[haematologica]
2004;89:11-20

Fanconi's anemia cell lines show distinct mechanisms of cell death in response to mitomycin C or agonistic anti-Fas antibodies

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Background and Objectives. Fanconi's anemia (FA) cells are characteristically hypersensitive to bifunctional alkylating agents, notably mitomycin C (MMC), causing increased programmed cell death (PCD). FA cells also have abnormalities in mitochondrial function. We hypothesized that the abnormalities in PCD are mitochondrially mediated. We examined mitochondrial function in FA cells, comparing the intrinsic death pathway induced by MMC with the extrinsic pathway via Fas antibody, which can bypass the mitochondria.

Design and Methods. Normal and FA lymphoblastoid cell lines were treated with MMC or agonistic anti-Fas antibody. PCD was assessed using flow cytometry, Western blot analysis, and DNA gel electrophoresis.

Results. FA cells showed hypersensitivity to MMC, but slight resistance to Fas-mediated PCD. MMC induced chromatin condensation, but not apoptotic body formation. Fas induced classical apoptosis. MMC failed to induce mitochondrial depolarization, while some depolarization occurred with anti-Fas. These results suggest that MMC failed to induce caspase activity in FA cells. No cleavage of caspase 3 was observable and PCD was not inhibited by the caspase inhibitor zVAD-fmk. Fas-induced caspase 3 cleavage, and cell death was inhibited by zVAD-fmk. There were common downstream abnormalities in the execution phase of PCD, as both agonists failed to cleave PARP, or to induce nucleosomal fragmentation.

Interpretation and Conclusions. Our results suggest that mitochondrial function in FA cells is abnormal, resulting in necrotic or caspase independent PCD, but that further abnormalities may exist downstream of the mitochondria. This may have implications in explaining *in vivo* aspects of FA.

Key words: Fanconi's anemia, apoptosis, mitochondria, mitomycin C, Fas.

http://www.haematologica.org/journal/2004/1/11/

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Fanconi's anemia (FA) is a rare autosomal recessive disorder, characterized by the variable presence of a range of congenital abnormalities, a reduced life expectancy due to the onset of bone marrow failure, and a high predisposition to develop leukemia, and solid tumors, notably squamous cell carcinoma.[1] Somatic cell studies reveal that FA cells have a complex phenotype, characterized *in vitro* by oxygen sensitivity, increased spontaneous chromosomal fragility, and extreme sensitivity towards bifunctional alkylating agents, notably mitomycin C (MMC), and diepoxybutane.[2] FA cells exhibit phenotypic abnormalities in redox metabolism, apoptosis, cell cycle control, cytokine response, cell signaling, as well as DNA repair.[3] Eight complementation groups have been identified (A-G), and six of the eight genes cloned (*FANCA, FANCC, FANCD2, FANCE, FANCF*, and *FANCG*) are novel with little or no homology to known genes.[4] Group D is now known to consist of two genes, D1 and D2, and recently the D1 gene has been identified as the tumor suppressor gene *BRCA2*.[5] In spite of extensive investigation, the precise functions of the FA genes still remain unknown. However, the common clinical and cellular features found in FA patients suggest that FA proteins may interact in a common pathway. With the exception of FANCD2, which is exclusively nuclear, the other FA proteins appear to be compartmentalized between the nucleus and the cytosol. FANCC is predominantly cytosolic[6] with a relatively minor nuclear presence,[7] and FANCA, FANCF, and FANCG are present in both the cytosol and nucleus. A complex of FANCA, FANCC, and FANCG proteins can relocate from the cytosol to the nucleus,[8] and further association with FANCE and FANCF triggers the mono-

ubiquination of the FANCD2 protein.[9] FANCD2 co-localizes with BRCA1, BRCA2 and RAD51 to nuclear foci, suggesting a role for FA proteins in surveillance of chromosomal integrity, and DNA repair. However FA proteins are also cytosolic, and their association with a growing list of cytosolic proteins implicate them in different cellular pathways upstream of nuclear involvement and provide insight into the pleiotropic FA phenotype. The FANCC protein interacts with a variety of different proteins. These include the cell cyclin cdc2,[10] molecular chaperones GRP94[11] and HSP70,[12] NADPH cytochrome P450 reductase,[13] involved in xenobiotic biotransformation, glutathione S-transferase (GSTP1)[14] involved in redox metabolism, and STAT1[15] involved in cell signal transduction. FANCA is a phosphoprotein which associates with the IκB kinase (IKK) signalsome via interaction with IKK2,[16] promoting NF-κB signaling, which can have pro- and anti-apoptotic effects. FANCG, also a phosphoprotein, is localized to both the cytosol and nucleus and relocates from the nucleus to the cytosol and mitochondria in MMC-treated cells.[17] FANCG interacts with CYP2E1, a member of the P450 superfamily associated with production of ROS and bioactivation of carcinogens, possibly implicating FANCG in a redox mechanism, and protection against DNA oxidative damage.[18]

FA lymphoblastoid cells (LCL) treated with MMC do not undergo classic apoptotic cell death, and exhibit both qualitative and quantitative abnormalities.[19,20] There is absence of apoptotic body formation, caspase-3 activation and PARP cleavage, as well as absence of oligosomal DNA laddering. MMC alkylates genomic and mitochondrial DNA,[21] forming DNA adducts, and interstrand crosslink formation is a major cause of MMC toxicity. However MMC toxicity may also be due to the cyclic generation of high intracellular levels of reactive oxygen species (ROS), generated by MMC bound to DNA.[22-24]

Several reports indicate abnormal mitochondrial structure and function in FA cells.[25-27] Since MMC does not induce the classic features of apoptosis in FA cells, and mitochondria have a central role in the intrinsic pathway of apoptosis, this may implicate mitochondria in the abnormal cell death process in FA cells. Fas-induced cell death utilizes the extrinsic pathway of apoptosis. This involves trans-membrane activation of the death-inducing signaling complex (DISC), involving activation of caspase-8, which can initiate apoptosis, bypassing the mitochondria, and directly activate caspase-3.[28]

In this study we compared the response of FA cells to the extrinsic and intrinsic pathways of apoptosis. We show that different mechanisms of cell death are induced by MMC and anti-Fas antibody, indicating abnormal mitochondrial function in the cell death mechanism in FA cells.

