Fanconi Anemia Genes Act to Suppress a Cross-Linker–Inducible p53-Independent Apoptosis Pathway in Lymphoblastoid Cell Lines

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Hypersensitivity to cross-linking agents such as mitomycin C (MMC) is characteristic of cells from patients suffering from the inherited bone marrow failure syndrome, Fanconi anemia (FA). Here, we linked MMC hypersensitivity of Epstein-Barr virus (EBV)-immortalized FA lymphoblasts to a high susceptibility for apoptosis and p53 activation. In MMC-treated FA cells belonging to complementation group C (FA-C), apoptosis followed cell cycle arrest in the G2 phase. In stably transfected FA-C cells, plasmid-driven expression of the wild-type cytoplasmic FAC protein relieved MMC-dependent G2 arrest and suppressed p53 activation. However, in both FA and non-FA lymphoblasts, p53 seemed not to be instrumental in the induction of MMC-dependent apoptosis, since overexpression of a dominant-negative p53 mutant failed to affect cell survival. In addition, no differences in the level of Bcl-2 expression, an inhibitor of apoptosis, were detected between FA and non-FA cells either in the absence or presence of MMC. Our findings suggest that FAC and the other putative FA gene products may function in a yet to be identified p53-independent apoptosis pathway.

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FANCONI ANEMIA (FA) is an autosomal recessive disease characterized by developmental abnormalities (thumb and radius hypoplasia, microcephaly, growth delay, and kidney abnormalities), hyperpigmentation of the skin (café-au-lait spots), and life-threatening bone marrow failure. In addition, FA patients have a dramatically increased risk of developing malignancies, mainly acute myeloid leukemia and squamous cell carcinoma. Cultured FA cells exhibit an increased sensitivity to cross-linking agents such as mitomycin C (MMC) and diepoxybutane. Because of an increased level of spontaneous chromosomal aberrations in cultured cells, FA, like ataxia telangiectasia (AT) and Bloom syndrome (BS), is known as a chromosomal instability disorder. In FA, cell-fusion experiments have revealed four complementation groups, A to D; recently, a fifth group was identified. In contrast to the UV-sensitivity diseases xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy, where disturbances in excision repair and transcription have been documented in detail, the molecular bases of chromosomal instability disorders are still unknown.

The FA group C gene, FAC (according to the nomenclature recommended by Lehmann et al.), cloned by Strathdee et al., is the first chromosomal instability disease gene isolated. The gene encodes a 63-kD polypeptide with no known sequence motifs that could provide a clue to its function. Since functionally active FAC protein apparently localizes to the cytoplasmic compartment of cells, the protein is unlikely to be directly involved in DNA repair.

Besides cross-linker hypersensitivity, cell cycle kinetic studies in primary fibroblasts and lymphocytes derived from FA patients revealed a characteristic spontaneous delay and arrest in G2. This phenomenon may explain the poor proliferative properties of primary FA cells. Since reduction of the oxygen tension corrected this G2 delay in primary cell cultures and decreased the rate of spontaneous chromosomal breaks in lymphocyte cultures, it has been suggested that a cellular mechanism involved in detoxification of oxygen radicals may be responsible for the FA phenotype. However, oxygen hypersensitivity seems to be a secondary manifestation of the primary FA defect, since FA fibroblasts transformed with SV40 large T antigen had lost this feature.

DNA damage, such as that caused by MMC, UV light, and ionizing radiation, can lead to cell cycle arrest in either the G1 or G2 phase, which is thought to facilitate DNA repair prior to DNA replication and mitosis, respectively. An important inducer of G1 arrest is the tumor suppressor protein, p53 (for review, see Zambrini and Levine). Treatment of cells with a wide variety of DNA-damaging agents, including MMC, is known to induce accumulation of p53. AT and BS genes have been suggested to be involved in the pathway leading to p53 activation, since ionizing radiation–induced p53 accumulation was delayed or absent in AT and BS cells.

Another function of p53 is to initiate apoptosis, which is thought to occur when cells cannot cope with the DNA damage and thus avoid their own survival while suffering from an excessive mutational load. Sustained p53 accumulation has been shown to induce apoptosis in a variety of cell types. However, lymphoid cells obtained from transgenic mice lacking p53 (p53-knockouts) were recently shown to readily undergo apoptosis upon exposure to DNA-damaging agents, indicating that p53-independent mechanisms can also trigger apoptosis.

The proto-oncogene, bcl-2, a functional homolog of the Caenorhabditis elegans gene, ced-9, has been identified as an inhibitor of apoptosis in mammalian cells. Constitutive expression of the cytoplasmic membrane protein, Bcl-2, enhanced survival by suppressing cytotoxic agent–induced apoptosis in lymphoid cells, which appeared to be mediated through an as yet unknown mechanism downstream of p53.

