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Fanconi's anemia cells are relatively resistant to H_2O_2-induced damage

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Abstract

Background and Objective. **Fanconi's anemia (FA) is a rare autosomal recessive syndrome characterized by skeletal abnormalities, late onset bone marrow failure and susceptibility to neoplasias. Reduced defense against oxidative stress is thought to be one of the cell damaging mechanisms. We investigated *in vitro* the effects of oxidative stress on red blood cells (RBC) and on hematopoietic progenitor growth of normal donors and of FA patients.**

Design and Methods. **The effects of hydrogen peroxide (H_2O_2) on RBC and hematopoietic progenitors were studied *in vitro* by erythrophagocytosis assay and by hematopoietic progenitor colony assay, respectively.**

Results. **In an erythrophagocytosis assay using normal monocytes, RBC from nine FA patients showed increased binding index (defined as the percentage of monocytes with adherent or phagocytosed RBC) compared to that obtained with RBC from nine normal controls. Upon exposure to H_2O_2, the binding index of normal RBC increased, while that of FA RBC remained unchanged. In a set of different experiments, H_2O_2 treatment of peripheral blood mononuclear cells (PBMNC) caused a significant decrease of the number of colonies from circulating progenitor cells in all normal subjects; the inhibition was dose-dependent and direct as proven by using normal purified $CD34^+$ cells. In nine FA patients colony assays from intact cells showed a decreased number of circulating progenitors as compared to normal subjects; however, H_2O_2 treatment of FA PBMNC did not cause any further decrease of the plating efficiency.**

Interpretation and Conclusions. **Untreated FA cells behave as normal cells after exposure to the toxic effects of H_2O_2. However, since H_2O_2 exposure is inoffensive to circulating FA RBC and hematopoietic progenitors, it seems that a selection for cells resistant to further oxidative stress has taken place in the residual hematopoiesis of FA patients. We may surmise that the survival of cells that have suffered from oxidative damage may have increased the risk of their leukemic transformation.**

Key words: Fanconi's anemia, oxidative stress, hematopoietic progenitors

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Fanconi's anemia (FA) is a rare autosomal recessive disease leading to progressive bone marrow failure in about 90% of patients.[1] The risk of developing leukemia or other cancers is about 20-fold higher in FA patients than in normal subjects.[2] Documented hypersensitivity to bifunctional DNA cross-linking agents such as diepoxybutane (DEB) and mitomycin C (MMC) is essential for the diagnosis of FA.[3-6] Eight subtypes (FA-A to FA-H) have been identified so far through cell fusion and complementation analysis.[7,8] This finding suggests that at least eight genes are involved in a pathway that, when defective, causes chromosomal instability and cell death, leading to bone marrow failure. The cDNA for FA-C (FAC) and FA-A (FAA) genes have been cloned;[9-11] they encode new proteins that are unrelated to each other or to other known proteins. FAC and FAA proteins are localized in the cytoplasmic cell compartment,[11,12] but the FAA-FAC complex can migrate into the nucleus.[13] Expression of the FAC and FAA genes in FAC-deficient[14] and FAA-deficient[15] lymphoblastoid cell lines, respectively, can correct the susceptibility to MMC-mediated chromosomal breakage. Furthermore, $CD34^+$ cells from FAC patients transduced by a FAC retroviral vector[14,16] and $CD34^+$ cells from FAA patients transduced by a FAA retroviral vector[15] increased colony growth in the absence and presence of MMC. However, the basic cellular mechanism primarily disturbed in FA is not yet known. It has been speculated that defects in DNA repair, or in cell cycle regulation, or oxygen hypersensitivity could be the ultimate cause of the FA phenotype. Hypersensitivity of FA cells to oxidative stress is usually considered as a secondary manifestation of the primary FA defect, since FA fibroblasts transformed with SV40 large T antigen lose this feature.[17] However, there is increasing evidence of a connection between defects in DNA repair and in cell cycle regulation with oxygen hypersensitivity in FA cells. The impaired *in vitro* growth of FA cells is due to a cell cycle prolongation and arrest in the G2 phase,[18] and has been found to be dependent upon oxygen concentration.[19] Reactive oxygen species scavenging enzymes, such as superoxide dismutase (SOD) and catalase, and antioxidant agents showed a protective effect on the frequency of chromosomal abnormalities in FA cells.[20-24] SOD, catalase and glutathione peroxidase were found to be increased in FA

