Sperm protein 17 (Sp17) is a suitable target for immunotherapy of multiple myeloma

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Sperm protein 17 (Sp17) is a protein recently identified as a novel cancer-testis (CT) antigen in multiple myeloma (MM). Because this tumor antigen demonstrates a very restricted normal tissue expression, Sp17 may be an excellent target for tumor vaccine of MM. In this study, we determined the ability to generate Sp17-specific HLA class I-restricted cytotoxic T lymphocytes (CTLs) from the peripheral blood of 4 patients with MM, 3 consecutive Sp17+ patients, and 1 Sp17− patient. Dendritic cells were generated from monocytes of 4 patients with MM and used to present a recombinant Sp17 protein to autologous T cells. Following 4 rounds of antigen stimulation, the CTLs were tested for their ability to kill autologous targets in an Sp17-dependent and HLA-class I-restricted manner in standard cytotoxicity assays. Despite previous chemotherapy and the immunosuppression so often associated with MM, CTL generation was successful in all 4 patients, irrespective of the Sp17 status of their tumors. Most importantly, the CTLs were able to lyse autologous tumor cells that expressed Sp17. Tumor cell lysis in all cases appeared to be mainly mediated by perforin and could be blocked by concanamycin A. We conclude that Sp17 is a suitable target for immunotherapy of MM. Our findings provide the basis for a clinical study aimed at inducing a cellular immune response directed at Sp17+ MM.

Introduction

Therapeutic approaches for multiple myeloma (MM) remain a challenge. Although conventional chemotherapy or high-dose chemotherapy followed by autologous hematopoietic stem cell rescue has improved the overall response rate and survival of these patients, only a small proportion of patients enjoys long-term disease-free survival.1 Most patients die of disease relapse. Present therapeutic approaches aim at reducing the relapse rate by using maintenance chemotherapy or immunotherapy. Because immunotherapy is more specific and less toxic, it is an ideal approach. The idiotypic protein produced by myeloma cells is clone-specific and has previously been used.2-6 However, the clinical results have been uniformly disappointing.

T lymphocyte targets on myeloma cells that may also be suitable molecules for immunotherapy include MUC-1,7 mutant ras oncogene protein,8 and the new class of tumor antigens, known as cancer-testis (CT) antigens.9 The CT antigens are normal testicular antigens expressed aberrantly in tumor cells. Their restricted normal tissue expression makes them ideal molecules for immune targeting. CT antigens are expressed in some myeloma cells,10-11 Anti-MAGE-A3 cytotoxic T-lymphocyte (CTL) clones raised from healthy donors could lyse myeloma cells in an Sp17-dependent and HLA class I-restricted manner.11

We recently identified the sperm protein 17 (Sp17) as a novel CT antigen in MM.12 Sp17 is a spermatozoa protein of unknown function and is involved in acrosomal reaction during fertilization.13 It has been the target of investigation in the last few years as a form of immunocontraception. Sp17 is expressed in the tumor cells from nearly 30% of patients with MM. Sp17 expression is found at both the messenger RNA and protein levels and its normal tissue expression is limited to only testis and not any other normal tissues,12 including fibroblasts and normal leukocytes,14 suggesting that a Sp17-based tumor vaccine will be tumor-specific with very limited toxicities to normal tissues. To determine if Sp17 contains functional CTL epitopes suitable for T-cell targeting, we have also recently generated a recombinant Sp17 protein and successfully used it to induce Sp17-specific HLA class I-restricted CTL from the peripheral blood of a healthy donor.15 We found that these CTLs could lyse HLA class I-matched tumors from patients with MM, indicating that Sp17 contains functional CTL epitopes recognized by recombinant Sp17 protein-primed and propagated CTLs.

