Identification of MAGE-1 and MAGE-4 Proteins in Spermatogonia and Primary Spermatocytes of Testis

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Abstract

The MAGE genes encoding tumor rejection antigens recognized by CTLs are expressed at the mRNA level in various cancers and in the testis but not in the other normal tissues. The expression of MAGE-1 or MAGE-4 protein in the testicular cells was studied immunohistochemically with the antibodies to the recombinant MAGE-1 or MAGE-4 protein. Both proteins were identified in the nucleus and cytoplasm of spermatogonia and in primary spermatocytes but not in spermatids or Sertoli's cells. Therefore, MAGE proteins are normal tissue antigens compartmentalized in particular testicular cells playing an important role in the early phase of the spermatogenesis.

Introduction

The MAGE-1 or MAGE-3 gene codes for tumor antigens on HLA-A1 and HLA-Cw1601 or HLA-A1 and HLA-A2, which are recognized by CTLs, respectively (1-6). The other MAGE genes [MAGE-4 (MAGE-4a and MAGE-4b) and MAGE-6] also encode a potential tumor antigen on HLA-A1 (2). The MAGE-1, MAGE-2, MAGE-3, MAGE-4, MAGE-6, and MAGE-12 genes are expressed in many different solid cancers, whereas neither normal cells nor normal tissues other than testis express the MAGE gene at the mRNA level (1-7). MAGE-3 and MAGE-4 genes are also expressed in placenta (6). These results suggest MAGE gene products are appropriate target molecules for the specific immunotherapy of cancer. However, there was no information available on either location of MAGE proteins in the testis or their biological functions. The present study has investigated the expression of MAGE-1 and MAGE-4 proteins in the cells of testis by the immunohistochemical staining with the Ab to the MAGE-1 and MAGE-4 proteins.

Materials and Methods

Tissue Samples and Tissue Sections. Normal testes were obtained at the time of castration of two patients with prostatic cancer (ages 70 and 72 years) at our hospital. Tissue sections were prepared by the AmEx method reported previously (8). Briefly, tissues were fixed in acetone at −4°C overnight, then cleared in methyl benzoate and xylene, and embedded in ordinary paraffin at 58-60°C.

MAGE-1 and MAGE-4b Recombinant Proteins. Plasmid clones containing 1031-bp MAGE-1 and 1062-bp MAGE-4b cDNA fragments cloned from K562 leukemic cell line (MAGE-1) and RPMI 1788 B-cell line (MAGE-4b), respectively, were used as templates for the preparation of GST-fusion proteins as reported previously (7). The accession number of MAGE-1 or MAGE-4 in GenBank is M77481 or D32077, respectively, in which MAGE-4b was named as MAGE-41 (2, 9).

Polyclonal and mAb Anti-MAGE. Rabbits (Kuroda, Kumamoto, Japan) were immunized with GST-MAGE-1 or GST-MAGE-4b fusion protein and MPL plus TDM emulsion (Ribi ImmunocChem, Hamilton, MT) as an adjuvant 4 times at 1-week intervals. IgG fraction was purified from rabbit sera with HPLC (Pharmacia LKB, Uppsala, Sweden) and Affi-Prep protein A, MAPS analytical cartridge (Bio-Rad, Richmond, CA). Anti-MAGE-4b mAb (IgG1, R5 mAb) was made by immunizing BALB/c mice with purified GST-MAGE-4b fusion protein as reported (7).

MAGE-1 Peptides. A series of 30 MAGE-1 oligopeptides according to the predicted amino acid sequences of MAGE-1 protein (6) were synthesized on solid phase with the use of F-moc for transient N-hetero-terminal protection with the use of a 9050 Peptide synthesizer (Milligan/Biosearch, Burlington, MA), purified by C18 (μ-Bondaparticle column; Nihon Millipore, Tokyo, Japan) reverse-phase HPLC. Each peptide was a 15-mer that shared the same amino acids with the following peptide at positions 11-15. Epitope analysis was performed by the ELISA with the use of peptide-coated 96-well microplates (CovaLink; Nunk, Roskilde, Denmark) as reported (7).

Immunoblot Analysis. Details of the method for the immunoblot analysis was described previously (7). MAGE proteins were detected with the enhanced chemiluminescence mixture immunoblot analysis (Amersham, Aylesbury, United Kingdom) according to the manufacturer’s instruction. K562 and HEL leukemic cell lines (for MAGE-1 protein) and RPMI 1788 B-cell line (for MAGE-4 protein) were used as the positive control. PBMC from a healthy donor were used as the negative control.

