Genotypic and Phenotypic Comparisons of *de Novo* and Acquired Melphalan Resistance in an Isogenic Multiple Myeloma Cell Line Model

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ABSTRACT

Cancer cell adhesion confers a transient, *de novo* drug-resistant phenotype referred to as cell adhesion-mediated drug resistance (CAM-DR). In this report, we extend the CAM-DR phenotype to primary specimens from patients with myeloma, providing further evidence that CAM-DR is a viable clinical form of drug resistance. To examine mechanisms of cellular resistance to melphalan, we compared genotypic and phenotypic profiles of acquired and *de novo* melphalan resistance in an isogenic human myeloma cell line. Acquired melphalan resistance (8226/LR5) was associated with decreased drug-induced DNA damage and a complex gene expression profile showing that genes involved in the Fanconi anemia DNA repair pathway are increased in the LR5 cells compared with drug-sensitive or adherent cells. In contrast, cells adhered to fibronectin accumulate similar amounts of DNA damage compared with drug-sensitive cells but are protected from melphalan-induced mitochondrial perturbations and caspase activation. Levels of the proapoptotic protein Bim were significantly reduced in adherent cells. Gene expression changes associated with *de novo* resistance were significantly less complex compared with acquired resistance, but a significant overlap in gene expression was noted involving cholesterol synthesis. We propose that myeloma cell adhesion promotes a form of *de novo* drug resistance by protecting cells from melphalan-induced cytotoxic damage and that this transient protection allows cells to acquire a more permanent and complex drug resistance phenotype associated with a reduction in drug induced DNA damage.

INTRODUCTION

Multiple myeloma is a disease that typically responds to initial treatment; however, the disease is not cured by chemotherapy, and invariably drug resistance emerges (1, 2). Traditional *in vitro* unicellular models of melphalan resistance have identified several acquired melphalan resistance mechanisms, including (*a*) reduced drug uptake, (*b*) reduced DNA damage, and (*c*) changes in glutathione levels (1, 3, 4). However, it is unclear at present whether these mechanism(s) play a causative role in clinical drug resistance. Moreover, it is not known whether drug resistance mechanisms identified after chronic drug exposure (acquired drug resistance) allow for tumor cell survival after initial drug treatment (*de novo* drug resistance).

Evidence supporting the importance of understanding the influence of the tumor microenvironment on drug sensitivity has been reported by Teicher *et al.* (5). These investigators showed that *in vivo* selection of EMT-6 cells with alkylating agents produce a drug-resistant phenotype that is operative only *in vivo*. The tumor microenvironment consists of soluble factors (cytokines) as well as cell surface receptors (cell adhesion molecules), both of which can influence cellular fate

after cytotoxic exposure. More recently, our laboratory and others have shown that adhesion of tumor cell lines to FN[1] via β1 integrins contributes to a reversible, *de novo* drug resistance termed CAM-DR (6–12). Adhesion via β1 integrins is known to activate a network of signal transduction pathways that influence cell survival, growth, and differentiation (13–16). Although the signaling pathway(s) causative for drug resistance have not been entirely delineated, several intracellular targets have been identified that are influenced by β1-integrin adhesion and may contribute to inhibition of programmed cell death induced by either cytotoxic drugs or cell surface death receptors (*e.g.,* CD95). These targets include the following: alterations in the nuclear pool of topoisomerase IIβ, increased p27kip1 levels, and changes in the availability of Flip$_1$ binding to Fas-associated death domain (7, 8, 17). All of these changes occur before toxic or stressful insult; we therefore propose that cell adhesion to FN predisposes cells to be resistant to apoptosis and that this condition represents a form of *de novo* drug resistance.

In this report, we compared *de novo* and acquired resistance to melphalan-induced cell death in the human myeloma cell line, RPMI 8226. More specifically, we compared functional resistance mechanism(s) and corresponding gene expression profiles associated with acquired and *de novo* melphalan resistance. The functional endpoints measured included (*a*) levels of resistance compared with drug sensitive cells, (*b*) relative melphalan-induced interstrand cross-links, (*c*) mitochondrial depolarization, and (*d*) activation of effector caspases. Our findings show that acquired resistance to melphalan functionally correlates with reduced melphalan induced interstrand cross-links and a complex array of gene expression changes involving DNA repair genes, cell cycle checkpoints, transporters, detoxifying molecules, and apoptotic genes. By comparison, myeloma cells adhered to FN exhibit a reversible resistance to melphalan-induced death by reducing melphalan-induced mitochondria depolarization and caspase activation. However, in contrast to cells with acquired melphalan resistance, no changes in melphalan-induced cross-links were observed in FN-adhered cells compared with drug-sensitive cells. Changes in the transcriptsome when 8226 myeloma cells were adhered to FN were less complex than cells with acquired melphalan resistance; however, significant similarities in gene expression profiles were observed between cells with *de novo* and acquired melphalan resistance. The most striking similarity in gene expression profiles was the up-regulation of molecules involved with cholesterol synthesis. We propose that the changes in gene expression profile noted for *de novo* drug resistance (associated with FN adhesion) represent genomic changes that predispose cells to survive initial drug exposure and ultimately acquire a complex melphalan-resistant phenotype.

