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An oxidative mechanism of interferon induced priming of the Fas pathway in Fanconi anemia cells☆

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Abstract

Hematopoietic progenitor cells from children with Fanconi anemia of the C complementation group (FA-C) are excessively apoptotic and hypersensitive to various extracellular cues including Fas-ligand, tumor necrosis factor-α and double-stranded RNA. Interferon (IFN)-γ is known to augment apoptotic responses of these factors. The ''priming'' effect of IFN-γ is not fully explained. In view of the strong evidence that FA cells are intolerant of oxidative stress, we tested the notion that IFN-priming involves the induction of reactive oxygen species (ROS) in two FA-C B-lymphocyte cell lines and in peripheral blood neutrophils and mononuclear cells of FA patients. We also investigated whether the combination of IFN-γ and Fas created an intracellular environment that promoted apoptosis. Significantly lower doses of IFN-γ induced ROS accumulation in neutrophils and mononuclear cell of FA patients compared to cells of normal individuals. Enhanced ROS accumulation and decreased intracellular glutathione levels were observed in FA-C B-cell lines primed with IFN-γ and treated with agonistic anti-Fas antibody than in isogenic control cells corrected with FANCC. The above treatment also induced caspase-3 and -8 activation as well as apoptosis. That antioxidants reduced the priming effect of IFN-γ in Fas and IFN-γ-treated FA lymphoblast cells, demonstrates that ROS represent a critical effector mechanism for the exaggerated responses to IFN-γ characteristic of FA-C cells.

Keywords: Fanconi anemia; IFN-γ; Anti-Fas Ab; Apoptosis; ROS; GSH

1. Introduction

FA is an autosomal recessive disorder characterized by cellular hypersensitivity to cross linking agents, bone marrow failure, diverse congenital anomalies and a marked increase in the incidence of acute myelogenous leukemia ([1,2] and reviewed in [3]). Hematopoietic progenitor cells from mice nullizygous at the FA group C (FANCC) locus and children with FA of the C complementation group (FA-C) are hypersensitive to the apoptotic inducing effects of IFN-γ [4–6]. It is believed that IFN-γ induces apoptosis through up-regulation of the Fas death receptor [7–9]. IFN-γ is also known to augment apoptotic responses of TNF-α and double stranded RNA [10,11]. Conversely overexpression of FA-C gene (FAC) in mice reduced sensitivities to Fas ligation [12]. The ''priming'' effect of IFN-γ has not been fully explained.

FA cells are reportedly hypersensitive to oxygen [13,14], although the molecular basis of this sensitivity is yet unknown. Defective hematopoiesis and hepatic steatosis was observed in mice with combined deficiencies of FANCC and Cu/Zn superoxide dismutase, suggesting that the altered redox state present in the mice is responsible for impaired hematopoiesis [15]. Other studies have shown that FANCC and NADPH cytochrome P450 reductase interact and it is suggested that FANCC serves as an

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Abbreviations: DCFH-DA, 2′,7′-dichlorofluorescein-diacetate; DHA, dehydroascorbic acid; FA, Fanconi anemia; FCS, fetal calf serum; GSH, glutathione; HBSS, Hanks balanced salt solution; HE, hydroethidine; IFN, interferon; LDH, lactate dehydrogenase; MNC, mononuclear cells; PB, peripheral blood; PBS, phosphate buffered saline; ROS, reactive oxygen species; TNF, tumor necrosis factor.

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intracellular antioxidant [16]. In a recent study a novel interaction between FANCG and CYP2E1 (a member of the P450 superfamily that is associated with the production of ROS) was identified and it is proposed that FANCG participates in protection against oxidative DNA damage [17].

In view of the strong evidence that FA cells are intolerant of (a) oxidative stress and (b) apoptotic inducing cytokines, we asked whether these responses are linked. Using two FA lymphoblast cell lines, neutrophils and MNC isolated from the PB of FA patients and normal healthy volunteers, we report that: (a) IFN-γ induces enhanced ROS accumulation in neutrophils and MNC of FA patients and in HSC536/N and PD149L lymphoid cells; (b) IFN-γ and anti-Fas Ab induce decreased intracellular GSH levels in HSC536/N and HSC536/FANCC cells; (c) IFN-γ and anti-Fas Ab induce caspase-3 and -8 activation as well as apoptosis in HSC536/N cells; (d) ascorbic acid suppresses the priming effect of IFN-γ in Fas and IFN-γ-treated FA cells.

2. Materials and methods

2.1. Epstein–Barr virus (EBV)-transformed cell lines

The EBV-transformed lymphoblast cell line HSC536/N (gift of Manuel Buchwald, The Hospital for Sick Children, Toronto, Ont., Canada) was derived from PB cells of a child with FA-C. In the cells of this patient, one FAC allele was deleted and the other carries a leucine-to-proline substitution at amino acid position 554.

HSC536N/FANCC cells were derived by transducing the HSC536/N cells with a retrovirus encoding both FAC and neomycin phosphotransferase as previously described [6,18]. PD149L cells are EBV-transformed lymphoblasts derived from a child with FA-C whose mutant is IVS4+4 A to T, a mutation that results in an in-frame deletion of 37 amino acids encoded by exon IV of FANCC (gift of Grover Bagby MD, Oregon Health Science University). PD149/FANCC cells were derived by transducing the PD149L cells with a retrovirus encoding both FANCC and neomycin phosphotransferase. The lymphoblast cell lines were grown in RPMI 1640 (Biological Industries) supplemented with 15% FCS (Hyclone Lab.), 1% glutamine, 100 units/mL penicillin and 100 mg/mL streptomycin at 37° and 5% CO_2, in a humidified atmosphere.

