Streptococcal preparation OK-432 promotes the capacity of dendritic cells (DCs) to prime carcinoembryonic antigen (CEA)-specific cytotoxic T lymphocyte responses induced with genetically modified DCs that express CEA

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Abstract. Cancer immunotherapy using dendritic cells (DCs) adenovirally transduced with the whole tumor-associated antigen (TAA) gene is an effective approach. Streptococcal preparation OK-432 is useful for stimulating DCs in terms of maturation. In this study, we established carcinoembryonic antigen (CEA)-specific cytotoxic T lymphocytes (CTLs) using *in vitro* stimulation with adenovirally modified human DCs that express CEA. We investigated whether OK-432 stimulation could be more effective in inducing CEA-specific CTLs compared with other typical stimuli. DCs adenovirally transduced with the CEA gene were cultured under various conditions with tumor necrosis factor (TNF)-α, lipopolysaccharide (LPS), or OK-432. A cytotoxicity assay using peripheral blood mononuclear cell (PBMC)-derived CTLs was performed in a 4 h-^{51}Cr release assay. OK-432 stimulated immature DCs to acquire a mature phenotype and to produce significant amounts of T-helper 1 cytokines. In all groups (immature DCs, TNF-α/DCs, LPS/DCs, OK-432/DCs), CEA-specific CTLs were generated. OK-432-stimulated DCs (HLA-A24) induced the most potent cytotoxic activity against CEA-expressing targets (A24) but not against controls. OK-432/DCs were able to induce markedly potent CTLs specific to target cells pulsed with CEA652 peptide (HLA-A24-restricted peptide), although others failed to induce potent CTLs. In conclusion, the CTL induction protocol using adenovirally modified DCs that express CEA after maturation with OK-432 showed a potent antitumor activity against CEA-expressing target cells, and is therefore promising for clinical applications as a cancer vaccine therapy.

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Introduction

Dendritic cells (DCs) are the most potent stimulators of immunity, and DCs pulsed with tumor antigen *ex vivo* have applications in tumor immunotherapy (1). Carcinoembryonic antigen (CEA) is a heavily glycosylated oncofetal antigen that is overexpressed in human adenocarcinomas, especially in colon, pancreas, breast, and lung tumors (2). The tissue expression pattern makes CEA a potential target for tumor-specific immunotherapy. Antigenic peptides derived from CEA have been proven to elicit a cytotoxic T lymphocyte (CTL) response in the context of major histocompatibility complex (MHC) class I molecules (2-4). We performed a pilot study of DCs pulsed with CEA-specific peptides for patients with colorectal carcinoma. However, no clinical responses were found, although CEA-specific CTL responses were observed in a few patients (5). Therefore, we employed an alternative strategy for inducing antitumor immunity using DCs adenovirally transduced with the whole tumor-associated antigen (TAA) gene (6-10). In this approach, TAAs are endogenously processed and presented by MHC class I and II pathways in the context of the potent immune-stimulatory machinery of the DCs and induce polyclonal CTL responses (6,11). Ourselves and other groups have already demonstrated that human DCs transduced with a recombinant adenovirus encoding the CEA gene were able to effectively induce CEA-specific CTLs *in vitro* (10,12,13). We consider this strategy to be useful for clinical applications as a cancer vaccine therapy for patients with CEA-expressing gastrointestinal tumors.

Previous studies have clearly revealed that the maturity of the applied DCs has a significant impact on the outcome of the vaccination. Mature DCs are believed to induce T cell immunity, whereas the immature development stages of DCs are thought to induce T-cell anergy or regulatory T cells (14-16). The known DC maturation stimuli include lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α, interferon

(IFN)-α, CD40 ligand, poly I:C (a synthetic double-stranded RNA), and OK-432. However, there is no consensus on the best methods for DC maturation in such a gene-based vaccination strategy. OK-432, a penicillin-inactivated, lyophilized preparation of an avirulent human strain of *Streptococcus pyogenes*, has been used safely in Japan for >20 years as a cancer immunotherapy drug. OK-432 augments the activity of neutrophils, macrophages, lymphocytes, and natural killer (NK) cells. It modulates DC maturation through Toll-like receptor (TLR) 4 and the $ß_2$ integrin system to enhance antigen-specific CTL responses (17,18). OK-432-stimulated DCs can produce interleukin (IL)-12 and interferon (IFN)-γ, which are potent cytokines that mediate T-helper 1 (Th1) cell development (14,17-19). Our previous studies emphasized that the Th1 immune reaction was the most important to activate CTLs in a DC-based immunotherapy (7,8). Therefore, we considered that OK-432 is one of the best DC maturation stimuli.

In this study, we investigated whether OK-432 stimulation would be more effective in inducing CEA-specific CTLs compared with the other typical stimuli. Furthermore, we assessed the feasibility of this strategy using adenovirally modified DCs expressing CEA after maturation by OK-432.

