Mutagenesis vol.16 no.3 pp.283–288, 2001

Hydrogen peroxide: effects on DNA, chromosomes, cell cycle and apoptosis induction in Fanconi's anemia cell lines

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Fanconi's anemia (FA) is an inherited autosomal recessive syndrome; cells from FA patients are very sensitive to crosslinking agents and to oxygen. Epstein–Barr virus (EBV)-transformed lymphoblasts belonging to different FA complementation groups and normal EBV-transformed lymphoblasts were studied for their response to treatment with the oxidizing agent hydrogen peroxide (H_2O_2). The analysis of 8-hydroxy-2′-deoxyguanosine (8-OHdG) content in the DNA of untreated cells showed an increased basal level of damage in cells from the complementation groups FA-C and FA-E. H_2O_2-induced 8-OHdG was higher in FA than in normal cell lines. The removal of 8-OHdG after H_2O_2 treatment was significantly reduced in the cells from complementation group E. However, all FA cell lines showed a normal ability in the resealing of DNA breaks, at least soon after treatment. All cell lines were also equally efficient in the removal of damaged pyrimidines. Compared with normal cells, FA cell lines showed an increase in the baseline level of micronuclei, but not in the number of micronuclei induced by H_2O_2. Micronuclei in FA cells originated prevalently from chromosomal fragmentation and, at a minor extent, from chromosome loss. After H_2O_2 treatment, FA cell lines accumulated in G_2 phase to a greater extent than normal lymphoblasts. However, reversion of mutation in FA-A and FA-C cells did not result in the correction of this phenotype. In cells evaluated for apoptosis no ladder formation was found in FA-C, FA-E and corrected FA-C cells. In conclusion, among the FA cell lines examined, only FA-E showed a defect in the repair of H_2O_2-induced damage. On the other hand, differences found in the cell cycle and apoptosis might be due to irreversible changes occurring in FA cell lines as a consequence of the primary defect.

Introduction

Fanconi's anemia (FA) is a rare inherited autosomal recessive syndrome with a complex genotype including no fewer than eight (FA-A to FA-H) complementation groups (Carreau and Buchwald, 1998). While four genes (FA-A, FA-C, FA-F and FA-G) have been identified and cloned (Strathdee *et al.*, 1992; Lo Ten Fo *et al.*, 1996; de Winter *et al.*, 1998), and two others (FA-D and FA-E) have been mapped (de Winter *et al.*, 2000), their functions remain obscure. The protein encoded by FA-G, identical to XRCC9 (de Winter *et al.*, 1998), and that encoded by FA-F, revealed high homology with the prokaryotic RNA-binding protein ROM (de Winter *et al.*, 2000) and an apparent involvement in DNA binding. A role of these proteins in DNA repair has been proposed (Garcia-Higueira *et al.*, 2000), but this hypothesis has not yet found experimental evidence despite the fact that a defect in FA has been commonly associated with a defect in the DNA cross-links repair mechanism. The function of the FA-A and FA-C proteins is also still unknown.

The FA phenotype is characterized by a number of cellular and clinical features, including chromosomal instability, sensitivity to cross-linking agents, proneness to pancytopenia and cancer (D'Andrea and Grumpe, 1997). Tumors are mainly acute myeloblastic leukemias of various subtypes, but solid tumours have also been reported.

Increased chromosomal aberrations and micronuclei have been reported in untreated FA cells (Raj and Heddle, 1980; Dallapiccola *et al.*, 1985) and the evaluation of the former after cross-linking treatment is currently used as a diagnostic test (D'Andrea and Grompe, 1997). An increase of chromosomal aberrations was also reported after exposure of FA lymphocytes to H_2O_2 or hyperoxic conditions (Joenje *et al.*, 1981; Dallapiccola *et al.*, 1985).