2.2. Human MNC and neutrophils

Blood was obtained from five consenting healthy volunteers and under a protocol of informed parental consent from two patients with FA-C and one patient with FA-A. Low density MNC and neutrophils were isolated, using single density gradient centrifugation, as previously described by us [19]. The cells were washed and resuspended in Iscoves modified Dulbecco's medium (Gibco) supplemented with 15% FCS, 1% glutamine, 100 units/mL penicillin and 100 mg/mL streptomycin.

2.3. Uptake of DHA

Ascorbic acid (Sigma Chemical Co.) was dissolved in phosphate buffer (KH_2PO_4 0.1 M, Na_2HPO_4 4 mM, EDTA 0.5 mM), pH 5.6, and incubated with ascorbate oxidase as previously described by us [20]. The oxidized ascorbic acid, DHA, was added at a final concentration of 150 mM to the FA lymphoblast cell lines and to PB neutrophils and MNC that were resuspended in incubation buffer (15 mM Hepes, pH 7.6, 135 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.05 mM DTT). The cells were incubated with DHA for 10 min at 37°, washed and resuspended in medium.

2.4. Measurement of ROS

The oxidation of DCFH-DA (Molecular Probes) and HE (Polysciences) were analyzed. DCFH-DA and HE measure H_2O_2 and O_2^-, respectively [21]. An adaptation of the method of Jayanthi *et al.* was used [22]. Cells (2×10^6 mL^{-1}) were pre-incubated in the presence or absence of 150 μM DHA as above, washed and stained with 5 μM DCFH-DA or HE. Cells were washed, resuspended in Hank's balanced salt solution (HBSS) and plated at a density of 3750 cells/well of a 96-well plate. To each well were added 0.01–1.0 ng/mL IFN-γ (R&D Systems), 100 ng/mL anti-Fas Ab (Immunotech) or a combination of IFN-γ and anti-Fas Ab. Fluorescence was measured over a 180 min time period with a spectrofluorometer (FL-600 Microplate Fluorescence Reader) with excitation at 488 nm and emission at 525 nm. The fluorescence at each time point was expressed as relative fluorescence values normalized to the initial reading.

2.5. Intracellular GSH and GSSG assay

The mutant FA-C cell lines HSC536/N and PD149L and the corrected counterparts were pre-incubated in incubation buffer in the presence or absence of 150 μM DHA for 10 min, washed and plated at a concentration of 4×10^6 mL^{-1} in 24-well plates. IFN-γ (1.0 ng/mL) and anti-Fas Ab (25–100 ng/mL) were added individually or in combination for 120 min, cells were washed twice with cold (4°) PBS, and immediately lysed with 100 μL of 5% 5-sulphosalicylic acid [23]. After centrifugation at 12,000 *g* for 10 min, supernatants were collected for GSH and GSSG assays. In several experiments cells were pre-incubated with 50 μM Z-VAD-FMK (a general caspase inhibitor, R&D Systems) for 60 min at 37° before the addition of 100 ng/mL anti-Fas Ab for 120 min. The total cellular GSH and GSSG concentrations were assayed using the method of Meister as described previously [24].

2.6. Determination of Fas expression

Cell surface expression of the Fas antigen was measured by flow cytometry using UB-2 (Immunotech) a mouse IgG anti-Fas Ab that does not induce apoptosis in target cells. The four lymphoblast cell lines (1×10^6 of each cell line) were washed with cold PBS containing 0.1% NaN_3 and incubated with binding buffer containing 1.0 mg/mL of γ-globulin (Sigma) on ice for 15 min to block nonspecific binding. The cells were then washed, incubated with 10 μg/mL anti-Fas (UB-2) Ab or isotype-matched control IgG (Dako) for 30 min at 4°. Following incubation the cells were washed and reacted with FITC-goat anti-mouse $F(ab)_2$ (Sigma) for 30 min at 4°, at which time they were washed and analyzed on a flow cytometer (Becton Dickinson) [25].

2.7. Fluorogenic assay for caspase-3 and -8 activity

The FA cell lines were pre-incubated with DHA as above and cultured in medium in the presence of 1.0 ng/mL IFN-γ or 100 ng/mL anti-Fas Ab added individually or in combination for 0–120 min. At various time points cells were collected, washed, resuspended in 50 mM Tris–HCl buffer pH 7.4, 1 mM EDTA, 10 mM EGTA and lysed by three successive freeze–thaw cycles at dry ice/37°. Cell lysates were centrifuged at 15,000 *g* for 5 min, and the supernatants were stored at −70°. The protein concentration of each sample was estimated using the Bradford Bio-Rad protein assay. For caspase-3 and -8 activity a total of 50 μg protein was incubated with 50 μM ac-DEVD-AMC and ac-IETD-AMC, respectively (both from BIOMOL Research Lab.) at 37°, for 30 min in the dark. The release of 7-amino-4-methylcoumarine was monitored by a spectrofluorometer using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. In some experiments caspase-3 and -8 inhibitors (ac-DEVD-cho, BIOMOL Research Lab. and Z-IETD-FMK, R&D Systems, respectively, 50 μM each) were directly added to the medium 60 min prior to the addition of IFN-γ and anti-Fas Ab [26].

2.8. Immunoblotting

HSC536/N and HSC536/FANCC cells were pre-incubated in the presence or absence of 150 mM DHA and exposed to a combination of 1.0 ng/mL IFN-γ and 100 ng/mL anti-Fas Ab for 0–120 min as described above. The cells were harvested, the cell pellets were solubilized with a solubilizing solution, and samples of 50 μg protein were subjected to electrophoresis on a 12% SDS–PAGE as previously described [27]. Caspase-3 and-8 were detected by incubating blots with anti-caspase-3 polyclonal Ab or anti-caspase-8 mAb (from PharMingen and Cell Signaling Tech., respectively) (each Ab was diluted 1:1000). The blots were incubated with secondary antibodies as previously described [27] and Ab-reactive proteins were detected using enhanced chemiluminescence reagents (Amersham).

