Resistance to apoptosis in Fanconi's anaemia

An ex vivo study in peripheral blood mononuclear cells

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Abstract Fanconi's anaemia (FA) is a rare autosomal recessive disease characterised by progressive pancytopoenia, a diverse assortment of congenital malformations, an increased sensitivity to reactive oxygen species and a predisposition to the development of malignancies. In the present study, we assessed the propensity to undergo apoptosis of peripheral blood mononuclear cells (PBMC) from Italian FA patients. Cells were challenged by 2-deoxy-D-ribose (dRib) or TNF-α plus cycloheximide as agents that induce apoptosis by interfering with cell redox status and mitochondrial membrane potential (MMP), and PBMC from FA patients resulted to be less prone to die than those from healthy subjects. The decreased susceptibility of FA cells to undergo apoptosis was also evident when another parameter highly correlated with the apoptotic process, i.e. MMP, was measured. Moreover, when *N*-acetylcysteine was added to dRib-treated PBMC, a strong protection was evident either in PBMC from control subjects or from FA patients. These data indicate that an alteration of unknown nature of the mechanisms favouring apoptosis is present in freshly collected cells from FA patients, and that such alteration could contribute to the pathogenesis of the disease, and particularly to the increased susceptibility to cancer.

Key words: Fanconi's anaemia; Apoptosis; Mitochondria; Deoxy-D-ribose, 2-; Tumour necrosis factor-α

1. Introduction

Fanconi's anaemia (FA) is a rare autosomal recessive disease (≈1 out of 350000) characterised by pancytopoenia, excess rate of chromosomal breakages, and an increased incidence of non-lymphoid leukaemias and solid tumours [1]. More than five complementation groups have been recognised (currently classed as FA-A to FA-E) and two genes (*FAC* and *FAA*) have been located to chromosomes 9 and 16, respectively, and cloned. *FAD* gene has been located to chromosome 3 [2,3]. The fifth complementation group (FA-E) is currently regarded as a heterogeneous set of genetic subtypes [4]. FA cells are highly susceptible to DNA cross-linking agents such as mitomycin C (MMC) or diepoxybutane (DEB), resulting in increased chromosomal instability characterised by peculiar rearrangements. [5–7]. Moreover, alteration of cell cycle kinetics, such as G2 phase prolongation and arrest, as well as a disregulation of reactive oxygen species (ROS) production and/or antioxidant systems, seem to be a general feature of FA cells [8,9]. Overproduction of cytokines such as TNF-α has also been reported in FA either in vivo or in vitro [10,11]. Taking into account the intriguing relationship which exists between cell proliferation and cell death [12,13], as well as the role played by ROS and TNF-α in cancer progression and apoptosis [14–16], we thought it worthwhile to assess the propensity of FA cells to undergo apoptosis. The data presented here indicate that peripheral blood mononuclear cells (PBMC) from Italian FA patients, mostly belonging to complementation group A [17], are less prone to die when exposed to 2-deoxy-D-ribose (dRib) or TNF-α plus cycloheximide (CHX), two conditions which induce apoptosis by interfering with cell redox status and mitochondrial membrane potential (MMP) [18,19].

2. Material and methods

2.1. Patients

The diagnosis of FA was confirmed by means of DEB test [20]. A total of seven patients (one female and six males) were recruited in this study. The patients were aged 5–18 years (mean ± SD, 12.3 ± 1.6). Four of seven patients were clarified as homozygous for the *FA-A* gene, whereas two of seven patients, although being positive by DEB test, provided MMC-resistant lymphoblastoid cell lines. The haematological, genetic and clinical data of the patients are reported in Table 1. Ten normal, apparently healthy, age-matched volunteers (mean 12.7 ± 1.3) were recruited as controls. In both cases the informed consent of the parents was obtained.

