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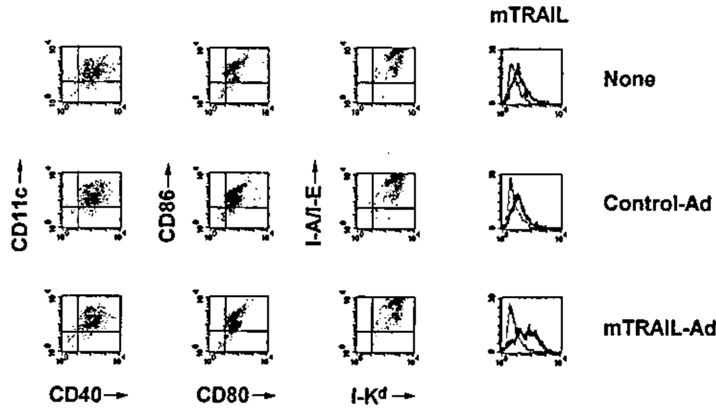

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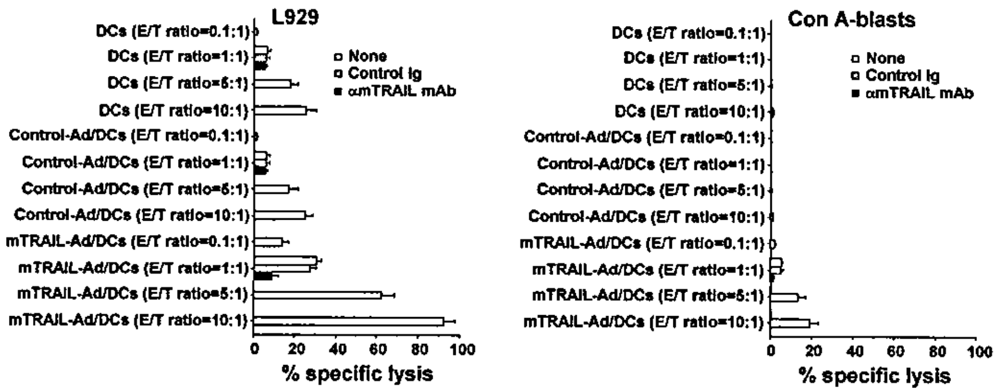

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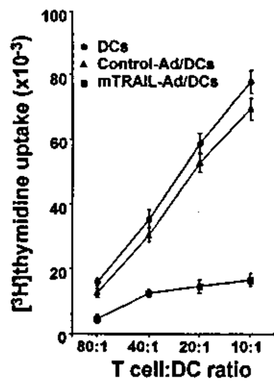

