Bax expression and apoptotic cell death in Fanconi anaemia peripheral blood lymphocytes

G. A. Baruque^{*,†}, M. A. Bitencourt[‡], R. Pasquini[‡],

M. T. L. Castelo-Branco[§], J. C. Llerena Jr.^{†,¶} and V. M. Rumjanek^{*}

**Instituto de Bioquímica Médica/CCS – UFRJ, †Instituto de Biofísica Carlos Chagas Filho/CCS – UFRJ, ‡Serviço de Transplante de Medula Óssea/Hospital de Clínicas – UFPR, §Departamento de Histologia e Embriologia/ICB-CCS/UFRJ, ¶Departamento de Genética Médica/Instituto Fernandes Figueira – FIOCRUZ/RJ, Brazil*

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Abstract. *Objective:* Deregulated apoptosis might be involved in some of the features of Fanconi anaemia (FA). The possibility that the pro-apoptotic Bax protein could be involved in an increased susceptibility to apoptosis in FA patients was investigated. *Materials and methods:* Intracellular Bax expression, Bcl-2 expression (an anti-apoptotic protein) and cell death were analysed in 26 FA peripheral blood lymphocyte samples. *Results:* Most FA samples (69%) displayed increased levels of Bax and were more susceptible to both spontaneous apoptosis and mitogen activation-induced cell death. Two subgroups were identified: one presented elevated levels of Bax ($n = 18$), whereas the other ($n = 8$), had Bax levels lower than controls. Two subgroups based on Bcl-2 expression were also identified: one with normal and another with high Bcl-2 expression. No inverse correlation was found between Bcl-2 levels and Bax expression. A clear difference in susceptibility to induced cell death could be observed between control and FA samples. The best correlation was observed between high levels of Bax and mitogen-induced apoptosis of cells; these displayed characteristics of necrosis secondary to apoptosis, suggesting that the intrinsic apoptotic pathway was being activated. *Conclusion:* Despite increased susceptibility to cell death induction, there was no correlation between Bax levels, chromosome breakage, haematological parameters or androgen therapy. The importance of apoptosis and Bax expression in the clinical development of FA awaits clarification.

INTRODUCTION

The clinical course of Fanconi anaemia (FA) includes haematological complications that culminate in bone marrow failure and susceptibility to development of cancer (D'Andrea & Grompe 1997). It has been suggested that these bone marrow abnormalities derive, at least in part, from excessive apoptosis in committed progenitor cells (Pang *et al.* 2001). However, decreased

susceptibility to undergo apoptosis has been described in the peripheral blood of FA patients, mainly belonging to complementation group A (Monti *et al.* 1997). Mutations in the gene characteristic of complementation group A accounts for 65% of all cases of the disease, and in Brazil 30% of the FA patients studied present the 3788–3790 *del* mutation (Magdalena *et al.* 2005). Individual FA proteins interact in many pathways other than their better known role in DNA repair (Tischkowitz & Dokal 2004). Among FA proteins, it has been reported that the protein Fanconi anaemia complementation group C has an anti-apoptotic function (Cumming *et al.* 1996), as well as that of Fanconi anaemia complementation group D2 (Liu *et al.* 2003). On the other hand, classical anti-apoptotic proteins of the Bcl-2 family, such as Bcl-2 and Bcl-X_L, have not been found to be greatly modified in FA cells (Kruyt *et al.* 1996; Ferrer *et al.* 2003). Despite the apparent resistance to apoptosis, an increased susceptibility to cross-linking agents is a characteristic of FA cells (Ishida & Buchwald 1982; Auerbach *et al.* 1989), and it has been reported that this hypersensitivity is associated to a mechanism of cell death involving both necrotic and apoptotic features (Ferrer *et al.* 2003). Furthermore, the pro-apoptotic molecule Bax has been evaluated in FA lymphoblastoid cells and a 18-kDa Bax variant was found to be clearly associated to cisplatin sensitivity (Ferrer *et al.* 2003). Observations by our group (Baruque *et al.* 2005) have suggested that Bax expression in FA cells is more indicative of apoptotic susceptibility than expression of the death receptor Fas. We have thus studied the possibility that Bax could be involved in an increased susceptibility to apoptosis in FA patients' cells. Intracellular expression of Bax and the anti-apoptotic molecule Bcl-2, as well as apoptotic levels, and a possible correlation with chromosome breakage, were analysed in 26 FA peripheral blood lymphocyte samples.

MATERIALS AND METHODS

Patients and peripheral blood mononuclear cell samples

Peripheral blood mononuclear cell (PBMC) samples were obtained from 26 FA patients from the Hospital de Clínicas, Curitiba, Brazil (Table 1) after FA diagnosis (Auerbach *et al.* 1989). PBMCs of 14 normal controls were obtained from healthy volunteers; this study was approved by the local ethical committee. At the time of sampling, some of the FA patients were undergoing androgen therapy. In all cases, cells were separated by Ficoll-Hypaque gradient, were washed and resuspended in Roswell Park Memorial Institute culture medium (Sigma, St. Louis, MO, USA), containing 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 µg/mL) and 10% heat-inactivated foetal calf serum (Gibco, Grand Island, NY, USA). Previous erythrocyte elimination had been performed by treatment with Hespan (1 : 8 mL of peripheral blood, Sigma) to discard contamination by haemolytic samples, which is frequently observed in FA samples.