2.2. Cell culture and induction of apoptosis

PBMC from FA patients and control subjects were separated by Ficoll-Hypaque sedimentation (Nycomed, Pharma AS, Oslo, Norway) and cultured at a density of 10^6 cells/ml in RPMI-1640 culture medium, containing 2 mM L-glutamine, penicillin (100 U/ml) streptomycin (100 μg/ml) and 10% heat-inactivated fetal calf serum (Gibco, Paisley, Scotland, UK). Cell suspensions were seeded into culture

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Abbreviations: FA, Fanconi's anaemia; PBMC, peripheral blood mononuclear cell(s); dRib, 2-deoxy-D-ribose; TNF-α, tumour necrosis factor-α; CHX, cycloheximide; MMP, mitochondrial membrane potential; NAC, *N*-acetylcysteine; DEB, diepoxybutane; MMC, mitomycin C; ROS, reactive oxygen species; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP nick end labeling; PI, propidium iodide; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide

This paper is dedicated to the late Antonio Pagano, the beloved son of G.P., who recently lost his final battle against FA.

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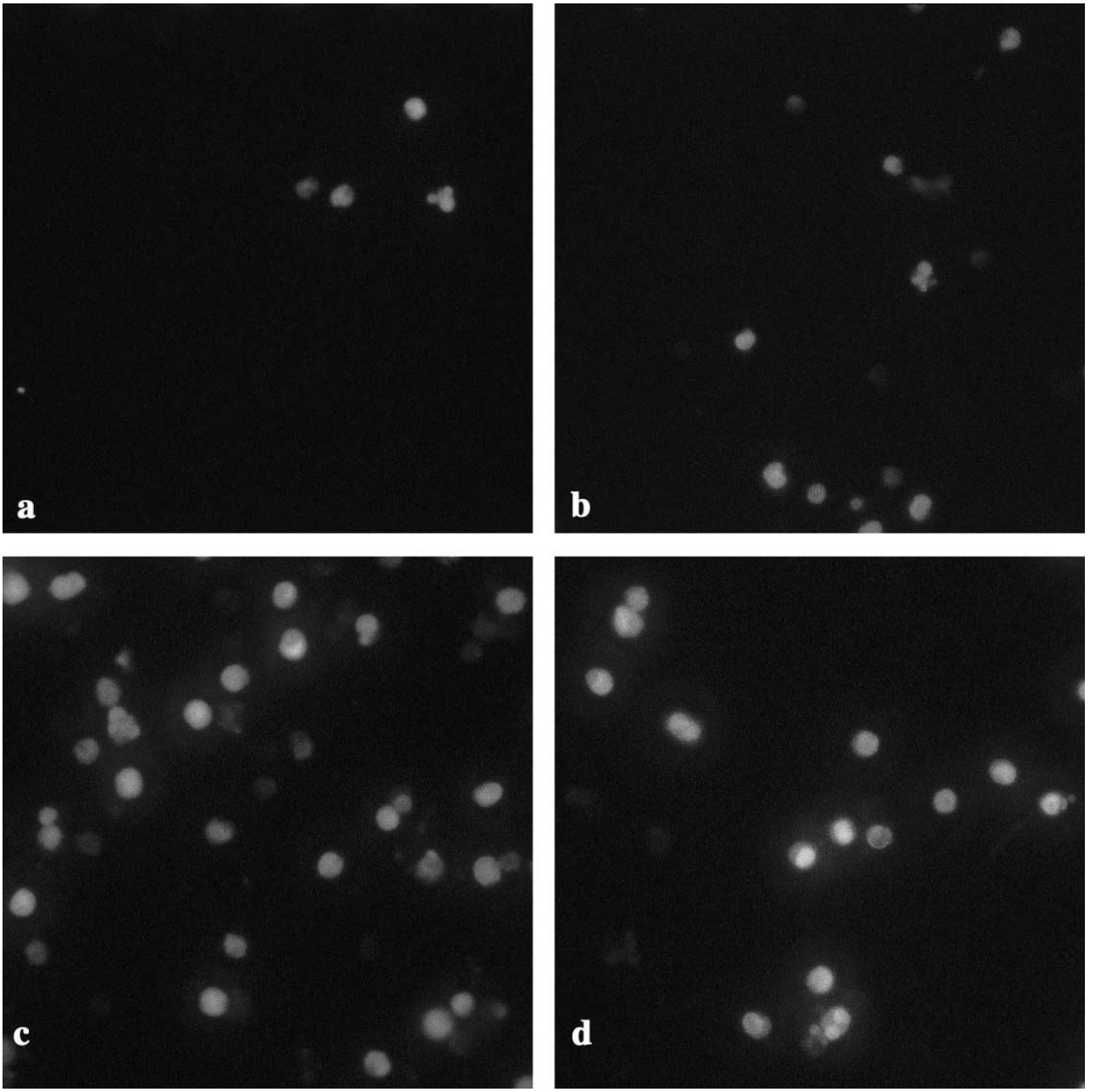

