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Induction of *MAGE* Genes in Lymphoid Cells by the Demethylating Agent 5-Aza-2′-deoxycytidine

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***MAGE* genes encoding tumor antigens recognized by cytotoxic T lymphocytes are appropriate target molecules for specific immunotherapy of cancer. We have investigated whether the demethylating agent 5-aza-2′-deoxycytidine (DAC) induces *MAGE-1, -2, -3,* and *-6* in normal and malignant lymphoid cells. DAC induced these *MAGE* genes in both PHA/interleukin-2 (IL-2)-activated T cells from healthy donors and *MAGE*-negative T and B cell leukemias in most cases. It also induced *MAGE-1* in IL-2-dependent T cell clones and all *MAGE* genes tested in Epstein-Barr virus-transformed B cell lines. Expression of MAGE-1 protein in the cells was confirmed by western blot analysis with anti-MAGE-1 polyclonal antibody. Therefore, demethylation is a potent stimulus to induce *MAGE* genes in both normal and malignant lymphoid cells.**

Key words: *MAGE* gene — Lymphoid cell — DAC — Anti-MAGE-1 antibody — Western blot analysis

MAGE-1 gene encodes tumor antigens recognized by cytotoxic T lymphocytes on HLA-A1, -Cw1601, and probably other HLA-A molecules.[1-4] *MAGE-3* also encodes tumor antigens on HLA-A1 and -A2.[5] These *MAGE* genes are frequently expressed in various cancers.[5-9] In contrast, no normal cells except for the testicular cells express *MAGE* genes.[10] Therefore, *MAGE* gene products are appropriate target molecules for specific immunotherapy of cancer. However, mechanisms involved in *MAGE* gene expression in the cells remain to be fully addressed. Normal cells of skin basal layer transiently express *MAGE-1* during wound healing.[11] The demethylating agent 5-aza-2′-deoxycytidine (DAC), but not other agents, up-regulated *MAGE-1* in melanoma cells.[12] We have investigated whether DAC induces *MAGE-1, -2, -3,* and *-6* genes and MAGE-1 protein in both normal and malignant lymphoid cells in order to better understand the mechanisms of their expression in the cells. DAC induced *MAGE* genes in both normal and malignant lymphoid cells in most cases.

MATERIALS AND METHODS

Normal cells, leukemia cells and cell lines Heparinized peripheral blood was collected from healthy volunteers and patients with adult T cell leukemia (ATL) from the Miyazaki Prefectural Hospital. Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood as reported.[9] The surface markers of the cells were studied by flow cytometric analysis using various monoclonal antibodies (mAbs) including anti-CD3, anti-CD19, anti-CD14, anti-HLA class I mAbs (W6/32) (Becton-Dickinson, Mountain View, CA). Patients' PBMC that consisted of more than 50 percent leukemia cells were collected prior to the treatments and cryopreserved in a liquid nitrogen tank until use. Five T cell clones (TCC) (U16, U64, V230-80, V230-81, V230-83) were used in this study. These $CD3^+CD4^+CD8^-$ TCC were established from the ocular fluid of patients with human T cell lymphotropic virus type 1 (HTLV-I) uveitis, and their characterization was previously reported.[13] All these TCCs required both exogenous interleukin-2 (IL-2) and feeder cells in the culture for their proliferation.

PBMC from healthy donors were incubated for 4 days with 10 μg/ml of PHA and 100 U/ml of human recombinant IL-2 (kindly provided by Dr. T. Yoshida, Shionogi Research Lab., Osaka), followed by incubation for an additional 8 days with PHA and IL-2 in the presence or absence of 1 μg/ml of DAC.[12] The majority ($>95\%$) of these PHA/IL-2-activated cells were phenotypically $CD3^+$ T cells. For induction of *MAGE* genes, PBMC of ATL patients were incubated with or without 1 μg/ml of DAC for 8 days. TCCs were also incubated with 100 U/ml of IL-2 in the absence or presence of 1 μg/ml of DAC for 8 days.