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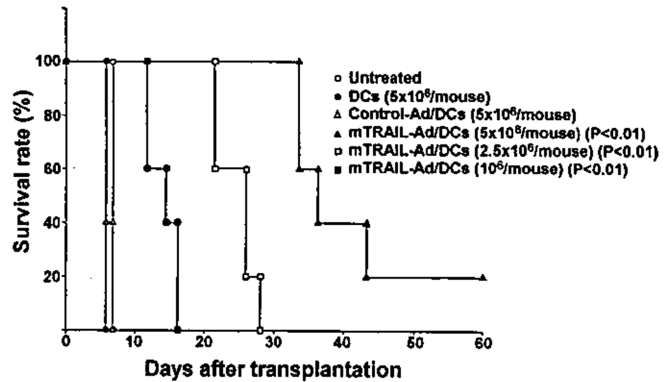
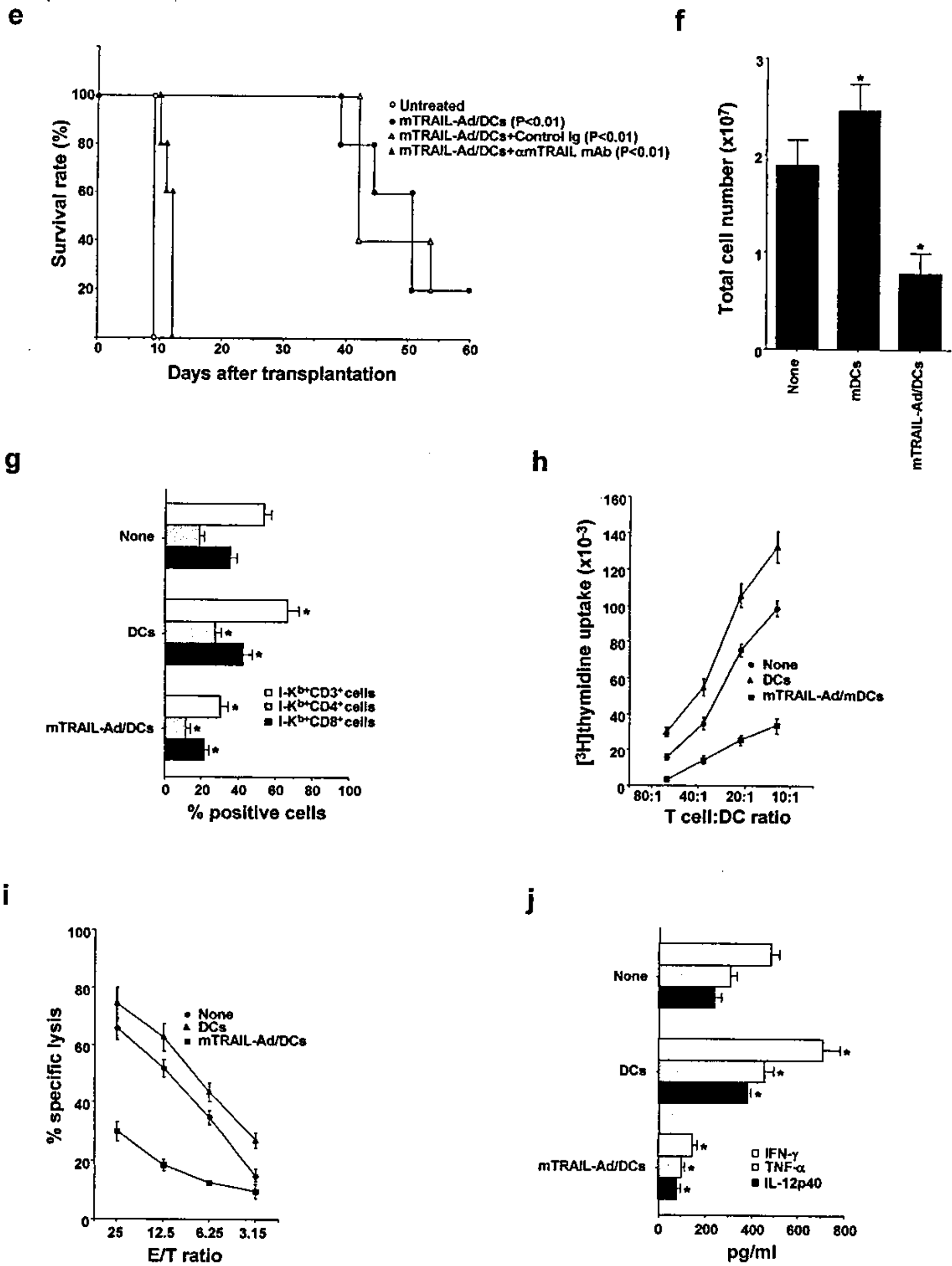


