13 Peiper M, Goedegebuure PS, Linehan DC, Ganguly E, Douville CC and Eberlein TJ: The HER2/neu-derived peptide p654-662 is a tumor-associated antigen in human pancreatic cancer recognized by cytotoxic T lymphocytes. Eur J Immunol *27*: 1115-1123, 1997.
14 Yang D, Nakao M, Shichijo S, Sasatomi T, Takasu H, Matsumoto H, Mori K, Hayashi A, Yamana H, Shirouzu K and Itoh K: Identification of a gene coding for a protein possessing shared tumor epitopes capable of inducing HLA-A24-restricted cytotoxic T lymphocytes in cancer patients. Cancer Res *59*: 4056-4063, 1999.
15 Oken MM, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET and Carbone PP: Toxicity and response criteria of the Eastern Cooperative Oncology Group. Am J Clin Oncol *5*: 649-655, 1982.
16 Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, Van Oosterom AT, Christian MC and Gwyther SG: New guidelines to evaluate the response to treatment in solid tumors. J NCI *92*: 205-216, 2000.
17 Romani N, Gruner S, Brang D, Kampgen E, Lenz A, Trockenbacher B, Konwalinka G, Fritsch PO, Steinman RM and Schuler G: Proliferating dendritic cell progenitors in human blood. J Exp Med *180*: 83-93, 1994.
18 Shimizu K, Yamaguchi Y, Ohta K, Miyahara E and Toge T: Analysis of T cell receptors reactive with squamous cell carcinoma antigen SART-1 presented by HLA-A24 molecule. Oncology Reports *9*: 599-605, 2002.
19 Apostolopoulos V, Karanikas V, Haurum JS and McKenzie IF: Induction of HLA-A2-restricted CTLs to the mucin 1 human breast cancer antigen. J Immunol *159*: 5211-5218, 1997.
20 Hiltbold EM, Ciborowski P and Finn OJ: Naturally processed class II epitope from the tumor antigen MUC1 primes human CD4+ T cells. Cancer Res *58*: 5066-5070, 1998.
21 Zaremba S, Barzaga E, Zhu M, Soares N, Tsang KY and Schlom J: Identification of an enhancer agonist cytotoxic T lymphocyte peptide from human carcinoembryonic antigen. Cancer Res *57*: 4570-4577, 1997.
22 Nukaya I, Yasumoto M, Iwasaki T, Ideno M, Sette A, Celis E, Takesako K and Kato I: Identification of HLA-A24 epitope peptides of carcinoembryonic antigen which induce tumor-reactive cytotoxic T lymphocyte. Int J Cancer *80*: 92-97, 1999.
23 Kawakami Y, Eliyahu S, Jennings C, Sakaguchi K, Kang X, Southwood S, Robbins PF. Sette A, Appella E and Rosenberg SA: Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with *in vivo* tumor regression. J Immunol *154*: 3961-3968, 1995.
24 Fisk B, Blevins TL, Wharton JT and Ioannides CG: Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J Exp Med *181*: 2109-2117, 1995.
25 Miyagi Y, Imai N, Sasatomi T, Yamada A, Mine T, Katagiri K, Nakagawa M, Muto A, Okouchi S, Isomoto H, Shirouzu K, Yamana H and Itoh K: Induction of cellular immune responses to tumor cells and peptides in colorectal cancer patients by vaccination with SART3 peptides. Clin Cancer Res *7*: 3950-3962, 2001.
26 Kawabuchi Y, Yamaguchi Y, Ohshita A, Minami K and Toge T: Host-oriented peptide evaluation using whole blood assay for generating antigen-specific cytotoxic T lymphocytes. Anticancer Res *24*: 1193-1200, 2004.
27 Miyahara E, Yamaguchi Y, Minami K, Hihara J, Noma K, Toge T, Takafuta T and Fujimura K: T-cell receptor V beta gene usage of human cytotoxic T-cell clones obtained from gastric cancer patients. Anticancer Res *19*: 2057-2066, 1999.
28 Ballmer-Weber BK, Dummer R, Kung E, Burg G and Ballmer PE: Interleukin 2-induced increase of vascular permeability without decrease of the intravascular albumin pool. Br J Cancer *71*: 78-82, 1995.
29 Boldt DH, Mills BJ, Gemlo BT, Holden H, Mier J, Paietta E, McMannis JD, Escobedo LV, Sniecinski I and Rayner AA: Laboratory correlates of adoptive immunotherapy with recombinant interleukin-2 and lymphokine-activated killer cells in humans. Cancer Res *48*: 4409-4416, 1988.
30 Simon RM, Steinberg SM, Hamilton M, Hildesheim A, Khleif S, Kwak LW, Mackall CL, Schlom J, Topalian SL and Berzofsky JA: Clinical trial designs for the early clinical development of therapeutic cancer vaccine. J Clin Oncol *19*: 1848-1854, 2001.
31 Aruga A, Yamauchi K, Takasaki K, Furukawa T and Hanyu F: Induction of autologous tumor-specific cytotoxic T cells in patients with liver cancer. Int J Cancer *49*: 19-24, 1991.
32 Iwasaki M, Mukai T, Gao P, Park WR, Nakajima C, Tomura M, Fujiwara H and Hamaoka T: A critical role for IL-12 in CCR5 induction on T cell receptor-triggered mouse CD4+ and CD8+ T cells. Eur J Immunol *31*: 2411-2420, 2001.
33 Kedl RM, Schaefer BC, Kappler JW and Marrack P: T cells down-modulate peptide-MHC complexes on APCs *in vivo*. Nature Immunol *3*: 27-32, 2002.
34 Mine T, Gouhara R, Hida N, Imai N, Azuma K, Rikimaru T, Katagiri K, Nishikori M, Sukehiro A, Nakagawa M, Yamada A, Aizawa H, Shirouzu K, Itoh K and Yamana H: Immunological evaluation of CTL precursor-oriented vaccines for advanced lung cancer patients. Cancer Sci *94*: 548-556, 2003.
35 Schaft N, Willemsen RA, de Vries J, Lankiewicz B, Essers BWL, Gratama JW, Figdor CG, Bolhuis RLH, Debets R and Adema GJ: Peptide fine specificity of anti-glycoprotein 100 CTL is preserved following transfer of engineered TCRab genes into primary human T lymphocytes. J Immunol *170*: 2186-2194, 2003.

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Generation of Antigen-Presenting Cells Using Cultured Dendritic Cells and Amplified Autologous Tumor mRNA

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Key Words
Dendritic cells · Tumor mRNA · Cytotoxic T cells · Peptide · Malignant melanoma of the esophagus

Abstract
Novel antigen-presenting cells (APCs) were generated using cultured dendritic cells (DCs) and amplified tumor mRNA, and the potential of tumor antigen-reactive T cell induction by the tumor RNA-introduced DCs (DC/tumor RNA) was analyzed in a patient with melanoma antigen-encoding gene (MAGE3)-positive malignant melanoma of the esophagus. DCs were generated from an adherent fraction of peripheral blood mononuclear cells in the presence of granulocyte macrophage colony-stimulating factor and interleukin-4. Tumor mRNA was purified from tumor tissue, amplified in vitro using a T7 RNA polymerase system, and then introduced into DCs by electroporation (150 V/150 μF or 100 V/200 μF). The gene introduction efficiency was 44–55% as measured by enhanced green fluorescent protein reporter gene expression, and the viability of RNA-introduced DCs was approximately 80%. DC/tumor RNA could induce tumor antigen-reactive cytotoxic T lymphocytes (CTLs) in an mRNA-specific manner, but had no effect on the self-antigen-reactive T cells. DC/tumor RNA could induce the polyspecific antigen-reactive CTL responses mediated by both human leukocyte antigen class I and class II molecules, whereas MAGE3 peptide-pulsed DCs induced only the monospecific MAGE3-reactive CTL responses mediated by human leukocyte antigen class I molecules, showing the superiority of the DC/tumor RNA over the DC/peptide. It is suggested that the use of DC/tumor RNA as antigen-presenting cells may be more effective, convenient and practical for the DC-based anti-cancer immunotherapy.

Introduction

The identification of the melanoma antigen-encoding gene (MAGE) by Van der Bruggen et al. [1] has contributed greatly to the molecular understanding of antigen presentation and recognition in the immune system [2]. When the immune system recognizes the tumor, tumor-associated antigens (TAAs) are internalized, processed and presented on antigen-presenting cells (APCs) as antigenic epitope peptides in the context of human leukocyte antigen (HLA) molecules [3]. Recently, dendritic cells (DCs) have been the focus of anti-tumor immunotherapy, because they are professional APCs and can ini-

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tiate a primary immune response by recruiting and activating naive T cells [4, 5]. Moreover, functional DCs can be easily generated in vitro from progenitors in the peripheral blood using granulocyte macrophage colony-stimulating factor and interleukin-4 (IL-4) [6]. Many clinical trials employing DCs in anti-tumor immunotherapy have been reported [7–16]. In these trials, available sources of TAAs include identified peptides, native or modified proteins, tumor cell lysates and tumor cells. Previously, we reported using tumor antigen-specific CTLs induced by autologous DCs interacting with identified peptides in a clinical trial [17]. Results of these trials provided strong evidence for the ability of the DCs to induce autologous tumor-specific CTL responses in vivo and in vitro and to stimulate clinically beneficial anti-tumor immune responses.

However, a wider use of DC therapy for tumor patients is limited by the availability of identified TAAs and HLA phenotypes or sufficient tumor tissues for TAA preparation. Gilboa et al. [18, 19] have shown that murine and human DCs transfected with mRNA encoding antigens can stimulate potent CTL responses in vitro and in vivo. Treatment of tumor-baring mice with DCs transfected with tumor RNA led to a significant reduction in metastases or survival benefit [20]. Use of the RNA form as TAAs has one significant advantage since it can be amplified in sufficient amounts from only a few tumor cells by polymerase chain reaction (PCR) [21]. In addition, the transfection of autologous unfractionated tumor mRNA into DCs has the clinical benefit that we must not identify TAAs and HLA phenotypes when educating naive T cells to tumor-specific CTLs in vitro and in vivo.

In this study, we attempted to generate novel APCs by introducing cultured DCs with amplified RNA encoding antigens by electroporation and analyzed them for anti-tumor immune responses in vitro. First, the optimal electroporation conditions for introducing cultured immature DCs with mRNA encoding enhanced green fluorescent protein (EGFP) were tested. Secondly, we tested whether the functional APCs could be generated using in vitro amplified mRNA and cultured DCs in healthy volunteers. Finally, we generated the novel APCs using in vitro amplified autologous tumor-extracted mRNA and cultured autologous DCs in a patient with malignant melanoma of the esophagus, in whom MAGE3 peptides were identified as TAAs. The tumor mRNA-introduced DC system was compared with the MAGE3 peptide-pulsed DC system for the potential to induce anti-tumor immune responses in vitro.

Materials and Methods

Cells and Tissue Materials

Peripheral blood mononuclear cells (PBMCs) from 3 healthy volunteers and a HLA-A24 patient with malignant melanoma of the esophagus were obtained, after receiving written informed consent, by the Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, N.J., USA) density gradient separation method. Subjects underwent subtotal esophagectomy, and tissue materials (malignant melanoma and normal esophageal mucosa) were obtained and snap frozen in liquid nitrogen. A human breast cancer cell line BT-474 from the American Type Culture Collection was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Paisely, UK).

Reagents

The following HLA-A24 restricted synthetic peptides (>90% pure) purchased from TaKaRa (Shiga, Japan) were used, i.e. $MAGE3_{76-84}$ peptides (NYPLWSQSY), $MAGE3_{113-121}$ peptides (VAELVHFLL), carcinoembryonic antigen $(CEA)_{10-19}$ peptides (RWCIPWQRLL) and $CEA_{101-108}$ peptides (IYPNASLLI) [22, 23]. Mouse monoclonal antibodies to human HLA-ABC (HLA class I), HLA-DR (HLA class II) and control immunoglobulin were from Pharmingen (San Diego, Calif., USA).

RNA Extraction, Amplification and in vitro Transcription

Total RNA from the tissue materials or PBMCs was extracted using the RNeasy kit (Qiagen, Valencia, Calif., USA) according to the manufacturer's protocol. Total RNA was reverse transcribed using the Smart Race cDNA amplification kit (Clontech, Calif., USA). Briefly, first-strand full-length cDNA synthesis was primed with a modified oligo(dT) primer (5′-AAGCAGTGGTATCAACGCAGAGTAC $(T)_{30}N_{-1}$ N-3′, N = A, C, G or T; N_{-1} = A, G or C) and a Smart II A oligonucleotide (5′-AAGCAGTGGTATCAACGCAGA TACGCGGG-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′-CTAATACGACTCACTCACTATAGGGAAGCAGTGGTATCAACGCAGA-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 s × 1 cycle, 95°C for 15 s/65°C for 30 s/68°C for 6 min × 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 mMessage mMachine high-yield capped RNA transcription kit (T7 Kit; Ambion, Tex., 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 [24, 25]. Total RNA (1 μg) was extracted from about 5 mg of tissue 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 PCR amplification and in vitro transcription.