Immunohistochemical Staining. AmEx-processed tissue sections of testis were deparaffinized with xylene for 20 min, immediately immersed in acetone for 20 min, and then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 5 min at room temperature. The tissue sections were washed in PBS and then stained immunohistochemically by the streptavidin-biotin-peroxidase method (10). In brief, the sections were incubated for 15 min in 3% H2O2 to block endogenous peroxidase activity, rinsed in PBS, and incubated for 20 min in 10% goat (or rabbit) normal serum for the polyclonal Ab (for mAb). The slides were incubated with the Ab for 60 min at room temperature, rinsed in PBS, and incubated for 10 min with biotinylated anti-rabbit IgG (or anti-mouse IgG) for polyclonal Ab (for mAb), followed by washing in PBS. They were incubated with peroxidase-labeled streptavidin (Histofine SAB-PO kit; Nichirei, Tokyo, Japan) for 5 min, rinsed in PBS, and incubated with 3,3'-diaminobenzidinetetrahydrochloride (DAB substrate kit; Nichirei) for 5-10 min. Nuclear counterstaining was performed with Mayer’s hematoxylin solution (Muto, Tokyo, Japan). The serial sections of each sample were incubated as the negative control with IgG of preimmune rabbit serum for polyclonal Ab or an irrelevant mouse mAb (IgG1) for the R5 mAb.

Results and Discussion

The expression of MAGE-1 or MAGE-4 gene in the testes at the mRNA level was initially confirmed by the specific primers for MAGE-1 or MAGE-4 gene and the semiquantitative reverse transcription-PCR method (7). The testes from two donors expressed the MAGE-1 and MAGE-4 genes (data not shown).

The anti-MAGE-1 Ab mainly reacted to a number 26 peptide (positions 251-265; YRQVPDSDPARYEFL) and number 9 and 25 epitopes (positions 251-265; YRQVPDSDPARYEFL) and number 9 and 25 peptide sequences (positions 251-265; YRQVPDSDPARYEFL) and number 9 and 25 peptide sequences (positions 251-265; YRQVPDSDPARYEFL) and number 9 and 25 peptide sequences (positions 251-265; YRQVPDSDPARYEFL) and number 9 and 25 peptide sequences (positions 251-265; YRQVPDSDPARYEFL). Amino acid sequence of the number 26 peptide was different from the corresponding part of any of
MAGE-2, MAGE-3, MAGE-4a, MAGE-4b, MAGE-6, and MAGE-12 proteins reported (6, 9). Indeed, this Ab showed no apparent cross-reactivity to any of the peptides of the other MAGE proteins corresponding to the number 26 peptide of MAGE-1 protein (Fig. 1B). Further, this Ab showed no apparent cross-reactivity to the other MAGE proteins (MAGE-2, MAGE-3, MAGE-4, MAGE-6, and MAGE-12) based on the results from the immunoblot analysis (data not shown).

Both the monoclonal and polyclonal anti-MAGE-4 Ab primarily recognized one (positions 119–133) and two oligopeptides (positions...
Fig. 3. MAGE-1 and MAGE-4 proteins in the testicular cells. Immunohistochemical studies were performed to identify the cells expressing MAGE proteins among the testicular cells using the three Abs: A, stained with IgG of the anti-MAGE-1 polyclonal Ab (× 200); B, stained with IgG of anti-MAGE-4b polyclonal Ab (× 200); C, stained with IgG of the preimmune rabbit serum (× 200); D, stained with anti-MAGE-4b mAb (× 200); E, stained with an irrelevant mouse mAb (× 200). Spermatogonia (solid arrow) were reactive to all these Abs and were adjacent to the basement membrane of the seminiferous tubules and smaller than the primary spermatocytes. Primary spermatocytes (open arrow) reactive to all these Abs and were adjacent to the basement membrane and were larger than the other cells with large nuclei. Primary spermatocytes not reactive to these Abs were located far from the basement membrane with relatively small nuclei. Spermatids (solid arrowhead) were not reactive to any of these Abs and were located far from the basement membrane and smaller than the primary spermatocytes or spermatagonia with mostly irregular shaped nuclei. Sertoli's cells (open arrowhead) were also not reactive to these Abs and were not adjacent to the basement membrane with oval- or spindle-shaped nuclei. A-C, the testis of Case 1 in which the typical spermatids were rarely observed. D and E, the testis of Case 2.

119-133 and 259-273), respectively, among a series of 31 different MAGE-4b oligopeptides (data not shown). The amino acid sequences of these two peptides were identical to those of MAGE-4a and MAGE-4b but differed from those of the other MAGE proteins (MAGE-1, MAGE-2, MAGE-3, MAGE-6, and MAGE-12). These Abs showed no apparent cross-reactivity to the other MAGE proteins (MAGE-1, MAGE-2, MAGE-3, MAGE-6, and MAGE-12) based on the results from both the immunoblot analysis and the ELISA as reported (7).