Although our findings in a healthy donor suggested the potential of Sp17 as a tumor vaccine, it remained to be determined if CTL generation was possible in the autologous hosts in patients with MM. Patients with MM are often immunologically suppressed, due to a combination of the disease process and treatment. Their immune repertoire is also often contracted following chemotherapy. It is therefore possible that CTL generation may not be possible in these patients. In addition, it also remained to be determined if Sp17-specific CTL precursors were present in the immune repertoire of these patients, especially those with Sp17+ tumor cells. Theoretically, these T cells may be deleted from the immune repertoire in patients with Sp17+ tumor cells as a form of immunocontraception.
of escape from tumor immunosurveillance. In this present study, we therefore extended our investigations to 4 patients with MM to determine the feasibility of Sp17 as a tumor vaccine in these patients.

**Patients, materials, and methods**

**Patients and materials**

Four patients with MM were studied, 3 consecutive patients positive for Sp17 and 1 negative for Sp17. All patients were previously treated with combination chemotherapy, consisting of either oral melphalan and prednisone or vincristine-Adriamycin-dexamethasone combination chemotherapy. The HLA phenotypes of these patients were: patient 1 (A1, A2; B7, B8; Cw7); patient 2 (HLA-A1, A11; B8, B18; Cw7); patient 3 (HLA-A1, A11; B51, B44); and patient 4 (HLA-A24, A29; B6, B44). Anti–Syndecan-1 (CD138) antibody-sorted tumor cells were obtained from 3 patients with MM. All clinical materials were obtained with the patients’ consents and approval from the local ethics committee.

**Isolation of peripheral blood mononuclear cells and generation of dendritic cells**

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized venous blood by Ficoll-Hypaque (Sigma, St Louis, MO) density gradient centrifugation. Dendritic cells (DCs) were generated from peripheral blood monocytes as described previously. Briefly, PBMCs were seeded into 6-well culture plates containing 3 mL RPMI 1640 and 10% fetal calf serum (FCS) at 5 to 10 × 10^6/well. After 2 hours at 37°C, nonadherent cells were removed and the adherent cells were cultured at 37°C in RPMI 1640 supplemented with 10% FCS, 800 IU/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Immunex, Seattle, WA), and 1000 IU/mL interleukin 4 (IL-4; Genzyme, Cambridge, MA). After 7 days of culture, a combination of mature and immature DCs was harvested for pulsing with Sp17 recombinant protein.

**DC pulsing**

Following culture, the DCs were washed twice and added to 50-mL polypropylene tubes. Recombinant Sp17 protein described previously was used for these experiments. The cationic lipid DOTAP (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used to deliver the Sp17 recombinant protein because we previously found that DCs with DOTAP-delivered antigens efficiently promoted CD8 responses. Briefly, the recombinant protein was mixed with the lipid at room temperature for 20 minutes and added to DCs at 37°C in an incubator with occasional agitation for 3 hours. The cells were washed twice before being used as antigen-presenting cells.

**In vitro generation of Sp17-specific CTLs**

Fresh PBMCs were cocultured with antigen-pulsed DCs at a ratio of 10:1 in RPMI 1640 supplemented with 10% autologous serum, IL-2 (10 IU/mL), and IL-7 (5 ng/mL) and incubated at 37°C. IL-2 was added to the culture every 3 to 4 days thereafter. Irradiated autologous PBMC feeder cells and Sp17 recombinant protein (50 μg/mL) were added to the culture every week. The cells were harvested after 3 rounds of stimulation and used for cytotoxicity assays.

**Cytotoxicity assays**

Standard 4-hour ^51^Cr-release assays were performed to determine the cytotoxic activity of the Sp17-stimulated T cells. Target cells (at an effector-target cell [E/T] ratio of 20:1) used included autologous DCs, with or without Sp17, and CD138 antibody-enriched autologous tumor cells. To determine the HLA dependency of the cytotoxicity, the following antibodies were added to the cocultures: HLA-class I (W6/32) and HLA-class II (L243) antibodies at a concentration of 25 μg/mL. All experiments were set up in quadruplicate and repeated at least once. SDs were determined based on the quadruplicates. For all targets (including fresh myeloma cells), cell viability was more than 90% with the maximum releases in excess of 2000 cpm and the spontaneous release less than 30% of the maximum release.