Materials and methods

Cell lines. Autologous Epstein-Barr virus-transformed B-lymphoblastoid cell lines (EBV-LCLs) were generated using standard methods (20) from healthy donor peripheral blood mononuclear cells (PBMCs) (HLA-A02/24) transformed by EBV using a B95-8 kit and maintained in TIL media (Immuno-Biological-Laboratories, Gunma, Japan). The human gastric cancer cell lines MKN1 (HLA-A24/26) and MKN45 (HLA-A24/24) (Takara, Shiga, Japan), and the human colon cancer cell line HT29 (HLA-A24/24) (Shionogi Pharmaceutical Co., Osaka, Japan) were cultured in RPMI-1640 (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 μg/ml streptomycin (Invitrogen).

Generation of immature 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 μg/ml streptomycin, and 1 mM MEN sodium pyruvate, 0.1 mM MEN non-essential amino acids, and 2 mM L-glutamine (Bio Whittaker) was used (complete medium). DCs were prepared from PBMCs as described previously (5,21). 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 $4x10^6$/ml in complete medium and incubated for 1.5 h in 75 cm^2 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 IL-4 (kindly provided by Ono Pharmaceutical Co., Tokyo, Japan). After 7 days, the non-adherent cells were removed and used as immature DCs.

Generation of recombinant adenoviral vectors. The CEA cDNA fragment was excised from the plasmid pL107.1 containing the complete human CEA cDNA, and pAxCACEA was generated as described previously (8). The recombinant AxCACEA was generated by the COS-TPC method as described previously (22). The recombinant AxCALacZ expressing a LacZ reporter gene was also generated by the COS-TPC method.

Gene transduction of DCs. DCs were transfected with AxCACEA using the centrifugal method (9). Briefly, DCs were mixed with AxCACEA 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 μg/ml streptomycin, and they were centrifuged at 2000 x g at 37˚C for 2 h. The DCs were then washed twice with PBS, counted, and placed in 6-well plates at a density of $1.0x10^6$ cells/well in 3 ml of complete medium supplemented with 100 ng/ml of rh TNF-α (BD PharMingen, San Diego, CA), 100 ng/ml of LPS (*Escherichia coli* 055:B5, Sigma, St. Louis, MO), or 0.1 KE/ml of OK-432 (Chugai Pharmaceutical Co., Tokyo, Japan) (23,24). After 48 h, these DCs were used for the experiments.

Synthetic peptides. The 9-mer peptide CEA652 (TYACFVSNL), derived from CEA, was synthesized according to standard solid phase methods and purified by high-performance liquid chromatography (Takara). CEA652 was identified as a HLA-A2402-restricted epitope peptide that is able to induce CTLs specific to CEA-expressing tumor cells (5,14). The purity (>90%) and identity of peptides were determined by mass spectrometry analysis.

Induction of CEA-specific CTLs and cytotoxicity assay. Immature DCs (HLA02/24) were transfected with AxCACEA at MOI of 100, and then cultured under various conditions, medium alone, 100 ng/ml of rh TNF-α, 100 ng/ml of LPS or 0.1 KE/ml of OK-432. After 48 h, these DCs were irradiated (5,000 rads) and used as stimulator cells. Autologous PBMCs (HLA02/24) were used as responder cells. On day 0, $4x10^6$ responder cells and $2x10^5$ 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 in a total volume of 1 ml/well. On day 2, complete medium containing 20 U/ml of rh IL-2 (Shionogi) was added in 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 2-3 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, LacZ cDNA-transduced LCLs, and HLA-A24 positive human cancer cell lines, MKN45, HT29, and MKN1 were used as target cells. CEA652-loaded LCLs, LCLs incubated with 10 μg/ml CEA652 peptide overnight at 37˚C, were also used as a model target (5,14). The abbreviations used are: TNF-α/DC, TNF-α stimulated

Table I. The expression of costimulatory molecules on DCs.

	%			
	Immature DC	TNF-α/DC	LPS/DC	OK-432/DC
CD80	9.1±1.3	91.4±4.1[a]	85.2±6.8[a]	86.2±7.5[a]
CD83	5.7±1.0	74.2±5.4[a]	68.7±8.5[a]	72.1±4.5[a]
CD86	92.4±3.1	99.7±0.3[b]	99.4±0.5[b]	99.7±0.3[b]

The expression of cell surface antigens was analyzed by flow cytometry. The values show the percentage of positively stained cells. Results are shown as the mean ± SD of 3 different donors. [a]Significantly different from immature DC (CD80, CD83; $p<0.0001$). [b]Significantly different from immature DC (CD86, $p<0.001$).

DCs; LPS/DC, LPS stimulated DCs; OK-432/DC, OK-432 stimulated DCs; DC-AxCACEA, DCs transfected with AxCACEA; LCL-AxCACEA, EBV-LCLs transfected with AxCACEA; LCL-CEA652, CEA652 peptide-loaded EBV-LCLs.

