

Melanoma Antigen A4 Is Expressed in Non–Small Cell Lung Cancers and Promotes Apoptosis

Tobias Peikert,¹ Ulrich Specks,¹ Carol Farver,^{2,3,4} Serpil C. Erzurum,^{3,4} and Suzy A.A. Comhair^{3,4}

¹Thoracic Diseases Research Unit, Division of Pulmonary and Critical Care Medicine, Mayo Clinic College of Medicine, Rochester, Minnesota; Departments of ²Anatomic Pathology and ³Pulmonary, Allergy and Critical Care Medicine, The Cleveland Clinic Foundation; and ⁴Department of Pathobiology, Lerner Research Institute, Cleveland, Ohio

Abstract

A variety of *melanoma antigen A (MAGE-A)* genes are commonly detected in non–small cell lung cancers. Their biological function is not well characterized but may involve the regulation of apoptosis and cell cycle progression. We hypothesized that MAGE-A4 is involved in the regulation of apoptosis. To investigate this, expression of MAGE-A was evaluated. MAGE-A4 was expressed in 48% of non–small cell lung carcinomas. Ninety percent of lung carcinomas expressing MAGE-A4 were classified as squamous cell carcinomas and 10% were adenocarcinomas. Tumor-free surrounding lung tissue was negative for MAGE-A4. A molecular clone of *MAGE-A4* derived from human lung cancer was stably expressed in human embryonic kidney cells (293 cells) to evaluate effects on cell death. Overexpression of *MAGE-A4* increased apoptosis as measured by the apoptotic index ($P < 0.0001$) and caspase-3 activity ($P < 0.002$). Exposure to 25 $\mu\text{mol/L}$ etoposide, a chemotherapeutic agent, increased the apoptotic effect ($P < 0.0001$). Furthermore, we show that *MAGE-A4* silencing using a small interfering RNA approach results in decreased caspase-3 activity in the squamous cell lung cancer cell line H1703 by 58% ($P = 0.0027$) and by 24% ($P = 0.028$) in 293/*MAGE-A4* cells. These findings suggest that MAGE-A4 expression may promote tumor cell death, sensitize malignancies to apoptotic stimuli, such as chemotherapeutic agents, and therefore may represent a tumor suppressor protein. (Cancer Res 2006; 66(9): 4693-700)

Introduction

Melanoma antigens (MAGE) are a group of 55 closely related proteins (1). Based on differences in tissue-specific gene expression and gene structure, MAGE are classified into type 1 (*MAGE-A*, *MAGE-B*, and *MAGE-C*) and type 2 (*MAGE-D*) genes (2). Type 2 MAGE genes are almost universally expressed (2, 3). In contrast, type 1 MAGE expression is restricted to a small number of normal tissues, such as spermatocytes, placenta, and certain stages of the embryonal development (2, 3). In addition, type 1 MAGE expression has been documented in a broad variety of malignancies (4, 5). For example, non–small cell lung cancers frequently express a variety of type 1 MAGE genes (4–13).

Proteasomal degradation of type 1 MAGE proteins generates several small peptides, which are subsequently expressed on the cell surface in association with MHC class I molecules. These

peptides represent the target antigens for CTLs directed against MAGE-A-expressing cancer cells (2, 14–16). Based on these observations, type 1 MAGE protein–derived peptides are currently studied as targets for the development of cancer vaccines for non–small cell lung cancer and other malignancies (11).

Despite these efforts, the biological function of type 1 MAGE proteins remains poorly understood. MAGE-A3 has been shown to bind *in vitro* to murine pro-caspase-12, thereby blocking auto-activation of caspase-9 and downstream activation of caspase-3 (17). By this mechanism, MAGE-A3 is thought to inhibit apoptosis triggered by insults to the endoplasmic reticulum (17). In contrast, a recombinant 107 amino acid carboxyl-terminal fragment of MAGE-A4 was found to induce apoptosis *in vitro* through p53-dependent and p53-independent pathways (18). Furthermore, this carboxyl-terminal fragment of MAGE-A4 inhibited p21-mediated cell cycle arrest by blocking p21 up-regulation through inhibition of the binding of the poxvirus and zinc finger domain transcription factor Miz-1 to the p21 promoter (18). Interestingly, no change in apoptosis was observed when the intact MAGE-A4 protein was transiently expressed in the absence of apoptotic stimuli (18).

We hypothesized that MAGE-A4 expression represents a cellular defense mechanism that promotes removal of MAGE-A4-expressing cells by activation of the caspase pathway and induction of apoptosis.

Here, we show that MAGE-A4 is commonly present in squamous cell carcinomas, rarely found in adenocarcinomas, and absent in tumor-free surrounding lung. Overexpression of MAGE-A4 leads to increased apoptotic index and activation of caspase-3 in 293 cells *in vitro*. Moreover, we show that *in vitro* MAGE-A4 silencing in the squamous cell lung cancer cell line H1703 and MAGE-A4-overexpressing 293 cells (293/*MAGE-A4*) is associated with decreased caspase-3 activity. Exposure to etoposide augments cell death in the setting of MAGE-A4 overexpression, suggesting that the susceptibility of cancer cells to chemotherapeutic agents is enhanced by MAGE-A4 expression.

Subjects and Methods

All tissue samples were obtained following surgical resection of non–small cell lung cancers at the Cleveland Clinic Foundation (Cleveland, OH). Demographic information, clinical characteristics, and histologic characteristics of the enrolled patients were collected at study entry. Tumors were staged by cell type, differentiation, and the revised TNM (T = primary tumor, N = regional lymph nodes, M = metastasis) classification (19). Parenchymal lung tissue received from the operating room was processed immediately. If technically feasible, tissue samples were macroscopically separated into tumor and tumor-free lung tissue. All samples were frozen in liquid nitrogen until further processing.

The study was approved by the institutional review board of the Cleveland Clinic Foundation, and all patients provided informed consent before enrollment.

