Cell Adhesion to Fibronectin (CAM-DR) Influences Acquired Mitoxantrone Resistance in U937 Cells

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Abstract

Cell adhesion to fibronectin is known to confer a temporarily related cell adhesion–mediated drug resistance (CAM-DR). However, it is unknown whether cell adhesion during drug selection influences the more permanent form of acquired drug resistance. To examine this question, we compared the acquisition of mitoxantrone resistance in U937 cells adhered to fibronectin versus cells selected in a traditional suspension culture. Our data show that acquired drug resistance levels of resistance to mitoxantrone are 2- to 3-fold greater for cells adhered to fibronectin compared with cells in suspension culture. We also compared mechanism(s) of resistance associated with drug selection in suspension versus fibronectin-adherent cultures. Drug resistance in both suspension and fibronectin-adherent cultures correlated with reduced drug-induced DNA damage and diminished topoisomerase II levels and activity; however, mechanisms regulating topoisomerase II levels differed depending on culture conditions. In suspension cultures, a reduction in topoisomerase II levels was detected at both RNA and protein levels. Furthermore, the decreased expression of topoisomerase II mRNA levels correlated with decreased expression of NF-YA. In contrast, in spite of no changes in NF-YA or topoisomerase II RNA expression, topoisomerase II protein levels were decreased in fibronectin-adherent, drug-resistant cells. In addition, topoisomerase IIα protein levels (but not RNA levels) were reduced in drug resistance cells selected on fibronectin; however, no change in topoisomerase IIα was observed in cells selected with mitoxantrone in suspension culture. Taken together, our results suggest that the development of drug resistance models must consider interactions with the microenvironment to identify clinically relevant targets and mechanisms associated with acquired drug resistance. (Cancer Res 2006; 66(4): 2338-45)

Introduction

Acquisition of drug resistance remains a major obstacle for the successful treatment of many tumors. The problem of acquired drug resistance has classically been studied in vitro by exposing cancer cell lines to increasing doses of drug (1, 2). These drug-resistant models have been instrumental in identifying mechanisms that modulate drug response and, in some cases, have aided in the identification of drug targets. However, these models lack consideration of the role of the tumor microenvironment in the emergence of the drug-resistant phenotype. Recently, several investigators, including our own laboratory, have shown that the tumor microenvironment can modulate drug response (3–8). Specifically, we reported that adhesion of hematopoietic cancer cell lines via β1 integrins to fibronectin causes a temporal resistance to diverse cytotoxic insults (4, 5, 9–12). In addition, we showed that cellular adhesion results in reduced DNA damage induced by topoisomerase II inhibitors and alterations in the nuclear pool of topoisomerase II (5). We hypothesize that cell adhesion–mediated drug resistance (CAM-DR) is a form of de novo drug resistance promoting cell survival during initial drug exposure and influences the eventual acquisition of a multidrug-resistant phenotype.

To address the question of how cell adhesion influences acquired drug resistance, we compared the levels and mechanisms of drug resistance when the human histiocytic lymphoma cell line U937 was selected for mitoxantrone resistance either while adherent to fibronectin (U937/FN series) or in a traditional suspension culture (U937/Sus series). Mitoxantrone is a DNA intercalating agent and is known to stabilize topoisomerase II-DNA complexes (13, 14). Drug-induced DNA-topoisomerase II complexes are referred to as the “cleavable complex,” and the majority of evidence indicates that this is the initiating lethal event of this cytotoxic (15). Previous reports have indicated that selection of tumor cells with mitoxantrone typically results in drug-resistant cells that overexpress the ABC transporter BCRP and/or alter the drug target topoisomerase II (16–18).

In this study, we show that adhesion of U937 cells to fibronectin increased levels of acquired mitoxantrone resistance compared with drug resistance acquired by selection in routine suspension culture. The overall drug-resistant phenotype between the two models was similar with reduced drug-induced DNA double-strand breaks associated with reduced topoisomerase II levels. However, the regulation of reduced topoisomerase II levels was different, depending on whether cells were selected in suspension culture or adhered to fibronectin.

