Overexpression of **MAGE/GAGE** Genes in Paclitaxel/Doxorubicin-resistant Human Cancer Cell Lines

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**ABSTRACT**

Previous studies directed at identifying paclitaxel resistance genes in a paclitaxel-resistant subclone of the human ovarian cancer cell line SKOV-3 identified a novel cancer testis antigen, Taxol resistance-associated gene 3 (TRAG-3). Because investigation suggested that TRAG-3, located on chromosome Xq28, does not directly participate in the paclitaxel-resistant phenotype, it was hypothesized that TRAG-3 might be linked to a neighboring gene that is directly involved in the drug-resistant phenotype, or alternatively, overexpression of TRAG-3 might be attributable to coregulation with other cancer testis antigens. To distinguish between these two hypotheses, expression of the genes that flank TRAG-3 was evaluated, namely the Centrin 2 gene and several members of the MAGE gene cluster. Northern analysis demonstrates overexpression of MAGE2 but not Centrin 2. Extension of this analysis to other neighboring and non-neighboring representative cancer testis antigens reveals overexpression of MAGE3, MAGE6, MAGE11, and MAGE12, as well as GAGE-2, GAGE-4, GAGE-5, GAGE-6, and GAGE-7 (clustered on Xp11) in SKOV-3 TR , as compared with SKOV-3. In addition, Affymetrix-based analysis of gene expression in SKOV-3 subclones with variable paclitaxel resistance demonstrates MAGE gene overexpression occurs early in the development of the paclitaxel-resistant phenotype, whereas GAGE gene overexpression occurs somewhat later. Evaluation of additional breast and ovarian cancer cell lines reveals MAGE/GAGE overexpression in both paclitaxel- and doxorubicin-resistant cell lines, whereas gemcitabine-resistant subclones of several ovarian cancer cell lines, including SKOV-3 GR , reveals no change in MAGE/GAGE expression. To determine whether MAGE gene overexpression contributes directly to the drug-resistant phenotype, MAGE2 or MAGE6, cDNA was introduced into the paclitaxel-sensitive human ovarian cancer cell line OVCAR8. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity analysis of both MAGE2 and MAGE6 transfectants demonstrates a 4-fold increase in resistance to paclitaxel and 2-fold increase in resistance to doxorubicin but not to other drugs, such as topotecan and cisplatin, through a nonmultidrug resistance-1 mechanism. MAGE2 or MAGE6 overexpression also induces a growth advantage in OVCAR8-transfected cells. These studies suggest that the in vitro acquisition of paclitaxel and doxorubicin resistance can be associated with increased expression of a variety of both neighboring and non-neighboring cancer testis antigens genes. This does not appear to be a consequence of random genetic instability or genomic amplification of the X chromosome. These antigens, because of limited expression in normal tissues, may be suitable targets for immunotherapy and novel therapeutic strategies in the treatment of chemotherapy-resistant epithelial tumors.

**INTRODUCTION**

MDR² is a major obstacle to the effective treatment of essentially all epithelial malignancies. The mechanisms responsible for the development of MDR are incompletely defined but clearly attributable to multiple mechanisms (1–3). Paclitaxel is one of the most widely used chemotherapeutics for ovarian, breast, and lung cancers; unfortunately, cellular resistance to paclitaxel, either intrinsic or acquired, severely limits the therapeutic potential of this drug (4, 5). A more thorough understanding of the mechanisms of MDR could lead to the development of specific biological and pharmacologic treatments for a wide variety of drug-resistant malignancies.

A previous study of a paclitaxel-resistant subclone of the human ovarian cancer cell line, SKOV-3, identified a novel cancer testis antigen, TRAG-3 (GenBank accession no. AF080246), that is overexpressed in 80% of melanoma cell lines and a subset of paclitaxel-resistant cell lines (6, 7). TRAG-3 is located on chromosome Xq28 and flanked by the MAGE gene cluster (which includes MAGE2, 3, 4, 5, 10, and 12) and the CENT2 gene (Refs. 8 and 9; Fig. 1). Transfection of TRAG-3 into paclitaxel-sensitive cell lines does not lead to paclitaxel resistance, suggesting that TRAG-3 does not directly participate in the paclitaxel-sensitive phenotype. However, TRAG-3’s consistent overexpression in many paclitaxel-resistant cell lines led

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²The abbreviations used are: MDR, multidrug resistance; TRAG-3, Taxol resistance-associated gene 3; IRES, internal ribosomal entry site; CMV, cytomegalovirus; ORF, open reading frame; CENT2, centrin 2; RT-PCR, reverse transcription-PCR; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
to the hypothesis that overexpression of linked neighboring genes might explain paclitaxel resistance.