MATERIALS AND METHODS

Cell Culture. The 8226 human multiple myeloma cell line was obtained from the American Type Culture Collection (Rockville, MD) and maintained as described previously (6). The 8226 myeloma-resistant cell line, 8226/LR5,

[1] The abbreviations used are: FN, fibronectin; CAM-DR, cell adhesion-mediated drug resistance; FANC, Fanconi anemia; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA.

was passaged weekly in medium containing 5 μM melphalan (3). Cell adhesion experiments were conducted as described previously (6, 7).

Drugs and Antibodies. Melphalan was obtained from Sigma (St. Louis, MO), and stock solutions were dissolved in acid–ethanol. Antibodies to caspase-8, -7, and -9 were obtained from Cell Signaling (Beverly, MA), antibodies to caspase-3 were kindly provided by H-G. Wang (H. Lee Moffitt Cancer Center, Tampa, FL; Ref. 18), antibodies to Bim were obtained from Calbiochem (La Jolla, CA), and antibodies to β-actin were obtained from Sigma.

Melphalan-Induced Apoptosis and Mitochondrial Perturbations. After 24 h of adhesion to FN, cells were exposed to drug for 2 h, and extracellular drug was removed with two washes of RPMI containing 5% fetal bovine serum. Annexin V staining was used to measure apoptotic cells after drug exposure (24 h later) as described previously (6). The means and SDs from a representative experiment performed in triplicates are shown. Mitochondrial integrity was analyzed by flow cytometry using $DiOC_6$ staining (Molecular Probes) as described previously (17). Pairwise statistical comparisons were performed by Student's t test ($n = 9$/group).

Western Blot Analysis of Pro- and Cleaved Caspases. Cells grown in suspension or adhered to FN were treated with melphalan as described above. Four h after drug treatment, samples were washed twice with ice-cold PBS and incubated for 15 min at 4°C in Triton X-100 lysis buffer [30 mM Tris-HCl (pH 7.5), 137 mM NaCl, 25 mM NaF, 1% Triton X-100, 15% glycerol, 2 mM sodium orthovanadate, 25 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml pepstatin A]. Protein lysates were quantified with Bio-Rad reagent, and 30–60 μg of cellular lysates were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membrane. Protein levels were examined with antisera specific to caspase-8, -7, -9, and -3, and β-actin and visualized with Lumi-Light chemiluminescence (Roche, Indianapolis, IN).

Melphalan-Induced Apoptosis of Patient Specimens. To determine whether CAM-DR occurred in patient specimens as well as myeloma cell lines, we developed a double-immunofluorescence assay to simultaneously detect apoptotic plasma cells. After obtaining Institutional Review Board consent, we isolated mononuclear cells from bone marrow aspirates obtained from patients with stage II and III myeloma by Ficoll-Hypaque centrifugation and placed them in αMEM medium. We used 1×10^6 cells/ml and adhered them to FN or placed them in suspension (0.1% polyHEMA-coated wells) for 2 h in serum-free αMEM medium. After 2 h of adhesion, fresh medium containing 15% fetal bovine serum was added, and cells were incubated for an additional 12–16 h. After overnight adhesion, cells were treated with 200 μM melphalan for 2 h, extracellular drug was removed, and cells were maintained in drug-free medium for an additional 24 h before fixation. Plasma cells were identified by positive κ or λ staining (Vector Laboratories, Burlingame, CA), and apoptotic plasma cells were identified by terminal deoxynucleotidyltransferase-mediated nick end labeling analysis labeling using a commercially available kit (Intergen Company, Purchase, NY). Fluorescence microscopy (Vysis, Downers Grove, IL) was used to count 500 total plasma cells/slide. Results after cell adhesion were compared with mononuclear cells exposed to melphalan in suspension culture.

Alkaline Comet Assay. The alkaline comet assay was used to detect melphalan-induced DNA cross-links in 8226 myeloma cells. Cells cultured in suspension or adhered to FN were treated with various doses of melphalan or vehicle control for 2 h. After drug treatment, single-strand breaks were induced, by irradiating appropriate samples at 900 rad (MARK I model 68A irradiator). After drug treatment and irradiation, 5000 cells were placed in a microcentrifuge tube containing 1 ml of cold PBS, and the alkaline comet assay was performed as described by Kent *et al.* (19). Fifty images were randomly captured per slide, and images by fluorescence microscopy were quantified using Optimus software as described previously (8, 19).

The percentage of cross-linking was calculated as follows:

Relative cross-linking

$$= [1 - (\text{comet moment}_{\text{drug treated}} - \text{comet moment}_{\text{control}})/$$

$$\text{comet moment}_{\text{900 rad}} - \text{comet moment}_{\text{control}})] \times 100$$

The data shown are the means of three independent experiments ($n = 50$ images for each dose of each independent experiment). An ANOVA model was used to quantify the relationship between the response variable and the two independent variables.