Materials and Methods

Selection of drug-resistant cell lines. The U937 human histiocytic lymphoma cell line was obtained from the American Type Culture Collection (Rockville, MD). Before exposing U937 cells to mitoxantrone, cells were adhered to either fibronectin-coated plates or cultured in suspension for 2 hours at a density of 3.75 × 10⁶/mL in serum-free media as previously described (5). Following 2 hours of cellular adhesion in serum-free RPMI, fibronectin plates were gently washed to remove unadhered cells, and RPMI containing 10% fetal bovine serum (FBS) was added back. Suspension cultures were centrifuged, and cells were suspended in fresh RPMI containing 10% FBS. Following the addition of serum containing

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cells were exposed to ddH2O vehicle control (VC), while either cultured in drug-free media for 1 week before performing experiments. Trypan blue staining in both suspension and fibronectin populations was then used to determine viability. If the viability in both suspension and fibronectin cultures was >65%, both cultures were reexposed to mitoxantrone as described above. If cell viability was <65% in either the fibronectin-adhered or suspension populations, both populations were allowed to expand in suspension in drug-free media. Therefore, the timing, dose, and exposure to mitoxantrone were identical for both drug-resistant selection conditions. After ~100 days, drug-resistant variants emerged, which were exposed twice a week with 10 nmol/L mitoxantrone for 1 hour. These cell lines are referred to as either U937/SusMR1 (selected in suspension culture) or U937/FNMR1 (selected while adherent to fibronectin). At this time point, the MR1 variants were exposed to 20 nmol/L mitoxantrone as described above. Following another 80 days of selection, stable variants emerged referred to as either SusMR2 or FNMR2. After the selection of the MR2 cell lines, cultures were maintained by adding either 20 nmol/L mitoxantrone or vehicle control for 1 hour once a week while the cells were adherent to fibronectin or cultured in suspension. Drug-resistant variants were cultured in drug-free media for 1 week before performing experiments.

To control for changes that resulted from recurrent cell adhesion, U937 cells were exposed to ddH2O vehicle control (VC), while either cultured in suspension media (SusVC) or adhered to fibronectin (FNVC). Therefore, two separate drug-sensitive cell lines (SusVC and FNVC) were developed and used for comparison with each other and their respective drug-resistant cell lines SusMR and FNMR.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assays were used to compare levels of drug sensitivity in the cell line variants. Cells were centrifuged and resuspended in RPMI containing 10% FBS at a concentration of 100,000/ml for vehicle control cell lines and 200,000/ml for drug-resistant cell lines. Cells were treated with varying concentrations of drug for 1 hour. Following drug exposure for 1 hour, plates were washed once with RPMI containing 10% FBS. After a 72-hour incubation at 37°C, the MTT assay was done as previously described (5). IC50 values were calculated by linear regressions derived from the linear portion of the survival curve.

Microarray analysis. Microarray analysis was used to examine and compare gene expression between drug-sensitive cell lines (SusVC and FNVC) and drug-resistant cell lines (SusMR and FNMR). RNA was isolated, and Affymetrix U133A GenChip arrays were processed as previously described (11). The cell line selected with vehicle control in suspension media was the reference sample for drug-resistant cell lines (SusMR and FNMR). Cells selected with vehicle control while adherent to fibronectin (FNVC) served as the reference sample for the drug-resistant cell lines selected while adherent to fibronectin (FNMR1 and FNMR2). Gene expression, which was detected as absent in both test and reference sample by MAS 5.0 was not considered for further analysis. Gene expression changes of 1.8-fold in both the MR1 and MR2 cell lines compared with the reference sample, was necessary to be considered for further analysis. Venn diagrams were used to determine changes in gene expression that were common to both selection models, as well as to identify changes in gene expression that were exclusive to the culture condition.