Design and Methods

Tissue culture

Human Epstein-Barr virus (EBV)-transformed LCL from defined complementation groups were maintained in RPMI 1640 with Glutamax-1 containing 10% fetal calf serum and supplemented with penicillin/streptomycin (Life Technologies, Paisley, UK). Cells were cultured in a humidified incubator in ambient oxygen with 5% CO_2 at 37°C. Cell viability was determined using the trypan blue exclusion assay.

Cell lines

HSC536 (FA-C) LCL were a gift from Professor Manuel Buchwald. FA-C LCL express a functionally defective FANCC protein due to a leucine for proline substitution at position 554.[29] A genetically corrected FA-C cell line (referred to as FA-C+C) was made by transfection of an *FANCC* cDNA in the EBV-based expression vector pREP4,[29] which also contained a hygromycin resistance gene. HSC72 (FA-A) LCL produce very little FANCA mRNA.[30] HSC72 cell lines that contained a hygromycin resistance gene expression vector with no insert (FA-A), or a genetically corrected cell line (FA-A+A) containing *FANCA* cDNA in the DR_2 expression vector were provided by Dr Hans Joenje. Tansfected cell lines were maintained long term in the presence of 200 mg/μL hygromycin (Sigma). A control cell line AB0048 (normal) derived from a normal Caucasian male was obtained from ECAAC, Porton Down, UK.

Induction and inhibition of cell death

Mitomycin C (2 mg, Sigma), was reconstituted in high quality water (≥ 18 mM) to 1 mM. Further dilutions were made in the same quality water. The solutions were sterilized by 0.22 μM filtration and added to cells at a final concentration of 25 nM or 100 nM. Cells were incubated in standard culture conditions for 4 days. Anti-Fas antibody (CD95-Immunotech) was added to the cells at a dose of 250 μg/mL for 24 hrs. The broad spectrum caspase inhibitor zVAD-fmk (caspase-1 inhibitor V, Calbiochem), was reconstituted as a 50 mM stock solution in DMSO. zVAD-fmk was added to cells at a final concentration of 100 μM on day 0, and equal amounts were added on each of the three subsequent days.

Western blotting

Proteins were detected in whole cell extracts by Western blotting. The LCL (3×10^6) were lysed in buffer (20 mM Tris pH 8.0, 40 mM $Na_4P_2O_7$, 50 mM NaF, 5 mM $MgCl_2$, 10 mM EGTA, 1% Triton-X100, 0.5% sodium deoxycholate, 0.1% SDS) containing broad spectrum protease inhibitors (Complete Mini tablets, Roche). The protein concentration of cell lysates was determined

using the BCA method (Pierce). Proteins (4-10 μg) were loaded onto 5% stacking SDS-PAGE gels and separated on 10-12% resolving gels, after electrophoresis at 150V for 1.5 hrs in a *Ready Gel Cell®* mini system (Bio-Rad). Proteins were electroblotted at 100V for 1 hr onto Hybond ECL nitrocellulose membrane (Amersham) using a Bio-Rad transfer cell. Membranes were washed in TBS blocking buffer (50 mM Tris-HCl pH 7.5, 188 mM NaCl) containing 0.1-0.2% Tween 20 (TBST) and supplemented with 5-8% dried skimmed milk (MARVEL, Premier Beverages) for 1hr with agitation.

Immunoblotting was performed with primary antibodies (Human PARP polyclonal antibody diluted 1:2000 (Upstate #06-557), caspase-3 polyclonal antibody diluted 1:2000 (BD Pharmingen #551150), caspase-8 polyclonal antibody diluted 1:2000 (BD Pharmingen #559932), anti-actin diluted 1:1000 (Sigma #A5060), followed by horseradish peroxidase conjugated secondary antibody (Upstate #12-324 diluted 1:3000). Enhanced chemiluminescence (ECL Amersham) was used for development followed by exposure to X-ray (Amersham Hyper ECL) film.

Cell death assays

7-AAD staining

The level of apoptosis in cells was quantified using 7-amino actinomycin-D (7-AAD, Sigma) staining.[31] The cells were washed twice in PBS (containing 0.05% sodium azide, 1% fetal calf serum) and then resuspended in 450 μL of wash solution; 50 μL of 7-AAD (200 μg/mL) were added, and the solution was incubated at 4°C in the dark for 20 mins. Cells were pelleted at 1000 rpm, fixed in freshly made 2% paraformaldehyde (Sigma), and analyzed on a FACScan analyzer (Becton Dickinson, Mountain View, CA, USA) within 30 min of fixation using Lysis II software (Becton Dickinson). Scattergrams of 7-AAD fluorescence (FL-3) versus forward scatter (FSC) were used to define two regions representing populations of live and apoptotic (including dead) cells and the proportion of cells within each region was determined.

Mitochondrial depolarization

Cells were incubated under standard culture conditions in 24-well plates. JC-1 (5,5', 6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide, Molecular Probes) was added to 500 μL of cells at a final concentration of 10 μg/mL.[32] The cells were incubated for 10 min at 37°C, then transferred to ice. Mitochondrial depolarization was assayed by FACscan analysis (Becton Dickinson). Forward and side scatter were used to exclude debris and dead cells. Cells were excited at 488 nm and green (530 nm) and red (585 nm) fluorescence were collected in the FL1 and FL2 channels respectively. FL1-FL2 compensation was 4%, FL2-FL1 compensation was 12%. A total of 20,000 events were acquired, and data analyzed using Cell Quest software (Becton Dickinson). Green fluorescence (FL1) represents the monomeric form of JC-1, and red fluorescence corresponds to the J-aggregate form of JC-1. Relative cell numbers were counted for the polarized and depolarized populations, from which the percentage of depolarized cells was determined.

