The FA/BRCA pathway is involved in melphalan-induced DNA interstrand cross-link repair and accounts for melphalan resistance in multiple myeloma cells

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Melphalan, a DNA cross-linker, is one of the most widely used and effective drugs in the treatment of multiple myeloma (MM). In this report, we demonstrate that enhanced interstrand cross-link (ICL) repair via the Fanconi anemia (FA)/BRCA pathway contributes to acquired drug resistance in melphalan-resistant myeloma cell lines, and disruption of this pathway reverses drug resistance. Using the alkaline comet assay (single-cell gel electrophoresis), we observed that melphalan-resistant cells have reduced ICL formation and enhanced ICL repair compared with melphalan-sensitive cells. Cell-cycle studies demonstrated that enhanced ICL repair released cells from melphalan-induced cell-cycle delay. Using siRNA to knock down FANCF in 8226/LR5 and U266/LR6 drug-resistant cells demonstrated a direct relationship between ICL repair capacity and drug sensitivity. Overexpression of FANCF in 8226/S and U266/S drug-sensitive cells partially reproduced the drug-resistant phenotype. These data show that enhanced DNA repair via the Fanconi anemia/BRCA pathway is involved in acquired melphalan resistance. Our findings provide for a new target to enhance response to DNA cross-linking agents in cancer treatment. (Blood. 2005;106:698-705)
Materials and methods

Cell lines and drugs

The RPMI 8226 and U266 human multiple myeloma cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cell lines were grown in RPMI 1640 medium (CellGro; MediaTech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Omega Scientific, Tarzana, CA), 1% penicillin/streptomycin, and 100 mM l-glutamine (Gemini Bio-Products, Calabasas, CA). The melphalan-resistant cells 8226/LR5 and U266/LR6 were passaged weekly in media containing 5 μM or 6 μM melphalan, respectively.2

Methyl-thiazol tetrazolium (MTT) cytotoxicity assay

Cells were seeded at 8000 to 25,000 cells/well in 96-well plates (Becton Dickinson, Lincoln Park, NJ). To establish a dose response to melphalan (Sigma, St Louis, MO), cells were incubated with melphalan for 96 hours in 2-fold serial dilutions ranging from 1 × 10⁻⁵ M to 3.9 × 10⁻⁴ M. The melphalan-induced growth inhibition assays were performed as previously described.27

Real-time quantitative reverse-transcriptase–polymerase chain reaction (RT-PCR)

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) and used for cDNA synthesis (Invitrogen first-strand cDNA synthesis kit; Invitrogen, Frederick, MD). Expression of 11 genes involved in drug-sensitive and drug-resistant cells. All other statistical comparisons were made using the Student t test.

Results

Resistant cells have less melphalan-induced growth inhibition and apoptosis

MTT and apoptosis assay were used to compare melphalan sensitivity in the drug-sensitive (8226/S, U266/S) and drug-resistant (8226/LR5, U266/LR6) myeloma cell lines. Similar to our previously published reports,2,3 melphalan-resistant cells 8226/
LR5 and U266/LR6 have increased survival (Figure 1A) and decreased drug-induced apoptosis (Figure 1B) compared with their drug-sensitive parental cells 8226/S and U266/S.

Melphalan-resistant myeloma cells have enhanced expression of the FA/BRCA pathway

Using the Affymetrix oligonucleotide microarray (Affymetrix, Santa Clara, CA) to examine gene expression profile (GEP), we recently reported that the melphalan-resistant myeloma cell line 8226/LR5 showed significant increases in the expression of the FANCF and RAD51C genes and reduced DNA cross-links compared with the drug-sensitive 8226/S cells. Because the Affymetrix HG-133A chip does not include the FANCD2 and FANCL genes, we used a custom-designed real-time PCR microfluid card to examine 11 genes of the FA/BRCA pathway in 8226/S and U266/S myeloma cells and their drug-resistant variants, 8226/LR5 and U266/LR6, respectively. The results revealed that expression of multiple components of the FA/BRCA pathway were upregulated in drug-resistant 8226/LR5 and U266/LR6 cells compared with drug-sensitive 8226/S and U266/S cells, respectively (Figure 2A). The expression levels of BRCA1, BRCA2, FANCA, FANCC, FANCF, FANCL, and RAD51C were at least 2-fold increased in 8226/LR5 compared with 8226/S. The expression of FANCL in U266/LR6 is at least 3-fold higher than U266/S drug-sensitive cells (Figure 2A).