fibroblasts,[23] suggesting that these detoxification enzymes are increased in order to eliminate an excess of toxic radicals. A high concentration of SOD has been shown to suppress the cytotoxic effect of MMC on FA cells *in vitro*[25,26] and *in vivo*.[27] More recently, FA leukocytes have been reported to release large amounts of oxygen radicals,[28] and FA plasma to exert clastogenic activity through the intermediacy of superoxide radicals.[29-31] These findings suggested that FA cells are either more susceptible to oxidative DNA damage or less efficient in repairing it.[32] According to recent reports, even the increased sensitivity of FA cells to MMC seems to be mediated by oxygen free radical generation rather than by DNA crosslinking.[33,34] The involvement of FA genes in hematopoietic differentiation and the possible role of reactive oxygen-induced damage in the pathogenesis of FA led us to investigate the effects of H_2O_2, the most toxic oxygen compound, on circulating red blood cells and hematopoietic progenitors from normal donors and FA patients.

Materials and Methods

Specimen collection

Peripheral blood (PB) samples were obtained from 49 normal subjects and nine Italian patients with FA. Bone marrow (BM) was obtained from two healthy volunteers in the course of a bone marrow donation. All blood and bone marrow samples were obtained after informed consent. The diagnosis of FA was established by the characteristic cytogenetic findings after DEB exposure.[35] The patients' clinical features are summarized in Table 1.

PB and BM mononuclear cell isolation

PB and BM were collected in heparin (Vister) or in ethylene-diamine-tetracetic acid (EDTA, Sigma) for colony and erythrophagocytosis assays, respectively. Mononuclear PB and BM cells were isolated by density gradient centrifugation using lymphocyte separation medium (Flow). After washing in Iscove's modified Dulbecco's medium (IMDM, Life Technologies), cells were resuspended in IMDM supplemented with 20% FCS (Life Technologies). Macrophage and monocyte depletion was obtained using adherence to plastic (2 h at 37°C with 5% CO_2).

Separation of CD34⁺ cells

$CD34^+$ BM cells were separated using affinity chromatography (Cellpro). Briefly, nonadherent BM cells were incubated at room temperature with a murine anti-human CD34 IgM mAb, washed in phosphate-buffered saline (PBS, Live Technologies), and then incubated with streptavidin-conjugated goat $F(ab')_2$ anti-mouse IgM. After washing with PBS supplemented with 2% human albumin, cells were applied to an affinity column containing biotin-coated beads, and the $CD34^+$ cell fraction was eluted with PBS. An aliquot of the eluted cells was stained with phycoerythrin-conjugated anti-CD34 HPCA-2 monoclonal antibody (Becton Dickinson) to assess the purity of the eluted cells.

Red and mononuclear cell treatment by H_2O_2

RBC at a hematocrit of 1.5% were incubated in PBS with and without 10 mM H_2O_2 for 1 h at 37°C. Macrophage-depleted PBMNC were incubated at a concentration of 5×10^5 cells/mL for 2 h at 37°C in PBS in the absence and in the presence of 1 and 4 mM H_2O_2; in preliminary experiments higher concentrations of H_2O_2 (8 and 10 mM) almost completely inhibited colony formation from normal PBMNC (data not shown). $CD34^+$ BM cells were incubated at a concentration of 1×10^3 cells/mL for 2 h at 37°C in PBS in the absence and in the presence of 4 and 10 mM H_2O_2. After three washings with PBS, H_2O_2-treated red or mononuclear cells were used for erythrophagocytosis or colony assay, respectively.

Erythrophagocytosis assay

Isolated normal PBMNC at a concentration of 1×10^6/mL were plated in 0.2 mL aliquots on a coverslip kept in a Petri dish, and monocytes were allowed to adhere to plastic (1 h at 37°C with 5% CO_2). After multiple washing, the coverslips with adherent normal monocytes were overlayed with the RBC to be tested, and incubated for 90 minutes at 37°C in 5% CO_2. Then the coverslips were washed, fixed with glutaraldehyde and observed for adherent or phagocytosed RBC by a phase contrast microscope. Two indices were determined: the binding index (B%), defined as the percentage of monocytes with adherent or phagocytosed RBC, and the ingestion index (Ii), defined as the number of phagocytosed RBC per monocyte with adherent or phagocytosed RBC.[36] Each experiment was performed in triplicate with intact or H_2O_2-treated RBC.