To investigate the possible role of FA genes in the pathways that control cellular survival strategies, we set out to document the unique hypersensitivity of FA cells to MMC in terms of p53 activation and apoptosis induction. For this purpose, we used Epstein-Barr virus (EBV)-immortalized lymphoblasts derived from FA patients belonging to different complementation groups and healthy individuals. In ad-

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dition, we used a complementation group C cell line that had been corrected for MMC sensitivity by stable transfection with an episomal vector expressing wild-type FAC. We found that FA cells are highly susceptible to MMC-, but not to gamma ray- or UV-, induced G2 arrest and subsequent apoptosis. Moreover, although the FA genes appeared to function in a pathway that suppresses MMC-dependent p53 activation, apoptosis occurred independently of p53. Thus, FA gene products may function in pathways involved in apoptosis and cell cycle regulation, either directly or indirectly, through DNA repair.

**MATERIALS AND METHODS**

Cell lines and cell treatments. FA and non-FA lymphoblastoid cell lines used in this study (Table 1) were cultured at 37°C in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MD) containing 10% newborn calf serum (HyClone, Logan, UT) under an atmosphere of 5% CO2 in air. Stably transfected cell lines were grown in the same medium supplemented with hygromycin (final concentration, 200 μg/mL; Boehringer, Mannheim, Germany). Cell counting was performed with a Coulter counter (Coulter Immunology, Hialeah, FL). Treatment with MMC (Kyowa Hakko Kogyo Ltd, Japan) was performed according to one of two protocols: (1) exponentially growing cells were exposed for 1 hour to MMC concentrations between 0 and 30 μM, washed in Earle’s balanced salts (GIBCO-BRL), and cultured in the presence of MMC for indicated periods; or (2) cells in parallel cultures were grown, starting with 5 × 105 cells, in the continuous presence of low concentrations of MMC, between 0 and 100 μM as indicated, and harvested at the time that untreated cells had undergone three cell divisions as determined by cell counting (typically after 3 to 5 days). To assess growth inhibition, the final cell count of untreated cultures was set at 100%.

A 60Co source (3.4 Gy/min) was used for exposure of cells to various dosages of gamma rays. Irradiated cells were resuspended in fresh medium at 4 × 106/mL and cultured for 1 or 2 days, as indicated.

For UVC-irradiation exposure, 2 × 106 cells were sedimented and resuspended in 4 mL phosphate-buffered saline (PBS) followed by irradiation in a petri dish (60 cm2) using a germicidal lamp (254 nm) at 10 μW/cm2, and then resuspended in culture medium.

**Plasmids.** pDRFAC was constructed by subcloning the 2-kb BamHI-XbaI fragment derived from pFACC3 in the EBV-based episomal expression vector pDR2 (Clontech Laboratories Inc, Palo Alto, CA) containing the Rous sarcoma virus, LTR.

pDR53m was constructed by subcloning the 1.8-kb BamHI fragment from pC53-SCX31 in pDR2. pC53-SCX3 encodes a mutated p53 protein in which the valine at position 143 is substituted for alanine (p53-143A).

**Stable transfections.** QIAGEN Plasmid Kit (QIAGEN Inc, Chatsworth, CA)-purified DNA was used to produce stably transfected cells by electroporation. Briefly, exponentially growing cells were pelleted and resuspended in RPMI 1640 medium (GIBCO-BRL) containing 10% normal calf serum (HyClone) at a concentration of 5 × 106 cells/mL. Electroporation was performed in a 4-mm cuvette containing 800 μL cell suspension using the EasyJet (Eurogentec, Seraing, Belgium) electroporation apparatus in twin-pulse mode, with a setting for the first pulse at 750V/25 μF/201 Ohm, and for the second pulse at 125V/3,000 μF/999 Ohm. After 24 hours’ recovery time in 10 mL fresh medium, cells obtained from two independent transfections with pDR2-based constructs were grown separately in medium containing 200 μg/mL hygromycin for at least 3 weeks to select for hygromycin-resistant cell populations. Since in pilot experiments similar results were obtained in at least two independently selected stable transfecants containing the same expression vector construct, in most experiments only one selected population was used. In addition, parental and corresponding control cell lines that had been stably transfected with pDR2 alone behaved similarly in all experiments.

**Apoptosis assay and cell cycle analysis.** Apoptotic cells were assayed using propidium iodide (PI) staining and subsequent flow cytometry analysis. The method used was essentially as described previously. Briefly, 5 × 106 cells were fixed in 70% ethanol for 24 to 48 hours at −20°C. After washing with PBS, cells were permeabilized in PBS/0.1% Nonidet P-40 for 10 minutes on ice followed by another wash in PBS. Cells were stained in PBS containing PI 50 μg/mL (Calbiochem, La Jolla, CA) and RNase A 25 μg/mL (Sigma, St Louis, MO) for 30 minutes at room temperature. For each measurement, 105 cells were analyzed for fluorescence, which was recorded by a FACScan flow cytometer (Becton Dickinson, Mountain View, CA); cell debris was excluded on the basis of forward and side light-scattering properties. Cell cycle distribution, including apoptotic cells represented by a “sub-Gi” peak, was determined using the Cellfit program (Becton Dickinson) supplied by the manufacturer.

