The Effect of the Fanconi Anemia Polypeptide, FAC, Upon p53 Induction and G2 Checkpoint Regulation

By Gary M. Kupfer and Alan D. D’Andrea

Fanconi anemia (FA) is an autosomal recessive disease marked by developmental defects, bone marrow failure, and cancer susceptibility. FA cells are hypersensitive to DNA cross-linking and alkylating agents and accumulate in the G2 phase of the cell cycle in response to these agents. FA cells also display genomic instability, suggesting a possible defect in the p53 pathway. To test the effect of heterologous expression of FAC cDNA on drug-induced cytotoxicity, G2 accumulation, and p53 induction in FA cells, we compared two isogenic FA cell lines: HSC536N (mock), a FA type C cell line sensitive to mitomycin C (MMC), and the same cell line transfected (corrected) with wild-type FAC cDNA (HSC536N [+FAC]). HSC536N (+FAC) cells showed a 30-fold increase in resistance to MMC concentration. Similarly, increases in resistance were observed following exposure to cisplatin, carboplatin, and cyclophosphamide. In addition, HSC536N (+FAC) cells showed a twofold lower G2 accumulation following MMC treatment. To analyze the possible interaction of FAC with the p53 pathway, we analyzed p53 induction in mock and corrected cell lines following exposure to MMC. HSC536N (mock) cells induced p53 at lower MMC concentrations than HSC536N (corrected). Caffeine, a known G2 checkpoint inhibitor, not only inhibited G2 accumulation seen in both cell lines but also caused the resistant HSC536N (+FAC) to become as sensitive to MMC as HSC536N (mock) cell line. We conclude that the FAC protein has a specific cytoprotective effect and may function as a cell cycle regulator of the G2 phase of the cell cycle.

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described and was generously supplied by M. Buchwald (Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada). All lymphoblast lines were maintained as suspension cultures in RPMI medium supplemented with 15% heat-inactivated fetal calf serum (growth medium). Cells were grown at 37°C in a humidified atmosphere containing 5% CO2. pREP4 vector (Stratagene, La Jolla, CA) and pREP4-wild type FAC11 were transfected (20 μg), respectively, into HSC536N cells (106 cells), by electroporation, as previously described. Selection with hygromycin (0.1 mg/mL) in RPMI medium was initiated 48 hours after electroporation. The subsequently generated cell lines, HSC536N + vector (mock) and HSC536N + vector-FAC (+FAC) were maintained in culture under hygromycin selection at 0.1 mg/mL.

Drug sensitivity assay. Lymphoblasts (106 cells) were cultured continuously for 4 days, as is standard for this assay, in growth medium (200 μL) containing the indicated concentrations of drug. The number of viable cells was then measured by the XTT (3'-l-phenylaminocarbonyl-3,4-tetrazolium-bis[4-methoxy-6-nitrobenzenesulfonic acid]) assay, a measure of cell proliferation and viability as indicated by respiratory capacity, as previously described.17 G2 accumulation assay and apoptosis detection. Lymphoblasts (5 to 10 × 105 cells) were incubated in the continuous presence of drug for 48 hours. At 24 hours, cells were then washed in phosphate-buffered saline (PBS) and resuspended in 1 mL of a propidium iodide (PI) solution containing 0.1% citrate, 0.03% NP-40, and 0.7 μmol/L PI. The incubation was performed at 4°C overnight or at 37°C for 30 minutes. Approximately 10,000 cells were analyzed for fluorescence intensity by FACScan (Becton Dickinson, San Jose, CA). The percentage of cells in each phase of the cell cycle was determined by analysis of the resultant DNA flow histogram with the computer program CellFIT (Becton Dickinson). Counts from the immediate sub-G1 region were also measured by FACScan at 48 hours of drug exposure, the earliest time point at which we see apoptosis in these cell lines, and were reported as the apoptotic fraction of the total population counted.

Cell irradiation. Lymphoblasts (1 × 106 cells in 5 mL of media) were irradiated using a gamma source-Cesium 137 machine. Cells received 1,000 cGy over 50 seconds. Cells were procured 2 hours later for Western blot analysis.

