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Overexpression of MDR1 and survivin, and decreased Bim expression mediate multidrug-resistance in multiple myeloma cells

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ABSTRACT

Multidrug resistance represents a major obstacle for the chemotherapy of a wide variety of human tumors. To investigate the underlying mechanisms associated with resistance to anti-cancer drugs, we established anti-cancer drug-resistant multiple myeloma (MM) cell lines RPMI8226/ADM, RPMI8226/VCR, RPMI8226/DEX, and RPMI8226/L-PAM, the 50% inhibitory concentration values of which were 77-, 58-, 79-, and 30-fold higher than their parental cell lines, respectively. The resistant cell lines overexpressed MDR1 and survivin, or showed decreased Bim expression. These results indicated that regulating these factors with inhibitors might be a viable approach to increasing the susceptibility of quiescent MM cells to chemotherapy.

1. Introduction

Multiple myeloma (MM) is a hematological cancer characterized by the presence of malignant plasma cells that are predominantly located in the bone marrow. Despite the emergence of new treatments, MM remains an incurable disease with a median survival of 4–5 years. Current treatments are aimed at reducing the malignant cell mass and at overcoming disease-related complications. Although the initial chemotherapy treatments are mostly successful, drug resistance often develops during disease progression, which leads to death. Thus, it is important to identify the mechanism by which cancer cells develop resistance.

Although several prognostic factors have been identified for newly diagnosed myeloma and for patients with relapsed or refractory disease [1,2], the genetic factors that may influence responses to therapy, especially in the latter setting, have been less well defined. One possible factor is the presence of variant multiple drug-resistant (MDR) genotypes, which have been associated with differential expression of the multidrug resistance protein 1 (MDR1) gene and the protein product P-glycoprotein, also known as the ATP-binding cassette transporter B1 (ABCB1). MDR1 is a transmembrane protein that acts as an energy-dependent drug efflux pump for chemotherapeutic drugs that are commonly used against hematologic malignancies [3]. Therapeutic resistance may also be mediated by another MDR-associated protein, also known as ABCC1 (MRP1), ABCG2 (BCRP), and lung resistance protein 1 (LRP1). Like MDR1, it confers resistance to anthracyclines, vinca alkaloids, alkylating agents, and glucocorticoids [4,5]. Moreover, MDR1 overexpression in myeloma cells might contribute to treatment failure in patients receiving proteasome inhibitors [6] since these transporters conferred bortezomib resistance in some pre-clinical models [7,8]. In addition, Bcl-2 and related proteins are key regulators of apoptosis that are expressed in hematologic malignancies [9]. Bcl-2 is known to be constitutively overexpressed in approximately 80% of follicular lymphomas and 20% of diffuse B-cell lymphomas [10,11]. Overexpression of anti-apoptotic family members is associated with apoptosis inhibition and chemotherapy resistance [12–17]. However, the main factors of drug resistance in MM cells remain unidentified.

The aim of the present study was to identify the main factors that are associated with MDR by using established MM cell lines, namely RPMI8226/ADM, RPMI8226/VCR, RPMI8226/DEX, and RPMI8226/L-PAM.

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2. Materials and methods

2.1. Materials

Melphalan was purchased from Sigma (St. Louis, MO, USA), and dissolved in dimethyl sulfoxide (DMSO). Dexamethasone was purchased form Wako (Tokyo, Japan), and dissolved in DMSO. These reagents were dissolved in phosphate buffer saline (PBS; 0.05 M, pH 7.4), filtrated through syringe filters (0.45 μm, IWAKI GLASS, Tokyo, Japan) and used for various assays described below.

Adriamycin and vincristine were purchased from Sigma. Verapamil was purchased form Wako. These reagents were dissolved in phosphate buffer saline (PBS; 0.05 M, pH 7.4), and used for various assays described below.

2.2. Cell culture

MM cell line RPMI8226 was obtained from Health Science Research Resources Bank (Osaka, Japan). The cells were cultured in RPMI1640 medium (Sigma) supplemented with 10% fetal calf serum (Gibco, Carlsbad, CA, USA), 100 μg/ml penicillin (Gibco), 100 U/ml streptomycin (Gibco), and 25 mM HEPES (pH 7.4; Wako) in an atmosphere containing 5% CO_2.