Microarray Analysis. Cells were adhered to FN or grown in suspension for 24 h as described previously (6). RNA was isolated by RNeasy columns according to the manufacturer's instructions (Qiagen). Double-stranded cDNA was prepared with the Life Technologies, Inc. Superscript system using T7-$(dT)_{24}$ primers to prime the first strand synthesis. cRNA was synthesized and labeled with biotin by *in vitro* transcription using the Enzo Bioarray high-yield RNA transcript labeling kit. Control oligonucleotides BioB, BioC, BioD, and Cre, prokaryotic labeled RNAs, were added to the sample, and hybridization was carried out for 14–16 h. After hybridization, the GeneChip arrays (Affymetrix HG-133A) were washed and stained with phycoerythrin-conjugated biotin. Chips were subsequently scanned at 570 nm with a GeneChip System confocal scanner. Scanned output files were visually inspected for hybridization artifacts and then analyzed by Affymetrix Microarray Suite 5.0 software.

Signal intensity was scaled to an average intensity of 500 before comparison analysis. The MAS 5.0 software uses a statistical algorithm to assess increases or decreases in mRNA abundance in a direct comparison between two samples. This analysis is based on the behavior of 11 different oligonucleotide probes designed to detect the same gene. With the programmed default values, probe sets that yielded a change with $P < 0.004$ were identified as changed (increased or decreased), and those that yielded a change with $P = 0.004–0.006$ were identified as marginally changed. The data were additionally screened with use of the calculated signal intensities to include only those probe sets for which the change in signal intensity correlated with the change identified by the MAS 5.0 software. Four independent experiments were performed, and gene lists were further trimmed to only contain genes that performed similarly in at least three of the four experiments performed. Finally, the master lists for both FN-adhered and LR5 cells were used to determine genes that were similarly changed in the acquired and *de novo* drug resistance model.

RESULTS

Adhesion of the 8226 Myeloma Cell Line to FN Inhibits Melphalan-Induced Apoptosis. Annexin V positivity was used to detect apoptotic cells after melphalan treatment of the parental RPMI 8226 myeloma cell line (8226/Sus), the parental 8226 cell line adhered to FN (8226/FN), and the melphalan-selected 8226 cell line in suspension (8226/LR5). These experiments allowed us to compare resistance levels associated with *de novo* and acquired resistance in an isogenic model system. As depicted in Fig. 1, the parental 8226 cell line adhered to FN and the drug-resistant variant 8226/LR5 cell line were both significantly resistant ($P < 0.05$ at each dose tested, Student's t test; $n = 9$/group) to melphalan-induced cell death compared with the parental 8226 cell line maintained in suspension medium (8226/Sus).
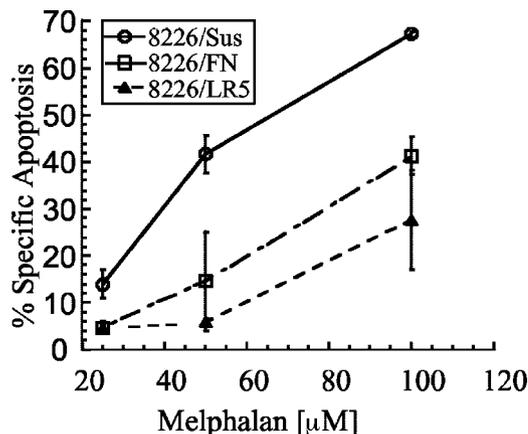

Fig. 1. Parental 8226 cells adhered to FN and the 8226/LR5 cell line are significantly ($P < 0.05$) protected from melphalan-induced apoptosis at all doses tested. Shown are the results of a representative experiment performed in triplicate. The experiment was repeated three times, and similar results were obtained. *Bars*, SD.

These data show that adhered cells and cells with acquired melphalan resistance are similarly protected from melphalan-induced apoptosis.

Adhesion of Myeloma Cells from Patient Specimens to FN Inhibits Melphalan-Induced Apoptosis. To determine whether the CAM-DR phenotype is a potential mechanism of clinical *de novo* drug resistance, we measured melphalan-induced cell death in primary patient specimens. Plasma cells were identified by either positive κ or λ staining, and apoptotic myeloma cells were identified by the terminal deoxynucleotidyltransferase-mediated nick end labeling assay (Fig. 2*A*). Nine of 10 patient specimens were protected from melphalan-induced cell death when attached to FN (Fig. 2*B*). The mean percentage of apoptosis of plasma cells obtained from bone marrow aspirates and adhered to FN before treatment with 200 μM melphalan was 27.7% (95% confidence interval, 17.6–32.8%) compared with 55.6% (95% confidence interval, 44.1–67.2%) apoptosis for plasma cells maintained in cell suspension ($P < 0.05$, Student's paired t test). These data further support the potential role of FN-mediated adhesion in mediating clinical *de novo* drug resistance.