2.9. Quantification of apoptotic cells

A portion of HSC536/N and HSC536/FANCC cells cultured under various conditions were quantified for apoptosis using the method of TUNEL (Terminal transferase, Boehringer) as previously described by us [28] and fluorescence microscopy or a Zeiss confocal laser-scanning microscope (Oberkochen). Cells with nuclear fragmentation or green fluorescence or both were scored as apoptotic. At least 300 cells from randomly selected fields were counted. Plasma membrane integrity was monitored by release of cytosolic LDH, using the LDH cytotoxicity detection kit (Boehringer).

Statistical significance was determined by using the Student's *t*-test and Wilcoxon signed ranks test.

3. Results

3.1. IFN-γ treatment induces ROS accumulation in the lymphoblast cell lines. Effect of DHA

We determined intracellular ROS levels using two compounds, DCFH-DA and HE, which measure intracellular H_2O_2 and O_2^-, respectively. HSC536/N, HSC536/FANCC, PD149L and PD149/FANCC cells were preloaded with DCFH-DA and HE and treated with 1.0 ng/mL IFN-γ or 100 ng/mL anti-Fas Ab added individually or in combination. Exposure of HSC536/N (Fig. 1A) and PD149L cells (Fig. 1B) to IFN-γ induced an immediate and marked augmentation in oxidation of DCFH-DA which increased with time. A moderate augmentation in oxidation of DCFH-DA was induced in HSC536/FANCC cells while no augmentation was observed in PD149/FANCC cells, thus indicating that HSC536/N and PD149L cells accumulate higher levels of ROS than the corrected counterparts (Fig. 1A and B). In recent studies we have shown that DHA decreases ROS accumulation in HL-60 promyelocytic cells [20,28], thus we investigated the effect of DHA on ROS accumulation in the lymphoblast cell lines. In preliminary studies we determined ascorbic acid accumulation in HSC536/N and HSC536/FANCC cells. The intracellular concentrations of ascorbic acid in HSC536/N and HSC536/FANCC cells following their incubation with 150 μM DHA for 10 min were 2.1 ± 0.12 and 1.6 ± 0.13 ng/10^6 cells, respectively (mean ± SE of two experiments performed in triplicates). Pre-incubation of HSC536/N and PD149L cells with 150 μM DHA significantly ($P < 0.05$) decreased fluorescence intensity, induced by IFN-γ, in the cells (Fig. 1A and B). No protective effect of DHA was observed with HSC536/FANCC cells. While IFN-γ induced ROS accumulation,

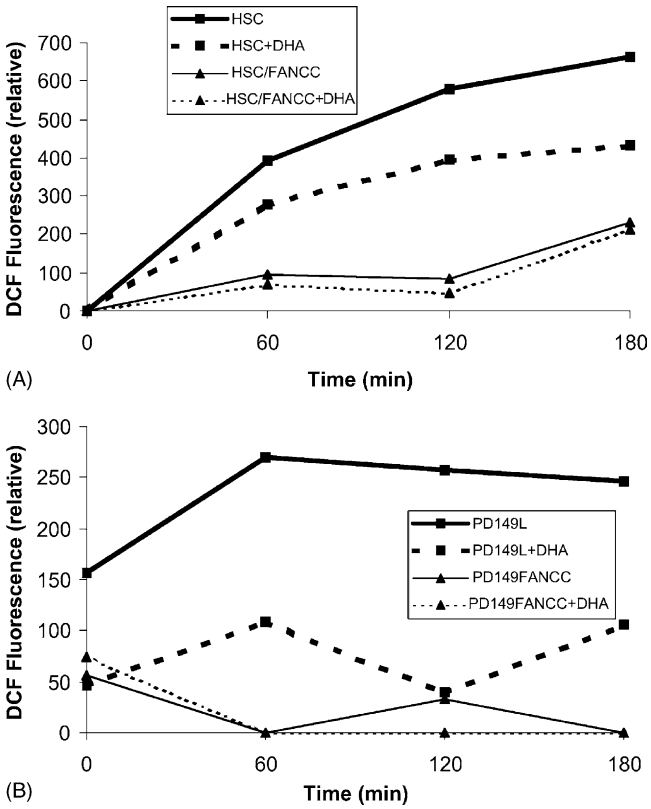

Fig. 1. Time course changes in DCF fluorescence in (A) HSC536/N and HSC536/FANCC cells and (B) PD149L and PD149/FANCC cells exposed to 1.0 ng/mL IFN-γ. The cells were pre-incubated in buffer in the presence or absence of 150 mM DHA. Following washing the cells were incubated for 15 min with DCFH-DA, cultured in wells and IFN-γ was added. ROS generation was determined as described in Section 2. Graphic data are representative of at least four experiments.

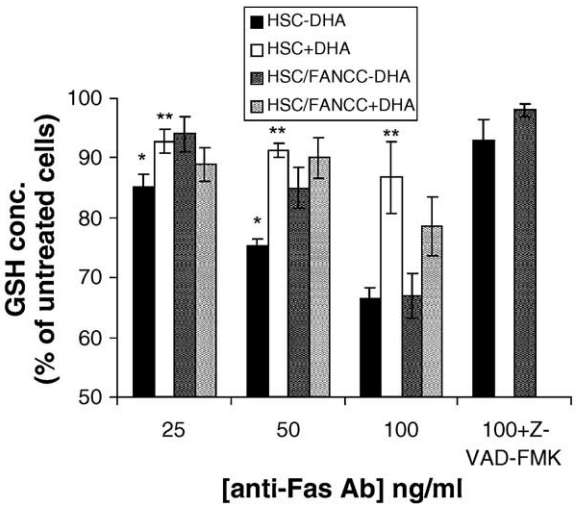

Fig. 2. Loss of intracellular GSH in HSC536/N and HSC536/FANCC cells exposed to various concentrations of anti-Fas Ab. Cells (4×10^6 mL^{-1}) were pre-incubated in buffer or 150 mM DHA left untreated or exposed to increasing concentrations of anti-Fas Ab (25–100 ng/mL) for 120 min. Following incubation cells were collected and intracellular GSH levels were determined as described in Section 2. In some experiments cells were pretreated with 50 mM Z-VAD-FMK before the addition of 100 ng/mL anti-Fas Ab for 120 min. Data values show mean ± SE from three independent experiments. (*) $P < 0.05$ HSC536/N vs. HSC536/FANCC cells. (**) $P < 0.05$ HSC536/N + DHA vs. HSC536/N cells-DHA.