2.3. Establishment of acquired resistance to anti-cancer drugs

Over a period of 6 months, RPMI8226 cells in culture continuously exposed to increasing concentrations of adriamycin, vincristine, dexamethasone, or melphalan. Commencing with IC50 of these drugs for a particular RPMI8226 cells, the exposure dose was progressively doubled every 10–14 days until 7–8 dose doublings had been successfully achieved. The established resistant cell lines were then maintained in continuous culture with maximally achieved dose of anti-cancer drugs that still allowed cellular proliferation, and the resistant phenotype has been stable for at least 1 year under drug-free conditions.

2.4. Trypan blue dye exclusion assay

The effect of various anti-cancer drugs on cell survival/proliferation was determined using the trypan blue dye exclusion assay. Prior to each experiment, cells (3×10^3 cells/well) were plated onto 96-well plates. After culturing for 24 h, the cells were exposed to anti-cancer drugs for various times. Equal volumes of cell suspension and 0.4% trypan blue solution were mixed gently, loaded into a hemocytometer, and the viable cells (unstained) and dead cells (stained blue) were counted. Each experiment was performed in triplicate. Results are reported from an average of at least 5 independent experiments.

2.5. Annexin-V apoptosis assay

Apoptosis was measured using an Annexin V-FITC apoptosis detection kit (Becton Dickinson, Bedford, MA, USA), according to the manufacturer's protocol. In brief, cells were washed twice in PBS and then resuspended in binding buffer containing Annexin V-FITC and PI. The cells were incubated for 15 min at room temperature and then analyzed using a BD-LSR (Becton Dickinson) flow cytometer.

2.6. Measurement of caspase-9, caspase-8, and caspase-3 proteolytic activities

Caspase-9, caspase-8, and caspase-3 enzyme activities were measured by proteolytic cleavage of the fluorogenic substrate LEHD-AFC, IETD-AFC, and DEVD-AFC using the Caspase-9 Fluorometric Assay Kit, Caspase-8 Fluorometric Assay Kit, and Caspase-3 Fluorometric Assay Kit (BioVision Inc., CA, USA). Cells were treated with adriamycin, vincristine, dexamethasone, and melphalan for 48 h. The cells were collected, washed in PBS, and lysed in lysis buffer provided by the kit. For the assay, a solution of cell lysates containing 50 μM substrate was incubated at 37 °C for 1 h. The release of AFC from the substrate was measured fluorimetrically using a fluorescence spectrophotometer (F-4010, Hitachi, Tokyo, Japan) with an excitation wavelength of 400 nm and an emission wavelength of 505 nm. The results were corrected for protein content of the lysates and are expressed as the change in proteolytic cleavage of the substrate (pM) for 1 h per mg protein. The protein content of the cell lysate was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA).

2.7. Western blotting

Cells treated under various conditions were lysed with a lysis buffer containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 100 mM NaF, 1% NP-40, 1 μg/ml leupeptin, 1 μg/ml antipain, and 1 mM phenylmethylsulphonyl fluoride (PMSF). The protein content in the cell lysates was determined using a BCA protein-assay kit. The extracts (40 μg of protein) were fractionated on polyacrylamide-SDS gels and transferred to PVDF membranes (Amersham). The membranes were blocked with a solution containing 3% skim milk and incubated overnight at 4 °C with each of the following antibodies: anti-MDR1 antibody, anti-BCRP antibody, anti-MRP1 antibody, amti-LRP1 antibody, anti-Bcl-2 antibody, anti-Bcl-xL antibody, anti-survivin antibody, anti-Bim antibody, and anti-Bax antibody (Santa Cruz Biotechnologies, CA, USA). Subsequently, the membranes were incubated with horseradish peroxidase-coupled anti-rabbit IgG sheep antibodies (Amersham) for 1 h at room temperature. The reactive proteins were visualized using ECL-plus (Amersham) according to the manufacturer's instructions. As an internal standard, anti-β-actin mouse monoclonal antibody (Sigma) was used as the primary antibody to detect β-actin protein.

