Identification and Intracellular Location of MAGE-3 Gene Product

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Abstract

The human MAGE-3 gene encodes a melanoma antigenic epitope recognized by specific cytotoxic T lymphocytes, but its gene product has not been identified thus far. We produced a recombinant MAGE-3 gene product by expression cloning of the entire reading frame in the context of a fusion protein characterized by a 10-histidine tail, allowing purification by metal chelation on a nickel Sepharose column. The semipurified product was used to generate MAGE-3-specific monoclonal antibodies. One reagent could identify by immunoblotting the native MAGE-3 gene product as a Mr, 48,000 protein in lysates of cell lines showing evidence of MAGE-3 gene expression. No apparent cross-reactivity with recombinant or native MAGE-1 gene product was observed. Immunohistochemistry shows that, closely resembling the MAGE-1 gene product, MAGE-3 is a cytoplasmic protein.

Introduction

The gene cloning of human tumor-associated antigens, recognized by HLA class I-restricted CTL, could represent a milestone in tumor immunology (1). The antigens of the MAGE family are particularly interesting since their expression is shared by different types of tumors (2–4). The identification of this gene family required transfection of DNA libraries from cells able to stimulate tumor-specific CTL into cell variants insensitive to the killing or into COS-7 cells. In the latter case, cotransfection of the MHC gene encoding the appropriate restriction element was also required (5, 6). These approaches, which proved extremely successful in the identification of CTL peptide targets, do not provide information concerning the nature of the gene products encompassing antigenic epitopes or their intracellular location. Serological reagents recognizing such gene products are thus necessary for further molecular characterization of tumor-associated antigenic determinants and for the evaluation of the potential clinical relevance of specific active immunotherapy protocols. We report here on the identification and intracellular location by a mAb of the melanoma antigen MAGE-3 gene product.

Materials and Methods

Materials and Methods

Cell Lines. MZ-2 (5, 6), D10, and A375 cell lines are a gift of Dr. Carrel (Ludwig Institute, Lausanne, Switzerland). SK3 and WM115 cells were obtained from American Type Culture Collection (Rockville, MD), while HBL and S7 cell lines were kindly provided by Drs. Ghanem (Free University of Brussels, Brussels, Belgium) and Shibahara (Tohoku University, Sendai, Japan), respectively. RE, a primary melanoma cell line, was a gift of Dr. Siegert (Zentrum für Lehr und Forschung, Basel, Switzerland).

Cloning Procedures. Total cellular RNA was extracted from the MZ-2 cell line and reverse transcribed as described previously (7). cDNA was amplified by 30 cycles of PCR by using the following primers, embracing the entire reading frame of MAGE-3 gene (6) and introducing XhoI restriction sites at both ends: sense, 5'-CGGGCTCGAGATGCCTCTTGAGCAGAGGAGT-3'; and antisense, 5'-CGCGCTCGAGCTCTTCCCCCTCTCTCAAAACCC-3'. The PCR product thus obtained was XhoI restricted and ligated with a similarly restricted and dephosphorylated pET 16b (Novagen, Madison, WI) expression vector, allowing the inducible production of a fusion protein characterized by a 10-histidine tail on the NH2 terminus of the gene product of interest. Such construct was transformed into Escherichia coli, HB 101, and sequenced (see "Results").

Expression and Purification of the MAGE-3 Gene Product. The purified plasmid was transformed into expression strain E. coli BL21 (DE3). After appropriate PCR screening demonstrating successful transformation, protein expression could be induced by IPTG according to the producer's instruction (Novagen). Cultures (100 ml) were induced by 3-h treatment with 1 mM IPTG and lysed under denaturing conditions in a lysis buffer consisting of 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9), and 8 M urea. The lysate was passed on a nickel chelating column and eluted at pH 4. Production and purification of the recombinant protein were monitored by SDS-PAGE and Coomassie blue staining.

Production of Monoclonal Antibodies. BALB/c mice were immunized i.p. at 2-week intervals with 100 μg of semipurified material, together with complete Freund's adjuvant, incomplete adjuvant, and in the absence of adjuvants. Three days after the last boost, animals were sacrificed, and fusions were performed as described previously (7). Hybridoma supernatants were screened by ELISA.