Immunoblot analysis. Cells treated in the indicated fashion were procured after 2 hours following irradiation and after 12 and 36 hours after MMC treatment. Previous experience indicated that p53 induction did not occur before 8 to 12 hours of MMC treatment, and apoptosis occurred at 48 hours of drug exposure. Therefore we wished to analyze a second timepoint before widespread cell death. Cells were viable by trypan blue exclusion until 48 hours of drug exposure. The cells were washed with PBS, lysed in 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% Triton-X (Sigma, St Louis, MO), and protease inhibitors aprotinin (2 μg/mL), pepstatin (1 μg/mL), leupeptin (2 μg/mL), and phenylmethylsulfonyl fluoride (1 mmol/L), and supernatants collected. The protein concentrations were assessed by Bradford assay and 150 μg of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels. The proteins were then transferred onto nitrocellulose membrane and probed with an anti-p53 antibody (monoclonal antibody 2; Oncogene Science, Uniondale, NY).

RESULTS

Transfection with the FAC cDNA selectively corrects functional cross-linking agent cytotoxicity of HSC536N. To show that FAC-corrected and uncorrected cell lines are representative models of primary cells from normal controls and FA patients, respectively, a wild-type FAC cDNA construct, pREP1, FAC, was transfected into HSC536N cells. Hygromycin resistant cells were selected and compared to a cell population transfected with pREP4 alone. Figure 1 depicts cell survival as measured by the XTT assay to show cytotoxicity of MMC, cisplatin, carboplatin, cyclophosphamide, asparaginase, cytosine arabinoside, etoposide, doxorubicin, actinomycin D, vincristine, H2O2, and gamma radiation. HSC536N (+FAC), the corrected cell line, was less sensitive to MMC with an IC50 of 0.06 μmol/L, whereas HSC536N (mock), the mutant cell line, was more sensitive with an IC50 of 0.002 μmol/L, consistent with previous reports.11,12 IC50 values for the other agents are listed in Table 1. Only the functional cross-linking agents (MMC, carboplatin, cisplatin, cyclophosphamide) revealed differential cytotoxicity in the two cell lines. Potent free radical producers (gamma radiation, H2O2, etoposide, doxorubicin) did not cause differential cytotoxicity. These results are consistent with observed cytotoxicity of primary blood lymphocytes from FA patients versus those of normal controls.6,5 Although only one representative experiment is displayed, similar results were obtained on three separate XTT assays with the same cells.

Transfection with the FAC cDNA corrects drug-induced G2 accumulation of HSC536N. FA cells have prolonged G2 transit times and accumulate excessively in G2 after cell irradiation. In this study, we chose to test the effect of a conservatively high dose of ionizing radiation, 4,000 rad, on FAC-corrected and uncorrected cell lines. Radiation exposure resulted in a substantial increase in G2 cells in both cell lines, with an average increase in FAC-corrected cell lines of 20% over mock controls. A representative experiment is displayed, similar results were observed on three separate XTT assays with the same cells.
The mutant cells consistently had greater G2 content even to correct G2 accumulation, we treated HSC536N (mock) and assayed for p53 protein levels, although only the 12 h timepoint is depicted. Preliminary experiments showed that MMC, like irradiation, can induce p53 induction resulting from DNA damage.23,24 To test the ability of wild type FAC cDNA to correct G2 accumulation, we treated HSC536N (mock) and HSC536N (+FAC) with 0.1 μmol/L MMC or L-cisplatin. HSC536N (mock) cells increased G2 content to 46% and 48% in the presence of MMC and cisplatin, respectively, whereas HSC536N (+FAC) increased G2 content to 22% and 24% (Fig 2). Untreated HSC536N (mock) cells had G2 content from 10% to 15%, whereas untreated HSC536N (+FAC) cells had G2 content from 5% to 8%. The mutant cells consistently had greater G2 content even when untreated, as reported by others.23,39 Similar results were obtained on three separate FACS analysis with the same cells.