Hematopoietic cell culture

Colony assays of hematopoietic progenitors were carried out in methylcellulose medium. Briefly, macrophage-depleted PBMNC or $CD34^+$ BM cells were plated at a concentration of 5×10^5 cells/mL and 1×10^3/mL respectively, in basal condition and after 2 hours incubation with H_2O_2. The culture medium contained 0.8% methylcellulose (Fisher), 10% FCS, 1% bovine serum albumin (Boehringer), and the following growth factors: 10% phytohemagglutinin-leukocyte conditioned medium (PHA-LCM), 20% plasma from a pancytopenic patient, 50 U/mL recombinant granulocyte-macrophage colony stimulating factor (rGM-CSF, Amgen), and 3 U/mL recombinant erythropoietin (EPO, Ortho). Identification and scoring of myeloid (CFU-GM), erythroid (BFU-E), and mixed (CFU-GEMM) colonies were performed *in situ* by inverted microscopy on the basis of

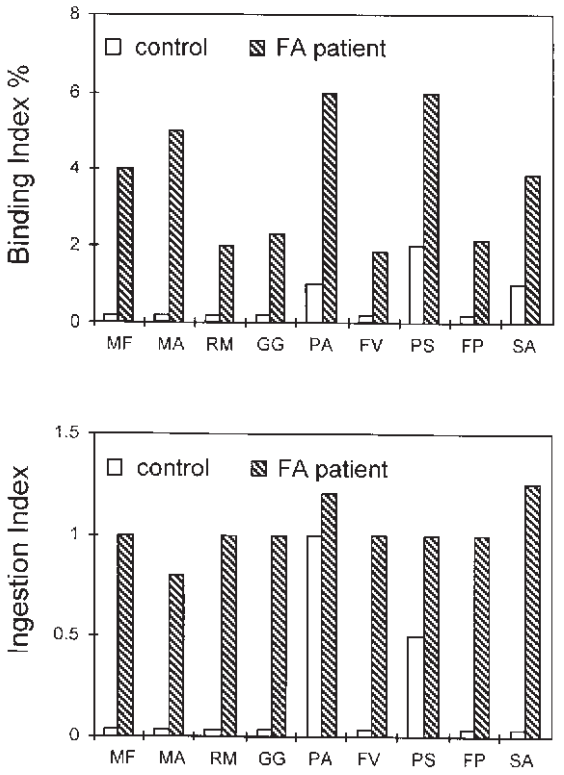

Figure 1. Erythrophagocytosis assay of untreated FA RBC in comparison with simultaneous normal control.

their characteristic morphology after 14 days incubation at 37°C in 5% CO_2. All assays were performed in quintuplicate. All experimental procedures were performed in endotoxin-free plastic ware (Corning).

Statistical analysis

Statistical analysis was performed using the Wilcoxon rank sum test on paired or unpaired samples, as suitable, and linear regression. Statistical significance was accepted for any $p < 0.05$.

Results

In vitro phagocytosis of RBC by normal monocytes

Each experiment was performed challenging the same batch of normal monocytes with RBC from an FA patient and a normal donor. RBC from nine FA patients showed increased binding and a higher ingestion index as compared to normal RBC (mean B%: 3.67 vs. 0.44, p=0.008; mean Ii: 1 vs. 0.17, p=0.008) (Figure 1). The erythrophagocytosis assay carried out in 40 additional normal controls confirmed the results obtained in the nine simultaneous controls (mean B%: 0.45; mean Ii: 0.3).