Diepoxybutane sensitivity assay

Increased chromosomal breakage and radial formation of FA cells were analysed by culturing peripheral blood in the presence of phytohaemagglutinin with or without 0.1 µg/mL of diepoxybutane. Samples were coded, and metaphase cells were scored blind before analysis for chromosomal breakage, including the formation of radials. A chromosome breakage index was obtained by dividing the number of breaks per cell over the number of cells with alterations. FA results were compared to those from healthy controls run in parallel (normal patients range: 0.00–0.10; Fanconi anaemia patients range: 1.06–23.9).

Table 1. Laboratory data of patients with Fanconi anaemia

Patients	Age (years)	Chromosome breakage index	Erythrocytes ($\times 10^6/\text{mm}^3$)	Leucocytes ($\times 10^3/\text{mm}^3$)	Lymphocytes ($\times 10^3/\text{mm}^3$)	Platelets ($\times 10^3/\text{mm}^3$)	Androgen therapy
1	9	2.64	2.19	3.43	2.74	37	2
2	13	2.40	2.12	2.41	1.28	19	1
3	12	2.92	2.34	2.87	1.78	40	2
4	9	2.76	2.93	4.23	0.63	57	1
5	10	2.88	2.25	1.71	1.23	60	2
6	7	2.56	3.07	4.25	2.25	52	2
7	29	1.44	3.06	3.92	1.22	38	2
8	5	*	4.05	9.66	3.96	110	2
9	5	5.44	2.86	3.2	2.56	10	1
10	9	2.16	*	*	*	*	2
11	12	3.36	3.06	10.5	1.41	26	1
12	11	4.20	3.05	1.39	1.11	10	2
13	6	2.64	2.23	2.45	1.72	24	2
14	4	1.84	2.62	5.44	1.69	24	2
15	12	2.40	1.65	3.75	2.33	21	2
16	18	3.08	2.53	1.89	1	32	1
17	7	1.56	2.03	2.15	1.51	*	1
18	12	2.00	2.74	1.57	1.08	2	2
19	3	12.76	4	7.93	5.15	144	2
20	8	3.96	0.91	2.39	0.72	16	*
21	7	3.52	1.71	3.01	2.11	23	*
22	15	5.92	3.29	2.23	1.09	29	1
23	22	3.33	*	*	*	*	2
24	12	1.46	2.31	3.51	2.6	37	2
25	18	2.4	*	*	*	*	2
26	17	8.42	3.7	5.12	2.2	74	2

*Data not available; 1, receiving androgen therapy; 2, not receiving androgen therapy.

Staining for Bax and Bcl-2

Samples containing 5×10^5 – 1×10^6 cells were washed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde and were permeabilized for 60 min at 4 °C with 0.1% saponin in PBS containing 0.1% sodium azide plus 10% foetal calf serum and 10% human serum. Cells were then washed in saponin solution and were incubated for 30 min at 4 °C with 20 µg/mL anti-Bax monoclonal antibody, 21 kDa isoform (rabbit anti-human Bax, DAKO, Carpinteria, CA, USA), or 20 µg/mL anti-Bcl-2 monoclonal antibody (mouse anti-human-Bcl-2, Pharmingen, San Diego, CA, USA). The cells were washed in 0.1% saponin in PBS containing 0.1% sodium azide and were incubated for 30 min at 4 °C, with the secondary antibody swine anti-rabbit IgG-fluorescein isothiocyanate (FITC) (1 : 100 v/v) (DAKO) for Bax, and with goat anti-mouse IgG-FITC (1 : 100 v/v) (Sigma) for Bcl-2 staining, respectively. Stained samples were then analysed using flow cytometry. Results were expressed as the difference between the median fluorescence intensity (MFI) of cells stained with specific antibody plus secondary antibody and the MFI obtained subjected to secondary antibody (Δ MFI), only.

Cell culture and activation induction

Patients' blood samples were obtained in Curitiba and were immediately sent by mail, at room temperature, to Rio de Janeiro where they were processed and put into culture 24 h after

collection. A similar procedure was made with regard to the samples from healthy individuals. Then, samples containing 5×10^5 cells were cultured in 96-well plates for 20 h with or without phytohaemagglutinin (5 µg/mL) (Sigma) in Roswell Park Memorial Institute medium containing 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 µg/mL) and 10% heat-inactivated foetal calf serum (Gibco). Cells were then kept at 37 °C in atmosphere of 5% CO₂.