DNA fragmentation

DNA was extracted for fragmentation studies using the method described by Newell *et al.*[33] Cells were lysed and pelleted at 4°C. Small molecular weight DNA fragments were purified using phenol:chloroform:*iso*-amyl alcohol (1:1:24, Sigma) extraction at 4°C, and DNA fragments were precipitated using NaCl and isopropanol. The DNA fragments were pelleted at 12,000g for 10 min, washed with 70% ethanol, dried, and resuspended in 10 μL of TE (containing 0.5 mg/mL RNAse A, Sigma). The resuspended fragments were heated at 65°C for 10 min to denature the RNAse, and loaded onto a 0.85% agarose gel in TAE. The gel was electrophoresed for 90 min at 7 V/cm, and migration of the DNA ladder was visualized by ethidium bromide staining under UV light.

Results

Quantitation of apoptosis

FA and control cell lines were treated with 250 μg/mL anti-Fas for 24 hrs, or with 25 nM MMC for 4 days, and the level of cell death was determined using 7-AAD staining and flow cytometry.[19] Live cells with an intact membrane show low fluorescence, dead cells with loss of membrane integrity show maximum levels of fluorescence, whereas cells undergoing mid-apoptosis exhibit intermediate levels of fluorescence. The percentage cell death was defined as the cell fraction undergoing apoptosis and committed to die, added together with the dead cell population. Therefore the figures for cell death are higher using 7-AAD staining than using trypan blue staining, which only measures dead cells. Preliminary studies with anti-Fas on Jurkat cells demonstrated induction of apoptosis at 6 hrs, as judged by morphology (Figure 2). After 24 hrs DNA laddering also became apparent, so cells were treated for 24 hrs in subsequent experiments.

We had previously demonstrated increased cell death in FA-C cells treated with 25 nM MMC.[19] We extended this study to include FAA and FAA+A cells lines. FA-C and FA-A LCL showed greater sensitivity towards MMC than did normal cells, as expected, and this was normalized in their respective genetically corrected cell lines (Figure 1A). In contrast, when treated with anti-Fas antibody, normal LCL showed markedly greater cell death than did either of the FA cell lines (Figure 1B).

FA cells show agonist-dependent cell death morphology

Classically cells undergoing apoptotic cell death exhibit identifiable morphologic changes.[34] These changes include membrane blebbing, nuclear condensation, cytosolic vacuolization, and apoptotic body formation with degraded chromatin. The FA-C cell line treated with MMC showed marked chromatin condensation, with a lack of apoptotic body formation (Figure 2A), as previously reported.[19] The FA-A cell line treated with MMC showed similar marked chromatin condensation, and a lack of apoptotic bodies (Figure 2B). Interestingly the genetically corrected cell lines for both FA-A and FA-C, although corrected for MMC sensitivity, did not show apoptotic morphology (Figures 2C-D), when compared to the normal LCL or Jurkat cells (Figures 2E-F).

FA cells treated with anti-Fas antibody demonstrated a markedly different morphology compared to cells treated with MMC. FA-A, FA-C, as well as FA-A+A and FA-C+C cells all showed classic apoptotic morphology, with the notable presence of apoptotic bodies.

Mitochondrial depolarization differs in MMC and anti-Fas treated cells

To ascertain whether mitochondria are involved in the cell death process in FA cells, cells were treated with either MMC or anti-Fas, stained with the mitochondrial specific lipophilic cationic probe JC-1, and analyzed by flow cytometry. A collapse of the inner transmembrane potential ($\delta\Psi_m$), is associated with cessation of oxidative phosphorylation and the opening of a large conductance channel called the mitochondrial permeability transition (PT) pore. The change in $\delta\Psi_m$ was quantified as the percentage of cells shifting to green-orange fluorescence, seen as a drop in FL-2 fluorescence. MMC treatment had very little effect on $\delta\Psi_m$ in either FA or control cells (Figure 3). In comparison, anti-Fas triggered measurable depolarization most notably in normal, FA-A+A, and Jurkat cells, with less depolarization observed in FA-A and FA-C cells.

Anti-Fas but not MMC induces caspase activation in FA cells

Proteolytic activation of caspase-3 activation produces several cleavage products, notably 20, 17, and 12 kD fragments. Full activation of caspase-3 results in cleavage of the intermediate 20 kD fragment to the biologically active 17 kD fragment. Western blot analysis detected both the 20 and 17 kD fragments in both FA and genetically corrected cell lines treated with anti-Fas, suggesting partial caspase-3 activation in these cells (Figure 4A). Jurkat cells showed complete caspase-3 activation with only the fully active 17 kd fragment. Although 25 nM MMC induced significant cell death in FA-A and FA-C LCL, there was no detectable presence

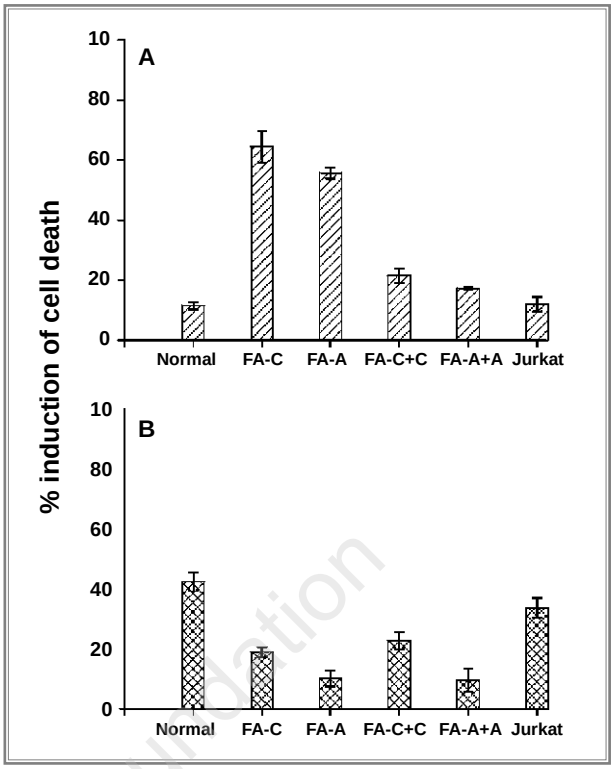

Figure 1. Induction of cell death in FA cells. Cells were treated with 25 nM MMC for 4 days (A), or 250 µg/mL agonistic anti-Fas antibody (B) for 24 hrs, then stained with 7-AAD, and cell death measured using flow cytometry. Results from treated cells were corrected for baseline levels of cell death. Results are from 3 experiments and are the mean ± SEM.

of caspase-3 activation products in these or in their genetically corrected cell lines. Unexpectedly the normal LCL cell line, which did appear to undergo normal apoptosis (apoptotic body formation and DNA laddering), did not show activation of caspase-3. Three other normal LCL cell lines also did not show MMC-induced caspase-3 activation (*data not shown*).

