MAGE-A Inhibits Apoptosis in Proliferating Myeloma Cells through Repression of Bax and Maintenance of Survivin

Tricia Nardiello1, Achim A. Jungbluth2, Anna Mei1, Maurizio DiLiberto3, Xiangao Huang3, Ania Dabrowski1, Valéria C.C. Andrade1, Rebecca Wasserstrum1, Scott Ely2, Ruben Niesvizky4, Roger Pearse4, Morton Coleman4, David S. Jayabalan3, Nina Bhardwaj1, Lloyd J. Old2, Selina Chen-Kiang3, and Hearn Jay Cho1

Abstract

Purpose: The type I Melanoma Antigen Genes (MAGEs) are commonly expressed in cancers, fueling speculation that they may be therapeutic targets with oncogenic potential. They form complexes with RING domain proteins that have E3 ubiquitin ligase activity and promote p53 degradation. MAGE-A3 was detected in tumor specimens from patients with multiple myeloma and its expression correlated with higher frequencies of Ki-67+ malignant cells. In this report, we examine the mechanistic role of MAGE-A in promoting survival of proliferating multiple myeloma cells.

Experimental Design: The impact of MAGE-A3 expression on survival and proliferation in vivo was examined by immunohistochemical analysis in an independent set of tumor specimens segregated into two groups: newly diagnosed, untreated patients and patients who had relapsed after chemotherapy. The mechanisms of MAGE-A3 activity were investigated in vitro by silencing its expression by short hairpin RNA interference in myeloma cell lines and primary cells and assessing the resultant effects on proliferation and apoptosis.

Results: MAGE-A3 was detected in a significantly higher percentage of relapsed patients compared with newly diagnosed, establishing a novel correlation with progression of disease. Silencing of MAGE-A showed that it was dispensable for cell cycling, but was required for survival of proliferating myeloma cells. Loss of MAGE-A led to apoptosis mediated by p53-dependent activation of proapoptotic Bax expression and by reduction of survivin expression through both p53-dependent and -independent mechanisms.

Conclusions: These data support a role for MAGE-A in the pathogenesis and progression of multiple myeloma by inhibiting apoptosis in proliferating myeloma cells through two novel mechanisms. Clin Cancer Res; 17(13); 4309–19. ©2011 AACR.

Introduction

The type I Melanoma Antigen Genes (MAGE-A, B, and C) belong to the cancer-testis antigen (CTAg) group of tumor-associated genes. CTAgs are expressed in a broad range of human cancers, but their normal expression is restricted to immunologically “privileged” tissues, including developing germ cells and trophoblastic tissue such as placenta (1). For these reasons, CTAgs have been investigated as targets for therapeutic tumor vaccines (2). Their widespread expression in cancer also suggests that CTAgs may have oncogenic activity and may be a novel class of therapeutic targets amenable to pharmacologic intervention. We previously reported that MAGE-A3 and CT7 (MAGE-C1) were detected in more than 70% of tumor specimens from patients with stage III (Durie-Salmon staging) multiple myeloma (MM; ref. 3), an incurable malignancy of plasma cells that is the second most common hematologic cancer (4). The patients in this study were a heterogeneous group, including newly diagnosed, relapsed, and refractory cases. Expression of these type I MAGEs correlated with higher frequencies of Ki-67+ cells, a marker for proliferating cells, and with advanced stage of disease. The exceptionally high frequencies of expression and clinical correlations suggested that type I MAGEs play a role in the pathogenesis or progression of MM by promoting survival of proliferating cells.