**Immunoblotting.** Cell extracts were prepared by lysis of PBS-washed cells in (1:1 cold sample buffer (106 cells/10 μL) containing 25 mmol/L Tris hydrochloride, pH 6.8, 1% (w/v) sodium dodecyl sulfate (SDS), 5% (vol/vol) glycerol, 2.5% 2-mercaptoethanol, and 0.25 mg/mL bromophenol blue, followed by sonication for 10 seconds on ice. After boiling for 5 minutes, cell extracts (representing 106 cells per sample) were loaded and electrophoresed on a 10% SDS-polyacrylamide gel and transferred to immobilon-P membrane (Millipore Corp, Bedford, MA). After blocking with 5% dry milk in TBS (10 mmol/L Tris hydrochloride, pH 7.5, and 150 mmol/L NaCl) for 1 hour at room temperature, the membrane was incubated overnight at 4°C with the antihuman p53 mouse monoclonal antibody Do-7 (Dako, Glostrup, Denmark) or antihuman bcl-2 oncprotein mouse monoclonal antibody 124 (Dako), diluted 1:3,000 in TBS containing 1% bovine serum albumin (BSA), Sigma). Subsequently, after washing four times in TBST (TBS supplemented with 0.05% Tween 20; Sigma), the membrane was incubated for 1 hour with biotinylated Fab′ fragments of rabbit antimonoclonal IgGs (Dako) diluted 1:3,000 in TBS/BSA, washed three times in TBST, and incubated with a 1:3,000 dilution of peroxidase-conjugated streptavidin (Dako) in TBS/BSA for 1 hour; all steps were performed at 4°C. Blots were developed by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham, Arlington Heights, IL).

Quantification of p53 levels was performed by densitometry using a Charge Coupled Device camera and a Cybertype Image Processing system (Cybertype, Berlin, Germany).
RESULTS

**FAC suppresses MMC-induced apoptosis in FA-C cells.** To assess whether apoptosis is involved in the hypersensitivity of FA cells to the growth-inhibitory effect of MMC, we investigated MMC-induced apoptosis in FA lymphoblasts belonging to complementation group C and in the same cells expressing FAC. For this purpose, we used HSC536 cells, which have been shown to express a defective FAC protein due to substitution of a leucine for a proline at position 554. We generated stably transfected HSC536 cells containing either the episomal EBV-based expression vector pDR2 alone or pDR2 inserted with the cDNA encoding wild-type FAC protein. In growth-inhibition assays, the wild-type FAC–expressing cell line HSC536FAC was greater than 1 order of magnitude more resistant to MMC than the control cell line HSC536P (Fig 1A). In the same experiment, apoptosis was determined by flow cytometry of PI-stained permeabilized cells. In this assay, apoptotic cells show up in a sub-G1 peak, since chromatin condensation and DNA cleavage, which is characteristic of apoptotic cells, reduces the PI fluorescence intensity to a level less than that of diploid cells. Quantification of the fraction of apoptotic cells in untreated cell cultures showed a background level of less than 5% in both cell lines. However, upon MMC exposure in HSC536P cells, the fraction of apoptotic cells started to increase at 3 nmol/L MMC and continued to increase in a dose-dependent way, whereas in the corrected cell line apoptosis was not observed at concentrations less than 30 nmol/L. Fluorescence microscopy and determination of internucleosomal DNA cleavage by agarose gel electrophoresis confirmed that the occurrence of the sub-G1 peak correlated with the occurrence of cells displaying subnuclear bodies and DNA-cleavage patterns characteristic of apoptotic cells (not shown).

These findings demonstrate that hypersensitivity of HSC536 cells to MMC is correlated with excessive induction of apoptosis, which is suppressed by expression of wild-type FAC.

**MMC-induced apoptosis in lymphoblasts is preceded by G2 arrest.** The cellular response to chronic low-dose MMC, such as applied in Fig 1, is compound, since it reflects the cellular recovery from cytotoxic lesions and the response to new lesions being continuously introduced. Application of a pulse treatment with higher-dose MMC would simplify interpretation of the data, since only the recovery response would be observed. To determine whether the latter protocol could be used to distinguish FA from non-FA cells in apoptosis assays, HSC536P and HSC536FAC cells were treated with various high concentrations of MMC (1 to 30 μmol/L) and MMC-dependent apoptosis was examined after 3 days' recovery time. As observed earlier in these cell lines, the background level of apoptotic cells was again approximately 5% (Fig 2A). In HSC536P cells treated with 1 μmol/L MMC, approximately 15% of the cell population became apoptotic, whereas in FAC-corrected cells 10 μmol/L MMC was required to induce a similar level of apoptosis. Up to 10 μmol/L MMC, a dose-dependent increase in the fraction of apoptotic cells was observed in HSC536P cells, whereas at 30 μmol/L MMC, both cell lines exhibited similar levels of apoptosis (~60%), indicating that this high concentration no longer distinguishes a FA from a non-FA phenotype. In addition, MMC treatment appeared to induce a small population of polyploid cells (>G2) in HSC536P cells, whereas in FAC-corrected cells, which had an elevated background level of polyploid cells, MMC exposure had no such effect (Fig 2B).