In this study, we evaluated whether TRAG-3 overexpression is associated with overexpression of neighboring genes (in which case both MAGE cluster genes and CENT2 could be overexpressed) or, alternatively, if TRAG-3 overexpression was linked to overexpression of both contiguous (MAGE) and non-contiguous (GAGE) cancer testis genes (10), in which case it would be hypothesized that acquired drug resistance in these cells was associated with up-regulation of cancer testis genes presumably through activation of a common regulatory pathway. Analysis of SKOV-3 and SKOV-3TR demonstrates that although CENT2 is not overexpressed, there is overexpression of both contiguous (MAGE) and non-contiguous (GAGE) cancer testis genes. This result suggests that paclitaxel resistance is associated with generalized overexpression of cancer testis antigens. Further evaluation of other paclitaxel-resistant, as well as doxorubicin-resistant, cell lines demonstrates this pattern of overexpression is generalizable to these MDR-1 substrates, whereas evaluation of gemcitabine-resistant cell lines reveals no change in MAGE/GAGE expression. To determine the potential contribution of the MAGE genes to the drug resistance phenotype, a paclitaxel-sensitive cell line, OVCAR8, was transfected with two different plasmids containing the full-length coding region of two MAGE genes located within the cluster, namely human MAGE2 or MAGE6. Two independent OVCAR8 clones from both the MAGE gene transfections demonstrated that MAGE2 and MAGE6 could induce a drug resistance phenotype.

The association of MAGE/GAGE gene overexpression with paclitaxel and doxorubicin resistance may provide new insights into MAGE/GAGE function, as well as new therapeutic targets for treating multidrug-resistant malignancies.

**MATERIALS AND METHODS**

**Cell Culture.** The human ovarian cancer cell lines SKOV-3 and MDA435 were obtained through the American Type Tissue Collection (Rockville, MD). Their paclitaxel-resistant subclones were established as reported previously (11). Briefly, three SKOV-3 subclones were selected to be paclitaxel resistant by continuous culture in media containing stepwise increases in paclitaxel concentration over a period of 8 months. The resistant subclones were designated SKOV-3\_0.003TR, SKOV-3\_0.03TR, and SKOV-3\_0.3TR after selection in 0.003, 0.03, and 0.3 μM paclitaxel, respectively. SKOV-3\_0.3TR has been described previously under the designation SKOV-3TR and is referred to as such in this study. An MDA435\_TR subclone was established in an identical fashion. Dr. Patricia Donahoe provided the human ovarian cancer cell lines OVCAR8, OVCAR5, and IGROV1 (Massachusetts General Hospital, Boston, MA). Three gemcitabine-resistant cell lines, SKOV-3GR, OVCAR5GR, and IGROV1GR, were established by culturing in continuously increasing gemcitabine, analogous to the paclitaxel selection. The human multiple myeloma cell line 8226/S and its doxorubicin-resistant subclones 8226/Dox40 and 8226/MDR10V were provided by Dr. William S. Dalton (Ref. 12; University of South Florida, H. Lee Moffitt Cancer Center, Tampa, FL). The melanoma cell line K4 was provided by Dr. Hensin Tsao (Massachusetts General Hospital). All cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (all obtained from Life Technologies, Inc., Grand Island, NY). Chemotherapeutic agents were purchased from commercial sources.

**Primer Design.** TRAG-3 is located on chromosome Xq28 and flanked by the MAGE gene cluster and CENT2 gene. A search of GenBank identified all known MAGE, GAGE, and CENT2 sequences recorded in the databases of the National Center for Biotechnology Information, NIH (Bethesda, MD). In contrast to TRAG-3 and CENT2, both MAGE and GAGE are gene families with numerous members (Table 1). The MAGE cluster is located on chromosome Xq28, and the GAGE cluster is located on Xp11. All primers, synthesized by Life Technologies, Inc., were constructed according to the obtained sequence information to generate primer specificity. Primer design and restriction enzyme identification were facilitated by sequence analysis using the Wisconsin Package (Genetics Computer Group, Inc., Madison, WI).