Blocking of CTL activity. Blocking of the CTL activity against LCL-AxCACEA was achieved by incubating either effector or target cells with anti-human CD4 (RPA-T4), anti-human CD8 (RPA-T8), anti-human HLA-A,B,C (G46-2.6) (all from BD PharMingen), or anti-mouse IgG (control antibody, Dako, Glostrup, Denmark) antibodies at a dose of 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 ($2x10^5$) were incubated with specific antibodies in PBS for 30 min at 4˚C, rinsed twice, and then analyzed. The following antibodies were used for flow cytometry: FITC-conjugated anti-human CD4 mAb (RPA-T4), PE-conjugated anti-human CD8 mAb (RPA-T8), PE-conjugated anti-human CD80 mAb (L307.4), PE-conjugated anti-human CD83 mAb (HB15e), PE-conjugated anti-human CD86 mAb (2331) (all from BD PharMingen), and mouse anti-human CEA mAb (NCL-CEA-2) (Novocastra, Newcastle, UK). FITC-conjugated anti-mouse immunoglobulin G (IgG) (Dako) was used as a secondary antibody for the unconjugated mAb at 4˚C for 30 min.

Enzyme-linked immunosorbent assay (ELISA). DCs were seeded at a concentration of $1x10^5$ cells/well in a 48-well plate for 48 h in complete medium (1 ml/well). Thereafter, the supernatants were harvested and the IFN-γ and IL-12 (p40+p70) levels were measured using a human IFN-γ ELISA kit (Endogen, Inc., Woburn, MA) and human total IL-12 ELISA kit (Endogen). Each assay was performed on duplicate samples.

IFN-γ release assay. We examined the CEA-specific $CD4^+$ and $CD8^+$ T cell responses using a IFN-γ release assay following the methods described previously (25). CTL cultures after 3 cycles of restimulation *in vitro* by OK-432/DC-AxCACEA or OK-432/DC-AxCALacZ were isolated, and then $CD4^+$ and $CD8^+$ T cells were isolated from the *in vitro* primed CTLs using a magnetic cell sorting (MACS) system (Dynabeads M-450 and Detachabeads, Dynal Inc., Lake Success, NY). Our flow cytometric analyses showed that >99% of the targeted cells were specifically selected (data not shown). The positively selected $CD4^+$ and $CD8^+$ T cells ($5x10^4$) were each stimulated with target cells (LCL-AxCACEA or LCL-AxCALacZ, $5x10^3$), in a total volume of 200 μl of complete medium in a 96-well round-bottomed plate. Supernatants collected 24 h later were tested for IFN-γ release using an ELISA kit (Endogen).

Statistical analysis. StatView 5.0 software (Abacus Concepts, Inc., Berkeley, CA) was used for all statistical analyses. Statistical analysis was performed by ANOVA and Fisher's test. A P-value of <0.05 was considered to be significant.

The experimental procedure. This experiment was approved by the Committee of Recombinant DNA Experiments of Wakayama Medical University. All experiments were performed in accordance within the Guidelines of this Committee. We obtained informed written consents from 3 healthy donors before experiments.

Results

CEA expression on target cells. Our previous study showed that the optimal MOI for AxCACEA was 100 in terms of the viability of genetically modified DCs (10). Therefore, the MOI value for AxCACEA was fixed at 100 in this study. We evaluated the expression of CEA in target cells, which are all bound by HLA-A24. CEA expression on AxCACEA-infected autologous LCLs at a MOI of 100 demonstrated a 95% frequency, although this expression on LCL-AxCALacZ demonstrated a 4% frequency (data not shown). A higher frequency of CEA expression was found in MKN45 cells and HT29 cells, but detectable fluorescence was not detected in MKN1 cells (data not shown).

Stimulatory activity of DCs cultured with TNF-α, LPS, or OK-432. Cell surface antigens of immature DCs were determined by flow cytometry. The cells (~99%) showed high levels of CD11b and CD11c, >99% of the cells showed high levels of HLA class I and class II, and ~5% of the cells showed low levels of CD14, CD80 and CD83 (data not shown). To examine the effect of stimulators on the phenotype of DCs, they were cultured with rh TNF-α, LPS, or OK-432, and the expression of costimulatory molecules (CD80, CD83, CD86) on cultured DCs was determined by flow cytometry. As shown in Table I, all stimulators induced higher expression levels of costimulatory molecules than immature DCs (CD80 and CD83, $p<0.0001$; CD86, $p<0.001$). The expression of CD80, CD83, and CD86 on OK-432/DC-AxCACEA was also determined by flow cytometry in order to examine the effect of transduction with the CEA gene on the expression of costimulatory molecules in DCs. The results showed that adenoviral transduction with the CEA gene did not influence