The effect of MMC treatment on cell cycle distribution suggested that the onset of apoptosis was preceded by a sharp decrease of the G1 cell fraction with a simultaneous accumulation in the G2 phase (Fig 2A). To examine these phenomena in more detail, time course experiments were performed. Cells were treated with 1, 10, and 15 μmol/L MMC for 1 hour and analyzed after 8, 24, 48, and 72 hours' culture. Quantified cell cycle fractions are shown graphically in Fig 2B; during this time course, cell cycle fractions remained constant in the absence of MMC (not shown).

Within 24 hours, treatment with 1 μmol/L MMC resulted...
FA GENES SUPPRESS p53-INDEPENDENT APOPTOSIS

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PI fluorescence

Fig 2. MMC-induced cell cycle changes and apoptosis in FA-C lymphoblasts and genetically corrected counterparts. (A) HSC536P and HSC536FAC cells were exposed to the indicated concentrations of MMC for 1 hour and assayed for apoptosis after 3 days' culture in fresh medium. PI-stained cells were analyzed by flow cytometry, allowing detection of apoptotic cells as sub-G1 material, indicated by horizontal bars. Percentages of apoptotic cells for each sample are indicated. (B) Time course for the effect of MMC on cell cycle distribution. HSC536P and HSC536FAC cells treated with 1, 10, or 15 μmol/L MMC for 1 hour were fixed after the periods indicated. PI-stained cells were analyzed by flow cytometry, and percentages of cells present in the different phases of the cell cycle were quantified.

in a sharp decrease in the fraction of HSC536P cells in G1 and a simultaneous accumulation in G2 (~70%). At later time points, the fraction of cells in G1 and G2 became equal, but the absolute number of cells in each fraction was reduced 10% to 15% as a result of many cells having undergone apoptosis. Thus, upon treatment with 1 μmol/L MMC, HSC536P cells progress from G1 to G2, and following accumulation in G2, either partially transit to G1 or exit the cell cycle to undergo apoptosis. At 10 and 15 μmol/L MMC and 24 hours posttreatment, the majority of these cells accumulated in G2, whereas apparently part of the cells underwent apoptosis directly from G1, as illustrated by the approximately 10% increase of the sub-G1 fraction and a similar reduction of G2-arrested cells, as compared with the situation found at 1 μmol/L MMC treatment. Whether G2-arrested cells undergo apoptosis directly or following transition to the G1 phase cannot be inferred from this experiment.

In HSC536FAC cells, 1 μmol/L MMC hardly affected the cell cycle distribution; after 24 hours, a small decrease in G1 and an increase in G2 were observed, which returned to normal values at 48 hours posttreatment. In contrast to the situation in HSC536P cells, massive apoptosis induction at 10 and 15 μmol/L MMC in HSC536FAC cells was still accompanied by a fraction of cells (20% to 30% after 72 hours) successfully completing mitosis. Furthermore, in both cell lines, MMC treatment appeared to stimulate progression from G1 to G2, as demonstrated by the increase in S-phase cells 8 hours posttreatment.

In conclusion, wild-type FAC expression in HSC536 cells reduces MMC-induced accumulation in G2 and suppresses...
the onset of apoptosis, as well as progression to polyploidy, in this cell fraction.

**High-susceptibility for MMC-induced apoptosis is common to FA cells and specific for MMC.** To examine whether hypersensitivity to low-dose MMC-induced apoptosis is a common characteristic of the FA cellular phenotype, we determined MMC-induced apoptosis in FA lymphoblasts representing complementation groups A, B, C, and D. As controls, we used a normal human and a FA-A heterozygous cell line (Table 1). Cells were pulse-treated with MMC and assayed for apoptosis after 3 days. In the cell lines, background apoptotic levels were not increased following treatment with 0.1 μmol/L MMC (Fig 3A). However, in all FA cell lines studied, approximately 10% of the cells became apoptotic after treatment with 1 μmol/L MMC, whereas a 10-fold—higher concentration was required to induce a similar level of apoptosis in control lymphoblasts. In addition, PI fluorescence histograms for these cell lines were similar to those depicted in Fig 2A, with histograms for FA and wild-type cell lines being similar to those for HSC536P and HSC536FAC cells, respectively (results not shown). Cultures of cells with wild-type MMC sensitivity were appreciably affected in cell cycle distribution only at the two highest MMC concentrations. However, the elevated level of polyploid cells found in untreated HSC536FAC cells was not observed in wild-type cell lines (results not shown).

