Generation of tumor-reactive effector lymphocytes using tumor RNA-introduced dendritic cells in gastric cancer patients

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Abstract. Anti-tumor effector cells were generated by stimulating peripheral blood lymphocytes with cultured dendritic cells (DCs) and mRNA extracted from the gastric cancer cell line MKN45 or ascites tumor cells of gastric cancer patients. DCs were generated from an adherent fraction of peripheral blood mononuclear cells (PBMCs) in the presence of GM-CSF and IL-4. mRNA was extracted from tumor cells and subjected to T7-amplification. The DCs were electroporated (150 V/150 μF) with amplified mRNA and used after maturation with TNF-α to stimulate PBMCs to generate tumor RNA-introduced DC-activated killer (TRiDAK) cells. It was found that tumor RNA could efficiently be introduced into cultured DCs by electroporation (55% efficiency, 78% viability), and tumor RNA-introduced DCs could reproducibly stimulate lymphocytes to be tumor-reactive TRiDAK cells. The TRiDAK cells expressed an IFN-γ response specific for tumor cells, but not for normal cells. Mock DCs or normal cell RNA-introduced DCs did not induce any killer cells. RNA-specific recognition of the effector cells generated was demonstrated using an amplified EGFP-mRNA system. The tumor killing activity of TRiDAK cells was inhibited not only with the anti-HLA class I antibody but also with the anti-HLA class II antibody as well as the anti-TCR antibody. TRiDAK cells reactive with autologous tumor cells could be generated in a CEA-positive gastric cancer patient with malignant ascites, in whom effector cell generation using DCs and CEA peptides had failed. These results suggest that TRiDAK cell generation is safe, feasible, and active in gastric cancer patients with malignant ascites, and is superior to other effector cell generation systems using DCs and epitope peptides. The adoptive immunotherapy of cancer using TRiDAK cells may be warranted in a clinical setting. This is the first study investigating anti-tumor effector cell generation using cultured DCs and tumor mRNA from gastric cancer cells.

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Abbreviations: AK, activated killer; APCs, antigen presenting cells; CEA, carcinoembryonic antigen; CD, cluster of differentiation; CTLs, cytotoxic T-cells; DCs, dendritic cells; DNA, deoxyribonucleotide; DMSO, dimethyl sulfoxide; EGFP, enhanced green fluorescence protein; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot; FBS, fetal bovine serum; GM-CSF, granulocyte-macrophage colony stimulating factor; HLA, human leukocyte antigen; IFN, interferon; IL, interleukin; LAK, lymphokine-activated killer; MAGE, melanoma antigen-encoding gene; mRNA, messenger ribonucleotide; MMC, mitomycin C; PDAK, peptide-pulsed dendritic cell-activated killer; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; RPMI, Roswell Park Memorial Institute; TCR, T cell receptor; TAAs, tumor-associated antigens; TILs, tumor-infiltrating lymphocytes; TNF, tumor necrosis factor; TRiDAK, tumor RNA-introduced dendritic cell-activated killer

Key words: dendritic cells, tumor mRNA, cytotoxic T cells, gastric cancer, TRiDAK cells

Introduction

Gastric cancer is one of the most frequent malignancies worldwide, accounting for 10.4% of cancer deaths in 2000 (1). Although recent progress in the diagnosis of gastric cancer using endoscopy has enabled the detection of gastric tumors at relatively earlier stages, the prognosis of gastric cancer patients at an advanced stage still remains poor. The efficacies of other therapeutic modalities, such as chemotherapy, are limited; the overall 5-year survival rate of advanced tumors ranges from 5 to 15% (2). Hence, the development of novel therapeutic strategies is urgently needed for the treatment of patients with advanced gastric cancer.

Identification of the melanoma antigen-encoding gene (MAGE) by van der Bruggen *et al* (3) has contributed greatly to the molecular understanding of antigen presentation and recognition in the immune system (4). Many CTL epitopes have been reported as targets for gastric cancer; Her-2/neu (5), NY-ESO-1 and MAGE (6), immediate early response gene X-1 (7), immunoglobulin superfamily 11 (IGSF11) (8), and survivin (9). However, a clinical study of vaccine trials for patients with gastric cancer has still demonstrated unsatisfactory results (10). One possible reason for these poor clinical results may have to do with the heterogenic nature of gastric cancer antigens as well as many clinical tumors. To deal with the heterogeneity of clinical tumors, Chen *et al* (11) have indicated the necessity for multiple vaccine preparation. Mine *et al* (12) have demonstrated notable tumor responses

in clinical trials only with CTL precursor-oriented multiple vaccines. These observations indicate that multiple epitopes should be targeted to augment the clinical responses of immunotherapy for patients with gastric cancer.

It has been shown that murine and human DCs transfected with tumor-derived mRNA can stimulate multi-specific potent CTL responses *in vitro* and *in vivo* (13-15). The use of the RNA form as a tumor-associated antigen has significant advantages since 1) tumor RNA may code multiple tumor antigens, 2) the antigens and HLA phenotypes must not be identified when educating naïve T cells to tumor specific CTLs, 3) it can be amplified in sufficient amounts from only a few tumor cells by polymerase chain reaction (16). We have been engaged in the adoptive immunotherapy of cancer using autologous activated lymphocytes including lymphokine-activated killer (LAK) cells, tumor-infiltrating lymphocytes (TILs), tumor-sensitized lymphocytes, and peptide-pulsed DC-activated killer (PDAK) cells (8,17). The above studies encouraged us to use DCs introduced with tumor mRNA in order to advance the immunotherapeutic approaches using autologous activated lymphocytes. Here, we show the successful generation of effective autologous lymphocytes reactive with autologous tumors in gastric cancer patients with malignant ascites.