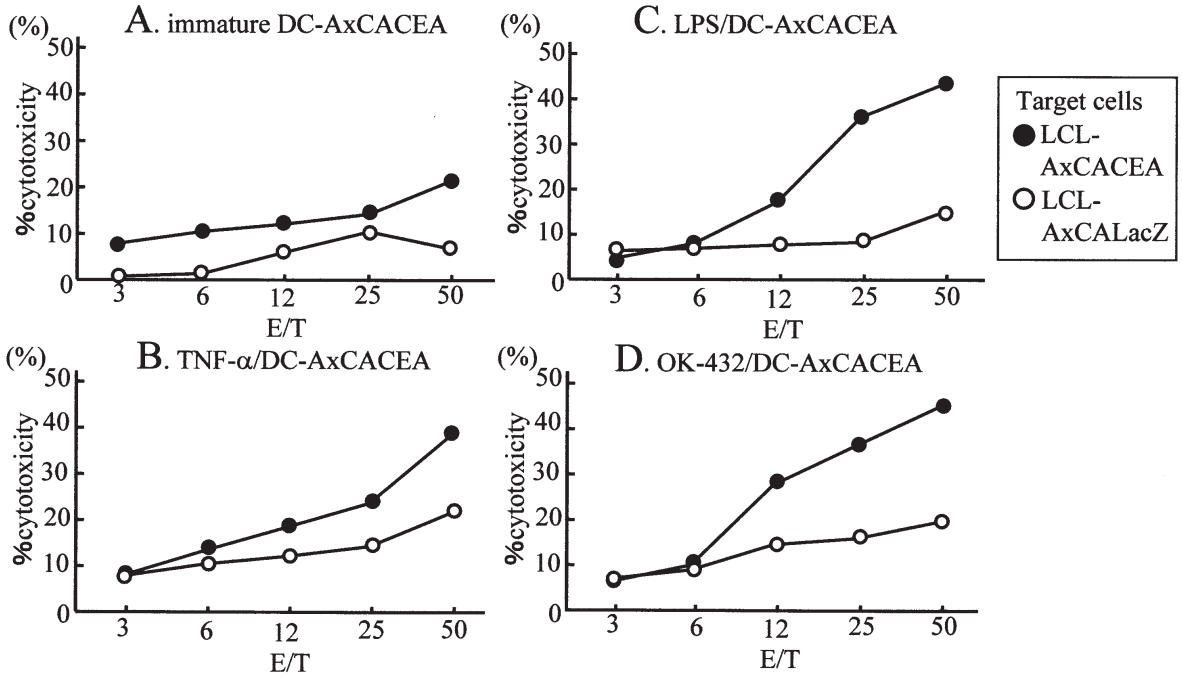

Figure 1. Cytotoxic activity of CTLs against CEA cDNA-transduced LCLs induced by CEA cDNA-transduced DCs. CEA cDNA-transduced DCs were cultured under various conditions (A. immature DC-AxCACEA: medium alone; B. TNF-α/DC-AxCACEA: 100 ng/ml of rh TNF-α; C. LPS/DC-AxCACEA: 100 ng/ml of LPS; D. OK-432/DC-AxCACEA: 0.1 KE/ml of OK-432). After 48 h, DCs were used as stimulator cells and autologous PBMCs were used as responder cells. The CTL culture after 3 cycles of restimulation *in vitro* was used as an effector to test the lysis of the following targets: LCL-AxCACEA (●) and LCL-AxCALacZ (○). Experiments were performed from 3 different donors to confirm the reproducibility of the results, and similar results were obtained.

Table II. IFN-γ and IL-12 production from DCs.

	pg/1x10^5			
	Immature DC	TNF-α/DC	LPS/DC	OK-432/DC
IFN-γ	9±3	72±9	65±5	307±20[a]
IL-12	0	0	589±36	15540±807[a]

DCs were cultured at a concentration of 1x10^5 cells per well for 48 h. Then supernatants were harvested and assayed by ELISA. Results are shown as the mean ± SD of 3 different donors. [a]Significantly different from TNF-α/DC, LPS/DC and immature DC (IFN-γ, IL-12; $p<0.0001$).

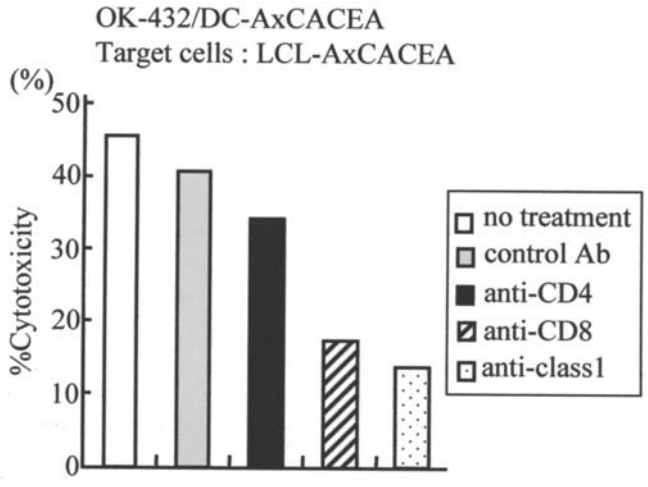

Figure 2. Blocking of CTL activity. To assess the functional phenotypes of CTLs induced by the OK-432/DC-AxCACEA, the effector cells or target cells were incubated with anti-CD4, anti-CD8, anti-HLA-class I, or anti-mouse IgG (control Ab) antibodies for 30 min at 4˚C before a 4-h ^{51}Cr-release assay. LCL-AxCACEA was used as a target (E/T ratio=50).

the expression of surface markers for DC maturation (data not shown).