Drastic reductions of cell viability and alterations in cell cycle progression have been reported in FA cells incubated under increased oxygen tension (Ruppitsch *et al.*, 1997). In contrast, hypoxia (5% oxygen) reduces chromosomal breaks (Joenje *et al.*, 1981), increases cell survival and removes G_2 arrest (Poot *et al.*, 1996). Moreover this condition reduces mutation frequency in plasmids transfected in FA cells (Bredberg *et al.*, 1995). Many authors reported an alteration in the level of anti-oxidating agents such as superoxide dismutase (SOD), catalase and glutathione *S*-transferase (GST) in FA cells (Joenje *et al.*, 1978, 1979; Takeuchi and Morimoto, 1993). Addition of anti-oxidating enzymes was found to decrease the level of spontaneous or H_2O_2-induced chromosomal breaks (Dallapiccola *et al.*, 1985; Nordenson *et al.*, 1997). Korkina *et al.* (1992) reported a 13-fold increase in luminol-dependent chemiluminescence (LDCL) in white blood cells obtained from FA homozygotes compared with controls, whereas FA heterozygotes displayed a lesser, yet significant, increase. These data suggest overproduction and/or an impaired detoxification of reactive oxygen species (ROS).

8-Hydroxy-2′-deoxyguanosine (8-OHdG), one of the DNA lesions due to oxidizing agents, has been studied extensively as an indicator of oxidative DNA damage. A significant increase in the baseline level of 8-OHdG, evaluated by HPLC-EC, was found in leukocytes from FA patients and from their parents (Degan *et al.*, 1995). Takeuchi and Morimoto (1993) reported that the increase in the formation of 8-OHdG in FA cells exposed to H_2O_2 was two to three times higher than in normal cells.

The increased sensitivity of FA cells to oxidants underpins claims that the high cytotoxicity of the crosslinking agent, mitomycin C (MMC), could actually be due to the generation

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of oxygen free radicals during the metabolism of this compound in FA cells at physiological oxygen concentration (Clarke *et al.*, 1997). MMC metabolism in these conditions generates oxygen free radicals, whereas under conditions of hypoxia the cross-linking reaction is favored (Pritsos *et al.*, 1997). Overexpression of tireodoxin, an important intracellular antioxidant, reduces the cytotoxicity and the clastogenic action of crosslinking agents in FA cells (Ruppitsch *et al.*, 1998), but at the same time is ineffective on spontaneous chromosomal damage in the same cells. These observations argue in favor of a major role of the oxidative versus the crosslinking action of MMC and other crosslinking agents in determining cytotoxicity in FA cells.

Cell cycle analysis in MMC-treated FA cells showed an increased proportion of cells that arrest in the G_2 phase compared with normal cells. It is not clear whether this increased arrest is due to the normal response of cells to an increased DNA damage as suggested by some authors (Heinrich *et al.*, 1998), or to an altered response as suggested by others (Kupfer and D'Andrea, 1996). Unlike the G_2 arrest induced by X-ray treatment, the MMC-induced G_2 arrest in FA cells is removed by caffeine (Seyschab *et al.*, 1994).

Studies on apoptosis induction with different chemical and physical agents have produced conflicting results. After treatment with D-ribose, UV or X-ray, FA cells were found to undergo apoptosis to a lesser extent compared with normal cells (Rosselli *et al.*, 1995; Monti *et al.*, 1997; Ridet *et al.*, 1997), while MMC (Kruyt *et al.*, 1996) and nitrogen mustard (HN_2) (Marathi *et al.*, 1996) induced an increase in apoptosis.

Treatment with γ-interferon (γ-INF) has been reported to induce apoptosis in both FA cells and cells derived from knock-out mice for FA-C gene (Whitney *et al.*, 1996; Rathbun *et al.*, 1997). γ-INF induction of apoptosis is due to its ability with enhanced Fas expression. This protein is active in association to its ligand (FasL), an analog of TNF, whose expression is increased in FA patients (Schultz and Shaidi, 1993). FasL expression is increased in Jurkat cells treated with H_2O_2 (Bauer *et al.*, 1998), which is known to activate different apoptotic pathways depending on the dosage and, most likely, on the cell type (Gardner *et al.*, 1997).

In the present work we investigated FA cell lines belonging to five complementation groups (FA-A, FA-B, FA-C, FA-D and FA-E), as well as FA-A and FA-C cell lines corrected by the reversion of the corresponding mutant genes. We studied the repair of different types of H_2O_2-induced damage (8-OHdG, damaged pyrimidines, DNA breaks), as well as chromosomal damage, cell cycle changes and apoptosis. The aim of the study was to ascertain whether the reported sensitivity of FA cells to ROS is due to a defect in the repair of oxidative damage or whether other cellular functions are involved. We also sought to investigate if differences in the response to oxidants exist among the different complementation groups.