Sato et al., Figure 5



Sato et al., Figure 5

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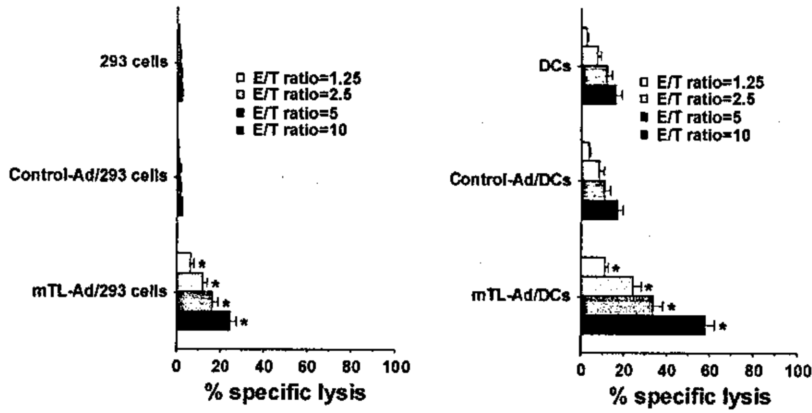

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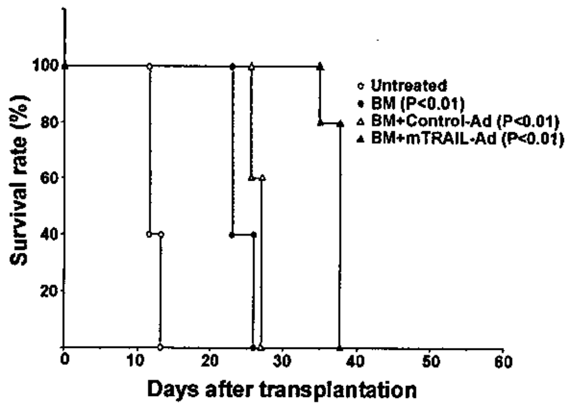

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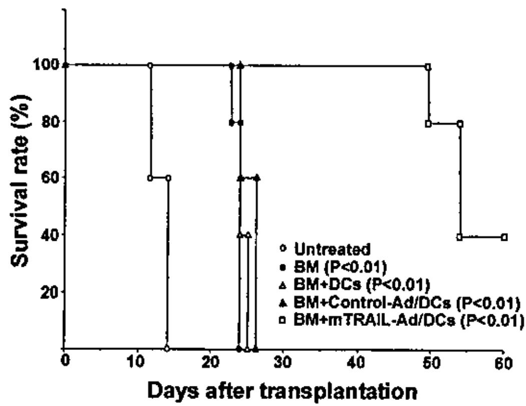

Sato et al., Figure 6

Frequent immune responses to a cancer/testis antigen CAGE in patients with microsattelite instability positive endometrial cancer

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Abbreviations used are: SEREX, Serological identification of tumor antigens by cDNA expression cloning; MSI, microsattelite instability; AEH, atypical endometrial hyperplasia; HNPCC, hereditary non-polyposis colon cancer.

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ABSTRACT

Purpose: Identification of cancer testis antigens useful for diagnosis or immunotherapy of cancers was attempted by cDNA expression cloning with patients' sera (SEREX).

Experimental design: cDNA expression libraries made from testis or endometrial cancer cell lines were screened using sera from patients with endometrial cancer or melanoma patients immunized with dendritic cells pulsed with autologous tumor lysates. Tissue specific expression by RT-PCR and Northern blot analysis and immunogenicity by Western blotting of the bacterial recombinant antigen with sera from cancer patients were evaluated.

Results: A cancer testis antigen CAGE was isolated by 2 independently performed SEREX. CAGE was expressed in various cancer cell lines including endometrial cancer, colon cancer, and melanoma, in 7 of 10 endometrial cancer tissues and in 1 of 3 atypical endometrial hyperplasia, but not in normal tissues including endometrium except testis. The protein expression on cancer cells was confirmed. By Western blot analysis with the recombinant CAGE protein, anti-CAGE IgG antibody was detected in sera from 5 of 45 endometrial cancer, 2 of 24 melanoma, and 2 of 33 colon cancer patients, but not in sera from healthy individuals. By ELISA analysis, anti-CAGE antibody was detected in 12 of 45 endometrial cancer, 2 of 20 melanoma, and 4 of 33 colon cancer patients. Intriguingly, anti-CAGE antibody was highly positive in 7 of the 13 (53.8%) MSI-H patients with endometrial cancer, but negative in 20 non-MSI-H patients (P=0.001).