Immunoblot analysis was performed to detect the expression of
MAGE-1 and MAGE-4 proteins in the testes with the use of these Abs (Fig. 2). Polyclonal anti-MAGE-1 Ab was reactive to the positive control (K562 and HEL) and the testes from two donors but failed to react to the negative control (PBMC). This Ab recognized a M, 42,000 protein in the K562 cell line and a M, 46,000 protein as a major band and an additional M, 42,000 protein in the HEL cell line and a M, 38,000 protein as a major band and the additional M, 42,000 and 45,000 proteins in the testes. The M, of cellular MAGE-1 protein in melanomas was reported as 46,000 (11, 12). We observed that this polyclonal Ab recognized only a M, 46,000 band in the MAGE-1 mRNA-positive ovarian tumors but recognized a M, 38,000 protein as a major band and additional M, 42,000 and 45,000 proteins in the brain tumors (data not shown).

Both the polyclonal and monoclonal anti-MAGE-4 Abs were reactive to the positive control (RPMI 1788) and the testes but failed to react to the negative control (PBMC). The polyclonal Ab recognized a M, 45,000 protein in RPMI 1788 cells and a M, 45,000 protein as a major band and the additional M, 28,000, 35,000, and 40,000 proteins in the testes. The R5 mAb also showed the similar pattern of the reactivity, although its reactivity was weaker than that of the polyclonal Ab. Both Abs also recognized these four proteins in most of the MAGE-4-positive lung cancers but recognized only a M, 45,000 band in either the recombinant MAGE-4b protein or a small cell lung cancer cell line (S-1) as reported (7). Levels of MAGE gene expression both at the mRNA and protein levels in the testis of Case 1 were lower than those of Case 2.

Although the detailed studies are under investigation, these M, 38,000–46,000 proteins or M, 28,000–45,000 proteins might be either the MAGE-1 or MAGE-4 protein by itself, respectively, with different levels of glycosylation. Alternatively, M, 38,000–45,000 MAGE-1 or M, 28,000–40,000 MAGE-4 might be a part of the MAGE-1 or MAGE-4 protein, respectively, processed by low molecular weight proteins in the cytoplasma possessing proteasome activity.

A band around M, 50,000 was evaluated as nonspecific because it was found in all of the cells including PBMC (Fig. 2) and MAGE-1 or MAGE-4 mRNA-negative tumors (data not shown).

Immunohistochemical studies were performed to identify the cells expressing MAGE-1 and MAGE-4 proteins in the testes with the use of these Abs. The same results were obtained in the testes of two donors and the representative results were shown in Fig. 3. Both MAGE-1 and MAGE-4 proteins were observed in the nucleus and the cytoplasm of the spermatogonia but not in spermatids or Sertoli’s cells (A, stained with the polyclonal anti-MAGE-1 Ab; B, stained with the polyclonal anti-MAGE-4b Ab; C, stained with IgG of preimmune rabbit serum; D, stained with the anti-MAGE-4b mAb; and E, stained with an irrelevant mouse mAb). MAGE-1 and MAGE-4 proteins were also detectable in the primary spermatocytes adjacent to the basement membrane with relatively large nuclei but not in the primary spermatocytes far from the basement membrane with relatively small nuclei.

Spermatogenesis is a complex process. The process may be divided into three phases based on functional considerations: (a) the proliferative phase (spermatogonia) in which cells undergo rapid divisions; (b) the meiotic phase (spermatocytes) in which genetic material is recombined and segregated; and (c) the differentiation phase (spermatids) in which spermatids transform into cells equipped to reach and fertilize the egg (13). Therefore, MAGE-1 and MAGE-4 proteins are normal tissue antigens compartmentalized in the particular testicular cells playing an important role in the early phase of the spermatogenesis. The young spermatocytes, specifically preleptotene spermatocytes, seem positive for the MAGE proteins based on their location and morphology, although the other approaches shall be performed to identify the specific types of the MAGE-positive cells among the various stages of spermatocytes (13). Our results showed that spermatids were negative for MAGE-1 or MAGE-4 protein. However, because spermatids have very little cytoplasm, the other approaches would also be needed to clarify this issue.

We observed the expression of MAGE-4, but not MAGE-1 gene, at the mRNA level in the two placentas in agreement to a previous report (6). However, neither the MAGE-1 nor the MAGE-4 protein was detectable in any of these placenta by the immunoblot analysis (data not shown). Additionally, none of these Abs immunohistochemically showed the apparent reactivity to any of the cells in the placentas (data not shown). Detailed studies using different placent al specimens are under investigation.

The recent studies have demonstrated that human tumor antigens recognized by CTLs are not truly foreign but rather normal differentiation antigens expressed in the compartmentalized tissues (14–18). MAGE-1 gene is expressed in normal skin during wound healing (19). This study has identified the MAGE protein-positive testicular cells, and confirmed the MAGE proteins are one of the normal tissue antigens recognized by the host CTL. These results would be important for discovering the biological functions of MAGE proteins and also for understanding the nature of cancer antigens recognized by the immune system.

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References


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