The full integrity of the upstream FA complex promotes monoubiquitination of FANCD2 following DNA cross-linker treatment, therefore formation of the monoubiquitinated FANCD2 (FANCD2-L) isoform has been used to clinically diagnose FA patients. To determine whether the upstream portion of the FA/BRCA pathway is intact, we examined FANCD2-L at various time points after drug treatment in the 4 cell lines. The results revealed that the expression of FANCD2-L was significantly increased within 2 hours of melphalan treatment (Figure 2B), and a maximum increase of FANCD2-L occurred within 24 hours after melphalan treatment (Figure 2B lower panel). These data suggest that proteins involved in the upstream FA complex (FANCA, B, C, E, F, G, L) are functionally complete in all 4 cell lines. Data also suggest that increased formation of FANCD2-L may be due to the enhanced expression of the FA complex proteins, and elevated levels of the FA/BRCA pathway may contribute to enhanced ICL repair capacity in drug-resistant cells. To examine this possibility, we compared DNA ICL formation and removal in drug-sensitive and -resistant myeloma cells.

Melphalan-resistant cells have reduced DNA ICL formation and enhanced ICL removal compared with drug-sensitive cells

We recently reported that, within 2 hours following drug exposure, melphalan-induced cross-links were significantly reduced in the...
drug-selected 8226/LR5 cell line. In this study, using alkaline comet assay, we compared the kinetics of DNA ICL formation and removal between melphalan-sensitive (8226/S, U266/S) and melphalan-resistant (8226/LR5, U266/LR6) cells. When cells were treated with 25 μM, 50 μM, and 100 μM melphalan, cross-links were significantly reduced in 8226/LR5 and U266/LR6 compared with drug-sensitive 8226/S and U266/S cells, respectively (Figure 3A-B, ANOVA P < .05). To examine the ICL removal, melphalan-treated cells were examined at 10-hour and 24-hour time points. Similar amounts of ICL were induced in drug-sensitive and drug-resistant cells at the 10-hour time point, when 8226/S, 8226/LR5, U266/S, and U266/LR6 were treated with 25 μM, 50 μM, 25 μM, and 60 μM melphalan, respectively (Figure 3C). At 25 hours after melphalan treatment, 37% and 22% of ICLs were removed in 8226/LR5 and U266/LR6 cells, respectively, while no significant ICL removal was observed in drug-sensitive 8226/S and U266/S cells (Figure 3C, t test P < .05).

**Melphalan has less effect on cell-cycle progression in 8226/LR5 cells compared with drug-sensitive 8226/S cells**

To measure dose-response effects of melphalan on cell-cycle progression, both 8226/S and 8226/LR5 cells were treated with 10 M or 25 μM melphalan for 2 hours, cultured in drug-free medium, and collected at 24 hours and 48 hours. Serial DNA histograms revealed that within 24 hours, high-dose melphalan treatment (25 μM) resulted in a greater accumulation of both 8226/S and 8226/LR5 cells in early S phase compared with low-dose (10 μM) treatment (Figure 4A). High-dose melphalan (25 μM)-treated cells required more time to eliminate the melphalan-induced G2 block, and fewer cells were released from the drug-induced growth inhibition, compared with low-dose melphalan–treated 8226/S and 8226/LR5 cells (Figure 4A). However, the 8226/LR5 cells showed less growth inhibition compared with 8226/S cells, when cells were treated with either 10 μM or 25 μM melphalan (Figure 4A). BrDU/PI staining was performed to evaluate cell-cycle progression over time following drug exposure (Figure 4B). Within 12 hours after 10-μM melphalan treatment, 8226/S cells predominantly accumulated in early S phase (S1, S2) compared with 8226/LR5 cells, which accumulated in late S phase (S3, S4) (Figure 4B-C). Figure 4D shows further detail regarding S phase cell-cycle accumulation at 24 hours using FlowJo 4.4.4 analysis. At the 48-hour post–10-μM melphalan treatment time point, the DNA content of 8226/LR5 cells accumulated at G2/M and G1 phase, while the DNA content of the drug-sensitive 8226/S cells accumulated at late S and G2/M phases and less G1 accumulation was observed. By the 72-hour time point, 8226/LR5 cells had recovered from the drug-induced cell-cycle delay; whereas, the drug-sensitive 8226/S cells remained arrested in the G2/M phase (Figure 4B).