MMC induces p53 levels in HSC536N at lower concentrations than in FAC cDNA-complemented cells. Previous studies have shown that MMC, like irradiation, can induce the tumor suppressor, p53, in normal cells.40 A recent report suggested that FA cells are defective in p53 induction.35 To test for differences in MMC-induced p53 levels, we next treated HSC536N (mock) and HSC536N (+FAC) cells with increasing doses of MMC, as indicated (Fig 3). Cells were harvested after 12 and 36 hours of continuous drug exposure and assayed for p53 protein levels, although only the 12 hour timepoint is depicted. Preliminary experiments showed that p53 induction caused by MMC did not occur until 12 hours (data not shown). In contrast to published reports,35 p53 induction was greater in HSC536N (mock) cells at 0.1 μmol/L (lane 4) when measured at 12 hours. No p53 induction was observed at doses below 0.1 μmol/L in mutant cells and below 1 μmol/L in corrected cells. This difference was also evident after 36 hours (data not shown). To show a wild-type p53 effect, we analyzed HSC536N (mock) and HSC536N (+FAC) cells for apoptosis in response to MMC and radiation. HSC536N (mock) cells had a greater percentage of apoptotic cells in response to 0.1 μmol/L MMC (Table 2), consistent with the higher induction of p53 at this drug concentration.

Ionizing radiation induces p53 levels equally in both HSC536N and FAC cDNA-complemented cells. Because HSC536N (mock) cells induced p53 at lower MMC concentrations, we next tested p53 induction in response to a range of gamma radiation doses (Fig 4). Both cell lines induced equivalent amounts of p53 over the radiation dose range indicated. No differences in p53 amounts were observed at later timepoints (data not shown). FACS analysis up to 24 hours showed that at least partial G1/S arrest occurred (data not shown).

Caffeine blocks FAC-induced correction of MMC sensitivity. Previous studies have shown that caffeine can inhibit a G2 checkpoint and decrease G2 phase transit times.27,28 Because of the selective G2 effect of caffeine, we performed cytotoxicity and G2 accumulation assays on HSC536N (mock) and HSC536N (+FAC) (as in Figs 1 and 2) in the presence or absence of caffeine. Caffeine blocked G2 accumulation caused by MMC in both HSC536N (mock) and HSC536N (+FAC) (Fig 5). HSC536N (mock) cells treated with MMC alone accumulated markedly in G2, as was seen in previous data (Fig 1). All values in the caffeine treated samples were corrected against a caffeine-only treated control, which was normalized to 100%.

Caffeine blocks induction of p53 in HSC536N and in FAC cDNA-complemented cells. Caffeine is known to decrease p53 induction resulting from DNA damage.41 We therefore tested for p53 induction in response to MMC treatment in the presence or absence of caffeine (Fig 7). Caffeine reduced p53 induction caused by MMC in both HSC536N (mock) and HSC536N (+FAC). In the absence of caffeine, p53 was again induced at lower concentrations of MMC in HSC536N (mock) cells (lane 2), consistent with previous data (Fig 3).

Table 1. Drug or Radiation Dose Level Causing 50% Kill in HSC536N (mock) and HSC536N (+FAC) Cell Lines

<table>
<thead>
<tr>
<th>Agent</th>
<th>IC50 (μmol/L) HSC536N (mock)</th>
<th>IC50 (μmol/L) HSC536N (+FAC)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMC</td>
<td>0.002</td>
<td>0.06</td>
<td>30</td>
</tr>
<tr>
<td>CDOP</td>
<td>0.01</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>0.5</td>
<td>5.0</td>
<td>10</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>0.5</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>L-Asparaginase</td>
<td>6 μM</td>
<td>6 μM</td>
<td></td>
</tr>
<tr>
<td>ARA-C</td>
<td>0.02</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>VP-16</td>
<td>0.05</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.01</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.09</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>H2O2</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>0.06</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>X-ray</td>
<td>250 cGY</td>
<td>250 cGY</td>
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</table>

Values listed are doses required to cause 50% cell kill in each respective cell line and are obtained from the XTT cytotoxicity data in Fig 1. All concentrations are in micromoles per liter unless indicated otherwise. Fold change is calculated by IC50 HSC536N (+FAC)/IC50 HSC536N (mock).