Detection of MAGE Family Gene Expression. Total cellular RNA was extracted from the cell lines under investigation, reverse transcribed, and tested in 25 cycles of PCR for the presence of MAGE-1, MAGE-2, and MAGE-3 transcripts by taking advantage of specific pairs of primers (4). PCR products were run on agarose gels in the presence of ethidium bromide and photographed under UV transillumination.

Immunoblotting. Cultured cells were lysed in a buffer consisting of 150 mM NaCl, 1% NP40, and 50 mM Tris (pH 8) supplemented with phenylmethylsulfonyl fluoride (50 μg/ml) and SDS-PAGE run in reducing conditions. The gels were then blotted on nitrocellulose membranes by semidy transfer (Trans-Blot; Bio-Rad, Hercules, CA). Unspecific binding was blocked by incubation in 0.15% casein in TBS, and membranes were incubated overnight in the presence of the relevant hybridoma supernatants, extensively washed, and treated with a horseradish peroxidaseabeled goat anti-mouse IgG second step antibody. A chemiluminescence-based detection system (ECL; Amersham, Aylesbury, United Kingdom) was used to reveal specific binding.

Immunohistochemistry. Acetone-fixed cytospin preparations of melanoma cell lines showing evidence of MAGE-3 gene expression were incubated with undiluted supernatants of the indicated hybridomas for 30 min at room temperature. The alkaline phosphatase/anti-alkaline phosphatase method was used with a rabbit-anti-mouse link antibody and a commercial alkaline phosphatase/anti-alkaline phosphatase complex (30 min each with appropriate washings; both from DAKO A/S, Glostrup, Denmark). Control incubations included replacement of the specific mAb by an anti-MAGE-1 mAb or isotype-matched irrelevant mAb and usage of cell lines not expressing MAGE-1, MAGE-2, or MAGE-3 genes.
Identification of MAGE-3 Gene Product

Expression Cloning and Immunodetection of rMAGE-3 Gene Product. MAGE-1 and MAGE-3 genes were cloned into an inducible expression vector. A, Coomassie blue-stained SDS-PAGE run of lysates obtained from noninduced, transformed E. coli colonies (Lanes 1 and 3, respectively) or after a 3-h induction with IPTG (Lane 2, MAGE-1; Lane 4, MAGE-3). B, the respective semipurified products (Lane 1, MAGE-1; Lane 2, MAGE-3). In C, rMAGE-1 was run on Lanes 1 and 3, whereas rMAGE-3 was run on Lanes 2 and 4. Gels were blotted and assayed with an anti-MAGE-1 mAb (77B; Lanes 1 and 2) or with an anti-MAGE-3 (57B; Lanes 3 and 4). Specific binding of mAbs was detected by goat anti-mouse IgG. For more details, see “Materials and Methods.”

Results

Expression Cloning and Purification of the MAGE-3 Gene Product. The MAGE-3 gene was amplified by 30 cycles of PCR from MZ-2 cell line cDNA by taking advantage of the primers described above, encompassing the entire reading frame and adding appropriate XhoI restriction sites at both ends. The PCR product was purified, XhoI restricted, and ligated with dephosphorylated pET 16b vector. The ligation mixture was transformed in E. coli, strain HB 101. Positive clones were identified by PCR, plasmids were restriction mapped, and colonies containing the insert in the desired orientation were expanded and sequenced. One missense mutation (A per G in position 991) was detected in all clones, resulting in substitution of valine by leucine in position 279, at difference with the published sequence (6). The plasmid was subsequently transformed into the BL21(DE3) expression host. Positive clones were expanded and tested for induction of gene expression upon IPTG treatment. As shown in Fig. 1A, Lane 4, IPTG induction resulted in the production of a protein exhibiting an apparent M, 50,000, slightly larger than the previously described (7) rMAGE-1 gene product (Fig. 1A, Lane 2). Since the insert encoded a fusion protein, endowed with a 10-histidine tail, allowing purification on nickel Sepharose, bacterial lysates were passaged on a metal chelation column. The retained material was eluted at pH 4 and SDS-PAGE run, resulting in a semipurified product (Fig. 1B, Lane 2) that was used for mice immunization.