Reverse transcriptase-PCR. Reverse transcriptase-PCR (RT-PCR) was used to validate expression of genes changed during selection with mitoxantrone. These genes include topoisomerase IIα and topoisomerase IIβ and the multidrug transporter BCRP. In addition, based on our previous work examining the contribution of the Fanconi anemia pathway in acquired melphanal resistance, we chose to validate Fanconi anemia complementation group E (FANCE; refs. 11, 19). RNA was extracted from log growth cells with RNAeasy columns (Qiagen, Valencia, CA) per manufacturer’s instructions. First-strand cDNA synthesis was carried out with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Frederick, MD) per manufacturer’s instructions. For BCRP expression, the PCR reaction was run on an Applied Biosystems Geneamp system 9700 (Foster City, CA). Briefly, 50 ng of first-strand cDNA reaction mixture were amplified using the following primers: BCRP upstream primer (5′-3′), TTAGATT- GAAGCCAAAGC and BCRP downstream primer (5′-3′), TAGGCAATTGT-GAGGAAAAA.

The reaction mixture was initially heated to 94°C. Then 25 reaction cycles of 30 seconds at 94°C followed by 30 seconds at 50°C and 30 seconds at 72°C occurred. Finally, the reaction mixture was kept at 72°C for 3 minutes.

For the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) loading controls, the following primers were used: GAPDH upstream primer (5′-3′), CAAAAGGGTCATCATCTCTGTC and GAPDH downstream primer (5′-3′), GAGGGGGCATCCAGCTTTC.

The reaction consisted of 25 reaction cycles (30 seconds at 94°C followed by 30 seconds at 55°C and then 30 seconds at 72°C).

Real-time PCR primers for topoisomerase Iα and topoisomerase Iβ and FANCE were obtained from Ambion (Austin, TX). The gene expression level was normalized using the endogenous control gene GAPDH. Real-time PCR reactions were done using ABI 7900 Sequence Detection System (Applied Biosystems).

Comet assay. The neutral comet assay was used to detect drug-induced protein associated DNA double-strand breaks as previously described (5). Briefly, cell lines were exposed to either 1 μmol/L mitoxantrone or ddH2O at 37°C for 1 hour before performing the comet assay. To detect non–protein-associated DNA double-strand breaks, cells were lysed for 1 hour at 4°C in the absence of proteinase K; samples were then processed for DNA double-strand breaks as previously described (5). The comet moment for cells treated with vehicle control was subtracted from the drug-treated sample. The data shown are the means with 95% confidence intervals as determined by ANOVA (51 images for each dose of each independent experiment, n = 3 independent experiments).

Drug accumulation. Alteration in intracellular drug concentration as a possible mechanism of drug resistance was examined by measuring cellular accumulation of [3H]mitoxantrone in all cell lines. Briefly, for each cell line 1 × 106 cells were exposed to 2.5 μmol/L [3H]mitoxantrone (specific activity, 8.1 μCi/mmol) for 1 hour. Following drug exposure at 37°C for 1 hour, samples were washed thrice in cold PBS before the addition of scintillation fluid. The samples were counted on a Beckman scintillation counter (Palo Alto, CA). The data were normalized to cpm [3H]mitoxantrone per one million cells. The experiment was done in triplicate and repeated twice.

Topoisomerase II levels and activity. Alterations in the activity and/or levels of topoisomerase II were measured in the drug-resistant cell lines and compared with the drug-sensitive cell lines. Nuclear and whole-cell extracts were prepared as previously described (5). For immunoblotting, 20 μg of fresh nuclear extract from suspension and fibronectin-adhered samples were separated on a 5% to 15% gradient SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The blot was probed with either a topoisomerase IIα, topoisomerase IIβ, or topoisomerase I monoclonal antibody (BD Biosciences, San Jose, CA). The band of interest was detected by chemiluminescence (Pierce Biochemicals, Rockland, IL).

Catalytic activity of topoisomerase II was measured as the decatenation of networks of kDNA per manufacturer’s instructions (Topogen, Columbus, OH). Briefly, 1 μg of nuclear protein extract and 100 ng of kDNA were incubated in a total volume of 20 μL at 37°C for 5 minutes. The reaction buffer consisted of the following: 50 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 10 mmol/L MgCl2, 1.0 mmol/L ATP, 0.5 mmol/L DTT, and 30 μg/mL bovine serum albumin. The reaction was terminated by the addition of 5 μL of 2.5% SDS.