As anti-Fas induced caspase-3 activation, we looked for activation of caspase-8. Jurkat cells treated with anti-Fas showed complete disappearance of the 57 kD inactive proform (Figure 4B); however, the antibody used only weakly detected the 43/41 kD activation products. Anti-Fas-treated normal LCL showed a marked reduction in the proform and the appearance of the double band activation product. Activation was also detected in the FA cell lines but to a less marked extent.

Caspase involvement in FA cell death was also investigated using the broad spectrum caspase inhibitor zVAD-fmk. This inhibitor has a short half life, and pilot experiments determined that zVAD-fmk had to be added daily for a full effect (*data not shown*). The presence of zVAD-fmk did not reduce MMC-induced cell death in FA or control cells (Figure 5A). In contrast zVAD-fmk had a significant effect on anti-Fas-induced

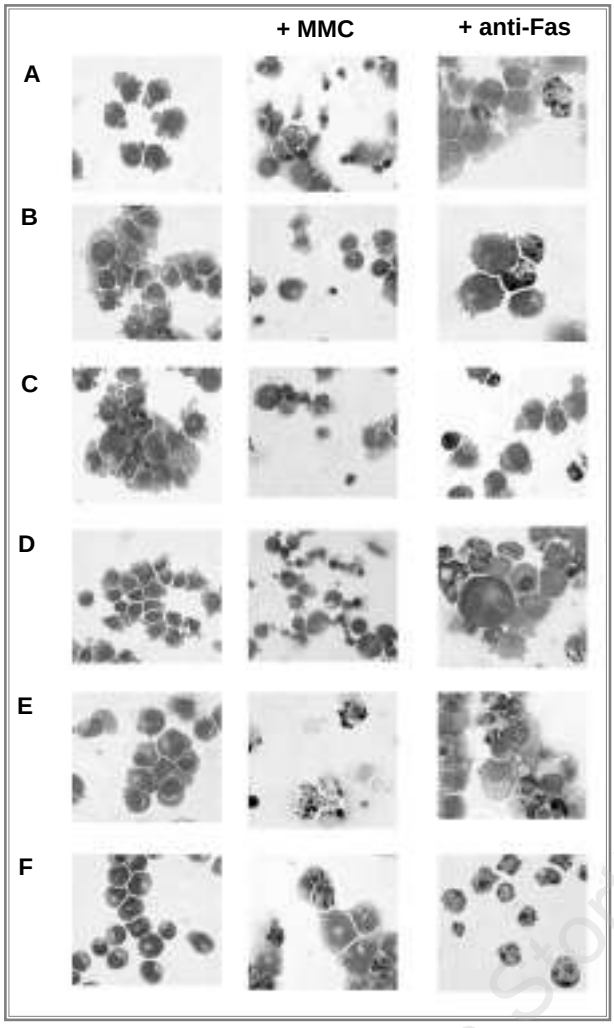

Figure 2. Morphology of FA cells undergoing cell death. Giemsa stained cytospin preparations of cells untreated, and treated with 25 nM MMC or 250 μM agonistic anti-Fas antibody. (A): FA-C, (B): FA-A, (C): FA-A+A, (D): FA-C+C, (E) normal, (F) Jurkat cells.

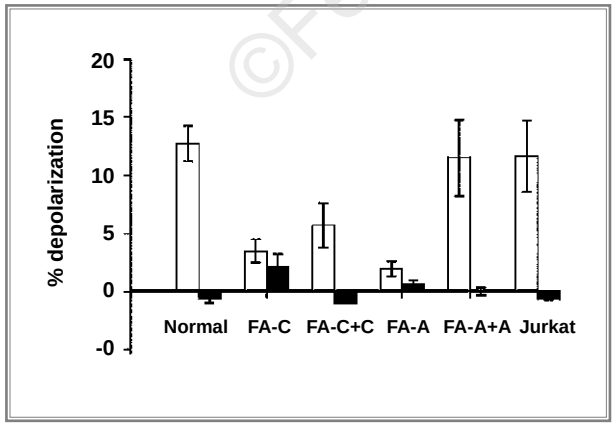

Figure 3. Mitochondrial depolarization in MMC or anti-Fas treated cells. Cells were treated with MMC (■) or anti-Fas (□), and stained with JC-1. Mitochondrial depolarization was measured using flow cytometry. Results are expressed as the percentage induction of mitochondrial depolarization, corrected for baseline levels, and expressed as the mean ± SEM from 3 experiments.

apoptosis (Figure 5B). Normal, FA-C, Fa-C+C, and Jurkat cells all showed a reduction of anti-Fas induced cell death to basal levels in the presence of zVAD-fmk. Interestingly, Fa-A cells reacted quite differently. zVAD-fmk significantly increased cell death in the presence of anti-fas, compared to anti-Fas alone. This apparent toxicity of zVAD-fmk in FA-A cells was re-examined using a range of concentrations of zVAD-fmk from 25 μM to 200 μM. The inhibitor showed a dose-dependent toxicity, which increased in the presence of anti-Fas.