**Flow cytometric analysis of intracellular cytokines**

This was carried out as previously described. Briefly, the T-cell population was tested 4 weeks after priming, after rest for 6 days following the last antigen stimulation. The T cells were incubated at 37°C for 6 hours in RPMI 1640 plus 10% FCS, 50 μg/mL Sp17 recombinant protein, and 500 ng/mL ionomycin. Brefeldin A (10 μg/mL) was added for the final 3 hours of incubation. Controls (nonactivated cultures) were incubated in the presence of Brefeldin A only. The cells were harvested, washed, and fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes at room temperature, after which they were washed and stored overnight in PBS at 4°C. For intracellular staining, the cells were washed and permeabilized by incubation in PBS plus 1% bovine serum albumin and 0.5% saponin (Sigma) for 10 minutes at room temperature. Activated and control cells were stained with fluorescein isothiocyanate–labeled anti–interferon γ (IFN-γ), phycoerythrin (PE)–labeled anti–IL-4, and isotype-matched control antibodies (Becton Dickinson, Palo Alto, CA) and analyzed by flow cytometry.

**Results**

**Successful generation of Sp17-specific CTLs from PBMCs of patients with MM**

To determine the feasibility of generating Sp17-specific CTLs from patients with MM, we primed PBMCs from 4 patients with Sp17-pulsed DCs. We successfully generated Sp17-specific CTLs after 3 rounds of T-cell stimulation with the recombinant protein. When used as effector cells in cytotoxicity assays, we reproducibly demonstrated that these T cells were able to efficiently kill autologous DCs pulsed with the Sp17 recombinant protein (Figure 1). Target lysis for the autologous DCs was Sp17 dependent because no significant target lysis was observed when autologous DCs without prior pulsing with Sp17 protein were used as targets in the cytotoxicity assays (P < .0001; Figure 1). Because both target cells (DCs with or without Sp17) were cultured in FCS-containing medium, these results also suggest that the cytotoxicity was specific and not directed at FCS. In patient 1, we also used Epstein-Barr virus (EBV)–transformed autologous lymphoblastoid cell lines (LCLs) as targets in the cytotoxicity assay and found similar pattern of target cell killing (Figure 2), suggesting the successful generation of Sp17-specific CTLs in these patients. These results also indicate that recombinant Sp17 protein when
used with autologous DCs can prime T cells from patients with MM, irrespective of the Sp17 status. Target lysis was HLA class I restricted and could be blocked by antibodies directed against monomorphic HLA class I molecules. In contrast, HLA class II antibodies did not affect the cytotoxic activity of the T cells (P < .00001; Figure 1). These results point to the involvement of CD8 T cells in the CTL activity against Sp17-pulsed autologous target cells. These results also suggest the presence in the immune repertoire of Sp17-specific T cells in these patients.

Recombinant protein-primed CTLs lyse Sp17⁺ autologous tumor cells

To investigate the feasibility of using a Sp17-based tumor vaccine to treat MM, we then investigated the relevance of the Sp17-primed CTLs in the context of autologous tumor cell lysis. We first used reverse transcription–polymerase chain reaction (RT-PCR) to confirm the Sp17 status of the tumor cells from these 4 MM patients. Tumor cells from patients 1, 2, and 3 tested positive for the expression of Sp17 protein by the LCLs (S.H.L., data unpublished, 2001). Chemistry staining of EBV-transformed LCLs did not show the expression of Sp17 transcripts. Autologous tumor cell lysis could also be blocked in the context of autologous tumor cell lysis. We observed autologous target lysis was tumor-specific and was not just directed at any B-lineage cells because when EBV-transformed LCLs were used as targets for CTLs from patient 1, only a very low level of killing of the Sp17⁺ LCLs was observed (Figure 2). This finding is not unexpected because immunocytochemistry staining of EBV-transformed LCLs did not show the expression of Sp17 protein by the LCLs (S.H.L., data unpublished, 2001). Similar patterns of results have also been observed in our previous work involving the use of healthy donor-derived Sp17-specific CTLs against HLA-matched LCLs pulsed with Sp17 recombinant protein. Tumor cell lysis could also be blocked in cold target competition assays when unlabeled Sp17-pulsed autologous LCLs were added to the tumor cells at a ratio of 40:1 in cytotoxicity assays by Sp17-specific CTLs (P < .00001; Figure 4). This blocking of tumor cell lysis was not observed when Sp17⁻ LCLs were added to the cytotoxicity assays. Tumor cell lysis was also not mediated by natural killer cells because no significant target lysis was observed when K562 cells were used as targets (data not shown). Autologous tumor cell lysis could also be blocked by a monoclonal antibody directed at monomorphic HLA class I molecules but not HLA class II molecules (Figure 3). Until the availability of specific antibodies directed at the HLA-peptide complex, our results suggest the in vivo processing and presentation of Sp17 CTL peptides in association with the HLA class I molecules in Sp17⁺ tumor cells. Furthermore, they also demonstrate that Sp17 recombinant protein could be used to prime and propagate CTLs that recognize Sp17 peptides of the concentration and configuration similar to those presented on the surface of Sp17⁺ autologous tumor cells.