Fas ligation did not (data not shown). The data obtained using HE show the same pattern (data not shown).

3.2. Expression of Fas on FA lymphoblast cell lines

Having observed higher ROS levels in HSC536/N and PD149L cells than in the corrected counterparts, following their exposure to IFN-γ, we wanted to rule out different Fas expression on the four cell types. The mean fluorescence intensity of HSC536/N and of HSC536/FANCC cells was found to be 71.8 and 68.5, respectively, percent positivity of cells 96.8 and 97.2%, respectively. The mean fluorescence intensity of PD149L and PD149/FANCC cells was found to be 158.3 and 139.8, respectively, percent positivity of cells 98.2 and 98.8%, respectively, thus indicating that most of the cells express equal levels of the protein. Exposure of the cell lines to IFN-γ did not affect Fas expression on the cells (data not shown).

3.3. Anti-Fas Ab treatment induces a decrease in intracellular GSH. Effect of DHA

When cells are oxidatively stressed, GSH depletion is commonly observed [29]. Thus, we investigated the effect of anti-Fas Ab (25–100 ng/mL) and IFN-γ (1.0 ng/mL) added individually or in combination, on intracellular GSH levels. The intracellular GSH content in HSC536/N cells was observed to fall after exposure to various concentrations of anti-Fas Ab, in a dose-dependent manner. HSC536/N cells were more sensitive than HSC536/FANCC cells, following exposure the former cells to 25 or 50 ng/mL anti-Fas Ab, their GSH concentration was 85 ± 2 and $75 \pm 1.2\%$ of untreated cells, respectively ($P < 0.05$). No significant fall was observed in HSC536/FANCC cells exposed to 25 ng/mL anti-Fas Ab and a slight decrease following exposure to 50 ng/mL anti-Fas Ab (94.3 ± 3 and $85 \pm 3.5\%$ of untreated cell, respectively) (Fig. 2). A similar decrease of intracellular GSH was observed in HSC536/N and HSC536/FANCC cells following their exposure to 100 ng/mL anti-Fas Ab. Exposure of PD149L or PD149/FANCC cells to 100 ng/mL anti-Fas Ab did not affect intracellular GSH concentrations (data not shown). Reasoning that caspases may cause a decrease in intracellular GSH levels we preincubated HSC536/N and HSC536/FANCC cells with Z-VAD-FMK, a caspase inhibitor [30]. Fig. 2 shows that irrespective of the presence of 100 ng/mL anti-Fas Ab, intracellular GSH levels were fully maintained when Z-VAD-FMK was included in the pre-incubation medium. This indicates that the decline in GSH concentration observed after Fas ligation is activated (directly or indirectly) by a member of the caspase family. Exposure of the FA cell lines to 1.0 ng/mL IFN-γ did not affect GSH concentrations. Exposure to a combination of 25–100 ng/mL anti-Fas Ab and IFN-γ resulted in

a decrease no greater than with anti-Fas Ab alone (data not shown).

To investigate whether the decrease in intracellular GSH levels in HSC536/N and HSC536/FANCC cells is accompanied by damage to the plasma membrane, the release of cytosolic LDH was determined. Following exposure to 100 ng/mL anti-Fas Ab the loss of LDH in HSC536/N and HSC536/FANCC cells was 5 ± 0.5 and $3 \pm 0.4\%$, respectively, thus indicating that plasma membrane integrity was maintained and no leakage of cytosolic LDH into the culture medium was detected.

We investigated whether loading of FA cells with ascorbic acid will blunt the decline in intracellular GSH levels and will affect intracellular concentrations of GSSG in cells exposed to anti-Fas Ab. Fig. 2 and Table 1 indicate that pre-incubation of HSC536/N and HSC536/FANCC cells with DHA significantly attenuated GSH loss while no effect on intracellular GSSG concentrations could be observed. Exposure of the cells to 1 ng/mL IFN-γ or a combination of 100 ng/mL anti-Fas Ab and IFN-γ did not affect GSSG levels (data not shown).

3.4. Activation of caspase-3 and -8 by IFN-γ and anti-Fas Ab. Effect of DHA

We investigated whether exposure to IFN-γ and anti-Fas Ab plays a role in activation of caspase-3 and -8 in the FA lymphoblast cell lines and whether pre-incubation with DHA will reduce caspase activation. Dose and time-dependent studies were performed. Fluorogenic studies performed on lysates of these cells revealed that exposure of HSC536/N cells to 10–100 ng/mL anti-Fas Ab for 120 min enhances caspase-3 activity in a dose-dependent manner (Fig. 3A). Although exposure of the cells to 1.0 ng/mL IFN-γ alone did not induce caspase-3 activation (data not shown), exposure to a combination of IFN-γ and 100 ng/mL anti-Fas Ab induced an additional activation of caspase-3 (1.2-fold increase, $P < 0.05$) compared to anti-Fas Ab alone (Fig. 3A). There is substantially more activation of caspase-3 in HSC536/N cells than in the HSC536/FANCC cells following treatment with 10–100 ng/mL anti-Fas Ab or a combination of anti-Fas Ab and IFN-γ (Fig 3A).