Generation of MAGE-3-specific Monoclonal Antibodies. After repeated immunizations, as described in “Materials and Methods,” fusions were performed, and the hybridoma supernatants were differentially screened on lysates of wild-type and MAGE-3 gene-transformed bacterial colonies in a purposely designed ELISA test. Two mAb (both IgGl) were obtained, showing exclusive reactivity on the transformed bacteria. 57B mAb displayed a clearly higher reactivity than 46R mAb in ELISA tests (data not shown). The specificity within the MAGE gene family was then assessed in Western blots. As shown in Fig. 1C, Lane 4, 57B mAb recognized the immunizing MAGE-3 gene product, but not rMAGE-1 (Fig. 1C, Lane 3). Conversely, a previously described (7) anti-MAGE-1 mAb recognized rMAGE-1 but not rMAGE-3 (Fig. 1C, Lanes 1 and 2, respectively). Both anti-MAGE-1 and anti-MAGE-3 mAbs also recognized bands of higher or lower molecular weight, likely to be aggregates and shorter transcription products of the genes under investigation. Both mAbs also recognized MAGE-1 and MAGE-3 gene products of the MZ-2 cell line lysates (Fig. 2, Lane 5). Interestingly, 46R mAb although binding rMAGE-3 (Fig. 2, Lane 4), but not rMAGE-1 (Fig. 2, Lane 3), was unable to recognize native protein in MZ-2 lysates (Fig. 2, Lane 5).

Identification of Native MAGE-3 Gene Product by 57B mAb. Western blots performed by using membrane-immobilized MZ-2 cell line lysates showed that 57B mAb recognizes a protein exhibiting an apparent molecular weight of M, 48,000, clearly distinguishable from MAGE-1 gene product, as recognized by 77B mAb (Ref. 7; Fig. 2, Lanes 1 and 2). Recognition of MAGE-3 by 57B mAb was demonstrated in Western blots. As shown in Fig. 1C, Lane 4, 57B mAb recognized the immunizing MAGE-3 gene product, but not rMAGE-1 (Fig. 1C, Lane 3). Conversely, a previously described (7) anti-MAGE-1 mAb recognized rMAGE-1 but not rMAGE-3 (Fig. 1C, Lanes 1 and 2, respectively). Both anti-MAGE-1 and anti-MAGE-3 mAbs also recognized bands of higher or lower molecular weight, likely to be aggregates and shorter transcription products of the genes under investigation.
**Detection of MAGE-3 Gene Product in Melanoma Cell Lines.**

Established melanoma cell lines were screened for the expression of the MAGE gene family by 25 cycles of reverse PCR (Fig. 3, lower panel). In these conditions, MZ-2 and RE cell lines scored positive for MAGE-1, MAGE-2, and MAGE-3 (Fig. 3, Lanes 2 and 5), whereas D10 and A375 were positive for MAGE-2 and MAGE-3 but negative for MAGE-1 (Fig. 3, Lanes 1 and 6). HBL and WM115 cell lines only scored positive for MAGE-3. No transcripts of these components of the MAGE gene family were detectable in lines S7 and SK3. MAGE-3 gene product immunodetection (Fig. 3, upper panel) by 57B mAb fully matched this gene expression pattern. Interestingly, however, only a relatively weak positivity could be observed in WM115 lysate (Fig. 3, Lane 8). No differences in molecular weight could be detected among MAGE-3 gene products of different cell lines, as compared with the original MZ-2 cells.