DNA Cross-Links Are Reduced in Cells with Acquired Drug Resistance but Not Adherent Cells. Our laboratory previously reported, using the alkaline elution assay, that melphalan resistance in the cell line with acquired drug resistance (8226/LR5) correlated with reduced DNA cross-links (3). In this study, the alkaline comet assay was used to determine whether cellular adhesion also reduced the amount of melphalan-induced DNA-cross-links. The comet assay indicated that melphalan-induced cross-links were significantly reduced in the drug-selected 8226/LR5 cell line (Fig. 3*A*; ANOVA $P < 0.05$); the means, SDs, and confidence intervals for the results represented by the data points in Fig. 3 are given in Table 1. In contrast, cells adhered to FN accumulated similar numbers of melphalan-induced cross-links (ANOVA, $P > 0.05$) compared with 8226 cells treated in suspension (Fig. 3*B* and Table 1). These data suggest
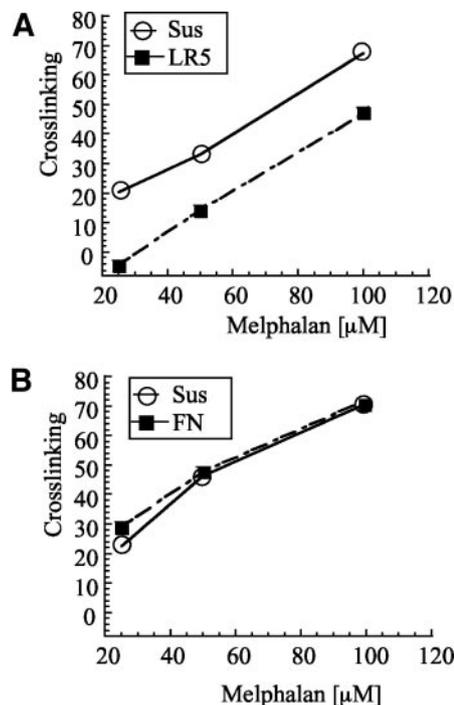
Fig. 3. *A*, the alkaline comet assay was used to detect melphalan-induced cross-links. Similar to the results we reported previously (3) with the alkaline elution technique, the 8226/LR5 cell line (■) showed significant reductions (for testing cell line differences: $P < 0.05$, ANOVA) in the relative number of melphalan cross-links compared with the parental cell line (Sus; ○). Shown is the ANOVA-based mean value from three independent experiments. *B*, 8226 cells adhered to FN showed no alterations in melphalan-induced cross-links compared with cells treated in suspension (for testing cell line differences: $P < 0.05$, ANOVA). Shown is the ANOVA-based mean value from three independent experiments. The means, SDs, and confidence intervals for these results are given in Table 1.

Fig. 2. *A*, dual-staining immunofluoroscopy was used to identify apoptotic myeloma cells in patient bone marrow specimens. Myeloma cells are stained *red*, and apoptotic nuclei are *green*. *B*, for each patient sample 500 plasma cells were scored for apoptosis. Nine of 10 patient samples tested exhibited the CAM-DR phenotype.

Table 1 *ANOVA-based mean (SE) values and 95% confidence intervals for comet assays showing results for suspended cells compared with the drug-selected (LR5) and FN-adherent resistant cell lines*

Shown are results for three independent experiments.

Dose (μM)	Suspended cells *vs.* drug-selected cells		Suspended cells *vs.* FN-adherent cells	
	8226/LR5	8226/SUS[a]	8226/FN	8226/SUS
25				
Mean (SE)	−4.49 (16.687)	20.31 (9.579)	29.11 (0.888)	22.40 (9.458)
95% confidence interval	−37.19 to 28.22	1.53–39.08	27.27–30.86	3.86–40.93
50				
Mean (SE)	14.02 (8.648)	33.12 (6.218)	47.73 (2.141)	46.27 (5.850)
95% confidence interval	−2.93 to 30.97	20.93–45.30	43.53–51.93	34.80–57.73
100				
Mean (SE)	47.32 (9.121)	67.31 (4.110)	72.03 (2.09)	70.67 (6.175)
95% confidence interval	29.44–65.20	59.25–75.36	67.93–76.12	58.57–82.77

[a] SUS, suspended cells.

that antiapoptotic target(s) associated with resistance in FN-adhered cells reside downstream of the formation of melphalan-induced DNA cross-links.