Emerging data support the concept that type I MAGEs promote tumor cell survival. Type I MAGEs were shown to interact with the tumor suppressor p53 in sarcoma and non–small cell lung cancer cell lines, inhibiting p53 transactivation activity and conferring resistance to etoposide-induced apoptosis (5). In this setting, MAGE-A2 did not
Translational Relevance

Multiple myeloma is an incurable blood cancer. MAGE-A3 is a cancer-associated gene that is commonly expressed in multiple myeloma and correlates with higher frequencies of proliferating malignant cells. The results presented here show that expression of MAGE-A3 in patient specimens also correlated to relapse of multiple myeloma, suggesting a pathogenic role in progression of disease. Silencing of MAGE-A3 in myeloma cell lines and patient cells resulted in activation of intrinsic apoptosis due to regulation of Bax and survivin by p53-dependent and -independent mechanisms. MAGE-A3 seemed to contribute to progression of disease by promoting resistance to apoptosis in proliferating multiple myeloma cells. Therefore, MAGE-A3 is a promising therapeutic target in multiple myeloma, and strategies targeting it, such as MAGE-A3-specific tumor vaccines currently in clinical trials, may result in myeloma cell apoptosis and prevent relapse.

see seem to regulate transcription of the p53 gene itself. However, Yang and colleagues reported that MAGE proteins interacted with the RING domain protein TRIM28/Kap1 and suppressed transcription of p53 in human and mouse melanoma cell lines, and that silencing of type I MAGEs also induced apoptosis (6). Silencing of MAGE-A3 or CT7 expression by siRNA transfection seemed to reduce viability in human myeloma cell lines (HMCL; ref. 7), but the underlying mechanism was not explored. More recently, Doyle and colleagues used biochemical methods to show that MAGE/RING domain protein complexes function as E3 ubiquitin (Ub) ligases (8). Several MAGE proteins associated with TRIM28/Kap1, and the MAGE-A2 and -C2 complexes were capable of polyubiquitylating p53, targeting it for proteasomal degradation. This report of a biochemical function for MAGE was a groundbreaking revelation; however, it remains to be determined whether this activity is responsible for the effects of type I MAGE on survival and whether p53 is the sole or dominant target substrate in myeloma cells.

To investigate the role of type I MAGEs in the pathogenesis and progression of MM, we examined their expression by immunohistochemistry (IHC) in an independent set of samples from 2 critical clinical milestones: newly diagnosed, untreated patients and patients who had relapsed after chemotherapy. Our data revealed that both the number of patients expressing MAGE-A3 in their tumor cells and the percentage of MAGE-A3-positive tumor cells increased with progression of disease and correlated with higher frequencies of Ki-67+ myeloma cells. Silencing of MAGE-A in HMCL and primary patient cells did not impair cell-cycle regulation, but it did result in apoptosis through p53-dependent activation of Bax and Bak genes and by repression of survivin through p53-dependent and -independent mechanisms. We propose that MAGE-A plays a critical role in the survival of proliferating MM cells through regulation of antiapoptotic mechanisms.

Materials and Methods

Patient samples

Specimens from patients diagnosed with MM (9) were obtained at the Weill-Cornell Medical Center or New York University Clinical Cancer Center under Institutional Review Board–approved sample collection protocols (WCMC IRB #1000-422 and NYU IRB #06-523) in accordance with the Declaration of Helsinki.

Immunohistochemical analysis

IHC of bone marrow biopsies was carried out as described previously (10). Briefly, primary antibodies were as follows: M3H67 (MAGE-A3), CT7-33 (CT7), CT10 (CT10), MA454 (MAGE-A1), 57B (MAGE-A4), and E978 (NY-ESO-1), all provided by the Ludwig Institute for Cancer Research. Slides were blinded to the pathologist in regards to newly diagnosed versus relapsed status. Images were acquired as JPEG format files with a Nikon Coolpix 990 digital camera. Minor adjustments of brightness, contrast, and color solely for the purpose of legibility were made with Adobe Photoshop 7.0 for Windows (Adobe Systems Inc.).

Plasma cell proliferation index assay

Plasma cell proliferation index (PCPI) assay was carried out as previously described (11). To compute the PCPI, 500 cells with a positive membranous CD138 signal were counted; each of these cells was scored as proliferating (containing a brown Ki-67+ nucleus) or nonproliferating (containing a blue, counter-stained, Ki-67–negative nucleus). The PCPI was reported as a percentage.

Cell lines

MM.1r and H929 were from ATCC. ARP-1 was provided by S. Chen-Kiang. Cells were cultivated at 37°C, 5% CO2 in R10 media [RPMI-1640 (Mediatech Cellgro), 10% heat-inactivated FBS (Sigma-Aldrich), 1% 1 mol/L HEPES buffer (Mediatech Cellgro) and 20 µg/mL gentamycin (Invitrogen Gibco)].