Requests for reprints: Tobias Peikert, Division of Pulmonary and Critical Care Medicine, Mayo Building, East 18, 200 First Street Southwest, Rochester, MN 55905. Phone: 507-284-2495; Fax: 507-266-4372; E-mail: peikert.tobias@mayo.edu.

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The limited amount of tissue available precluded the use of all specimens in every experiment. The number of samples used in each analysis is specified in the text.

Cell culture. 293 cells, adenovirus-transformed human embryonic kidney cells [American Tissue Culture Collection (ATCC), Manassas, VA], were cultured in DMEM-F12 medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Rockville, MD) and 1% penicillin/streptomycin (Cambrex, East Rutherford, NJ). A375 cells, human melanoma cells (ATCC), were cultured in DMEM medium (BioWhittaker) supplemented with 10% FBS (Life Technologies) and 1% penicillin/streptomycin (Cambrex). The non-small cell lung cancer cell lines NCI-H1703 (ATCC), NCI-H2122 (ATCC), and H441 (ATCC) were cultured in RPMI 1640 (ATCC) supplemented with 10% FBS (Life Technologies) and 1% penicillin/streptomycin (Cambrex).

RNA and protein extraction. For RNA extraction, lung tissue specimens were homogenized with the PowerGen tissue homogenizer (Fischer Scientific, Brightwaters, NY). Total RNA from tissue homogenate was extracted using the GTC [4 mol/L guanidinium thiocyanate, 25 mmol/L sodium citrate (pH 7.0), 0.5% Sarkosyl, and 0.1 mol/L β -mercaptoethanol]-CsCl gradient method (20).

For protein extraction, tissue specimens were snap frozen in liquid nitrogen and manually crushed with a hammer. This was followed by homogenization with a hand tissue homogenizer (Fischer Scientific) in ice-cold lysis buffer (40 mmol/L HEPES, 1.25 μ g/mL leupeptin, 2.5 μ g/mL aprotinin, 1.25 μ g/mL pepstatin, 0.125 mg/mL pefabloc, 0.09 mmol/L DTT, and 1% NP40; all from Boehringer Mannheim, Indianapolis, IN) and ultrasonication. After 5 minutes of centrifugation at $13,000 \times g$ (4°C), the supernatant was collected and stored at -80°C until further use.

Cell lysates of 293 cells and A375 cells were resuspended in ice-cold lysis buffer [50 mmol/L Tris (pH 7.9), 50 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, 0.5% NP40, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μ g/mL leupeptin, 10 μ g/mL pepstatin A, 200 μ mol/L NaOV, and 20 μ L/mL aprotinin; all from Boehringer Mannheim] followed by a 30-minute incubation on ice and 30 minutes of centrifugation at $13,000 \times g$ (4°C). Supernatant was collected and stored at -80°C until further use. Total protein concentration was measured by bicinchoninic protein assay (Pierce, Rockford, IL).

Evaluation of *MAGE-A4* expression by PCR. cDNA was reverse transcribed from 1 μ g RNA using reverse transcriptase mMLV RT and random hexamers (Invitrogen, Carlsbad, CA). Based on the reported human *MAGE-A4* cDNA sequence, the PCR primers (F, forward primer; R, reverse primer) F-*MAGE-A4* (5'-GAGCAGACAGGCCAACCG-3') and R-*MAGE-A4* (5'-AAGGACTCTGCGTCAGGC-3') were used (21). After preheating at 94°C for 5 minutes, the PCR cycles (35 repeats) were as follows: 1 minute at 94°C , annealing at 68°C for 2 minutes, and extension at 72°C for 2 minutes; this was followed by a final extension for 2 minutes at 72°C . PCR products (expected product 426 bp) were evaluated by agarose gel (1%) electrophoresis. β -Actin cDNA was amplified as control.

Cloning of *MAGE-A4*. PCR primers were based on the human sequence of *MAGE-A4* (BC017723). PCR was done using the following two nested reactions: F-*MAGE-A4*-S65 (5'-CCCTGTGAGGAGTCAAGGTTCT-3') and R-*MAGE-A4*-S1660 (5'-1660 ACGAAGCCAAGGAAATCCAG-3') for the first PCR and F-*MAGE-A4*-S128 (5'-ACAGAGGAGCACCAAGGAGAAG-3') and R-*MAGE-A4*-S1608 (5'ATAGACTGAGGCATAAGGCGG-3') for the nested PCR and the c-DNA of a pulmonary squamous cell carcinoma (T29) as the template. The PCR product (1,501 bp) was cloned into the TA cloning vector PCR2.1 (Invitrogen). After purification, *MAGE-A4* was subcloned into the overexpression vector pcDNA 3.1/Zeo 5.0 kb (Invitrogen). The entire length of both corresponding DNA strands of insert was sequenced using Sequenase 2.0 (United States Biochemical, Cleveland, OH).

The length of the amplified insert, pulmonary *MAGE-A4* (p*MAGE-A4*) Genbank accession number AY954624, is 1,501 bp. The open reading frame (ORF) starts at position 92 and extends to position 1,045. With the exception of three single nucleotide substitutions, the ORF of the cloned sequence corresponded to the published sequence for *MAGE-A4* (BC017723; ref. 22). These single nucleotide changes involved the following changes:

adenine (A) to guanine (G) in position 175, cytosine (C) to thymine (T) in position 402, and guanine (G) to adenine (A) in position 608 of p*MAGE-A4*. Sequencing analysis of the PCR products obtained by amplification of cDNA from T29 confirmed the changes in position 175 and 608 to be single nucleotide polymorphisms (data not shown). These changes did not result in alterations of the amino acid sequence of the protein. The single nucleotide change in position 402 of p*MAGE-A4* was determined to be a PCR artifact. This substitution resulted in the exchange of the serine in position 104 to a phenylalanine. Because this alteration is localized outside the conserved carboxyl-terminal MAGE homology domain and does not involve the carboxyl-terminal region linked to the biological function of MAGE-A4, we disregarded this amino acid change.