Fig. 3B indicates that exposure to a combination of 1.0 ng/mL IFN-γ and 100 ng/mL anti-Fas Ab strongly enhances caspase-3 activity in a time-dependent manner. The activation was detected by 15 min and increased up to 120 min (the time the experiment was terminated).

Table 1
Intracellular concentrations of GSH and GSSG in HSC536/N cells

Treatment	GSH	GSSG
HSC536/N	45.5 ± 1.9	1.56 ± 0.6
HSC536/N + DHA	45.2 ± 0.9	1.59 ± 0.7
HSC536/N + anti-Fas Ab	29.5 ± 1.5*	1.35 ± 0.4
HSC536/N + anti-Fas Ab + DHA	39.6 ± 2.0**	1.60 ± 0.8
HSC536/FANCC	43.4 ± 1.5	1.25 ± 0.5
HSC536/FANCC + DHA	43.8 ± 1.5	1.30 ± 0.6
HSC536/FANCC + anti-Fas Ab	28.6 ± 0.8*	1.10 ± 0.3
HSC536/FANCC + anti-Fas Ab + DHA	34.3 ± 1.7**	1.50 ± 0.5

HSC536/N and HSC536/FANCC cells (10×10^6 cells) were pre-incubated in the presence or absence of DHA and exposed to 100 ng/mL anti-Fas Ab for 2 hr. Amount of GSH (nmol/10×10^6 cells) and GSSG (nmol/10×10^6 cells) were determined as described in Section 2. Values are expressed as mean ± SE of six independent experiments.

* $P < 0.05$, cells exposed to anti-Fas Ab vs. cells.

** $P < 0.05$, cells exposed to anti-Fas Ab + DHA vs. cells exposed to anti-Fas Ab.

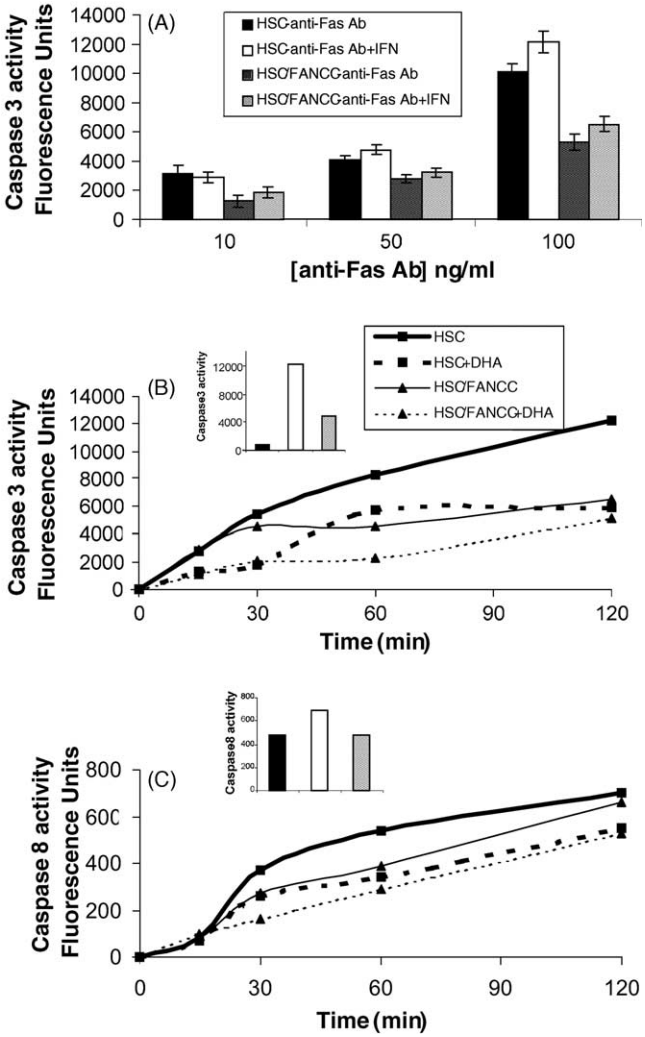

Fig. 3. Caspase-3 (A and B) and caspase-8 (C) cleavage in response to treatment with IFN-γ and anti-Fas Ab. Fluorogenic assay on lysates from HSC536/N and HSC536/FANCC cells. Dose-dependent studies (A). The cells were incubated with increasing concentrations of anti-Fas Ab or a combination of anti-Fas Ab and 1 ng/mL IFN-γ and activation of caspase-3 was determined as described in Section 2. Time-dependent studies (B and C). The cells were pre-incubated in the presence or absence of DHA, exposed to a combination of 1.0 ng/mL IFN-γ and 100 ng/mL anti-Fas Ab for various time periods and activation of the two caspases was determined as described in Section 2. Effect of caspase inhibitors: cells were pre-incubated with ac-DEVD-cho (caspase-3 inhibitor, inset in A) and with Z-IETD-FMK (caspase-8 inhibitor, inset in B) prior to a 120 min exposure to a combination of 1.0 ng/mL IFN-γ and 100 ng/mL anti-Fas Ab. (■) Cells incubated in medium, (□) cells exposed to IFN-γ and anti-Fas Ab, (▨) cells exposed to an inhibitor and IFN-γ and anti-Fas Ab. Graphic data are representative of at least four experiments.

Treatment of HSC536/N cells with ac-DEVD-cho (a caspase-3 inhibitor) prior to a 120 min exposure to IFN-γ and anti-Fas Ab inhibited caspase-3 activation (inset in Fig. 3B). IFN-γ and anti-Fas Ab treatment induced a mild activation of caspase-3 in HSC536/FANCC cells. After incubation with a combination of IFN-γ and anti-Fas Ab for 120 min the activity of caspase-3 in HSC536/N cells was 1.8-fold higher than in HSC536/FANCC cells ($P < 0.05$). Exposure of PD149L and PD149/FANCC cells to IFN-γ and anti-Fas Ab did not induce caspase-3 activation.