Results

Drug resistance. MTT assays were done to compare levels of acquired drug resistance between the two culture conditions used in the selection process. Comparing the IC50 values from five independent experiments showed that the FNMR2 cell line was

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significant more resistant to mitoxantrone compared with the SusMR2 cell line (Fig. 1). Because MTT assays were done in suspension media for all cell lines, including the FNMR2, these data indicate that cell adhesion at the time of acute drug exposure was not required to show the increased drug resistance for FNMR2 compared with the SusMR2 cell line. Furthermore, there was no difference in drug sensitivity for cells repeatedly adhered to fibronectin without drug selection pressure (FNVC) compared with cells maintained in suspension without selection pressure (SusVC), thereby showing that repeated exposure to fibronectin alone was not sufficient to confer acquired drug resistance. These data are consistent with our previous report showing that the de novo CAM-DB phenotype associated with fibronectin is rapidly reversed when cells are released from fibronectin adhesion (20).

**Microarray analysis.** Microarray analysis was done to assess the differences as well as similarities in gene expression profiles (GEP) when cells were selected with drug in suspension cultures compared with adherent cultures. Spotfire was used to analyze the data. Genes that changed in the same direction by at least 1.8-fold in the MR1 and MR2 cell lines compared with the appropriate VC cell line were selected for further analysis. Using these criteria, 441 probe sets were altered in suspension cultures, 371 probe sets were altered in fibronectin adhered cultures, and 71 probe sets changed in common between the two culture conditions (see Fig. 2A).

Genes that were exclusively changed in either the suspension or adherent cultures with acquired resistance to mitoxantrone were further screened for genes known to contribute to DNA damage or drug-induced apoptosis. Cell lines (SusMR1 and SusMR2) selected in suspension resulted in a 2-fold reduction in topoisomerase IIβ (see Fig. 2B). In contrast, microarray analysis showed only a modest 1.4-fold reduction in topoisomerase IIβ in the two FNMR drug-resistant variants. Recently, Lok et al. showed that the transcription factor NYA binds to the promoter region of topoisomerase IIβ and is critical for regulating the expression of topoisomerase IIβ (21). Microarray analysis revealed a 3.4- and 4.5-fold decrease in NYA in the SusMR1 and SusMR2, respectively, compared with the parental cell line. In contrast, there were no detectable decreases in NYA in the FNMR drug-resistant cell lines. Together, these data suggest that decreased NYA expression may be critical for the observed decrease in topoisomerase IIβ expression in SusMR2 variants, and that this regulation is limited to cells selected for resistance in suspension compared with cells adhered to fibronectin.

Cells selected with mitoxantrone while adherent to fibronectin showed a 2-fold increase in FANCE expression. FANCE is a member of the Fanconi anemia pathway (see Fig. 2B). We recently showed that overexpression of FANCp is a determinant of reduced melphalan-induced interstrand cross-links and acquired drug resistance (11, 19). There is no current evidence linking the Fanconi anemia pathway to sensitivity or resistance to topoisomerase II inhibitors. However, recent evidence has shown that two topoisomerase II inhibitors doxorubicin and mitoxantrone can form DNA adducts (22–24). Thus, further studies are warranted to determine whether the Fanconi anemia pathway contributes to the removal of mitoxantrone-DNA adducts.

We recently showed that changes in gene expression predictive for increased cholesterol synthesis represented a common fingerprint between de novo and acquired melphalan resistance (11). As a result of these findings, we screened GEP lists generated in Fig. 2A for changes in gene expression related to cholesterol homeostasis. In the FNMR2 cell line, we observed several changes that would be predictive of increased cholesterol synthesis (HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1; SIE, squalene epoxidase) and transport (NPC1, Niemann-Pick disease, type CI; see Fig. 2B). Further studies are warranted to determine whether the cholesterol pathway represents a marker of drug resistance and/or has functional consequences in mediating the emergence of drug resistance.