**RT-PCR.** TRIzol-extracted total RNA was DNase treated to remove contaminating genomic DNA according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). RT-PCRs were performed using Titan One Tube RT-PCR systems following the manufacturer’s protocol (Roche, Indianapolis, IN). MAGE, GAGE, and CENT2 RT-PCR primers are listed in
Table 2: RT-PCR primers

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<th>Primer sequence</th>
<th>GenBank accession</th>
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<td></td>
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<td></td>
<td></td>
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<tr>
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<td>XM.018108</td>
<td>260 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense: GAGCCCTCTACGGATTGTC</td>
<td></td>
<td></td>
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<td>MAGE6</td>
<td>Sense: TGGAGACCAGAGGCCCC</td>
<td>U10691</td>
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<tr>
<td></td>
<td>Antisense: CCATCAGGACCATCTCA</td>
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</table>

Table 2. RT-PCR successfully amplified each cDNA from both SKOV-3_3TR and human melanoma cell line K4 (data not shown).

DNA Sequencing. MAGE, GAGE, and CENT2 RT-PCR products were TA cloned into the pcR 2.1 vector (Invitrogen) according to the manufacturer’s instructions. Plasmids, prepared using S.N.A.P MiniPrep Kits (Invitrogen), were sequenced, with subsequent nucleic acid homology searching conducted via Blast searching (National Center for Biotechnology Information, NIH). DNA sequencing of the cDNA fragments (data not shown) confirmed the specificity of the MAGE, GAGE, and CENT2 RT-PCR products.

Northern Analysis. MAGE, GAGE, and CENT2 probes were prepared from the clones described above after sequence confirmation. The cDNA inserts were cut using EcoRI (Promega, Madison, WI) and purified using QIAEXII Gel Extraction Kit (Qiagen, Inc., Chatsworth, CA). Probes were 32P-labeled with Megaprime DNA Labeling System (Amersham Pharmacia Biotech, Piscataway, NJ). Total RNA was TRizol extracted as above. RNA was separated by electrophoresis in 1.2% agarose/formaldehyde gels (−5 μg of total RNA per lane), transferred to Hybond N-plus Nylon membranes (Amersham Pharmacia Biotech), and UV cross-linked. A 1-h prehybridization step was performed in Rapid-hyb buffer (Amersham Pharmacia Biotech). Probes were hybridized to the blots in the same buffer for 2 h and then washed twice at room temperature with 2× SSC-0.1% SDS for 15 min and twice at 65°C with 0.2× SSC-0.1% SDS for 15 min. Blots were exposed to autoradiography X-ray film with an intensifying screen. To confirm the amounts of RNA loaded in each lane, blots were hybridized with a β-actin probe.

High-density Oligonucleotide Array Expression Analysis. Total RNA, TRizol extracted from SKOV-3, and three paclitaxel-resistant subclones (SKOV-3_0.03STR, SKOV-3_0.05STR, and SKOV-3_0.3STR) were subjected to cDNA array analysis. Arrays were performed using Affymetrix HG-U95Av2 arrays, which contain 12,386 probes representing ~9,600 known human genes. Expression levels of numerous MAGE and GAGE genes were analyzed using GeneChip 3.1 (Affymetrix, Santa Clara, CA). Arrays were performed at the Gene Array Technology Center at Partners Healthcare (Brigham and Women’s Hospital, Boston, MA). Hierarchical clustering was performed using ClusFavor 6.0 (Baylor College of Medicine, Houston, TX).