Fig. 1. In situ DNA strand break-labeling (TUNEL) for detection of apoptotic cells after 48 h of incubation with dRib, revealed by fluorescence microscopy. Only positive nuclei appear coloured. a,b: Spontaneous apoptosis in control subjects and in FA patients, respectively. c,d: dRib-induced apoptosis, in control subjects and in FA patients, respectively. Magnification: 63×.

plates in presence or absence of dRib (final concentration 10 mM) or TNF-α plus CHX (50 U/ml and 16 μM, respectively) and then incubated for 24 or 48 h at 37°C in humidified atmosphere of 5% CO_2 in air. For studies on the protective effect of *N*-acetylcysteine (Sigma Chemical Co., St. Louis, MO), PBMC were incubated with dRib plus NAC (10 mM) for 24 and 48 h. Finally, cells were collected and analysed by different assays.

2.3. Apoptosis assessment

2.3.1. TdT-mediated dUTP nick end labeling. DNA fragmentation associated with apoptosis was detected by the TdT-mediated dUTP nick end labeling (TUNEL) technique using an in situ cell death detection kit (Boehringer Mannheim, Germany). dRib-treated PBMC were harvested and fixed in freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) on slides. After permeabilisation (with 0.1% Triton X-100, 0.1% sodium citrate), slides were incubated with TUNEL mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP. Finally, slides were analysed by fluorescence microscopy (Axioscope, Zeiss, Germany). Only the apoptotic cells appeared coloured, while the non-apoptotic cells showed an absence of labelled nuclei.

2.3.2. DNA content. Apoptosis quantification was evaluated by flow cytometry as reduced fluorescence of the propidium iodide (PI) in the apoptotic nuclei, according to Nicoletti et al. [21], as previously described [19]. Briefly, the 200×*g* centrifuged cell pellet (10^6 cells) was gently resuspended in 500 μl of hypotonic fluorochrome solution (PI 50 mg/ml in 0.1% sodium citrate plus 0.1% Triton X-100 in bidistilled water; Sigma Chemical Co.). Cells were analyzed by flow cytometry analysis after a minimum of 20 min of incubation in this solution.

2.4. Mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was measured by the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1, Molecular Probes, Eugene, OR),

Table 1
Haematological, genetic and clinical data in the FA patients whose PBMC were tested for their propensity to undergo apoptosis

Patient (no.)	Hb (g/dl)	WBC ($\times 10^9$/l)	PMN (%)	Plt ($\times 10^9$/l)	% Aberrant cells[a]		Complementation group	Malformations	On-going therapies
					(spontaneous)	(DEB-induced)			
1	9.3	4.7	23	219 000	26	100	ND	Bone, skin, kidney eyes, microcephaly	Androgens, EPO, steroids, transfusions
2	13.5	4.0	27	140 000	21	85	A	None	None
3	11.6	3.1	16	29 000	18	86	A	Kidney, microcephaly	None
4	14.2	3.2	33	75 000	20	62	A	Skin	None
5	5.9	2.8	33	27 000	18	68	A	Skin	Transfusions
6	12.6	2.5	30	103 000	31	86	ND	Bone	None
7	13.2	3.2	63	67 000	10	81	MMC-res.	Bone, microcephaly	None

[a]Normal range: spontaneous, 0–11%; DEB-induced, 0–17%.

with a method that has already been applied to apoptotic cells [22,23]. Cell suspension was adjusted to a density of 0.5×10^6 cells/ml and incubated in complete medium for 10 min at room temperature in the dark with 10 μg/ml JC-1. At the end of the incubation period cells were washed twice in cold phosphate-buffered saline (PBS), resuspended in a total volume of 400 μl and analyzed.

