

Cancer-testis antigens MAGE-C1/CT7 and MAGE-A3 promote the survival of multiple myeloma cells

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Supplementary Methods

Immunoblotting

For some experiments myeloma cell lines were sorted based on their expression of CD138 using a FACSria cell-sorting system (BD Biosciences, Heidelberg, Germany). Whole cell protein extracts were prepared from cell lines using RIPA lysis buffer [phosphate-buffered saline with 1% Igepal CA-630, 0.5% sodium-deoxycholate, and 0.1% sodium dodecylsulfate (SDS)] containing a cocktail of protease inhibitors (Sigma, Steinheim, Germany). Lysates of human testis, which served as a positive control, were obtained from Abcam (Cambridge, UK). Proteins (30 µg/lane) were electrophoresed on 4% to 12% NuPAGE SDS-polyacrylamide gels (Invitrogen, Karlsruhe, Germany) under reducing conditions. Proteins were blotted on Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA), which had been blocked overnight at 4°C with Top-Block (Fluka, Buchs, Switzerland), and were incubated with 1 µg primary monoclonal antibody for 4 h at room temperature. Next, secondary horseradish peroxidase-labeled anti-mouse monoclonal antibody (R&D Systems, Minneapolis, MN, USA) was applied for 1 h at room temperature. Monoclonal antibodies for the analyses of cancer testis (CT) antigen expression were kindly provided by the New York branch of the Ludwig Institute for Cancer Research (monoclonal antibody CT7-33 for MAGE-C1/CT7, monoclonal antibody M3H67 for MAGE-A3, monoclonal antibody E978 for NY-ESO-1) and by Dr. Spagnoli from the University of Basel in Switzerland (monoclonal antibody 57B for MAGE-A4). A monoclonal antibody directed against ropporin-1 was obtained from Abnova (Taipei City, Taiwan). Monoclonal antibodies directed against caspases were obtained from Santa Cruz (Heidelberg, Germany; caspase-3, -8, -9) or from Abcam (Cambridge, UK; caspase-12). ACTB, as a marker for protein loading, was determined using an appropriate monoclonal antibody (Santa Cruz). Following application of a peroxidase-linked species-specific sheep anti-mouse antibody (R&D Systems), protein bands were visualized with electrochemiluminescence (ECL) western blotting detection reagent (Amersham) and were quantified using Quantity One software (BioRad, Munich, Germany). Recombinant full-length MAGE-A2, MAGE-A3, MAGE-A4, and MAGE-A12

(Abnova) proteins were used to determine the specificity of monoclonal antibody M3H67.

Gene silencing using transfection with stealth interfering RNA

Non-targeting green fluorescent protein (GFP)-coupled stealth interfering RNA (RNAi), scrambled control RNAi, and stealth RNAi targeting MAGE-A3 or MAGE-C1/CT7 were purchased from Invitrogen. Specific down-regulation of CT antigens was achieved with two out of three RNAi targeting, respectively, MAGE-A3 (5'-UCG UCG GAA AUU GGC AGU AUU UCU U-3'; 5'-AAG AAA UAC UGC CAA UUU CCG ACG A-3' and 5'-GAG GAG CUG AGU GUG UUA GAG GUG U-3'; 5'-ACA CCU CUA ACA CAC UCA GCU CCU C-3') and MAGE-C1/CT7 (5'-CAC CUU GCU AGA GAG UGA UUC CUU G-3'; 5'-CAA GGA AUC ACU CUC UAG CAA GGU G-3' and 5'-AAA GUA GCC CGU GUA CCU GCU GAU G-3'; 5'-CAU CAG CAG GUA CAC GGG CUA CUU U-3'). Importantly, sense siRNA constructs targeting MAGE-A3 were confirmed to contain contiguous complementary sequences exclusively aligning with MAGE-A3, but not MAGE-A6, mRNA.

Myeloma cell lines were transfected using the cationic lipid-based reagent Lipofectamine 2000 (Invitrogen). For each condition 3×10^5 cells were washed and resuspended in 100 µL Optimem I medium (Gibco, Karlsruhe, Germany). Next, 50 pmol of stealth RNAi with or without 1 µL Fluorescent Control (Invitrogen) were added to the cells and incubated for 10 min at room temperature. Lipofectamine 2000 was gently mixed before being used and was diluted 1:20 in Optimem I medium without serum followed by incubation at room temperature for 10 min. Fifty microliters of the Lipofectamine 2000 dilution was then added to the cells and incubated at room temperature for 25 min. Next, cell suspensions were transferred to a 24-well plate (Greiner Bio-One, Frickenhausen, Germany) and incubated at 37°C for 4 h. Afterwards, 1.5 mL complete medium were added and cells were cultured at 37°C for another 72 h. Cells were stained with nuclear and dead cell stain (RNAi Basic Control Kit-Human; Invitrogen) and transfection efficiency was evaluated using 40x bright-field microscopy. Images were obtained using a digital camera (Canon, Krefeld, Germany) and Adobe