PARP cleavage is not a feature of FA cells undergoing cell death

PARP cleavage by effector caspases is frequently used as a biomarker for apoptosis. Jurkat cells treated with 68 μM etoposide showed substantial PARP cleavage generating the 85 kD cleavage product. Neither the FA cell lines, the genetically corrected, or the four normal cell lines showed PARP cleavage after MMC treatment, consistent with the absence of caspase 3 activation (Figure 6A). The Western blots for PARP consistently showed the presence of a single or double band of slightly higher molecular weigh, in both treated and untreated cells, which was considered unrelated to the 85 kD PARP cleavage product. MMC at 25 nM induced 50-60% cell death in FA cells, but normal LCL 10-15% cell death, so it is possible that there was an insufficient level of cell death to detect PARP cleavage. To obtain equitoxic doses, normal LCL were treated with a range of concentrations of MMC up to 150 nM, but this still did not result in detectable PARP cleavage (Figure 6C).

Anti-Fas initiated apoptosis in FA and control cells, inducing apoptotic changes including caspase-3 activation. However, as with MMC, PARP cleavage could not be detected in these cells, whereas anti-Fas treatment of Jurkat cells resulted in the complete cleavage of PARP (Figure 6B).

FA cells do not undergo nucleosomal DNA fragmentation

FA cells were treated with 100nM MMC for 4 days, and DNA extracts were analyzed by electrophoresis. This showed qualitative differences between normal and FA cells (Figure 7A). Normal LCL showed a marked 180 bp band, with a ladder of bands at multiples of 180bp with the intensity of the bands decreasing: this is typical of cells undergoing apoptosis. FA-A cells produced a partial DNA ladder, with very low levels of the 180 bp fragment, but higher molecular weight DNA ladder repeats, and a marked predominance of large DNA fragments. FA-C LCL showed almost no nucleosomal ladder, but there was a smear of higher molecular weight DNA, as we had previously reported.[19] Surprisingly, the genetically corrected FA-C+C LCL did not correct the qualitative ladder abnormalities in FA-C LCL. The FA-A+A LCLs

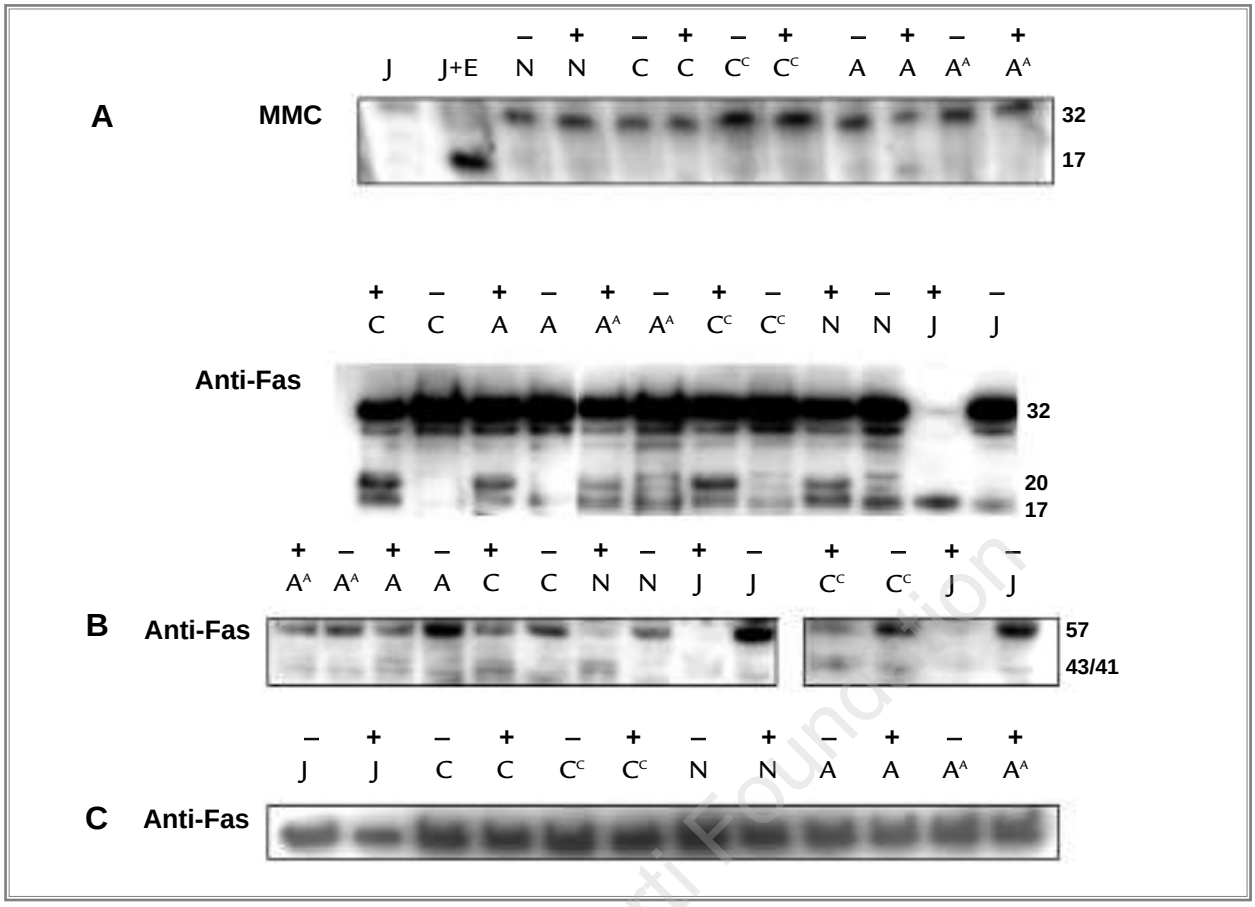

Figure 4. Caspase cleavage detected by Western blotting. Cells were untreated (-), or treated (+) with 25 nM MMC for 4 days, or 250 µg/mL agonistic anti-Fas antibody for 24 hrs, proteins extracted and caspase activation detected by Western blotting. Protein band sizes are in kD. Cells: (J): Jurkat; (N): normal; (C): FA-C; (C^c): FA-C+C; (A): FA-A; (A^A): FA-A+A (a) Caspase-3 cleavage, (b) Caspase-8 cleavage, (c) Actin.

showed partial correction, with the appearance of small molecular weight nucleosomal fragments, as well as high molecular weight DNA fragmentation. Cells untreated with MMC showed no appreciable DNA fragmentation (*data not shown*).

