P-Glycoprotein Expression in Plasma-Cell Myeloma Is Associated With Resistance to VAD

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Tumor cell-associated expression of multidrug resistance (MDR) was quantitated in 22 patients with DNA-aneuploid myeloma using 2-parameter flow cytometry with monoclonal antibody (MoAb) C-219 for the detection of cytoplasmic p-170 and propidium iodide for nuclear DNA content. The proportion of cells expressing p-170 and the intensity of p-170-related fluorescence were determined for each patient. Among the 14 patients treated with vincristine-adriamycin-dexamethasone (VAD), the proportion of p-170-positive cells distinguished sensitive from resistant disease (P < .01). Among a subgroup of seven patients with MDR analysis available prior to VAD therapy, two subsequent nonresponders had high proportions of C-219–reactive cells. The presence de novo of high proportions of p-170–expressing cells in another still untreated patient and in a further individual with resistance to dexamethasone and interferon (not associated with MDR) warrants systematic analysis of p-170 expression prior to therapy to determine its clinical implications for response to MDR-associated drugs as combined in the VAD regimen. Concurrent MDR expression by aneuploid tumor cells and cells in the diploid subcompartment may represent involvement of diploid cells in the myeloma disease process.

METHODS

Patients. Twenty-two patients with a diagnosis of DNA-aneuploid plasma-cell myeloma were selected for study (Table I). Response to the VAD regimen (with continuous infusions of vincristine and doxorubicin and pulses of high-dose dexamethasone) was defined as ≥75% tumor cytodestruction in patients with serum myeloma protein and disappearance of Bence Jones proteinuria. After obtaining consent, heparinized marrow aspirates were subjected to Ficoll-Hypaque density separation (SD 1.077 g/mL). Aliquots of the mononuclear cells were analyzed by FCM for cytoplasmic immunoglobulin and tumor-cell DNA ploidy. The remaining sample was fixed in 70% methanol at −20°C for at least 18 hours and stored at −20°C for up to 1 week.

Cell lines. The myeloma cell line RPMI 8226 and two adriamycin-resistant variants, 8226/R6 and 8226/R40, were obtained from Dr. V. Ing, Ontario. A third variant, resistant to 1 µg/mL adriamycin (8226/R1µg), was derived from 8226/R40 by culturing in the presence of 1 µg/mL of the drug. These cell lines were routinely cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and periodically tested for resistance to adriamycin. The human lymphoid cell line CCRF-CEM and its vinblastine-resistant variant VLB1000 (resistant to 1,000 ng/mL) were obtained from Dr. V. Ling, Ontario. VLB1000 cells were maintained in media containing 1 µg/mL vinblastine. Exponentially growing cells were used for all experiments.
**Flow cytometry.** Methanol-fixed cells were washed twice in phosphate-buffered saline (PBS), resuspended in 100 μL of C-219 MoAb diluted to 10 μg/mL in PBS containing 1% normal rabbit serum (PBS/NRS). Following one-hour incubation at 4°C with periodic gentle shaking and two washes with PBS + 1% BSA (PBS/BSA), cells were reacted for one hour at 4°C with 100 μL of FITC-conjugated rabbit antimouse IgG serum (Dako) diluted 1:50 in PBS/NRS, subsequently washed in PBS/BSA then in PBS, after which the cells were incubated 30 minutes at 37°C with 350 U RNase (Worthington) in 800 μL PBS. Finally, propidium iodide was added to a final concentration of 20 μg/mL, and 10,000 cells were analyzed with an EPICS V flow cytometer (Coulter Electronics, Hialeah, FL). A matching control was prepared for each patient by incubating cells with isotype-matched normal mouse immunoglobulin (IgG2a) instead of the C-219 antibody. The human cell line VLB100, resistant to 1,000 ng/mL of vinblastine, served as positive control. Spheres (2% maximum fluorescence) were used as a calibration standard. Specificity of the reaction was confirmed by analyzing sensitive and resistant myeloma cells with a second MoAb, MRK-16, which recognizes the surface domain of p-170 (kindly provided by Dr Tsuruo) and by immunoblotting. Staining with MRK-16 was similar to that with C-219 except that fresh, unfixed cells were used. MRK-16 was diluted to 30 μg/mL in PBS containing 4% human donor serum and, following secondary antibody reaction, the cells were fixed for one hour in 70% ice-cold ethanol. The cells were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountainview, CA).