Materials and methods

Reagents. The reagents used in this study were: HLA-A2402 restricted synthetic peptides (>90% pure), including CEA10(10) (RWCIPWQRLL), CEA101(9) (IYPNASLLI), CEA234(9) (LYGPDAPTI), CEA268(10) (QYSWFVNGTF), CEA318(9) (VYAEPPKPF), CEA425(9) (TYYRPGVNL), CEA426(10) (YYRPGVNLSL), CEA590(9) (LYGPDTPII), CEA604(10) (SYLSGANLNL), CEA652(9) (TYACFVSNL), FLU38(10) (RFYIQMCTEL), (19,20), HER2 780 (PYVSRL LGI) (21) (Takara Bio Inc., Ohtsu, Japan); mouse monoclonal antibodies (mAbs) to human HLA-ABC (HLA-class I), HLA-DR (HLA-class II), and control Ig (Pharmingen, San Diego, CA, USA), CD4, CD8, CD25, TCRαß (Becton Dickinson, San Diego, CA); interleukin (IL)-2 (Sionogi, Tokyo), IL-4, IL-7, granurocyte-macrophage colony stimulating factor (GM-CSF), and TNF-α (IBL, Gunma, Japan).

Cancer cells and peripheral blood mononuclear cells. A human gastric cancer cell line MKN45 (HLA-A24) was purchased from the American Type Culture Collection and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Life Technologies, Paisely, UK). Peripheral blood mononuclear cells (PBMCs) and ascites cells were obtained by a Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ) density gradient separation method (100%, 75/100%) at 800 g for 25 min from 3 healthy donors and 6 gastric cancer patients with malignant ascites after their written informed consent was obtained. PBMCs and ascites lymphocytes (tumor-infiltrating lymphocytes, TILs) were collected from a 100% interface, and ascites tumor cells from a 75% interface. The cells were washed 3 times with RPMI-1640 medium and subjected to further experiments. The tumor cells were frozen in fetal bovine serum supplemented with 10% DMSO until used as a target for the cytotoxicity assays.

RNA extraction, amplification, and in vitro transcription. Total RNA from MKN45, ascites tumor cells, or PBMCs was extracted using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Total RNA was reverse transcribed using the Smart Race cDNA amplification kit (Clontech, CA, USA). Briefly, first strand full-length cDNA synthesis was primed with a modified oligo(dT) primer (5'-A AGCAGTGGTATCAACGCAGAGTAC $(T)_{30}N_{-1}$ N-3', N=A,C,G, or T; N-1 =A,G, or C) and a SMART II A Oligonucleotide (5'-AAGCAGTGGTATCAACGCAGATACGCG GG-3') and reverse-transcribed using Power Script reverse transcriptase for 1.5 h at 42˚C. For the full-length cDNA amplification, Universal primer mix A [Long; 5'-CTAATAC GACTCACTCACTATAGGGAAGCAGTGGTATCAACG CAGA-3', Short; 5'-CTAATACGACTCACTATAGGGC-3' (underline indicates T7 promoter sequence)], Nested Universal primer mix A (5'-AAGCAGTGGTATCAACGCAGAGT-3'), the Advantage DNA polymerase mix, and the following cycling parameters were used: 95˚C for 60 sec x 1 cycle, 95˚C for 15 sec / 65˚C for 30 sec / 68˚C for 6 min x 20 cycles, and 4˚C hold. The quality of cDNA was evaluated on ethidium bromide-stained 1.2% agarose gels. *In vitro* transcription was performed using the Message Machine high yield capped RNA transcription kit (T7 kit) (Ambion, TX, USA). Briefly, the transcription mix, ribonucleotide mix, amplified cDNA, and T7 RNA polymerase were mixed and incubated at 37˚C for 4 h. The DNA template was degenerated by incubating with DNase I at 37˚C for 15 min (22,23). Total RNA (1 μg) was extracted from about 5 mg of cell materials and used for the synthesis of 50 μg of first strand full-length cDNA, and the latter was stored at -20˚C. The full-length cDNA was used to synthesize 500 mg of mRNA by polymerase chain reaction (PCR) amplification and *in vitro* transcription.