In both suspension and fibronectin drug-selected cell lines, we noted several changes in genes known to positively or negatively affect apoptosis (BCL-2, MCL-1, TUCAN = Card8, CDKN1B = p27Kip1, TNFSF13 = April, caspase-1, TNFRSF6 = FAF1, BNIP3). It is currently unclear based solely on GEP what effect the culmination of these changes would have on drug-induced apoptosis (see Fig. 3). This observation is similar to what we and others have reported with GEP analysis of acquired drug-resistant cell lines (11, 25).

Finally, drug selection while cells were adherent to fibronectin or cultured in suspension resulted in a significant number of common changes (71 gene probes). The number of changes that would be predicted to be common by chance alone is 7.4 (expected by chance = probe sets changed SusMR2 × probe sets changed FNMR / total number probes sets). Thus, the 71 common changes in gene expression are unlikely to be the result of chance alone and may represent a common cluster between the two drug-resistant models. However, based on current available knowledge, only one gene was identified that could be considered as directly linked

![Figure 1](http://cancerres.aacrjournals.org/content/66/4/2340/F2.large.jpg)

**Figure 1.** U937 cells either adhered to fibronectin or cultured in suspension were selected with mitoxantrone as described in Materials and Methods. The FNMR2 cell line is 2.3-fold more resistant than the SusMR2 cell line (P < 0.05, Student’s t test). Representative MTT experiment. The mean IC50 values and 95% upper (UCI) and lower (LCI) confidence intervals (n = 5 independent experiments) of the four cell lines are as follows:

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mean IC50 (nmol/L)</th>
<th>LCI</th>
<th>UCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>SusVC</td>
<td>15.24</td>
<td>8.99</td>
<td>21.49</td>
</tr>
<tr>
<td>SusMR2</td>
<td>64.76</td>
<td>34.82</td>
<td>94.73</td>
</tr>
<tr>
<td>FNVC</td>
<td>17.16</td>
<td>13.4</td>
<td>20.92</td>
</tr>
<tr>
<td>FNMR2</td>
<td>147.32</td>
<td>102.35</td>
<td>192.33</td>
</tr>
</tbody>
</table>

The cell lines are defined as follows: SusVC, U937 cells cultured in suspension and exposed to vehicle control (ddH2O); SusMR2, U937 cells selected with mitoxantrone while cultured in suspension; FNVC, U937 cells cultured adherent to fibronectin and exposed to vehicle control; FNMR2, U937 cells selected with mitoxantrone while adherent to fibronectin.
to cell survival (see Fig. 2B), serum/glucocorticoid regulated kinase (SGK). Similar to AKT, SGK can phosphorylate and inactivate forkhead transcription factors, such as FKHRL1. Brunet et al. recently showed that AKT and SGK have different phosphorylation site preferences on FKHRL1 and thus may coordinately regulate FKHRL1 (26). Further studies are warranted to determine whether increased expression of SGK contributes to acquired drug resistance.

**Drug accumulation.** Mitoxantrone selection in some cell lines results in overexpression of BCRP (18). Microarray analysis did not show overexpression of any functional drug transporters. To confirm these findings, we did both a functional assay for drug
accumulation and RT-PCR for BCRP expression. As shown in Fig. 3A, there were no changes in intracellular mitoxantrone levels between the four cell lines. RPMI 8226 myeloma cells selected for mitoxantrone resistance overexpress BCRP and were used as a positive control for detection of BCRP expression (see Fig. 3B; ref. 17). In contrast to 8226 mitoxantrone-resistant cells, no detectable levels of BCRP were noted in any of the U937 drug-resistant cell lines. Together, these data indicate that the reduction in topoisomerase IIα and topoisomerase IIβ protein levels are predominately regulated post-transcriptionally in the fibronectin-adhered drug selected cell lines, whereas in the suspension mitoxantrone–selected FNM2 cell line is likely related to the reduction in both topoisomerase IIα and topoisomerase IIβ in the FNM2 cell line, compared with only a reduction in topoisomerase IIβ in the SusMR2 cell line.