Photoshop CS3 imaging software (Adobe Systems Inc., San Jose, CA, USA). Transfection efficiency was generally 70-80% and cell death less than 5% at 24 h post transfection as determined by fluorescent microscopy.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay

For screening purposes, the relative numbers of viable cells were assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Myeloma cells were seeded into a 96-well plate (Costar, Cambridge, MA, USA) at a concentration of 3000 cells/well with a final volume of 100 μ L and were cultured overnight at 37°C. Next, 10 μ L of MTT (ATCC bioproducts, Wesel, Germany) were added to each well and cells were incubated for another 4 h at 37°C. One hundred microliters of detergent reagent (ATCC Bioproducts) were added and absorbance was read at 465/540 nm using a spectrophotometer (Tecan, Mannedorf, Switzerland). For some experiments, cells were treated with 10 nM bortezomib or 20 μ M melphalan for 12 h before the MTT assay was performed.

Measurement of apoptosis

Apoptosis was analyzed applying a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Terminal desoxynucleotidyl transferase was used to label DNA breaks with fluorescein-dUTP (TUNEL technique; APT110 Kit, Millipore). Cells were fixed for 1 h in 1% paraformaldehyde then washed twice in phosphate-buffered saline and fixed for 1 h on ice and for 18 h at -20°C using 70% ethanol. Following two washes, cells were incubated in staining solution (10 μ L TdT reaction buffer, 0.75 μ L TdT enzyme, 8 μ L fluorescein-dUTP, 32.25 μ L distilled water) for 1 h at 37°C. Cells were washed and incubated in propidium iodide/RNase A solution for 30 min at room temperature. Analysis by flow cytometry or by fluorescent microscopy was performed within the next 3 h. Cells were separated according to their annexin V staining by flow cytometry as recommended by the manufacturer of the flow cytometer (BD Biosciences, San Jose) after 72 h of specific siRNA transfection.

Colony formation assay

Myeloma cell lines Molp-8 and RPMI-8226 were plated at

1000 cells/mL of methylcellulose medium (StemCell Technologies, Cologne, Germany) and 1 mL/well in a 6-well culture dish (Nunc, Langensfeld, Germany). Plates were incubated at 37°C and colonies consisting of more than 40 cells were counted 7 to 10 days after starting the culture.

Analysis of cell proliferation

Cell proliferation was measured by staining with carboxy-fluorescein diacetate succinimidyl ester (CFSE) or using a plate-based system. In the Biotrak™ enzyme-linked immunosorbent assay (ELISA) proliferation assay (Amersham Biosciences) myeloma cells were pulsed with 10 μ M bromodeoxyuridine (BrdU) for the last 18 h of culture. Following fixation, peroxidase-labeled anti-BrdU, which binds to the BrdU incorporated into newly synthesized cellular DNA, was added. Resulting immune complexes were detected by a substrate reaction, and absorbance was read at 450nm using a microtiter plate spectrophotometer (SLT Labinstruments). For FACS analysis, myeloma cells were stained intracellularly with CFSE at a final concentration of 700 ng/mL and CFSE-related green fluorescence intensity was measured by flow cytometry after 0, 12, 24, 48, and 72 h of culture.

Cell adhesion assay

Myeloma cells were resuspended 72 h post-transfection in RPMI-1640 plus 0.2% bovine serum albumin (adhesion medium) at 5×10^6 /mL and were incubated for 30 min at 37°C followed by two washes. Cells were then resuspended in adhesion medium and were seeded in triplicate into 96-well plates (Costar) coated with fibronectin (Sigma; 20 μ g/mL) and were incubated for 4 h at 37°C. Unbound cells were removed by four washes with RPMI 1640 (Invitrogen,) and absorbance with Chromogen Substrate Solution (Alpco, Salem, NH, USA) was read at 450/620 nm using a spectrophotometer (SLT Labinstruments).

Statistical analysis

Statistical analyses were performed using SPSS software. The Mann-Whitney U test was used to calculate differences between different experimental conditions. Differences were considered statistically significant if the P value was less than 0.05.