Materials and methods

Cell lines

All the cell lines used in this study were Epstein–Barr virus (EBV)-immortalized lymphoblasts. LB was established from a healthy donor. FA cell lines were: FA-A (HSC72 and EUFA-274-L cell lines, both belonging to complementation group A), FA-B (HSC230, complementation group B), FA-C (HSC536, complementation group C), FA-D (HSC62, complementation group D), FA-E (93-V-130, complementation group E). FA-A COR (EUFA COR) and FA-C COR (HSC536 COR), spontaneous *in vitro* revertants of cell lines EUFA-274-L and HSC536, respectively, were kindly provided by Hans Joenje. All FA cell lines were obtained from Hans Joenje (Free University, Amsterdam, The Netherlands).

Chemicals

Mitomycin C was supplied by Kyowa Italiana (Milan, Italy) and hydrogen peroxide by Astrochimica (Opera, Italy).

HPLC and 8-OHdG quantification

Cells were washed twice in PBS and suspended in 1 ml of SE buffer (75 mM NaCl and 25 mM EDTA pH 8.0) and the DNA was purified (Degan *et al.*, 1995). Following Nuclease P1 and alkaline phosphatase (Boehringer, Milan, Italy) hydrolysis, samples were filtered through 0.22 μm cellulose acetate Centricon filter units, and 20–100 μg of DNA per sample were injected in HPLC. The separation of 8-OHdG and normal deoxynucleosides was performed in a LC-18-DB Supelco column (150×4.6 mm, Supelco, Bellefonte, PA) equipped with a LC-18 guard column cartridge. The solvent system consisted of an isocratic mixture of 90% 50 mM potassium phosphate, pH 5.5, and 10% methanol at 1 ml/min flow rate. UV detection was performed at 254 nm and electrochemical analysis was carried out by a PED detector (Pulsed Electrochemical Detector, Dionex, Sunnyvale, CA) in the amperometric mode, with a glass-carbon electrode using an Ag/AgCl reference electrode. The levels of 8-OHdG were referred to the amount of deoxyguanosine (dG) detected by UV absorbance at 254 nm. The amount of DNA was determined by a calibration curve versus known amounts of calf thymus DNA. The levels of 8OHdG were expressed as the number of 8-OHdG adducts per 10^5 dG bases (8-OHdG/10^5dG).

Single cell gel electrophoresis (SCGE)

SCGE was performed as previously described (Collins *et al.*, 1995). Briefly, cells were suspended in 1% low melting point agarose (Bio-Rad, Milan, Italy) in PBS, pH 7.4 and stratified onto a fully frosted microscope slide (Richardson Supply Co., London, UK) pre-coated with a layer of normal melting point agarose. After 5 min on ice, slides were immersed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, 1% Triton X-100) at 4°C for at least 1 h. Slides were then placed in a electrophoresis tank containing 0.3 M NaOH and 1 mM Na_2EDTA for 40 min at 4°C. At the same temperature, slides were run in the same buffer for 30 min at 25 V. The slides were then washed with 0.4 M Tris–HCl, pH 7.5 before staining with 20 μg/ml ethidium bromide (Boehringer). Slides were scored under an Axioplan microscope (Zeiss, Oberkoken, Germany) equipped with fluorescence and then photographed. Tail length was measured on negatives using a Peak Scale Lupe 7×. At least two independent experiments were run. To measure the amount of damaged pyrimidines, sites sensitive to endonuclease III (obtained from Andrew Collins, Rowett Institute, Aberdeen, UK) were evaluated. Endonuclease III treatment was performed as described (Collins *et al.*, 1995). Briefly, after lysis and before unwinding, the slides were washed three times for 5 min each in endonuclease buffer (40 mM HEPES–KOH, 0.1 M KCl, 0.5 mM Na_2EDTA, pH 8) and incubated with 50 μl of either buffer alone or buffer containing endonuclease III (1 mg/ml) for 30 min at 37°C in a moist chamber. Experiments were carried out in at least a triplicate independent cultures.