Stable transfection of the p*MAGE-A4* gene. The p*MAGE-A4*/pcDNA 3.1/Zeo 5.0 kb vector was transfected into 293 cells by using the mammalian cell transfection kit (Stratagene, La Jolla, CA) and cultured in DMEM-F12 with 200 μ g/mL zeocin (Invitrogen; ref. 23). Transfection with the vector without insert was used as negative control. *MAGE-A4*-transfected 293 cells (293/*MAGE-A4*) and 293 control cells (293/vector) were lysed for protein extraction or sedimented on to glass slides using Cytospin (Shandon, Pennsylvania, PA). Cell samples were air dried and fixed with a freshly prepared paraformaldehyde solution [4% in PBS (pH 7.4)] for 30 minutes at room temperature. MAGE-A4 expression was evaluated by Western blot or immunohistochemistry.

Microarray analysis. Detailed protocols for data extraction from Affymetrix oligonucleotide array and documentation on the sensitivity and quantitative aspects of the method have been described (24, 25). In brief, one Affymetrix Hu95A GeneChips containing 12,400 unique genes was used for each human lung tissue sample (tumor-free lung or tumor). For comparison across GeneChips, the data on each GeneChip was normalized to a total fluorescence of 300, representing the total cRNA hybridized to the GeneChip. MICROSUITE 5.0 (Affymetrix) was used for data extraction. Data analysis was carried out on all 13 microarrays representing samples of eight individuals. Raw data was normalized using GeneSpring Software (Silicon Genetics, Redwood City, CA) using the default variables recommended by the software. The data from probe sets representing genes with a $P > 0.05$ were considered to have failed the Affymetrix Detection criteria, called absent, and eliminated from all 13 microarrays. Clustering and experimental tree building were used to compare the overall expression patterns among lung tumor-free tissue and lung tumors.

Cell viability and apoptosis detection. Cell viability was assessed by bright-field microscopy using the trypan blue dye (0.4%; Sigma-Aldrich, St. Louis, MO) exclusion method. The mean survival was determined by examining four different low-power fields. Cell proliferation and viability was also measured by bio-reduction of MTS tetrazolium, CellTiter 96_{Aqueous} (Promega, Madison, WI).

Caspase-3 activity was measured by a spectrofluorometric assay (Promega). This assay measures release of fluorescent R110 generated by cleavage of the profluorescent substrate Z-DEVD-R110 by active caspase-3/7. Rhodamine 110 fluorescence was quantified by UV spectrofluorometry with an excitation wavelength of 499 nm and an emission wavelength range of 521 nm.

The apoptotic index was calculated by using a 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) staining. A minimum of 500 cells were counted by fluorescent light microscopy at a $\times 20$ magnification and categorized as apoptotic if they displayed chromatin fragmentation, nuclear condensation, and/or formation of pyknotic bodies.

Western blot analysis. Protein expression of MAGE-A proteins in tissue homogenates and cell lysates was evaluated by Western blotting (26). In brief, β -mercaptoethanol reduced and heat denatured protein samples (50 μ g/lane) were separated electrophoretically in a 12% SDS-PAGE (Bio-Rad, Hercules, CA) and blotted on nitrocellulose membrane. The mouse monoclonal anti-MAGE-A1 antibody 6C1 (Novocastra, Newcastle upon Tyne, United Kingdom) was used for immunoblotting at a dilution of 1:100. Protein expression of β -actin, as a marker for protein loading, was determined by using a mouse monoclonal anti- β -actin antibody (Sigma, St. Louis, MO) at 1:2,000 dilution. The peroxidase-linked species-specific

sheep anti-mouse antibody (Amersham, Arlington Heights, IL) was used as secondary antibody at a dilution of 1:5,000. Signal detection was accomplished by ECL Western blotting reagents (Amersham).

Northern blot analysis. *MAGE-A4* mRNA expression was assessed by separation of equal amounts of total RNA (2.5 µg) by electrophoresis through a 1% agarose gel in the presence of 2.2 mol/L formaldehyde. Separated RNA species were transferred to nitrocellulose. A 654 bp *MAGE-A4* probe generated by restriction enzyme digestion (*Bgl*II, Invitrogen) of the p*MAGE-A4*/pcDNA 3.1/Zeo 5.0 kb vector was labeled with [α -³²P]dATP (Amersham Bioscience, Piscataway, NJ) by a random primer method (Invitrogen). The probe (1.5 × 10⁶ cpm/mL) was added to the hybridization solution (ExpressHyb, BD Bioscience, Palo Alto, CA) and incubated with the membrane for 1 hour at 68°C. After hybridization, the membranes were washed four times with 2× SSC solution [where 1× solution contained 150 mmol/L NaCl and 15 mmol/L sodium citrate (pH 7.0)] with 0.05% SDS at room temperature for 40 minutes followed by 0.1× SSC with 0.1% SDS solution at 50°C for 40 minutes. The blots were visualized by autoradiography (27).

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections (5 µm) or cytopspins were stained with the mouse monoclonal anti-MAGE-A1 antibody 6C1 (Novocastra) at a dilution of 1:50. The automated biotin-avidin peroxidase system (Ventana-ES-320) with amino-ethyl-carbonyl (Ventana, Tucson, AZ) was used as a chromogen. Positive controls for MAGE-A consisted of melanoma tissue sections and cytopspins of the melanoma cell line, A375. Negative controls were obtained by staining each tissue section with the secondary antibody alone and by staining cytopspins of 293/vector cells. A specialized pulmonary pathologist (C. Farver) was responsible for the interpretation of immunohistochemical staining.