Conclusion: CAGE may be useful for immunotherapy and diagnosis of various cancers particularly MSI positive endometrial cancer.

INTRODUCTION

Identification of human tumor antigens is important not only for analysis of anti-tumor immune responses and development of immunotherapy, but also for development of diagnostic methods(1). Various methods for the identification of tumor antigens have recently been applied, including cDNA expression cloning with tumor reactive T cells and patients' serum IgG Ab, as well as reverse immunology strategy which evaluates induction of T cells against candidate molecules identified by various techniques such as systematic gene expression analysis and cDNA subtraction.

Among the representative tumor antigens recognized by T cells, cancer testis antigens which express in various cancers and the limited normal tissues including testis and placenta, are good candidates as tumor specific common antigens for use in the immunotherapy. Cancer testis antigens have previously been isolated by various methods. MAGE1 was first isolated by cDNA expression cloning with melanoma reactive T cells(2), and NY-ESO-I was isolated by cDNA expression cloning (SEREX) with serum from a patient with esophageal cancer (3). CT15, 16, and17 were isolated DNA homology search using public gene databases(4), and MAGEC-1 were isolated by cDNA subtraction (RDA) between testis cDNA library and normal tissues(5).

A cancer testis antigen CAGE was originally isolated by SEREX with serum from a patient with gastric cancer(6). By RT-PCR analysis, it was reported to be frequently expressed in gastric cancer, cervical cancer, lung cancer, and liver cancer. However, expression of the CAGE protein in

tumor cells has not yet been evaluated and serum IgG Ab was detected only in serum of a single patient with gastric cancer. Thus, further analysis of the CAGE protein and its immunogenicity in various cancers remained to be investigated.

In this study, we isolated CAGE by screening a testis cDNA library with sera from melanoma patients who were frequently immunized with dendritic cells pulsed with autologous tumor lysates(7), and by screening an endometrial cancer cDNA library with sera from patients with endometrial cancer. Through evaluation of the tissue specific expression and immunogenicity by screening serum IgG Ab specific for the recombinant CAGE protein, we revealed that CAGE was expressed frequently in various cancers including endometrial cancer and melanoma, and serum IgG antibody was frequently detected in sera from patients with microsatellite instability (MSI) positive endometrial cancers, indicating the possible use of CAGE for immunotherapy of endometrial cancer and melanoma as well as for diagnosis of MSI positive cancers.

MATERIALS AND METHODS

Patients. In this study, sera of 7 endometrial cancer patients, one esophageal cancer patient, and 3 melanoma patients were used for SEREX method. Clinical stage of each patients of 7 endometrial cancer patients were one was stage II, 3 were stage III, and 3 were stage IV. One esophageal cancer patient was stage III, and all of 3 melanoma patients were stage IV. These 3 melanoma patients were frequently (8-10 times) immunized with dendritic cells pulsed with autologous tumor lysates, but no effective regression of disease was seen, and all were died within 6 months(7). In addition to these sera, all sera of other cancer patients and healthy individuals, cancer tissues, and normal endometrial tissues used in this study were obtained with informed consent and written agreement.

Cell Lines and Tissues. The human endometrial cancer cell line SNG-II(8), ovarian clear cell adenocarcinoma cell lines RMG-I(9) and RMG-II(10), were established by our group. The human endometrial cancer cell lines Hec-Ib(11) was kindly provided by Dr Kuramoto (Kitasato University, Kanagawa, Japan), and Ishikawa line(12) was kindly provided by Dr Nishida (Kasumigaura national hospital, Ibaraki, Japan). Ishikawa, Hec-Ib SNG-II, RMG-I, and RMG-II cell lines were cultured in F12 (Sigma Chemical Co., St Louis, MO) supplemented with 10 % FCS and 100 μg/ml kanamycin. The melanoma cell lines SKmel23, 888mel, A375mel, Groves mel, 501mel, 586mel, 526 mel, and 501Amel, the lung cancer cell lines LU99, EBC1, and RERF-LC-MA, the renal cell cancer cell lines Saito, RCC6, RCC7, and RCC8, the bladder cancer cell line KU7, the prostate cancer cell line PC3,