Preparation of mRNA encoding EGFP reporter gene. A pEGFP-N1 Vector (Clontech, CA, USA) was doubly digested with the restricted enzymes *Hin*dIII (TaKaRa, Shiga, Japan) and *Not*I (TaKaRa) into 0.8 Kbp- and 3.9 Kbp-fragments. The 0.8 Kbp-digested fragment (EGFP-dsDNA) was amplified with EGFP-forward primer (5'-CGGAACAAGGGAGC TTCGAATTCTGC-3'), EGFP-reverse primer (5'-TGAGT CAAGGGCTAGCTTTACTTGTACAG-3'), and DNA polymerase, using the following cycling parameters: 94˚C for 2 min x 1 cycle, 94˚C for 60 sec / 33˚C for 60 sec / 72˚C for 60 sec x 8 cycles, and 94˚C for 60 sec / 61˚C for 60 sec / 72˚C for 60 sec x 25 cycles. The fragments were ligated with the T7 promoter sequence (5'-GACTCGTAATACGA CTCACTATAGGGCCCT-3') at the 5'-end and with the poly (dA) sequence (5'-GACTCAAAGGGA$(A)_{24}$CCTAAATCG TATGTGTATGATACATA-3') at the 3'-end using Topo tools (Invitrogen, CA, USA). The resulting product was amplified by PCR and followed by *in vitro* mRNA transcription using the Message Machine high yield capped RNA transcription kit (T7 kit) (Ambion, TX, USA). The final product was used as mRNA encoding the EGFP reporter gene (EGFP-mRNA).

Generation of DCs from peripheral blood progenitors. Human DCs were generated according to Romani *et al* (24) with minor modifications. PBMCs were cultured in serum-free

RPMI-1640 at 37˚C and 5% CO_2. After 2 h, the non-adherent cells were removed. The adherent cells were re-suspended in RPMI-1640 medium supplemented with 1 mM L-glutamine, 2% autologous serum, 800 U/ml GM-CSF and 500 U/ml IL-4 in a humidified incubator at 37˚C and 5% CO_2 for 5 days. This immature DC preparation was used for subsequent RNA introduction in order to generate antigen presenting cells (APCs).

Generation of APCs. RNA was introduced into the cultured immature DCs either by passive pulsing or electroporation. The passive pulsing procedure was modified from Heiser *et al* (23). Briefly, 10 μg of RNA was added to $2x10^5$ cells in 200 μl of serum-free RPMI-1640 medium and incubated for 45 min at 37˚C and 5% CO_2 in a humidified incubator. Electroporation was done using the Gene Pulser II as directed (Bio-Rad, CA, USA). Approximately $2x10^5$ cells ($1x10^6$ cells/ml) in 200 μl of serum-free RPMI-1640 medium were placed in a 4 mm gap chamber along with 10 μg of RNA. The mixture was placed in the Gene Pulser II and electroporated at an electrical setting of 150 voltage and 150 μF (25). Subsequently, RNA-introduced DCs were allowed to mature in the presence of 1000 U/ml TNF-α in RPMI-1640 complete medium for 2-3 days. Phenotypic analysis of the matured DCs showed >85% HLA class I^+, >75% HLA-DR^+, >95% $CD80^+$, >75% $CD86^+$, >65% $CD83^+$ and <20% $CD14^+$. These mature RNA-introduced DCs were treated with 50 μg/ml mitomycin C (MMC, Kyowa Hakkou Pharmaceutical Co., Ltd., Tokyo), washed 4 times with RPMI-1640 medium and used as APCs. In some experiments, peptide-pulsed DCs were generated from mature DCs by pulsing them with 20 μg/ml of an antigenic epitope peptide for 2 h and used as APCs (26).

Induction of TRiDAK and PDAK cells. In order to induce tumor-reactive effector cells, the non-adherent cells of PBMCs were stimulated in the presence of 10 μg/ml of IL-7 with MMC-treated tumor RNA-introduced DCs, which were prepared as described above, for 5-7 days at a responder: stimulator ratio of 20:1. In some experiments, this stimulation process was repeated every 7 days. After two stimulations, the cells were further expanded on a flask coated with anti-CD3 antibody (1 μg/ml) in the presence of 40 U/ml of IL-2. The medium of the culture was half-changed every 3-4 days. The effector cells induced were designated as tumor RNA-introduced dendritic cell-activated killer (TRiDAK) cells. The mature DCs were pulsed with antigenic epitope peptides (20 μg/ml) for 2 h, washed, and used for stimulating non-adherent cells of PBMCs to generate other effector cells designated as peptide-pulsed DC-activated killer (PDAK) cells (17,18,26).

Enzyme-linked immunosorbent assay (ELISA) and immunospot (ELISPOT) assay for IFN-γ. Antigen recognition of the effector cells, including the LAK cells, TILs, TRiDAK and PDAK cells, was detected by ELISA or ELISPOT assay (R&D Systems Inc., Minneapolis, USA) specific for IFN-γ. In ELISA, effector cells ($1x10^6$/ml) were stimulated for 72 h either with MMC-inactivated normal cells, autologous tumor cells, or DCs treated with/without antigen peptide or tumor RNA at a responder/stimulator ratio of 20, and the culture supernatant was subjected to the assay in triplicate according to the manufacturer's instructions. For the ELISPOT assay, effector cells ($4x10^4$) were cultured either with MMC-inactivated normal cells, tumor cells, or antigen-presenting DCs at a responder/stimulator ratio of 20 for 48 h using the ELISPOT kit in triplicate, and IFN-γ spots were visualized according to the manufacturer's instructions.