Primary myeloma cells

Patient 1 (Pt #1) cells were cultivated in X-VIVO 15 medium (Lonza Walkersville, Inc.) supplemented with 10% heat-inactivated pooled human AB serum (Omega Scientific, Inc.), 20 µg/mL gentamicin, and 1 µg/mL recombinant human interleukin-6 (R&D Systems) at 37°C, 5% CO2. These cells were greater than 90% CD138+ by flow cytometry. Experiments were conducted on cells between passages 3 and 5 in tissue culture.

Genomic p53 sequencing

Genomic DNA was isolated from Pt #1 cells by using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer’s instructions. TP53 exons 2–11 were
amplified and sequenced by using the International Agency for Cancer Research (IARC) direct sequencing protocol: http://www-p53.iarc.fr/Download/TP53_DirectSequencing_IARC.pdf. Amplified exons were sequenced at the New York University Sequencing Core Facility.

**Lentiviral short hairpin RNA transduction**

Lentiviral short hairpin RNA (shRNA) construct particles targeting MAGE-A3 (shMA, TRCN0000128375 and TRCN0000129750; Sigma Aldrich), nontarget (shNT) control (SHC002V) containing a scrambled sequence, and a construct encoding green fluorescent protein (GFP; SHC003V) were used for these experiments. Briefly, cells were diluted to 10^5 cells/mL in R10 and incubated overnight at 37°C. Cells were then harvested and resuspended in R10 media without antibiotics supplemented with 8 µg/mL polybrene (hexadimethrine bromide; Sigma-Aldrich) and aliquoted into a 96-well round bottom plate at 30,000 cells per well. Lentivirus particles were added at a multiplicity-of-infection (MOI) of 5 × 10^3 for MM.1R and a MOI of 15 × 10^3 for ARP-1 and Pt #1. Untreated cells were included as additional negative controls. Cells were incubated for 18 hours at 37°C, then the cells were washed 3 times with prewarmed R10 media without antibiotics to remove excess virus and polybrene. Incubations were continued until the time points noted in individual experiments. Transduction efficiency typically exceeded 60% as measured at 24 and 48 hours by flow cytometry for GFP fluorescence. For apoptosis assessments, typically exceeded 60% as measured at 24 and 48 hours. For substrate-specific Ub assay, was assessed at 48 hours. For substrate-specific Ub assay, was assessed at 48 hours.

**Western blotting**

Protein lysates were prepared by freeze-thawing cells in lysis buffer [350 mmol/L NaCl, 20 mmol/L HEPES (pH 7.9), 0.02% NP-40, 1 mmol/L MgCl₂, 1 mmol/L DTT, 20% glycerol, 2 mmol/L sodium orthovanadate, 10 mmol/L β-glycerol phosphate (all from Sigma-Aldrich), 1 × protease inhibitor (Calbiochem)]. Protein concentration was determined by Bradford assay (Bio-Rad) according to the manufacturer’s instructions. Protein concentration was determined by Bradford assay (Bio-Rad) according to the manufacturer’s instructions. Lysate (10–20 µg) was run on an SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore). Blocking and antibody dilutions were all done in 5% nonfat dry milk (NFDM) in TBS-T [10 mmol/L Tris base, 150 mmol/L NaCl, 0.01% (v/v) Tween 20; Sigma-Aldrich], with the exception of the pRb blots, for which 5% bovine serum albumin (BSA) + 0.4% NFDM in TBS-T was used for blocking. 1% BSA in TBS-T for primary antibody dilution, and 1% BSA + 0.4% NFDM for secondary antibody dilution. Blots were visualized with Supersignal West Femto Substrate (Thermo Scientific).