Micronucleus assay

Cells were resuspended in hypotonic solution (0.075 M KCl) and fixed in methanol:acetic acid (3:1). Slides were stained with 3% Giemsa for scoring of total micronuclei. For each sample at least 2000 cells were scored blindly. To distinguish micronuclei generated by chromosome loss from those generated by chromosomal fragmentation, slides were denatured and hybridized, with an all human centromeres α-satellite probe (Oncor, Gaithersburg, MD), as previously reported (Pinkel *et al.*, 1986), and according to manufacturer's instruction with minor modifications. Detection was performed using Cy3-avidin (Amersham); nuclei were counter stained with DAPI (Boehringer). Slides were scored under an Axioplan microscope (Zeiss) equipped with fluorescence and suitable filters. Presence or absence of fluorescent signals was used to classify micronuclei as generated by chromosome loss or chromosomal fragmentation, respectively. At least three independent experiments were done for micronuclei scoring. Experiments for evaluation of the presence of centromeric sequences in micronuclei were run in at least duplicate independent cultures.

Evaluation of apoptosis

Apoptosis was evaluated as previously reported (Herrmann *et al.*, 1994) with minor modification. Fragmented DNA, was extracted in lysis buffer (10 mM Tris, 1 mM EDTA, 0.2% Triton X-100, pH 7.5) and centrifuged (13 000 *g*, 10 min). The supernatant was precipitated by isopropanol. The pellet was resuspended in TE (10 mM Tris, 1 mM EDTA, pH 7.4) and digested with RNase (100 μg/ml). DNA was run on a 2% agarose gel.

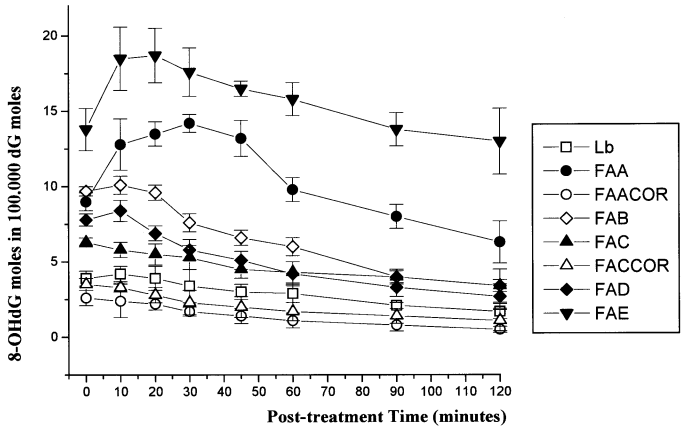

Fig. 1. Kinetic of removal of 8-OHdG from DNA in the different FA cell lines after treatment with 20 mM H_2O_2. Means and standard deviation (SD) bars from a minimum of three independent experiments are shown.

Table I. Basal level of 8-OHdG (mol/10^5 mol dG) in the different FA cell lines and level of damage induced by treatment with 10 and 20 mM H_2O_2[a]

	Basal mean (SD)	10 mM H_2O_2 mean (SD)	20 mM H_2O_2 mean (SD)
LB	1.8 (0.5)	3.2 (0.5)	3.9 (0.7)
FA-A	1.2 (0.6)	4.5 (0.6)	9.0 (1.0)
FA-A COR	1.3 (0.5)	2.3 (0.7)	2.6 (0.1)
FA-B	1.6 (0.3)	6.3 (1.2)	9.2 (0.5)
FA-C	3.4 (0.3)*	5.7 (0.3)	6.3 (0.7)
FA-C COR	2.1 (0.4)	2.9 (0.4)	3.5 (0.7)
FA-D	1.7 (0.4)	6.2 (1.2)	7.8 (1.2)
FA-E	6.7 (1.4)*	11.5 (0.3)	13.8 (2.1)

[a]A minimum of three experiments were performed for each data point.
*Statistically significant differences ($P < 0.05$).

Determination of cell cycle phases

Cells, previously fixed in ethanol, were treated with RNase (1 mg/ml in PBS), washed twice in PBS containing 1% Tween-20 and stained with propidium iodide (20 µg/ml in PBS). The analysis was performed using a FACScan (Becton Dickinson Italia, Milan).

Cell treatment

For the quantification of 8-OHdG cells were treated with H_2O_2 for 30 min at 37°C in complete medium with doses reported in Results.