Cytotoxicity assay. To examine the cytotoxic activity of effector cells, a conventional 4-h ^{51}Cr release assay was performed. Target cells, including normal cells, tumor cells and DCs, were labeled with ^{51}Cr (100 μCi) for 2 h, washed 3 times, and plated onto round-bottomed 96-well microtiter plates at a concentration of $1x10^4$ cells/0.1 ml. Effector cells were added over the target cells at various concentrations in a final volume of 0.2 ml. In some experiments, this incubation was performed in the presence of 10 μg/ml anti-HLA class I, class II, -TCR antibodies or control Ig. After 4 h of incubation at 37˚C, the release of ^{51}Cr in the supernatant was measured by an automated gamma counter (Aloka, Tokyo, Japan). The mean percentage of the specific lysis of the triplicate wells was calculated by the following formula: (experimental release - spontaneous release)/(maximum release - spontaneous release) x100. The experimental release was obtained from the wells of the effector cells plus the target cells, and the spontaneous release was obtained from the wells of the target cells alone and was around 15% of the maximum release, which was obtained from the wells in which 2% Triton X-100 was added over the target cells instead of effector cells.

Flow cytometric analysis. EGFP-mRNA and the Cellstain double staining kit (Dojindo, Kumamoto, Japan) were used to evaluate the RNA introduction efficiency into immature DCs (24). Briefly, the EGFP expression rate in EGFP-mRNA-introduced DCs (DC/EGFP-mRNA) was assessed 48 h after electroporation by flow cytometric analysis using FACSCalibur (Becton-Dickinson, NJ, USA). The cell viability rate was assessed by calcein and propidium iodide (PI) double staining and flow cytometric analysis. Immediately after electroporation, the DCs were stained using the Cellstain double staining kit according to the manufacturer's protocol. Prior to flow cytometric analysis, Calcein-acetyoxymethyl (Calcein-AM) and PI were added at a final concentration of 2 and 4 μM, respectively, directly into the DCs suspended in RPMI-1640 complete medium and incubated for 15 min at 37˚C. The fluorescence of calcein in viable cells was read at a 490 nm excitation and 530 nm emission setting. The fluorescence of PI in the dead cells was read at a 530 nm excitation and 590 nm emission setting. The percentage of the EGFP-mRNA introduction efficiency was calculated according to the formula: (EGFP expression rate) x (cell viability rate) x100. Flow cytometry was also performed to determine the phenotypes of the TRiDAK cells ($5x10^5$) using anti-CD4, -CD8, and -CD25 antibodies.

Statistics. Results are expressed as the mean ± SD. Statistical analysis was conducted by unpaired Student's t-test using StatView software (version 5) on a Macintosh computer. A value of $p<0.05$ was considered statistically significant.

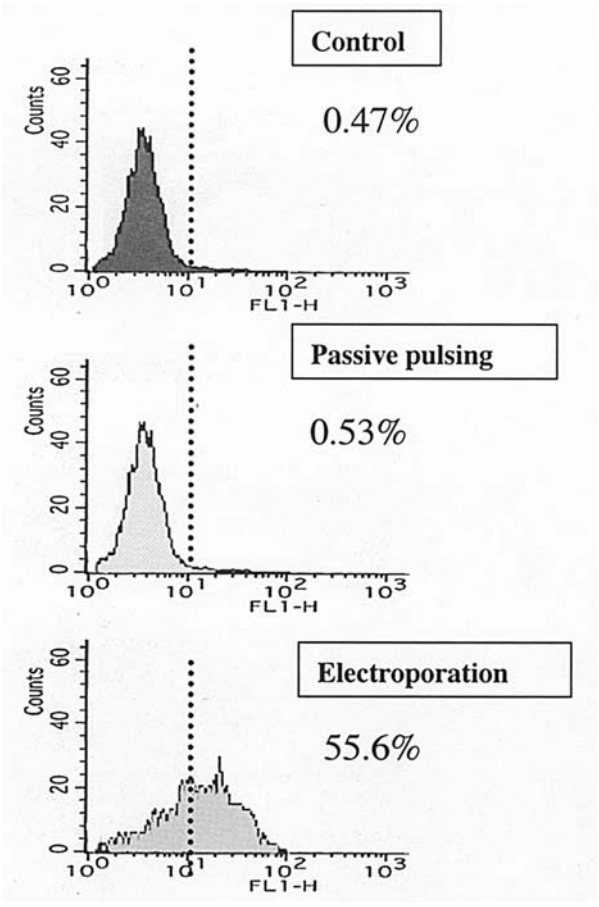

Figure 1. Introduction of EGFP mRNA into cultured DCs by electroporation. DCs were induced from an adherent fraction of PBMCs with GM-CSF and IL-4. EGFP mRNA was introduced into the DCs either by passive pulsing or electroporation at an electrical setting of 150 V and 150 μF. The viability of EGFP mRNA-introduced DCs was 78.7%. Gene introduction efficiency was assessed by flow cytometry.