Gene silencing. *MAGE-A4* small interfering RNA (siRNA) was synthesized by Ambion (Austin, TX). The sense and antisense *MAGE-A4* siRNA were 5-CCGUGAAAUAGGUGAGAUAtt-3 (sense) and 5-UAUCUCACCUAAUUUCACGGTg-3 (antisense). Silencer-negative control no. 1 siRNA (siRNA control; Ambion) was used as a control. NCI-H1703 and 293/*MAGE-A4* cells were transfected in either 10 cm cell culture plates (Western blot) or 96-well plates (Apo-One assay) at 30% to 40% confluence in full growth medium in the absence of antibiotic supplements by using the LipofectAMINE 2000 reagent (Invitrogen) according to the instructions of the manufacturer. For the silencing experiments, cells were transfected with 10, 30, and 50 nmol/L *MAGE-A4* siRNA or with 10 or 50 nmol/L Silencer Negative Control no. 1 siRNA. Forty-eight hours after transfection, cells were washed, trypsinized, and harvested for evaluation of protein expression or caspase-3 activity was measured (28).

Statistical analysis. Demographic and clinical data are summarized by mean ± SD. Caspase-3 activity and apoptotic index were reported as mean ± SE. The paired *t* test (continuous variables) and the χ^2 test (nominal variables) were used to compare clinical and experimental data. *P* < 0.05 was considered statistically significant. The statistical software used was JMP (SAS Institute, Inc., Cary, NC). Microarray gene expression data was analyzed with GeneSpring software and *P* values were calculated for the Welch *t* test and the Benjamini-Hochber correction for false discovery rate.

Results

Demographic and clinical data. There were 39 lung tissue samples collected from 22 patients between 1993 and 2001. Thirty-four samples were taken as paired samples of tumor and tumor-free lung tissue from 17 individuals. The mean age of the patients was 67 ± 9 years and 64% of patients were men. The majority of patients were either current or previous smokers (91%) and their mean cigarette exposure was 45 ± 28 pack-years. The tumor samples included 10 squamous cell carcinomas, 10 adenocarcinomas, and 1 bronchoalveolar cell carcinoma. As expected for a surgical series, most of our patients were either TNM stage I or II (86%). Statistical analysis did not reveal any significant differences

with regard to demographic and clinical characteristics between the patients providing tumor tissue or tumor-free lung.

***MAGE-A4* mRNA expression.** To investigate whether *MAGE-A4* was expressed in tumor and/or tumor-free lung tissue, we analyzed gene chip data obtained from two adenocarcinomas, four squamous cell carcinomas, one bronchoalveolar cell carcinoma, and six tumor-free lung tissue samples. A two-way hierarchical clustering algorithm of expression data successfully distinguished squamous from adenocarcinomas and tumor-free lung tissue, generating a phylogenetic tree that appropriately represented the clinical relationship between the three tissue types included (25). Further analysis showed that compared with tumor-free lung tissue, 20 genes were exclusively expressed in squamous cell carcinomas (Table 1). Interestingly, a large number of these genes (15%) were identified as members of the MAGE family (*MAGE-A1*, *MAGE-A3*, and *MAGE-A4*; Table 1). *MAGE-A4* had the highest expression level.

To confirm the results obtained by gene chip analysis, the *MAGE-A4* mRNA expression of five squamous cell carcinomas, five adenocarcinomas and 10 tumor-free lung tissue samples were analyzed by reverse transcription-PCR (RT-PCR; Fig. 1). *MAGE-A4* mRNA was confirmed in all squamous cell carcinomas, but was absent in all tumor-free lung tissue specimens. However, one adenocarcinoma expressed *MAGE-A4* mRNA. DNA sequencing confirmed that the PCR product represented the expected 426 bp nucleotide overlapping exons 2 and 3 of the *MAGE-A4* gene. Sequencing of several small bands of varying size observed in tumor-free lung tissue specimens revealed these to be either nonspecific or to represent amplified genomic DNA (data not shown). These results suggest that *MAGE-A4* mRNA is predominantly expressed in squamous cell lung cancers.

***MAGE-A* protein expression.** To investigate if *MAGE-A* mRNA expression is translated into MAGE-A protein, we analyzed lung tissue homogenates by Western blot and paraffin-embedded lung tissue sections by immunohistochemistry. Unfortunately, the close structural homology of these proteins precludes the development of antibodies targeting specific MAGE-A family members (29). The anti-MAGE-A1 antibody 6C1, which cross-reacts with MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, and MAGE-A12 is commonly used for evaluation of *MAGE-A4* protein expression (29). MAGE-A protein expression was detected in three of four squamous cell carcinomas and none of the adenocarcinomas (*n* = 4) or tumor-free lung tissues (*n* = 7; Fig. 2A and B).

Because lung tissue samples contain respiratory epithelium, blood vessels, connective tissues, and inflammatory cells, immunohistochemical staining was done to confirm Western blot analysis results and to identify specific cells expressing MAGE-A. Immunohistochemical staining revealed a predominantly nuclear localization of MAGE-A proteins in squamous cell carcinomas, whereas no staining was observed in adenocarcinomas or tumor-free lung tissue (Fig. 2C and D).

***MAGE-A4* cloning and stable transfection.** To evaluate the physiologic function of the *MAGE-A4* protein and to avoid possible confounding effects of mutations associated with carcinogenesis, we established a stable expression system using the widely used and well-characterized nonmalignant 293 cell line. The level of *MAGE-A4* expression of 293/*MAGE-A4* as assessed by Northern blotting and Western blot analysis was comparable with the squamous cell carcinoma non-small cell lung cancer cell line (H1703). *MAGE-A4* was detected at lower levels in the melanoma cell line A375 (Fig. 3A). Furthermore, subcellular localization of

Table 1. Genes expressed in squamous cell carcinomas and absent in tumor-free lung tissue