Cells of $MAGE^-$ lymphoid cell lines (5 T cell leukemia lines: HPB-MLT, HUT-102, MT-2, RPMI-8402, SALT-3; 6 B cell leukemia lines: ABL-2, BALL-1, Raji, Namalwa, NALM-1, NALM-6; and 4 Epstein-Barr virus (EBV)-transformed B cell lines: BANB-1, NAKB-1, SS-B, AY-B)[9] were also incubated with 1 μg/ml of DAC

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for 8 days. *MAGE*$^+$ K562 and HEL leukemia cell lines were used as positive controls in these studies, as reported.[9] A *MAGE*$^-$ melanoma cell line (M36) established in our laboratory was used as a control tumor cell line for the induction experiment with DAC. Three myeloid tumor cell lines (ML-1, ML-2, ML-3), and 4 monocyte tumor cell lines (HL60, KG-1, U937, THP-1) were also incubated with 1 μg/ml of DAC for 8 days.

***MAGE* gene expression at the mRNA level** Methods for detection of *MAGE-1, -2, -3,* and *-6* genes have been reported.[8, 9] The primers used in the study were 5′-CGGCCGAAGGAACCTGACCCAG-3′ and 5′-GCTGGAACCCTCACTGGGTTGCC-3′ for *MAGE-1* gene; 5′-AAGTAGGACCCGAGGCACTG-3′ and 5′-GAAGAGGAAGAAGCGGTCTG-3′ for *MAGE-2*; 5′-TGGAGGACCAGAGGCCCCC-3′ and 5′-GGACGATTATCAGGAGGCCTGC-3′ for *MAGE-3*; 5′-TGGAGGACCAGAGGCCCCC-3′ and 5′-CAGGATGATTATCAGGAAGCCTGT-3′ for *MAGE-6*; and 5′-CTTCGCGGGCGACGATGC-3′ and 5′-CGTACATGGCTGGGGTGTTG-3′ for *β-actin* gene. Amplification was performed for 35 cycles (1 min at 94°C and 4 min at 72°C for *MAGE-1* and *-3*, 1 min at 94°C, 2 min at 67°C and 2 min at 72°C for *MAGE-2,* and 1 min at 94°C, 2 min at 68°C and 2 min at 72°C for *MAGE-6*). The methods for semi-quantitative evaluation of the results of RT-PCR were previously reported.[8, 9]

Anti-MAGE-1 polyclonal antibody (Ab) and Western blot analysis Detailed characterization of the polyclonal anti-MAGE-1 Ab has been reported in the previous manuscript.[10] In brief, the anti-MAGE-1 Ab mainly reacted to a single peptide of MAGE-1 protein (positions 251 to 265, YRQVPDSDPARYEFL). The amino acid sequence of this peptide is different from that of the corresponding region of any of MAGE-2, -3, -4, -6, and -12 proteins.[10] This Ab showed no apparent cross-reactivity to the other MAGE proteins (MAGE-2, -3, -4, -6, and -12) based on the results of Western blot analysis. Methods for detection of the MAGE-1 protein by western blot analysis have been described previously.[10]

RESULTS

The kinetics of DAC-induced expression of *MAGE-1* gene was initially investigated by incubation of a TCC (VI-230-80) and M36 melanoma cell line with 1 μg/ml of DAC for up to 8 days (Fig. 1). NALM-1 or K562 cells was used as a negative or positive control, respectively. Neither the TCC nor M36 cells expressed *MAGE-1* without DAC treatment (day 0 of VI-230-80 or M36 in Fig. 1). DAC induced *MAGE-1* in these cells at day 3, and the level of expression was highest at day 8 of incubation (Fig. 1). Subsequently, cells were incubated with 1 μg/ml

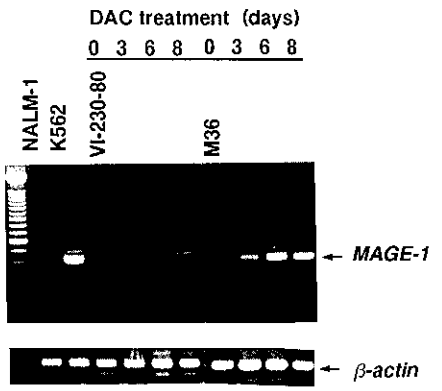

Fig. 1. Induction of *MAGE* genes in the cells. An HTLV-I-infected TCC (VI-130-80) and M36 melanoma cell line were incubated for 8 days with or without 1 μg/ml of DAC, and *MAGE-1, -2, -3,* and *-6* expression was examined on days 3, 6, and 8. Results of *MAGE-1* and *β-actin* expression are shown in this figure.