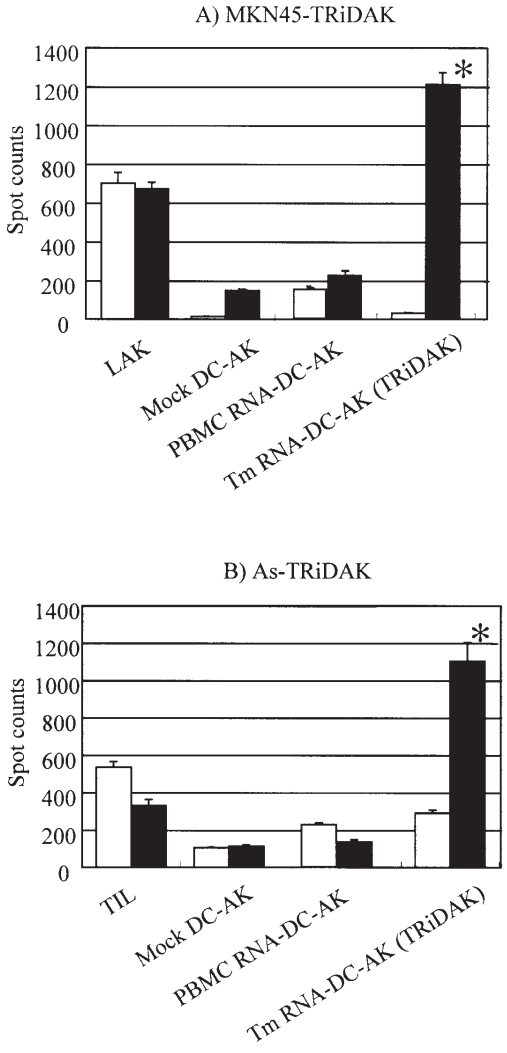

Figure 2. Tumor recognition by TRiDAK cells. Effector cells were generated by using cultured DCs and mRNAs from A) a gastric cancer cell line (MKN45) or B) ascites tumor cells (AS), as indicated. Tumor recognition was assessed by ELISPOT assay in the presence (black boxes) or absence (open boxes) of tumor cells. A significant difference between the values in the presence and absence of tumor cells, $^{*}p<0.01$.

Results

Efficient introduction of mRNA into cultured DCs by electroporation. First, RNA introduction into cultured DCs from healthy donors was confirmed using EGFP mRNA (Fig. 1). As compared to the control, passive pulsing of the EGFP mRNA showed 0.53% gene introduction efficiency. However, the EGFP mRNA introduction into DCs using electroporation demonstrated 55.6%. Viability of the DCs at this condition was determined to be 78.7%, as described in Materials and methods. This was highly reproducible in at least two other independent experiments.

Tumor specific recognition by TRiDAK cells. Using the above tumor RNA-introduced DCs, TRiDAK cells were generated and their tumor reactivity was evaluated using ELISPOT assay (Fig. 2A and B). When using the RNA of an MKN45 cell line and PBMCs from healthy donors (MKN45-TRiDAK), the TRiDAK cells showed approximately 1,200 IFN-γ spots in the presence of MKN45 tumor cells but <50 spots in the absence of the tumor cells (Fig. 2A). LAK cells that were generated with an anti-CD3 plus IL-2 culture system without tumor antigens, effector cells activated with mock DCs (mock DC-AK), or effector cells activated with normal cell-derived RNA-introduced DCs (PBMC RNA-DC-AK) demonstrated no MKN45-specific IFN-γ spots (Fig. 2A). Similar results were obtained when ascites tumor cells and cultured autologous DCs were employed in the investigation (Fig. 2B). TRiDAK cells generated from gastric cancer patients (AS-TRiDAK) showed a significant production of IFN-γ spots in the presence of autologous ascites tumor cells, but not without tumor cells. However, TILs from autologous malignant ascites, mock DC-stimulated effector cells (mock DC-AK), or normal RNA-introduced DC-stimulated effector cells (PBMC RNA-DC-AK) failed to show tumor-specific IFN-γ spot production. These were reproductively confirmed in two experiments using an MKN45 tumor cell line and donor PBMCs, and in 5 of 6 experiments from gastric cancer patients with malignant ascites (Table I). The tumor-specific reactivity of TRiDAK cells was augmented when stimulation with tumor RNA-introduced DCs was repeated twice in the healthy donor

Table I. IFN-γ spots of TRiDAK cells in the presence of tumor cells.

TRiDAK cells	Stimulation	Tumor (-)	Tumor (+)
MKN45/donor 1	x1	45	1271
MKN45/donor 2	x1	34	266
	x2		400
As-1	xl	303	1098
As-2	xl	149	411
As-3	xl	127	274
As-4	xl	135	523
As-5	xl	262	275
As-6	xl	27	52
	x2		278

TRiDAK cells were generated using cultured DCs and mRNAs from an MKN45 gastric cancer cell line or ascites tumor cells. Tumor recognition was assessed by ELISPOT assay in the presence or absence of tumor cells.

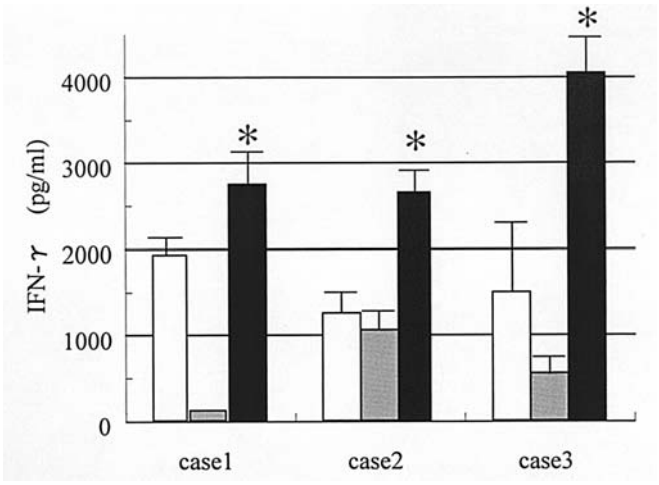

Figure 3. TRiDAK cells do not recognize normal PBMCs. TRiDAK cells were generated using cultured DCs and mRNAs from ascites tumor cells. The target recognition of TRiDAK cells was assessed by IFN-γ production in the supernatant at an effector-to-target ratio of 10. The target cells used were none (white), autologous PBMCs (gray), and autologous tumor cells (black). A significant difference from the values against the control or PBMC targets, *p<0.05.