Fig. 3C indicates that exposure of HSC536/N and HSC536/FANCC cells to a combination of 1.0 ng/mL IFN-γ and 100 ng/mL anti-Fas Ab resulted in enhanced activation of caspase-8. Treatment of HSC536/N cells with Z-IETD-FMK (a caspase-8 inhibitor) prior to a 120 min exposure to IFN-γ and anti-Fas Ab inhibited caspase-8 activation (inset in Fig. 3C).

We analyzed the protective effect of ascorbic acid loading on ac-DEVD-AMC cleavage. Fig. 3B and C indicate a significant decrease in caspase-3 and -8 activity, respectively, in HSC536/N and HSC536/FANCC cells that were pre-incubated with DHA and exposed for 120 min to a combination of IFN-γ and anti-Fas Ab ($P < 0.05$).

Immunoblots performed on lysates of these cells demonstrate the cleaved form of caspase-3 and -8 at 60–120 min in HSC536/N and HSC536/FANCC cells (Fig. 4A and C, respectively). Pre-incubation with DHA partially protected caspase-3 and -8 activation in both cell types (Fig. 4B and D, respectively).

3.5. IFN-γ and anti-Fas Ab induce apoptosis in HSC536/N and HSC536/FANCC cells. Effect of DHA

The rate of apoptotic cells increased from 2.8 ± 0.01 to $21.2 \pm 1.4\%$ in HSC536/N cells following their exposure to a combination of 1.0 ng/mL IFN-γ and 100 ng/mL anti-Fas Ab for 120 min (as analyzed by TUNEL assay) (Fig. 5A and C). Pre-incubation with 150 μM DHA significantly ($P < 0.05$) protected apoptosis induced by the above treatment ($15.6 \pm 0.5\%$) (Fig. 5A and D). Exposure of HSC536/FANCC cells to a combination of IFN-γ and anti-Fas Ab induced apoptosis only in $13.3 \pm 0.6\%$ of cells (Fig. 5A), thus indicating that these cells are less sensitive than HSC536/N cells to the apoptotic inducing effect of IFN-γ and anti-Fas Ab. Pre-incubation of HSC536/FANCC cells with DHA did not protect against apoptosis (Fig. 5A).

3.6. IFN-γ treatment induces enhanced ROS accumulation in FA neutrophils and MNC. Effect of DHA

Having observed enhanced ROS accumulation in FA lymphoblast cells exposed to INF-γ we sought to determine the effect of IFN-γ on ROS accumulation in neutrophils

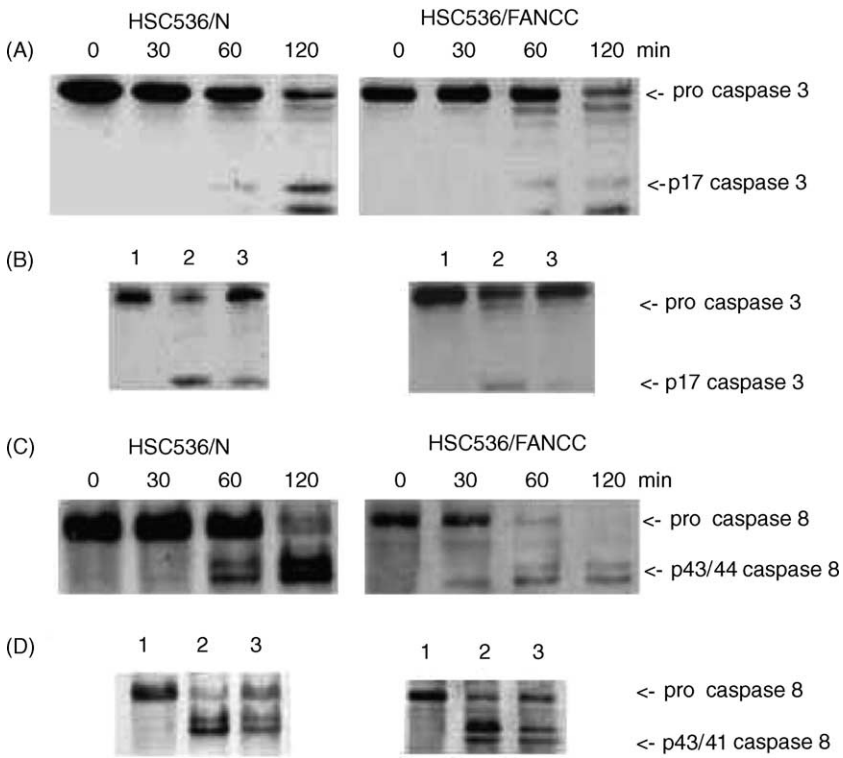

Fig. 4. Western blot analysis of caspase-3 (A) and -8 (C) activation. Time-dependent studies. HSC536/N and HSC536/FANCC cells were treated with a combination of 1.0 ng/mL IFN-γ and 100 ng/mL anti-Fas Ab for 0–120 min. Protective effect of DHA on caspase-3 (B) and -8 (D) activation. HSC536/N and HSC536/FANCC cells were pre-incubated in the absence (lanes 1 and 2) or presence (lane 3) of DHA and treated with a combination of 1.0 ng/mL IFN-γ and 100 ng/mL anti-Fas Ab for 120 min (lanes 2 and 3).