the breast cancer cell line MDA231, leukemia cell lines HL60, K562, and Molt 4 were cultured in RPMI1640 (Sigma Chemical) supplemented with 10 % FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The esophageal cancer cell lines TE8 and TE10 were cultured in DMEM (Sigma Chemical) supplemented with 10 % FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The pancreatic cancer cell line PK59 was cultured in a complete medium consisting of RPMI 1640 supplemented with 10 % FBS, 2 mM L-glutamine, 10 mM HEPES, 6 μg/liter epidermal growth factor, 150 units/liter insulin, 0.5 mg/liter hydrocortisone, 10 mg/liter transferin, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Melanocyte was cultured in serum-free MM-4 medium (Morinaga, Yokohama, Japan). NIH-3T3 cell line was cultured in DMEM supplemented with 10 % CS (Calf serum), 100 IU/ml penicillin, and 100 μg/ml streptomycin. Normal tissues used in RT-PCR were obtained from Clontech (Palo Alto, CA). Normal endometrium, endometrial cancer tissues, and atypical endometrial hyperplasia tissues were obtained from surgical operation with informed consent and stored at -80°C until use.

Construction of cDNA Libraries. We used two kinds of cDNA library in this study, testis cDNA library and endometrial cancer cDNA library. Total RNA of testis was obtained from Clontech and that of endomeytrial cancer was isolated from the endometrial cancer cell lines Ishikawa, Hec-Ib, and SNG-II by guanidine isothiocyanate and CsCl gradient ultracentrifugation. These endometrial cancer cell lines, SNG-II, Hec-Ib, and Ishikawa are frequently used for research work, Ishikawa is established from well differentiated endometrioid adenocarcinoma and expresses both estrogen receptor and progesteron receptor(11, 12), SNG-II is established from well differentiated

endometrioid adenocarcinoma and expresses CA125 antigen, Hec-Ib is established from moderately differentiated endometrioid adenocarcinoma and expresses progesteron receptor(11). We mixed these three kinds of total RNAs of endometrial cancer cell lines for constructing the library. We purified poly(A)+RNA with latex beads, synthesised cDNA by RT-PCR, and inserted cDNA into the bacteriophage expression vector λ-Zap express (Stratagene, La Jolla, CA) as disclibed(13). Testis cDNA library and endometrial cancer cDNA library consisted of $2.5x10^6$ and $1.2x10^6$ primary recombinants.

Immunoscreening of the cDNA Library with Sera. SEREX method was carried out as described previously(14). The testis cDNA library was screened with mixed sera of one esophaguial cancer patient and three melanoma patients who received immunization with dendritic cells pulsed with autologous tumor lysates. The endometrial cancer cDNA library was screened using sera of 7 endometrial cancer patients. The positive clones were picked up and PCR was conducted by Ex Taq kit (Takara, Kyoto, Japan) and then the PCR products were sequenced on ABI Prism 3100 automated sequencer (Perkin-Elmer, Branchburg, NJ).

Expression of CAGE gene in Tumor Cell Lines or Tissues. Total RNA was isolated from cell lines by guanidine isothiocyanate and CsCl gradient ultracentrifugation, and total RNA from normal tissues was purchased from Clontech. Total RNA from endometrial cancer tissues and normal endometrial tissues was obtained by TRIzol method (Invitrogen, Calsbad, CA) for a higher yield and treated with DNaceI (Takara) for avoiding DNA contamination. Reverse transcription was performed

using Super Script II reverse transcriptase (Invitrogen) and gene-specific PCR was performed as follows by Ex-Taq DNA polymerase (TAKARA).. The primers for CAGE were 5'-CTTCCAACCGTATGTAGGCGAG (forward), 5'- CTCCTTGCGTCTTTGTCCAGGT (reverse) and used in RT-PCR consisting of initial denaturation at 94°C for 2 min and 35 amplification cycles of 30 sec at 94°C, 30 sec at 56°C, and 1.5 min at 72°C, followed by 5 min at 72°C. The primers for GAPDH were 5'- TGAACGGGAAGCTCACTGG (forward), 5'- TCCACCACCCTGTTGCTGTA (reverse) and used in RT-PCR consisting of initial denaturation at 94°C for 2 min and 25 amplification cycles of 30 sec at 94°C, 30 sec at 56°C, and 30 sec at 72°C, followed by 5 min at 72°C.