Next, we tested whether defective FA genes also affect the apoptotic response to gamma ray or UV irradiation, for which hypersensitivity has been demonstrated in AT and XP cells, respectively. FA and non-FA lymphoblasts, including HSC536-derived transfectants, were gamma- or UV-irradiated and the level of apoptosis was assayed at 1 or 2 days posttreatment. Both gamma ray and UV irradiation induced a dose-dependent increase in the fraction of apoptotic cells (Fig 3B and C). Except for some variation in the level of apoptosis found at fixed doses of gamma irradiation, no differences in apoptotic response were observed between FA and non-FA cells (Fig 3B). The FA phenotype apparently also did not interfere with the apoptotic response to UV irradiation, as depicted for HSC536-derived transfectants (Fig 3C). Thus, the specific hypersensitivity of FA cells to cross-linking agents extends to apoptosis.

**FA genes suppress MMC-induced p53 activation and do not affect Bcl-2 expression.** To investigate the molecular mechanism leading to apoptosis in normal and FA lymphoblasts, we focused on the expression of two predominant modulators of cell death, p53 and Bcl-2.

First, we examined p53 expression by immunoblotting in normal lymphoblasts and a panel of FA cell lines representing complementation groups A to D, including HSC536P and HSC536FAC cells, following continuous exposure to various low concentrations of MMC. Interestingly, as shown by representative experiments in Fig 4A and C, treatment with 3 or 10 nmol/L MMC resulted in a clear increase in p53 expression in all FA lymphoblasts examined except for HSC230 (FA-B) cells, which exhibited only a small increase in p53 levels, whereas at least 10-fold—higher concentrations were required to induce p53 expression in cell lines with wild-type MMC sensitivity. The weak p53 response in HSC230 cells appeared to be characteristic for the FA-B subtype, since VU178 showed a similar response (not shown). The observation that HSC536FAC cells accumulate p53 only after treatment with 100 nmol/L MMC, resembling the situation found in wild-type HSC93 and VU12 cells,
whereas in the FA control cell line HSC536P p53 activation was readily detected following treatment with 3 nmol/L MMC, shows that FAC suppresses MMC-induced p53 activation.

Next, we investigated p53 and Bcl-2 expression in lymphoblasts recovering from high-dose MMC treatment, i.e., 1 hour of treatment with 1 to 30 μmol/L MMC. Time course experiments in these cells showed that p53 accumulated within 8 hours after MMC treatment, reaching a maximum at 24 hours, followed by a decrease at 48 hours (not shown). Figure 4B shows p53 accumulation in lymphoblasts 24 hours posttreatment. A small increase in p53 expression was detected in wild-type VU12 cells exposed to 2.5 and 5 μmol/L MMC, whereas treatment with 10 and 30 μmol/L MMC strongly induced p53 accumulation. In contrast, 1 μmol/L MMC was sufficient to induce strong p53 accumulation in FA cell lines VU56 and VU202. These findings were confirmed in other wild-type and FA cell lines but not in FA-B cells, which again showed only a weak p53 response (not shown). In addition, similar Bcl-2 expression levels were detected in wild-type and FA cells, which was not affected by MMC treatment (Fig 4B). This indicates that hypersensitivity of FA cells to MMC-induced apoptosis is not due to an altered expression level of the cell-death inhibitor, Bcl-2.

Taken together, these results show that MMC-induced p53 accumulation is suppressed by FAC genes, although this was much less obvious in the case of FA-B cells. Moreover, FAC appears to function in a cytoplasmic pathway that by an as-yet-unknown mechanism antagonizes MMC-dependent p53 accumulation.

**Gamma or UV irradiation–induced p53 activation is not affected by FAC.** To investigate whether FAC might also be involved in pathways leading to gamma ray– or UV-induced p53 accumulation, we studied p53 activation in HSC536P and HSC536FAC cells. Cells were exposed to various dosages of gamma or UV radiation, whereafter p53 induction was monitored. As depicted in Fig 5A and B, essentially no dose-dependent differences in p53 accumulation were detected in these cell lines, although UV-induced p53 accumulation seemed to reach higher levels in HSC536P as compared with HSC536FAC cells. Similar observations were made in other FA and non-FA cells treated with both types of radiation (not shown). In addition, as observed in MMC-treated lymphoblasts, Bcl-2 expression was not affected by either gamma or UV irradiation in these cells.
These findings indicate that FA gene products do not function in the pathway leading to gamma ray- or UV-induced p53 accumulation.