Adhesion to FN Protects Cells from Melphalan-Induced Mitochondrial Depolarization and Activation of Effectors Caspase-3, -7, and -9. To determine the molecular ordering of resistance associated with CAM-DR, we investigated events downstream of melphalan-induced interstrand cross-links and determined the effects of cellular adhesion on melphalan-induced mitochondrial depolarization. The cationic dye $DiOC_6$ was used to determine whether adhesion protects against melphalan-induced depolarization of the mitochon-
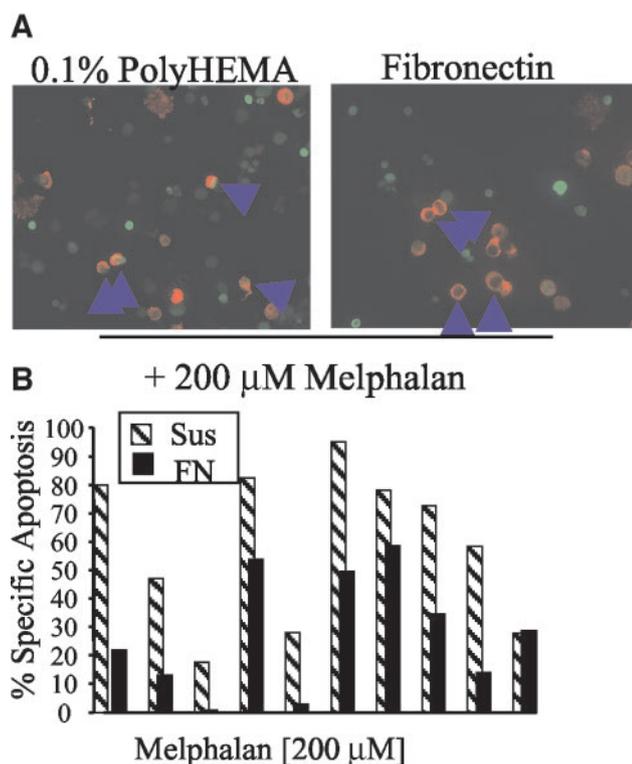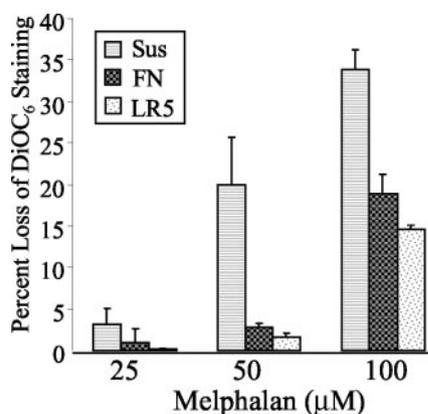
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Fig. 4. Both 8226 cells adhered to FN and the cell line with acquired drug resistance (8226/LR5) were significantly protected from melphalan-induced mitochondrial perturbations ($P < 0.01$, Student's t test) compared with 8226 cells treated in suspension.

drial membrane potential. Adhesion of 8226 cells to FN significantly protected cells from melphalan-induced mitochondrial depolarization (Fig. 4). Similarly, the acquired drug-resistant cell line LR5 was also protected from melphalan-induced mitochondrial perturbations. Caspase activity was measured to confirm that the inhibition of mitochondrial depolarization correlated with the functional inhibition of effector caspases. As shown in Fig. 5, for both the LR5 cells and cells adhered to FN, the melphalan-induced activation of caspase-3, -7, and -9 was reduced compared with drug-sensitive cells in suspension. Taken together, these data indicate that mechanisms underlying adhesion-mediated melphalan resistance occur downstream of DNA damage but before the induction of mitochondria depolarization and activation of effector caspases.

Microarray Analysis. Oligonucleotide microarray analysis was used to identify signal transduction pathways and gene products involved in melphalan resistance. Comparisons between *de novo* resistance associated with cell adhesion and acquired drug resistance were made. Cells maintained in suspension were designated as the reference population, and selected genes were considered to be significantly altered in gene expression when similar results were obtained in at least three of four experiments. On the basis of this selection criterion, 1,764 probe sets (1,479 unique genes) of a total of 22,215 Affymetrix probe sets [HG-133A chip, comprising ~12,000 characterized probe sets and 10,000 expressed sequence tags (ESTs)] were considered changed in the LR5 cell line compared with drug-sensitive 8226/S cells. In comparison, 72 probe sets (69 unique genes) were considered to be significantly changed when 8226/S cells were adhered to FN. Considering the unique differences in the functional phenotype, we noted that of the 72 probe sets that were changed when cells were adhered to FN, 25 of those sets (21 unique genes) were also represented in the cell line with acquired drug resistance. The unique and common lists were then further screened for genes that functionally correlated with the drug-resistant phenotype.