Preparation of Recombinant His-tagged CAGE and Production of Anti-CAGE polyclonal antibodies. His-tagged CAGE were generated. Due to the fact that the ORFs of genes of DEAD box family are highly homologous, if full length CAGE protein is used for antibody generation, it will produce non-specific antibodies. Therefore we selected a part that is specific for CAGE as the target sequence for pET16a partial construct, from codon1261 to 1873 in the ORF. The PCR products contained sits of the restriction enzymes *BamH1*(5') and *Sal1*(3'). The primers for partial protein were 5'-taaaaggatccTATTTGAAAGATCCTATGAT (forward), 3'-taaaaagtcgacTCAACTTAAAAAATAAAACT (reverse). The PCR product was digested with *BamH1* and *Sal1,* cloned into the pET16a (Novagen, Darmstadt, Germany) which was modified to contain multi cloning sites, and then expressed in the E. coli, AD494(DE3)pLys S (Novagen). The recombinant CAGE proteins were purified using the affinity resin HiTrap Chelating (Amersham Pharmacia). The number of amino acids of recombinant His-tagged CAGE protein was 235 and

predictive molecular weight was 31.1kDa. The rabbit anti-CAGE polyclonal antibody of this recombinant CAGE protein was made by Protein Purification Company (Tochigi, Japan).

pcDNA3.1 construction and transfection. pcDNA vector (Invitrogen) was used for construction of CAGE. PCR was conducted by using the following primers to generate full length of ORF of the CAGE gene with the sites of *BamHI* on 5' end and *NotI* on 3'end; 5'-taaaaaggatccATGTCCCACTGGGCCCCAGAG (forward), 3'-taaaaagcggccgcTCAACTTAAAAAATAAAACT (reverse). Then the PCR product was digested by *BamHI* and *NotI* cloned into the pcDNA3.1 vector. The pcDNA3.1-CAGE was transfected by Lipofectamine method (Invitrogen) into 5.0×10^5 NIH-3T3 cells on 100 mm culture plate. After 48 h incubation at 37°C, the transfected cells were collected and lysised by SDS sample buffer.

Protein Expression of CAGE in Tumor Cell Lines and Normal Cell Line. The expression of the CAGE protein was evaluated by Western Blotting with generating rabbit anti-CAGE antibodies. The human endometrial cell lines Ishikawa and SNGII, melanoma cell lines 888mel and normal melanocyte cell line were diluted into 1.0×10^4 cells/μl in SDS sample buffer. 5.0×10^4 cells were loaded per lane, elacterophoresed on 10 % SDS-PAGE gel and then transferred to nitrocellulose membrane (Hybond Extra C; Amersham Pharmacia). After blocking , the membrane was incubated overnight at 4°C with 1:1000 diluted anti-CAGE antibodies in 5 % skim milk solution, the membrane was washed in TBST, incubated for 2 h with 1:4000 diluted goat anti-Rabbit Fc antibody conjugated

with alkaline phosphatase (Cappel), and then washed in TBST. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used for the enzymatic detection.

Immunoscreening of CAGE by Western Blotting. One μg of recombinant CAGE protein was loaded per gel, and electerophoresed on 10% SDS-PAGE gel. After transferred to the membrane, it was cut into 10 strips and the strips were incubated overnight at 4°C with 1:100 diluted serum samples or 1:4000 diluted monoclonal anti-His antibody (Amersham Pharmacia). The strips were washed in TBST, incubated for 2 h with 1:4000 diluted goat anti-human Fc antibody conjugated with alkaline phosphatase (Cappel) or goat antimouse Fc antibody conjugated with alkaline phosphatase (Cappel).