2.8. Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated using TRIZOL (Invitrogen; Carlsbad, CA). One microgram of purified total RNA was used for the real-time PCR analysis with the SuperScript First-Strand Synthesis System (Invitrogen). cDNA was subjected to quantitative real-time PCR by using SYBR Premix Ex *Taq* (Takara Biomedical; Siga, Japan) and the ABI Prism 7000 detection system (Applied Biosystems; Foster, CA) in a 96-well plate according to the manufacturer's instructions. The PCR conditions for GAPDH, MDR1, survivin, and Bim were 94 °C for 2 min; followed by 40 cycles of 94 °C for 0.5 min, 50 °C for 0.5 min, and 72 °C for 0.5 min. The following primers were used: MDR1, 5′-GGC TCC GAT ACA TGG TTT TCC-3′ (5′-primer) and 5′-TTC AGT GTG CGA TCT TCC CAG C-3′ (3′-primer); survivin, 5′-CTG CCT GGC AGC CCT TTC TCA A-3′ (5′-primer) and 5′-AAT AAA CCC TGG AAG TGG TGC A-3′ (3′-primer); Bim, 5′-ATG GCA AAG CAA CCT TCT GA-3′ (5′-primer) and 5′-CGC ATA TCT GCA GGT TCA GCC-3′ (3′-primer); and GAPDH, 5′-GAC ATC AAG AAG GTG GTG AA-3′ (5′-primer) and 5′-TGT CAT ACC AGG AAA TGA GC-3′ (3′-primer). As an internal control for each sample, the GAPDH gene was used for standardization. Cycle threshold (Ct) values were established, and the relative difference in expression from GAPDH expression was determined according to the $2^{-\Delta\Delta Ct}$ method of analysis and compared to the expression in control cells.

2.9. RNA interference

The double-stranded survivin small interfering RNAs (siRNAs; HSS179403) were synthesized and purified by Invitrogen. Stealth™ RNAi negative control duplex (low GC content) (Invitrogen) was used as a negative control. Transfection of siRNAs was performed according to the manufacturer's protocol by using the LipofectAMINE™ 2000 reagent (Invitrogen). Briefly, 4 μl of 20 μM siRNA was mixed with 200 μl of Opti-MEM. LipofectAMINE™ 2000 (4 μl) was diluted in 200 μl of Opti-MEM and incubated at room temperature for 5 min. After incubation, the diluted LipofectAMINE™ 2000 was mixed with the diluted siRNA and further incubated for 20 min at room temperature. Total 400 μl of the siRNA-LipofectAMINE™ 2000 complex was applied to each well of the cultured cells at approximately 50–70% confluence in 6-well microplates.

2.10. Statistical analysis

All results are expressed as means and S.D. of several independent experiments. Multiple comparisons of the data were done by ANOVA with Dunnet's test. p values less than 5% were regarded as significant.

3. Results

3.1. Sensitivity of anti-cancer MDR cell lines to cytotoxic agents

No significant difference in the growth of RPMI8226 cells was observed compared to that of RPMI8226/ADM, RPMI8226/VCR, RPMI8226/DEX, or RPMI8226/L-PAM cells (Fig. 1A–D). We also found that adriamycin, vincristine, dexamethasone, and melphalan did not induce cell death in anti-cancer drug-resistant cell lines (Fig. 1A–D). Fig. 1E–H shows the growth inhibitory effect of adriamycin, vincristine, dexamethasone, and melphalan on the parent RPMI8226 cell line and its resistant sublines RPMI8226/ADM, RPMI8226/VCR, RPMI8226/DEX, and RPMI8226/L-PAM. The IC_{50} values of adriamycin, vincristine, dexamethasone, and melphalan in RPMI8226 cells were 0.356 μM, 6.113 nM, 5.530 μM, and 1.851 μM, respectively, compared to 27.524 μM, 356.56 nM, 439.62 μM, and 55.71 μM in RPMI8226/ADM, RPMI8226/VCR, RPMI8226/DEX, and RPMI8226/L-PAM cells (77-fold, 58-fold, 79-fold, and 30-fold higher resistance, respectively).

Next, we investigated whether anti-cancer drugs induce apoptosis and activate caspases in anti-cancer drug-resistant cell lines. In parent cells, anti-cancer drugs induced apoptosis and activated caspases, but these events did not occur in the resistant sublines RPMI8226/ADM, RPMI8226/VCR, RPMI8226/DEX, and RPMI8226/L-PAM (Fig. 2A and B).

The RPMI8226/ADM, RPMI8226/VCR, RPMI8226/DEX, and RPMI8226/L-PAM cells exhibited cross-resistance to other