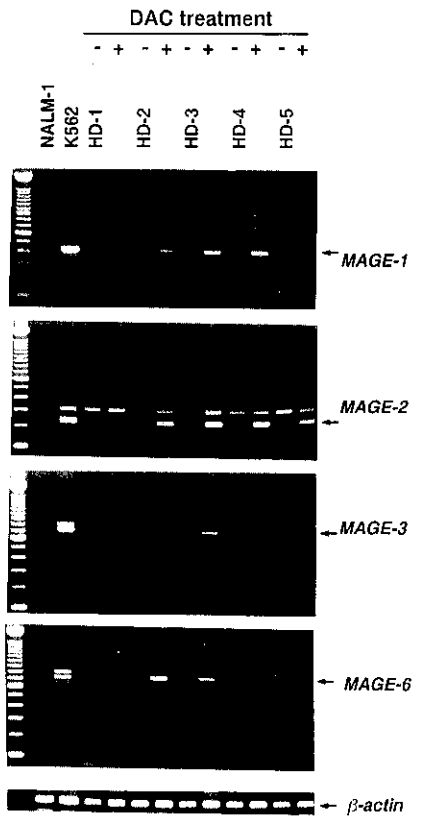

Fig. 2. Induction of *MAGE* genes in PHA/IL-2-activated T cells. PHA/IL-2-activated T cells from five healthy donors were tested for their expression of *MAGE-1, -2, -3,* and *-6* after incubation for 8 days with or without 1 μg/ml of DAC. The other results are summarized in Table I. All *MAGE* genes were induced in PBMC of HD-2, -3, and -4. None of the *MAGE* genes was induced in PBMC of HD-1. *MAGE-1* and *-2* were induced in HD-5.

Table I. Induction of *MAGE* Tumor Antigens in Normal and Malignant Cells

	Cells	DAC treatment	Expression of *MAGE* genes (positive/tested)			
			MAGE-1	*MAGE-2*	*MAGE-3*	*MAGE-6*
T cells	Fresh normal PBMC	+	0/5	0/5	0/5	0/5
	PHA/IL2-activated	−	0/5	0/5	0/5	0/5
	normal T cells	+	4/5	4/5	3/5	3/5
	T cell clones	−	0/5	0/5	0/5	0/5
		+	4/5	0/5	0/5	1/5
	Leukemia cell lines	−	0/5	0/5	0/5	0/5
		+	5/5	5/5	4/5	5/5
	PBMC from ATL	−	0/2	0/2	0/2	0/2
		+	1/2	2/2	1/2	1/2
B cells	Leukemia cell lines	−	0/6	0/6	0/6	0/6
		+	5/6	5/6	2/6	3/6
	EBV-transformed	−	0/4	0/4	0/4	0/4
	normal B cell lines	+	4/4	4/4	4/4	2/4
Monocytes	Leukemia cell lines	−	0/4	0/4	0/4	0/4
		+	2/4	1/4	1/4	4/4
Myeloid cells	Leukemia cell lines	−	0/3	0/3	0/3	0/3
		+	3/3	3/3	2/3	2/3

of DAC for 8 days, and were tested for their expression of *MAGE* genes in the following experiments.

Fresh PBMC did not express any *MAGE* genes (data not shown). PHA/IL-2-activated T cells from 5 healthy donors were tested for their expression of *MAGE-1, -2, -3,* and *-6* after incubation with or without DAC (Fig. 2). None of them expressed any of the *MAGE* genes without DAC treatment. DAC induced *MAGE-1, -2, -3,* and *-6* in PHA/IL-2-activated T cells from 4, 4, 3, and 3 of 5 healthy donors, respectively (Fig. 2). The results are summarized in Table I.