Clonogenic capacity of hematopoietic progenitors from FA patients

Numbers of myeloid, erythroid and mixed colonies from PBMNC were extremely low in all nine FA patients tested (Table 1), and were not affected by *in vitro* addition of danazol or stem cell factor (data not shown). By pooling data in Table 1 and other data obtained in the same patients with additional experiments during the follow-up (data not shown) we found a correlation between Hb level and number of BFU-E ($r=0.65$; $p < 0.05$) and CFU-GEMM ($r = 0.67$; $p < 0.05$), as well as between WBC and number of CFU-GM ($r = 0.67$; $p < 0.05$) and CFU-GEMM ($r = 0.73$; $p < 0.05$).

In vitro effects of H_2O_2 on RBC and hematopoietic progenitors from normal subjects and FA patients

In an erythrophagocytosis assay the exposure of normal RBC (n=11) to H_2O_2 10 mM caused an increase of B% (from 0.36 to 4.09, p=0.002) and Ii (from 0.05 to 0.91, p=0.002). The exposure to the same concentration of H_2O_2 turned out to be inof-

Table 1. Clinical features of Fanconi's anemia patients.

Case	*Age/ sex*	*Age at diagnosis*	*Skin/skeletal abnormalities*	*Parent consanguinity*	*Hgb g/dL*	*WBC ×10⁻³/mL*	*Plt ×10⁻³/mL*	*BFU-E**	*CFU-GM**	*CFU-GEMM**
MF	11/M	5	yes	no	11.1	2.8	105	15.4	20.6	8.2
MA	11/M	5	yes	no	12.7	4.8	107	11	14.8	3.2
RM	8/F	4	yes	yes	10.7	2.7	25	6.2	11.4	2
GG	6/M	4	yes	no	12.6	7.8	56	20	30	11.4
PA	14/M	11	yes	no	8.3	2.5	42	7.6	6.4	0
FV	16/F	11	yes	yes	11.1	3.3	118	19	24.4	5.2
PS	17/F	8	yes	yes	9	3.0	91	0.6	1.2	0
FP	34/F	33	no	no	10.5	2.5	39	3.4	6.6	0
SA	10/F	5	yes	yes	8.5	2.8	52	5.8	0	2

**Number of colonies/5x10⁵ PBMNC plated. Control values, obtained from 10 normal subjects, were BFU-E: 28-134, CFU-GM: 52-114, and CFU-GEMM: 10-26.*