DNA damage.23,24 To test the ability of wild type FAC cDNA to correct G2 accumulation, we treated HSC536N (mock) and HSC536N (+FAC) with 0.1 μmol/L MMC or 1 μmol/L cisplatin. HSC536N (mock) cells increased G2 content to 46% and 48% in the presence of MMC and cisplatin, respectively, whereas HSC536N (+FAC) increased G2 content to 22% and 24% (Fig 2). Untreated HSC536N (mock) cells had G2 content from 10% to 15%, whereas untreated HSC536N (+FAC) cells had G2 content from 5% to 8%. The mutant cells consistently had greater G2 content even when untreated, as reported by others.23,25,39 Similar results were obtained on three separate FACS analysis with the same cells.

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Fig 2. Transfection with FAC cDNA corrects drug-induced G2 accumulation of HSC536N. HSC536N (mock) and HSC536N (+FAC) cells were either untreated or treated with 0.1 μmol/L MMC for 24 hours. Cells were stained with PI and analyzed by FACS as described in the Materials and Methods. The percentage of cells in each phase of the cell cycle was determined by analyzing data with the computer program CELLFIT (Becton Dickinson).
DISCUSSION

We have compared two isogenic cell lines, one mutant for FAC (HSC536N [mock]) and the other complemented with wild type FAC cDNA (HSC536N [+FAC]) to delineate differences in biological activity and thereby to infer a function of the FAC polypeptide. By comparing these cell lines in parallel, we have validated their use as a functional model of patient-derived primary cells. In contrast to previous work, both cell lines were equally capable of inducing p53 in response to ionizing radiation, but HSC536N (mock) cells were hyperresponsive to MMC. In addition, we have shown that caffeine blocked FAC-induced correction of MMC sensitivity and reduced p53 induction and G2 accumulation in both cell lines, suggesting a role for the FAC polypeptide at a DNA repair-related G2 checkpoint.

To show the specificity of the FAC gene product for certain types of DNA damage, we have presented evidence that mutant FA(C) cells are hypersensitive only to functional alkylating and cross-linking agents. FA has been classified as a disorder with an undescribed DNA repair defect. In the face of the cytoplasmic localization of the FAC polypeptide, a direct role in DNA repair is less likely. Some investigators have suggested that FAC may act in the cytoplasm as a scavenger of free radicals. However, early studies stated DNA cross-linking and alkylating agent specific hypersensitivity in FA cells. Indeed, our data imply that only specific drug classes unmask the phenotypic differences between mutant and corrected cells, while other classes of drugs, such as topoisomerase-targeting drugs, do not, as seen in other studies. Also, drugs such as doxorubicin and etoposide, which are potent free radical producers and for which free radical scavengers reduce cytotoxicity, did not reveal a cytotoxic difference. Indeed, hydrogen peroxide did not cause any difference as well.

Diagrams:
- Fig 3. MMC induces p53 levels in HSC536N at lower concentrations than in FAC cDNA-complemented cells. HSC536N (mock) and HSC536N (+FAC) cells were treated with varying doses of MMC and were harvested for Western blot analysis 12 hours after treatment, as described in the Materials and Methods, using a monoclonal anti-p53 antibody (Ab-2, Oncogene Science) against a human carboxy epitope. Mock transfected and +FAC cells are depicted. MMC doses: 0 (lane 1), 0.001 (lane 2), 0.01 (lane 3), 0.1 μmol/L (lane 4), 1.0 μmol/L (lane 5), and 10 μmol/L (lane 6).

- Fig 4. Ionizing radiation induces p53 levels equally in both HSC536N and in FAC cDNA-complemented cells. HSC536N (mock) and HSC536N (+FAC) cells were treated with varying doses of ionizing radiation and were procured 2 hours after treatment for Western blot analysis, as described in the Materials and Methods, using a monoclonal anti-p53 antibody against a human carboxy epitope (Ab-2, Oncogene Science). Mock cells and +FAC cells are depicted. Ionizing radiation doses: 0 (lane 1), 100 (lane 2), 250 (lane 3), 500 (lane 4), 1,000 (lane 5), and 2,500 cGy (lane 6).

Table 2. Percentage of Apoptotic Cells

<table>
<thead>
<tr>
<th></th>
<th>HSC536N (mock)</th>
<th>HSC536N (+FAC)</th>
</tr>
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<tbody>
<tr>
<td>0.1 μmol/L MMC</td>
<td>2%</td>
<td>2%</td>
</tr>
<tr>
<td>1.0 μmol/L MMC</td>
<td>32%</td>
<td>9%</td>
</tr>
<tr>
<td>100 cGy</td>
<td>44%</td>
<td>43%</td>
</tr>
<tr>
<td>1,000 cGy</td>
<td>8%</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td>13%</td>
<td>8%</td>
</tr>
</tbody>
</table>

Values are obtained from cell cycle analysis and represent sub-G1 region, as analyzed by CellFit program on Becton Dickinson Flow Cytometer.

