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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Received September 17, 2007; Accepted November 15, 2007

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-51Cr 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.

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-γ), 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 β2 integrin system to natural killer (NK) cells. It modulates DC maturation through the activity of neutrophils, macrophages, lymphocytes, and natural killer (NK) cells. It modulates DC maturation through Toll-like receptor (TLR) 4 and the β2 integrin system to natural killer (NK) cells. It modulates DC maturation through the activity of neutrophils, macrophages, lymphocytes, and natural killer (NK) cells.

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 centrifugation 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 1.0x10⁶ 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 (HLA-A02/24) 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 (HLA-A02/24) 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 Technne 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 ⁵¹Cr 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, TNF-ε, DC, TNF-α, and TNF-ε.
Table I. The expression of costimulatory molecules on DCs.

<table>
<thead>
<tr>
<th></th>
<th>Immature DC</th>
<th>TNF-α/DC</th>
<th>LPS/DC</th>
<th>OK-432/DC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD80</td>
<td>9.1±1.3</td>
<td>91.4±1.4</td>
<td>85.2±6.8</td>
<td>86.2±7.5</td>
</tr>
<tr>
<td>CD83</td>
<td>5.7±1.0</td>
<td>74.2±5.4</td>
<td>68.7±8.5</td>
<td>72.1±4.5</td>
</tr>
<tr>
<td>CD86</td>
<td>92.4±3.1</td>
<td>99.7±0.3</td>
<td>99.4±0.5</td>
<td>99.7±0.3</td>
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</table>

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 (CD80, CD83; p<0.0001). "Significantly different from immature DC (CD86, p<0.001)."

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 using a FACSCalibur (Becton-Dickinson, Mountain View, CA) with CellQuest software. The cells (2x10⁶) 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-huCD8 mAb (RPA-T8), PE-conjugated anti-human CD86 mAb (L304.7, PE-conjugated anti-human CD83 mAb (HLB15e), 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 1x10⁶ 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 total human 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 (5x10⁴) were each stimulated with target cells (LCL-AxCACEA or LCL-AxCALacZ, 5x10³), 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).
We further examined the secretion of Th1-type cytokines (IFN-\(\gamma\) and IL-12) from DCs after stimulation with various stimuli. The OK-432 stimulation induced IFN-\(\gamma\) and IL-12 secretion in DCs at a high level, and the levels were significantly higher than that in DCs stimulated with TNF-\(\alpha\) 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.

Table II. IFN-\(\gamma\) and IL-12 production from DCs.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Immature DC</td>
<td>9±3</td>
</tr>
<tr>
<td>TNF-(\alpha)/DC</td>
<td>72±9</td>
</tr>
<tr>
<td>LPS/DC</td>
<td>65±5</td>
</tr>
<tr>
<td>OK-432/DC</td>
<td>307±20 a</td>
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</tbody>
</table>

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. aSignificantly different from TNF-\(\alpha\)/DC, LPS/DC and immature DC (IFN-\(\gamma\), IL-12; \(p<0.0001\)).

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-\(\alpha\)/DC-AxCACEA: 100 ng/ml of rh TNF-\(\alpha\); 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.

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 51Cr-release assay. LCL-AxCACEA was used as a target (E/T ratio=50).

CEA-specific CTL responses induced by CEA gene-transduced DCs. We investigated the effect of DC maturation factors TNF-\(\alpha\), 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 I 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

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

We further examined the secretion of Th1-type cytokines (IFN-\(\gamma\) and IL-12) from DCs after stimulation with various stimuli. The OK-432 stimulation induced IFN-\(\gamma\) and IL-12 secretion in DCs at a high level, and the levels were significantly higher than that in DCs stimulated with TNF-\(\alpha\) 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.
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...
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).

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**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-1β, 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 adeno-virally 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 shown 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 adeno-virally 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.

**Acknowledgements**

This study was supported by Grant-in-Aid no. 15591354 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**References**


