of the transduction of the GM-CSF gene on the apoptosis of genetically modified DCs induced by TGF β -1. DCs or genetically modified DCs were incubated with either medium alone or rh TGF β -1 (40 ng/ml) for 48 h. Thereafter, apoptotic cells were quantified using the TUNEL assay. The values show the percentage of apoptotic cells in 10,000 cells. Experiments were performed from 3 different donors.

the extension of the lifespan of those cells. The augmenting effect on the cytotoxic activity of CTLs against CEA-expressing target cells stimulated with DCs that simultaneously express CEA and GM-CSF could therefore be mediated by the prolongation of the lifespan of these DCs *in vitro*.

Recent studies have revealed that the maturity of DCs was involved in apoptosis of DC itself. Factors that stimulate DC maturation, such as the CD40 ligand, receptor activator of NF- κ B (RANK) ligand, tumor necrosis factor α (TNF- α), lipopolysaccharide (LPS), IL-12, IL-15 and CpG are shown to inhibit apoptosis in DCs via up-regulations of Bcl-2, Bcl-x_L, and the cellular inhibitors of apoptotic proteins (28,29). However, the results of the present study showed that adenoviral co-transduction with the GM-CSF gene did not influence the expression of surface markers for DC maturation, such as CD80, CD83, CD86, because OK-432, which was used as maturation stimulus of DCs after adenoviral gene transduction, fully matured DCs. On the other hand, CCR7 expression on DCs was markedly enhanced by adenoviral transduction with the GM-CSF gene. Our previous study also demonstrated that GM-CSF gene transfer to DCs enhanced the expression of CCR7 on these DCs, and led to improved migratory capacity of DCs to draining lymph nodes (8). Indeed, CCR7 expression enhances migration of stimulated DCs to lymphatic vessels and lymph nodes as well (30,31). In addition, CCR7 has also been reported to induce anti-apoptotic signaling in DCs. It has been demonstrated that the stimulation of DCs with CCR7 ligands, CCL19 and CCL21, activates phosphatidylinositol 3'-kinase and its downstream effector Akt1 (PI3K/Akt1), thereby regulating the survival of the DCs, and thus resulting in the inhibition of apoptosis of mature DCs (32). Therefore, *in vivo*, when genetically modified DCs simultaneously expressing TAA and GM-CSF move to lymph nodes, CCR7 may play an important role in maintaining the viability of these DCs, because CCL19 and CCL21 are present at high levels in T-cell zones in the lymph nodes (31,32).

Tumor-induced immunosuppressive factor, TGF β has been reported to be involved in the apoptotic death of DCs (13). Tumor-derived TGF β -1 induces apoptosis of DCs in

the sentinel lymph node (13). Therefore, the lifespan of DCs in lymphoid tissue is reduced in cancer patients (13). The present study showed that the transduction of the GM-CSF gene into DCs strongly suppressed the incidence of apoptosis of DCs caused by TGF β -1. This suggests that the vaccination strategy, using DCs expressing TAA and GM-CSF for the cancer patients can overcome the immunosuppressive conditions in lymphoid tissues, and thus induce a potent antitumor effect.

The present study demonstrated the first trial of co-transduction of the CEA gene and the GM-CSF gene into human PBMCs-derived DCs using recombinant adenovirus vectors. The immunotherapy using these genetically modified DCs may provide the following advantages: i) adenoviral toxicity to DCs can be reduced; ii) the lifespan of the vaccinated DCs can be prolonged; and iii) GM-CSF may prevent DCs from apoptosis induced with tumor-derived TGF β -1 in regional lymph nodes and tumor tissues. Therefore, this strategy may be promising for clinical application as an effective cancer vaccine therapy for patients with CEA-expressing gastrointestinal tumors.

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