Cytogenetic Analysis. SKOV-3 and SKOV-3_3TR were grown in T75 flasks and harvested for karyotyping. Fifty microliters of working solution, a 50:50 mixture of Colcemid and BrdUrd, was added overnight to facilitate chromosome arrest during metaphase. Arrested cells were incubated in a hypotonic solution and subsequently fixed with a 3:1 methanol/acetic acid solution. The Colcemid working solution contains 0.1 gram of BrdUrd (Sigma, St. Louis, MO), 33 ml of 0.8% sodium citrate, 30 ml of HBSS, and 3.5 ml of Colcemid (Life Technologies, Inc.). Slides were prepared and G banded using standard cytogenetic protocols, and images were captured using Applied Imaging’s (Santa Clara, CA) Cytovision software.

pIRES_MAGE2 and pIRES_MAGE6 Expression Vector Construction. Cloneetech’s (Palo Alto, CA) mammalian expression vector pIRESnneo contains the IRES of the encephalomyocarditis virus, which permits the translation of two ORFs from one mRNA. The expression cassette of pIRESngeo contains the human CMV major immediate early promoter/enhancer (pCMV) followed by a multiple cloning site and synthetic intron known to enhance the stability of the mRNA. A 950-bp cDNA fragment containing the full ORF of human MAGE2 was amplified by RT-PCR from the RNA of SKOV-3_3TR, a paclitaxel-resistant cell line that highly overexpresses MAGE2. RT-PCR primers were forward 5’-ATAAGAATCCGGCGCATACTTTCGCAGTCAGAGGAAGG-3’ to introduce a NotI site as underlined and reverse 5’-GGTGGATCCTACTTCCTTCCCTCTTCTCAAAGG-3’ to introduce a BamHI site as underlined. A 944-bp cDNA fragment containing the full ORF of human MAGE6 was amplified from the RNA of SKOV-3_3TR. RT-PCR primers for MAGE6 were forward 5’-CCGGCGCGCCATGGCTCTCTTGTAGCAGAGGAAGG-3’ to introduce a NotI site as italized and reverse 5’-CGAAATTTCACTTCACCTCCCTTCCCTTCAAGG-3’ to introduce an EcoRI site as italized. The resulting MAGE2 or MAGE6 RT-PCR product was cloned to pCR 2.1 vector using Invitrogen’s Original TA Cloning Kit. After sequence confirmation, MAGE2 or MAGE6 was cut from the pCR 2.1 vector, purified, subcloned to the multiple cloning site of expression vector pIRESngeo, and subsequently sequenced to confirm the correct ORF. Expression of MAGE2 or MAGE6 cDNA was under the control of the pCMV.

Transfection and Production of Stable Cell Lines. Transfections were performed using Lipofect Amine Plus reagents (Life Technologies, Inc.) as follows: ~5 × 10^5 OVCARB cells were plated into 90-mm tissue culture dishes and cultured overnight. Before transfection, the growth medium was replaced with serum-free RPMI 1640 and cultured for 3 h. Lipofect Amine reagent containing 5 μg of pIRESngeo, pIRE_MAGE2, or pIRE_MAGE6 was combined with Plus reagent and applied to the cells. After culture for 4 h, the media were replaced with RPMI 1640 containing 10% fetal bovine serum. G418 sulfate (Invitrogen) selection (400 μg/ml) was started at 24-h post-transfection. The selection medium was changed ev-
RT-PCR and Northern analysis. MAGE2

Lane 7, 8226/MDR 10 V. B, CENT2 control.

Expression (Fig. 2). Northern evaluation of SKOV-3 TR for A

After dissolving the resulting formazan product with acid-
mg/ml in PBS) were added to each well and incubated for 4 h.

taining increasing concentrations of paclitaxel, doxorubicin, to-

GAGE2, OVCAR8, OVCAR8 pIRES, OVCAR8 MAGE2, or OVCAR8 MAGE6

RESULTS

Expression of MAGE/GAGE in Evolving Paclitaxel Resistance of SKOV-3. To determine the relative expression of the MAGE and GAGE genes in evolving paclitaxel resistance, three paclitaxel-resistant clones of SKOV-3 were isolated during prolonged exposure to incrementally increasing concentrations of paclitaxel. These four lines (parental line and three drug-resistant lines) were evaluated in triplicate by affimetry array and subjected to self-organizing map analysis to identify genes that are upregulated early versus late in evolving paclitaxel resistance (14).