Thus, selection with mitoxantrone in a suspension culture resulted in a cell line with reduced topoisomerase IIβ RNA levels that correlated with reduced topoisomerase IIβ protein levels. In contrast, topoisomerase IIβ protein levels were dramatically decreased in the FNM2 cell line despite only a modest 1.4-fold reduction in RNA levels. In addition, in the FNM2 cell line topoisomerase IIα protein levels were decreased, although there were no detectable changes in RNA levels as determined by microarray analysis or real-time RT-PCR (data not shown). Together, these data indicate that the reduction in topoisomerase IIα and topoisomerase IIβ protein levels are predominately regulated post-transcriptionally in the fibronectin-adhered drug selected cell lines, whereas in the suspension mitoxantrone–selected cell lines...
culture, topoisomerase IIβ levels were transcriptionally attenuated. Finally, there were no detectable differences in topoisomerase II levels or activity between the drug-sensitive SusVC and FNVC cell lines.

Because it is feasible that increased proteasome mediated degradation of topoisomerase II is a mechanism underlying the reduction in topoisomerase II protein levels in the FNMR2 cell line, we screened the U937/FNMR microarray list for changes that may contribute to this finding. As shown in Fig. 2B, we observed a 2.3-fold decrease in ubiquitin-specific protease 20 (USP20), a 9-fold increase in the Neddd4-like ubiquitin ligase (WWP2) and a 1.8-fold increase in the Neddd4 binding partner (NB4B1), in the FNMR2 variant compared with the FNVC parental cell line. Further studies are warranted to determine whether these changes are causative for the reduced topoisomerase II protein levels in the FNMR2 drug-resistant cell line.

**Mitoxantrone induced DNA damage.** The neutral comet assay was used to measure mitoxantrone-induced protein and non–protein-associated DNA double-strand breaks. As shown in Fig. 5A, both drug-resistant variants showed significantly decreased drug-induced protein-associated DNA double-strand breaks compared with the respective parental cell lines. In addition, cells selected on fibronectin (FNMR2) showed a further reduction (P < 0.0167, Student’s t test corrected for multiple testing 0.05 of 4 independent tests) in DNA damage compared with cells selected in suspension (SusMR2). Surprisingly, we also observed a significant 1.7-fold reduction (P < 0.0167, Student’s t test) in mitoxantrone-induced DNA damage between cells that were maintained on fibronectin (FNVC) without the addition of drug compared with cells maintained in suspension (SusVC). This reduction in DNA damage in the FNVC cell line did not correlate with increased survival as measured by MTT cytotoxicity assays, or changes in topoisomerase II levels or activity. Together, these data suggest that reduction in drug-induced DNA damage in the FNVC cell line is not the result of altered topoisomerase II, and that the damage is not processed as being lethal. Mitoxantrone is an intercalating agent and could potentially disrupt multiple DNA-protein interactions. Our data suggest that total DNA drug–induced DNA damage does not always predict drug sensitivity. DNA damage measured in the presence of proteinase K in the lysis buffer measures both protein and non–protein-associated DNA damage. To test whether the FNVC cell line showed reduced mitoxantrone induced non–protein-associated DNA damage, we measured the DNA double-strand breaks in the absence of proteinase K. As shown in Fig. 5B, the FNVC cell line showed a significant 6-fold reduction (P < 0.0167, Student’s t test) in non–protein-associated mitoxantrone-induced DNA damage compared with the SusVC cell line. These data suggest that cultivating cells attached to fibronectin over time preferentially reduces the amount of non–protein-associated DNA damage induced by mitoxantrone. We previously showed that preadhesion for 2 hours reduced the amount of mitoxantrone- and etoposide-induced DNA double-strand breaks (5). The reduction in drug-induced DNA damage correlated with increased cell survival and decreased ability to salt extract topoisomerase IIβ. However, considering our findings in the FNVC cell line, future studies will determine what effect preadhesion has on drug-induced non–protein-associated mitoxantrone-induced DNA damage.