For SCGE cells were treated with 100 µM H_2O_2 for 30 min on ice in complete medium. Cells were washed in PBS and resuspended in complete medium to allow them to recover. For micronucleus test, cell cycle and apoptosis, H_2O_2 or MMC treatment was carried out at 37°C for 48 h in complete medium at the doses reported in Results.

Results

Induction of DNA damage by hydrogen peroxide

DNA damage and repair in FA cells treated with H_2O_2 were evaluated by measuring both 8-OHdG in DNA (HPLC-EC analysis) and DNA breaks in intact nuclei (SCGE assay).

The analysis of the 8-OHdG content in the DNA of untreated cells showed an increased basal level of DNA oxidative damage in the cells from the complementation groups FA-C and FA-E (Table I). H_2O_2 treatment resulted in an increase of the 8-OHdG level in the DNA from all cell lines. All FA cell lines were more susceptible to this agent than normal lymphoblasts. The integration of the kinetic data for the repair of 8-OHdG showed that the ability to remove this adduct was reduced for all FA cell lines examined; this difference, however, was statistically significant only for FA-A and FA-E

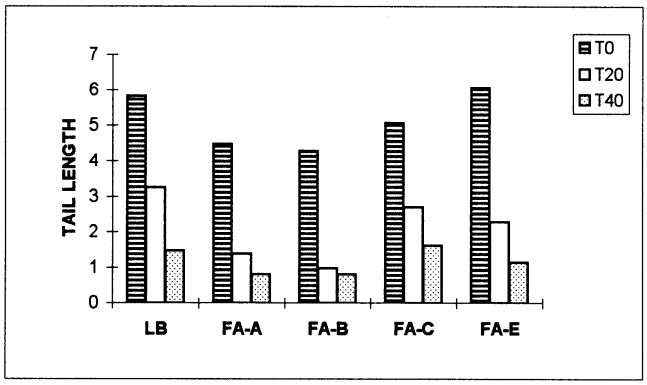

Fig. 2. Mean tail length of cells treated with 100 µM H_2O_2 at 0, 20 and 40 min from the end of the treatment. Mean of at least two experiments.

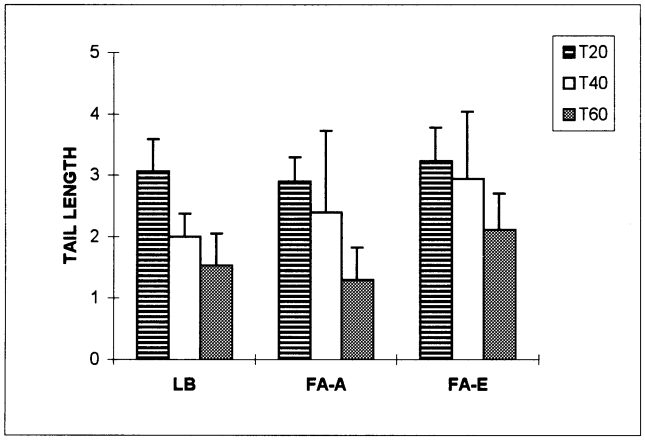

Fig. 3. Repair kinetic of damaged pyrimidines in normal and FA cells treated with 100 µM H_2O_2 and submitted to endonuclease III digestion (mean tail length ± SD). Mean of at least three experiments.

(Figure 1). It is noteworthy that the two corrected cell lines behaved quite similarly to controls, in both the induction and repair phases of the process.

DNA breaks in FA and normal cells treated with H_2O_2, were evaluated by the SCGE assay (Figure 2). The tail length of comets was broadly similar both immediately after the treatment and at two post-treatment times, indicating that damage was induced to a similar extent and repaired at a similar rate in all cell lines.

To investigate if the removal of a specific H_2O_2-induced damage could be defective in FA cells, a modification of the comet assay was used. Sites sensitive to endonuclease III, an enzyme that recognizes damaged pyrimidines, were measured at different times after treatment (Figure 3). Linear regression analysis showed that all cell lines were able to efficiently remove the damaged pyrimidines. The rate of removal was somewhat lower in FA-E compared with the other cell lines, but this difference was not statistically significant.