Preparation of mRNA-Encoding EGFP Reporter Gene

A pEGFP-N1 Vector (Clontech) was doubly digested with restricted enzymes *Hind* III and *Not* I (both from TaKaRa) into 0.8- and 3.9-kbp fragments. The 0.8-kbp digested fragment (EGFP ds-

DNA) was amplified with EGFP forward primer (5′-CGGAACAAGGGAGCTTCGAATTCTGC-3′), EGFP reverse primer (5′-TGAGTCAAGGGCTAGCTTTACTTGTACAG-3′) and DNA polymerase, using the following cycling parameters: 94°C for 2 min × 1 cycle, 94°C for 60 s/33°C for 60 s/72°C for 60 s × 8 cycles, and 94°C for 60 s/61°C for 60 s/72°C for 60 s × 25 cycles. The fragments were ligated with the T7 promoter sequence (5′-GACTCGTAATACGACTCACTATAGGGCCCT-3′) at the 5′-end and with poly(dA) sequence (5′-GACTCAAAGGGA$(A)_{24}$CC-TAAATCGTATGTGTATGATACATA-3′) at the 3′-end using Topo tools (Invitrogen, Calif., USA). The resulting product was amplified by PCR and followed by in vitro mRNA transcription using the mMessage mMachine high-yield capped RNA transcription kit (T7 Kit; Ambion). The final product was used as mRNA encoding EGFP reporter gene (EGFP mRNA).

Generation of DCs from Peripheral Blood Progenitors

Human DCs were generated according to Romani et al. [6] with minor modifications. PBMCs were cultured in serum-free RPMI-1640 at 37°C and 5% CO_2. After 2 h, the nonadherent cells were removed. The adherent cells were resuspended in RPMI-1640 medium supplemented with 1 m*M* *L*-glutamine, 2% autoserum, 800 U/ml granulocyte macrophage colony-stimulating factor (Osteogenetics GmbH, Germany), and 500 U/ml IL-4 (Osteogenetics GmbH) 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 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 the report of Heiser et al. [25]. Briefly, 10 μg of RNA was added to 2 × 10^5 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 Gene Pulser II, as directed (Bio-Rad, Calif., USA). Approximately 2 × 10^5 cells (1 × 10^6 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 various electrical settings [26, 27]. Subsequently, RNA-introduced DCs (DC/RNA) were allowed to maturate in the presence of 1,000 U/ml tumor necrosis factor-α in RPMI-1640 complete medium for 2–3 days. Phenotypic analysis on the matured DCs showed >85% HLA class I+, >75% HLA-DR+, >95% CD80+, >75% CD86+, >65% CD83+ and <20% CD14+. These mature DC/RNA were treated with 50 μg/ml mitomycin C (Kyowa Hakkou Pharmaceutical Co., Ltd., Tokyo), washed three times with RPMI-1640 medium and used as APCs. In some experiments, peptide-pulsed DCs (DC/peptide) were used as APCs. DC/peptide were generated from mature DCs by pulsing them with 20 μg/ml of an antigenic epitope peptide for 2 h [17].

Flow Cytometric Analysis

EGFP mRNA and 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, N.J., 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 μ*M*, 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 490 nm excitation and 530 nm emission setting. The fluorescence of PI in the dead cells was read at 530 nm excitation and 590 nm emission setting. The percentage of the EGFP mRNA introduction efficiency was calculated according to the following formula: (EGFP expression rate) × (cell viability rate) × 100.

Induction of Effector Cells

In order to induce effectors, the nonadherent cells of PBMCs that had been cultured in RPMI-1640 complete medium supplemented with 20 U/ml IL-2 (Genzyme, Cambridge, UK) were stimulated with APCs, which were prepared as above, for 5–7 days in a responder:stimulator ratio of 10:1. This stimulation process was repeated three times every 7 days.

Cytotoxicity Assay

The calcein-AM cytotoxicity assay was used to determine cytotoxicity [28]. Briefly, target cells (approximately 10^6 cells/ml) were incubated with 10 μ*M* calcein-AM in RPMI-1640 complete medium for 30 min at 37°C with occasional shaking. Only the live target cells can produce insoluble fluorescent product calcein from calcein-AM in cytoplasm. Therefore, the live target cells were labeled, treated with 50 μg/ml mitomycin C for 30 min, and washed three times with RPMI-1640 medium prior to cytotoxicity assays. Effectors and calcein-labeled targets with various effector/target (E/T) ratios were cocultured in U bottom 96-well plates in triplicates for 4 h at 37°C in a total volume of 200 μl. Supernatant samples were measured using Fluoroskan Ascent (Labsystems, Chesire, UK; exciting filter: 485 ± 9 nm; band-pass filter: 530 ± 9 nm). Data were expressed as arbitrary fluorescent units. Specific lysis (%) of the cells was calculated as follows: [(test release – spontaneous release)/(maximum release – spontaneous release)] × 100. The maximum and spontaneous release represents calcein release from the targets in medium with and without 2% Triton X-100, respectively. Each measurement was done in at least six replicate wells.

Enzyme-Linked Immunosorbent Assay for Interferon-γ

Antigen recognition of CTL precursors in PBMCs was detected by interferon (IFN)-γ secretion after stimulation with generated APCs. PBMCs (1 × 10^5 cells) were cocultured with the APCs (5 × 10^3 cells) in 96 flat bottom plates in triplicates for 72 h at 37°C in a total volume of 200 μl. In some experiments, effector cells were stimulated with APCs in the presence of 10 μg/ml anti-HLA class I, class II antibodies or control IgG. Supernatant samples were tested for IFN-γ secretion by enzyme-linked immunosorbent assay (ELISA) (Quantikine human IFN-γ; R&D Systems, Inc., Minn., USA) according to the manufacturer's protocol. Measurements are presented as picogram/milliliter IFN-γ released by 10^5 PBMCs per 72 h.

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 p value <0.05 was considered statistically significant.

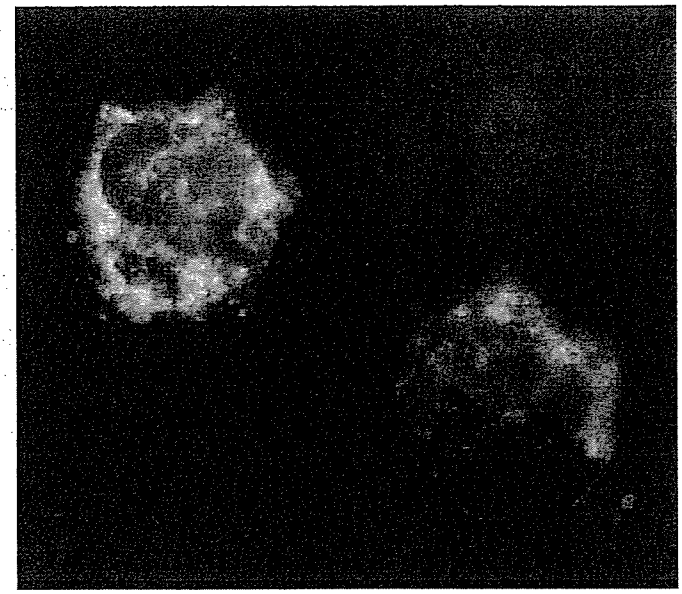

Fig. 1. Fluorescent microscopic images of cultured dendritic cells introduced with EGFP mRNA. Cultured DCs were introduced with mRNA encoding EGFP using electroporation at a capacitance of 150 μF and a voltage of 150 V, and fluorescent microscopy was examined.

Table 1. Efficiencies of RNA introduction by electroporation into dendritic cells and their viabilities

Capacitance, μF		Voltage, V				
		0	100	150	200	250
Expt. 1						
0	efficiency, %	0.4				
	viability, %	90.1				
150	efficiency	–	33.9	55.6	57.3	44.5
	viability	–	80.0	78.7	59.8	42.0
200	efficiency	–	54.7	42.5	28.6	–
	viability	–	86.4	73.1	46.2	–
Expt. 2						
0	efficiency, %	0.5				
	viability, %	91.5				
150	efficiency	–	27.1	43.8	34.3	18.7
	viability	–	79.0	82.1	64.5	45.5
200	efficiency	–	44.0	31.1	13.2	–
	viability	–	83.7	76.6	51.2	–

Cultured DCs were introduced with mRNA encoding EGFP using electroporation at various electrical settings. Efficiencies of RNA introduction into DCs and their viabilities were investigated as described in 'Materials and Methods'. A setting of 0 V and 0 μF indicates the passive pulsing.

Results

Optimization of mRNA Electroporation into Cultured DCs

In order to optimize the mRNA-based electroporation, we used cultured immature DCs from healthy volunteers and an EGFP mRNA reporter gene. Following electroporation at various electrical settings, EGFP expression rate and cell viability rate in the electroporated DCs were assessed as described in 'Materials and Methods'. Of all the electrical settings tested, a voltage of either 150 or 100 V combined with a capacitance of 150 or 200 μF were found to be optimal. At these settings, introduction efficiencies of 55.6 and 54.7% and 43.8 and 44.0%, with cell viabilities of 78.7 and 86.4% and 82.1 and 83.7% were observed (table 1). On the other hand, cultured immature DCs, which were passively pulsed with EGFP mRNA, demonstrated an introduction efficiency of only 0.4 and 0.5% (table 1). Figure 1 shows the finding of fluorescent microscopy of the EGFP expression in DC/EGFP mRNA.

Antigen-Presenting Capacity of RNA-Introduced DCs

To evaluate the antigen-presenting capacity of the RNA-introduced DCs (DC/RNA), PBMCs from healthy volunteers were stimulated in vitro with the DC/RNA, and IFN-γ secretion in the supernatant was determined. First, we compared IFN-γ secretion by stimulating PBMCs with DCs introduced with four different RNAs: (1) autologous total RNA from PBMCs (auto-tRNA), (2) allogeneic total RNA from the BT474 cell line (BT474 tRNA), (3) allogeneic amplified mRNA from the BT474 cell line (BT474 mRNA), and (4) xenogenic amplified EGFP mRNA. Here, passive pulsing was used as an RNA delivery system into cultured DCs. Experiments were performed independently in 3 volunteers and repeated three times. Representative data of similar results are shown in figure 2. It was observed that stimulation of PBMCs with DC/BT474 tRNA, DC/BT474 mRNA, and DC/EGFP mRNA resulted in significant IFN-γ secretions from PBMCs. On the other hand, DC/auto-tRNA, as well as mock DCs, failed to stimulate PBMCs to secrete IFN-γ (fig. 2a). Next, to test whether the RNA delivery system could influence the antigen-presenting capacity of DC/RNA, two different delivery systems of passive pulsing and electroporation were compared. BT474 mRNA was used as a transgene. PBMCs secreted twice as much IFN-γ when stimulated with electroporation than pas-

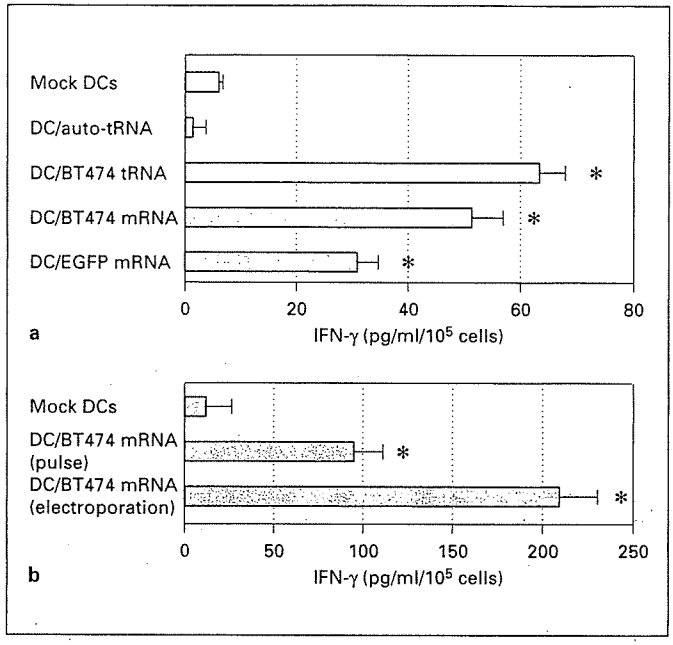

Fig. 2. Priming of CTL precursors in PBMCs using DC/RNA in vitro. PBMCs from healthy volunteers were stimulated with autologous mock DCs (control), DC/auto-tRNA, DC/BT474 tRNA, DC/BT474 mRNA or DC/EGFP mRNA (**a**), and with mock DCs (control), passive pulsing-based DC/BT474 mRNA (pulse) or electroporation-based DC/BT474 mRNA (electroporation) (**b**), for 72 h. The responder (PBMCs):stimulator (DC/RNA) ratio was 20:1. Supernatants were tested for IFN-γ secretion by ELISA. Experiments were performed independently in 3 volunteers and repeated three times. Representative data of similar results are shown. * $p < 0.05$, significant differences from the value of mock DC.