Phenotypes and cytokine profiles of CTL lines

Flow cytometry was used to evaluate the phenotypes of the CTL lines we generated. Compared to unstimulated CTLs from the same patients, there was a predominance of CD8⁺ T cells and a paucity of CD4⁺ T cells in the CTL lines following in vitro restimulation with the recombinant Sp17 protein (Figure 5). These T cells were mainly CD56⁺. Taken together with the results of the cytotoxicity assays, our results support the involvement of CD8⁺ T cells in the CTL activities. Two-color flow cytometric analysis for intracellular IFN-γ and IL-4 following Sp17 recombinant protein restimulation of the T cells was carried out to determine the cytokine profile of the CTL line. As expected, the CTL line produced predominantly IFN-γ and very little IL-4, a pattern in keeping with a Tc1 cytokine profile (Figure 6).
Sp17-specific CTLs lysed target cells through perforin-mediated pathway

To determine the mode of target cytotoxicity mediated by the Sp17-specific CTLs, we treated the effector cells with either concanamycin A (CMA) or Brefeldin A. CMA selectively inhibits the perforin-mediated target lysis pathway, whereas Brefeldin A inhibits Fas-mediated target apoptosis. We observed no significant effect of Brefeldin A on the ability of the effector cells to kill autologous tumor cells (Figure 7), CMA treatment of the effector cells, on the other hand, nearly completely abrogated the cytotoxic ability of the CTLs to lyse autologous tumor cells (Figure 7), suggesting that autologous tumor cell lysis by the Sp17-specific CTLs was mediated primarily through perforin.

Discussion

The list of human tumor antigens recognized by human immune system is growing rapidly. Of these tumor antigens, CT antigens have received considerable attention because of their unique expression pattern and their potential as targets for tumor vaccines. CT antigens share the following characteristics: expression predominantly in testis and not in other normal somatic tissues and gene activation and expression in a wide range of human tumor types. The reasons for their aberrant expression in tumor cells are not clear but could be linked to an overall DNA demethylation so often associated with tumor development and progression. It is therefore not surprising that the proportion of tumor cells expressing MAGE, BAGE, GAGE, and LAGE-1/NY-ESO-1 family genes increases with advancing disease in MM.11

Following our recent identification of Sp17 as a novel CT antigen in MM12 and the demonstration that Sp17-specific CTLs could be generated from the PBMCs of all 4 patients, irrespective of the Sp17 status of their tumor cells. However, CTL activities against Sp17-pulsed autologous DCs were more efficient in the 3 patients who expressed Sp17 in their tumor cells than in the patient who did not do so. It is therefore possible that Sp17-specific T cells in patients with Sp17+ tumor cells were already primed in vivo. Because myeloma cells seldom express adequate immune costimulatory molecules, it is unlikely that myeloma cells primed these T cells. More likely, these T cells were cross-primed by antigen-presenting cells that endocytosed any soluble or processed Sp17 released as a result of myeloma cell death.