The following cells were incubated for 8 days with or without DAC, and were tested for *MAGE* expression; cells of 5 different TCCs, PBMC from 2 ATL patients (Fig. 3A), 5 different T cell leukemia lines (Fig. 3B), 6 B cell leukemia lines and 4 EBV-transformed B cell lines (Fig. 3C), 4 monocytic leukemia lines and 3 myeloid leukemia lines (Fig. 3D). Representative results for the *MAGE-1* gene are shown in Fig. 3. The results are summarized in Table I. DAC induced *MAGE-1* in the most of the cell lines tested (Fig. 3). It also induced *MAGE-2, -3,* and *-6* genes in most of these cells except for TCC and monocyte cell lines. DAC failed to induce *MAGE-2,* and *-3* in TCCs. It induced *MAGE-2* and *-3* in only 1 of 4 monocyte cell lines.

The kinetics of DAC-induced expression of MAGE-1 protein was investigated by incubation of M36 cell line with 1 μg/ml of DAC for up to 8 days (Fig. 4). The polyclonal anti-MAGE-1 Ab recognized 43-kDa MAGE-1 protein in the K562 cell line (lane 3, positive control), and 43 and 46 kDa proteins in the cells of HEL cell line (lane 4, positive control) in western blot analysis. None of these bands was observed in PBMC from a healthy donor (lane 2, negative control), or in M36 (lanes 5 and 6) with or without incubation for 3 days with DAC. DAC induced 43 kDa bands in M36 (lanes 7, 8 and 9) after 4, 6 and 8 days of incubation with DAC. Several other bands (45, 48, and 51 kDa) were observed in all the cells tested, and therefore were evaluated as non-specific bands, as reported.[9] Based on these results, cells were incubated with 1 μg/ml of DAC for 8 days, and then tested for expression of MAGE-1 protein in the following experiments.

PHA/IL-2-activated T cells from 5 healthy donors and cells of 5 MAGE-1$^-$ (representative runs of HD-3, HPB-MLT and SALT-3 are shown in lanes 3, 4 and 8 of Fig. 5) T cell leukemia lines (HPB-MLT, HUT-102, MT-2, RPMI-8402, and SALT-3) were incubated with DAC. Up to 6 days of incubation with DAC, MAGE-1 protein was undetectable in both HBP-MLT (lane 6 of Fig. 5) and SALT-3 (lane 9 of Fig. 5). DAC induced *MAGE-1* mRNA in PHA/IL-2-activated T cells from 4 of 5 healthy donors (not HD-1), but MAGE-1 was difficult to detect at the protein level (Fig. 5, HD-3: lane 4). DAC induced a 46-kDa MAGE-1 protein in all 5 T cell lines incubated for 8 days with 1 μg/ml of DAC. Representative runs of HPB-MLT and SALT-3 are shown (Fig. 5, lanes 7 and 10, respectively).

DISCUSSION

We have shown here that DAC induced *MAGE-1* gene in normal and malignant lymphoid cells and also in myeloid-monocyte cell lines in most cases. The other