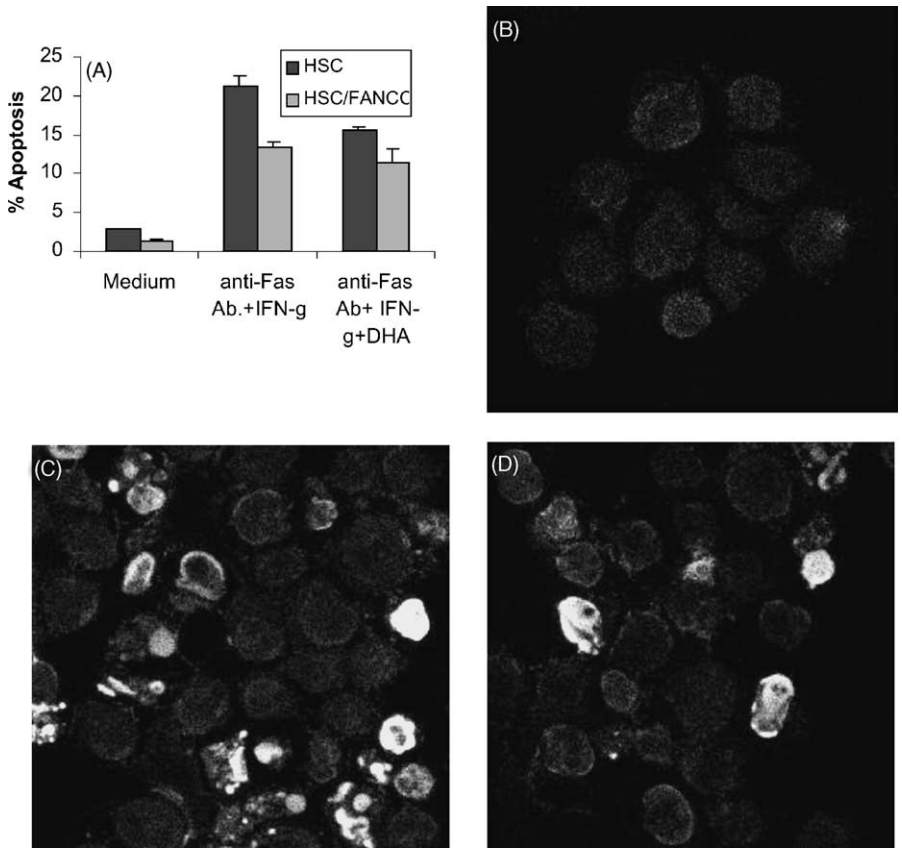

Fig. 5. Protective effect of DHA against apoptosis induced by treatment with IFN-γ and anti-Fas Ab. HSC536/N and HSC536/FANCC cells were pre-incubated in the presence or absence of DHA and exposed to a combination of 1.0 ng/mL IFN-γ and 100 ng/mL anti-Fas Ab for 120 min before cells were harvested and analyzed for apoptosis using the TUNEL assay. (A) At least 300 cells from randomly selected fields were counted and the percentage of apoptotic cells was determined using a fluorescence microscope. Data represent mean ± SE of three experiments performed in duplicate. (B–D) Morphological features of apoptosis induced by a combination of anti-Fas Ab and IFN-γ in HSC536/N cells. The cells were incubated in medium (B) or pre-incubated in buffer in the absence (C) or presence (D) of DHA before exposure to a combination of 1.0 ng/mL IFN-γ and 100 ng/mL anti-Fas Ab for 2 hr. Following performing the TUNEL assay cells were analyzed by confocal laser-scanning microscope. Magnification: 750×.

and MNC isolated from FA patients. We obtained PB from three FA patients, parallel studies were performed using PB obtained from five normal healthy volunteers. We tested the hypothesis that IFN-γ induces ROS accumulation at lower doses of IFN-γ in neutrophils and MNC of FA patients when compared to cells isolated from normal volunteers. The results described in Fig. 6A and B confirm this notion. A marked augmentation in oxidation of DCFH-DA occurred upon exposure of neutrophils (Fig. 6A) and MNC (Fig. 6B) from FA patients to low doses of IFN-γ (0.01–0.05 ng/mL). The effect was significantly greater at these low doses of IFN-γ than in cells isolated from normal volunteers ($P < 0.05$). Having observed IFN-γ hypersensitivity in neutrophils and MNC of the FA patients, we next examined whether pre-treatment with DHA will reduce ROS accumulation in these cells. The histogram shown in Fig. 6C represents neutrophils and MNC, respectively, pretreated with DHA (or buffer) for 10 min and exposed for 3 hr to 1.0 ng/mL IFN-γ. Pre-incubation of both types of cells, isolated from FA patients and from healthy volunteers, with DHA markedly reduced the augmented oxidation of DCFH-DA, induced by IFN-γ.

4. Discussion

Hematopoietic progenitor cells from children with FA-C are excessively apoptotic and hypersensitive to various extracellular cues including Fas ligand, TNF-α and double stranded DNA [10,11]. Studies have shown that IFN-γ primes cells for apoptosis after Fas ligation [11,31]. The ‘‘priming’’ effect of IFN-γ is not fully explained.

Several lines of evidence also point to an abnormality in the response of FA cells to ROS. Catalase deficiency [32] and reduced thioredoxin content (a central intracellular antioxidant) in FA cells were recently reported [33]. High intracellular levels of ROS and a disturbed oxygen metabolism were documented [32,34–37]. Thus, we investigated whether IFN-γ induced ‘‘priming’’ of the Fas ligation in FA-C cells induces an oxidative mechanism. The results presented here, performed with FA isogenic EBV-transformed cell lines (Fig. 1) and with PB neutrophils and MNC of FA patients (Fig. 6) show that IFN-γ (by itself) enhanced ROS production. Moreover, we have found that IFN-γ induces ROS accumulation at lower doses of IFN-γ in neutrophils and MNC of FA patients compared to