Cytotoxicity Assay. In vitro cytotoxicity assays were performed by MTT assay as described previously (13). MTT was obtained from Sigma. Briefly, 2 × 10^5 cells/well were plated in 96-well plates. Culture medium was RPMI 1640 containing increasing concentrations of paclitaxel, doxorubicin, topotecan, or cisplatin. After culture for 7 days, 10 μl of MTT (5 mg/ml in PBS) was added to each well and incubated for 4 h. After dissolving the resulting formazan product with acid-isopropanol, the absorbance (A_{590}) was read on a BT 2000 Microkinetics Reader (Bio-Tek Instrument, Inc., Winooski, VT) at a wavelength of 490 nm. The IC_{50} is defined as the drug concentration required inhibiting A_{590} to 50% of the control value. The absorbance values were normalized assigning the value of the parent line in media without drug to 1 and the value of the no-cell control to 0. Experiments were performed in duplicate.

RESULTS

Expression of MAGE/GAGE in Paclitaxel, Doxorubicin, or Gemcitabine-resistant Cell Lines. SKOV-3 and SKOV-3_TR were evaluated for expression of MAGE2 and CENT2 by RT-PCR and Northern analysis. MAGE2 is overexpressed in SKOV-3_TR (Fig. 2A), whereas there is no change in CENT2 expression (Fig. 2B). Northern evaluation of SKOV-3_TR for additional cancer testis antigens also demonstrates overexpression of MAGE6. Because both TRAG-3 and the members of the MAGE gene cluster are cancer testis genes, we hypothesized that the drug-resistant phenotype in these cells might also be asso-

ciated with overexpression of a second noncontiguous cancer testis gene cluster, namely the GAGE cluster located on Chromosome Xp11. To evaluate this possibility, Northern blots were also probed with GAGE2. MAGE/GAGE expression was also evaluated in additional paclitaxel- or doxorubicin-resistant cell lines (Fig. 2A). Specifically, MAGE2 and MAGE6 are overexpressed in the paclitaxel-resistant breast cancer cell line MDA435_TR and doxorubicin-resistant multiple myeloma cell lines 8226/DOX40 and 8226/MDR_{10V}. As with SKOV-3_TR, CENT2 is not overexpressed in these paclitaxel- or doxorubicin-resistant cell lines (Fig. 2B). Evaluation of gemcitabine-resistant ovarian cancer cell lines SKOV-3_{GR}, OVCAR5_{GR}, and IGROV1_{GR} demonstrates no change in MAGE/GAGE expression (data not shown).

Cytogenetic Analysis of SKOV-3 and SKOV-3_TR. To ascertain the relationship between MAGE/GAGE overexpression and genetic instability, SKOV-3 and SKOV-3_{TR} were karyotyped. Both SKOV-3 and SKOV-3_{TR} demonstrate four copies of the X chromosome, suggesting that changes in the MAGE/GAGE expression are not caused by duplication of the X chromosome (data not shown).

Effect of MAGE2 or MAGE6 Transfection on in Vitro Growth of Transfected Cells. The overexpression of multiple cancer testis antigens suggested the possibility that these genes might have a direct role in drug resistance. To begin a preliminary exploration of the potential role of the MAGE A cluster genes in the drug-resistant phenotype, we evaluated two arbitrarily selected genes in this cluster, namely MAGE2 and MAGE6. These genes were selected based on their overexpression in various tumor- and paclitaxel-resistant cell lines. Transfection of MAGE2 or MAGE6 into OVCAR 8 with subsequent cloning of transfecants demonstrates overexpression of the respective genes. Analysis of these same transfecants demonstrates no change in MDR1 gene expression as compared with the parental line or empty vector transfecant control (Fig. 4). An assay measuring cell growth demonstrates a modest increase in cell numbers in intermediate term culture as compared with transfection with empty pRES vector (Fig. 5), suggesting that both MAGE2 and MAGE6 either increase cellular proliferation or, alternatively, decrease apoptosis in this cell line.
Table 3  MAGE/GAGE fold overexpression in paclitaxel-resistant cell lines as compared with SKOV-3