Micronucleus assay

The micronucleus assay was used to evaluate chromosomal damage induced by H_2O_2. The results, reported in Table II, showed that the basal micronucleus level is about one order of magnitude higher in FA cell lines than in normal lymphoblasts. This finding has been previously reported in fibroblasts from FA patients (Raj *et al.*, 1980) and is in agreement with the increased level of chromosomal breaks usually found in

Table II. Micronuclei induced by H_2O_2 in normal and FA lymphoblasts

	Control		200 μM		Net increase of μN
	M.I.[a]	(μN/1000 cells)	M.I.[a]	(μN/1000 cells)	
LB	10	3	12	20[c]	17
FA-A	16.3	15.15[b]	13.5	30.6[c]	15.45
FA-C	12.7	13.15[d]	10.3	28.68[c]	15.53
FA-C COR	12.7	2[e]	10.7	19.7[c]	17.7

[a]M.I., mitotic index, mitosis/1000 cells.
[b]χ^2 test, LB versus FA: $P < 0.0001$.
[c]χ^2 test, control versus treated: $P < 0.0001$.
[d]χ^2 test, LB versus FA: $P < 0.0002$.
[e]χ^2 test, LB versus FA: not significant.

Table III. Micronuclei containing α-centromeric sequences (C+ μN) in normal and FA lymphoblasts treated by 200 μM H_2O_2

	Control % C+ μN	H_2O_2 % C+ μN	Statistics[a]
LB	50	30	$P < 0.001$
FA-A	33.5	26	N.S.[b]
FA-C	38	30	N.S.[b]
FA-C COR	51.1	28	$P < 0.004$

[a]χ^2 test of control versus H_2O_2 -treated.
[b]N.S., not significant.

FA cells. In FA-C cells corrected for the defective gene, a normal micronucleus level is restored.

After H_2O_2 treatment, micronucleus frequencies were 1.5-fold higher in FA than in normal or FA-C corrected cells, but no difference was found in the net increase (controls subtracted) of micronuclei among all cell lines examined. Mitotic index did not vary noticeably among the studied cell lines, nor was it influenced by the H_2O_2 treatment.

Micronuclei can be generated by either chromosomal fragments or missegregating whole chromosomes. To investigate if differences in the origin of micronuclei might exist between FA and normal lymphoblasts, samples were examined for the presence of α-centromeric sequences in the micronuclei, which were then distinguished as C– micronuclei, chromosomal fragments, and C+ micronuclei, containing chromosomal centromeric regions. Results reported in Table III show that normal and FA-corrected cells contain an equal proportion of C– and C+ micronuclei. However, most of the micronuclei in untreated FA cell lines and in H_2O_2-treated normal cells originated from chromosomal fragments.

Cell cycle and apoptosis

The action of H_2O_2 on cell cycle and its capacity to induce apoptosis were determined by FACS analysis and by DNA ladder formation, respectively.

In the FACS analysis, the difference in the percentage of cells in the G_2 phase of the cell cycle between control and treated cells was evaluated. MMC was used as a positive control. As expected (Table IV), FA cell lines were affected by MMC to a greater degree if compared with normal cell lines. The reversion of mutant genes restored the normal phenotype. A different situation was found with H_2O_2. First, the differences between normal and FA cell lines were not as sharp. Moreover, the induced G_2 delay was more marked in the corrected FA than in the corresponding FA cell lines.

When cells were evaluated for DNA ladder formation, no ladder was seen in FA-C, FA-E and corrected FA-C cell lines, while normal, FA-A and corrected FA-A cell lines showed the typical DNA ladder, indicative of fragmentation (Figure 4).

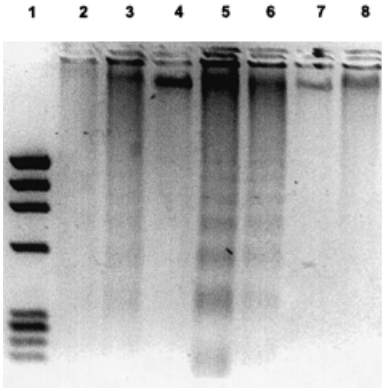

Fig. 4. Apoptotic ladder of cell lines treated with 500 μM H_2O_2 after 48 h from the beginning of the treatment. Lane 1, ϕX174/*Hae*III; lane 2, LB untreated; lane 3, LB; lane 4, FA-E; lane 5, FA-A; lane 6, FA-A cor; lane 7, FA-C; lane 8, FA-C-cor. Only LB control ladder is shown.