2.5. Statistical analysis

Statistical analysis was performed by an unpaired Student's *t* test. A *P* value lower than 0.05 was considered statistically significant.

3. Results

As expected, dRib was able to induce apoptosis in PBMC from control subjects and FA patients, as assessed by TUNEL. The propensity of PBMC from FA patients to undergo apoptosis was apparently lower than that of control subjects (Fig. 1d,c). In contrast, spontaneous apoptosis was similar in the two groups (Fig. 1a,b).

Using flow cytometry, it was possible to better quantify this phenomenon and to perform a time-course study. A marked and significant difference between FA patients and control subjects was observed after 48 h of culture (Fig. 2a). The decreased susceptibility of FA cells to undergo apoptosis was also evident when another parameter, related to the apoptotic process, i.e. the decrease of MMP, was measured. Also in this case, the number of cells with depolarised mitochondria following dRib exposure was higher in control subjects than in FA cells, and this phenomenon was particularly evident after 48 h of culture (Fig. 2b). In a previous paper, we showed that the dRib-induced apoptosis and decrease of MMP were fully prevented by the addition of NAC, an agent capable of reversing the dRib-induced exhaustion of GSH intracellular pool [19], to the culture. When NAC was added to dRib-treated PBMC a strong protection was evident not only in cells from control subjects but also in cells from FA patients (Fig. 3a,b).

To test whether the lower propensity to undergo apoptosis was a general characteristic of FA cells, and taking into account the derangement of TNF-α level characteristic of the syndrome, we exposed cells from FA patients to TNF-α plus CHX, a well-known experimental model of apoptosis [18]. Owing to the shortage of cells it was possible to perform this study only in cells from several FA children. The percentage of either apoptotic cells or cells with depolarised mitochondria was lower in FA patients in comparison to control subjects, and a significant difference was observed for apoptosis at 48 h (Fig. 4a,b).

4. Discussion

In spite of the recent progress in localising and cloning some of the *FA* genes, a number of biological problems remain open, mainly concerning the role of such genes in bone marrow impairment, chromosomal instability and cancer proneness.

An alteration of the propensity to undergo apoptosis could contribute to the FA phenotype and could be related to the above-mentioned major features of the syndrome. Previous reports on apoptosis in FA provided somewhat contradictory results. Rey et al. showed that MMC induced apoptosis in lymphoblastoid cells, but the level of DNA fragmentation 48 and 96 h after MMC treatment was approximately the

same in cells from control subjects and FA patients [24]. The authors argued that the FA defect did not lead to abnormal apoptotic cell death. It is interesting to note that this conclusion was reached on the basis of results obtained using a limited number of lymphoblastoid cell lines (one belonging to complementation group A, another to complementation group D, and one unclassified). Treating cells with γ-rays, one of the several DNA-damaging agents capable of inducing apoptosis in cell culture [25], Rosselli et al. showed that 5 and 10 Gy induced significantly less apoptosis in FA lymphoblastoid cell lines belonging to groups C and D than in control cells. This finding was related to a strong reduction of p53 inducibility either by γ-irradiation or MMC treatment [26]. In the same cell lines the spontaneous frequency of apoptotic cells was slightly more elevated in FA than in normal cell lines. Recently, using eight lymphoblastoid cell lines (belonging to groups A, B, C and D) and MMC, γ- and UV irradiation as apoptotic inducers, Kruyt et al. found that FA lymphoblastoid cell lines were more responsive only when MMC was used. This result was apparently specific, being the same cell lines normally sensitive to γ- and UV-induced apoptosis. Moreover, all the MMC-treated cell lines except one exhibited a clear increase in p53 expression [27]. These discrepancies can be partially explained by the fact that in all the above-mentioned investigations a limited number of EB-virus transformed FA lymphoblastoid cell lines have been used, and no ex vivo experiments were performed on cells taken directly from patients. These considerations are particularly appropriate for studies aimed to a quantitative assessment of apoptosis, an intriguing event related to cell proliferation and transformation.