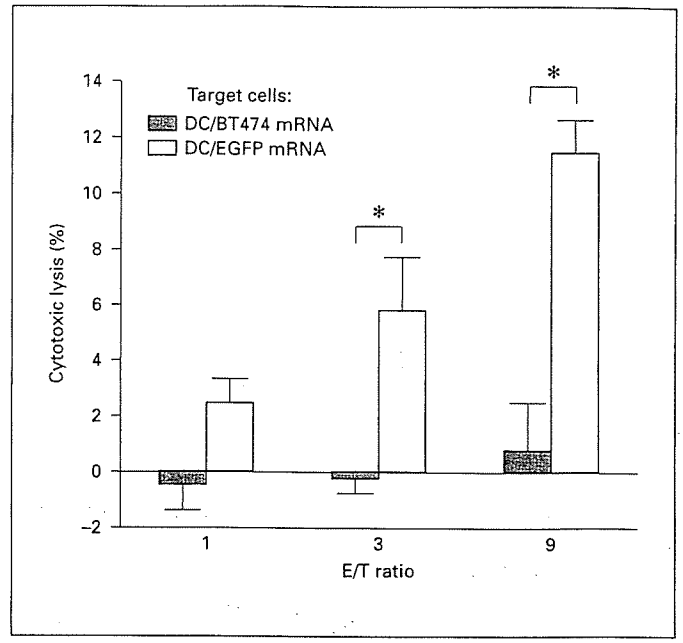

Fig. 3. RNA-specific cytotoxic activity of CTLs induced with DC/RNA. CTLs were induced with DC/EGFP mRNA and cytotoxicity assay was performed against DC/EGFP mRNA or DC/BT474 mRNA as targets at various E/T ratios. Cytotoxicity assay was assessed by calcein-AM release assay. Experiments were repeated three times, and representative data of similar results are shown. * $p < 0.05$.

sively pulsed DC/BT474 mRNA, showing a significant difference ($p < 0.05$) (fig. 2b).

EGFP-Specific CTL Induction by DC/EGFP mRNA

To assess whether DC/RNA can induce CTL responses specific to introduced RNA, cytotoxicity assays were conducted using one CTL line as an effector and two different DC/RNAs as targets. The effector CTLs (CTL/EGFP) were induced by stimulating PBMCs from healthy volunteers with DC/EGFP mRNA. Target cells used were DCs introduced with DC/EGFP mRNA and DC/BT474 mRNA. Experiments were repeated three times, and representative data of similar results are shown in figure 3. It was observed that the effector CTL/EGFP was capable of recognizing and lysing only DC/EGFP mRNA in a dose-dependent manner. However, CTL/EGFP did not at all recognize or lyse DC/BT474 mRNA.

Tumor-Specific CTL Induction by Autologous DC/Tumor RNA

To demonstrate whether DCs that were introduced with amplified autologous tumor mRNA can induce the tumor-specific CTL responses, effector CTLs were induced in a patient with malignant melanoma of the esophagus. Effector cells, designated as control CTL, CTL/muc and CTL/mel, were generated by stimulating patient's PBMCs with patient's mock DCs, patient's normal mucosa mRNA-introduced DCs (DC/muc-RNA) and patient's melanoma mRNA-introduced DCs (DC/mel-mRNA), respectively. The DC/mel-mRNAs were substituted for the tumor cells as the target cells. Experiments were repeated three times, and representative data of similar results are shown in figure 4. It was observed that the cytotoxic activity of CTL/mel showed 73% at an E/T ratio of 80 against the target cells and decreased in a dose-dependent manner. However, the cytotoxic activities of control CTL and CTL/muc were only 13 and 11% at an E/T ratio of 80 (fig. 4a). There were significant differences in the cytotoxic activities between control CTL, CTL/muc and CTL/mel ($p < 0.05$).

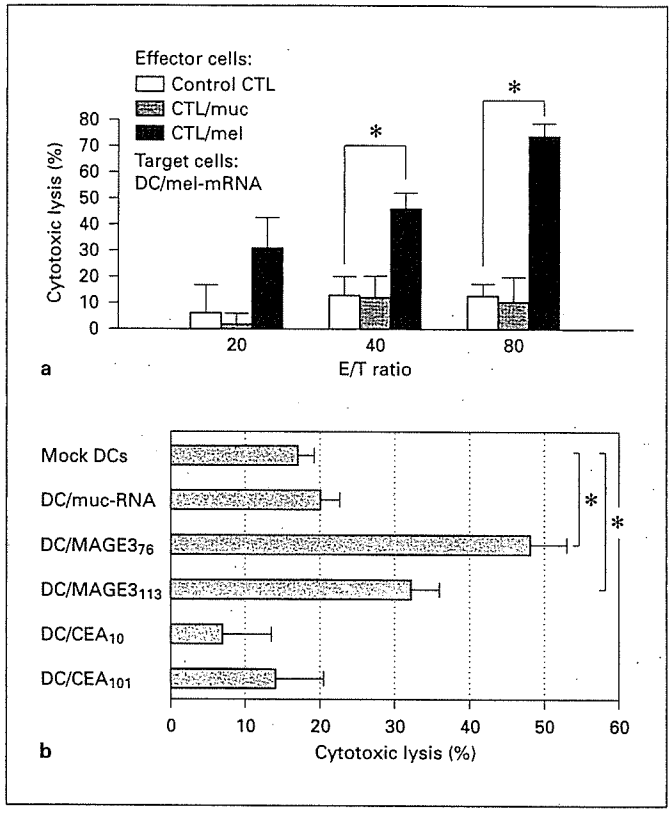

Fig. 4. Cytotoxic activity of CTLs induced with DC/mel-mRNA. Effector CTLs were induced from patient's PBMCs with autologous mock DCs, autologous DC/muc-RNA or autologous DC/mel-RNA. Effector CTLs were described as control CTL, CTL/muc or CTL/mel, respectively. DC/mel-RNA were used as the target cells. Cytotoxicity assay was performed at various E/T ratios (**a**). Cytotoxic activity of CTL/mel was tested against mock DCs, DC/muc-RNA, MDC/MAGE3$_{76}$, DC/MAGE3$_{113}$, DC/CEA$_{10}$ and DC/CEA$_{101}$. The E/T ratio was 40/1 (**b**). Experiments were repeated three times, and representative data of similar results are shown. * $p < 0.05$

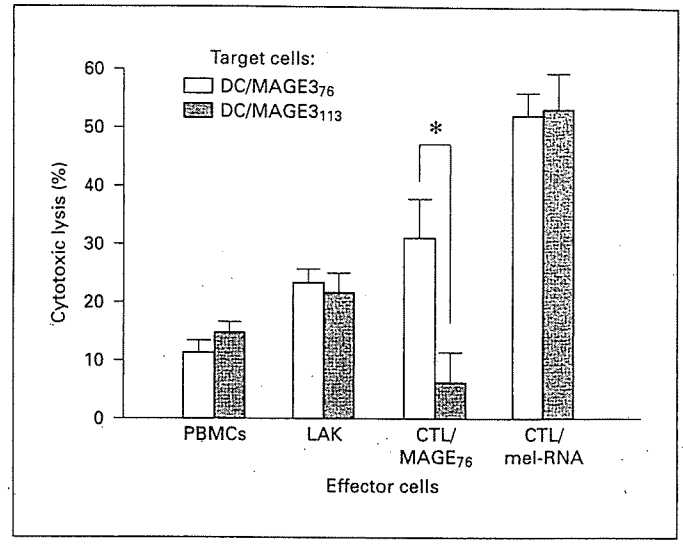

Fig. 5. Comparison of cytotoxic activity between CTL/peptide and CTL/RNA. Four different effector cells were used: (1) unstimulated PBMCs, (2) LAK cells induced with 400 U/ml IL-2, (3) CTL/peptide induced with DC/MAGE3$_{76}$ and (4) CTL/RNA induced with DC/mel-RNA. DC/MAGE3$_{76}$ and DC/MAGE3$_{113}$ were used as targets. Cytotoxicity assay was performed at an E/T ratio of 50/1. Experiments were repeated three times, and representative data of similar results are shown. * $p < 0.05$.

Next, antigen peptide specificity of the CTL/mel generated in the patient was analyzed. In this melanoma, the epitope peptides of TAAs were MAGE3$_{76-84}$ and MAGE3$_{113-121}$, but not the CEA$_{10-19}$ or CEA$_{101-108}$ peptides, when identified using the host-oriented peptide evaluation approach described in our previous report (data not shown) [29]. Therefore, we evaluated the cytotoxic activity of the CTL/mel against target cells of mock DCs, DC/muc-RNA, MAGE3$_{76-84}$ peptide-pulsed DCs (DC/MAGE3$_{76}$), MAGE3$_{113-121}$ peptide-pulsed DCs (DC/MAGE3$_{113}$), CEA$_{10-19}$ peptide-pulsed DCs (DC/CEA$_{10}$), and CEA $_{101-108}$ peptide-pulsed DCs (DC/CEA$_{101}$). It was observed that the CTL/mel showed 47 and 32% cytotoxic activity against DC/MAGE3$_{76}$ and DC/MAGE3$_{113}$, respectively. However, the CTL/mel showed only 16, 18, 7, and 14% cytotoxic activity against the mock DCs, DC/muc-RNA, the DC/CEA$_{10}$, and DC/CEA$_{101}$, respectively (fig. 4b). There were significant differences between the cytotoxic activities against mock DCs and DC/MAGE$_{76}$ or DC/MAGE$_{113}$ ($p < 0.05$).

Polyspecific CTL Induction by DC/Tumor RNA

Next, the TAA-presenting potential of CTLs generated using DC/tumor RNA was evaluated. The patient's PBMCs were stimulated with the DC/MAGE3$_{76}$ and DC/mel-RNA to generate the effector CTLs that were designated as the CTL/MAGE3$_{76}$ and the CTL/mel-RNA, respectively. Patient's PBMCs alone and the lymphokine-activated killer (LAK) cells that were stimulated with 400 U/ml recombinant human IL-2 were used as the control effector cells. Patient's DC/MAGE3$_{76}$ and DC/MAGE3$_{113}$ were used as the target cells. Experiments were repeated three times, and representative data of similar results are shown in figure 5. It was observed that the PBMCs showed only 11 and 14% cytotoxic activity, and the LAK cells showed 23 and 21% cytotoxic activity

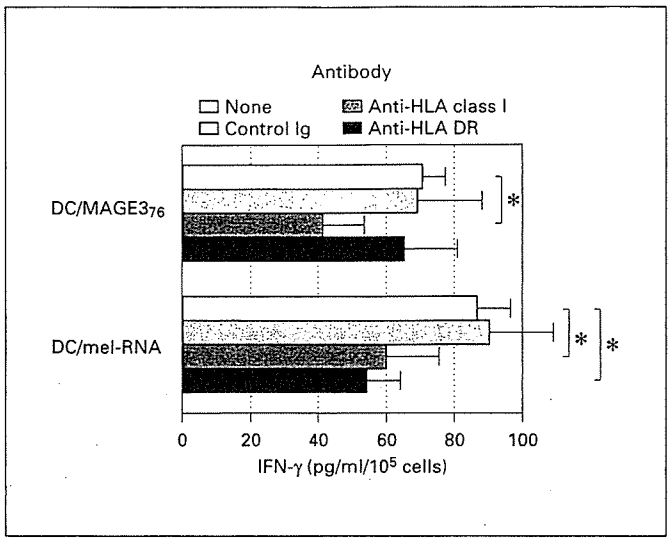

Fig. 6. Involvement of HLA class I and class II pathways in the effector cell induction with DC/peptide and DC/RNA. PBMCs were stimulated with DC/MAGE3$_{76}$ and DC/mel-mRNA in the presence of various antibodies indicated: (1) no immunoglobulin, (2) control IgG, (3) anti-HLA class I and (4) anti-HLA-DR antibodies. The responder:stimulator ratio was 20:1. Each supernatant sample was tested for IFN-γ secretion by ELISA. Experiments were repeated three times, and representative data of similar results are shown. * $p < 0.05$.

against the DC/MAGE3$_{76}$ and the DC/MAGE3$_{113}$, respectively. The CTL/MAGE3$_{76}$ showed a 31% cytotoxic activity against the DC/MAGE3$_{76}$, but only a 6% cytotoxic activity against the DC/MAGE3$_{113}$. The cytotoxic activity of the CTL/MAGE3$_{76}$ was significantly higher against the DC/MAGE3$_{76}$ than against the DC/MAGE3$_{113}$, whereas the CTL/mel-RNA demonstrated cytotoxic activity against both the DC/MAGE3$_{76}$ and the DC/MAGE3$_{113}$, showing an activity of 51 and 52%, respectively. There was no significant difference between the cytotoxic activity of CTL/mel-RNA against DC/MAGE3$_{76}$ and DC/MAGE3$_{113}$.

Involvement of HLA Class I and II Pathways in Stimulation of PBMCs by DC/mel-RNA

To determine the involvement of HLA class I or class II pathways when stimulating PBMCs with two different APCs, blocking studies with monoclonal antibodies were performed. The patient's PBMCs were stimulated with the DC/MAGE3$_{76}$ and the DC/mel-RNA in the presence or absence of the antibodies indicated, and IFN-γ secretion in the supernatant was determined. Experiments were repeated three times, and representative data of similar results are shown in figure 6. The control immunoglobulin had no effect on the IFN-γ secretion of PBMCs when stimulated with DC/MAGE3$_{76}$ or DC/mel-RNA. The addition of anti-HLA class I, but not the anti-HLA-DR monoclonal antibodies resulted in a significant inhibition of IFN-γ secretion of PBMCs stimulated with DC/MAGE3$_{76}$. However, when stimulated with DC/mel-RNA, not only the addition of anti-HLA class I but also of anti-HLA-DR monoclonal antibodies resulted in a significant inhibition of IFN-γ secretion of PBMCs.