**Immunoprecipitation**

For substrate-specific ubiquitination, 25 µg lysate was incubated with 0.5 µg p53-specific monoclonal antibody (mAb) or IgG isotype control on ice for 1 hour, then coincubated with Protein A Sepharose beads (Pierce) that had been blocked for 1 hour with 10% BSA (Sigma-Aldrich) at room temperature. Immune complexes were then fixed in 100 µL of 4% paraformaldehyde (Sigma-Aldrich) and analyzed by flow cytometry.

**RNA isolation**

RNA isolation was done as previously described (3).

**Quantitative real-time RT-PCR**

Quantitative real-time RT-PCR (qRT-PCR) was carried out as previously described (3). Taqman primer sets (Applied Biosystems Inc.) for the genes of interest in these experiments were as follows: β-actin (Hs00181698_m1), cyclin B1 (Hs00168719_m1), p53 (Hs01034249_m1), p21 (Hs99999142_m1), cyclin B1 (Hs01030103_m1), p27 (Hs00153277_m1), survivin (Hs03063352_s1), MAGE-A3 (Hs00366532_m1), MAGE-A1 (Hs00607097_m1), CT7 (Hs00193821_m1), cyclin B2 (Hs00270424_m1), cyclin A2 (Hs00996789_g1). Relative expression was calculated according to the geometric mean of the reference primers (β-actin and cyclin B1) by using the formula: relative expression = 2^-ΔΔCt(sample) = ΔΔCt(reference control), where ΔΔCt = Ct(test) – Ct(reference control), the control is untreated cells, and Ct is the cycle threshold to reach a threshold fluorescence intensity of 0.2. Results were normalized to the shNT control in each set of samples and depicted as the mean of triplicate samples ± SEM.
[0.05% Tris (pH 7.4), 0.15 mol/L NaCl, 1% (w/v) Na Deoxycholate (Sigma-Aldrich), 1% (v/v) Triton X-100, 0.1% (w/v) SDS (MP Biomedical)]. Immunoprecipitation products were analyzed by Western blot with p53 or Ub (MMS-264R, Covance) mAb.

Statistics and data presentation

Statistical analysis was carried out with Excel 2008 for Mac (Microsoft Corporation) and with Prism 5.0 (GraphPad Software) for Mac OSX (Apple Inc.). Figures were prepared with Prism 5.0, Adobe Photoshop CS3, and Illustrator CS3 (Adobe Systems Inc.) for Mac OSX (Apple Inc.). Densitometry of Western blots was carried out with ImageJ 1.44o (Adobe Systems Inc.) for Mac OSX (Apple Inc.). Figures were prepared with Microsoft Corporation and with Prism 5.0 (GraphPad Software) for Mac OSX (Apple Inc.). Figures were prepared with Microsoft Corporation and with Prism 5.0 (GraphPad Software) for Mac OSX (Apple Inc.).

Results

MAGE-A3 expression correlates with progression of disease in MM patients

Expression of MAGE-A3 and other CTAg proteins was assessed by IHC in bone marrow biopsy specimens from 2 critical clinical milestones: newly diagnosed, untreated MM patients (n = 46) and patients who relapsed after chemotherapy (n = 35; Fig. 1A). Demographic data (age and sex distribution) and clinical characteristics were similar between the 2 groups and were representative of the general MM patient population (Table 1). Karyotypic abnormalities were detected by either conventional cytogenetics or FISH in more than half of each group. High-risk cytogenetic abnormalities, including chromosome 13 abnormalities, t(4;14), and complex cytogenetics, accounted for approximately one third of the tested patients in each group.

The frequency of MAGE-A3 expression was significantly higher in relapsed (77.1%) versus newly diagnosed patients (35.6%, P < 0.0003; Fig. 1B), and the grading of MAGE-A3 expression (percentage of MAGE-A3-positive MM cells) also increased in the relapsed patients (Fig. 1C). In contrast, although CT7 (MAGE-C1) and CT10 (MAGE-C2) were also commonly detected, their frequencies of expression were not increased in relapsed patients (Fig. 1B), although the grading of CT7 expression seemed to increase with progression of disease (Supplementary Fig. S1A). Other CTags (MAGE-A1, MAGE-A4, and NY-ESO-1) were detected in less than 20% of patients in either group. Proliferation in new and relapsed specimens was examined by PCPI, defined as the percentage of myeloma cells expressing both the plasma cell marker CD138 and the proliferation marker Ki-67 (11). As expected, the average PCPI was significantly higher in relapsed patients (19.0 ± 3.5%) compared with newly diagnosed (6.9 ± 1.3%, P < 0.0002; Fig. 1D; ref. 12). Therefore, although CT7 and MAGE-A3 were the predominant type I MAGEs expressed in MM, MAGE-A3 was distinguished by unique correlations with progression of disease and higher frequencies of Ki-67+ myeloma cells.

