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Epigenetic modulation of MAGE-A3 antigen expression in multiple myeloma following treatment with the demethylation agent 5-azacitidine and the histone deacetlyase inhibitor MGCD0103

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Abstract

Background aims. Immunotherapy targeting MAGE-A3 in multiple myeloma (MM) could eradicate highly aggressive and proliferative clonal cell populations responsible for relapse. However, expression of many cancer-testis antigens, including MAGE-A3, can be heterogeneous, leading to the potential for tumor escape despite MAGE-A3-induced immunity. We hypothesized that a combination of the hypomethylating agent 5-azacitidine (5AC) and the histone deacetylase inhibitor (HDACi) MGCD0103 (MGC) could induce MAGE-A3 expression in MAGE-A3-negative MM, resulting in recognition and killing of MM cells by MAGE-A3-specific cytotoxic T lymphocytes (CTL). *Methods.* Gene expression analyses of MAGE-A3 expression in primary MM patient samples at diagnosis and relapse were completed to identify populations that would benefit from MAGE-A3 immunotherapy. MM cell lines were treated with 5AC and MGC. Real-time polymerase chain reaction (PCR) and Western blotting were performed to assess MAGE-A3 RNA and protein levels, respectively. Chromium-release assays and interferon (IFN) secretion assays were employed to ascertain MAGE-A3 CTL specificity against treated targets. *Results.* Gene expression analysis revealed that MAGE-A3 is expressed in MM patients at diagnosis (25%) and at relapse (49%). We observed *de novo* expression of MAGE-A3 RNA and protein in MAGE-A3-negative cell lines treated with 5AC. MGC treatment alone did not induce expression but sequential 5AC/MGC treatment led to enhanced expression and augmented recognition by MAGE-A3-specific CTL, as assessed by ^{51}Cr-release assays ($P = 0.047$) and enzyme-linked immunosorbent assay (ELISA) for IFN-γ secretion ($P = 0.004$). *Conclusions.* MAGE-A3 is an attractive target for immunotherapy of MM and epigenetic modulation by 5AC, and MGC can induce MAGE-A3 expression and facilitate killing by MAGE-A3-specific CTL.

Key Words: *5-azacitidine, cancer-testis antigen, demethylation, epigenetics, histone deactylase inhibitor, hypomethylation, MAGE-A3, MGCD0103, multiple myeloma*

Introduction

Currently available therapies offer poor long-term outcomes for multiple myeloma (MM) patients with a high-risk genetic signature (1). This group accounts for 15% of newly diagnosed patients and 75% of those with relapsed disease. Approaches to intensify therapies in this group of patients have led to cumulative toxicity and host exhaustion and have not improved overall survival (OS). Therefore, new therapeutic approaches that are both non-toxic and non-cross-resistant with chemotherapy are desperately needed for these patients. One potential answer lies in immune therapies targeting tumor-specific antigens, which may eradicate chemoresistant tumor-cell clones without inducing significant toxicities. For example, vaccination with tumor-specific antigens and transfer of tumor-specific T cells is safe and has induced clinical responses in lung cancer and melanoma (2,3).

Targets of particular interest are the cancer-testis antigens (CT-Ag), whose expression in normal tissues is restricted to immunoprivileged sites such as the testes, ensuring that immune responses generated toward these antigens will be non-toxic to normal tissues. CT-Ag expression is common in MM (4–8) and has been linked to poor prognosis (9,10).

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CT-Ag expression in cancer is probably the result of global hypomethylation, specifically of CpG islands at promoter sites (11–13). This phenomenon may also explain reports of coordinate expression of multiple CT-Ag in malignancies, including MM (8,14–16).

One potential concern in targeting CT-Ag for immunotherapy is that CT-Ag-negative clones could lead to tumor escape (17–20). Interestingly, *de novo* induction of CT-Ag expression has been achieved with hypomethylating agents such as 5-aza 2-deoxycytidine (DAC) and its nucleoside analog 5-azacitidine (5AC) (20–27), which incorporates into RNA and, to a lesser extent, DNA (12,28). 5AC has been approved by the food and drug administration (FDA) for use in myelodysplastic syndrome (MDS). Phase I and II clinical trials investigating the use of 5AC in MM have been initiated (protocol ID NCT00761722 and NCT00412919). Further increases in hypomethylating agent-induced gene expression have been achieved with histone deactylase inhibitors (HDACi) such as trichostatin A, valproic acid and MGCD0103 (MGC) via hyperacetylation of the histone core (12,29,30).