Mechanism of apoptosis induction in FA and non-FA lymphoblasts is p53-independent. Accumulation of p53 is known to be associated with DNA damage and to mediate G1/S cell cycle arrest, which may facilitate DNA repair. On the other hand, when a cell cannot cope with the genomic insult, p53 can induce cell death. Our results indicate that functional FA gene products can suppress induction of p53, since in FA lymphoblasts p53 accumulation is detected at MMC concentrations that do not induce p53 expression in wild-type cells. In addition, expression of wild-type FAC in HSC536 cells repressed p53 activation, leading to a MMC concentration–dependent accumulation of p53 similar to that seen in wild-type lymphoblasts. Although the increase in p53 expression appeared to correlate with the occurrence of apoptotic cells, MMC-induced cell cycle arrest at the G1/S border was not apparent in the lymphoblastoid cell lines studied (Fig 2B). This suggests that p53 does not properly exert its G1/S checkpoint function in these EBV-transformed lymphoblasts, even though it still might be involved in mediating apoptosis. However, the weak MMC-dependent p53 response found in FA-B cells does not reflect the strong induction of apoptosis (Figs 3A and 4A and C), and may suggest that other mechanisms could be involved in mediating apoptosis. Moreover, it is questionable whether p53 can exert its proper function in EBV-transformed lymphoblasts. Analogous to the situation found in SV40-immortalized fibroblasts, where the function of p53 is abrogated by complex formation with the oncogene protein large T antigen, p53 function in EBV-transformed lymphoblasts may be impaired by EBV-encoded proteins EBNA-5 and BZLF1, which have been reported to interact with p53. In fact, BZLF1 has been shown to inhibit p53-dependent transactivation in lymphoid cells.

To investigate whether p53 mediates MMC-induced apoptosis in EBV-transformed FA and non-FA lymphoblasts, we stably transfected a panel of cell lines with the expression vector pDR2 containing cDNA encoding the dominant-negative-acting mutant p53-143a, and examined MMC-dependent growth inhibition and apoptosis. Among other defects in function, this mutant form of p53 has lost its ability to suppress cell growth and fails to either activate or repress transcription, and in addition, is thought to inactivate wild-type p53 by hetero-oligomer formation.

In immunoblotting experiments (Fig 6), we found a threefold to sixfold overexpression of p53-143a in stably transfected cells as compared with the level of p53 detected in control cell lines transfected with the empty expression vector, as determined by densitometry. If p53 would mediate MMC-induced apoptosis in EBV-transformed lymphoblasts, expression of mutant p53 in FA lymphoblasts would be expected to enhance their MMC resistance. However, as depicted for HSC93, HSC536, and VU56 cells (Fig 7A and B), p53-143a-expressing transfectants of both normal and FA cells did not behave differently in either growth-inhibition or apoptosis assays, indicating that wild-type p53 function is not essential for MMC-induced apoptosis in lymphoblastoid cells.

DISCUSSION

In this study, we show that hypersensitivity of EBV-immortalized FA lymphoblasts to the growth inhibitory effect of low-dose MMC is due to an excessive accumulation in the
G2 phase of the cell cycle that precceeds massive induction of apoptosis. As illustrated by restored expression of wild-type FAC in stably transfected FA-C lymphoblasts, this protein suppressed both low-dose MMC–induced G2 accumulation and apoptosis (Figs 1 and 2). At high-dose MMC pulse treatment (30 μmol/L), similar levels of apoptotic cells were detected in both FA-C and FAC-corrected stably transfected cell cultures. Time course experiments showed that MMC-induced G2-accumulated cells either transit to G1 or exit the cell cycle to undergo apoptosis. The percentage of cells in G1 was inversely correlated with the MMC dose. Already at low-dose MMC, only a minor proportion of FA-C cells were in G1, accompanied by a strong increase in the level of apoptotic cells, in contrast to FAC-corrected cells, which showed a similar response only at an approximately 10-fold higher MMC concentration. The time course experiments do not allow us to conclude whether the cells undergo apoptosis directly from the G2-arrested state or shortly after transition to G1. Furthermore, time course studies showed MMC-induced accelerated G1/S transition in lymphoblasts, as indicated by an increase in S-phase cells 8 hours posttreatment (Fig 2B). In primary FA lymphocytes, high sensitivity to MMC-induced G2 delay and arrest within this compartment has been reported previously, but accelerated G1/S transition was not observed. Therefore, our finding in EBV-transformed cells may be related to the presence of virally encoded proteins.