Table II. Cytotoxic activity and phenotype of TRiDAK cells.

TRiDAK	Cytotoxicity (%)	Phenotype (%)		
		CD4	CD8	$CD4^+CD25^+$
Case 1	5	98.4	0.4	98.3
Case 2	72	40.0	59.9	58.7
Case 3	98	19.8	66.4	22.4

TRiDAK cells were generated using cultured DCs and mRNA from ascites tumor cells. Cytotoxicity assay was performed using a ^{51}Cr-releasing assay, and phenotypic analysis was done using flow cytometry.

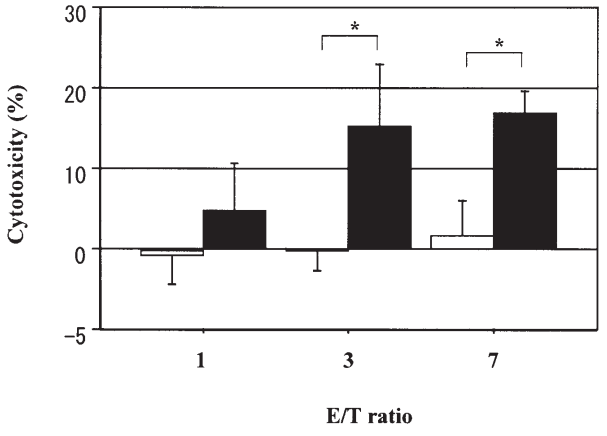

Figure 4. RNA-specific cytotoxic activity of TRiDAK cells. Effector cells were generated using cultured DCs and mRNA either from gastric cancer cells (white) or an EGFP mRNA (black). Cytotoxicity assay was performed against EGFP mRNA-introduced DCs as a target at various effector-to-target ratios. The significant difference, *p<0.05.

system and in the system of patients with malignant ascites (Table I).

TRiDAK cells do not recognize normal cells. When using TRiDAK cells in cancer immunotherapy, the reactivity of TRiDAK cells with normal cells is of importance. The reactivity of TRiDAK cells with normal cells was investigated (Fig 3). From 3 independent experiments, the TRiDAK cells reproducibly showed significant productions of IFN-γ only in the presence of autologous tumor cells (~2,700-4,000 pg/ml). However, the TRiDAK cells did produce quite low levels of IFN-γ (~200-1,000 pg/ml) in the presence of autologous PBMCs. Significant differences were found between these values ($p<0.05$).

RNA-specific cytotoxic activity of effector cells stimulated with EGFP mRNA-introduced DCs. The RNA specificity of effector cells stimulated with RNA-introduced DCs was examined (Fig. 4). Effector cells activated with EGFP mRNA-introduced DCs were capable of lysing target cells, which were EGFP mRNA-introduced DCs, in a dose-dependent fashion. However, TRiDAK cells that were generated with gastric cancer-derived RNA-DCs could not kill the target of EGFP mRNA-introduced DCs. There were significant differences in the values of cytotoxic activity against EGFP mRNA-introduced DCs as a target between effector cells activated with EGFP mRNA-introduced DCs and TRiDAK cells from gastric cancer cells ($p<0.05$).

Involvement of HLA class I, class II, or T-cell receptor molecules in cytotoxicity machinery of TRiDAK cells. To determine the involvement of HLA class I, class II, or T-cell

Table III. Comparison between PDAK cells and TRiDAK cells.

Effector cells	IFN-γ (pg/ml) Tumor (-)	Tumor (+)
Healthy donor		
Flu-PDAK	100	112
CEA652(9)-PDAK	98	640
Ascites patient		
Flu-PDAK	52	40
CEA-PDAK		
CEA10(10)	-	31
CEA101(9)	-	29
CEA234(9)	-	43
CEA268(10)	-	33
CEA318(9)	-	37
CEA425(9)	-	44
CEA426(10)	-	50
CEA590(9)	-	25
CEA604(10)	-	38
CEA652(9)	59	64
HER2-PDAK	-	61
TRiDAK	124	1147

PDAK cells were generated using cultured DCs and HLA-A24-restricted CEA, Her2, or Flu-epitope peptides, as indicated, in a healthy donor (HLA-A24) and a patient As-4 in Table I who had HLA-A24 and a positive serum CEA level. TRiDAK cells were generated using DCs and mRNA from autologous ascites tumor cells. Target recognition of PDAK and TRiDAK cells was assessed by IFN-γ production in the presence or absence of the ascites tumor cells.

receptor molecules in the cytotoxic machinery of TRiDAK cells, blocking experiments with monoclonal antibodies were performed in a cytotoxicity assay (Fig. 5). TRiDAK cells lysed gastric cancer cells reproducibly, and this was not affected by the addition of a control antibody. However, the addition of either anti-HLA class I, class II, or T-cell receptor antibodies in the cytotoxicity assay abrogated ~70-95% cytotoxic activity of the TRiDAK cells generated. There were significant differences between these values ($p<0.01$).