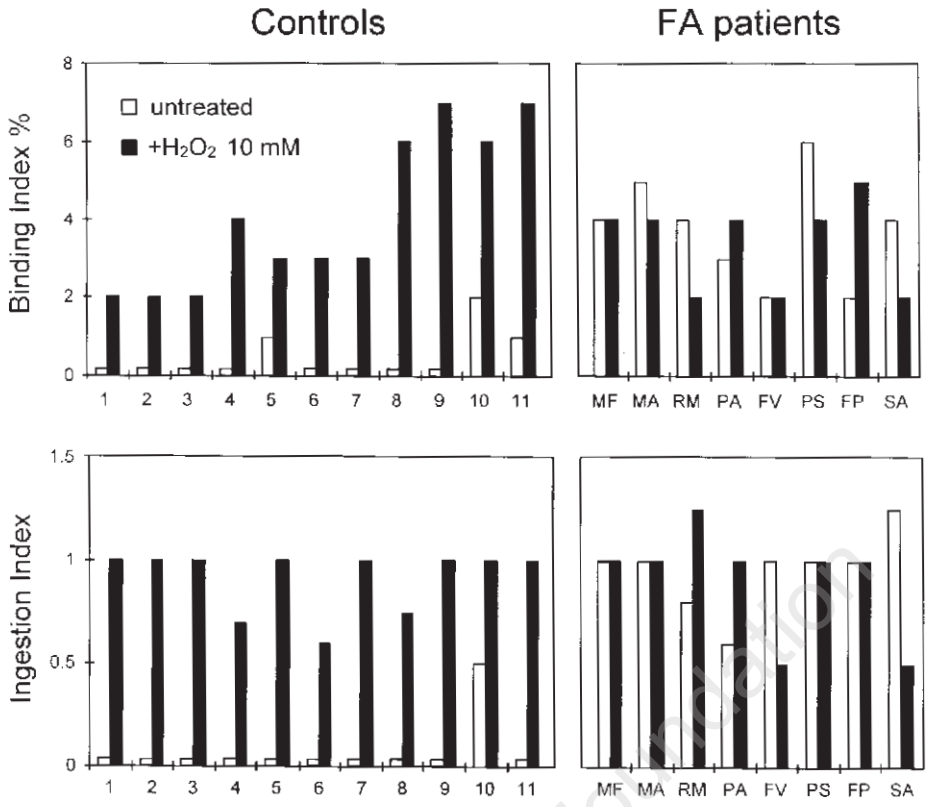

Figure 2. Erythrophagocytosis assay of untreated and H_2O_2-treated RBC from normal subjects and FA patients. H_2O_2 treatment altered the RBC membrane of normal RBC thus causing increased B% and Ii, but had no additional effect on FA RBC.

fensive to RBC from FA patients (n=8: B% from 3.75 to 3.38, Ii from 0.97 to 0.9; p >0.5) (Figure 2).

Treatment of macrophage-depleted PBMNC from normal donors with H_2O_2 1 and 4 mM caused a dose-dependent inhibition of myeloid, erythroid and mixed colonies (Table 2). To test whether H_2O_2 directly inhibited normal progenitor growth, we examined its effects on colony formation from enriched $CD34^+$ BM cells (80-90% purity). H_2O_2 at the concentration of 4 and 10 mM decreased normal colony formation by $CD34^+$ cells in a dose dependent manner, in agreement with the data obtained using total PBMNC (Table 3). By contrast, treatment with H_2O_2 1 and 4 mM only marginally reduced the number of colonies from macrophage-depleted PBMNC of FA patients (Table 4). The percentage of colonies still growing after treatment with H_2O_2 4 mM was significantly lower in normal subjects than in FA patients: BFU-E, 41.8% vs. 86.9%, p<0.02; CFU-GM, 28.5% vs. 69.8%, p<0.01; CFU-GEMM, 18.1% vs. 80.9%, p <0.002 (Wilcoxon rank sum test on unpaired samples).

Table 2. Hematopoietic progenitor growth from normal PBMNC in basal conditions and after H_2O_2 treatment.

	BFU-E	*CFU-GM*	*CFU-GEMM*
	/5×10⁵ cells plated		
Untreated (n=14)	53.1±8.4	54.7±4.9	11.2±1.3
H_2O_2 1 mM (n=7)	32.6±5^	48.5±7.2^	10.9±1.9^
H_2O_2 4 mM (n=14)	23.9±6.3*	16.2±3*	2.3±0.7*

*Values represent mean numbers±SEM. Each assay was performed in quintuplicate. Statistical analysis (Wilcoxon rank sum test on paired samples): ^p<0.05 and *p<0.005, as compared to the paired untreated cells.*

Discussion

Hemophagocytosis is a well known feature of FA bone marrow, even in early stages of the disease;[37] in keeping with this finding, we found that more FA RBC were phagocytosed than normal RBC by normal monocytes in an *in vitro* erythrophagocytosis assay. On the other hand, a reduced number of

Table 3. Hematopoietic progenitor growth from normal CD34+ BM cells in basal conditions and after H_2O_2 treatment.

	BFU-E	CFU-GM
	/10^3 cells plated	
Untreated	103±9.1	69±8.8
H_2O_2 4 mM	53±6.7	36±6.4
H_2O_2 8 mM	14.7±3.6	13±3.1

Values represent mean±SEM from two independent experiments. Each culture was performed in duplicate.

Table 4. Hematopoietic progenitor growth from FA PBMNC in basal conditions and after H_2O_2 treatment.

	BFU-E	CFU-GM	CFU-GEMM
	5×10^5 cells plated		
Untreated (n=8)	8.6 ± 2.2	10.7 ± 3.1	2.5 ± 1
H_2O_2 1 mM (n=4)	10.6 ± 2.4	13.2 ± 3.6	3 ± 1.4
H_2O_2 4 mM (n=8)	6.5 ± 1.7	7.7 ± 2.7	2 ± 0.6

Values represent mean numbers±SEM. Each assay was performed in quintuplicate. Statistical analysis (Wilcoxon rank sum test on paired samples): BFU-E, CFU-GM, and CFU-GEMM after treatment with H_2O_2 1 and 4 mM always produced $p > 0.5$ as compared to the paired untreated cells.

