Fanconi anemia (FA) cells are hypersensitive to cytotoxicity, cell cycle arrest, and chromosomal aberrations induced by DNA cross-linking agents, such as mitomycin C (MMC) and nitrogen mustard (HN2). Although MMC hypersensitivity is complemented in a subset of FA cells (complementation group C [FA-C]) by wild-type FAC cDNA, the cytoprotective mechanism is unknown. In the current study, we tested the hypothesis that FAC protein functions in the suppression of DNA interstrand cross-link (ISC)-induced cell cycle arrest and apoptosis. Comparison of HN2-induced cell cycle arrest and apoptosis with those of its non-cross-linking analogs, diethylaminoethyl chloride and 2-dimethylaminoethyl chloride, delineated the DNA ISC specificity of FAC-mediated cytoprotection. Overexpression of wild-type FAC cDNA in FA-C lymphoblasts (HSC536N cell line) prevented HN2-induced growth inhibition, G2 arrest, and DNA fragmentation that is characteristic of apoptosis. In contrast, cytoprotection was not conferred against the effects of the non-cross-linking mustards. Our data show that DNA ISCs induce apoptosis more potently than do DNA monoadducts and suggest that FAC suppresses specifically DNA ISC-induced apoptosis in the G2 phase of the cell cycle.

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and whether such a process is ISC-specific. Therefore, we have evaluated the cytoprotective effect of FAC complementation by comparing growth inhibition, cell cycle arrest, and apoptosis induced by HN2 with the perturbations produced by non-cross-linking congeners of HN2, 2-diethylaminoethyl chloride (DEM), and 2-dimethylaminoethyl chloride (DMM). All three agents are similar in that they produce predominately N7-alkyl guanine monoadducts in DNA.

In contrast to HN2, DMM and DEM cannot produce DNA ISC's because they lack a second alkylating chloroethyl moiety.

The comparison of the cytotoxic effects of HN2 with those of its closely related analogs delineated the lesional specificity of FAC-mediated cytoprotection and suggested that the FAC protein protects against HN2 cytotoxicity by preventing ISC-induced apoptosis.

MATERIALS AND METHODS

Drugs. HN2 was purchased from Sigma Chemical (St Louis, MO). A stock solution of 50 mmol/L HN2 was prepared in 0.1 N HCl and diluted in culture medium to achieve the desired concentrations. The HCl concentration never exceeded the nontoxic dose of 100 mmol/L. DEM and DMM, purchased from Aldrich Chemical (Milwaukee, WI), were dissolved in serum-free medium immediately before use.

Cell culture. The FA-C mutant lymphoblast cell line, HSC536N, was purchased from National Institute of General Medical Sciences human genetic mutant cell repository (Camden, NJ). The repository designation for this cell line is GM13021. HSC536N + pFAC3 cells were a generous gift from Dr M. Buchwald, Hospital for Sick Children (Toronto, Ontario, Canada). These cells are HSC536N cells that constitutively overexpress the wild-type FAC cDNA.

Normal wild-type human lymphoblasts were obtained from C.E. Walsh, Sick Children (Toronto, Ontario, Canada). These cells are the HSC536N cells, possibly due to a nonspecific mechanism, such as elevated thiol levels.

Growth inhibition assay. Parental HSC536N (mutant) cells, HSC536N + pFAC3 (corrected) cells, and normal wild-type cells were treated with various doses of mustards, and nuclei were counted 3 days later. Growth inhibition induced by HN2, DEM, and DMM is illustrated in Fig 1. The effective dose of HN2 necessary to produce a 50% reduction in cell growth (ED50) in the mutant cells was 150 nmol/L. Overexpression of the wild-type FAC cDNA increased the HN2 ED50 by 15-fold to 150 nmol/L, approaching that of the wild-type control cells. Consistent with this observation, the wild-type cells are significantly more resistant to the bifunctional mustard (Fig 1A), suggesting that these cells are generally more resistant to the monofunctional mustards than both the mutant and corrected FA cells. This observation, together with the somewhat greater resistance of the wild-type cells to the bifunctional mustard (Fig 1A), suggests that these cells are, in general, more resistant to alkylating agents than are the HSC536N cells, possibly due to a nonspecific mechanism, such as elevated thiol levels.