We further examined the secretion of Th1-type cytokines (IFN-γ and IL-12) from DCs after stimulation with various stimuli. The OK-432 stimulation induced IFN-γ and IL-12 secretion in DCs at a high level, and the levels were significantly higher than that in DCs stimulated with TNF-α or LPS (Table II, $p<0.0001$). These results suggested that DCs were activated by OK-432, and activated DCs were strong inducers of Th1-type cytokines.

CEA-specific CTL responses induced by CEA gene-transduced DCs. We investigated the effect of DC maturation factors TNF-α, LPS, and OK-432 on CEA-specific CTL induction. The lymphocytes stimulated by all factors/DC-AxCACEA showed cytotoxic activity against LCL-AxCACEA, while they showed no cytotoxicity against LCL-AxCALacZ (Fig. 1). The cytotoxic activity of PBMC-derived CTLs against LCL-AxCACEA induced by stimulation with OK-432/DC-AxCACEA was blocked by anti-CD8 antibody and anti-HLA class 1 antibody, suggesting that these genetically modified DCs induced an MHC class I restricted CEA-specific CD8$^+$ CTL response (Fig. 2). It is important to analyze whether the established CTLs recognize the HLA-A24-restricted tumor

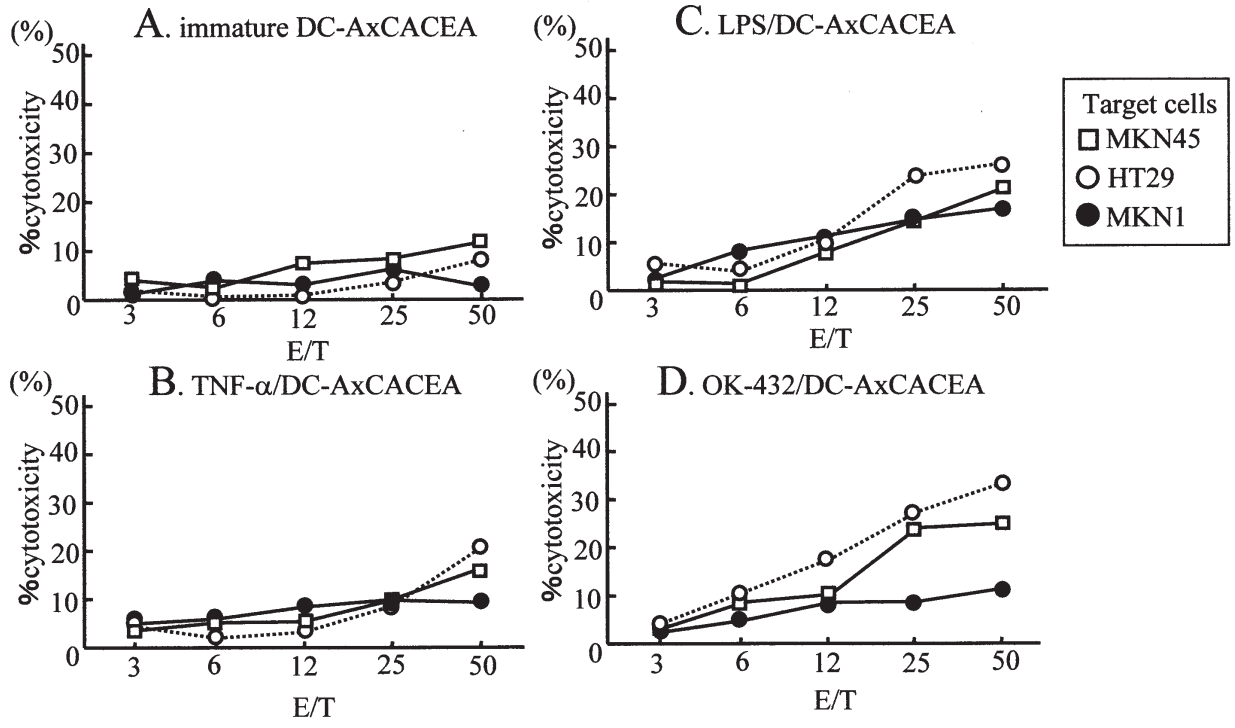

Figure 3. Cytotoxic activity of CTLs against tumor cells that endogenously express CEA as induced by CEA cDNA-transduced DCs. CTL cultures after 3 cycles of restimulations *in vitro*, as described in Materials and methods, were used as effectors to test the lysis of the following targets: MKN1 (●), MKN45 (□), and HT29 (○). Experiments were performed from 3 different donors to confirm the reproducibility of the results, and similar results were obtained.