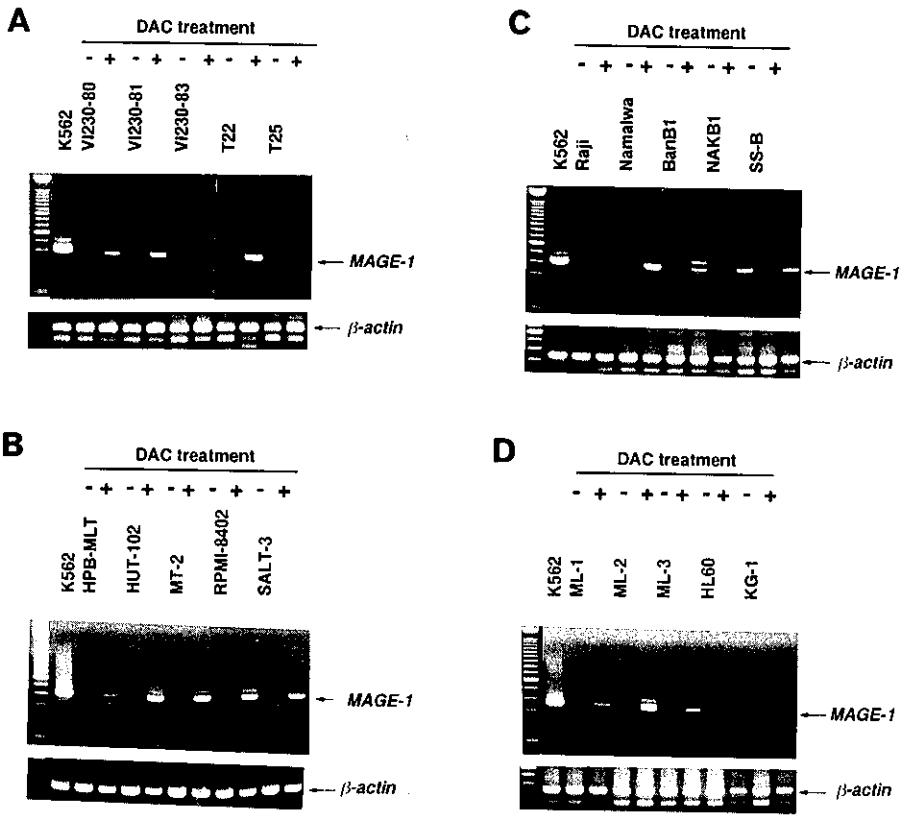

Fig. 3. Induction of *MAGE* genes in cell lines. The following cells were incubated for 8 days with or without DAC, and were tested for *MAGE* gene expression; cells of 5 different TCCs, PBMC from 2 ATL patients, 5 different T cell leukemia cell lines, 6 B cell leukemia cell lines, 4 EBV-transformed B cell lines, 3 myeloid leukemia cell lines and 4 monocyte leukemia cell lines. Representative results for *MAGE-1* are shown in these figures. The results are summarized in Table I. A, *MAGE-1* was induced in 4 of 5 TCC (results for 3 TCC are shown) and in PBMC from 1 (T22) of 2 ATL patients. The level of *MAGE-1* in the other ATL patient (T25) after DAC treatment was very low and was evaluated as negative by semi-quantitative analysis. B, *MAGE-1* was induced in all 5 T cell leukemia cell lines. C, *MAGE-1* was induced in 5 out of 6 B cell leukemia cell lines (not Raji). The results for Namalwa and Raji are shown. *MAGE-1* was induced in all 4 EBV-transformed cell lines (results for 3 cell lines are shown). D, *MAGE-1* was induced in all 3 myeloid leukemia cell lines. *MAGE-1* was induced in 2 (U937 and THP-1) of 4 monocyte leukemia cell lines, and the results for HL-60 and KG-1 were shown. In these cells, levels of *MAGE-1* after DAC treatment were very low and were evaluated as negative by semi-quantitative analysis.

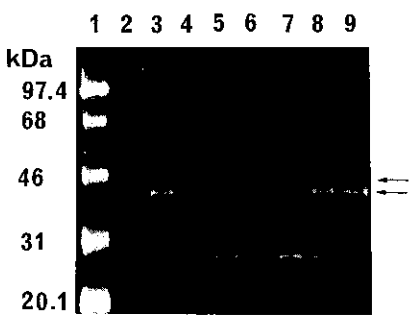

Fig. 4. Induction of MAGE-1 protein in the cells. M36 melanoma cell line was incubated for 8 days with or without 1 μg/ml of DAC, and MAGE-1 protein expression was examined on days 3, 4, 6, and 8 by Western blot analysis with anti-MAGE-1 antibody. The molecular weight of each band was calculated based on the calibration curve from the migration of markers (lane 1). Untreated PBMC of a healthy donor (lane 2) were used as a negative control, and K562 (lane 3) and HEL (lane 4) cell lines as positive controls. DAC induced 43 kDa bands in M36 (lanes 7, 8 and 9) at 4, 6 and 8 days of incubation with DAC. Several other bands (45, 48, and 51 kDa) were observed in all the cells tested, and were evaluated as non-specific bands, as reported.[8]