Discussion

In this study, we have shown the generation of tumor antigen-reactive CTL using the tumor RNA-introduced DCs. Tumor mRNA that was obtained from tiny tumor mass could easily be amplified in vitro and efficiently introduced into DCs by an electroporation-based mRNA delivery system. Here, laser capture microdissection (LCM) may be of value to be introduced into our system, because LCM can avoid the contamination of normal cells when tumor RNA is extracted [30]. The LCM may augment the CTL induction in our system by obtaining pure tumor RNA, although no induction of CTLs reactive with normal tissue mRNA-introduced DCs was observed in this study even when normal RNA was used. The benefit of LCM remains to be addressed.

The approach using amplified tumor RNA and an electroporation-based mRNA delivery system has several advantages: (1) DCs can be introduced to levels comparable with transduction by recombinant viruses, such as poxviruses [31] or adenoviruses [32], without the problems associated with viral vectors [33, 34]; (2) DCs can be introduced with the total antigenic spectrum using mRNA extracted from the tumor tissues without prior identification of TAAs; (3) RNA can be amplified by PCR to provide an unlimited supply of TAAs from an often small amount of clinical tumor tissues [24], and (4) RNA has a short cellular half-life and lacks the potential to integrate into the host genome, and thereby, we can avoid the potential safety hazard in the context of clinical therapeutic trials [35, 36]. Our results showed that the electroporation-based mRNA delivery system had sufficient mRNA introduction efficiency and low cell toxicity against cultured immature DCs under optimal electrical settings (table 1). This observation is consistent with other reports showing that the electroporation-based mRNA delivery system is superior to the commonly used techniques of

lipofection or passive pulsing in providing better RNA transfection efficiency [26, 27].

By using DC/RNA, we could generate excellent effector cells, CTL/RNA, which were capable of recognizing the DC/RNA. This was also evidenced using the samples from a patient with malignant melanoma of the esophagus. Moreover, CTL/EGFP mRNA could only recognize DC/EGFP mRNA but not DC/BT474 mRNA, indicating the RNA-specific target recognition of CTL/RNA effector cells. This is consistent with other reports showing that exogenous DC/mRNA can prime precursors and induce antigen-specific CTLs in an introduced mRNA-specific manner [37, 38]. It indicates that tumor RNA which 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 DC/RNA are superior to DC/peptide in terms of tumor-reactive T cell activation. Actually, our results showed that the DC/mel-RNA induced polyspecific CTLs that were reactive with both $MAGE3_{76}$ and $MAGE3_{113}$, whereas the DC/$MAGE3_{76}$ induced monospecific CTLs reactive only with $MAGE3_{76}$. Since the malignant tumors have heterogeneity, CTLs induced with the DC/tumor RNA can reduce the chance of clonal tumor escape more effectively than CTLs induced with the TAA peptide-pulsed DCs. Heiser et al. [25] demonstrated that the amplified prostate tumor RNA-transfected DC-stimulated T cell responses were directed against the multiple TAAs, including the prostate-specific antigen and the telomerase reverse transcriptase. They also suggested that tumor RNA-transfected DCs might minimize the risk of clonal tumor escape [25].

Interestingly, IFN-γ response of PBMCs stimulated with DC/mel-RNA was inhibited not only with anti-HLA class I antibody but also with anti-HLA class II antibody, indicating that DC/tumor RNA cannot only stimulate potent CTL responses but also antigen-reactive CD4+ T cell responses. Nair et al. [19] and Weissman et al. [38] demonstrated that the antigenic mRNA transfection of DCs delivers encoded antigen to major histocompatibility complex class I and class II molecules; on the other hand, DC/TAA peptide can only stimulate potent CTL responses, but not CD4+ T cell responses, suggesting that the DC/tumor RNA is superior to the DC/peptide for a potent induction of the antigen-reactive CTLs and the antigen-reactive helper T cells. Furthermore, Zhao et al. [39] demonstrated that a short incubation of mRNA-transfected DCs with the antisense oligonucleotides (against the invariant chain) enhances the presentation of mRNA-encoded class II epitopes and the activation of CD4+ T cell responses in vitro and in vivo, and that immunization of mice with antisense oligonucleotide-treated DCs stimulates potent and longer-lasting CTL responses and enhances the anti-tumor efficacy of DC-based tumor vaccination protocols. More recently, Bonehill et al. [40] reported the presentation of MAGE-A3 antigen simultaneously in HLA class I and class II molecules by mRNA-electroporated DCs. The induction of CD4+ T cell responses plays an important role in the induction and persistence of HLA class I-restricted antigen-reactive CTLs. These observations indicate the importance of activating class II as well as class I pathways by DC/tumor RNA in DC-based tumor immunotherapy.

If tumor RNA contains numerous antigen genes and if DC/tumor RNA can stimulate numerous CTL precursors, the application of DC/tumor RNA for immunotherapy could potentially have several drawbacks. For example, unfractionated tumor mRNA contains self-antigen-encoding RNAs, and the use of DC/tumor mRNA in clinical trials can induce autoimmune toxicity by reducing tolerance to self-antigens. However, our results demonstrated that there was no induction of CTLs reactive with normal tissue mRNA-introduced DCs as targets. Several phase I clinical trials showed no apparent adverse effects or dose-limiting toxicities including autoimmune toxicity [41, 42]. Therefore, there is a strong possibility that the DC/tumor RNA may not stimulate the forbidden clones that react with self-antigens. This may augment the possible clinical application of DC/tumor RNA in tumor immunotherapy.

In summary, the use of autologous tumor mRNA-introduced DCs can stimulate the induction of anti-tumor immune responses against the multiple tumor-derived antigens without inducing autoimmunity against self-antigens. An immunotherapeutic approach using DC/tumor RNA permits broad applicability against various tumor-bearing patients without prior identification of HLA phenotypes and TAAs. This approach offers unlimited supply of tumor mRNA by in vitro amplification from a limited source of tumor tissue. Collectively, the approach using DC/mRNA offers novel possibilities for DC-based antigen-specific immunotherapy of cancer.

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References

1 Van der Bruggen P, Traversari C, Boon T, et al: A gene encoding an antigen recognized by cytotoxic T lymphocytes on a human melanoma. Science 1991;254:1643–1647.
2 Mescher MF: Molecular interactions in the activation of effector and precursor cytotoxic T lymphocytes. Immunol Rev 1995;146:177–210.
3 Lanzavecchia A, Sallusto F: Regulation of T cell immunity by dendritic cells. Cell 2001;106:263–266.
4 Banchereau J, Steinman RM: Dendritic cells and the control of immunity. Nature 1998;392:245–252.
5 Hart DNJ: Dendritic cells: unique leukocyte populations which control the primary immune response. Blood 1997;90:3245–3287.
6 Romani N, Gruner S, Schuler G, et al: Proliferating dendritic cell progenitors in human blood. J Exp Med 1994;180:83–93.
7 Hus FJ, Benike C, Levy R, et al: Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. Nat Med 1996;2:52–58.
8 Kugler A, Stuhler G, Ringert RH, et al: Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. Nat Med 2000;6:332–336.
9 Thurner B, Haendle I, Schuler G, et al: Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastatses in advanced stage IV melanoma. J Exp Med 1999;190:1669–1678.
10 Mackensen A, Herbst B, Lindemann A, et al: Phase I study in melanoma patients of vaccine with peptide-pulsed dendritic cells generated in vitro from CD34(+) hematopoietic progenitor cells. Int J Cancer 2000;86:385–392.
11 Morse MA, Deng D, Lyerly HK, et al: A Phase I study of active immunotherapy with carcinoembryonic antigen peptide (CAP-1)-pulsed, autologous human cultured dendritic cells in patients with metastatic malignancies expressing carcinoembryonic antigen. Clin Cancer Res 1999;5:1331–1338.
12 Murphy GP, Tjoa BA, Simmons SJ, Rogers MK, Kenny GM, Jarisch J: Higher-dose and less frequent dendritic cell infusions with PSMA peptides in hormone-refractory metastatic prostate cancer patients. Prostate 2000;43:59–62.
13 Burch PA, Breen JK, Vuk-Pavlovic S, et al: Primary tissue-specific cellular immunity in a phase I trial of autologous dendritic cells for prostate cancer. Clin Cancer Res 2000;6:2175–2182.
14 Murphy G, Tjoa B, Ragde H, Kenny G, Boynton A: Phase I clinical trial: T-cell therapy for prostate cancer using autologous dendritic cells pulsed with HLA-A0201-specific peptides from prostate-specific membrane antigen. Prostate 1996;29:371–380.
15 Schott M, Seissler J, Feldkamp J, von Schilling C, Scherbaum WA: Dendritic cell immunotherapy induces antitumor response in parathyroid carcinoma and neuroendocrine pancreas carcinoma. Horm Metab Res 1999;31:662–664.
16 Holtl L, Rieser C, Thurnher M, et al: Cellular and humoral immune responses in patients with metastatic renal cell carcinoma after vaccination with antigen pulsed dendritic cells. J Urol 1999;161:777–782.
17 Ohta K, Yamaguchi Y, Shimizu K, Miyahara E, Toge T: Novel system for generation cytotoxic effector lymphocytes using carcinoembryonic antigen (CEA) peptide and cultured dendritic cells. Anticancer Res 2002;22:2597–2606.
18 Boczkowski D, Nair SK, Synder D, Gilboa E: Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. J Exp Med 1996;184:465–472.
19 Nair SK, Boczkowski D, Morse M, Cumming RI, Lyerly HK, Gilboa E: Induction of primary carcinoembryonic antigen (CEA)-specific cytotoxic T lymphocytes in vitro using human dendritic cells transfected with RNA. Nat Biotechnol 1998;16:364–369.
20 Ashley D, Faiola B, Nair S, Hale LP, Bigner DD, Gilboa E: Bone marrow-generated dendritic cell pulsed with tumor extracts or tumor RNA induce anti-tumor immunity against central nervous system tumors. J Exp Med 1997;186:1177–1182.
21 Gilboa E, Nair SK, Lyerly H: Immunotherapy of cancer with dendritic cell-based vaccines. Cancer Immunol Immunother 1998;46:82–87.
22 Gaugler B, Van den Eynde B, Boon T, et al: Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. J Exp Med 1994;179:921–930.
23 Zimmermann W, Ortlieb B, Friedrich R, Von Kleist S: Isolation and characterization of cDNA clones encoding the human carcinoembryonic antigen reveal a highly conserved repeating structure. Proc Natl Acad Sci USA 1987;84:2960–2964.
24 Boczkowski D, Nair SK, Nam JH, Lyerly HK, Gilboa E: Induction of tumor immunity and cytotoxic T lymphocyte responses using dendritic cells transfected with messenger RNA amplified from tumor cells. Cancer Res 2000;60:1028–1034.
25 Heiser A, Gilboa E, Vieweg J, et al: Induction of polyclonal prostate cancer-specific CTL using dendritic cells transfected with amplified tumor RNA. J Immunol 2001;166:2953–2960.
26 Van Tendeloo VFI, Ponsaerts P, Berneman ZN, et al: Highly efficient gene delivery by mRNA electroporation in human hematopoietic cell: superiority to lipofection and passive palsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. Blood 2001;98:49–56.
27 Kalady MF, Onaitis MW, Padilla KM, Emani S, Tyler DS, Pruitt SK: Enhanced dendritic cell antigen presentation in RNA-based immunotherapy. J Surg Res 2002;105:17–24.
28 Neri S, Mariani E, Meneghetti A, Cattini L, Facchini A: Calcein-acetyoxymethyl cytotoxicity assay: standardization of a method allowing additional analyses on recovered effector cells and supernatants. Clin Diagn Lab Immunol 2001;8:1131–1135.
29 Kawabuchi Y, Yamaguchi Y, Ohshita A, Minami K, Toge T: Host-oriented peptide evaluation using whole blood assay for generating antigen-specific cytotoxic T lymphocytes. Anticancer Res 2004;24:1193–1200.
30 Kim CJ, Prevette T, Cormier J, et al: Dendritic cells infected with poxviruses encoding MART-1/Melan A sensitize T lymphocytes in vitro. J Immunother 1997;20:276–286.
31 Dietz AB, Vuk PS: High efficiency adenovirus-mediated gene transfer to human dendritic cells. Blood 1998;91:392–398.
32 Jenne L, Hauser C, Arrighi JF, Saurat JH, Hugin AW: Poxvirus as avector to transduce human dendritic cells for immunotherapy: abortive infection but reduced APC function. Gene Ther 2000;7:1575–1583.
33 Jonuleit H, Tuting T, Steitz J, et al: Efficient transduction of mature CD83+ dendritic cells using recombinant adenovirus suppressed T cell stimulatory capacity. Gene Ther 2000;7:249–254.
34 Lu D, Benjamin R, Kim M, Conry RM, Curiel DT: Optimization of methods to achieve mRNA-mediated transfection of tumor cells in vitro and in vivo employing cationic liposome vectors. Gene Ther 1994;1:245–252.
35 Ying H, Zaks TZ, Wang RF, et al: Cancer therapy using a self-replicating RNA vaccine. Nat Med 1999;5:823–827.
36 Heiser A, Gilboa E, Vieweg J, et al: Human dendritic cells transfected with RNA encoding prostate-specific antigen stimulate prostate-specific CTL responses in vitro. J Immunol 2000;164:5508–5514.
37 Heiser A, Maurice MA, Yancey DR, Coleman DM, Dahm P, Vieweg J: Human dendritic cells transfected with renal tumor RNA stimulate polyclonal T-cell responses against antigens expressed by primary and metastatic tumors. Cancer Res 2001;61:3388–3393.
38 Weissman D, Ni H, Kariko K, et al: HIV gag mRNA transfection of dendritic cells (DC) delivers encorded antigen to MHC class I and II molecules, causes DC maturation, and induces a potent human in vitro primary immune response. J Immunol 2000;165:4710–4717.
39 Zhao Y, Boczkowski D, Nair SK, Gilboa E: Inhibition of invariant chain expression in dendritic cells presenting endogenous antigens stimulates CD4+ T-cell responses and tumor immunity. Blood 2003;102:4137–4142.
40 Bonehill A, Heirman C, Tuyaerts S, Michiels A, Breckpot K, Brasseur F, Zhang Y, Van der Bruggen P, Thielemans K: Messenger RNA-electroporated dendritic cells presenting MAGE-A3 simultaneously in HLA class I and class II molecules. J Immunol 2004;172:6649–6657.
41 Heiser A, Coleman D, Gilboa E, et al: Autologous dendritic cells transfected with prostate-specific antigen RNA stimulate CTL responses against metastatic prostate tumors. J Clin Invest 2002;109:409–417.
42 Su Z, Gilboa E, Vieweg J, et al: Immunological and clinical responses in metastatic renal cancer patients vaccinated with tumor RNA-transfected dendritic cells. Cancer Res 2003;63:2127–2123.