**FANCE confirmation and melphalan sensitivity.** Microarray analysis revealed a 2.2-fold increase in the expression of FANCE in the FNMR2 variant, and we confirmed this finding with real-time RT-PCR (see Fig. 6A). FANCE is a member of the Fanconi anemia pathway, and mutations in family members correlates with increased sensitivity to cross-linking agents. We recently showed that increased expression of FANCE correlates with melphalan resistance in acquired drug-resistant cell lines selected with melphalan (11, 19). Based on our previous work, we asked whether increased expression of FANCE in the FNMR2 cell line conferred resistance to melphalan. Surprisingly, as shown in Fig. 6B, the FNMR2 cell line showed no change in melphalan sensitivity compared with the FNVC cell line. However, cells selected for mitoxantrone resistance in suspension showed a significant increase in sensitivity to melphalan compared with the SusVC cell line (P < 0.05, Student’s t test). Increased sensitivity to melphalan has been reported in cell lines with reduced topoisomerase II levels associated with resistance to topoisomerase II inhibitors (27, 28). It is therefore possible that the increase in FANCE prevents the increased sensitivity to melphalan normally associated with cells resistant to topoisomerase II inhibitors. Further studies are warranted to investigate the interaction between topoisomerase II activity and the Fanconi anemia/BRC pathway.

**Discussion**

The emergence of acquired drug resistance continues to impede progress in the treatment of many cancers. Our laboratory has previously shown that adhesion of hematopoietic tumor cells to fibronectin can confer a form of de novo drug resistance (4, 5, 20).
These reports include showing that adhesion of U937 cells to fibronectin results in a transient drug resistance associated with reduced drug-induced DNA damage induced by topoisomerase II inhibitors, a finding that correlated with alterations in the nuclear pool of topoisomerase IIβ. Because cellular adhesion alters the drug target and drug response, we hypothesized that cellular adhesion could alter the targets associated with acquired drug resistance. Specifically, in this report, we asked whether cellular adhesion to fibronectin altered (a) the level of resistance, (b) the GEP associated with resistance, and (c) mechanisms associated with drug resistance compared with cells selected for drug resistance in a typical suspension culture.

In this report, we show that selecting U937 cells while adherent to fibronectin resulted in a phenotype that showed reduced DNA damage and increased survival compared with cells selected in a suspension culture. These data suggest that selection of adherent cells promotes the emergence of a more aggressive resistance phenotype. Furthermore, all cell drug cytotoxicity assays were done in suspension cultures, indicating that acquired drug resistance does not require survival signals associated with cell adhesion for expression of the acquired drug-resistant phenotype. Teicher et al. showed that selection of resistance with a cross-linking agent in an in vivo model resulted in a phenotype that was operative only in vivo (29). Our results, using an in vitro U937 model, confirm the observation that the microenvironment influences drug response and the acquisition of drug resistance.

Gene expression profiling of the drug-resistant variants was used to determine whether changes in gene expression could account for the differences in levels of drug resistance. Microarray analysis showed that cells selected in suspension showed a 2-fold reduction in topoisomerase IIβ levels, whereas adherent cells only showed a modest reduction in topoisomerase IIβ mRNA levels. In addition, in the suspension culture condition, reduced topoisomerase IIβ levels correlated with a reduction in NF-YA levels, a known transcriptional activator of topoisomerase IIβ (21). These findings were specific for cells selected in a suspension culture, indicating that regulation of the drug target topoisomerase II was dependent on the culture condition.