Loss of MAGE-A does not impair cell cycling in HMCL and primary myeloma cells

To investigate the mechanisms by which MAGE-A3 contributes to progression in MM, we silenced its expression in the HMCL MM.1r and H929, both of which express wild-type p53, and in ARP-1, which bears homozygous deletions
of the TP53 loci on chromosome 17. We also used polyclonal primary cells (Pt #1) that had a monoallelic deletion of TP53 by FISH, and sequencing of the other TP53 allele revealed a loss-of-function mutation in the DNA-binding region of exon 7, rendering them p53-null (Supplementary Fig. S2A and B). MAGE-A3 was silenced with targeted lentiviral shRNA constructs (Supplementary Fig. S1B) that showed high transduction efficiency and low background toxicity. Two distinct constructs (shMA 129750 and 128375) silenced MAGE-A3 mRNA and protein expression compared with nontarget shRNA-transduced (shNT) and untreated negative control (Con) cells (Fig. 2A and B). Subsequent experiments were conducted with shMA 128375. This construct also silenced MAGE-A1 (Supplementary Fig. S1C), most likely due to the high degree of sequence conservation among the MAGE-A family (13, 14).

CT7 mRNA levels in ARP-1 and Pt #1 cells were not affected by shMA 128375 (Supplementary Fig. S1D). Because the potential activity of MAGE-A1 and other MAGE-A family members could not be formally excluded in these shRNA experiments, the target genes will be referred to collectively as "MAGE-A." However, it should be noted that MAGE-A1 was detected in less than 20% of patient samples by IHC (Fig. 1B).

MAGE-A may contribute to myeloma progression by promoting cell cycling, cell survival, or both. To investigate the role of MAGE-A in cell-cycle regulation, we labeled replicating DNA in MAGE-A–silenced and control cells with BrdU for 30 minutes. BrdU uptake and total DNA content per cell were then assessed by flow cytometry. Short pulses of BrdU provided a "snapshot" of cells in S-phase at a given time without skewing the distribution of cells in each phase of the cycle due to death. Silencing of MAGE-A led to a drastic decrease in the percentage of live cells by 72 hours in MM1.r, H929, and Pt #1 cells and by 96 hours in ARP-1 cells (Fig. 2C, red arrows). Of note, the reduction of viable MM1.r and H929 cells was accompanied by a decrease in the percentage of S-phase cells (Fig. 2C, green arrows), but this selective loss of S-phase cells was not observed in ARP-1 or Pt #1 cells.

The loss of MM.1r and H929 cells in S-phase was not accompanied by an increase in G1 cells (Fig. 2D), suggesting that the loss was due to cell death rather than a block in progression through the G1 cell-cycle checkpoints. Corroborating this observation, silencing of MAGE-A in MM.1r did not perturb the ratio of CDK4/CDK6-specific phosphorylation of Rb to total Rb compared with controls at 48 hours (Supplementary Fig. S3A), indicating that cell-cycle progression through mid-G1 was intact. Total Rb protein was reduced in MAGE-A–silenced cells, possibly reflecting the decrease in overall cell viability. Thus, although dysregulation of G1 progression via overexpression of CDK4 or CDK6 is central to loss of cell-cycle control in myeloma (13), these data confirmed that MAGE-A is not required for cell-cycle progression through G1. The CDK inhibitors p21Cip1 and p27kip1 can act in both G1 and G2–M to mediate cell-cycle arrest. The expression of p21 and p27 proteins also remained unchanged upon silencing of MAGE-A, despite an increase in