After treatment with anti-Fas for 24 hrs, normal LCL showed the expected ladder pattern, as seen with MMC. However, neither FA-A nor FA-C LCL, nor the genetically corrected cell lines, showed evidence of DNA laddering (Figure 7B).

Discussion

FA proteins are believed to play a role in the regulation of programmed cell death (PCD) and apoptosis.[35] Overexpression of the FANCC gene has been shown to inhibit apoptosis while abnormalities of PCD in FA LCL have been reported by ourselves and others,[19, 20] and could help to explain some of the *in vitro* and *in vivo* phenotypic aspects of FA. We previously studied the toxic effects of MMC on PCD in FA-C cells, and showed increased levels of cell death, but with lack of apoptotic body formation, and abnormal oligosomal DNA laddering.[19] These abnormal markers of cell death do not fit the profile of classical apoptosis, but have been described as necrosis-like PCD.[36]

The intrinsic pathway of apoptosis is co-ordinated by the release of pro-apoptogenic factors by the mitochondria. Many models of PCD involve mitochondrial control of alternative cell death pathways, and ATP levels can influence a change from an apoptotic to a necrotic pathway.[37] Mitochondrial morphology appears to be abnormal in FA cells,[27] and inhibitor studies have demonstrated deficiencies in mitochondrial encoded proteins and ATP production,[25] and markedly reduced oxygen consumption,[26] all indications of functional abnormality. Alkylating agents, including MMC, bind preferentially to mitochondrial DNA (mDNA),[21] and the FANCG protein has been reported to relocate from the nucleus to the mitochondria in MMC-treated cells.[17] These results led us to hypothesize that abnormalities in apoptosis in FA cells may be mediated at the level of the mitochondria. In order to test this hypothesis, we

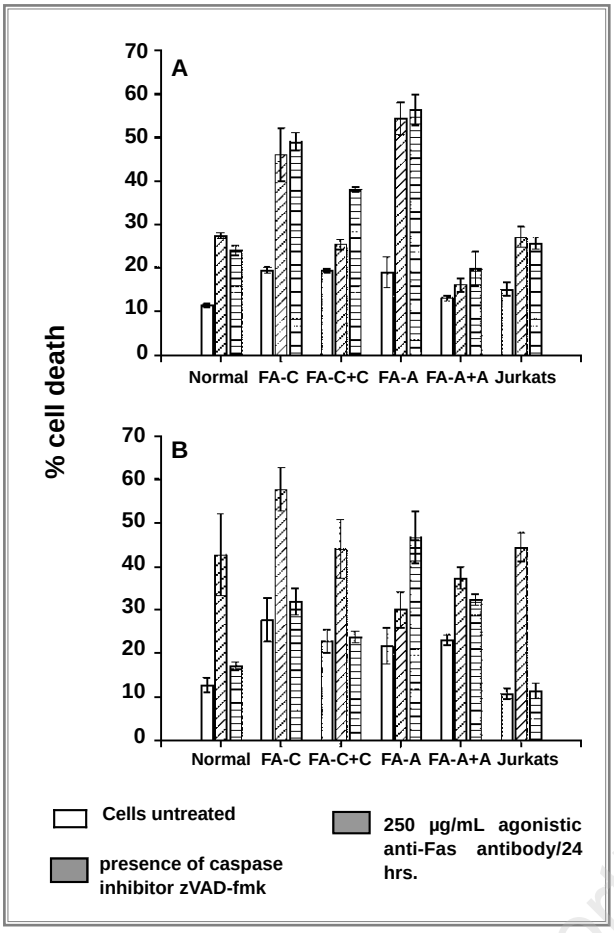

Figure 5 [left]. Cells were untreated, or treated with 25 nM MMC for four days, or 250 µg/mL agonistic anti-Fas antibody for 24 hrs, and in the presence of the caspase inhibitor zVAD-fmk. Results are from 3 experiments ± SEM. ***(a): MMC, (b): anti-Fas.***

compared the response of FA and control cells to MMC with their response to Fas ligation. The Fas cell death pathway, initiated by either Fas ligand or agonistic anti-Fas antibody binding to the Fas cell surface receptor, triggers the extrinsic pathway of apoptosis, with paramembrane activation of caspase-8 which can directly activate caspase-3, thereby bypassing the mitochondria.[28]

A small increase in cell membrane permeability is an early indicator that a cell is initiating apoptotic cell death.[31,38] MMC-treated FA-C and FA-A cells showed 3-6 fold greater increases in cell death, measured by 7-AAD permeability, than did control cells at ambient oxygen concentrations. In contrast, the effect of agonistic anti-Fas on cell death was reduced in the FA cell lines, as previously reported by Ridet *et al.*[39] FA cells are considered to be in a pro-oxidant state,[40] with increased levels of ROS.[41] Since Fas-mediated cell death is inhibited by the superoxide anion[42] this might explain the lower level of Fas induced death we observed.