ELISA for Detection of Anti-CAGE Antibodies. The recombinant CAGE protein was diluted in PBS buffer to a final concentration of 3μg /ml. The CAGE solution of 3μg /ml were dispensed into 96-well plates (100μl/well) and incubated overnight at 4°C. 1:100 diluted 100 μl of serum samples were added per well and incubated for 2 h at room temperature. After incubation with 100μl of 1:5000 diluted of goat anti-human IgG Fc labeled with horseradish peroxidase (Cappel), the plates were washed with PBST, and developed by tetramethylbenzidine solution for 20 min. After stopping the reaction by adding H_2SO_4, the absorbance was measured at 450 nm. All serum samples were run in duplicates and randomly dispensed on the plates. Sera from cancer patients and sera from healthy controls were tested simultaneously. Statistical analysis was conducted by using X^2 test.

MSI detection. MSI detection was performed as described previously(15). Genomic DNA was extracted from tumor tissues and peripheral lymphcytes. Flagments of microsatellite repeat loci *D2S123*, *D5S346*, *D17S250*, *BAT26*, *BAT25*, *MSH3*, *MSH6*, *TGFβRII*, *BAX*, *MBD4A10*, and *MBD4A6* were amplified PCR, using the above DNA as the template with that from peripheral lymphocytes serving as the normal control. If ≧30 % of the above markers showed MSI, the tumor was defined as MSI-H, based on the standard proposed at the Workshop of the National Cancer Institute Workshop(16). If the MSI rate was less than 30 %, the tumor was defined as MSI-L, and if MSI rate was zero, the tumor was defined as MSS.

Detection of serum CA602 antigen. Serum CA602(17) level was measured by enzyme immunoassay at Mitsubishi BML co. (Tokyo, Japan). Serum CA602 cutoff was set at 63 U/ml based on the values measured in normal individuals (10).

RESULTS

Isolation of cancer testis antigens by SEREX

To isolate cancer testis antigens, we have screened total 1.2×10^6 clones of a testis cDNA library with the mixture of sera from 3 melanoma patients who were frequently immunized with dendritic cells pulsed with autologous tumor lysates, and an esophageal cancer serum which was known to contain the MAGE cancer testis antigens (as a positive control for isolation of cancer testis antigens). Total 87 positive clones representing 26 distinct genes including cancer testis antigens such as MAGE1a, MAGE2b, MAGE4a, MAGE4b, MAGE6, MAGE9a, and NY-ESO-1, were isolated (Supplemental Table 1). We have also screened a cDNA library made from a mixture of mRNA of 3 endometrial cancer cell lines with sera from 7 patients with endometrial cancer, because endometrial cancer antigens have not yet been isolated by SEREX. Total 5.0×10^6 cDNA clones were screened, and 193 positive clones representing 59 distinct genes were isolated (Supplemental Table 2). By evaluating tissue specific expression using gene databases including SAGE databases and EST databases, and RT-PCR analysis, one of the isolated clones which showed cancer testis antigen like expression, and identified from both screening of testis and endometrial cancer libraries, was found to be a cancer testis antigen CAGE, which was originally identified by Cho et al. by SEREX with sera from gastric cancer patients(6). They reported that by RT-PCR analysis CAGE was frequently expressed in gastric cancer, cervical cancer, lung cancer, and liver cancer, but the serum recognition was only tested with one gastric cancer patient's serum, which was used for screening the cDNA

library. The expression of CAGE in other cancers including endometrial cancers and melanoma used in this study, and its immunogenicity in patients with various cancers remained to be investigated. Therefore, we attempted further analysis on the CAGE expression and its immunogenicity in various cancers.