Table 1. Demographic and staging data for MM patients in this study

<table>
<thead>
<tr>
<th></th>
<th>Newly diagnosed (n = 46)</th>
<th>Relapsed (n = 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>62.0 ± 1.5</td>
<td>57.7 ± 1.5</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>27</td>
<td>20</td>
</tr>
<tr>
<td>Female</td>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td>Heavy chain isotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>26</td>
<td>18</td>
</tr>
<tr>
<td>IgA</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>IgM</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>IgG + IgA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>IgD</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Light chain only</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Oligosecretory</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Light chain isotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kappa</td>
<td>31</td>
<td>23</td>
</tr>
<tr>
<td>Lambda</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>Not done</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Cyto genetics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>32</td>
<td>18</td>
</tr>
<tr>
<td>Del(20q)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Complex</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Complex including t(5;13)(q31;q14)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Inv(2)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Inv(9)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Y(–)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Complex t(1;8); t(11;14); t(12;27)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Not done</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>FISH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>15</td>
<td>19</td>
</tr>
<tr>
<td>Del (13q14)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>t(11;14)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Complex incl. del(13q14)</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Del(13)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>t(11;14)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>t(4;14)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Trisomy 11</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Trisomy 11 + del(13q14)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Trisomy/tetrasomy 11</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Trisomy 11/trisomy 17</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Complex</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Not done</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Serum β2-microglobulin, mg/dL</td>
<td>4.8 ± 0.6</td>
<td>4.7 ± 0.7</td>
</tr>
<tr>
<td>Serum albumin, mg/dL</td>
<td>3.6 ± 0.1</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>10.4 ± 0.4</td>
<td>10.2 ± 0.3</td>
</tr>
<tr>
<td>Serum creatinine, mg/dL</td>
<td>1.5 ± 0.2</td>
<td>2.2 ± 0.4</td>
</tr>
</tbody>
</table>
p21 mRNA (Supplementary Fig. S3B and C). Collectively, these data showed that MAGE-A is required to maintain myeloma cell survival, particularly survival of S-phase cells in the presence of p53, but is dispensable for cell cycling.

Silencing of MAGE-A triggers the intrinsic apoptosis pathway in proliferating myeloma cells

This led us to investigate the mechanisms by which silencing MAGE-A induced cell death. We first assessed apoptotic versus necrotic cell death in this system. Silencing of MAGE-A with shMA 128375 (Fig. 3A) or 129750 (Supplementary Fig. S1E) in HMCL and Pt #1 cells was associated with an increased number of apoptotic cells (annexin V–positive) in the total acquisition population, compared with controls. Analysis of the kinetics of cell death showed an inverse correlation between live cells and apoptosis in MM.1r, we investigated expression of p53-dependent mechanisms of MAGE-A inhibition of apoptosis by

MAGE-A regulates Bax and survivin through p53-dependent mechanisms

Having shown that MAGE-A antagonized intrinsic apoptosis in MM.1r, we investigated expression of pro- and antiapoptotic Bcl-2 genes in these cells. MAGE-A silencing in MM.1r lead to increased mRNA expression of proapoptotic Bax and Bak (Fig. 4A). Increased expression of Bax protein was also evident, but Bak protein was not significantly changed under these conditions (Fig 4B). Other Bcl-2 proteins (Bid, Bim, Mcl-1, Bcl-2, and Bcl-xl) similarly showed very little changes compared with controls. Bax is a target of p53 transcriptional activation, so we next examined p53 expression. Silencing of MAGE-A in MM.1r increased p53 protein without affecting mRNA expression (Fig. 4C). MAGE-A posttranslational regulation of p53 was confirmed by substrate-specific Ub assay, which showed loss of Ub-p53 (70 kD band; Fig. 4D) after silencing of MAGE-A compared with controls. Silencing of p53 by shRNA lentiviral transduction in addition to MAGE-A in MM.1r reverted Bax and Bak mRNA to control levels, indicating that MAGE-A repressed their expression by inhibiting p53 transactivation (Fig. 4B). We then examined survivin, a multifunctional protein that plays critical roles in survival and cell division in normal and cancer cells (15, 16). It can be transcriptionally repressed by p53 (17, 18). Silencing of MAGE-A in MM.1r resulted in a marked reduction in survivin mRNA and protein (Fig. 4A and B). Survivin mRNA was partly restored by silencing of p53 in addition to MAGE-A. Collectively, these findings showed that MAGE-A inhibits Bax transcription through inhibition of p53 and promotes survivin expression in part through p53-dependent mechanisms.