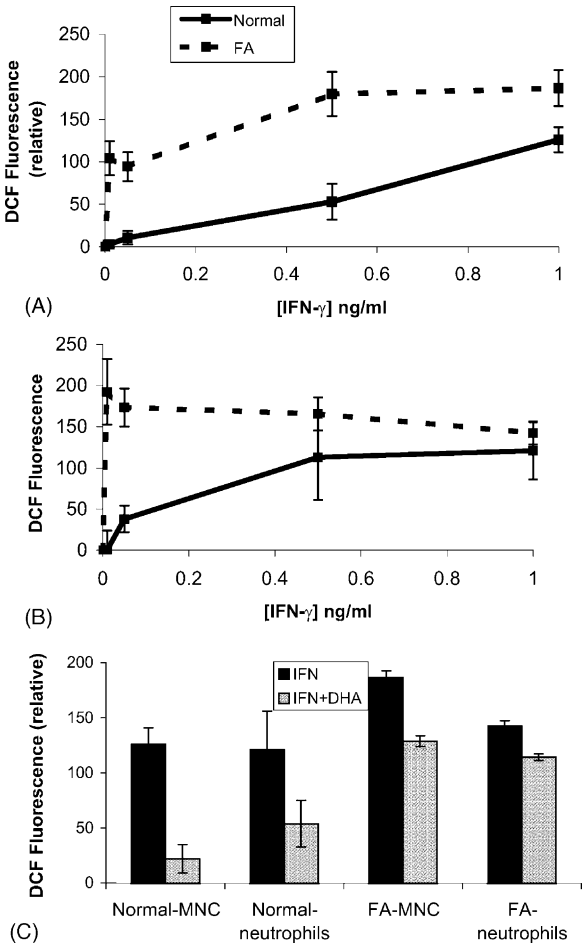

Fig. 6. Neutrophils and MNCs of FA patients are hypersensitive to IFN-γ. Effect of increasing concentrations of IFN-γ on ROS accumulation. (A) Neutrophils and (B) MNC isolated from the PB of two FA patients and four normal volunteers were incubated for 15 min with DCFH-DA, cultured in wells, 0.01–1.0 ng/mL IFN-γ was added for 3 hr and DCF fluoresce was determined. Results expressed as mean ± SE of experiments performed in triplicates. (C) Effect of DHA on ROS accumulation. The cells were pre-incubated in buffer in the presence or absence of 150 mM DHA. Following washing the cells were incubated for 15 min with DCFH-DA, cultured in wells and 1.0 ng/mL IFN-γ was added for 3 hr. ROS generation was determined.

leukocytes of normal individuals. In addition we found that HSC536/N and PD149L cells produced higher levels of ROS, than the genetically corrected counterparts, upon exposure to the same concentration of IFN-γ. HSC536/N cells were more sensitive than PD149L cells as they produced higher levels of ROS upon exposure to IFN-γ (Fig. 1). Although the two cell lines were derived from the PB cells of children with FA-C, the cells have different mutations and we speculate that the different respond of the cells might be related to the type of the mutation. Our results are in good agreement with Takeuchi and Morimoto who reported that FA cells show decreased ability to decompose H_2O_2 [32].

The present study also shows decreased levels of GSH in HSC536/N and HSC536/FANCC cells following their exposure to anti-Fas Ab while no effect on GSH levels was observed following exposure of PD149L cells to anti-Fas Ab.

Depletion of intracellular GSH from cells was found to induce apoptosis in different systems [38–41]. In a recent study Guaiquil *et al.* have shown that depletion of GSH rendered HL-60 cells sensitive to cell death induced by ROS [21]. It was suggested that cells deprived of GSH are more prone to undergo oxidative stress because their redox equilibrium is altered and their ability to scavenge various reactive oxygen intermediates is impaired [41,42]. Indeed, our study shows caspase-3 activation in HSC536/N cells exposed to anti-Fas Ab and even enhanced caspase activation and apoptosis in cells exposed to a combination of anti-Fas Ab and IFN-γ. The idea that IFN-γ requires additional factors to reveal the full apoptotic phenotype of FA cells might be explained in the following way: IFN-γ ''primes'' the cells by inducing the accumulation of increasing concentrations of ROS, but ROS alone does not induce apoptosis. Exposure of ''primed'' cells to anti-Fas Ab, reduces their capacity to protect themselves from oxidative stress (because of the decrease in GSH concentrations) thus forming an environment that promotes apoptosis. The observation that IFN-γ enhances ROS accumulation in PD149L cells while exposure to anti-Fas Ab does not decrease intracellular GSH levels nor does exposure to a combination of anti-Fas Ab and IFN-γ induce caspase-3 activation, supports our theory that IFN-γ requires additional factors to reveal the full apoptotic phenotype of FA cells.

No change in GSSG levels could be detected in HSC536/N and HSC536/FANCC cells following their exposure to IFN-γ and anti-Fas Ab. GSSG is known to be toxic to cells and is rapidly converted back to GSH by the enzyme GSH reductase (reviewed in [43]). It might be speculated that exposure of IFN-γ ''primed'' cells to anti-Fas Ab induces a temporal increase in GSSG concentrations that could not be detected by us.

Previous studies have shown that DHA reduces ROS production upon exposure HL-60 cells to oxidative stress or irradiation [21,25,26]. The present study indicates that pre-incubation with DHA results in reduced ROS production in neutrophils and MNC of FA patients and normal individuals and in the FA lymphoblast cells, exposed to IFN-γ. We have also found that pre-loading of the cells with ascorbic acid attenuated intracellular GSH loss and reduced caspase-3 and -8 activation. In agreement with Rathbun *et al.* [27] we have shown that a higher percentage of HSC536/N cells undergo apoptosis upon exposure to IFN-γ and anti-Fas Ab than cells that have been FANCC corrected. The enhanced apoptotic response observed in the former cells can be blunted by prior exposure to DHA.

Along with the findings that antioxidants exert a stabilizing influence on FA DNA and FA cells [34,35], this study raises the possibility that ascorbic acid might have therapeutic implications for FA patients.

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