**Intracellular Location of the MAGE-3 Gene Product.** Intracellular detection of MAGE-3 gene product was attempted in the MZ-2 cell line that originally provided DNA for the molecular cloning of the MAGE gene family. As shown in Fig. 4, a clear cytoplasmic reaction was obtained with anti-MAGE-3, 57B mAb (Fig. 4C). The staining appeared to be remarkably brighter than the faint positive reaction observed in the same cell line upon incubation with 77B anti-MAGE-1 mAb (Fig. 4B). Interestingly, however, in another MAGE-3-positive cell line (HBL), where no MAGE-1 or MAGE-2 gene...
expression could be observed, only scattered, strongly positive cells were detectable among faintly positive or negative cells (Fig. 4F). In this cell line, as expected, no staining could be obtained upon incubation with isotype-matched, anti-MAGE-1 77B mAb (Fig. 4E). No positive staining upon incubation with 57B anti-MAGE-3 mAb could be observed in cell lines where MAGE-3 gene expression was undetectable (data not shown).

Discussion

MAGE-3 is of particular relevance in the context of the MAGE gene family, since it is expressed in a large majority of melanomas, at difference with MAGE-1 (6). In contrast, in other neoplasms, including breast cancers and non-small cell lung carcinomas, the two genes appear to be mostly coexpressed, although only in a minority of cases (3, 4). Most interestingly, MAGE-3-specific CTL could be generated in healthy donors upon in vitro sensitization with synthetic peptides (8). Furthermore, a nonamer encoded by the MAGE-3 gene was found to efficiently bind HLA-A2 (9), thus suggesting the possibility of a clinical use in larger patient populations, in addition to HLA-A1+ individuals.

The MAGE gene family has been mapped to chromosome X (10), but no data suggestive of a functional role of its products have been reported thus far. The MAGE-1 gene product has been identified as a protein of an apparent molecular weight of M, 46,000 (11). We and others (7, 12) have identified the MAGE-1 gene product in the cytoplasm of melanoma cells, a location shared by other proteins containing putative HLA-class I-restricted antigenic epitopes (13).

In this work, we took advantage of the same technology successfully applied for the generation of MAGE-1-specific reagents to produce mAbs capable to identify and localize intracellularly MAGE-3 gene product. A fusion protein of an apparent M, 50,000, including the putative MAGE-3 gene product and a 10-histidine tail, was produced, purified, and used for the immunization of animals and for hybridoma screening. One of the two mAb thus obtained recognized the native protein, which exhibits an apparent M, 48,000. This protein was successfully identified in 6 of 6 established melanoma cell lines displaying MAGE-3 gene expression at the RNA level but in neither of the two lines where specific transcripts were undetectable. Despite the high (73%) sequence homology of the two genes (6), no obvious cross-reactivity with recombinant or native MAGE-1 protein could be observed. MAGE-3 protein shares some interesting features with the MAGE-1 gene product. An anomalous behavior under standard SDS-PAGE conditions is apparent in both proteins. The MAGE-1 gene codes for a protein of 309 residues, with a 34.3-kilodalton protein was successfully identified in 6 of 6 established melanoma cell lines displaying MAGE-3 gene expression at the RNA level but in neither of the two lines where specific transcripts were undetectable. Despite the high (73%) sequence homology of the two genes (6), no obvious cross-reactivity with recombinant or native MAGE-1 protein could be observed. MAGE-3 protein shares some interesting features with the MAGE-1 gene product. An anomalous behavior under standard SDS-PAGE conditions is apparent in both proteins. The MAGE-1 gene codes for a protein of 309 residues, with a 34.3-kilodalton

The cytoplasmic location also appears to be shared by both proteins. MZ-2 cells staining with anti-MAGE-3 mAb 57B yielded a markedly brighter positive reaction as compared with the faint reactivity induced by anti-MAGE-1 77B mAb. It is presently unclear whether this reflects quantitative differences in the cellular content of the two proteins or a mere differential affinity of the specific mAbs for their targets. Moreover, it is of interest that in the absence of MAGE-1 and MAGE-2 gene expression, the MAGE-3 gene product appears to be detectable with a relevant degree of intracellular heterogeneity. Further research is clearly warranted to clarify the issue of the interrelationships possibly occurring between different MAGE family genes at the protein level.

The identification of the MAGE-3 gene product might represent a further step towards the elucidation of the the function of the MAGE gene family products. In addition, 57B mAb could be of use in assaying the MAGE-3 protein content of clinical specimens or in the enumeration of positive neoplastic cells, especially in patients undergoing MAGE-3-specific, active immunotherapies (14).

References

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