MAGE-A promotes expression of survivin by p53-independent mechanisms

Because silencing of MAGE-A also induced apoptosis in p53-null ARP-1 and Pt #1, we investigated p53-independent mechanisms of MAGE-A inhibition of apoptosis by
examined expression of Bax, Bak, and survivin in these cells. MAGE-A silencing did not stabilize mutant p53 in Pt #1 cells (Fig. 5A). Bax and Bak mRNA or protein did not increase upon silencing of MAGE-A (Fig. 5B and C). In contrast, MAGE-A silencing resulted in marked decreases in survivin protein. The expression of survivin mRNA paralleled that of protein in ARP-1 cells. These data indicate that MAGE-A also promoted survivin protein expression through p53-independent mechanisms in myeloma cells.

Discussion

These results showed that MAGE-A inhibited apoptosis in proliferating MM cells through at least 2 novel mechan-
Figure 3. Silencing of MAGE-A results in activation of intrinsic apoptosis. HMCL and Pt #1 cells were transduced shMA or controls as previously described and apoptosis was assessed by staining with annexin V. A, total (ungated) acquisition populations at 72 or 96 hours were plotted by Annexin V-PE (apoptosis) vs. 7-AAD (necrosis) fluorescence. B, the percentages of viable cells (Live Gate as described in Fig. 2, solid circles) and annexin V+ cells in the ungated acquisition population (solid squares) were plotted over time for the MAGE-A silenced and control groups. C, HMCL and Pt #1 cells were stained with MitoTracker Red, which is fixed in mitochondria with intact membrane polarization, and analyzed by flow cytometry. Total (ungated) events were plotted by MitoTracker Red fluorescence and mitochondrial depolarization was assessed by decreased fluorescence (bars). D, lentivirus-transduced cells were incubated with 10 μmol/L Q-VD-OPh or DMSO vehicle control for 72 hours and apoptosis was assessed by staining with annexin V. E, caspase-3 and caspase-9 were analyzed by Western blot in at 48 hours for MM.1r and Pt #1 and 72 hours for ARP-1 cells.
isms: inhibition of p53-dependent upregulation of Bax and maintenance of survivin expression by p53-dependent and -independent mechanisms. These mechanisms were active in cell lines and primary MM cells. Furthermore, MAGE-A3 expression was unique among the CTAs characterized in that it was specifically correlated with progression of disease. Identification of these apoptotic mechanisms provides long-sought insight into the role of MAGE-A in the pathogenesis and progression of MM and validates them as functional therapeutic targets in this disease.

Silencing of MAGE-A resulted in loss of p53 ubiquitination and stabilization of p53 protein, increased expression of Bax, and apoptosis in p53−/− MM.1r cells. Progression through G1 and entry into S-phase seemed intact even as the majority of cells became apoptotic. Therefore, apoptosis in the S-phase population of MM.1r cells was likely a consequence of p53-dependent upregulation of Bax after loss of MAGE-A, because disproportionate S-phase apoptosis was not observed in p53-null ARP-1 or Pt #1 cells. These results strongly suggest that MAGE-A is an antagonist of p53-dependent proapoptotic transcriptional regulation in MM cells.

Survivin was downregulated in HMCL and primary MM cells after MAGE-A silencing regardless of p53 status. This is the first evidence of a functional link between type I MAGE and survivin and suggests that survivin is a principal mediator of MAGE-dependent resistance to apoptosis in MM. Interestingly, high levels of survivin mRNA in primary MM specimens from newly diagnosed patients were correlated with poor prognosis (19). Survivin expression is at its lowest in G1 and increases throughout the cycle, peaking in G2–M where it regulates the mitotic spindle checkpoint (15). Therefore, it is unlikely that loss of survivin made a significant contribution to S-phase apoptosis observed in MM.1r cells after MAGE-A silencing.