Spontaneous G2 delay observed in primary FA cells has been interpreted to reflect impaired DNA repair. However, under reduced oxygen tension this characteristic is lost, which has led to the hypothesis that FA cells are defective in a defense mechanism involved in detoxification of activated oxygen species. In contrast to primary FA fibroblasts and lymphocytes, FA fibroblasts transformed with SV40 large T antigen no longer exhibit oxygen hypersensitivity. However, since SV40-transformed fibroblasts still show MMC hypersensitivity, oxygen sensitivity seems to reflect a secondary consequence of the primary defect in FA. We have found oxygen hypersensitivity also to be absent in FA lymphocytes upon EBV immortalization (H. Joenje and F.A.E. Kruyt, unpublished results, May 1995); moreover, in the present study, we failed to observe significant differences in G2 cell fractions between untreated FA and non-FA cells (Fig 2 and results not shown), which is in contrast to the reported spontaneous G2 delay in nontransformed FA cells and is also likely to be a result of EBV immortalization.

Despite the above differences between primary and virus-transformed FA cells, both cell types are hypersensitive to cross-linkers. We found high susceptibility to MMC-dependent apoptosis to be a novel characteristic of FA lymphoblasts. In FA and non-FA cells, no differences were observed in the apoptotic response triggered by either gamma or UV irradiation (Fig 3B and C), treatments known to evoke hypersensitivity in AT and XP cells, respectively. This substantiates the notion that the genes defective in AT, XP, and FA, although all involved in maintaining genomic stability, clearly function in separate pathways.

Possible functions of FA gene products in the repression of MMC-induced p53 accumulation. Ionizing radiation and many cytotoxic drugs, including MMC, are known to induce accumulation of the tumor suppressor protein, p53, which typically leads to cell cycle arrest at the G1/S border, thus allowing DNA repair. The precise nature of signals that activate or inactivate p53 are presently unknown, although DNA damage is known to have a triggering function.

In this study, we found FA cells, representing complementation groups A, C, and D, to be highly susceptible to MMC-dependent p53 accumulation, ie, accumulation was detected at approximately 10-fold–lower MMC concentrations as compared with wild-type cells (Fig 4). In FA-B cells, we detected only a weak p53 response following MMC treatment, suggesting that FA-B cells are different in this respect from the other FA subtypes. Restored wild-type FAC expression in FA-C cells essentially normalized MMC-dependent p53 accumulation, whereas no such effect was detected following gamma or UV irradiation, although FAC expression seemed to reduce the level of UV-dependent p53 induction to some extent (Fig 5). These results indicate that FA gene products specifically act to suppress p53 activation in response to MMC.

Several mechanisms can be considered to explain the differences in p53 response, whereby FA gene products may function in a feedback mechanism that suppresses p53 activation. The notion that FA gene products are directly involved in DNA repair has been contradicted for FAC, which is localized to the cytoplasm. Moreover, FAC appears to be functionally active only in this cellular compartment, since a nuclear-directed fusion protein, containing FAC linked to the nuclear localization domain of the SV40 large T antigen, is unable to correct for MMC hypersensitivity in FA-C cells, whereas this potential is restored by mutational inactivation of the nuclear localization domain (H. Youssoufian, personal communication, March 1995). Therefore, two main alternatives may be suggested for FA gene function: (1) a nuclear target, eg, DNA, provides a signal for FA gene products to be activated, which induce an unknown cascade leading to activation of specific factors counteracting the cytotoxic effects of cross-linking agents; or (2) FA gene products operate in a cytoplasmic system designed to detect cross-linkers, leading to avoidance of cross-link damage.

The first possibility would require ‘‘inside-out’’ signal transduction, whereby a nuclear signal activates a cytoplasmic pathway, a mechanism that has been considered unlikely. Alternatively, DNA fragments originating from DNA repair activity may leak to the cytoplasm, thereby forming a signal for FA gene products to become activated. On the other hand, analogous to UV-induced signal transduc-
tion pathways, involving the detection of UV light at the cell membrane via an unknown receptor, leading to tyrosine kinase-mediated activation of immediate-early genes such as c-fos and c-jun (for review see Blattner et al35), a similar type of cascade might be involved in the cellular response to cross-linking agents. As in the case of the UV response, which is still functional in enucleated cells, indicating that a nuclear signal is not required,54 FAC may function in a MMC-dependent signal transduction pathway. Thus, cells may possess an extranuclear “cross-linker receptor” which is activated by cross-linkers and induces a response that counteracts the cytotoxic effects of cross-linking agents. FA gene products may either function in the detection of cross-linkers or be active in a signal transduction cascade induced by the activated cross-linker receptor, including enzymes that counteract cross-linker cytotoxicity such as specific DNA-repair proteins. Alternatively, these enzymes may function to detoxify cross-linking agents in the cytoplasmic compartment, as suggested by Youssoufiun.9 During evolution, a cytoplasmic defense system may have evolved that is specifically designed to protect DNA from naturally occurring cross-linkers, which are of both endogenous and exogenous origin (Joenje and Gille15 and references therein); examples of endogenously produced cross-linker agents include singlet oxygen and several dialdehydes resulting from lipid peroxidation.