<table>
<thead>
<tr>
<th>Gene</th>
<th>SKOV-3</th>
<th>SKOV-3</th>
<th>SKOV-3</th>
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<tr>
<td></td>
<td>0.003TR</td>
<td>0.03TR</td>
<td>0.3TR</td>
</tr>
<tr>
<td>MAGE2</td>
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<td>19.0</td>
<td></td>
</tr>
<tr>
<td>MAGE3</td>
<td>N</td>
<td>24.6</td>
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</tr>
<tr>
<td>MAGE6</td>
<td>N</td>
<td>19.0</td>
<td>30.0</td>
</tr>
<tr>
<td>MAGE11</td>
<td>N</td>
<td>2.5</td>
<td>9.6</td>
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<td>N</td>
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<td>GAGE-6</td>
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<td>9.6</td>
</tr>
<tr>
<td>GAGE-7</td>
<td>N</td>
<td>2.5</td>
<td>9.6</td>
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N denotes an insignificant fold change in expression between the listed cell line and SKOV-3.

Fig. 3 Hierarchical clustering of differentially expressed MAGE/GAGE genes in evolving paclitaxel resistance. Genes in the MAGE and GAGE gene families were specifically selected from the transcriptional array database. Clustering is based on the sorted raw data using the Euclidean distance function. Black, average expression; green, under-expression; red, overexpression. Brighter colors, a greater difference from the mean as compared with darker colors.

Drug Resistance in OVCAR8MAGE2 and OVCAR8MAGE6

As measured by MTT assay, cloned OVCAR8MAGE2 and OVCAR8MAGE6 are relatively resistant to paclitaxel and doxorubicin, whereas there is no significant change in relative resistance to topotecan or cisplatin (Fig. 6). The IC50 of both OVCAR8MAGE2 and OVCAR8MAGE6 are 4-fold higher for paclitaxel and 2-fold higher for doxorubicin as compared with the control OVCAR8pIRES, suggesting that MAGE2 or MAGE6 overexpression confers a moderate level of drug resistance.

DISCUSSION

The human MAGE genes were originally described in melanoma cell lines (14). The 12-member gene family, hMAGE-A, is expressed at a high frequency in various tumors (15). A sequencing effort directed at the human chromosomal region Xp21 led to the discovery of a second cluster, hMAGE-B (2, 16, 17). More recently, hMAGE-C and hMAGE-D were identified and localized to Xq26 and Xq27, respectively (18, 19). Antigens of MAGE genes are presented by either HLA-A1 or HLA-A2 on the surface of melanoma cells and can elicit a response from autologous CTLs (20–23). In addition, GAGE, BAGE, and PAGE have also been reported previously as cancer testis antigens. GAGE-1 was identified from the melanoma cell line MZ2-Mel (10). Subsequent screening of the MZ2-MEL cDNA library with a GAGE-1 probe identified five cDNAs, designated GAGE-2, -3, -4, -5, and -6 sharing 80–98% nucleotide identity with the GAGE-1 sequence. GAGE-1 differs from these other GAGE genes by the presence of a 143-bp insertion. The GAGE genes are located on chromosome Xp11. The GAGE genes also code for antigens presented by melanoma cells.
In this study, the expression level of MAGE/GAGE genes was examined in paclitaxel- and doxorubicin-resistant cell lines. The expression of MAGE/GAGE was significantly higher in the paclitaxel- and doxorubicin-resistant cells lines than in the drug naïve parental cell lines. Affymetrix analysis of SKOV-3 demonstrated that MAGE gene family overexpression appeared early in the development of the paclitaxel-resistant phenotype, whereas GAGE overexpression occurred later. This coordinated overexpression of several MAGE and GAGE genes suggests common regulatory elements and a possible functional relevance.

The finding of MAGE/GAGE overexpression in various paclitaxel- and doxorubicin-resistant cell lines, such as MDA435\textsubscript{TR}, 8226/DOX\textsubscript{40} and 8226/MDR\textsubscript{10}V, as compared with their sensitive parental cell lines was not anticipated. It is hypothesized that cancer testis antigen expression in tumors, in general, is attributable to a dedifferentiated state often associated with carcinogenesis. One possibility for increased expression of MAGE/GAGE genes in drug resistance is that they directly participate in the MDR phenotype. Alternatively, drug resistance may be associated with increased cellular dedifferentiation with changes in methylation status because hypomethylating agent 5-aza-2' deoxycytidine increases expression of several MAGE and GAGE genes as quantified by RT-PCR in malignant mesothelioma cells (24). This report represents, to our knowledge, the first analysis of MAGE/GAGE gene expression in paclitaxel-resistant cell lines.