Our previous publication, in combination with the present study, show that drug resistance in the LR5 cell line correlated with (*a*) reduced melphalan-induced interstrand cross-links, (*b*) increased cell doubling time, (*c*) increased glutathione levels, and (*d*) reduced mitochondrial perturbations (3). Accordingly, the master list was screened for genes that would affect DNA repair, drug transport, glutathione metabolism, cell cycle progression, and apoptosis. Table 2 lists the genes of interest and the direction of alteration that were unique for the LR5 cell line. In the LR5 cell line, several changes in genes reported to modulate the recognition and/or repair of DNA interstrand cross-links were observed, including increased expression of FANCF, UVRAG, RAD51 homologue C, and DNA ligase III. On the basis of the gene expression profile, genes involved in the FANCF DNA repair pathway were expressed at higher levels in the LR5 cells compared with drug-sensitive or adherent cells. We previously reported that the LR5 cell line has increased nonprotein sulfhydryl levels, which may detoxify melphalan and contribute to resistance (3). Furthermore, inhibition of γ-glutamylcysteine synthetase by buthionine sulfoximine partially reversed resistance to melphalan in the LR5 cell line (3). Consistent with these phenotypic observations, the gene expression profile of LR5 cells showed increased expression of several genes that regulate *de novo* glutathione synthesis, including increased expression of the catalytic subunit of glutamate-cysteine ligase (20).

In agreement with increased cell doubling time for the LR5 cell line, we observed changes in several genes that participate in G_1-S or G_2-S checkpoints. For example, we observed increased expression of both p27kip1 and p57kip2 in the LR5 cell line. Both of these cyclin-dependent kinase inhibitors can bind and effectively inhibit CDK2 activity, which would delay progression through the G_1 checkpoint (21). The change to the transcriptome of 8226 cells adhered to FN for 24 h is much less complex, with only 69 genes (represented by 72 probe sets) identified by our analysis. The most obvious single gene that might represent a positive functional correlation with the CAM-DR phenotype was repression of Bim (mean 1.4-fold decrease; $n = 4$). Bim is a BH3-only proapoptotic BCL-2 member, and transcriptional repression of Bim could contribute to mitochondrial protection after melphalan insult (22).

Immunoblot analysis of Bim confirmed results of the microarray analysis, with all detectable isoforms of Bim being reduced when 8226 cells were adhered to FN (Fig. 6). This reduction in Bim levels is not a shared property of the acquired drug resistance phenotype (Fig. 6, *Lane 3*) but could provide the transient protection necessary to allow the more permanent resistance to emerge.

Interestingly, 21 of the 72 probe sets changed in cells adhered to FN were similarly altered in the LR5 cell line. On the basis of the number of probe sets that were significantly altered in the LR5 (1764) and the FN sample (72), the predicted number of probe sets to be common by chance alone between the two sets is 5.7 [$(72 * 1,764/22,215) =$ predicted number in common by chance alone]. This common subset of probes (represented by 20 unique genes) is therefore unlikely to have
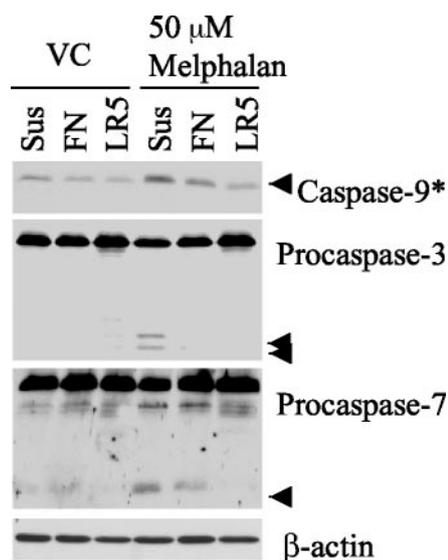

Fig. 5. Both 8226 cells adhered to FN and the cell line with acquired drug resistance (8226/LR5) were protected from melphalan-induced activation of caspase-3, -9, and -7. The experiment was repeated three times; shown is a representative experiment. *VC*, vehicle control. *, cleaved caspase 9.

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Table 2 *Genes that uniquely change in the LR5 cell line compared with the drug-sensitive cells in suspension that could impact DNA repair, glutathione synthesis, melphalan transport, and cell cycle progression*

Shown is the fold change $\pm$ SD as well as the mean signal for each condition ($n = 4$). No significant changes were observed in FN-adherent cells compared with suspended cells in these categories. Method of statistical comparisons is described in detail in the "Materials and Methods."

|	LR5 fold change	Signal		Description
		Sus[a]	LR5	
DNA damage				
	1.76 ± 0.82	229	360	Ligase III, DNA, ATP-dependent
	1.78 ± 0.24	1122	1994	RAD51 homologue C
	2.70 ± 0.25	282	751	FANC, complementation group F
	1.65 ± 0.45	276	449	UV-radiation resistance-associated gene
	0.40 ± 0.17	170	63	BRCA1-associated protein
Cell cycle				
	3.97 ± 3.70	129	545	Cyclin-dependent kinase inhibitor 1C (p57Kip2)
	1.46 ± 0.21	791	1135	Cyclin-dependent kinase inhibitor 1B (p27Kip1)
	0.82 ± 0.25	257	210	Cyclin D1 (parathyroid adenomatosis 1)
	0.85 ± 0.97	438	241	CDC25A
Amino acid transport				
	1.62 ± 0.71	420	632	Putative L-type neutral amino acid transporter
	0.82 ± 0.34	3997	3087	Solute carrier family 7 (cationic amino acid transporter system)
Glutathione				
	1.67 ± 0.40	924	1505	Glutamate-cysteine ligase, catalytic subunit