Regardless of the underlying mechanisms of FA gene function, the putative FAB gene product apparently functions differently from other FA gene products, as indicated by the atypical p53 response observed in FA-B cells. This function might involve a more direct role in cell cycle regulation. Recently, Digweed et al44 showed that overexpression of a small cyclin-related protein, designated SPHAR (for S-phase response), partially corrected the permanent repression of DNA synthesis observed in cross-linker–treated primary FA-A fibroblasts. In addition, in untreated stably transfected HSC536FAC cells, we found an increased proportion of polyploid cells as compared with the control transfec tant HSC536P and other nontransfected lymphoblast cell lines (Fig 2). This cell fraction likely represents polyploid cells resulting from endomitosis, a process in which a new round of DNA replication occurs before mitosis. Endomitosis has been a frequent finding in primary FA lymphocyte cultures,11 as well as in non-FA lymphocytes exposed to high levels of oxygen.45 We are currently exploring the possibility that FAC might be directly involved in G2/M transition.

Rosselli et al46 recently reported that FA lymphoblasts are defective in both MMC- and gamma-radiation–induced p53 accumulation. These results are in contrast to our data. The discrepancy might be explained by their use of a control cell line with an unusual p53 response. For example, short-term exposure to low-dose MMC (10 and 100 ng/mL, equivalent to 30 and 300 nmol/L MMC) for, at most, 16 hours failed to induce p53 accumulation in FA-C and FA-D cell lines, but did activate p53 in their control cell line. In our hands, 24-hour exposure to 100 nmol/L MMC did not induce p53 accumulation in both FA and non-FA cells, including HSC536P and HSC536FAC cells (not shown), suggesting that their control cell line was somehow hyperresponsive. The discrepancies concerning ionizing radiation–induced p53 activation might be explained in a similar way. Moreover, in our hands, the short-term low-dose protocol used by Rosselli et al is not suitable to demonstrate specific cross-linker hypersensitivity of FA cells. Instead, we used protocols that clearly distinguish FA from non-FA cells, i.e., chronic low-dose MMC treatment (≤100 nmol/L) or 1 hour of exposure to high MMC concentrations (1 to 30 μmol/L, pulse treatment), which revealed clear differences in p53 activation (Figs 4 and 5). Furthermore, we observed some variation in the sensitivity of FA and non-FA cells to gamma radiation–induced apoptosis, which was not related to the FA phenotype, suggesting that the somewhat enhanced resistance of the two FA cell lines to gamma-radiation–induced apoptosis as observed by Rosselli et al may be related to such variations.

**Role of FA genes in the suppression of p53-independent apoptosis.** Despite the MMC-dependent accumulation of p53, G1 arrest was not apparent in the lymphoblastoid cell lines used, as illustrated by cell cycle kinetic studies in the HSC536-derived stable transfectants (Fig 2). Furthermore, in FA-B cells, the weak MMC-dependent p53 response did not reflect the strong induction of apoptosis (Figs 3A and 4A). Since it has been reported that p53 function is impaired by interaction with the EBV-encoded proteins EBNA-5 and
BZLF1,23,38 these findings suggested that p53-independent mechanisms were mediating apoptosis. Indeed, overexpression of the dominant-negative mutant, p53-143ala, known to be impaired in both transactivation and transrepressor function (reviewed in Zambetti and Levine14), failed to affect MMC-dependent cell survival and apoptosis (Fig 7). This finding is in agreement with the results of Strasser et al.25 who showed apoptosis to be induced in MMC-treated lymphoid cells derived from p53-knockout mice. Although wild-type FA genes can act to prevent or avoid MMC-induced and p53-independent apoptosis in lymphoblasts, it remains possible that in primary FA cells elevated p53 levels will both mediate G1/S arrest and contribute to the induction of apoptosis. Whether FA gene products only counteract the cytotoxic effects of MMC at low concentrations, thereby preventing apoptosis, or are also linked directly to p53-independent apoptotic pathways remains to be elucidated. The latter possibility is especially attractive, since it would help to explain a number of clinical symptoms in FA. For example, FA patients typically suffer from bone marrow failure due to impaired hematopoiesis. Since cytokine-dependent repression and induction of apoptosis is known to play an important role in the maturation of cells belonging to the hematopoietic and immune system (for reviews, see Sachs and Lotem147 and Allen et al.48), bone marrow failure in FA may be related to an exaggerated tendency of hematopoietic progenitor cells to undergo apoptosis, which would implicate wild-type FA genes in hematopoietic-tissue homeostasis based on apoptotic mechanisms. Evidence for such a mechanism was recently provided by Segal et al.49 who demonstrated that normal hematopoietic progenitor cells exposed to antisense oligonucleotides complementary to FAC mRNA had inhibition of growth. In addition, in the present study, no aberrant expression levels of the apoptosis inhibitor Bcl-2 were found in FA lymphoblasts; Bcl-2 is known to oppose apoptosis in hematopoietic cells. Thus, FA genes might have a specific function to suppress apoptosis independently of Bcl-2.

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