Arrest of cells in G1 and in G2 in response to DNA damage is a well-described phenomenon in both mammalian cells and in yeast, presumably allowing DNA repair before DNA replication and mitosis, respectively. Several studies have established that FA cells have prolonged cell cycle time and greater accumulation in G2 after DNA damage. Our data suggest that FAC may function as a G2 checkpoint regulator, as FAC corrected G2 accumulation, as noted by Walsh et al.

Because FA cells exhibit genomic instability, we analyzed p53 induction caused by radiation and MMC in HSC536N (mock) and HSC536N (+FAC). p53 is a transcription factor that controls G1/S arrest and apoptosis in response to DNA strand breakage. p53 also controls G2/M checkpoint involved in spindle assembly. In our studies, both the mutant and corrected FAC cell lines exhibited induction of p53 in response to similar doses of ionizing radiation. In spite of this induction of p53, incomplete arrest at the G1/S boundary was seen in these cell lines, perhaps because of expression of EBV-encoded oncoproteins or because wild-type p53 induction is necessary but not sufficient for G1/S arrest. Isolated primary cells and cell lines do display G1/S arrest and apoptosis in response to radiation or MMC treatment. However, the mutant cell line exhibited greater p53 induction in response to MMC in a dose dependent fashion. Our results are in disagreement with a recent report that FAC lines are defective in p53 induction. This difference cannot be accounted for by kinetics or dose differences, as we have analyzed a range of these. Operator and equipment influences may account for 50% kill dose differences for MMC and...
By several arguments, the p53 polypeptide induced in the two cell lines appeared to be functional. First, HSC536N (mock) and HSC536N (+FAC) were capable of undergoing apoptosis, although this phenomenon can occur in p53-~cells. Second, radiation induced p53 equally in both cell lines and caused equal cytotoxicity, while greater induction and cytotoxicity were seen in HSC536N (mock) in response to MMC. Unlike the p53 observed in our studies, mutant p53 polypeptide exhibits excessive accumulation and prolonged half-life. Finally, the two cell lines were isogenic except for the transfected plasmid containing FAC cDNA, and the occurrence of a posttransfection p53 mutation is unlikely.

Caffeine is a known inhibitor of G2 checkpoints, postrepllication repair, and unlinks completion of DNA replication from commencement of mitosis. Caffeine also sensitizes and blocks G2 accumulation in previously radioresistant, p53 (-) cells. Others have reported that caffeine decreases G2 transit time in FA cells, has variable effects on G2 after radiation, and increases chromosomal breaks. Reports of caffeine effects on cytotoxicity have been variable with both potentiation of killing in normal cells as well as protection of killing by MMC in FA cells. In our experiments, caffeine inhibited not only G2 accumulation in both HSC536N (mock) and HSC536N (+FAC) cells in response to drug treatment but also blocked FAC-induced correction of MMC sensitivity. The increase in cytotoxicity of HSC536N (+FAC) cannot be accounted for by an increase in p53 levels. Indeed, caffeine caused a net reduction in p53 levels, likely inducing cell death by a p53-independent mechanism. Although these studies are inconclusive, one interpretation of our results is that the normal G2 checkpoint is triggered in response to cross-linking agents in both mutant and correct cell lines, but that a subsequent DNA repair step is mediated by FAC. Caffeine may interact either directly or indirectly with the FAC polypeptide, thereby blocking this DNA repair step.

We have attempted to show that FAC plays a specific role
in protecting against a defined drug class, namely cross-linking agents, rather than acting as a generic free radical scavenger. Furthermore our data suggest that FAC plays a role in cell cycle regulation. Of course, applicability of these results to the other FA complementation groups must await the cloning of their respective genes and analysis of isogenic mutant and complemented cell lines. Additional work will be necessary to determine the specific nature of the FAC G2 checkpoint.

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