**Immunoblotting.** Cells (4 × 10^6) were washed twice in PBS and extracted with 0.5 mL extraction buffer. Equal volumes (18 μL) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted to nitrocellulose membranes using BioRad's Mini Proton II and Mini Trans Blot apparatus according to the manufacturer's instruction. P-glycoprotein was detected with C-219 MoAb using a Protoblot Western Blot AP System (Promega, Madison, WI) according to kit instructions.

**Data analysis.** The proportion of tumor cells expressing p-170 was calculated by determining the proportion of cells with aneuploid DNA content that had p-170-related (green) fluorescence above that of matching control (specific fluorescence). The level of p-170 expression was determined from the intensity of specific p-170-related fluorescence in resistant cells was 4.7-fold higher than in sensitive cells. Panel A illustrates control cells reacted with isotype-matched mouse immunoglobulin.

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**Figure 1.** Flow cytometric determination of p-170 expression in CEM cells sensitive (B) or resistant to vinblastine (C). P-170-related fluorescence in resistant cells was 4.7-fold higher than in sensitive cells. Panel A illustrates control cells reacted with isotype-matched mouse immunoglobulin.

**Figure 2.** P-glycoprotein expression in adriamycin-resistant myeloma cells. RPMI 8226 cells were reacted with MoAb C-219 (panel A) or MRK-16 (panel B) as described in Methods and analyzed on a FACScan flow cytometer. 1: RPMI 8226 parent cells. 2-4: variants resistant to: 2 = 8 × 10^-8 mol/L (8226/R8), 3 = 4 × 10^-7 mol/L (8226/R40) and 4 = 1 μg/mL adriamycin.
related fluorescence on a 64-channel linear scale and was expressed as median fluorescence channel number of p-I70+ cells. Proportion of p-I70+ cells and intensity of specific p-I70-related fluorescence were determined for aneuploid myeloma tumor cells in each specimen, for the residual diploid cells, and for diploid \( G_{0/1} \) cells in remission and normal donor bone marrows.

RESULTS

An example of p-I70 FCM analysis is illustrated in Fig 1. The vinblastine-resistant VLB,000 cell line showed 4.6-fold higher p-I70 expression than its vinblastine-sensitive parent CEM. There was marked heterogeneity in p-I70 expression in both the resistant and parent cells. Similar heterogeneity in p-I70 expression was seen in the myeloma cell line RPMI 8226 and its adriamycin-resistant variants (Fig 2). The reactivity patterns of these cell lines with the two probes, C-219 and MRK-16, were remarkably similar and correlated directly with the level of drug resistance. Specificity and sensitivity of the antibody reaction and its quantitative reliability were also demonstrated by immunoblotting (Fig 3).

Heterogeneity in p-I70 expression was also observed in clinical samples, which is illustrated in Fig 4 for a case of hyperdiploid myeloma, demonstrating high levels of p-I70-related fluorescence. In this, as in several other cases with high p-I70 expression by aneuploid cells, the diploid cell subcompartment also showed marked p-I70 expression. There was a weak correlation between the proportion of aneuploid cells expressing p-I70 and the levels of p-I70 expression (Fig 5; \( r = .4 \), \( P = .1 \)). The frequency of p-I70-positive cells and the relative level of p-I70 expression were further examined in relationship to normal donor and remission marrow from myeloma patients. Among patients receiving VAD, markedly higher values in resistant compared with responding patients were observed for the proportion of p-I70-positive cells (\( P = .006 \)), while no significant difference was seen for the intensity of p-I70 expression (Fig 6). Similar high values were observed in three patients not previously exposed to MDR-inducing drugs, including one untreated patient with resistance to subsequent VAD, one among four still untreated patients, and a third patient with established resistance to interferon-alpha and dexamethasone. Seven morphologically normal bone marrows displayed low p-I70-related fluorescence intensity.