DNA fragmentation assay. Fragmentation of genomic DNA, a characteristic of apoptosis, was determined as described by Sellins et al44 with minor modifications. Briefly, equal numbers of drug-treated and untreated cells (5 to 10 × 106) were harvested, washed in phosphate-buffered saline, and lysed in 10 mmol/L Tris, 1 mmol/L EDTA, 0.2% Triton X-100, pH 7.4. Cell lysates were centrifuged at 12,000g for 10 minutes to separate small DNA fragments from intact chromatin. RNA and DNA in the supernatant were precipitated with 1/5 vol of 5 mol/L NaCl and 1 vol of isopropanol, and after centrifugation at 12,000g for 10 minutes, the pellets were resuspended in 20 to 60 μL of 10 mmol/L Tris, 1 mmol/L EDTA (pH 8). After the addition of 1/4 vol of loading buffer (15 mmol/L EDTA [pH, 8.0], 2% sodium dodecyl sulfate, 50% glycerol, and 0.5% orange G), the suspensions were incubated at 65°C for 10 minutes, chilled on ice, and again centrifuged at 12,000g for 10 minutes. Equivalent volumes of supernatant containing both RNA and DNA were electrophoresed in a 1.5% agarose gel, and photographed. In general, growth inhibition, cell cycle distribution, and DNA fragmentation were all measured in the same population of drug-treated cells.

RESULTS

The effect of the FAC protein on HN2-, DMM-, and DEM-induced growth inhibition. Parental HSC536N (mutant) cells, HSC536N + pFAC3 (corrected) cells, and normal wild-type cells were treated with various doses of mustards, and nuclei were counted 3 days later. Growth inhibition induced by HN2, DEM, and DMM is illustrated in Fig 1. The effective dose of HN2 necessary to produce a 50% reduction in cell growth (ED50) in the mutant cells was 150 nmol/L. Overexpression of the wild-type FAC cDNA increased the HN2 ED50 by 15-fold to 150 nmol/L, approaching that of the wild-type control cells. Consistent with this observation, in cells treated with 250 nmol/L HN2, overexpression of FAC increased viability determined by trypan blue exclusion by 54%, demonstrating that the FAC protein prevents cytotoxicity, as well as cytostatic effects of HN2.

The ED50 values for growth inhibition induced by DEM and DMM in FA-C mutant cells were 6.10 μmol/L and 8.65 μmol/L (Fig 1). Thus, DEM inhibits growth of these cells 610 times less effectively than does HN2, and DMM inhibits their growth 865 times less effectively. Overexpression of wild-type FAC in these cells failed to protect against monofunctional mustard-induced growth inhibition. Rather, overexpression of FAC conferred a 1.4-fold increased sensitivity, which is reflected in ED50 values of 4.5 μmol/L DMM and 6.0 μmol/L DEM. Consistent with this observation, viability of the corrected cells treated with 50 μmol/L DMM or 50 μmol/L DEM determined by trypan blue exclusion was approximately 50% lower than comparably treated mutant cells. It is notable that the wild-type cells are significantly more resistant to the monofunctional mustards than both the mutant and corrected FA cells. This observation, together with the somewhat greater resistance of the wild-type cells to the bifunctional mustard (Fig 1A), suggests that these cells are, in general, more resistant to alkylating agents than are the HSC536N cells, possibly due to a nonspecific mechanism, such as elevated thiol levels.
Fig 1. Growth-inhibitory effects of HN2, DMM, and DEM. Parental mutant HSC536N, corrected HSC536N + pFAC3, or normal wild-type cells were treated with the indicated doses of HN2, DMM, and DEM. Nuclei were counted 72 hours after drug treatment. Growth-inhibitory effects of HN2 (A), DMM (B), DEM (C) are expressed as percentages of values for untreated or vehicle-treated cells. Each data point represents the mean ± SD of at least triplicate determinations.