Cytotoxic activity and phenotypes of TRiDAK cells. The cytotoxic activity and phenotypes of TRiDAK cells were examined (Table II). TRiDAK cells that had only 5% cytotoxic activity expressed 98% of CD4 phenotype, but 0.4% of the CD8 phenotype. However, TRiDAK cells that expressed 98% cytotoxic activity showed 19.8% CD4 and 66.4% CD8 phenotypes. Two-color flow cytometric analysis using anti-CD4 and -CD25 antibodies indicated that most $CD4^+$ cells of TRiDAK cells expressed CD25 molecules.

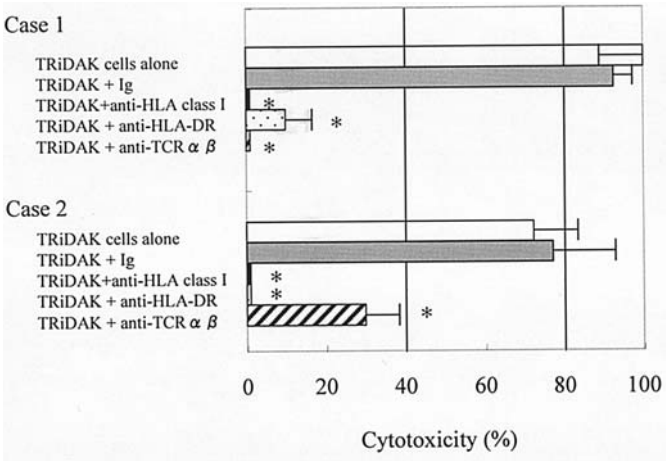

Figure 5. Involvement of HLA class I, II, and T-cell receptor molecules in target cell lysis of TRiDAK cells. TRiDAK cells were generated using cultured DCs and mRNAs from ascites tumor cells. Cytotoxicity assay was performed against autologous tumor cells at an effector-to-target ratio of 40 in the presence of antibodies. The antibodies used were none (white), mouse Ig (gray), anti-HLA class I (black), anti-HLA-DR (dotted), and anti-TCR (striped) antibodies. A significant difference from the value with control Ig, $^*p<0.01$.

Comparison between PDAK and TRiDAK cells. Effector cells were generated in a healthy donor (HLA-A24) or a gastric cancer patient with malignant ascites [As-4, HLA-A24, CEA(+)] either with a peptide-DC system (PDAK cells) or tumor RNA-DC system (TRiDAK cells), and tumor-specific IFN-γ production was examined (Table III). In the healthy donor experiment, the CEA-PDAK cells showed IFN-γ responses in the presence of the patient's ascites tumor cells, but not in the absence of the tumor cells. In the gastric cancer patient, however, 10 kinds of CEA-PDAK cells that were generated using an HLA-A24-restricted CEA peptide panel produced IFN-γ at a low level (25-64 pg/ml) in the presence of autologous tumor cells, comparable to that of control Flu-PDAK cells (40 pg/ml). The Her2-PDAK cells did not show any significant IFN-γ production (61 pg/ml). However, the TRiDAK cells that were generated using cultured autologous DCs and tumor mRNA produced 1,147 pg/ml of IFN-γ in the presence of the tumor cells, quite different from 124 pg/ml of IFN-γ in the absence of the tumor cells.

Discussion

In this study, we have shown the generation of tumor antigen-reactive effector cells, named TRiDAK cells, using the tumor RNA-introduced DCs and peripheral blood lymphocytes in both a gastric cancer cell line and gastric cancer patients with malignant effusion. Tumor RNA could efficiently be introduced into cultured DCs by electroporation. The approach using amplified tumor-RNA and an mRNA delivery system using electroporation has several advantages: 1) DCs can be introduced to levels comparable with transduction by recombinant viruses, such as poxviruses (27) or adenoviruses (28), without the problems associated with viral vectors (29), 2) DCs can be introduced with the total antigenic spectrum using mRNA extracted from cancer cells without prior identification of tumor-associated antigens (TAAs), 3) RNA can be amplified

by PCR to provide an unlimited supply of TAAs from a small amount of clinical tumor tissue (22), and 4) RNA has a short cellular half-life and lacks the potential to integrate into the host genome, thus, the potential safety hazard in the context of clinical therapeutic trials can be avoided (30). Although Schaft *et al* (31) demonstrated an optimized RNA transfection into DCs after rather than before their maturation, our results showed the efficient introduction of RNA into cultured immature DCs, consistent with other studies (32,33).