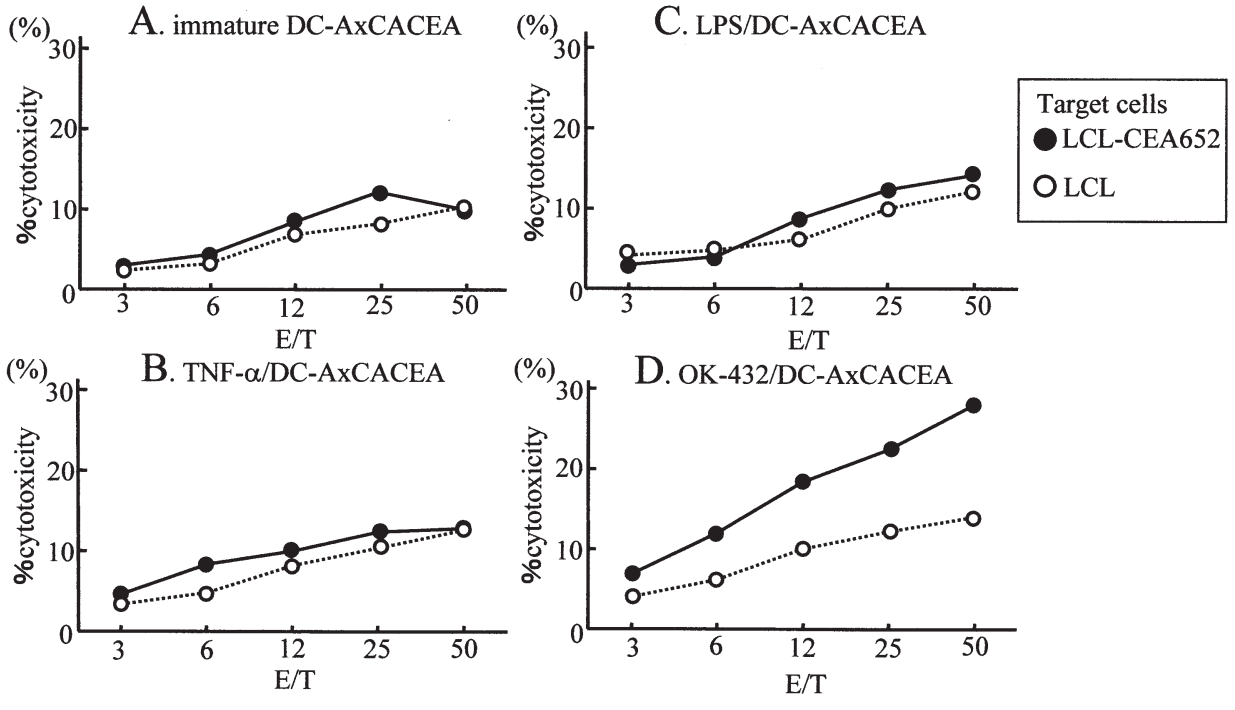

Figure 4. Cytotoxic activity of CTLs against CEA peptide-loaded LCLs induced by CEA cDNA-transduced DCs. CTL cultures after 3 cycles of restimulations *in vitro*, as described in Materials and methods, were used as effectors to test the lysis of the following targets: LCL-CEA652 (●) and LCL (○). Experiments were performed from 3 different donors to confirm the reproducibility of the results, and similar results were obtained.

cells expressing CEA as well as the CEA cDNA transduced autologous LCLs. As shown in Fig. 3, the CTLs induced by stimulation with OK-432/DC-AxCACEA showed cytotoxic activity only against CEA expressing tumor cells (MKN45, HT29), and importantly, other groups did not show significant activity. These results suggested the possibility that the CTLs