Having demonstrated the ability to generate Sp17-specific CTLs from MM patients, we then determined the clinical relevance of our findings in the context of autologous tumor cytotoxicity. Although we have demonstrated the expression of Sp17 transcripts and protein in myeloma cells, it remained to be determined if the protein is processed and presented in association with the major histocompatibility complex (MHC) molecules for target cell recognition by recombinant protein-primed CTLs. Our findings that Sp17-specific CTLs were able to kill autologous tumor cells in an Sp17-dependent manner support the notion that Sp17 produced by myeloma cells is, in fact, processed and presented on the tumor cell surface in association with the MHC molecules. Most importantly, Sp17 CTL epitopes for effector cell recognition were presented by myeloma cells. Therefore, Sp17 could be used as a tumor vaccine for patients with Sp17+ MM.

As predicted by results obtained using blocking antibodies to HLA molecules in cytotoxicity assays, the Sp17-specific CTLs were predominantly CD8 in phenotypes. These T cells also produced IFN-γ and very little IL-4. Furthermore, these T cells lysed autologous target cells via the perforin-mediated pathway and not through the induction of apoptosis. This is of major clinical relevance. Patients with MM often acquire resistance to Fas-mediated apoptosis as the disease progresses. This may arise in association with the development of multidrug resistance or a point mutation in the gene encoding the Fas protein. Therefore, CTLs that mediate target cell lysis via the induction of Fas-mediated apoptosis may be ineffective when resistance to apoptosis occurs. CTL cytotoxic mechanism mediated via perforin, on the treatment. It is also conceivable that Sp17-specific T cells may be deleted from the immune repertoire of these patients, especially in those that express Sp17 in their tumor cells, as a form of tumor immune escape mechanism. Demonstration of the ability to generate Sp17-specific CTLs that could kill autologous tumor cells will provide the basis for the use of Sp17 as a tumor vaccine for patients with MM.

Using autologous DCs pulsed with the Sp17 recombinant protein as targets in cytotoxicity assays, we observed that Sp17-specific T cells were, in fact, present in the immune repertoire of MM patients. Interestingly, Sp17-specific CTLs could be generated from the PBMCs of all 4 patients, irrespective of the Sp17 status of their tumor cells. However, CTL activities against Sp17-pulsed autologous DCs were more efficient in the 3 patients who expressed Sp17 in their tumor cells than in the one patient who did not do so. It is therefore possible that Sp17-specific T cells in patients with Sp17+ tumor cells were already primed in vivo. Because myeloma cells seldom express adequate immune costimulatory molecules, it is unlikely that myeloma cells primed these T cells. More likely, these T cells were cross-primed by antigen-presenting cells that endocytosed any soluble or processed Sp17 released as a result of myeloma cell death.

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other hand, would still be functional when resistance to apoptosis occurs. This may be of importance in determining the outcome of the tumor vaccine. Because MM that relapses following treatment with chemotherapy with or without autologous stem cell transplantation is likely to have been heavily pre-exposed to chemotherapy, it is likely for these tumor cells to develop defects in the Fas pathway.

In conclusion, we have demonstrated that Sp17 is a suitable target for a tumor vaccine for MM. A recent paper suggested that Sp17 might be expressed in normal leukocytes, casting doubt on the safety of Sp17 as a tumor vaccine. Based on the results presented in this manuscript showing the lack of killing of autologous monocyte-derived DCs and EBV-transformed LCLs, and our recent work using immunocytochemistry and immunohistochemistry failing to demonstrate that Sp17 protein is expressed by normal leukocytes, a phase II/I pilot study has been initiated in our institution for the use of Sp17-pulsed DCs for patients with residual disease following treatment for MM. It, however, remains to be determined whether the laboratory results reported here can be translated into clinical successes in our pilot study. In addition, although Sp17 appears a suitable target for immunotherapy of MM, it is only expressed in 26% of patients with myeloma; therefore the clinical applicability of the vaccine may be limited. Further work is still needed to identify other suitable myeloma antigens so that they can be targeted for patients whose myeloma cells do not express the Sp17 protein.

References

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