The effect of the FAC protein on HN2-, DMM-, and DEM-induced cell cycle arrest. Normal wild-type, FA-C mutant, and corrected cells treated with various doses of the mustards were stained with propidium iodide for analysis of DNA content by flow cytometry. The cell cycle distribution of the wild-type cells is shown in Fig 2A. Treatment of mutant cells with 0 to 250 nmol/L HN2 resulted in a dose-dependent increase of cells arrested in G2/M phase (Fig 2B). This increase was associated with a concomitant decrease in the G0/G1- and S-phase populations. Seventy-two hours after treatment with 250 nmol/L HN2, 60% to 72% of the mutant cells were in arrested G2/M. At a higher dose (500 nmol/L), the G2/M population was reduced by approximately 20%, with a corresponding increase in the G0/G1 population (data not shown), suggesting that cells were dying in G2/M. In addition, a small (~5%) population of tetraploid cells was seen only in HN2-treated mutant cells, suggesting that these cells had failed to go through mitosis. HN2-induced G2/M arrest and accumulation of tetraploid cells was essentially absent in the corrected, as well as the normal wild-type cells (Fig 2).

Changes in cell cycle distribution of the mutant and corrected cells over time are shown in Fig 3. Neither the untreated mutant nor corrected cells arrested spontaneously in G2/M during 96 hours of log-phase growth (data not shown). Within the initial 24-hour period after treatment with 250 nmol/L HN2, the proportions of both mutant and corrected cells in S phase increased by approximately 40% (Fig 3). Between 24 and 72 hours, the number of mutant cells in G2/M progressively increased until approximately 72% of cells were in G2/M at 72 hours. Wright-Giemsa staining verified that most mutant cells were arrested in G2, rather than in mitosis. In contrast to the mutant cells, the corrected cells gradually recovered from the transient S-phase arrest and progressed through mitosis to the next cycle.

DMM and DEM predominately produced an S-phase accumulation in both mutant and corrected cell lines (Fig 4). Seventy-two hours after treatment with 50 µmol/L DEM, the mutant and corrected cells in S phase had increased by 35% and 38%, respectively. Although similar proportions of mutant and corrected cells were in S phase, corrected cells arrested earlier than did mutant cells. Treatment of mutant...
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Fig 2. Dose-dependent effect of FAC protein on HN2-induced cell cycle arrest. Normal wild-type, HSC536N, or HSC536N + pFAC3 cells were treated with indicated doses of HN2, stained with propidium iodide 72 hours after drug treatment, and measured by flow cytometry to determine the relative DNA content. A, B, and C contain the histograms of DNA content of HN2-treated normal wild-type, HSC536N, and HSC536N + pFAC3 cells, respectively.

DISCUSSION

We have observed that overexpression of wild-type FAC cDNA in a mutant lymphoblast cell line decreases HN2-induced inhibition of cell growth. This effect was associated with reduced HN2-induced tetraploidy, G2 arrest, and apoptosis. Neither the mutant nor corrected cells spontaneously arrested in G2, which has been reported to occur in mononuclear FA cells after stimulation with phytohemagglutinin.48 Both FA-C mutant and corrected cells were similarly and transiently arrested in S phase during the initial 24 hours after drug treatment (Fig 3). Subsequently, the mutant cells accumulated in G2 (for up to 96 hours) and ceased to progress through mitosis. This prolonged G2 arrest was associated with DNA fragmentation characteristic of apoptosis. Overexpression of the wild-type FAC cDNA prevented the protracted G2 arrest and apoptosis. In contrast to these perturbations induced by HN2, the growth inhibition, S-phase arrest, and apoptosis produced by the monofunctional mustards DMM and DEM were similar in the mutant and corrected cells. We suggest that mutant HSC536N cells have increased sensitivity to bifunctional alkylating agents because of a greater susceptibility to ISC-induced cell cycle arrest and apoptosis. Whether this hypersensitivity to ISC-induced apoptosis is characteristic of all FA-C mutants, or is limited to the L554P mutation remains to be established.