The function of cancer testis antigens is currently under evaluation. Their expression pattern suggests a role in embryonic development, because they are not expressed in normal adult tissue, with the exception of the testis. Support for this hypothesis comes from a recent report that a novel group of MAGE proteins, magephins, has been found to regulate cell proliferation during gametogenesis in the mouse (25). MAGE genes have also been implicated in central nervous system development (26). Increased cell proliferation may be caused by decreasing rates of apoptosis because some MAGE D1 class proteins serve as binding partners for X-linked inhibitor of apoptosis protein and other inhibitors of caspase activity (27). In addition, MAGE proteins have been found to bind to several transcriptional activators and repressors (26). Expression of subsets of cancer testis antigens has been detected in melanoma, lung, colon, and breast cancers, suggesting that they may also play a role in tumor transformation or progression (14, 28–31). These data are consistent with the results reported here, namely that MAGE2 or MAGE6 transfection can increase the proliferation of tumor cells.

Review of the literature identified a report suggesting that overexpression of MAGE3 is associated with a doxorubicin-resistant phenotype (32). Although other MAGE and GAGE genes have not been linked to drug resistance, overexpression of MAGE genes has been correlated with a high risk of recurrence and are hence hypothesized to play a role in tumor progression (33–36). Serum levels of the MAGE4 protein are strongly associated with the development of hepatocellular carcinoma and can be a predictive marker of hepatocellular carcinogenesis in
cirrhotic patients (37). In another study, PAGE-1, a prostate-restricted cancer testis antigen, was found to be overexpressed in androgen-resistant LNCaP cells as compared with their androgen naïve parent line (29). Although the role these cancer testis antigens play in drug resistance is unknown, the demonstration that transfection of MAGE2 or MAGE6 causes moderate resistance to paclitaxel and doxorubicin provides evidence that the MAGE genes may directly participate in the drug resistance phenotype. The mechanism by which MAGE induces drug resistance is unknown, although this does not appear to be via MDR1-based mechanisms because Northern analysis demonstrates no change in MDR-1 expression with MAGE transfection. Of note, the GAGE-7 gene has been described as inducing resistance to paclitaxel and γ-irradiation in HeLa cells through resistance to apoptosis, generated, in part, through the Fas pathway (38).

Drug resistance in cancer is undoubtedly multifactorial (11). Although the magnitude of paclitaxel resistance observed in OVCAR5MAGE2 and OVCAR8MAGE6 transfectants is modest, these levels cannot be discounted as clinically irrelevant because these levels are consistent with clinically observed levels of drug resistance. Indeed, most patients with ovarian carcinoma who progress after cisplatin therapy have levels of drug resistance. Currently, the levels cannot be discounted as clinically irrelevant, although this does not appear to be via MDR1-based mechanisms because Northern analysis demonstrates no change in MDR-1 expression with MAGE transfection. Of note, the GAGE-7 gene has been described as inducing resistance to paclitaxel and γ-irradiation in HeLa cells through resistance to apoptosis, generated, in part, through the Fas pathway (38).

In conclusion, this study demonstrates that the MAGE/GAGE gene families are overexpressed in some drug-resistant cell lines. Transfection of MAGE2 and MAGE6 into a sensitive cell line can promote cell growth and induce moderate paclitaxel and doxorubicin resistance. Currently, MAGE/GAGE genes have been identified and manipulated as tumor antigens in immunotherapy approaches for melanoma. Similar approaches may be considered as adjuvant therapy for paclitaxel- or doxorubicin-treated breast or ovarian cancer patients who are likely to harbor subclinical occult disease.

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REFERENCES


14. van der Bruggen, P., Traversari, C., Chomez, P., Lurquin, C., De Plaen, E., Van den Eynde, B., Knuth, A., and Boon, T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human mela-


16. Lurquin, C., De Smet, C., Brasseur, F., Muscatelli, F., Martelange, V., De Plaen, E., Brasseur, R., Monaco, A. P., and Boon, T. Two members of the human MAGEB gene family located in Xp21.3 are


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