**Overexpression or silencing of FANCF shows a direct relationship among FANCF expression, DNA repair capacity, and drug sensitivity to melphalan**

To further examine the contribution of the FA/BRCA pathway to ICL repair and melphalan resistance, we transiently overexpressed FANCF in 8226/S and U266/S cells, and knocked down FANCF in 8226/LR5 and U266/LR6 cells. Overexpression of FANCF demonstrated by Western blot analysis in both drug-sensitive myeloma cell lines enhanced cell survival following melphalan treatment (Figure 5A). Conversely, knockdown of FANCF in melphalan-resistant 8226/LR5 and U266/LR6 cells reversed drug resistance (Figure 5B). We also performed the alkaline comet assay to determine whether knocking down FANCF was sufficient to reduce DNA ICL repair in the drug-resistant cell line. As shown in Figure 5C, a reduction in FANCF reduced the repair capacity of the 8226/LR5 and U266/LR6 cell lines. Taken together, these data show that despite increased expression in multiple genes in the FA/BRCA pathway, knocking down FANCF is sufficient to reverse, at least partially, the acquired drug-resistant phenotype in 8226/LR5 and U266/LR6 myeloma cells.

**Discussion**

DNA cross-linking agents, including melphalan, are important in the treatment of multiple myeloma and other cancers. The precise mechanisms contributing to melphalan resistance in multiple myeloma cells are poorly understood. This study demonstrates that overexpression of FA/BRCA pathway genes contributes to acquired resistance to melphalan for 2 multiple myeloma cell lines. In addition, this study provides evidence that the FA/BRCA pathway contributes to drug resistance via enhanced ICL repair, and release of cells from melphalan-induced growth inhibition.

We previously reported that acquired melphalan-resistant myeloma cells were cross-resistant to irradiation and also cross-resistant to other DNA cross-linkers, such as cisplatin and nitrogen mustard. This information, together with our recently published microarray data, led us to hypothesize that the FA/BRCA pathway may be involved in repair of melphalan-induced ICL and contributes to the drug-resistant phenotype. Using a custom-designed
real-time PCR microfluid card, we examined 11 genes involved in the FA/BRCA pathway. Expression of these genes was greater in drug-resistant cells compared with their drug-sensitive counterparts; however, greater differences were observed between the 8226 sensitive and resistant cells compared with the U266 sensitive and resistant cells. This may, in part, be due to the higher basal expression of FANC/BRCA genes in U266/S cells compared with 8226/S cells. The expression data correlated well with enhanced cell survival and reduced apoptosis following drug treatment. We observed that multiple components of the FANC/BRCA pathway were up-regulated in drug-resistant cells compared with drug-sensitive cells. These data suggest that genes involved in this pathway may be coregulated, and further studies are warranted to delineate the transcriptional regulation of this pathway.

Real-time PCR data showed at least 2-fold increase of FANCA, FANCC, FANCF, and FANCL in 8226/LR5 compared with 8226/S cells. Increased expression of FANCD2-L isoform in 8226/LR5 compared with 8226/S cells indicated that the upstream FA complex might facilitate FANCD2-L formation. In comparison, while some of the FA genes were less changed in U266/LR6 compared with the 8226/S-8226/LR5 pair, the increased posttranslational modification of FANCD2 may be related to the 3-fold increase of FANCL, which has E3 ubiquitin ligase activity. Within 24 hours after melphalan treatment, the FANC2D2-L/S ratio isoform in 8226/LR5 cells was significantly increased compared with drug-treated 8226/S cells.