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ORIGINAL ARTICLE

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Paclitaxel probably enhances cytotoxicity of natural killer cells against breast carcinoma cells by increasing perforin production

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Abstract Paclitaxel, a semisynthetic taxane, is one of the most active chemotherapeutic agents for the treatment of patients with breast cancer. We focused on the effect of paclitaxel on the cytotoxicity of natural killer (NK) cells. NK cells were purified by negative selection with magnetic beads from peripheral blood mononuclear cells of healthy volunteers. A human breast carcinoma cell line BT-474 and an NK cell–sensitive erythroleukemia cell line K562 were used as targets. Cytotoxicity of NK cells was determined by ^{51}Cr-release assay with labeled target cells. Paclitaxel (1–100 nM) did not affect cellular viability, and significantly enhanced cytotoxicity of NK cells in a dose-dependent manner. Although paclitaxel did not affect Fas-ligand expression of NK cells, paclitaxel induced mRNA and protein production of perforin, an effector molecule in NK cell–mediated cytotoxicity. Concanamycin A, a potent inhibitor of the perforin-mediated cytotoxic pathway, inhibited paclitaxel-dependent NK cell–mediated cytotoxicity. Furthermore, paclitaxel induced activation of nuclear factor κB (NF-κB) in NK cells. NF-κB inhibitor pyrrolidine dithiocarbamate significantly suppressed both paclitaxel-induced perforin expression and NK cell cytotoxicity. Our results show for the first time that paclitaxel enhances in vitro cytotoxicity of human NK cells. Moreover, our results suggest a significant association between enhanced NK cell cytotoxicity, increased perforin production, and NF-κB activation.

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Keywords Natural killer cells · Nuclear factor κB · Paclitaxel · Perforin

Abbreviations

CMA	Concanamycin A
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IL	Interleukin
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
NF-κB	Nuclear factor κB
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PDTC	Pyrrolidine dithiocarbamate
TLR	Toll-like receptor
TNF	Tumor necrosis factor

Introduction

Paclitaxel and docetaxel are two representative taxane-based chemotherapeutic agents for the treatment of patients with breast cancer [6, 10, 12, 30]. Paclitaxel is a semisynthetic taxane isolated from the Western yew, *Taxus brevifolia* [10, 12, 23, 30]. Paclitaxel is thought to induce apoptosis in carcinoma cells by binding to tubulin, inducing tubulin polymerization and microtubule formation, and blocking cell mitosis [22, 33, 43]. Judging from the results of recent studies, however, paclitaxel may induce apoptosis through different mechanisms, including c-Jun NH_2-terminal kinase activation, nuclear factor κB (NF-κB)[3] activation, p66 Shc phosphorylation, or mitogen-activated protein kinase pathway [3, 13, 21, 44, 46, 47], depending on cell lines and culture conditions. In addition, paclitaxel induces apoptosis in some cells and biological activation in other cells [24]. In murine models, paclitaxel has been shown

to be a lipopolysaccharide (LPS) mimetic [18]. For example, paclitaxel enhances secretion of interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α) in murine macrophages by stimulating signal pathways, such as toll-like receptor 4 (TLR4)/NF-κB, and gene expression indistinguishable from that of LPS [11]. Paclitaxel also increases the secretion of both IL-1β and TNF-α by human monocytes in vitro at drug concentrations achievable with clinical use of paclitaxel; however, the precise molecular mechanism remains unclear [1, 2].

The effect of paclitaxel on nature killer (NK) cell function appears to be quite different than that on macrophage and monocyte function. Taxanes including paclitaxel suppress NK cells in vitro [8, 9, 25]. For example, Chuang et al. [9] showed that paclitaxel inhibited the cytotoxicity of human NK cells against cell lines K562 (NK cell–sensitive erythroleukemia cell line) and OV-2774 (relatively NK cell–resistant ovarian cell line). Paclitaxel also increases cytotoxicity of NK cells [24, 41]. Tsavaris et al. [41] suggested that peripheral blood lymphocyte-derived NK cell activity increases in patients with advanced breast cancer undergoing chemotherapy with taxanes including paclitaxel. In addition, Tong et al. [39] showed that NK cytotoxicity of patients with advanced cancer did not differ before or after paclitaxel treatment. Thus, the effect of paclitaxel on NK cell activity is still controversial.

The main objective of the present study is to evaluate the effect of paclitaxel at clinically relevant concentrations (nanomole level) on cytotoxicity of purified NK cells. Here, we show for the first time that paclitaxel increases cytotoxicity of NK cells against a breast carcinoma cell line in vitro and that paclitaxel both induces NF-κB activation and increases production of perforin, which is one of the effector molecules that mediate cytotoxicity in NK cells.

Materials and methods

Reagents

Paclitaxel was purchased from the Bristol-Myers Squibb (Princeton, NJ, USA) and solubilized in RPMI 1640. Pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB translocation, was purchased from Sigma Chemical (St Louis, MO, USA). Perforin inhibitor concanamycin A (CMA) was purchased from Wako Pure Chemicals (Osaka, Japan).

Cells

Human breast adenocarcinoma cell line BT-474 and NK cell–sensitive erythroleukemia cell line K562 were maintained in complete medium composed of RPMI 1640 and 10% fetal bovine serum (FBS) (Sigma).

Preparation of human CD16$^+$ NK cells

Peripheral blood mononuclear cells (PBMCs) of healthy volunteers were isolated from heparinized peripheral blood by Histopaque-1077 (Sigma) density gradient centrifugation. NK cells were further purified by negative selection with magnetic beads coated with mouse monoclonal anti-CD3, anti-CD4, anti-CD14, and anti-CD19 antibodies (Dynabeads; Dynal, Oslo, Norway), resulting in CD16$^+$ NK cells with greater than 95% purity. Purity was confirmed by flow cytometric analysis with anti-CD16 monoclonal antibody (mAb) (Becton Dickinson).

Cytotoxicity assay

Cytotoxicity was determined by ^{51}Cr-release assay as described previously [19]. Briefly, target cells (1×10^6/ml) were incubated with 100 μCi ^{51}Cr for 60 min and then washed twice with complete medium to eliminate residual ^{51}Cr. The ^{51}Cr-labeled target cells (1×10^4/well) and effector cells (various cell density) were suspended in 200 µl of complete medium and incubated in a 96-well U-bottomed plate in triplicate at 37 °C. After 4 h, the radioactivity of the supernatant (100 µl) was measured by a gamma counter. The percentage of cellular cytotoxicity was calculated with the following formula: % specific lysis = (experimental cpm − spontaneous cpm) / (maximum cpm − spontaneous cpm) × 100. In some experiments, the effector cells were pretreated with 20 nM CMA for 2 h to inactive perforin [15, 16].

Fluorescence-activated cell sorting (FACS) analysis

Cell surface expression of Fas ligand was examined by a single-color immunofluorescence procedure with biotin-conjugated mouse antihuman Fas ligand antibody (Becton Dickinson) and goat antimouse IgG/FITC mAbs (Becton Dickinson). Intracellular expression of perforin was also determined by flow cytometry. Cells were examined with antihuman perforin/FITC mAbs (Ancell, Bayport, MN, USA) after fixation with 2% paraformaldehyde for 30 min and then permeabilization with 0.3% saponin for 10 min. In control samples, staining was performed with antihuman IgG/FITC mAbs (Becton Dickinson) as a negative control. The labeled cells were washed twice and then analyzed with a FACSCalibur flow cytometer (Becton Dickinson) and CELLQuest software (Becton Dickinson).

Reverse transcriptase polymerase chain reaction (RT-PCR)

Natural killer cells were incubated with various concentrations of paclitaxel (0–1,000 nM) at 37°C for 24 h and then washed with PBS (Wako) to eliminate paclitaxel. Total RNA was extracted from NK cells by the

guanidinium thiocyanate–phenol–chloroform extraction method [7]. RNA (3 μg) was reverse-transcribed to cDNA with the Superscript TM II RNaseH-reverse transcriptase system (Gibco BRL, Grand Island, NY, USA). The first strand cDNAs of perforin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were then amplified by 35 cycles of PCR (denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min) with the following specific primer sets: 5′-CGGCTCACACTCACAGG-3′ (perforin sense), 5′-CTGCCGTGGATGCCTATG-3′ (perforin antisense) [38], 5′-CCACCCATGGCAAATTCCATGGCA-3′ (GAPDH sense), and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (GAPDH antisense). PCR products were separated on ethidium bromide–containing 1.5% agarose gels. Expected RT-PCR product sizes were 369 bp for perforin and 593 bp for GAPDH. The intensity of the perforin and GAPDH bands was estimated with NIH image version 1.62 software (NIH Division of Computer Research and Technology, Bethesda, MD, USA).

Electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extracts of NK cells was performed as described previously [32]. Briefly, NK cells (5×10^6) were cocultured with various concentrations of paclitaxel for 24 h, washed once with PBS, and then collected by centrifugation. Collected NK cells were homogenized in hypotonic buffer and then incubated for 10 min on ice. Nuclei were collected by centrifugation at 800 *g* for 5 min, washed once with hypotonic buffer, and resuspended in low-salt buffer. An equal volume of high-salt buffer was added with vortex mixing. Nuclei were incubated for 30 min on ice and centrifuged at 1,800 *g* for 30 min, and the supernatants were collected. Nuclear protein extracts of NK cells were analyzed by EMSA for NF-κB nuclear translocation as described previously [32]. Briefly, nuclear protein extracts of $5–8\times10^6$ cells were incubated for 30 min at 37°C with binding buffer, poly (dI-dC) (Amersham Pharmacia Biotech, Uppsala, Sweden), and ^{32}P-labeled double-stranded oligonucleotide containing the binding motif of NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′; Promega, Madison, WI, USA). The sequence of Oct-1 probe was 5′-CTAGATATGCAAATCATTG-3′. These mixtures were loaded onto a 4% polyacrylamide gel and separated by electrophoresis in 0.25xTBE running buffer. The oligomer–protein complexes were visualized by autoradiography.

Statistical analysis

Student's *t* test was used for statistical analyses. All results with a *P* value less than 0.05 were considered statistically significant.