We showed that tumor RNA-introduced DCs could reproducibly stimulate lymphocytes to be tumor-reactive effector TRiDAK cells. TRiDAK cells produced IFN-γ spots only in the presence of tumor cells, not in their absence. Cultured mock DCs or normal cell RNA-introduced DCs failed to generate effector cells reactive with tumor cells. TRiDAK cells secreted IFN-γ when stimulated with tumor cells, but not with normal cells. These results indicate the nature of reactivity of TRiDAK cells specific for tumor cells, but not for normal cells, which is consistent with previous reports (13-16). Moreover, several phase I clinical trials using tumor mRNA-introduced DCs showed no apparent adverse effects or dose-limiting toxicities, including autoimmune toxicity (34). Therefore, there is a strong possibility that the tumor RNA-introduced DCs may not stimulate the forbidden clones that will react with self antigens. This may augment the possible clinical application of TRiDAK cells in the adoptive immunotherapy of cancer.

It was observed in this study that effector cells stimulated with EGFP mRNA-introduced DCs recognized EGFP-expressing cells, but the effector cells stimulated with irrelevant mRNA-introduced DCs did not, indicating the RNA-specific antigen presentation by DCs, and thereby RNA-specific antigen recognition by TRiDAK cells. This is consistent with other studies showing that an exogenous mRNA-DC system can prime precursors and induce antigen-specific CTLs in an introduced mRNA-specific manner (35,36). This indicates that tumor RNA that may contain numerous tumor antigen-coding genes must be able to stimulate numerous CTL precursors that have T cell receptors reactive with each tumor antigen, and suggests that an RNA-DC system is superior to a peptide-DC system in terms of tumor-reactive T-cell activation. Actually, our results showed that TRiDAK cells reactive with autologous tumor cells could be generated in a CEA-positive gastric cancer patient with malignant effusion, in whom we failed to generate effector cells even with CEA antigen peptides and cultured DCs. Moreover, in a study by Mine *et al* (12), notable tumor responses were demonstrated in peptide vaccine trials not using single but multiple peptides that were able to stimulate patients' PBMCs to produce IFN-γ, indicating the requirement of multiple epitope usage in the immunotherapy of clinical tumors. These results suggest that adoptive immunotherapy of cancer using TRiDAK cells may be superior to that using a peptide-DC culture system (26) because of the heterogenic nature of clinical tumors.

Tumor killing activity of TRiDAK cells was inhibited not only with the anti-HLA class I antibody but also with the anti-HLA class II antibody, indicating that tumor RNA-introduced DCs can not only stimulate $CD8^+$ CTL responses but also antigen-reactive $CD4^+$ T-cell responses. Nair *et al* (14) and Weissman *et al* (36) demonstrated that the antigenic mRNA transfection of DCs delivers encoded antigen to MHC class I and class II molecules, but that the peptide-DC system can stimulate only potent $CD8^+$ CTL responses, not $CD4^+$ T-cell responses. Bonehill *et al* (37) reported the presentation of the MAGE-A3 antigen simultaneously in HLA class I and class II molecules by mRNA-electroporated DCs, which is consistent with our observation. Zhao *et al* (38) demonstrated that a short incubation of mRNA-transfected DCs enhances the presentation of mRNA-encoded class II epitopes and the activation of $CD4^+$ T-cell responses *in vitro* and *in vivo* to stimulate potent and longer lasting $CD8^+$ CTL responses by enhancing the antitumor efficacy of DC-based tumor vaccination protocols. These observations together indicate the importance of activating class II as well as class I pathways by tumor RNA-DC system in the immunotherapy of cancer. Although activation of both the class I and class II pathways is certainly important, it should be noted that in our study, the TRiDAK cells from one gastric cancer patient that did not have any tumor killing activity expressed only the CD4 phenotype. This $CD4^+$ population was comprised of $CD4^+CD25^+$ cells, which have been known as activated T cells and regulatory T (T-reg) cells (39,40). The possible induction of T-reg cells during TRiDAK cell generation may also explain why TRiDAK cells did not react with normal cells, as shown above, because tumor mRNA encodes not only mutated oncogenic antigens but also some normal self-antigen proteins, and T-reg cells have been known to play a pivotal role in inhibiting host immune responses against self antigens. Antony *et al* (41) have demonstrated that $CD4^+$ helper T-cells can help break tolerance to persisting self antigens and treat established tumors through an IL-2-dependent mechanism, but requires the simultaneous absence of T-reg cells to be effective. Dudley *et al* (42) reported in the adoptive immunotherapy trial of malignant melanoma using TILs that drastic tumor responses are evidenced in the pre-treatment of patients with non-myeloablative lymphodepleting chemotherapy, which may cause the down-regulation of T-reg cells. Thus, gaining molecular and cellular understanding of the possible mechanism for the induction and regulation of T-reg cells is an important issue to be clarified in our TRiDAK cell generation system for future adoptive immunotherapy trials.

In summary, the use of autologous tumor mRNA-introduced DCs can generate anti-tumor effector cells, named tumor RNA-introduced DC-activated killer (TRiDAK) cells, without inducing autoreactive immune cells. To our knowledge, this is the first study investigating anti-tumor effector cell generation using cultured DCs and mRNA from gastric cancer cells. An adoptive immunotherapeutic approach using TRiDAK cells permits broad applicability against various tumor-bearing patients without prior identification of HLA phenotypes and epitope peptides. This approach offers an unlimited supply of tumor mRNA by *in vitro* amplification from a limited source of tumor cells. Collectively, the approach using the TRiDAK cell generation system offers novel possibilities for the antigen-specific immunotherapy of gastric cancer.

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