We also observed a significant delay in cell-cycle progression within 24 hours after melphalan treatment with most of the cells accumulating in S phase. This observation is consistent with the observation reported by Rothfuss and Grompe. They noticed that in human fibroblasts, FANCD2-L is specifically expressed during the S phase after drug treatment. It has also been reported that the FANC2D2-L/S ratio increased within 4 hours after treatment with photoactivated psoralen-inducing DNA ICLs, and a 3-fold increase of FANCD2-L occurred within 24 hours after melphalan treatment. Since the FANCD2 monoubiquitination event is important for normal cellular recovery from exposure to DNA cross-linkers, our data suggest that the increased formation of the FANC2D2-L isoform, which is activated by melphalan-induced ICL during the S phase, is also essential for melphalan resistance in myeloma cell lines.

It has been suggested that the ICL-induced stalled replication fork is the major signal that activates the FA pathway and induces G2/M arrest in FA cells. In human myeloma cell line 8226,
melphalan treatment induces a cell-cycle progression delay and G2/M arrest. Although equivalent doses of melphalan induce more ICL in drug-sensitive 8226/S cells compared with drug-resistant 8226/LR5 cells, a 2-fold increase in FANCD2-L was observed in 8226/LR5 compared with 8226/S (Figure 2B, 25-hour time point). Because only the monoubiquitinated FANCD2 (FANCD2-L) can be translocated to DNA damage foci to perform repair function, our data suggest that the efficiency of FANCD2-L formation is important for drug resistance. This efficiency in FANCD2-L formation in drug-sensitive and -resistant cell lines may be due to enhanced expression of upstream FA complex.

Approximately one third of the DNA-melphalan adducts are DNA interstrand cross-links. By studying gene-specific formation of DNA monoadducts and interstrand cross-links, Souliotis et al reported that maximum monoadducts were formed within 2 hours as a result of rapid binding of melphalan to nucleophilic site (N7-guanine) in one DNA strand, whereas DNA ICLs accumulate slowly and reach maximal levels within 8 hours through binding of a second chloroethyl group to another N7-guanine site in the cDNA strand. In our study, we were able to induce equivalent maximum ICLs in drug-sensitive and -resistant cells by increasing the melphalan dose exposure for drug-resistant 8226/LR5 and U266/LR6 cells compared with 8226/S and U266/S drug-sensitive cells. Interestingly, within 24 hours after melphalan treatment, approximately 37% and 22% of the ICLs were removed in the 8226/LR5 and U266/LR6 cells, respectively, while the drug-sensitive 8226/S and U266/S cells showed no significant removal of ICLs in this time frame. Our results strongly suggest that the FA/BRCA pathway contributes to the removal of ICLs during this time period. Furthermore, we demonstrated that the capacity to remove ICLs was significantly reduced in FANCF knockdown 8226/LR5 and U266/LR6 cells compared with control siLuc-transfected cells, LR5 siLuc (top) and LR6 siLuc (bottom), respectively. The mean values and standard deviations from 3 independent experiments are shown. Student t test was used for statistical analysis. *P < .05.