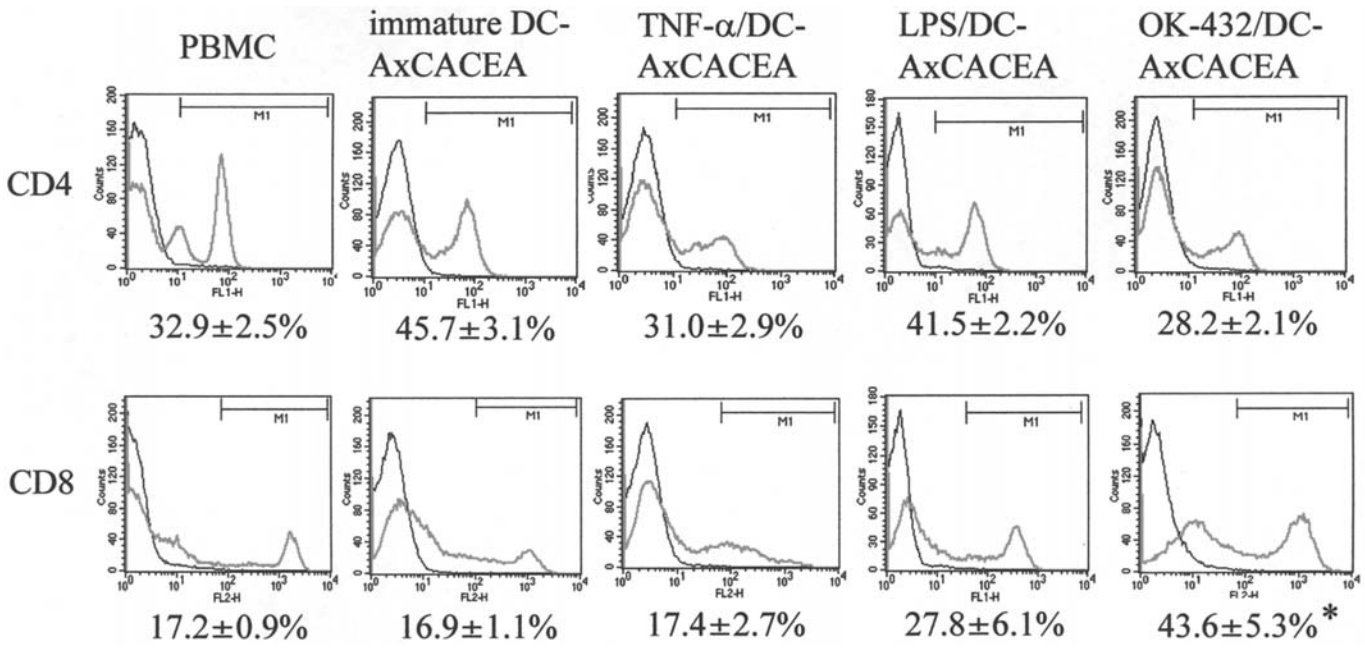

Figure 5. Cell surface antigens of CTLs. CTL cultures after 3 cycles of restimulation *in vitro* by genetically modified DCs expressing CEA were stained with FITC-conjugated anti-CD4 or anti-CD8 mAbs. Then, the expression of cell surface antigens was analyzed by flow cytometry. The values show the percentage of positively stained cells. Results are shown as the mean ± SD of 3 different donors. *Significantly different from immature DC-AxCACEA, TNF-α/DC-AxCACEA, LPS/DC-AxCACEA, and PBMCs (CD8, $p<0.001$).

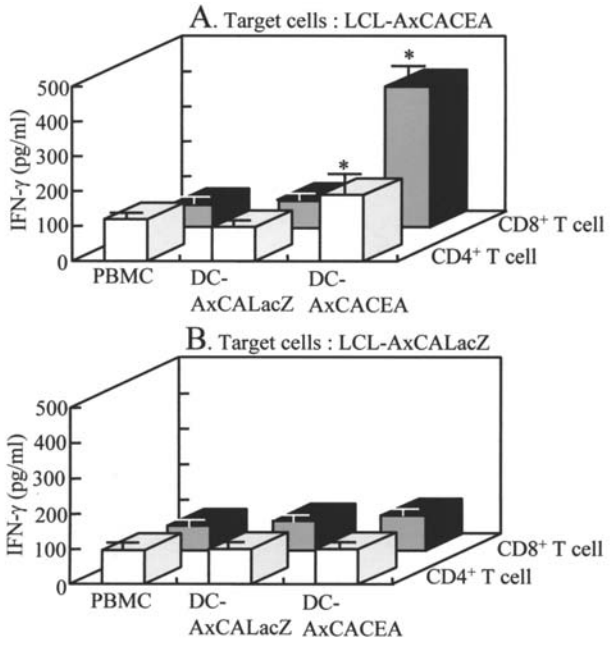

Figure 6. CEA-specific $CD4^+$ and $CD8^+$ T cell responses induced by *in vitro* stimulation using CEA cDNA transduced DCs. CTL cultures after 3 cycles of restimulation *in vitro* by OK-432/DC-AxCACEA or OK-432/DC-AxCALacZ were isolated, and then $CD4^+$ and $CD8^+$ T cells were isolated from the *in vitro* primed CTLs using a MACS system. Each of the responding lymphocytes was stimulated with LCL-AxCACEA (A) or LCL-AxCALacZ (B) in a 96-well round-bottomed plate. Supernatants collected 24 h later were tested for IFN-γ levels. Results are shown as the mean ± SD of 3 different donors. *Significantly different from DC-AxCALacZ and PBMC ($CD4^+$, $CD8^+$; $p<0.0001$).

induced by OK-432/DC-AxCACEA could recognize the CEA-specific peptide, which is bound by HLA-A24, although the CTLs induced by the DCs with the other stimuli could not recognize it. To investigate this possibility, CEA652 (HLA-A2402-restricted epitope peptide) -pulsed LCLs were used as a model target. As shown in Fig. 4, only the CTLs induced with OK-432/DC-AxCACEA showed cytotoxic activity against the peptide-pulsed targets but not the non-peptide-pulsed targets. These results suggested that OK-432 could induce markedly potent CTLs specific to the target cells pulsed with CEA652 peptide, although immature DCs and DCs stimulated with either TNF-α or LPS failed to induce potent CTLs.

Cell surface antigen in PBMC-derived CTLs. To understand the population of these PBMC-derived CTLs, we investigated the phenotypic analysis of CTLs preparations using flow cytometry. The results showed that there was no significant change in the population of $CD4^+$ cells in any of the groups, but the $CD8^+$ cell population in CTLs induced by OK-432/DC-AxCACEA increased significantly compared with that in the other groups (Fig. 5, $p<0.001$).