[a] Sus, suspended cells.
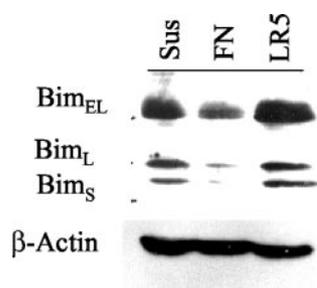
Fig. 6. Cells adhered to FN but not LR5 cells cultured in suspension have decreased protein levels of the proapoptotic molecule Bim. The experiment was repeated three times, and a representative experiment is shown.

occurred by chance ($P < 0.005$, χ^2) and probably represents a common cluster found in both drug-resistant models. The common subset (representing 20 unique genes) contributes to cholesterol metabolism, RNA processing, signal transduction, and cell adhesion. Shown in Table 3 are the subset of common genes that regulate cholesterol synthesis.

DISCUSSION

The failure to eradicate the entire tumor population with subsequent emergence of acquired drug resistance remains an obstacle for the successful treatment of many cancers, including multiple myeloma. In this report we provide evidence that tremendous functional genomic diversity and complexity are associated with acquired melphalan resistance in a isogenic human myeloma cell line. In contrast, *de novo* resistance associated with FN adhesion has a far less complex transcriptional adjustment. Because of this difference in inherent genomic

complexity associated with acquired drug resistance compared with *de novo* drug resistance, we propose that genes and signaling pathways associated with cell adhesion allow myeloma cells to survive initial drug exposure and eventually acquire a complex array of drug resistance mechanisms. As proof of principle for targeting mechanisms associated with CAM-DR, we showed in this report that the CAM-DR phenotype is operative in primary patient specimens. The implication of this model for chemotherapy is that targeting these transient drug resistance mechanisms may prevent acquired drug resistance and increase overall efficacy of drug treatment.

We used a combination of functional and genomic approaches to compare resistance mechanisms associated with *de novo* and acquired melphalan resistance in an isogenic myeloma cell line model. The functional phenotype was used to guide the identification of potentially relevant drug-resistant targets for both *de novo* and acquired melphalan resistance. Microarray analysis of the cell line with acquired melphalan resistance revealed complex changes to the transcriptome that could explain the observed phenotypic alterations. Changes in the 8226/LR5 transcriptome that could explain the decreased formation of melphalan-induced interstrand cross-links included increased expression of FANCF, UVRAG, RAD51 homologue C, and DNA ligase III. Cell lines containing mutations in FANC genes demonstrate hypersensitivity to DNA-damaging agents, including mitomycin C, *cis*-platinum, and nitrogen mustards (23). Our data suggest that the converse is also true, *i.e.,* that overexpression of FANCF may contribute to resistance to cross-linking agents. FANCF is a 42-kDa protein that localizes to the nucleus and has been shown to be part of a multiprotein complex containing FANCA, FANCC, FANCG, and FANCE (24). The formation of this nuclear complex is required for DNA damage-induced monoubiquination of FANCD2 and formation

Table 3 *Genes related to cholesterol metabolism that changed in the same direction in the models for acquired (LR5) and adherent (FN) drug resistance compared with the drug-sensitive cells*

Shown is the fold change $\pm$ SD as well as the mean signal for each condition ($n = 4$). Method of statistical comparisons is described in detail in the "Materials and Methods."

Fold change		Signal			Description
LR5	FN	Sus[a]	LR5	FN	
1.44 ± 0.51	1.53 ± 0.38	897	1240	1350	HMG-CoA reductase
1.96 ± 1.10	1.53 ± 0.53	1001	1671	1390	Squalene epoxidase
2.39 ± 1.83	1.80 ± 0.94	298	547	449	Membrane-bound transcription factor protease site 1
2.14 ± 0.80	1.51 ± 0.45	725	1608	1078	HMG-CoA synthase 1 soluble
3.69 ± 1.83	1.58 ± 0.58	384	1274	562	Lanosterol synthase

[a] Sus, suspended cells.

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of nuclear foci containing FANCD2, BRCA2, BRCA1, and RAD51 (24, 25). Importantly we observed several changes that potentially would converge on the FANC/BRCA pathway, including increased expression of RAD51 homologue C and decreased expression of BRCA1-associated protein. Further studies are warranted to determine the contribution of each gene in conferring the drug-resistant phenotype.