We have reported previously that potent immune responses to the CT-Ag MAGE-A3 can be induced by vaccination of a MM patient with MAGE-A3-positive disease with MAGE-A3 recombinant protein (31). We therefore wished to study whether the combination of demethylating agents and HDACi could optimize such therapy. We first analyzed expression of the CT-Ag MAGE-A3 in MM patients and correlated expression with validated disease subgroups identified by gene-expression profiling (GEP) (32) and survival. We then studied whether 5AC induced expression of MAGE-A3 in MM cells and whether any up-regulation could be enhanced with MGC. Finally, we assessed whether MAGE-A3/HLA-A*6801-specific cytotoxic T lymphocytes (CTL) could kill 5AC/MGC-treated targets.

Methods

Subject samples, GEP, subgroup and survival curve analyses

Normal tissue RNA (plasma cell, lung, uterus, kidney, stomach, brain, breast, spleen, prostate, skeletal muscle, testis, thymus, liver, ovary, heart and small intestine) were obtained from Clontech (Mountain View, CA, USA). Bone marrow was collected from healthy donors and patients with MM, after informed consent, in accordance with the Declaration of Helsinki. Approval was obtained from the University of Arkansas for Medical Sciences (UAMS, Little Rock, AR, USA) Institutional Review Board for sample procurement. CD138-positive plasma cells were purified using CD138 antibody (Ab)-coated magnetic beads (Miltenyi Biotec Inc., Auburn, CA, USA), as described previously (33). GEP (33), molecular subgroup (32), high-risk group classifications (34) and survival curve (32) analyses were performed as reported previously using samples obtained from MM patients uniformly treated with our clinical protocols UARK 98-026 [total therapy (TT) 2] and UARK 2003-033 (TT3) (35,36).

Cell lines and drug treatment

MM cell lines ANBL-6, OCI-MY1 and OCI-MY5 were kindly provided by Michael Kuehl, MD (Genetics Department, Medicine Branch, National Cancer Institute, Bethesda, MD, USA). RPMI-8226 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). LP1 was kindly provided by Facet Biotech (Redwood City, CA, USA) and ARK was developed in our laboratories at UAMS. The colorectal cell line COLO205, included in some experiments as a positive control for 5AC-induced MAGE-A3 expression (15), was obtained from ATCC. Cell lines were treated with vehicle (phosphate-buffered saline, PBS), 5AC (Celgene Corporation, Summit, NJ, USA) and/or MGC (Methylgene, Montreal, Quebec, Canada) for the treatment times and doses indicated.

Flow cytometry

Viability after treatment was assessed by staining with annexin V and propidium iodide as per the manufacturer's instructions (Vybrant apoptosis assay kit number 3; Invitrogen, Carlsbad, CA, USA). HLA-A*6801 cell-surface expression on the transfected LP1 cells was verified with an HLA-A*2/28 Ab directly conjugated to fluorescein isothiocyanate (FITC; One Lambda, Canoga Park, CA, USA), which recognizes HLA-A*6801. An appropriate isotype control was included. Intracellular staining for MAGE-A3 was performed after fixation and permeabilization of 10^6 LP1 A68 cells treated with and without 5AC, MGC or a combination of both, as per the manufacturer's instructions (BD Cytofix/Cytoperm fixation/permeabilization kit; BD Biosciences, San Jose, CA, USA). The MAGE-A3 protein was detected using an indirect labeling method with 5 μg purified primary murine IgG1 monoclonal Ab 57B, specific for MAGE-A3 protein (kindly gifted from Dr G. Spagnoli, University Hospital, Basel, Switzerland) and 0.5 μg secondary Ab rat anti-mouse IgG1–phycoerythrin (PE) (clone A85-1; BD Biosciences). Controls included a non-specific isotype murine IgG1 primary Ab with a secondary rat anti-mouse IgG1–PE Ab to confirm the absence of non-specific