Function	Genbank accession no.	Gene name	Ratio
Cancer testis antigens	U10688	<i>MAGE-A4</i>	22.8
	U03735	<i>MAGE-A3</i>	16.5
	M77481	<i>MAGE-A1</i>	9.6
Cell cycle	U73379	<i>Ubiquitin-conjugating enzyme</i>	28.5
	M15205	<i>Thymidine kinase 1, soluble</i>	22.7
	K02581	<i>Thymidine kinase 2, soluble</i>	7.0
	M86699	<i>TTK protein kinase</i>	14.3
	AF015254	<i>Serine/threonine kinase 12</i>	6.4
	AI375913	<i>Topoisomerase (DNA) 2α (170 kDa)</i>	7.6
	AB024704	<i>Microtubule-associated protein homologue</i>	15.9
Transcription factor	L07335	<i>SOX2</i>	4.7
Metastasis	L23808	<i>Matrix metalloproteinase 12</i>	7.8
Energy metabolism	J04469	<i>Creatine kinase, mitochondrial 1</i>	10.3
Tumor suppressor	U04313	<i>Serine (or cysteine) proteinase inhibitor</i>	36.4
Antioxidant	X53463	<i>Glutathione peroxidase 2</i>	17.4
Enzymes	U37519	<i>Human aldehyde dehydrogenase</i>	11.3
	AB001928	<i>Cathepsin V</i>	6.4
Protein	AI923984	<i>Small proline-rich protein</i>	16.7
Unclear function	U71207	<i>Eyes absent homologue 2 (Drosophila)</i>	3.6
	D79987	<i>Extra spindle poles-like 1</i>	4.1

NOTE: Members of the melanoma antigen family are in bold.

MAGE-A4 in 293/MAGE-A4 cells was compared with A375 cells by immunocytochemistry (Fig. 3B-D). Similar to the melanoma cell line A375, expressing MAGE-A endogenously, MAGE-A4 localized predominantly to the nucleus in 293/MAGE-A4 cells (Fig. 3B and D). In contrast, no MAGE-A4 expression was detected in 293 cells transfected with the expression vector alone and the adenocarcinoma non-small cell lung cancer cell lines H2122 and H441 (Fig. 3A and C).

MAGE-A4 expression induces apoptosis. Effects of MAGE-A4 overexpression on the apoptotic index and caspase-3 activation were evaluated. The apoptotic index and caspase-3 activity assay were measured in 293 cells stably transfected with MAGE-A4 (293/MAGE-A4) or vector alone (293/vector). The apoptotic index was significantly higher in 293/MAGE-A4 cells compared with 293/vector cells (apoptotic index: 293/MAGE-A4, $16.6 \pm 0.9\%$; 293/vector $7.1 \pm 1\%$; $P < 0.0001$; Fig. 4A-C). Furthermore, caspase-3 activity was also significantly higher in 293/MAGE-A4 cells [caspase-3 activity assay (RFU): 293/MAGE-A4, 253 ± 13 ; 293/vector 141 ± 8 ; $P < 0.002$; Fig. 4D].

To investigate if MAGE-A4 overexpression affects chemotherapeutic effects of etoposide, we exposed 293/MAGE-A4 cells and 293/vector cells to etoposide (25 $\mu\text{mol/L}$) for 24 hours. Treatment with etoposide resulted in significant increases of apoptotic index and caspase-3 activity in both 293/MAGE-A4 [apoptotic index: $15 \pm 1\%$ (DMSO) to $36 \pm 2\%$, $P < 0.0001$, caspase-3 activity (RFU): 253 ± 13 (DMSO) to 691 ± 17 , $P < 0.0001$] and 293/vector cells [apoptotic index: $8.5 \pm 0.6\%$ (DMSO) to $21 \pm 1\%$, $P < 0.0001$, caspase-3 activity (RFU): 157 ± 10 (DMSO) to 259 ± 11 , $P < 0.001$; Fig. 4C and D]. Similar to our observations at baseline, 293/MAGE-A4 cells had a higher apoptotic index (apoptotic index: 293/MAGE-A4, $35.6 \pm 1.9\%$; 293/vector, $21 \pm 1\%$; $P < 0.0001$) and caspase-3 activity [caspase-3 activity assay (RFU): 293/MAGE-A4, 691 ± 17 ; 293/vector, 259 ± 11 , $P < 0.0001$] than 293/vector cells following

exposure to etoposide (Fig. 4C and D). These results suggest that MAGE-A4 plays an important role in the regulation of apoptosis via the caspase pathway and that MAGE-A4 expression results in accelerated apoptosis in response to chemotherapy.

MAGE-A4 silencing is associated with decreased caspase-3 activity. To validate the proapoptotic effect of MAGE-A4 observed in the overexpression model, we silenced MAGE-A4 expression by using MAGE-A4-specific siRNA in squamous cell lung cancer cells and the MAGE-A4-overexpressing cells (Fig. 5A). Caspase-3 activity measured 48 hours following MAGE-A4 silencing was $58 \pm 3\%$ lower in H1703 cells ($P = 0.003$) and $24 \pm 6\%$ lower in 293/MAGE-A4 cells ($P = 0.028$) compared with cells with control scrambled siRNA (Fig. 5B).

Discussion

MAGE type 1 genes are frequently expressed in non-small cell lung cancers (4, 5, 7-13). Considering the limited specificity of the available anti-MAGE antibodies to distinguish different MAGE-A