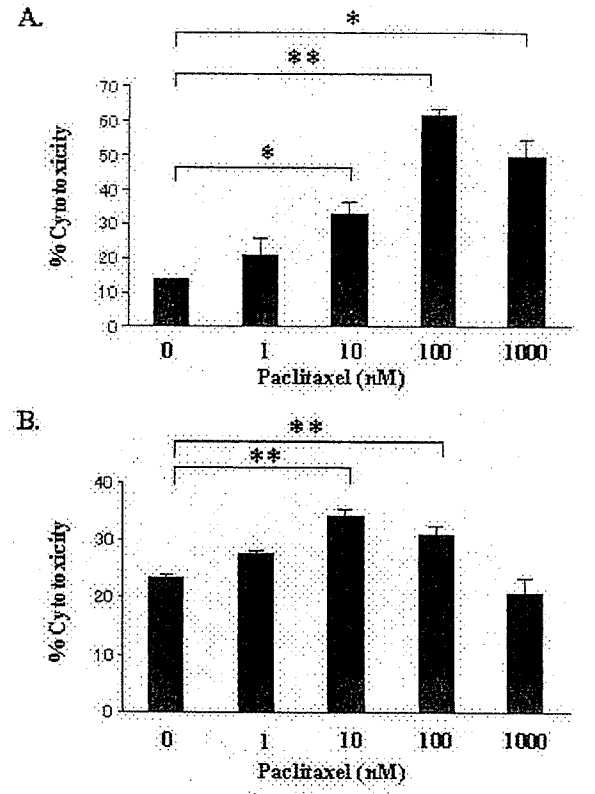

Fig. 1 Effects of paclitaxel on cytotoxicity of NK cells against BT-474 cells and K562 cells. NK cells, which were purified by negative selection with magnetic beads, were cocultured with various concentrations of paclitaxel (0–1,000 nM) at 37°C for 24 h and then washed to eliminate paclitaxel. Cytotoxicity was determined by ^{51}Cr-release assay at effector to target cell ratios of 10:1 and 5:1 (**a** and **b**, respectively). Paclitaxel enhanced cytotoxicity of NK cells against BT-474 cells and K562 cells (**a** and **b**, respectively) at relatively low concentrations. *Bars* SD. $^*P<0.05$ (significant difference from control); $^{**}P<0.01$

Results

Paclitaxel enhances cytotoxicity of NK cells

Natural killer cells were cocultured with various concentrations of paclitaxel (1–1,000 nM) at 37°C for 24 h. Then NK cells were washed twice with complete medium to eliminate residual paclitaxel and resuspended in fresh complete medium. Treatment of NK cells with paclitaxel at less than 1,000 nM did not affect cellular viability and total living cell numbers (data not shown). K562 cells are highly sensitive to NK cells, and BT-474 cells are relatively resistant to NK cells. To reveal the effect of paclitaxel on cytotoxicity of NK cells, we used effector to target ratios of 5:1 and 10:1 for K562 cells and BT-474 cells, respectively, throughout this study. Paclitaxel increased cytotoxicity of NK cells against BT-474 cells and K562 cells (Fig. 1a and b, respectively) at 1–100 nM, in

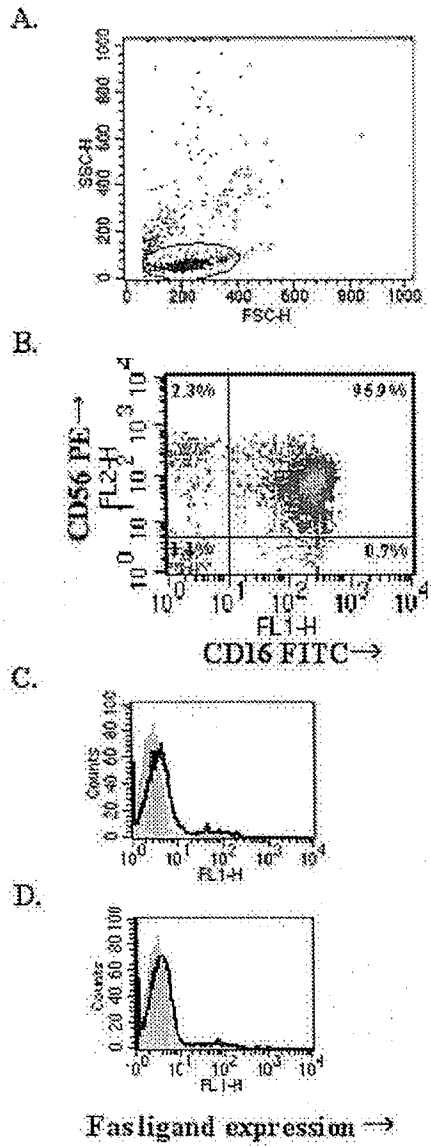

Fig. 2 Effects of paclitaxel on Fas ligand expression of NK cells. After NK cells were cultured with paclitaxel at various concentrations (0–100 nM) for 24 h, Fas ligand expression on NK cells was determined by FACS analysis. Flow cytometry was performed as described in "Materials and methods." Mean fluorescence intensity (MFI) was analyzed by CELLQuest software. **a** Representative cytogram obtained from NK cells purified by negative selection with magnetic beads and treated with 10 nM paclitaxel for 24 h is shown. *Circle* indicates lymphocytes region. **b** Purity of $CD16^+$ NK cells of gated cells (*circle*) was 96.6%. **c**, **d** Representative histograms (*open*) of NK cells treated with (**d**) and without (**c**) 10 nM paclitaxel are shown. *Filled histograms* indicate the staining with isotype control. Paclitaxel did not affect expression of Fas ligand. Similar results were obtained with NK cells from five different healthy volunteers

a dose-dependent manner. Similar results were obtained in 12 independent experiments with NK cells from five different healthy volunteers. Representative data are shown in Fig. 1.

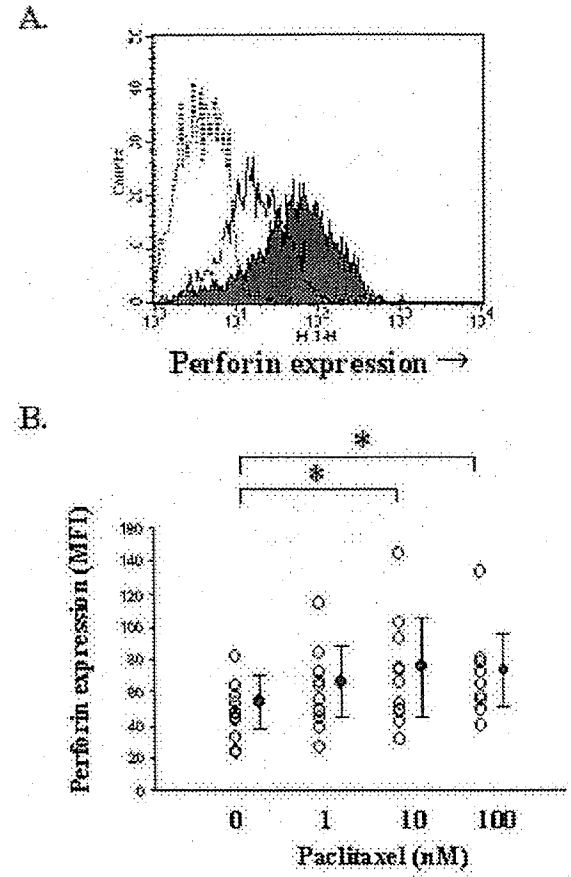

Fig. 3 Effects of paclitaxel on perforin expression of NK cells. NK cell perforin expression was determined by FACS analysis as described in Fig. 2 and "Materials and methods." **a** An *open* and *filled histogram* of perforin protein expression are indicated for nontreated NK cells and paclitaxel (10 nM) pretreated NK cells, respectively. *Dotted histogram* indicates the staining with isotype control. **b** Results from five healthy individuals showed peak perforin expression at 10–100 nM paclitaxel. *Bars* SD. $*P < 0.05$ (signifigant difference from control)

Paclitaxel does not affect Fas ligand expression of NK cells

Because two main mechanisms in NK cell cytotoxicity—i.e., the Fas/Fas ligand pathway and perforin/granzyme pathway—have generally been accepted, we first examined the effect of paclitaxel on Fas ligand expression on NK cells. NK cells treated with paclitaxel (1–100 nM) for 24 h did not affect expression of Fas ligand. A representative histogram of NK cells treated with 10 nM paclitaxel is shown in Fig. 2c, d. Similar results were obtained with NK cells from five different healthy volunteers.

Paclitaxel induces perforin production in NK cells

We next examined whether paclitaxel-induced NK cell cytotoxicity is related to perforin production. First, intracellular expression of perforin in NK cells was

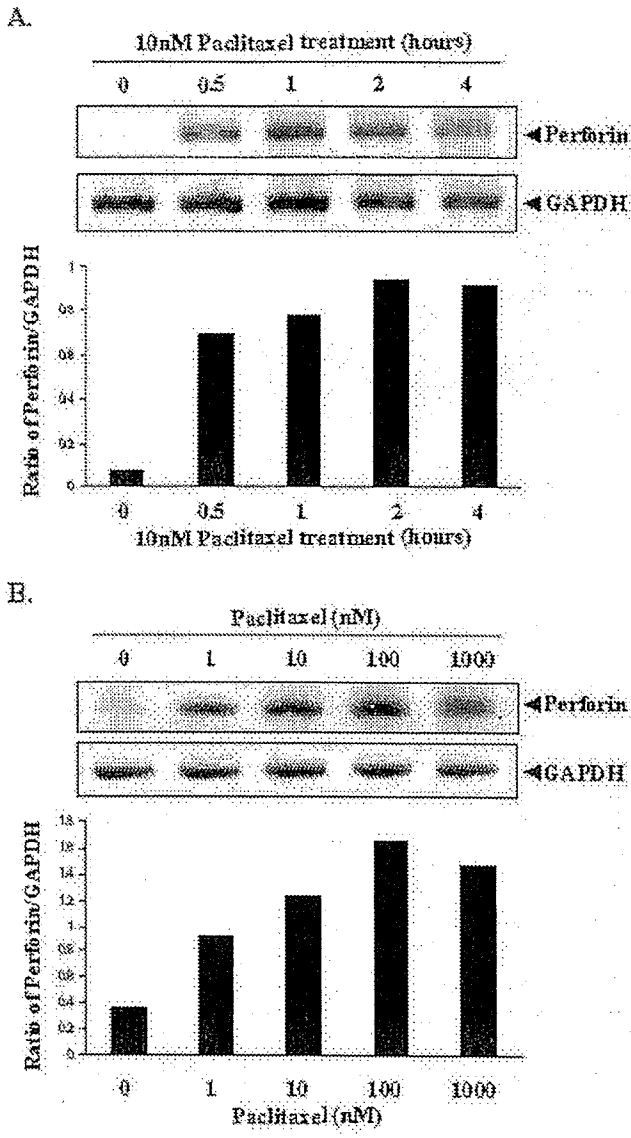

Fig. 4 Effects of paclitaxel on transcription of perforin mRNA in NK cells. a NK cells (2×10^6) were cultured with 10 nM paclitaxel at 37°C for various numbers of hours and then washed to eliminate paclitaxel. b NK cells were cultured with paclitaxel in various concentrations (0–1,000 nM) at 37°C for 4 h and then washed with PBS to eliminate paclitaxel. Transcription of perforin mRNA in NK cells was examined by RT-PCR. The level of perforin expression was quantified by densitometric scanning using NIH image software. GAPDH protein was blotted as a control

determined by FACS analysis as described in "Materials and Methods." A representative histogram of NK cells treated with 10 nM paclitaxel is shown in Fig. 3a (filled histogram). NK cells treated with paclitaxel (1–100 nM) for 24 h showed up-regulated intracellular expression of perforin in a dose-dependent manner (Fig. 3b). Similar results were obtained with NK cells from five different healthy volunteers.

Next, we examined the effect of paclitaxel on transcription of perforin mRNA in NK cells by RT-PCR. Paclitaxel increased transcription of perforin mRNA within 30 min after initial treatment and that increase continued for at least 4 h (Fig. 4a). When NK cells were treated with indicated concentrations of paclitaxel for 4 h, paclitaxel increased transcription of NK cell perforin mRNA in a dose-dependent manner (Fig. 4b). Similar results were obtained in five independent experiments with NK cells from three different healthy volunteers.

Paclitaxel induces NF-κB activation in NK cells

To examine why paclitaxel increases perforin expression of NK cells, we speculated that perforin expression is related to the NF-κB pathway. When NK cells were treated with 10 nM paclitaxel, increased nuclear translocation of NF-κB p65 was induced within 30 min after the initial culture (Fig. 5), indicating activation of NF-κB. Specificity of DNA binding was confirmed by a competition study with a 100-fold excess of unlabeled NF-κB oligonucleotide.

PDTC suppresses perforin production of NK cells induced by paclitaxel

First, the inhibitory effect of pyrrolidine dithiocarbamate (PDTC) on paclitaxel-induced NF-κB activation was confirmed. PDTC (10 μM) was added to NK cell cultures 1 h before treatment with 10 nM paclitaxel. Within 4 h, PDTC suppressed the nuclear translocation of NF-κB but not of Oct-1 (Fig. 6a). Similar results were obtained in five independent experiments with NK cells from three different healthy volunteers. Similarly, NK cells were pretreated with 10 μM PDTC for 1 h, and then 10 nM paclitaxel for 4 h to measure perforin mRNA expression, or for 24 h to measure perforin protein expression. PDTC suppressed perforin production at both mRNA and protein levels (Fig. 6b, c). Treatment of NK cells with 10 μM PDTC did not affect cellular viability and total living cell numbers (data not shown). Similar results were obtained in three independent experiments with NK cells from three different healthy volunteers.

PDTC suppresses cytotoxicity of NK cells

The NK cells were cultured with 10 nM paclitaxel with or without 10 μM PDTC at 37°C for 24 h. PDTC completely inhibited cytotoxicity of not only paclitaxel-treated NK cells but also nontreated NK cells against BT-474 cells and K562 cells (Fig. 7a, b, respectively). Treatment of NK cells with 10 μM PDTC did not affect cellular viability and total living cell numbers (data not shown). Similar results were obtained in five independent experiments with NK cells from three different healthy volunteers.