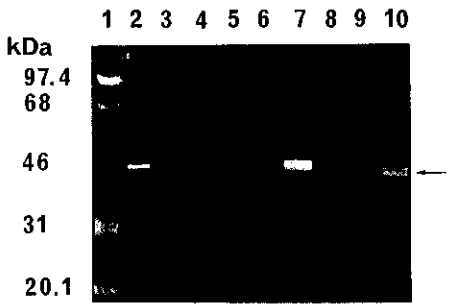

Fig. 5. Induction of MAGE-1 protein in T cells. PHA/IL-2-activated T cells from 5 healthy donors and cells of 5 *MAGE-1*$^-$ T cell leukemia lines (HPB-MLT, HUT-102, MT-2, RPMI-8402, and SALT-3) were incubated with DAC for 8 days. DAC induced a 46 kDa MAGE-1 protein in all 5 T cell lines, but this protein was not readily detectable in PHA/IL-2-activated T cells from all 5 healthy donors. Representative results (HD-3, SALT-3 and HPB-MLT) are shown in this figure. Lane 1: MW markers, lane 2: (HEL, positive control), lane 3: PHA/IL-2-activated PBMC (HD-3), lane 4: PHA/IL-2-activated PBMC (HD-3) cultured with DAC for 8 days, lane 5: HPB-MLT, lane 6: HPB-MLT cultured with DAC for 6 days, lane 7: HPB-MLT cultured with DAC for 8 days, lane 8: SALT-3, lane 9: SAL-3 cultured with DAC for 6 days, lane 10: SALT-3 cultured with DAC for 8 days.

MAGE genes were also induced in these cells with a few exceptions. These results suggest that demethylation is a potent stimulus to induce *MAGE* genes in most normal and malignant lymphoid cells. Weber *et al.*[12] reported that no normal diploid cell line could be induced with DAC to up-regulate *MAGE-1* expression, with the exception of tumor-infiltrating leukocytes. However, 4 out of 5 PHA/IL-2-activated normal T cells expressed *MAGE-1* after incubation with DAC in our experiment. This discrepancy might be explained by the difference in the period of DAC treatment or in the method used to stimulate lymphocytes. We activated lymphocytes with PHA for 3 days and then cultured them with IL-2 and DAC. It seems to be necessary to incubate normal cells or T cell clones for at least 8 days with DAC to induce *MAGE-1* mRNA, as shown in Fig. 1. The eukaryotic genome is methylated at the 5 carbon of cytosines that occur in 5′-CpG-3′dinucleotides.[14, 15] Demethylation seems to up-regulate the accessibility of DNA to transcription factors.[16] Incubation of cells with DAC may generate sufficient unmethylated DNA in the cells to allow binding of transcription factors to induce *MAGE* gene expression. Many developmentally regulated genes are under methylation control. Demethylation caused by gene targeting of methyltransferase in mice results in severe developmental abnormalities and early death.[15] These results suggest that *MAGE* genes are under methylation control. Genetic instability in cells might induce loss of this control, which in turn would result in the expression of *MAGE* genes in cancer cells. This hypothesis is in part supported by the fact that the *MAGE-1* promoter was found to exert transcriptional activity in tumor cells where the *MAGE-1* gene is not expressed.[17]

MAGE genes were not induced by DAC in PBMC of 1 of 5 healthy donors. *MAGE* genes were also not induced by DAC in a few malignant T, B and myeloid-monocyte cell lines. These results suggest that demethylation is not a sufficient stimulus to induce *MAGE* genes in all cases. In these cases, the levels of *MAGE* gene expression after DAC treatment were very low and were evaluated as *MAGE*$^-$ by a semi-quantitative RT-PCR analysis. Some factor(s) other than demethylation might be involved in expression of *MAGE* genes, either alone or in conjunction with demethylation.

In summary, this manuscript has demonstrated that demethylation is a potent stimulus for induction of *MAGE* genes in lymphoid cells in most cases. These results should lead to a better understanding of mechanisms of *MAGE* gene expression in cells.

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