CEA-specific $CD4^+$ and $CD8^+$ T cell responses induced by OK-432/DC-AxCACEA. To examine the CEA-specific $CD4^+$ and $CD8^+$ T cell responses in PBMC-derived CTLs induced by the stimulation with OK-432/DC-AxCACEA, their ability to synthesize IFN-γ upon specific restimulation with LCL-AxCACEA was tested. IFN-γ production of $CD8^+$ T cells induced by stimulation with OK-432/DC-AxCACEA was significantly higher than that from stimulation with control LacZ gene-transduced DCs (Fig. 6A, $p<0.0001$). Moreover, IFN-γ production of $CD4^+$ T cells induced from stimulation with OK-432/DC-AxCACEA showed similar results (Fig. 6A, $p<0.0001$). On the other hand, IFN-γ production upon restimulation with LCL-AxCALacZ was not detected (Fig. 6B).

Discussion

OK-432 has been reported to be useful for stimulating DCs in terms of maturation (14,17-19,23,24,26). In the present study, we also showed that OK-432 stimulated immature DCs to acquire a mature phenotype and to produce significant amounts of Th1-type cytokines such as IL-12 and IFN-γ. Previously, it has been reported that a mixture of prostaglandin E2 (PGE2) and inflammatory cytokines such as TNF-α, IL-1ß, IL-6, and IFN-α, termed the cytokine cocktail, is also a potent stimulant for DC maturation (27,28). Although DCs stimulated with the cytokine cocktail showed a phenotype similar to that of DCs stimulated with OK-432, the amounts of IL-12 and IFN-γ secreted from these DCs was much lower than that secreted from OK-432-stimulated DCs (data not shown). Therefore, OK-432 seems to be a more potent drug, especially in terms of the maturation of human DCs.

The generation of DCs that induce Th1-type responses is important for efficient anti-tumor immunity (7,8). In this study, we showed that OK-432 had immunomodulatory effects that promote Th1-type responses via activation of DCs and it had favorable characteristics to induce CTLs specific to TAA. This ability of DCs matured with OK-432 using DCs pulsed with peptide (14) or DCs fused with tumor cells (24,26) has been reported previously. This study is the first report that shows the utility of OK-432 in the CTL induction using adenovirally modified DCs expressing TAA. Our results showed that the CTLs induced by OK-432/DC-AxCACEA was able to recognize the CEA-specific peptide, CEA652, which is bound by HLA-A24. However, CTLs induced by the DCs stimulated with other stimuli (TNF-α and LPS) could not recognize CEA652. Accordingly, only the CTLs induced by stimulation with OK-432/DC-AxCACEA might be able to kill HLA-A24 positive tumor cells that endogenously express CEA. It has been demonstrated recently that MHC class I-peptide complexes and MHC class I molecules have a longer half-life in mature DCs as compared with immature DCs (26). Therefore, it might be possible that genetically modified DCs expressing CEA at the completely matured state express more stable MHC class I-CEA-specific peptide complexes resulting in efficient CEA-presentation in the context of costimulatory molecules.

We demonstrated that OK-432/DC-AxCACEA could efficiently induce the activation of CEA-specific $CD8^+$ CTLs. Furthermore, we showed that OK-432/DC-AxCACEA was sufficient to induce proliferation of $CD8^+$ CTLs. IL-12 has been reported to play an important role in the development of Th1-type T cells and expanding TAA-specific CTLs through IFN-γ production (29). Our previous studies clearly demonstrated that a vaccination therapy using DCs cotransduced with the TAA gene and the IL-12 gene was ideal for immunotherapy in terms of the activation of CTLs (7,8). We consider that activation and expansion of TAA-specific CTLs are mostly dependent on IL-12. In this study, OK-432 stimulation enhanced the production of IL-12 from DCs, and IL-12 might augment Th1-type immune responses, leading to an enhanced proliferation and activation of $CD8^+$ CTLs *in vitro*.

It is generally accepted that the priming of antitumor $CD8^+$ CTLs needs $CD4^+$ Th cells. We have showed that the $CD4^+$ Th-cell arm of the cellular immune response is a critical component of an effective antitumor response (7,8). Our previous animal study demonstrated that DCs transduced with the TAA gene endogenously expressed helper epitopes associated with MHC class II molecules, inducing tumor-specific $CD4^+$ T cells (6). In the present study, we showed in human that DCs transduced with the CEA gene activated not only CEA-specific $CD8^+$ T cells but also $CD4^+$ T cells.

In conclusion, OK-432 is a potent maturation stimulus for human DCs that influences the expression of TAA presentation-related molecules. Our CTL induction protocol using adenovirally modified DCs that express CEA after maturation with OK-432 showed a potent antitumor activity against CEA-expressing target cells. This strategy is promising for clinical applications as an effective cancer vaccine therapy for patients with CEA-expressing gastrointestinal tumors, and clinical trials along these lines are now under consideration.

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