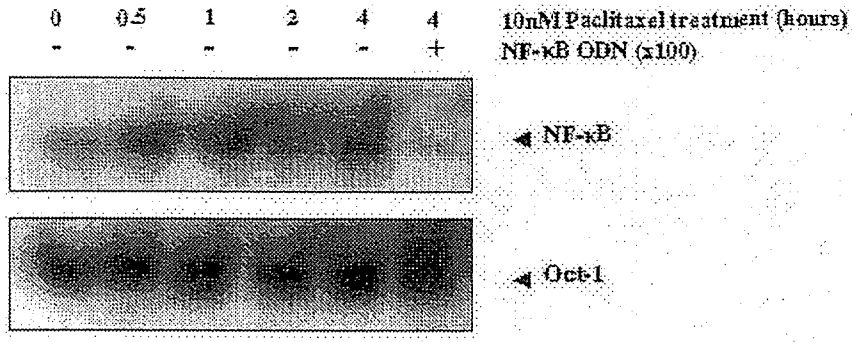

Fig. 5 Activation of NF-κB in NK cells treated with paclitaxel. Nuclear translocation of NF-κB was examined by EMSA. When NK cells were treated with 10 nM paclitaxel, NF-κB DNA binding increased within 30 min after the initial culture. In a competition study, nuclear extracts were preincubated with a 100-fold excess of unlabeled NF-κB oligonucleotide for 1 h and then with radiolabeled double-stranded oligonucleotide probe. Oct-1 protein was blotted as a control. Paclitaxel induced NF-κB activation in NK cells

CMA suppresses cytotoxicity of NK cells

The NK cells were incubated with or without 10 nM paclitaxel for 24 h. These NK cells were washed to eliminate paclitaxel and then pretreated with 20 nM CMA for 2 h before incubation with the target cells in the presence of CMA. CMA suppressed cytotoxicity of both nontreated NK cells and paclitaxel-treated NK cells against BT-474 cells and K562 cells (Fig. 8a, b, respectively). Treatment of NK cells with 20 nM CMA did not affect cellular viability and total living cell numbers (data not shown). Similar results were obtained in three independent experiments with NK cells from three different healthy volunteers.

Discussion

We provide evidence that paclitaxel at drug concentrations achievable in clinical settings can increase in vitro perforin production of NK cells and cause increased cytotoxicity against not only NK cell–sensitive K562 cells but also relatively NK cell–resistant breast carcinoma BT-474 cells. We also indicate that paclitaxel-mediated perforin production may be related to paclitaxel-induced NF-κB activation.

Paclitaxel increased perforin production at both mRNA and protein levels in NK cells, increasing their cytotoxicity (Fig. 1). Results from previous studies suggest there are two major mechanisms of NK-mediated cytotoxicity: the perforin/granzymes pathway [34, 40] and the Fas/Fas ligand pathway [26]. Since, paclitaxel did not induce a significant change in Fas ligand expression of NK cells (Fig. 2), we focused on the effect of paclitaxel on perforin-mediated cytotoxicity of NK cells. CMA is known to be a specific inhibitor of vacuolar type H^+-ATPase [46]. CMA inhibits the activity of perforin in dense granules, mostly because perforin degradation is accelerated by an increase in the pH of the lytic granules [14]. It has been postulated that CMA is a selective inhibitor, blocking only the perforin-based cytotoxicity and not affecting the Fas-based cytotoxicity [15]. Thus, in the present study, we used CMA to help determine the mechanism of paclitaxel-

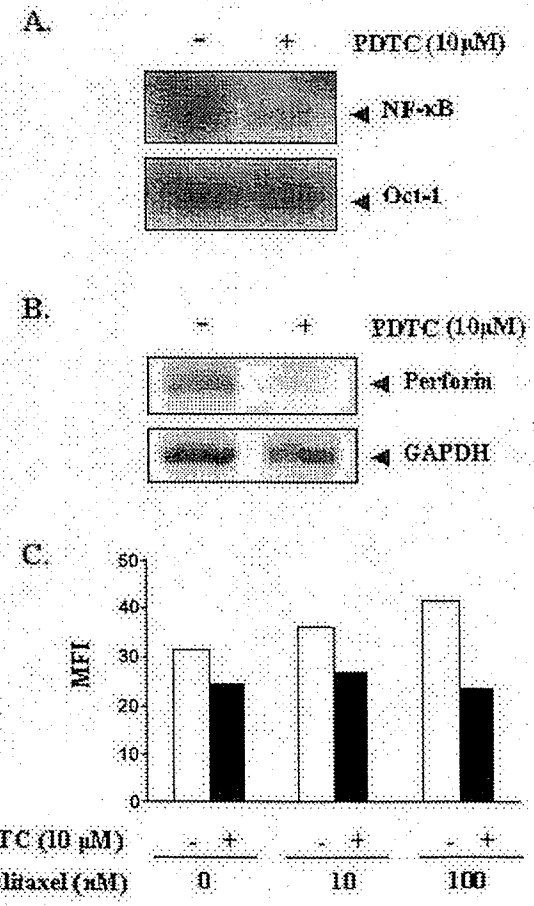

Fig. 6 Effects of NF-κB inhibitor PDTC on perforin expression in NK cells teated with paclitaxel. NK cells were pretreated with 10 μM PDTC for 1 h, and then with paclitaxel for 4 h to analyze NF-κB activation by EMSA, or for 24 h to analyze intracellular perforin expression by FACS. a PDTC suppressed the nuclear translocation of NF-κB but not of Oct-1. b, c PDTC suppressed perforin production at both mRNA and protein levels, respectively

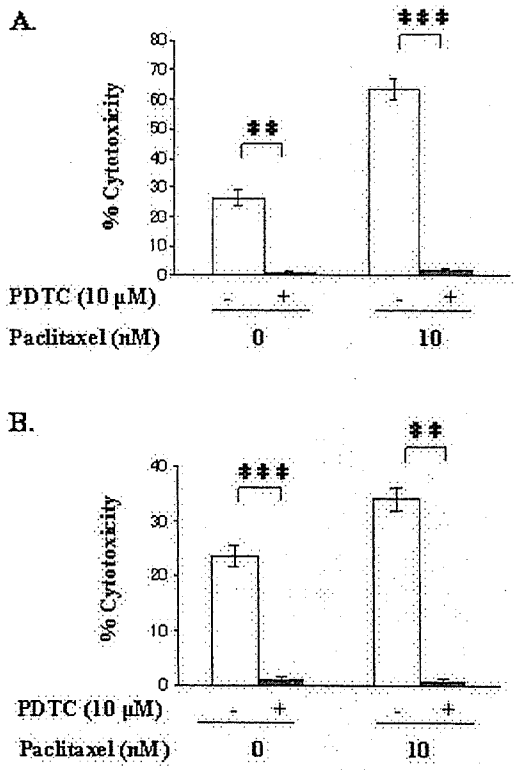

Fig. 7 Effects of PDTC on cytotoxicity of NK cells treated with paclitaxel. NK cells were pretreated with 10 μM PDTC for 1 h and then 10–100 nM paclitaxel for 24 h. Cytotoxicity was determined by ^{51}Cr-release assay at effector to target cell ratios of 10:1 (**a**) and 5:1 (**b**). Cytotoxicity of NK cells treated with or without paclitaxel against BT-474 cells (**a**) and K562 cells (**b**) was completely suppressed by PDTC. *Bars* SD. $^{**}P < 0.01$ (significant difference from control); $^{***}P < 0.001$

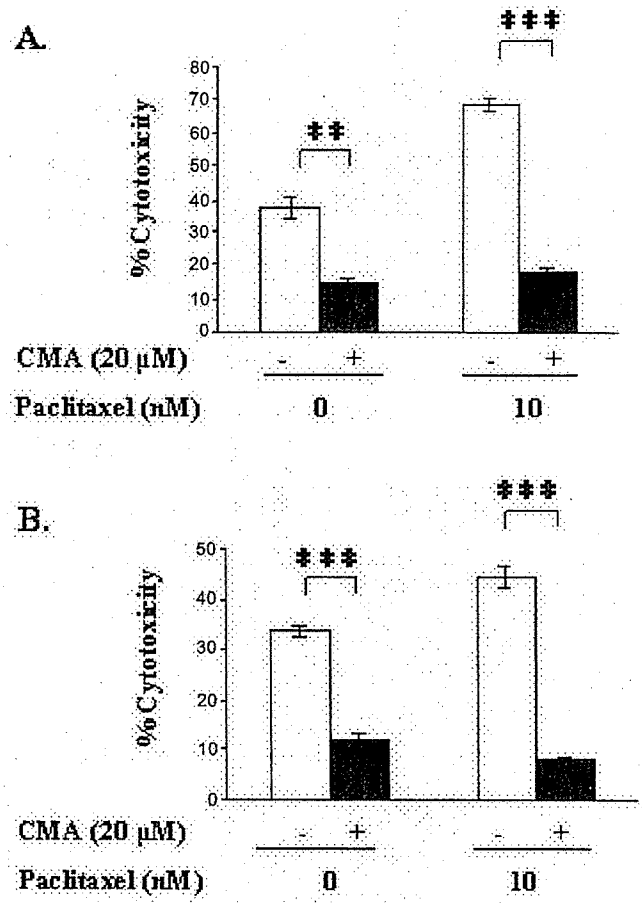

Fig. 8 Effects of perforin inhibitor CMA on cytotoxicity of NK cells treated with paclitaxel. Cytotoxicity was determined by ^{51}Cr-release assay at effector to target cell ratios of 10:1 (**a**) and 5:1 (**b**). NK cells were cultured with or without 10 nM paclitaxel at 37°C for 24 h and then washed to eliminate paclitaxel. These NK cells were treated with 20 nM CMA for 2 h before being cocultured with the target cells, BT-474 cells (**a**) and K562 cells (**b**). *Bars* SD; $^{**}P < 0.01$ (significant difference from control); $^{***}P < 0.001$

dependent cytotoxicity of perforin. CMA treatment suppressed cytotoxicity of both nontreated NK cells and paclitaxel-treated NK cells (Fig. 7). However, CMA could not completely inhibit paclitaxel-dependent cytotoxicity of NK cells. Although our present data indicate no significant role of Fas ligand in paclitaxel-induced NK cell cytotoxicity (Fig. 2), we can not rule out the existence of other cytotoxic pathways including the Fas/Fas ligand pathway. The perforin/granzymes pathway likely involves the formation of pores between NK cells and target cells by extracellular calcium-dependent polymerization of perforin. Consequently, granzymes, serin proteases responsible for downstream caspase activation, and DNA fragmentation are released into the target cell, resulting in target cell apoptosis [34, 40]. In the present study, we have data indicating that paclitaxel also induces granzyme expression of NK cells at both mRNA and protein levels (data not shown). However, the biological function of granzyme serine proteases released with perforin from the cytotoxic granules of NK cells is still controversial. Thus, it is not clear whether granzymes A and B play an essential role in cytotoxicity mediated by the perforin pathway [37]. Nonetheless, our study shows that perforin plays an important role in paclitaxel-dependent cytotoxicity of NK cells (Fig. 8).

How paclitaxel can induce increased perforin production in NK cells is an interesting question. Others showed signaling that indicated that the levels of perforin expression of NK cells are essentially controlled by IL-2Rβ [28, 31, 48]. Interestingly, DNA-binding activity of NF-κB can be induced by IL-2R signaling in T cells [5]. Valle Blázquez et al. [42] reported on a pharmacological inhibitor of NF-κB–impaired NK cell–mediated cytotoxicity. It was shown recently that NF-κB plays a critical role in perforin expression in NK cells [49]. In the present study, we used PDTC to examine the contribution of the NF-κB pathway to perforin production induced with paclitaxel. PDTC is a stable analog of dithiocarbamates and is one of the most widely used inhibitors of NF-κB signaling [4]. PDTC suppressed perforin production at both mRNA and protein levels (Fig. 6a, c). Thus, our

results strongly suggest that NF-κB plays a key role in paclitaxel-induced perforin production.

So, how does paclitaxel induce NF-κB activation in NK cells? In a murine system, paclitaxel induces NF-κB activation through TLR 4 [17]. We examined the expression of TLR 4 on NK cells by FACS analysis. Because we could not detect expression of TLR 4 on the cellular surface of either nontreated or paclitaxel-treated NK cells (data not shown), it is unclear whether activation of paclitaxel-dependent NF-κB is related to interaction between paclitaxel and TLR 4 in the human system as well.

Pyrrolidine dithiocarbamate completely inhibited not only paclitaxel-dependent NK cell–mediated cytotoxicity but also nontreated NK cell–mediated cytotoxicity (Fig. 7). Although PDTC completely inhibited paclitaxel-dependent perforin expression, PDTC did not affect intracellular levels of perforin in nontreated NK cells (Fig. 6c). As described above, the perforin/granzymes pathway is one of several cytotoxic pathways of NK cells. Binding of NK cells with target cells initiates a series of biochemical events responsible for the redistribution and secretion of granules such as perforin. Specific protein kinases (the primary kinases being Src family kinases and Syk) are central to NK cell functions, including granule release. Moreover, intervention to granule redistribution and release after target cell binding are regulated by members of the mitogen-activated protein kinase family, specifically extracellular signal-regulated kinase and p38 [29]. These molecules are directly or indirectly related to the NF-κB pathway through phosphorylation of IκB kinase [20, 27].