The presence of p-I70-positive diploid cells in aneuploid myeloma was further examined. As with the aneuploid tumor cell compartment, there were also higher proportions of p-I70-positive diploid cells in VAD-resistant \( v \) VAD-sensitive disease (medians of 50 vs 3%, \( P < .001 \)). The proportion of p-I70+ aneuploid and diploid cells were strongly correlated (\( r = .96 \), \( P < .0001 \); Fig 7). P-glycoprotein-expressing diploid cells did not express typical myeloma phenotypic features such as clg or the plasma cell antigen R1-3.26

DISCUSSION

While a well-established phenomenon in cell lines selected for resistance to high concentrations of certain drugs, the MDR phenotype has not yet been recognized as a major mechanism for clinical drug resistance. A number of problems have interfered with the assessment of the clinical
relevance of MDR, including (1) the complexity of treatment protocols employing usual combinations of MDR-related and MDR-unrelated drugs; (2) the difficulty in quantitating accurately tumor response to treatment; and (3) the lack of methods for quantitating p-170 expression on a per-tumor-cell basis in often heterogeneous clinical tissue samples. With DNA aneuploidy as a tumor cell marker in 2-parameter FCM analysis, marked heterogeneity in p-170 expression by tumor cells of individual patients was observed. Among the parameters examined, the proportion of p-170-positive cells correlated well with clinical response to VAD and may prove to be a suitable discriminator for prediction of initial response. While three available remission marrows with <1% aneuploid tumor cells revealed low MDR expression, we observed one patient with less than 75% cytoreduction from VAD who demonstrated high MDR expression (94% p-170+ among 10% residual aneuploid cells: data not shown). This observation suggests the potential usefulness of serial studies in patients undergoing VAD therapy to determine whether persistence or development of high p-170 expression by residual tumor cells confers a poor prognosis and hence requires alternative therapy.

Compared with the proportion of cells with p-170 expression, the intensity of p-170 fluorescence did not provide discrimination between VAD-sensitive and VAD-resistant patients, probably indicating that low levels p-170 expression are sufficient for the manifestation of clinical drug resis-

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**Fig 5.** Relationship between the proportion of aneuploid tumor cells expressing p-170 and the intensity of p-170-related fluorescence in these cells (6). The two parameters showed a weak correlation ($r = .4, P = .01$) suggesting a low degree of interdependence. Also shown for comparison are diploid cells from four normal donor marrows (ci).

**Fig 6.** MDR expression in aneuploid myeloma and in normal marrow. (A) Percent p-170+ expressing cells; (B) intensity of p-170-related fluorescence. Information on treatment and response is also provided. Patients were studied either prior to (c) or after treatment (6). Note difference in percentage of p-170-positive cells (A) between VAD-sensitive and VAD-resistant myeloma (left panel). High proportions of p-170+ cells were observed in two patients de novo and in a third with resistance to IFN plus dexamethasone (middle panel). Three patients were studied while in remission from VAD therapy.

**Fig 7.** Strong correlation between MDR expression by aneuploid tumor and residual diploid cells. For each patient, % p-170+ expressing aneuploid tumor cells and cells in the diploid cell compartment were determined ($r = .96, P < .0001$).
tance. Expression of p-170 de novo without prior exposure to agents known to induce the MDR phenotype suggests that FCM may be useful in identifying patients prior to therapy who are likely to have VAD-resistant myeloma.

The concurrent expression of MDR in diploid and aneuploid cells of patients with high MDR levels is reminiscent of observations with CALLA26 and H-ras p21,27 supporting further the notion of diploid tumor cells at an earlier stage of maturation,28 lacking typical phenotypic characteristics of myeloma cells (eg, clg, R1-3).

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