In similar studies reported recently by Kruyt et al,47 it was found that hypersensitivity to MMC-induced apoptosis extended to FA complementation groups A, B, C, and D, suggesting that it is a general characteristic of FA.

Because FAC failed to ameliorate the cytotoxic effects of DMM and DEM (two non-cross-linking mustards), the protein appears to confer ISC-specific cytoprotection. Kruyt et al47 drew a similar conclusion from observations that FA cells were not hypersensitive to γ-rays and UV light; however, the experiments we describe here are more definitive, because HN2, DMM, and DEM all predominately produce similar N7-guanine monoadducts in DNA. HN2, in addition to the monoadducts, produces bis(2-(guanin-7yl)-ethyl)methylamine- and N-(2-(3-adenylnyl)ethyl)-N-(2-(7-guaninyl)-ethyl)methylamine-DNA ISC6.22,23 Although the reactivity of HN2 with DNA is only three and six times greater than 4). Because such a population may represent cells undergoing apoptosis, we tested the hypothesis that wild-type FAC protein suppressed ISC-induced apoptosis. When measured at the time of maximal cell cycle arrest (72 hours after HN2 treatment), DNA fragmentation into multiples of 180-bp fragments occurred in a dose-dependent fashion only in the mutant cells (Fig 5). This fragmentation was not detected in either wild-type or corrected cells treated with up to 250 nmol/L HN2. Similar fragmentation of genomic DNA in wild-type, mutant, and corrected cells required 100-fold higher doses of the monofunctional mustards (Fig 6). No appreciable difference between mutant and corrected cells was apparent, while wild-type cells were somewhat less sensitive (consistent with the growth inhibition data in Fig 1). FAC-mediated protection against HN2-, but not DMM- or DMM-induced apoptosis, suggests that FAC suppresses only ISC-induced apoptosis.

and corrected cells with 50 μmol/L DMM led to similar (34% and 28%, respectively) increases in numbers of cells in S phase. In both cell lines, S-phase accumulation was dose-dependent throughout a concentration range of 2.5 to 50 μmol/L (data not shown).

FAC-mediated suppression of HN2-induced, but not DMM- or DEM-induced apoptosis. Flow cytometry showed a population with less than diploid DNA content among mutant cells treated with HN2 (Fig 2B) and among both mutant and corrected cells treated with DMM or DEM (Fig
that of DMM and DEM, respectively. HN2 inhibited cell growth 610 to 865 times, and induced apoptosis approximately 100-fold more effectively, than did the monofunctional mustards. Thus, the greater potency of HN2 cannot solely result from differential reactivities with DNA.

Bodell et al. and Roberts et al. drew similar conclusions from experiments comparing the cytotoxic and alkylating capability of monofunctional and bifunctional nitrogen and sulfur mustards. The ability to generate DNA ISCs appears to be the critical difference between HN2 and the monofunctional mustard. In light of these data, we propose that HN2 is a more potent cytotoxic agent than the monofunctional mustard, because DNA ISCs activate apoptosis more efficiently than do monoadducts in DNA. Furthermore, the cytoprotective effect of FA-C complementation in cells treated with HN2 (but not those treated with DMM or DEM) suggests that FAC either prevents the formation of DNA ISCs or enhances the removal of DNA ISCs.

Cellular thiols (eg, glutathione and metallothionine) react with cross-linking agents and quench formation of DNA ISCS. The functioning of FAC in a thiol-like fashion to prevent formation of HN2-induced DNA damage is consistent with the cytoplasmic localization of the protein. However, if this hypothesis were correct, then overexpression of FAC should have protected cells against the effects of monofunctional mustards, as well as HN2. Previous reports that MMC, HN2, and psoralen plus UV-A all produce similar levels of DNA ISCs in normal and FA cells are also inconsistent with this notion. Furthermore, we have observed that HN2 induced similar amounts of DNA ISCs in mutant and corrected HSC536N cells (unpublished data). Collectively, these data suggest that the mechanism of FAC-mediated cytoprotection is unrelated to the prevention of DNA ISC formation.