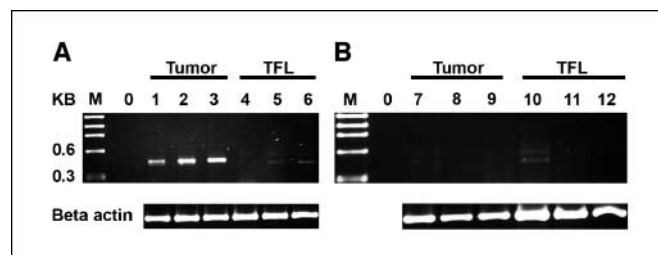
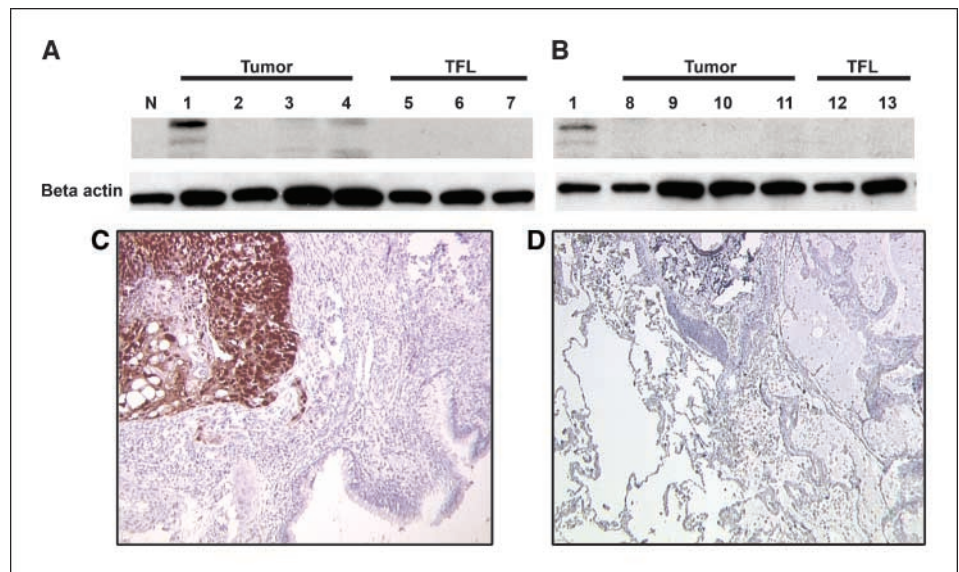


Figure 1. MAGE-A4 mRNA expression in lung cancer. RT-PCR confirmed MAGE-A4 mRNA expression in five of five squamous cell carcinomas, three samples shown, (A, lanes 1-3); one of five adenocarcinomas, three samples shown, (B, lanes 7-9); and none of 10 tumor-free lung tissue (TFL), six samples shown, (A, lanes 4-6 and B, lanes 10-12). 0, negative control.

Figure 2. MAGE-A protein expression. A, MAGE-A protein expression by Western blot using the anti-MAGE-A1 antibody 6C1 targeting MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, and MAGE-A12. MAGE-A proteins were identified in squamous cell carcinoma specimens (A, lanes 1-4) but were absent in adenocarcinoma (B, lanes 8-10) and tumor-free lung tissues (A, lanes 5-7 and B, lanes 12-13). The gels are representative of four squamous cell carcinomas, four adenocarcinomas, and seven tumor-free lung tissues. C and D, immunostaining of paraffin-embedded tissue by using the anti-MAGE-A1 antibody 6C1 for squamous cell carcinoma (C) and adenocarcinoma (D). Predominantly nuclear staining is observed within the squamous cell carcinoma (C). Staining is absent within the adenocarcinoma and the tumor-free, histologically normal lung tissue.

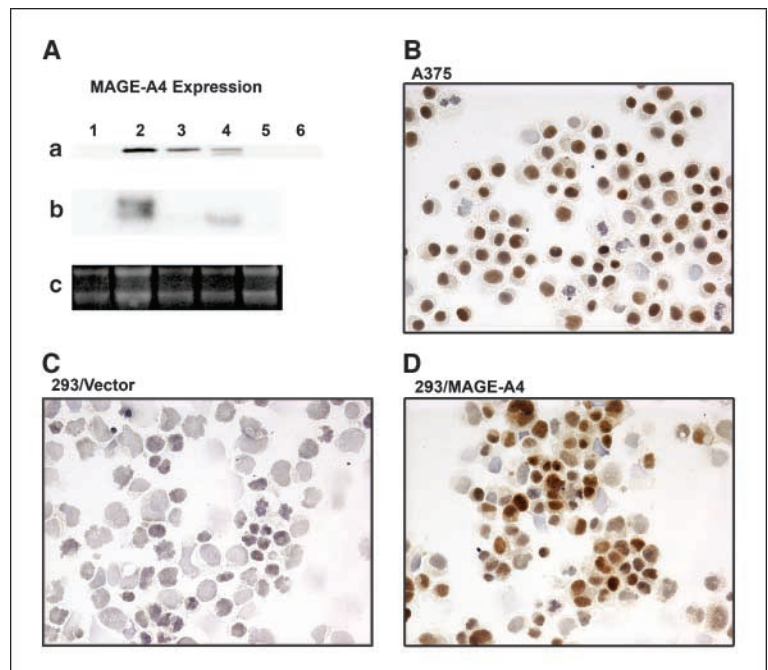


proteins, most investigators used microarray analysis, RT-PCR, and RNA *in situ* hybridization to characterize *MAGE-A* gene expression (29). Using these techniques, between 20% and 85% of non-small cell lung cancers express *MAGE-A1*, *MAGE-A2*, *MAGE-A3*, *MAGE-A4*, *MAGE-A12*, and *MAGE-B2* genes (4, 5, 7-13, 30, 31). Furthermore, Jang et al. (4) were able to detect *MAGE-A2*, *MAGE-A3*, and *MAGE-B3* mRNA by RT-PCR and RNA *in situ* hybridization not only in non-small lung cancers but also in tumor-free surrounding lung tissue and bronchial epithelial cells of patients at risk for lung cancer. Our findings corroborate the high prevalence of *MAGE-A* gene and protein expression in non-small cell lung cancers as well as their more frequent presence in squamous cell carcinomas compared with adenocarcinomas (5, 7, 9, 30, 32). In contrast to Jang et al. (4), we did not detect *MAGE-A4* mRNA or MAGE-A proteins in the tumor-free surrounding lung tissue analyzed by microarray

analysis, RT-PCR, Western blotting and immunostaining. This discrepancy is most likely attributable to the use of different gene probes and primer sets, especially because the mRNA identified by Jang et al. included mainly alternatively spliced variants. Moreover, in the absence of microscopic tissue dissection the presence of contaminating tumor cells within some of the tumor-free surrounding lung tissues samples cannot be excluded.