These results support a model in which MAGE-A promotes survival in proliferating MM cells through at least 2 distinct mechanisms that may act at different phases of the cycle: Bax in S-phase and survivin in G2–M. This model also reconciles the correlation between
MAGE-A3 and increases in the frequency of proliferating cells in patient samples as measured by Ki-67 expression (3, 20), despite the lack of evidence for a direct effect on cell-cycle progression in vitro. In a heterogeneous tumor mass, cells expressing MAGE-A3 are protected from apoptosis as they transit through the cell cycle, effectively increasing the percentage of MAGE-A3+ /Ki-67+ cells. Therefore, the correlations between type I MAGE, progression of disease, and proliferation in primary MM specimens are a consequence of survival of proliferating cells.

The lentiviral shRNA constructs in these experiments efficiently silenced MAGE-A1 as well as MAGE-A3, which is likely a reflection of the extensive sequence homology shared by this family (13). However, MAGE-A1 was only detected in a minority of samples in this study and in our previous set of patients (3), suggesting that MAGE-A3 plays a dominant role in vivo. Several MAGE-A family members and MAGE-C2 complex with TRIM28/Kap1 to form E3 Ub ligases, and p53 is one of their targets for proteasomal degradation (8). The results presented here indicate this activity reduces p53 protein in myeloma cells and decreases apoptosis. However, because apoptosis and downregulation of survivin were also observed after MAGE-A silencing in cells that lacked functional p53, it is likely that non-p53 substrates of MAGE-A3/TRIM28 Ub ligase activity contribute to inhibition of apoptosis by regulating survival factors such as survivin.

Silencing of CT7 by siRNA transfection was reported to decrease viability in MM cell lines (7). In our hands, silencing of MAGE-A by lentiviral shRNA transduction in ARP-1 and Pt #1 did not affect CT7 expression, but expression of CT7 in the absence of MAGE-A was not sufficient to prevent apoptosis. This discrepancy may be due to differences in RNA interference efficiency and greater background cell death with siRNA transfection. Although CT7 is commonly expressed in MM and its expression seems to be an early event in MM pathogenesis (3, 21), it is not known whether it has a similar spectrum of activity as MAGE-A family members. Further investigation is needed to illuminate the role of CT7 in MM pathogenesis.

These data indicate that MAGE-A is critical for the survival of proliferating MM cells and therefore a promising therapeutic target in this disease. This strategy is already under investigation in the form of MAGE-A3 tumor vaccines (22). Further investigation of the
mechanisms of MAGE-A3 inhibition of p53 and promotion of survivin expression in MM may reveal novel therapeutic targets that will be amenable to pharmacologic agents. These may be combined with vaccines in multitargeted, MAGE-specific therapy designed to induce apoptosis in proliferating MM cells or make them more susceptible to chemotherapy-induced apoptosis, resulting in durable remission of this currently incurable disease. Proof of principle in MM may lead to broader application in other cancers that express type 1 MAGE. These include lung, breast, prostate, and skin cancers, among the leading causes of cancer death.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

The authors thank Wayne Austin and Hasina Outtiz for their technical contributions.

Grant Support

V.C.C. Andrade is supported by scholarship 200024/2009-4 from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) of Brazil. H.J. Cho is supported by KO1-CA11917 from the NIH/NCI and a grant from the Multiple Myeloma Working Group Initiative of the Cancer Research Institute.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 8, 2010; revised April 7, 2011; accepted May 6, 2011; published OnlineFirst May 12, 2011.

References

MAGE-A Inhibits Apoptosis in Proliferating Myeloma Cells through Repression of Bax and Maintenance of Survivin

Tricia Nardiello, Achim A. Jungbluth, Anna Mei, et al.

Clin Cancer Res 2011;17:4309-4319. Published OnlineFirst May 12, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-10-1820

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2011/05/12/1078-0432.CCR-10-1820.DC1

Cited articles
This article cites 22 articles, 10 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/17/13/4309.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/17/13/4309.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.