Expression of the CAGE mRNA and protein in various cancers including melanoma and endometrial cancers

By RT-PCR analysis, CAGE was expressed in only testis among normal tissues and also in various cancer cell lines including lung cancer, renal cell cancer as previous reported (Fig 1A, B). Although it was previously reported that any of the melanoma, and breast cancer cell lines tested did not express CAGE, we observed that 4 of 7 melanoma, and one breast cancer cell line expressed CAGE. In addition, 2 of 3 endometrial cancer cell lines, one of 3 chronic myelogenous leukemia cell lines and one pancreatic cancer line were found to express CAGE (Fig 1B). We further revealed that 7 of 10 endometrial cancer tissues (4 in grade1, 4 in grade2, and 2 in grade3) and 1 of 3 atypical endometrial hyperplasia tissues expressed CAGE, whereas none of 8 normal endometrium (4 in the proliferation phase and 4 in the secretory phase) did not express CAGE (Fig. 2). The expression of CAGE did not have any correlation with the differentiation grade in endometrial cancer.

Since expression of the CAGE protein in tumor cells has not previously evaluated, we examined the CAGE protein in various cancer cell lines, including endometrial cancer and melanoma, by

Western Blot analysis with the anti-CAGE rabbit antibody which was produced by immunization with the bacterial recombinant CAGE protein as described in the Method section. This polyclonal antibody detected the predicted 82.5kDa band in lysates from NIH-3T3 cells transfected with pcDNA-CAGE, but not in lysates from untransfected NIH-3T3 cells, indicating specific recognition of CAGE (Fig. 3). The same specific bands were shown with this antibody in lysates from 2 endometrial cancer cell lines, Hec-Ib and Ishikawa, and one melanoma cell line 888mel, those are CAGE positive when evaluated by RT-PCR analysis, but were not shown in lysates from PCR negative cultured melanocytes, demonstrating that the CAGE protein was present in various cancer cell lines. Although we further attempted immunohistochemical study with this antibody to confirm the CAGE protein expression in fresh tumor tissues, reliable results could not be obtained by relatively strong background staining with the rabbit serum. We then evaluated subcellular localization of CAGE by immunohistochemical staining of COS cells transfected with pFLAG-CAGE with anti-FLAG M5 murine antibody, and found that the FLAG-CAGE fusion protein was present in nucleus (data not shown) as previously suggested (6).

Detection of anti-CAGE IgG antibody in sera from patients with various cancers including endometrial cancer and melanoma

Since the recognition of CAGE by serum from cancer patients was previously tested only with a single patient with gastric cancer, we have evaluated the recognition of CAGE by serum IgG antibody in patients with various cancers by Western blot analysis with the His-tagged bacterial recombinant

CAGE protein fragment. As a representative Western blot result was shown in Fig.4 and Table1, anti-CAGE IgG antibody was detected in sera from 5 of 45 endometrial cancer, 2 of 24 melanoma, and 2 of 33 colon cancer patients, but not detected in sera from 20 renal cell cancer, 18 prostate cancer, 12 pancreatic cancer patients, or 40 healthy individuals. The positive sera did not stain the negative control protein, recombinant His-tagged VEGFC, assuring the CAGE specific recognition of these positive sera (data not shown). The CAGE specific recognition was also confirmed by phage plaque assay with the same sera (data not shown).

We next attempted quantitative analysis of anti-CAGE IgG antibodies in sera from patients with various cancers using ELISA. By setting up the cutoff for positive anti-CAGE antibody at the average absorbance of the healthy individuals plus 2SD 0.058, positive sera were found in 12 of 45 (26.7%) endometrial cancer, 4 of 33 (12.5%) colon cancer, 2 of 20 (10.0%) melanoma patients, and 1 of 40 (2.5%) age matched healthy individuals, but not in 10 ovarian cancer patients (Fig. 5A). Among these positive patients, 5 endometrial cancer, 2 colon cancer, and 2 melanoma patients demonstrated high CAGE antibody titer over 0.12 OD, for whom positive bands was clearly detected in the Western blot analysis (Fig. 3).

Frequent detection of anti-CAGE serum IgG antibody in patients with MSI positive endometrial cancer

Correlation of the positive serum CAGE antibody with various clinocopathological features was