hematopoietic progenitors is associated with the progressive bone marrow failure of FA patients.[38,39] In our series of FA patients, we confirmed the low clonogenic ability of their hematopoietic progenitors. We found a correlation between patient's Hb level and the number of erythroid and mixed colonies, and a correlation between WBC count and the number of myeloid and mixed colonies. These findings confirm the previously described correlation between clinical status and *in vitro* erythropoiesis in FA patients.[38]

Several lines of evidence support either a direct defect in the removal of DNA cross-links or an impaired capacity of FA cells to remove reactive oxygen species resulting from the interaction with cross-linking agents.[40] Possible mechanisms for H_2O_2 toxicity include enhanced lipid peroxidation, direct DNA damage, altered calcium and sulphydryl homeostasis,[41] poly-ADP-ribosylation leading to post-transcriptional modification of proteins,[42] NADPH depletion,[43] and activation of apoptotic pathways.[44]

By an *in vitro* erythrophagocytosis assay, we found that phagocytosis of normal RBC increased after H_2O_2 exposure, while FA RBC were insensitive to the same treatment. In addition, we found that normal hematopoietic progenitors were susceptible to H_2O_2; this susceptibility was dose dependent and was not mediated by accessory cells, as it was confirmed in experiments using CD34+ purified cells. In contrast, FA hematopoietic progenitors were resistant to H_2O_2 treatment. A similar paradoxical behavior of FA cells was recently reported studying apoptosis; indeed, the role of FAC as an antiapoptotic gene seems well established.[45,46] In the mouse, after disruption of the FAC gene by homologous recombination, hematopoietic cells became hypersensitive to γ-IFN induced apoptosis.[47,48] Increased spontaneous apoptosis seems a feature of FA belonging to various complementation groups.[49] Nevertheless, apoptosis induced by specific stimuli was impaired in FA cells: (i) FA lymphoblastoid cells and FA peripheral lymphocytes were resistant to γ-irradiation-induced apoptosis as a result of p53 impairment;[49] (ii) FA lymphoblastoid cells were resistant to Fas-mediated apoptosis;[50] (iii) FA peripheral mononuclear cells were resistant to apoptosis induced by agents interfering with the cell redox status.[51] Since the relationship between oxidative damage and at least some apoptotic pathways is well established,[52,53] our results are in keeping with these findings.

The mechanisms of such resistance are not yet known. We might hypothesize that the continuous exposure of FA cells to the toxic effect of reactive oxygen species leads to selection of cells resistant to additional oxidative injury. Alternatively, the continuous exposure may induce expression/overexpression of protective antioxidant proteins such as mitogen-activated protein kinase[54] or various heat shock proteins.[55-57] A recent report that superoxide anion inhibits Fas-mediated apoptosis[58] suggests another possible explanation: a basal level of reactive oxygen species triggers spontaneous apoptosis in the oxidative stress-sensitive FA cells, while a higher level of reactive oxygen species, due to metabolic stress or experimental conditions, may inhibit apoptosis. Complementation studies and molecular analysis were not available in the cohort of patients presented. It is likely that most of them belonged to the FAA group, as do the majority of Italian patients.[59] Thus, it remains unknown whether resistance to H_2O_2 treatment bears any relationship to the type of molecular defect.

This study demonstrates that untreated FA cells behave as normal cells do after exposure to the toxic effect of H_2O_2. In conclusion our finding that H_2O_2 exposure at concentrations which damage normal cells was relatively inoffensive to FA RBC and hematopoietic progenitors indicates that circulating cells of FA patients are resistant to additional oxidative injury. A speculative hypothesis is that FA patients harbor a population of cells selected to resist further oxidative damage, which is responsible for the resid-

ual hematopoiesis; having already accumulated oxidative-mediated DNA damage, this population may be more susceptible to leukemia.

Contributions and Acknowledgments

RN performed the hematopoietic progenitor colony assays and data analysis. NM performed the erythrophagocytosis assays. CDG performed cell separation and participated in the experimental procedures. SF and BR jointly supervised the study. CS designed and co-ordinated the study. All authors contributed to the interpretation of the results and to writing the paper.

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Disclosures

Conflict of interest: none.

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Manuscript processing

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