The detailed mechanism by which paclitaxel increases NK cell cytotoxicity is not clear. Nonetheless, our new findings are likely to be useful for future cancer treatment. A recent study showed that patients with HER-2/neu-positive breast carcinoma responded better to a combination therapy with taxanes, including paclitaxel, and a mAb against HER-2, than with either agent alone [35]. NK cells undoubtedly play an important role in this anti-HER-2 antibody therapy by a mechanism involving antibody-dependent cellular cytotoxicity [36]. Thus, understanding the effects of paclitaxel on NK cell–mediated cytotoxicity is likely to be important for developing new therapeutic strategies with taxanes.

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References

1. Allen JN, Moore SA, Wewers MD (1993) Taxol enhances but does not induce interleukin-1 beta and tumor necrosis factor-alpha production. J Lab Clin Med 122:374–381
2. Bacus SS, Gudkov AV, Lowe M, Lyass L, Yung Y, Komarov AP, Keyomarsi K, Yarden Y, Seger R (1992) Taxol, a microtubule-stabilizing antineoplastic agent, induces expression of tumor necrosis factor-alpha and interleukin-1 in macrophages. J Leukoc Biol 52:119–121
3. Bacus SS, Gudkov AV, Lowe M, Lyass L, Yung Y, Komarov AP, Keyomarsi K, Yarden Y, Seger R (2001) Taxol-induced apoptosis depends on MAP kinase pathways (ERK and p38) and is independent of p53. Oncogene 20:147–155
4. Baeuerle PA, Henkel T (1994) Function and activation of NF-κB in the immune system. Annu Rev Immunol 12:141–179
5. Brach MA, Gruss HJ, Riedel D, Mertelsmann R, Herrmann F (1992) Activation of NF-kappa B by interleukin 2 in human blood monocytes. Cell Growth Differ 3:421–427
6. Chevallier B, Fumoleau P, Kerbrat P, Dieras V, Roche H, Krakowski I, Azli N, Bayssas M, Lentz MA, Van Glabbeke M (1995) Docetaxel is a major cytotoxic drug for the treatment of advanced breast cancer: a phase II trial of the Clinical Screening Cooperative Group of the European Organization of Research and Treatment of Cancer. J Clin Oncol 13:314–322
7. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
8. Chuang LT, Lotzová E, Cook KR, Cristofaroni P, Morris M, Wharton JT (1993) Effect of new investigational drug taxol on oncolytic activity and stimulation of human lymphocytes. Gynecol Oncol 49:291–298
9. Chuang LT, Lotzová E, Heath J, Cook KR, Munkarah A, Morris M, Wharton JT (1994) Alteration of lymphocyte microtubule assembly, cytotoxicity, and activation by the anticancer drug taxol. Cancer Res 54:1286–1291
10. Gelmon K (1994) The taxoids: paclitaxel and docetaxel. Lancet 344:1267–1272
11. Hayakawa M, Miyashita H, Sakamoto I, Kitagawa M, Tanaka H, Yasuda H, Karin M, Kikugawa K (2003) Evidence that reactive oxygen species do not mediate NF-κB activation. EMBO J 22:3356–3366
12. Holmes FA, Walters RS, Theriault RL, Forman AD, Newton LK, Raber MN, Buzdar AU, Frye DK, Hortobagyi GN (1991) Phase II trial of Taxol, active drug in the treatment of metastatic breast cancer. J Natl Cancer Inst 83:1797–1805
13. Huang Y, Johnson KR, Norris JS, Fan W (2000) Nuclear factor-κB/IκB signaling pathway may contribute to the mediation of paclitaxel-induced apoptosis in solid tumor cells. Cancer Res 60:4426–4432
14. Kataoka T, Takaku K, Magae J, Shinohara N, Takayama H, Kondo S, Nagai K (1994) Acidification is essential for maintaining the structure and function of lytic granules of CTL. J Immunol 153:3938–3947
15. Kataoka T, Shinohara N, Takayama H, Takaku K, Kondo S, Yonehara S, Nagai K (1996) Concanamycin A, a powerful tool for characterization and estimation of contribution of perforin- and Fas-based lytic pathways in cell-mediated cytotoxicity. J Immunol 156:3678–3686
16. Kataoka T, Yamada A, Bando M, Honma T, Mizoue K, Nagai K (2000) FD-891, a structural analogue of concanamycin A that does not affect vacuolar acidification or perforin activity, yet potently prevents cytotoxic T lymphocyte-mediated cytotoxicity through the blockage of conjugate formation. Immunol 100:170–177
17. Kawasaki K, Akashi S, Shimazu R, Yoshida T, Miyake K, Nishijima M (2000) Mouse toll-like receptor 4/MD-2 complex mediates lipopolysaccharide-mimetic signal transduction by Taxol. J Biol Chem 275:2251–2254
18. Kawasaki K, Nogawa H, Nishijima M (2003) Identification of mouse MD-2 residues important for forming the cell surface TLR4-MD-2 complex recognized by anti-TLR4-MD-2 antibodies, and for conferring LPS and Taxol responsiveness on mouse TLR4 by alanine-scanning mutagenesis. J Immunol 170:413–420
19. Kubo M, Morisaki T, Kuroki H, Tasaki A, Yamanaka N, Matsumoto K, Nakamura K, Onishi H, Baba E, Katano M (2003) Combination of adoptive immunotherapy with Her-

ceptin for patients with HER2-expressing breast cancer. Anticancer Res 23:4443–4450
20. Lee FS, Peters RT, Dang LC, Maniatis T (1998) MEKK1 activates both IκB kinase α and IκB kinase β. Proc Natl Acad Sci U S A 95:9319–9324
21. Lee LF, Li G, Templeton DJ, Ting JP (1998) Paclitaxel (Taxol)-induced gene expression and cell death are both mediated by the activation of c-Jun NH2-terminal kinase (JNK/SAPK). J Biol Chem 273:28253–28260
22. Manfredi JJ, Parness J, Horwitz SB (1982) Taxol binds to cellular microtubules. J Cell Biol 94:688–696
23. McGuire WP, Rowinsky EK, Rosenshein NB, Grumbine FC, Ettinger DS, Armstrong DK, Donehower RC (1989) Taxol: a unique antineoplastic agent with significant activity in advanced ovarian epithelial neoplasms. Ann Intern Med 111:273–279
24. Mehta S, Blackinton D, Manfredi M, Rajaratnam D, Kouttab N, Wanebo H (1997) Taxol pretreatment of tumor targets amplifies natural killer cell mediated lysis. Leuk Lymphoma 26:67–76
25. Munkarah A, Chuang L, Lotzová E, Cook K, Morris M, Wharton JT (1994) Comparative studies of taxol and taxotere on tumor growth and lymphocyte function. Gynecol Oncol 55:211–216
26. Nagata S, Golstein P (1995) The Fas death factor. Science 267:1449–1456
27. Nakano H, Shindo M, Sakon S, Nishinaka S, Mihara M, Yagita H, Okumura K (1998) Differential regulation of IκB kinase α and β by two upstream kinases, NF-κB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. Proc Natl Acad Sci U S A 95:3537–3542
28. Nelson BH, Willerford DM (1998) Biology of the interleukin-2 receptor. Adv Immunol 70:1–81
29. Perussia B (2000) Signaling for cytotoxicity. Nat Immunol 1:372–374
30. Rowinsky EK, Cazenave LA, Donehower RC (1990) Taxol: a novel investigational antimicrotubule agent. J Natl Cancer Inst 82:1247–1257
31. Salcedo TW, Azzoni L, Wolf SF, Perussia B (1993) Modulation of perforin and granzyme messenger RNA expression in human natural killer cells. J Immunol 151:2511–2520
32. Sasaki N, Morisaki T, Hashizume K, Yao T, Tsuneyoshi M, Noshiro H, Nakamura K, Yamanaka T, Uchiyama A, Tanaka M, Katano M (2001) Nuclear factor-κB p65 (RelA) transcription factor is constitutively activated in human gastric carcinoma tissue. Clin Cancer Res 7:4136–4142
33. Schiff PB, Fant J, Horwitz SB (1979) Promotion of microtubule assembly in vitro by taxol. Nature 277:665–667
34. Shresta S, Heusel JW, Macivor DM, Wesselschmidt RL, Russell JH, Ley TJ (1995) Granzyme B plays a critical role in cytotoxic lymphocyte-induced apoptosis. Immunol Rev 146:211–221
35. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 344:783–792
36. Sliwkowski MX, Lofgren JA, Lewis GD, Hotaling TE, Fendly BM, Fox JA (1999) Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). Semin Oncol 26:60–70
37. Smyth MJ, Street SEA, Trapani JA (2003) Granzymes A and B are not essential for perforin-mediated tumor rejection. J Immunol 171:515–518
38. Strehlau J, Pavlakis M, Lipman M, Shapiro M, Vasconcellos L, Harmon W, Strom TN (1997) Quantitative detection of immune activation transcripts as a diagnostic tool in kidney transplantation. Proc Natl Acad Sci U S A 94:695–700
39. Tong AW, Seamour B, Lawson JM, Ordonez G, Vukelja S, Hyman W, Richards D, Stein L, Maples PB, Nemunaitis J (2000) Cellular immune profile of patients with advanced cancer before and after taxane treatment. Am J Clin Oncol 23:463–472
40. Trapani JA, Smyth MJ (2002) Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol 2:735–747
41. Tsavaris N, Kosmas C, Vadiaka M, Kanelopoulos P, Boulamatsis D (2002) Immune changes in patients with advanced breast cancer undergoing chemotherapy with taxanes. Br J Cancer 87:21–27
42. Valle Blázquez M, Luque I, Collantes E, Aranda E, Solana R, Peña J, Muñoz E (1997) Cellular redox status influences both cytotoxic and NF-κB activation in natural killer cells. Immunology 90:455–460
43. Wahl AF, Donaldson KL, Fairchild C, Lee FYF, Foster SA, Demers GW, Galloway DA (1996) Loss of normal p53 function confers sensitization to Taxol by increasing G2/M arrest and apoptosis. Nat Med 2:72–79
44. Wang TH, Popp DM, Wang HS, Saitoh M, Mural JG, Henley DC, Ichijo H, Wimalasena J (1999) Microtubule dysfunction induced by paclitaxel initiates apoptosis through both c-Jun N-terminal kinase (JNK)-dependent and -independent pathways in ovarian cancer cells. J Biol Chem 274:8208–8216
45. Woo JT, Shinohara C, Sakai K, Hasumi K, Endo A (1992) Isolation, characterization and biological activities of concanamycins as inhibitors of lysosomal acidification. J Antibiot (Tokyo) 45:1108–1116
46. Yamamoto K, Ichijo H, Korsmeyer SJ (1999) BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G_2/M. Mol Cell Biol 19:8469–8478
47. Yang CP, Horwitz SB (2000) Taxol mediates serine phosphorylation of the 66-kDa Shc isoform. Cancer Res 60:5171–5178
48. Zhang BJ, Scordi I, Smyth MJ, Lichtenheld MG (1999) Interleukin 2 receptor signaling regulates the perforin gene through signal transducer and activator of transcription (Stat) 5 activation of two enhancers. J Exp Med 190:1297–1307
49. Zhou J, Zhang J, Lichtenheld MG, Meadows GG (2002) A role for NF-κB activation in perforin expression of NK cells upon IL-2 receptor signaling. J Immunol 169:1319–1325

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Research Paper

Three-dimensional two-layer collagen matrix gel culture model for evaluating complex biological functions of monocyte-derived dendritic cells

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Abstract

Dendritic cell-like cells (Mo-DCs) generated from peripheral blood monocytes with interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been used as tools to treat cancer patients (DC-vaccines). Because Mo-DCs have multiple antigen presentation-related functions, including phagocytosis, migration, cytokine production, and T cell stimulation, establishment of a method for simultaneously evaluating the various functions of Mo-DCs is important. We developed a new in vitro three-dimensional two-layer collagen matrix culture model that consists of a collagen gel containing Mo-DCs as the lower layer and a collagen gel containing necrotic GCTM-1 tumor cells and/or T cells as the upper layer. We used this system to observe simultaneously multiple functions of Mo-DCs by phase-contrast or fluorescence microscopy and to assess IL-12 secretion during more than 2 weeks of culture. We also observed interactions between Mo-DCs and necrotic GCTM-1 or T cells on an individual cell basis by time-lapse videomicroscopy. In addition, we collected Mo-DCs from the collagen gels by collagenase treatment and analyzed the expression of antigen presentation-related molecules such as HLA-DR, CD80, CD83, and CD86 on Mo-DCs. This model may be a useful tool for evaluation of the various functions of Mo-DCs used as DC vaccines and for studies of the complex behaviors of Mo-DCs in vivo.

Keywords: Mo-DC; Collagen matrix; Migration; Phagocytosis; IL-12 production

Abbreviations: DCs, dendritic cells; Mo-DCs, dendritic cell-like cells; IL, interleukin; MHC, major histocompatibility complex; GM-CSF, granulocyte macrophage colony stimulating factor; PBMCs, peripheral blood mononuclear cells; FBS, fetal bovine serum; PBS, phosphate-buffered saline; HSA, human serum albumin; BSA, bovine serum albumin; IFN-γ, Interferon-γ; ELISA, enzyme-linked immunosorbent assay; PI, propidium iodide; 2-D, two-dimensional; 3-D, three-dimensional.

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