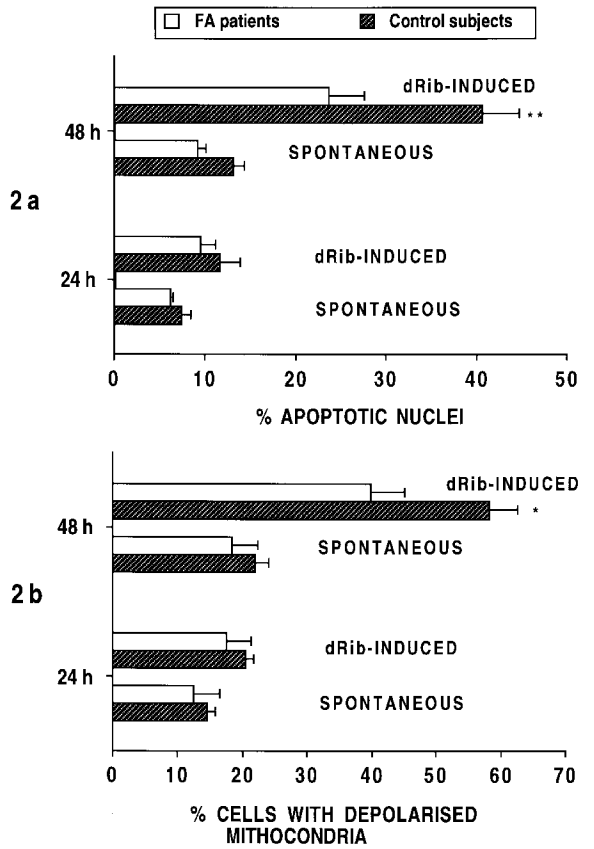

Fig. 2. Percentage of apoptotic cells (a) and cells with depolarised mitochondria (b), assessed by cytofluorimetric analysis. PBMC cultures were exposed to dRib for 24 or 48 h. A decrease in the percentage of apoptotic cells and cells with depolarised mitochondria was observed in FA cells treated with dRib at 48 h. Bars represents the mean ± SE of data concerning seven FA patients and 10 control subjects. $^{**}P < 0.01$, FA vs. control subjects.

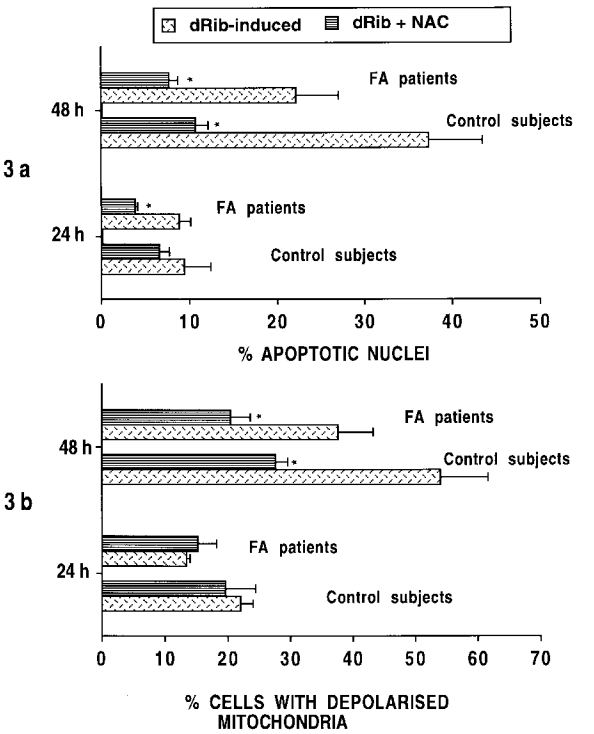

Fig. 3. Protective effect of *N*-acetyl-L-cysteine (NAC) in dRib-induced apoptosis (a) and mitochondria depolarisation (b) in PBMC from FA patients and control subjects, assessed by cytofluorimetric analysis. The protective effect of NAC on apoptosis was statistically significant only in FA cells at 24 h. A significant decrease concerning the percentage of apoptosis and of cells with depolarised mitochondria was observed at 48 h in FA patients and control subjects. Bars represents the mean ± SE of data concerning six FA patients and four controls subjects. $^{*}P < 0.05$: FA or control subjects+dRib vs. FA or control subjects+dRib+NAC.