In addition, we observed several changes in cell cycle checkpoints, including increased expression of p27kip1 and p57kip2 and decreased expression of CDC25A. All of these changes are consistent with an increased cell cycle transit time, which we reported previously for the LR5 cell line (3). Further studies are needed to determine whether these cell cycle checkpoints directly impact the FANC/BRCA pathway or represent an independent mechanism contributing to acquired melphalan resistance. Microarray analysis also revealed changes in gene expression that would be predictive for increased synthesis of glutathione. Again this finding is consistent with the previously published functional phenotype showing increased nonprotein sulfhydryl levels (3). Taken together, the genotypic data in the LR5 (acquired melphalan resistance) cell line indicate that multiple changes in the transcriptsome are likely to contribute to the overall drug resistance phenotype.

We noted changes in the expression of several pro- and antiapoptosis genes associated with acquired melphalan resistance. These changes included increased expression of BCL-Xl in the LR5 cell line, a result that was confirmed by RNase protection analysis (RPA) analysis (data not shown). Here it is less clear whether the net differences would be predictive for both cell survival or cell death. These results are similar to the observations of Reinhold *et al.* (26), who studied the topotecan-resistant human prostate cell line DU145. These investigators proposed a two-step model of resistance in which initially antiapoptotic genes emerge, ultimately allowing for changes with dual roles. These dual roles would presumably favor cell cycle progression in the presence of drug, at the expense of some apoptosis. Further analysis is required to determine whether the two-step model applies to melphalan selection.

Parental 8226 cells adhered to FN showed a change in expression of 72 probe sets, which corresponded to 69 unique genes. The most obvious gene that seemed to correlate with drug resistance was *Bim*. Decreased levels of both Bim RNA and protein were observed. Ectopic transfection of Bim is sufficient to induce apoptosis, with the splice variant Bim$_s$ demonstrating the most potent proapoptotic activity. Lymphocytes derived from Bim knockout mice are resistant to dexamethasone, gamma radiation, cytokine withdrawal, and ionomycin, but no changes in apoptosis were observed when cells were treated with either Fas ligand or phorbol 12-myristate 13-acetate (22). Further studies are needed to determine the effect of Bim expression in mediating melphalan-induced cell death.

We recently reported that adhesion of myeloma cells to FN for 8 h resulted in several changes in gene expression predictive of nuclear factor-κB activation, including increased expression of the antiapoptotic gene *cIAP2* (27). We also reported that by 24 h, the elevated levels of cIAP2 protein noted at 8 h returned to baseline values, a finding consistent with our current data showing no change in cIAP2 expression after 24 h of adhesion. Taken together, our data suggest that adhesion of myeloma cells to FN results in temporal changes in the balance of pro- and antiapoptotic genes in favor of cell survival.

Despite phenotypical differences between the models for acquired and *de novo* drug resistance, we observed a significant overlap in changes in the transcriptome. It is attractive to postulate that this fingerprint may be essential for initial survival, allowing for the acquisition of stable drug resistance. One functional gene cluster that coexists between the two resistant models is a change in cholesterol metabolism. The *de novo* and acquired drug resistance models both demonstrated an increase in several enzymes that would positively regulate cholesterol synthesis, including HMG-CoA reductase. HMG-CoA reductase is considered the rate-limiting enzyme in cholesterol synthesis and catalyzes the reduction of HMG-CoA to mevalonate. Inhibitors of HMG-CoA have previously been shown to act synergistically when used in combination with the interstrand cross-linking agent carmustine (28). Furthermore, a doxorubicin-selected 8226 cell line (8226/Dox 40) was ~7-fold more sensitive to HMG-CoA inhibitors (29). Together, these data suggest that HMG-CoA reductase may be an important target for both *de novo* and acquired drug resistance. Further studies are needed to determine whether increased total cholesterol synthesis contributes to the CAM-DR phenotype and predisposes myeloma cells to acquire melphalan resistance.

Our data suggest that adhesion to FN protects myeloma cells from melphalan-induced apoptosis by protecting mitochondria from drug-induced DNA damage and thereby blocking caspase activation. Gene expression profiling of FN-adhered cells indicated that the candidate genes involved in this *de novo* protection of cells include regulation of pro- and antiapoptotic genes in favor of cell survival as well as increased cholesterol synthesis. The mechanism(s) by which cholesterol and cholesterol synthesis may protect against mitochondrial damage need(s) to be determined, but it is interesting that this genetic alteration persisted in cells that acquired a more complex drug-resistant phenotype. Furthermore, we propose that cell adhesion promotes a form of *de novo* drug resistance that predisposes cells to acquire a more permanent and complex form of drug resistance that involves reducing the amount of melphalan-induced DNA damage. Gene expression profile changes that were unique to cells with acquired melphalan resistance included genes involved with DNA repair, especially the FANC pathway and genes associated with detoxification of melphalan. The genotypic changes associated with *de novo* drug resistance are significantly less complex than those for acquired melphalan resistance and suggest that preventing *de novo* melphalan resistance may be more therapeutically rewarding than trying to reverse the multiple mechanisms associated with acquired drug resistance.

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Genotypic and Phenotypic Comparisons of *de Novo* and Acquired Melphalan Resistance in an Isogenic Multiple Myeloma Cell Line Model

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