Overexpression of FANCA and FANCC in our genetically corrected cell lines reduced cell death by MMC to normal levels. FANCC has been ascribed an anti-apop-

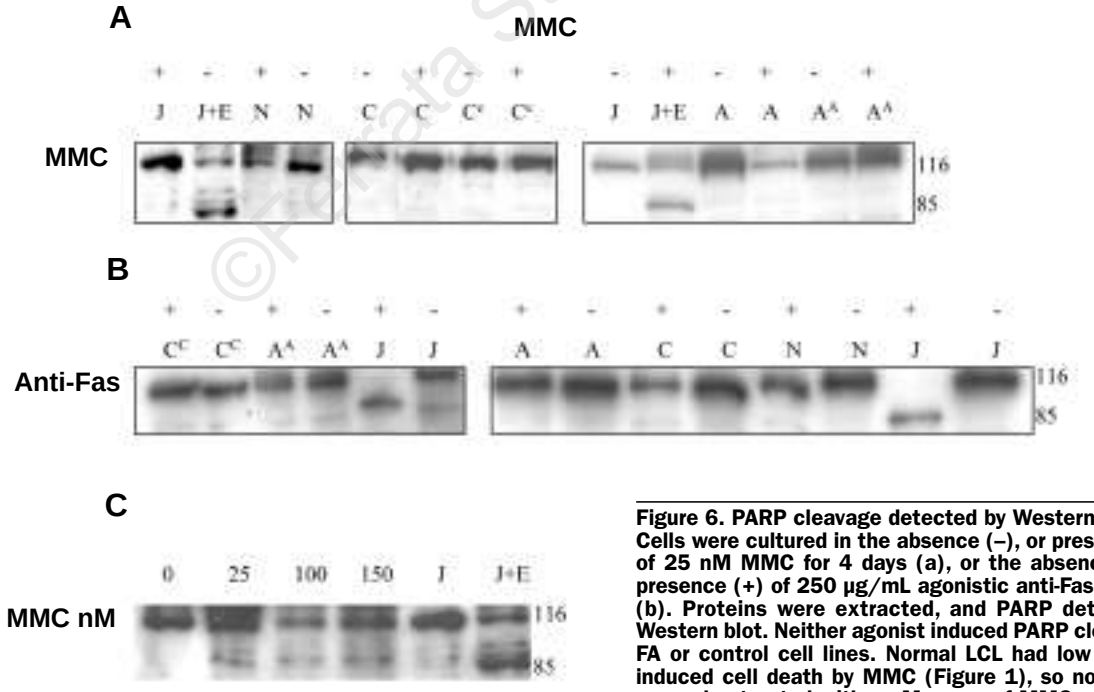

Figure 6. PARP cleavage detected by Western blotting. Cells were cultured in the absence (–), or presence (+), of 25 nM MMC for 4 days (a), or the absence (–), or presence (+) of 250 µg/mL agonistic anti-Fas antibody (b). Proteins were extracted, and PARP detected by Western blot. Neither agonist induced PARP cleavage in FA or control cell lines. Normal LCL had low levels of induced cell death by MMC (Figure 1), so normal LCL were also treated with a nM range of MMC concentrations in order to try to induce PARP cleavage (c). Jurkat cells (J) were treated with 68 µM etoposide for 5 hrs and the protein extracted used as a positive control for PARP cleavage. Protein band sizes are shown in kD.

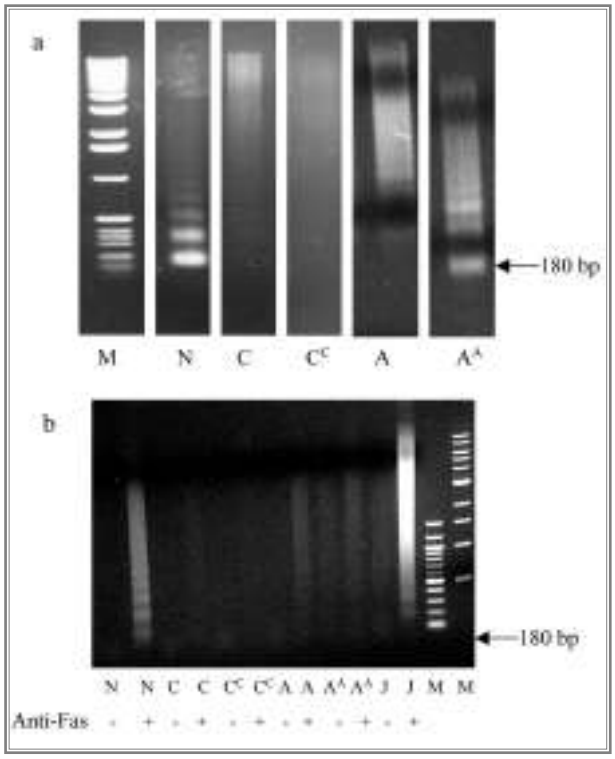

Figure 7. Nucleosomal fragmentation induced by MMC or anti-Fas. DNA fragments were extracted from cells treated with either 100 nM MMC (a), or treated (+), or untreated (–) with 250 µg/mL agonistic anti-Fas (b), and electrophoresed in 0.85% agarose gels. Lane (M) contains a DNA ladder size marker.

totic function in FA and non-FA cells,[35,43,44] and FANCA appears to have a similar function. Fas-mediated cell death was not decreased in our genetically corrected cell lines, indicating that cell death by the Fas pathway was occurring independently of FA gene expression. However, Wang *et al.*[43] have reported that FANCC overexpression in transgenic mice protects hematopoietic progenitors from Fas-mediated cell death. This may highlight differences between primary cells and cell lines, or between mice and humans, but their experiments also differed from ours in comparing normal expression with dramatic overexpression of FANCC, rather than normal with the mutant Fanconi's anemia phenotype.

In addition to direct caspase-3 activation, Fas can also act via the mitochondria by cleaving the Bcl-related protein Bid, which can translocate to the mitochondria and interact with other Bcl proteins, causing the collapse of the inner transmembrane potential, the opening of a large conductance channel and release of pro-apoptogenic proteins.[45] We detected a drop in mitochondria transmembrane potential in Fas-treated cells, indicating that caspase-3 activation may also be occurring via the mitochondria. However, this seems to be a minor and late effect.