Although the physiologic function of MAGE-A proteins remains largely unknown, there is increasing evidence to suggest their involvement in the regulation of apoptosis and cell cycle progression (17, 18). The type 2 MAGE proteins necdin and NRAGE have been shown to promote cell cycle arrest and inhibit apoptosis (33-37). Necdin shares 30% homology with MAGE and interacts with cell cycle promoters, such as SV40 big T protein, adenovirus E1A, and the transcription factor E2F1 (33, 38). It also

Figure 3. Stable expression of MAGE A4 in 293 cells. A, a, MAGE-A expression by Western blot with the anti-MAGE-A1 antibody 6C1. Lane 1, 293/vector; lane 2, 293/MAGE-A4; lane 3, A375; lane 4, H1703; lane 5, H2122; lane 6, H441 cell lysate (30 μ g). b, Northern blot using 654 bp *MAGE-A4* probe (2.5 μ g total RNA). c, RNA loading. B to D, immunostaining of cytospin preparations with the anti-MAGE-A1 antibody 6C1. Predominantly nuclear staining of the melanoma cell line A375 (B) and the 293/MAGE-A4 cell clone (D). In contrast, no staining was observed in sham-transfected 293/vector control cells (C).



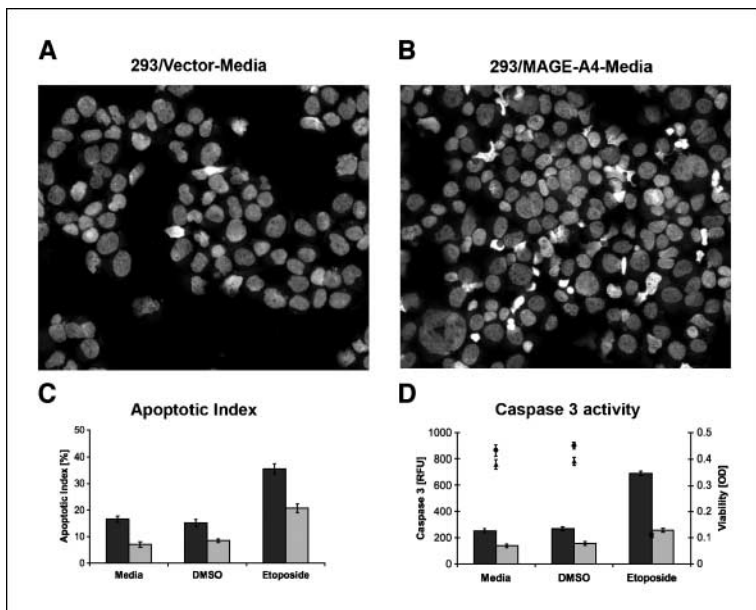


Figure 4. Increased apoptotic index and caspase-3 activity in 293/MAGE-A4 cells. *A* and *B*, DAPI-stained cytopsin preparations of untreated 293/vector control cells (*A*) and 293/MAGE-A4 cells (*B*). Nuclear changes suggestive of apoptosis, such as nuclear condensation, chromatin fragmentation, and pyknotic bodies were significantly more prevalent in 293/MAGE-A4 cells compared with 293/vector control. *C*, quantification of the apoptotic index in regular medium, DMSO (vehicle for etoposide) and 25 $\mu\text{mol/L}$ etoposide. *Black columns*, 293/MAGE-A4; *gray columns*, 293/vector. Apoptotic index was significantly higher in 293/MAGE-A4 cells compared with 293/vector cells before ($P < 0.0001$) and after exposure to 25 $\mu\text{mol/L}$ etoposide ($P < 0.0001$). There was no difference between cells exposed to medium compared with cells exposed to DMSO, the vehicle for etoposide. *D*, caspase-3 activity measured by Apo-One and viability measured by bioreduction of MTS tetrazolium, CellTiter 96_{AQUEOUS} in regular medium, DMSO (vehicle for etoposide), and 25 $\mu\text{mol/L}$ etoposide. *Black columns*, 293/MAGE-A4; *gray columns*, 293/vector. Viability: 293/MAGE-A4 (●); 293/vector (▲). Caspase-3 activity was significantly higher in 293/MAGE-A4 cells compared with 293/vector cells before ($P < 0.002$) and after exposure to 25 $\mu\text{mol/L}$ etoposide ($P < 0.0001$). There was no difference between cells exposed to medium compared with cells exposed to DMSO, the vehicle for etoposide.

inhibits p53-mediated apoptosis while augmenting the inhibitory effect of p53 on cell cycle progression (34).

In contrast, only few studies have investigated potential physiologic functions of type 1 MAGE proteins. Morishima et al. (17) showed that MAGE-A3 binds to murine pro-caspase-12 and inhibits its activation to caspase-12. Murine caspase-12 has been shown to induce apoptosis in response to insults targeting the endoplasmic reticulum, such as inhibition of glycosylation or endoplasmic reticulum-specific calcium ATPase. These observations suggest that MAGE-A3 protein expression protects tumor cells from such insults and, therefore, may provide a survival advantage to cancer cells (17). However, the physiologic relevance of these observations in humans remains uncertain especially because the proapoptotic effect of caspase-12 is likely small considering that caspase-12 deficient mice have only a moderate decrease (12%) in apoptosis and no human homologue of murine caspase-12 has been described (39, 40). MAGE-A proteins are also more frequently expressed in chemotherapy (paclitaxel) resistant compared with chemotherapy susceptible ovarian cancer, melanoma and multiple myeloma cell lines (41). These findings suggest that MAGE-A expression favors tumor cell survival and that MAGE-A proteins function as oncoproteins.

In contrast to these findings, a 107-amino-acid-long recombinant carboxyl-terminal fragment of MAGE-A4 was found to induce

apoptosis by both p53-dependent and p53-independent mechanisms (18). Although a fragment of similar size was present in cells overexpressing the entire MAGE-A4 molecule, apoptosis was not enhanced unless DNA damage was induced by exposure to adriamycin (18). In addition, MAGE-A4 can bind to the oncoprotein gankyrin, which has been implicated in the carcinogenesis of hepatocellular carcinoma (42). By binding to gankyrin, MAGE-A4 inhibits its carcinogenic effect (42). These observations in conjunction with the immunogenic effects of MAGE-A-derived peptides suggest that MAGE-A proteins function as tumor suppressor proteins rather than oncoproteins.