Table IV. Delta G_2[a] in normal, FA and FA corrected cells after treatment with 50 nM MMC and 500 μM H_2O_2.

	MMC		H_2O_2	
	24 h	48 h	24 h	48 h
LB	9.8	6.2	2.3	2.9
FA-A	23.5	18.2	8.4	5.4
FA-A COR	3.5	3.6	13.5	9.6
FA-C	41.2	27.8	4.6	13.1
FA-C COR	7.4	3.2	22.4	11.5
FA-E	–	31.4	7	6.4

[a]Increase over control of percentage of cells in the G_2 phase of cell cycle.

To evaluate if the behavior of FA-C cells was due to a defect in the apoptotic mechanism or was specific for H_2O_2 treatment, the effect of a different apoptotic agent, VP16, was tested: the effect on FA-C cells was not specific for H_2O_2, since no ladder formation was induced even by VP16 (data not shown).

Discussion

The results reported above all together suggest a complex picture for induction and repair of the oxidative damage from H_2O_2 in the different FA complementation groups. While an elevated basal level of 8-OHdG in FA-C and FA-E cells suggests a condition of spontaneous pro-oxidation FA-A and FA-E cell lines seem to have a diminished ability to remove the damage.

Our data agree in part with those reported by Lackinger *et al.* (1998), who compared the ability of different FA cell lines to reactivate a CAT plasmid transiently transfected in the cells after *in vitro* damage by $KMnO_4$. They reported a defective repair of bulk oxidative DNA damage in cells from complementation groups B–E. The differences from our data can be ascribed to the broad spectrum of damage induced by $KMnO_4$, or to the different nature of the repair system involved. Interestingly, in FA-A cells, the DNA damage actually increased during the first half an hour, as if an auto generating ROS production had been activated. The excess of oxidative damage seen in FA-A cells after H_2O_2 treatment suggests a

possible defective protection against oxidative stress that, as already reported (Takeuchi and Morimoto, 1993), may be related to a deficiency of the enzyme catalase.

No difference was found by SCGE in the initial damage level induced by H_2O_2 among the cell lines examined. The apparent discrepancy with data from HPLC analysis may be due to the different doses (a 200-fold difference) used in the two assays. Further differences may be ascribed to the fact that the SCGE assay evaluates the damage at the single cell, excluding dead cells from the estimate (Humar *et al.*, 1997), while the DNA analysis by HPLC is unable to discriminate likewise. Moreover, we cannot exclude the contribution of mitochondrial DNA to the level of damage found by HPLC, as already suggested (Pritsos *et al.*, 1997).

Some authors reported a normal background level of 8-OHdG evaluated as Fpg protein sensitive sites in immortalized FA lymphoblasts complementation group FA-A, FA-C and FA-D (Will *et al.*, 1998). The number of lesions induced by Ro19-8022 plus light and their repair were also similar in control and FA cells. A comparison of the dosages used in the different studies is not possible, since the treatments involved different agents. Moreover, using Fpg protein sensitive sites detection, Will *et al.* (1998) were unable to detect a difference in 8-OHdG content between FA and normal cells using purified foetal primary lung fibroblasts.

We found no significant differences in the ability to reseal the DNA breaks among the cell lines examined. This finding is not completely surprising since FA-C cells have been reported to have a normal ligation efficiency while showing a concomitant decrease in the fidelity of blunt-end DSB rejoining (Escarceller *et al.*, 1998).

An increased frequency of micronuclei in FA compared with normal cells was found in our experiments. This is in agreement with the micronuclei frequency reported by other authors in FA and normal fibroblasts (Rudd *et al.*, 1988) and may be related to the well established increase in chromosome aberrations in FA cells. Using FISH with DNA centromeric probes, we found that micronuclei containing acentric chromosomal fragments were ~50% in both normal and corrected FA-C, untreated cells and increased to 62–66% in FA-C and FA-A cells. Our data indicate that both chromosome loss and fragmentation are increased in FA compared with normal cells.