In the present study, apoptosis has been studied in freshly drawn PBMC taken from seven FA children, four of which are known to belong to complementation group A, as well as from 10 age-matched controls. dRib and TNF-α were used as they are able to trigger apoptosis in human quiescent PBMC by inducing oxidative stress [19,28], thus mimicking a situation likely to occur in vivo in FA patients [9]. Moreover, a target of dRib and TNF-α is the mitochondrion, an organelle critical for the occurrence of apoptosis [22,29,30]. In these experimental conditions, we found that spontaneous apoptosis was similar in FA and control cells, while dRib- and TNF-α-induced apoptosis was slightly but significantly reduced in FA cells. This conclusion is reinforced by the results obtained by studying the MMP, a parameter directly correlated to apoptosis in a variety of experimental systems including dRib and TNF-α induced apoptosis [29,31].

On the whole, our data indicate that freshly collected PBMC from FA patients are relatively resistant to the induction of apoptosis by agents that interfere with the redox status of the cell. How can this finding be reconciled with the data suggesting that FA cells are more sensitive to oxidants and are

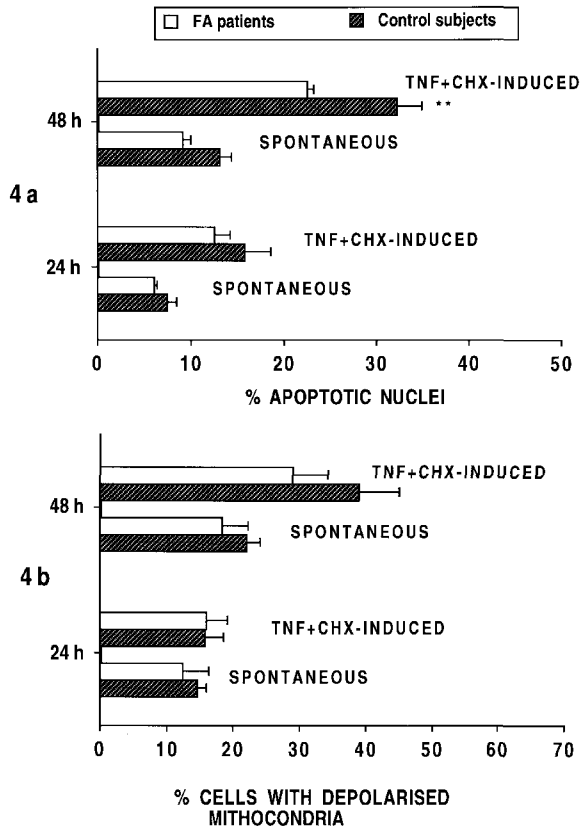

Fig. 4. Percentage of apoptotic cells (a) and cells with depolarised mitochondria (b), assessed by cytofluorimetric analysis. PBMC cultures were exposed to TNF-α+CHX for 24 or 48 h. A decrease in the percentage of apoptotic cells and of cells with depolarised mitochondria was observed in FA cells treated with TNF-α+CHX at 48 h. Bars represents the mean ± SE of data concerning four FA patients and three control subjects. $^{**}P<0.01$: FA vs. control subjects.

defective in antioxidant defence? Further studies are needed on this topic. A possible explanation is that the continuous in vivo exposure to oxidative stress can paradoxically make the cells more tolerant and resistant by inducing some antioxidant enzymes [32,33]. In particular, it has been shown that TNF-α is capable of inducing mitochondrial MnSOD, and thus protecting cells from cytotoxicity [34]. In conclusion, we suggest that an alteration of the mechanisms which favour apoptosis may contribute to the pathogenesis of FA, and particularly may be related to cancer proneness.

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