Surprisingly, neither the FNMR2 nor the SusMR2 cell line selected for overexpression of a drug transporter. Mitoxantrone will often select for increased expression of BCRP (18). However, in this study, we found no role for the ABC transporter BCRP in conferring mitoxantrone resistance. GEPs showed multiple changes in the apoptotic pathway in both drug-resistant cell lines. Although some changes in gene expression were predictive of increased cell survival, we did observe changes, like decreased expression of BCL-2, that would predict reduced cell survival. These observations make it difficult to predict what effect the culmination of these changes in apoptotic machinery would have on cell survival. Finally, in the FNMR2 cell line, we observed several changes that would predict for increased synthesis and trafficking of cholesterol. These changes included increased expression of 3-hydroxy-3-methylglutaryl-CoA synthase 1, squalene epoxidase, and NPC1. Recently, Li et al. showed that acute exposure to daunorubicin or 1-β-d-arabinofuranosylcytosine (ara-C) increased cholesterol levels in primary acute myelogenous leukemia (AML) specimens and cell lines. Importantly, in this same study, investigators showed that treatment with zaragozic acid sensitizes AML cells to daunorubicin- and ara-C–induced cell death. Zaragozic acid inhibits squalene synthase at the final branch step of the cholesterol synthesis pathway, indicating that reducing geranyl or farnesyl levels was not required to sensitize cells to either ara-C or daunorubicin (30). Taken together, their data indicate that reducing cholesterol levels is a viable strategy for increasing the efficacy of cytotoxics. Further studies are warranted to determine the mechanism of drug resistance associated with increased cholesterol levels and whether the gene expression profile of the cholesterol pathway is a good marker for predicting clinical response to cytotoxics.

Because gene expression profiling indicated that reduced topoisomerase IIβ levels might confer resistance in the SusMR2 cell line, we examined the protein levels of topoisomerase IIβ and topoisomerase IIα in the drug-resistant variants. In the SusMR2 cell line, reduced topoisomerase IIβ RNA correlated with decreased protein levels. In contrast, drug selection while cells were adhered to fibronectin showed reduced topoisomerase IIβ protein levels, but no detectable decrease in topoisomerase IIα or NF-YA RNA levels was noted. In addition, in the FNMR2 cell line a decrease in topoisomerase IIα protein levels was
observed. Again, no change in topoisomerase IIα RNA levels was detected in the FNMR2 cell line, suggesting that both topoisomerase IIα and topoisomerase IIβ are post-transcriptionally regulated in the FNMR2 cell line. Thus, although both drug selection conditions (with and without fibrotenin adhesion) resulted in mitoxantrone resistance associated with reduced topoisomerase II levels and activity, the mechanism regulating topoisomerase II levels is different between the two drug-resistant models. Together, our data indicate that increased levels of drug resistance in the FNMR cell line compared with the SusMR cell line is likely due to decreased topoisomerase IIα and topoisomerase IIβ protein levels compared with only reduced topoisomerase IIβ levels in the SusMR cell line. In addition, our results show that drug selection using a classic suspension culture resulted in attenuation of NF-YA a known transcriptional activator of topoisomerase IIβ. This finding correlated with reduced topoisomerase IIβ RNA and protein levels. In contrast, drug selection while cells were adhered to fibrotenin showed reduced topoisomerase IIβ protein levels but no detectable decrease in topoisomerase IIα or NF-YA levels.

Our previous work showed that preadhesion of U937 cells to fibrotenin altered the nuclear distribution of topoisomerase IIα. Although the mechanism underlying the altered nuclear trafficking of topoisomerase IIα is not known, the fact that nuclear trafficking of topoisomerase IIβ is altered before drug selection may favor protein degradation over transcriptional regulation of topoisomerase II in adherent cells. Possible mechanisms contributing to degradation of topoisomerase II include alterations in sumoylation, phosphorylation, and ubiquination of topoisomerase II. Microarray analysis of U937 cells selected for resistance while adherent to fibrotenin showed increased expression of Nedd4-like protein ligase and decreased expression of ubiquitin-specific protease 20. These observations warrant further study to determine whether these changes in gene expression contribute to degradation of topoisomerase II in the FNMR cell line. In conclusion, our studies show that models of acquired resistance need to consider the interaction of the tumor cell with the microenvironment, as this may be necessary to identify clinically relevant drug resistance targets.

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