H_2O_2 treatment increased the micronuclei frequency to a similar extent in normal and FA cells. However, a higher increase in H_2O_2-induced chromosomal aberrations in FA compared with normal peripheral blood lymphocytes was reported in another study (Dallapiccola *et al.*, 1985). Whether this discrepancy is due to different experimental conditions or to the different end points used in the two studies remains to be established. Most micronuclei induced by H_2O_2 originate from chromosomal fragmentation in both normal and FA cells. Therefore, H_2O_2 behaves in both cell types primarily as a clastogenic agent.

Evaluation of cell cycle parameters gave no evidence for an increased sensitivity of FA cells to hydrogen peroxide treatment. This result does not agree with data reported for FA cells (and verified also in our cells) after cross-linking agents treatment (Seyschab *et al.*, 1994; Kupfer *et al.*, 1996). Regardless of the efficiency of the repair of the oxidative DNA damage, it is not easy to attribute cross-linking agents FA cells sensitivity to ROS. Typically, a broad range of H_2O_2 concentrations induces a relatively narrow spectrum of damages compared with the effects elicited by MMC.

A reduced level of apoptosis has already been reported for FA-C cells treated by different agents, such as UV or X-rays (Rosselli *et al.*, 1995; Ridet *et al.*, 1997), as well as after treatment by MMC (Clarke *et al.*, 1997). In other cases FA-C cells were reported to undergo apoptosis to a greater extent than normal cells after cross-linking treatment (Kruyt *et al.*, 1996). Moreover, Kruyt reported that MMC-induced apoptosis in FA cells is p53 independent. It is important to bear in mind the method used to evaluate apoptosis: in the last paper apoptosis was evaluated by examining the ipo-diploid cell fraction in FACS histograms; in the papers by Rosselli, Ridet and Clarke SCGE or DNA-ladder formation assays were used; the FACS method may consider an excess of dead cells as apoptotic while at visual inspection (e.g. after Giemsa staining), FA-C cells and FA-E cells, looked very small and dark, but very few detectable apoptotic bodies could be found (not shown). This means that FA-C cells can die or undergo apoptosis following a pathway alternative to DNA-ladder formation. Our results concur with data recently published by Guillouf *et al.* (1999), who extensively studied apoptotic phenomena in MMC treated FA-C cells. They reported that FA-C cells are able to depolarize mitochondrial membrane, but fail in caspases activation. Moreover they found that MMC sensitivity was lost in corrected FA-C cells by transfection with the wild-type C gene, but that the apoptotic defect was retained. In another study (Monti *et al.*, 1997), FA patients' lymphocytes, treated with the apoptotic agent D-ribose, were found to depolarize mitochondrial membrane to a lower extent if compared with those of control subjects. As a consequence FA patients' lymphocytes showed a lower apoptosis rate.

In our hands FA-A cells and lines derived from them underwent apoptosis more than control cells; by contrast FA-C and FA-E never showed ladder formation. The unfolding mechanism of the apoptotic process cannot be regarded as a common pathway in FA cell lines derived from different complementation groups and these differences may reflect the different genotype. It is, however, surprising that reversion to normal phenotype, which normalizes baseline chromosomal breaks, 8-OHdG repair and MMC sensitivity, at least evaluated as cell cycle perturbation, is unable to restore a normal apoptotic mechanism. An explanation may be that mutated cells develop an alternative pathway to apoptosis, independent from both caspases activation and p53, and that this pathway is maintained also in revertant or transfected cells.

In conclusion, the data presented in this study show how a defective handling of oxidative damage is associated with the biochemical characteristics of FA-C and FA-E complementation groups cells. These same complementation groups also showed an alteration in the apoptotic pathway after treatment with different agents. Thus, FA proteins may not be just partners in a system involved in the processing of DNA repair, but might be involved in a pathway that signals to the cell cycle control in dependence to the cellular redox balance. These findings further suggest the complexity of the functions associated with the different activities codified by the genes that are associated with the Fanconi's phenotype.

Acknowledgements

The research was partially supported by the EC Concerted Action on DNA Repair and Cancer, MURST (Ministero dell' Università e della Ricerca Scientifica e Tecnologica), EUROS (no. BMH4-CT98-3107 DG12-SSMI).

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Received on August 14, 2000; accepted on January 8, 2001