MMC induced minimal mitochondrial depolarization and did not activate caspase-3 in any of our cell lines, implicating MMC in a caspase-independent mechanism of PCD, even in normal LCL. In addition we studied four other normal lymphoblastoid cell lines, which also did not show caspase-3 activation by MMC (*data not shown*). This might be due, in part, to the changes in gene expression in lymphoblastoid cells. Caspase-independent PCD was confirmed by inhibiting caspase activity with the broad spectrum caspase inhibitor zVAD-fmk which had no effect on cell death induced by MMC. Fas-mediated cell death in the cell lines studied, with the exception of FA-A cells, was inhibited by zVAD-fmk, confirming caspase involvement. Interestingly, these cells showed enhanced Fas toxicity in the presence of the caspase inhibitor. We have not investigated the mechanism involved, but inhibition of caspase activity using broad spectrum caspase inhibitors has been reported to enhance caspase independent death programs which can resemble apoptosis, apoptosis-like PCD, or necrosis.[46,47]

Cells undergoing apoptosis systematically degrade chromatin, which is then encapsulated in nuclear membrane-derived apoptotic bodies.[34] This ensures the controlled degradation of DNA and avoids the pro-inflammatory autoimmune response observed when DNA is released as a result of necrotic cell death. With MMC treatment we observed apoptotic body formation in normal, but not FA cell lines. In contrast, after Fas activation, apoptotic body formation was prominent in both normal and FA cell lines. Apoptotic body formation has been correlated with caspase-3 activation,[48] and unlike MMC, both were features in FA and control cells undergoing Fas-mediated apoptotic cell death. The contrasting effects of MMC and Fas on PCD upsteam of mitochondria were not seen in downstream events; in fact both agonists shared common effects. PARP, an abundant 116 kD nuclear protein, involved in surveillance of DNA damage, is cleaved and inactivated to an 85kD product by caspase-3 during apoptosis induced by many agents and in a variety of cell types,[49] and this has been linked to apoptotic body formation and nuclear fragmentation.[48] In spite of caspase-3 activation, PARP cleavage was absent in Fas-treated cells, and in all cell lines treated with MMC. The lack of detectable PARP cleavage in the four normal LCL studied which were treated with MMC might be due to the low level of apoptosis in these cells. However, PARP cleavage was still absent when normal LCL were treated with equitoxic doses of MMC. Increased PARP activity is associated with DNA repair in cells undergoing genotoxic damage resulting in single or double strand breaks. Sufficient damage will induce a switch from repair to PCD and PARP will normally be cleaved and inactivated in

order to preserve sufficient redox status of the cell to undergo PCD. However, if PARP is not cleaved by caspases, as in our FA cells, then rapid consumption of its substrate, nicotinamide adenine dinucleotide (NAD^+), will occur. NAD^+ levels have been reported to be normal in FA cells, but to become rapidly depleted after cell damage.[50] Both nutritional and *in vitro* depletion of NAD^+ has also been shown to inhibit the rejoining of DNA strand breaks[51] and increase chromosomal instability[52] in cultured cells, both reported features of FA cells.

DNA fragmentation was either absent or abnormal in the FA and genetically corrected cell lines treated with either agonist. DNA is degraded during apoptosis by the mitochondrial release of apoptosis-inducing factor (AIF) to produce large molecular weight fragments which are then further degraded by caspase-activated DNAse (CAD) into 180 bp oligonucleosomal fragments. High molecular weight DNA fragments were seen in MMC-treated FA cells, perhaps indicative of DNA degradation by AIF. The further degradation of DNA by CAD requires caspase activation, which was absent in MMC-treated cells, but was also absent in Fas-treated cells in which there was demonstrable caspase-3 activation, indicating a downstream mechanism. However, both genetically corrected cell lines failed to induce normal DNA fragmentation as seen in normal LCL.

FA cells are considered to be in a pro-oxidant state, and have increased endogenous levels of ROS. Since the mitochondria are the major source of ROS within a cell, this is consistent with the presence of mitochondrial abnormalities in FA. Furthermore, since Fas- mediated apoptosis can occur independently of the mitochondria, and may be inhibited by increased ROS, this is consistent with our observations that FA cells show normal or decreased sensitivity to Fas ligation *in vitro*. This does not exclude a role of Fas-mediated apoptosis *in vivo*, secondary to hypersensitivity of primary FA cells to pro-inflammatory cytokines. In contrast to Fas-mediated apoptosis, the toxicity of MMC towards FA cells is strongly oxygen-dependent. We and others have shown that the sensitivity of FA cells to MMC is normalized at low oxygen concentration. This creates an apparent paradox, as these conditions typically increase MMC DNA adduct formation, including the interstrand crosslink, and we have specifically confirmed this for the FA-A and FA-C cells used in this study using ^{32}P-post-labeling of MMC adducts (*manuscript in preparation*). We, therefore, believe that the increased cell death caused by MMC in these cell lines must be attributed to alternative toxic mechanisms. MMC is enzymically activated intracellularly to the active semiquinone, and at ambient oxygen conditions it can undergo futile cyclic generation of high levels of superoxide anion. Primary FA cells have been shown to have an oxygen sensitive phenotype, and MMC toxicity towards FA cells is exacerbated with increasing oxygen concentrations, indicative of increasing ROS levels.

The FA proteins appear to have important roles in both genotoxic and cytoplasmic stress. In the cytoplasm, they may interact with cytochrome P450 reductase, present in microsomes and mitochondria, and with HSP70, which has a role in mitochondrial protein transport, and thus in maintaining functional integrity of the mitochondria. We have shown here that abnormalities in apoptosis in FA cells are associated with the intrinsic, mitochondrial pathway rather than the extrinsic pathway. Thus elements of oxygen sensitivity, MMC sensitivity and abnormal apoptosis may all be integrated at the level of the mitochondrion. This has implications for therapy. While there is currently no obvious way to ameliorate DNA repair deficiencies in FA, abnormalities in apoptosis are proving amenable to therapy in a variety of disease models.

Contributions. AC: Involved in the experimental aspect of the project, conception, design, and analysis of data, also the primary author of the manuscript. FG: Involved in the experimental aspect of the project, as well as the analysis and interpretation of data. Contributed to the intellectual input to the manuscript, as well as correcting and approving the final version. JS: Involved in the experimental aspect of the project, revising and approving the final version of the manuscript. NM: Involved in the experimental aspect of the project, revising and approving the final version of the manuscript. TR: was involved in the initial project conception and design, as well as contributing to the writing, critical correction and approval of the submitted manuscript. The authors reported no conflict of interest.

Received on August 18, 2003, accepted November 10, 2003.

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