A number of retrospective studies investigated the prognostic significance of MAGE-A expression on patient survival and mortality. MAGE-A expression seems to be more prevalent in advanced tumors and has been associated with prolonged, decreased, and unchanged survival and mortality (5, 8, 30, 32, 43).

Considering that stimulation of *de novo* expression of cancer testis antigens with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine has been shown to augment adoptive immunotherapy in a murine tumor model (44), and that these antigens are not universally and homogeneously expressed in human malignancies (45), recent efforts to optimize the efficiency of cancer vaccines have focused on the pharmacologic induction of MAGE-A expression in human cancers (6, 46). To be able to anticipate

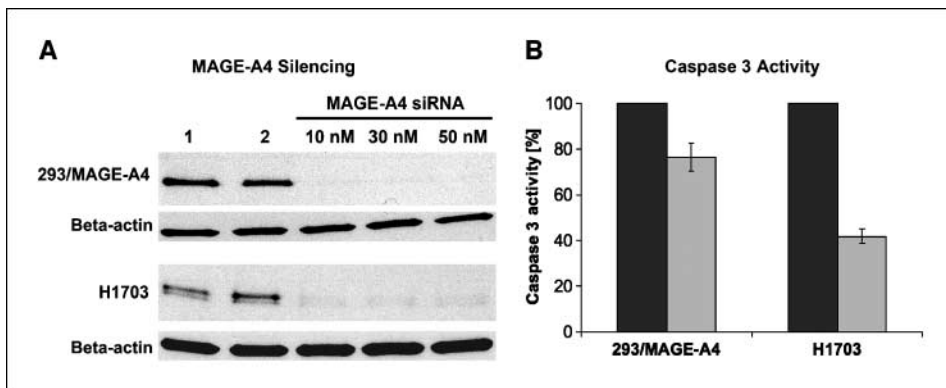


Figure 5. MAGE-A4 silencing results in increased caspase-3 activity. *A*, Western blot of successful MAGE-A4 silencing using MAGE-A4-specific siRNA at different concentrations. *Lane 1*, untreated control; *lane 2*, cells transfected with control siRNA (50 nmol/L). Fifty micrograms protein per lane. *B*, caspase-3 activity in 293/MAGE-A4 and H1703 cells 48 hours after transfection with either 10 nmol/L MAGE-A4 siRNA (*gray columns*) or 10 nmol/L control siRNA (*black columns*).

unintended adverse outcomes, it is crucial to precisely understand the physiologic function of these proteins. Therefore, we explored the effects of MAGE-A4 on apoptosis, which we found to be most significantly overexpressed *MAGE-A* gene in squamous cell lung cancers, on apoptosis. To our knowledge, we established the first stable transfection model for any MAGE-A protein in a nonmalignant human cell line and showed that stable overexpression of MAGE-A4 in the absence of other apoptotic stimuli enhances apoptosis by activating the caspase pathway. This proapoptotic effect is significantly enhanced by DNA damage as the result of etoposide exposure. The proapoptotic effect of MAGE-A4 was confirmed by experiments showing that MAGE-A4 silencing inhibits caspase activity in both the MAGE-A4-transfected 293 cells and the MAGE-A4-expressing squamous cell lung cancer cell line H1703. Our results support prior observations suggesting that MAGE-A4 functions as a tumor suppressor protein. Compared with Sakurai et al. (18), who saw a proapoptotic effect following exposure to adriamycin but no change at baseline, we also documented increased apoptosis in the absence of apoptotic stimuli. This discrepancy is likely due to differences in the experimental approach. More uniform protein expression in our stable transfection system may have enabled us to document a proapoptotic effect of MAGE-A4 in the absence of other apoptotic stimuli (18). The physiologic relevance of the observed proapoptotic effect of MAGE-A4 is supported by studies documenting a similar magnitude of proapoptotic effect following the introduction of p53 and BAX, well-known mediators of the apoptotic pathway, into various cell lines (47, 48). Taken together, our findings suggest that MAGE-A, specifically MAGE-A4, functions as a tumor suppressor protein rather than an oncoprotein in malignancies. Previous reports of antiapoptotic effects of MAGE-A3, the uncertain significance of MAGE-A expression as a prognostic marker, and the association of MAGE-A expression with resistance to chemotherapeutic agents could be attributable to functional differences within the MAGE-A family. In the absence of a functional human caspase-12 enzyme and in the presence of a high frequency of downstream mutations

within apoptotic pathways, it is difficult to draw definite conclusions regarding the biological function of MAGE-A proteins in humans from these observations (40).

Besides in various malignancies, MAGE-A protein expression has been documented in spermatocytes but their physiologic function in these cells remains unknown (49). Apoptosis is essential for normal spermatogenesis. It is required to maintain the appropriate balance between germ cells and Sertoli cells, it is crucial for the removal of abnormal spermatocytes, and it may be involved in the differentiation of these cells (50). Therefore, it can be speculated that the proapoptotic effect of MAGE-A4 may also contribute to the regulation of apoptosis in the testis.

In conclusion, MAGE-A proteins are commonly expressed in non-small cell lung cancers and are preferentially present in squamous cell carcinomas. MAGE-A expression was limited to tumor tissues and absent in the tumor-free surrounding lung. The data from our stable transfection model, the use of MAGE-A4-specific siRNA, and the previously reported findings by Sakurai et al. (18) suggest that MAGE-A4 has a proapoptotic effect and may function as a tumor suppressor protein. Moreover, our findings support that MAGE-A4 overexpression may sensitize malignancies to apoptotic stimuli such as chemotherapeutic agents. Lastly, this stable MAGE-A4 overexpression model will facilitate investigations of other potential biological functions of MAGE-A4.

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