Figure 5. Overexpression of FANCF in 8226/S and U266/S reduced melphalan response, whereas silencing FANCF in 8226/LR5 and U266/LR6 enhanced melphalan response, respectively. (A) MTT assay. Overexpression of FANCF in drug-sensitive 8226/S and U266/S cells enhanced cell survival. The data are presented as percent survival above control cells. The experiment was repeated at least 3 times. Representative results are shown. (ii) IC50 is the mean of 3 independent experiments and SD. Student t test was used for statistical analysis. *P < .05. The experiments were repeated 3 times. (ii) Empty vector–transfected 8226-PQC and U266-PQC cells served as controls for FANCF-transfected 8226-FANCF and U266-FANCF cells. β-Actin blot served as loading control. (B) Apoptosis assay. Transfection of siFANCF partially reversed melphalan resistance in 8226/LR5 and U266/LR6 cells. Annexin-V–FITC staining was used. The percentage of annexin-V–FITC-positive cells was labeled, and specific apoptosis has been calculated. The mean values and standard deviations from a representative experiment performed in triplicate are shown. Student t test was used for statistical analysis. *P < .05. (Bii) Western blot analysis of FANCD2 and FANCF showed reduced protein expression in 8226/LR5 and U266/LR6 cells transfected with siFANCF compared with cells transfected with siLuc as a control. β-Actin blot served as loading control. (C) Capacity to repair ICLs was reduced in FANCF knockdown LR5 and LR6 cells compared with control siLuc-transfected cells, LR5 siLuc (top) and LR6 siLuc (bottom), respectively. The mean values and standard deviations from 3 independent experiments are shown. Student t test was used for statistical analysis. *P < .05.
found (data not shown). Mechanisms associated with increased expression of FANCC in 8226/LR5 cells are undergoing further study.

We have shown that silencing FANCC in 8226/LR5 and U266/LR6 cells partially reversed drug resistance. We also showed that overexpression of FANCC in drug-sensitive cells could enhance drug resistance. It has been reported that FANCC plays an important role in stabilizing subunits of the FA complex and contributes to the proper function of this pathway.42 It has also been shown that the cellular level of FANCA, FANCC, and FANCG depends on the expression of FANCF.43 Overexpression of FANCF in parental 8226 and U266 cell lines induced less resistance compared with acquired melphalan-resistant cell lines, suggesting that other members of the FANC/BRCA complex contribute to melphalan resistance. Taniguchi et al reported that forced expression of FANCF in cisplatin-sensitive ovarian parental cells conferred a relative cisplatin resistance, however the resistance is less than the acquired cisplatin-resistant cells.38 Their data also show that overexpression of FANCF in acquired cisplatin-resistant cells increased resistance to cisplatin.38 They suggested that acquired cisplatin-resistant cells might have additional mechanisms involved in cisplatin resistance beyond the re-expression of FANCF. For example, overexpression of FANCC has been reported to inhibit EAS-induced apoptosis.44 This suggests that FA proteins might have other functions beyond assembly of the complex and participate in the drug-resistant phenotype. Taken together, we suggest that repeated exposure to DNA cross-linking agents increases expression of multiple components of the FA pathway, which contribute to the acquired drug resistance. Further investigations will analyze the role of individual FA proteins to the overall melphalan-resistance phenotype in myeloma cells.

Studying cell-cycle kinetics to diagnose Fanconi anemia patients has been reported.45 Exposing peripheral blood mononuclear cells ex vivo to DNA cross-linker treatment induces a significant G2-block in patients with FA.45 In our study, we observed that 10-μM melphalan treatment induces a cell-cycle progression delay in S phase within 24 hours and a G2 block within 48 hours. It has been shown that fibroblasts from FA patients require 3 times longer to recover from the ICL-induced cell-cycle arrest compared with fibroblasts from healthy subjects.33 In 8226/S cells, removal of melphalan-induced ICL is required for cells to progress from G2 block.35 We observed a higher degree of G2/M blockade in drug-sensitive 8226/S cells compared with 8226/LR5 cells following melphalan treatment. We also observed that 8226/LR5 cells require less time for cell-cycle progression from G2/M to the G1 phase compared with 8226/S cells; this is related to increased expression of the FA/BRCA pathway genes and successful removal of ICLs in 8226/LR5 cells compared with 8226/S cells. Overall, our data demonstrate that the FA/BRCA pathway contributes to melphalan resistance via enhanced ICL repair and reduced ICL-induced growth arrest via release of cells from G2/M block.

In summary, to our knowledge, our study is the first to show that enhanced expression of the FA/BRCA pathway is involved in melphalan-induced DNA ICL repair and is an important mechanism for acquired melphalan resistance in myeloma cells. This pathway also likely contributes to cross-resistance to other DNA cross-linking agents and irradiation. We propose that the FA/BRCA pathway represents a new target for preventing acquired drug resistance and improving cancer treatment.

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