Whether FAC increases the proficiency with which DNA ISCs are repaired in mutant cells is unknown. Although some FA cell lines are partially or completely unable to incise DNA ISCs, other lines have wild-type phenotypes for DNA ISC repair. Differing efficiencies of DNA ISC
Figure 5. Effect of the FAC protein on HN2-induced apoptosis. DNA fragmentation patterns in HSC536N, HSC536N + pFAC3, and normal cells 72 hours after treatment with the indicated dose of HN2 are illustrated. A 123-bp DNA molecular-weight ladder is shown in lane 1.

repair among the FA complementation groups (A, B, and D) may partially account for the apparently conflicting results. Attempts in our laboratory to identify differences in DNA ISC formation or repair in FA-C cell lines have been confounded by the very high doses of HN2 required to detect ISCs in denaturation-renaturation assays. Using an ethidium bromide fluorescence assay or gene-specific Southern blot assays, ISC formation and removal appeared equivalent in both mutant and corrected cells (data not shown). This result is perhaps not surprising, because such high HN2 doses (IC$_{50}$ × 10) likely saturate and mask any differences.

Walsh et al observed that overexpression of FAC prevented G2 arrest in cells treated with MMC and suggested that decreased accumulation of DNA damage allowed successful transition through G2/M checkpoint. Our observation that FAC overexpression prevents HN2-induced (but not monofunctional mustard-induced) cell cycle arrest and apoptosis is consistent with enhanced repair of DNA ISCs in G2 cells. The cytoplasmic localization of FAC protein suggests that the protein may play an indirect role in DNA repair, perhaps by activating other protein(s) that remove DNA ISCs. Alternatively, FAC could facilitate ISC repair in G2 by suppressing the activation of an apoptotic pathway. The data from several FAC gain- and loss-of-function experiments provide partial support for the latter hypothesis. Inactivation of FAC mRNA in human bone marrow by antisense oligonucleotides prevented differentiation of myeloid and erythroid cells. In mutant CD34+ hematopoietic progenitor cells, complementation by FAC led to increased clonogenicity of both untreated and MMC-treated cells. Furthermore, GM-CSF-dependent cells in which FAC mRNA was inactivated became hypersensitive to apoptosis induced by growth factor deprivation, suggesting a protective effect of FAC in suppressing apoptosis. We presume that the FA-C mutant cells have protracted G2 arrest (Figs 2 and 3) and hyperactivated apoptosis (Fig 5) due to the presence of unrepaired DNA ISCs. Suppression of apoptosis by FAC in the G2 phase may allow completion of postreplication repair of DNA ISCs and continuation through the G2/M checkpoint.

In all of the described experiments, the FAC corrected cells behaved essentially the same as the wild-type normal cells, with the qualification that the latter were generally more resistant to both monofunctional and bifunctional compounds, suggesting that they possess a nonspecific resistance mechanism, such as elevated thiols.

Figure 6. Effect of the FAC protein on DEM- and DMM-induced apoptosis. DNA fragmentation patterns in HSC536N (first 2 panels), HSC536N + pFAC3 (second 2 panels), and normal (last panel) cells 72 hours after treatment with the indicated doses of DEM or DMM are shown.
with the normal cell line, although indicative, are not as definitive as the information gained using the isogenic HSC536N cell system.

In conclusion, we propose that the FAC protein decreases the sensitivity of HSC536N mutant cells to cell cycle arrest and apoptosis by restoring competent DNA ISC repair. This protective effect, presumably due to restoration of DNA ISC repair, could be due to indirect activation of other protein(s) that directly participate in lesion removal. Alternatively, in the presence of DNA damage, the FAC protein could prevent the untimely activation of an apoptotic cascade, thereby allowing postreplicative repair of DNA ISC's in G2.

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