Assessment of apoptosis

Cell suspensions were stained 20 h after being cultured in the presence of phytohaemagglutinin (for activation-induced cell death – AICD) or culture medium (control) with Annexin V-FITC conjugate using the TACS™ Annexin V-FITC apoptosis detection kit (R & D Systems, Minneapolis, MN, USA). Stained cells were then analysed by flow cytometry.

Flow cytometric analysis

Fluorescence data were acquired on a FACScalibur Flow Cytometer (Becton & Dickinson, San Jose, CA, USA). Data on a minimum of 10^4 cells were analysed using Winmdi 2.8 software (Joseph Trotter, San Diego, CA, USA). For all measurements a gate was defined for the lymphocyte population only.

Statistical analysis

Data were analysed by Kruskal–Wallis and Dunnett T3 non-parametric tests with Spearman's rho comparisons test using SPSS 10.0 software for Windows (Chicago, IL, USA). A *P*-value of < 0.05 was considered to indicate a statistically significant difference.

RESULTS

Intracellular Bax expression was analysed in lymphocytes of 26 FA patients and 14 control samples. It was possible to observe differences in Bax expression between FA and control samples (Fig. 1b). FA patients could be divided into two groups of low (31%) ($P < 0.006$) and high (69%) ($P < 0.000$) Bax expression when compared to control samples. Statistically, the two groups were also different from each other ($P < 0.000$). No correlation between chromosome breakage and Bax expression was observed (Fig. 1c).

Generally, it is accepted that an inverse correlation between Bax levels and Bcl-2 levels in a cell, would tend to determine the degree of its susceptibility to apoptosis induction. However, in the present work, no such correlation was observed (Fig. 2). No inverse correlation between Bcl-2 and cell death was observed (Table 2), nor a correlation between Bcl-2 and chromosome breakage (data not shown).

Our previous work (Baruque *et al.* 2005) demonstrated increased cell death susceptibility among lymphocytes from FA samples. As expected, here a difference between FA and control samples was observed when total cell death was measured as a result of both spontaneous ($P < 0.016$) and activation-induced ($P < 0.000$) cell death (Fig. 3a,b).

Cell death was then analysed as the percentage of Annexin⁺ cells – ‘pure’ apoptosis (Fig. 3c,d), the percentage of cells incorporating propidium iodide (PI) – ‘pure’ necrosis (Fig. 3e,f), and the percentage of cells that were Annexin⁺ and at the same time incorporated PI – necrosis secondary to apoptosis (Fig. 3g,h). The percentage of FA cells presenting such characteristics was significantly different from controls both in spontaneous apoptosis ($P < 0.000$) and mitogen-induced apoptosis ($P < 0.000$). No significant difference was found when ‘pure’ necrotic cells were

Figure 1. Intracellular Bax expression in permeabilized lymphocytes from Fanconi anaemia (FA) patients and control samples. (a) Dot plot represents an example of a gated FA lymphocyte population and, in the histogram on the right hand side, lines correspond to auto-fluorescence control (grey line), secondary antibody control (black line) and anti-Bax stained lymphocytes (filled grey histogram). (b) Scatter plots represent the median of Δ MFI $\pm$ inter-quartile range of data concerning 26 FA patients and 14 control subjects. FA patients were separated based on levels of Bax expression, high ($n = 18$, $P < 0.006$) and low ($n = 8$, $P < 0.000$). Δ MFI represents median fluorescence intensity (MFI) of lymphocytes, stained with both anti-Bax antibody and FITC secondary antibody and FITC secondary antibody only. (c) Scatter plot represents the correlation between Bax expression and chromosome breakage in FA samples ($n = 25$).

Table 2. Bcl-2 levels and cell death

	Spontaneous*	AICD*
Bcl-2 ^{high}	4 (7)†	6 (7)†
Bcl-2 ^{normal}	11 (15)†	15 (15)†

*Spontaneous or activation-induced cell death (AICD) above control levels.

†Number of samples.

Figure 2. Intracellular Bcl-2 and Bax expression in permeabilized lymphocytes from Fanconi anaemia (FA) patients and control samples. (a) Scatter plots represent the median of $\Delta\text{MFI} \pm$ inter-quartile range of data. (b) Scatter plot of the correlation between Bcl-2 and Bax expression in control samples ($n = 12$). (c) Scatter plot of the correlation between Bcl-2 and Bax expression in FA samples ($n = 26$). ΔMFI represents median fluorescence intensity (MFI) of lymphocytes, stained with both primary and secondary antibodies minus MFI of cells stained just with FITC secondary antibody alone.

analysed. However, when 'pure' apoptotic cells were analysed a significant difference was observed when mitogen-induced cell death was studied ($P < 0.019$).

When necrosis secondary to apoptosis was studied in relation to the levels of Bax expression, spontaneous cell death was increased independent of the levels of Bax exhibited (Fig. 4a); a difference was only observed in mitogen-induced cell death. In this case, 93% of Bax high samples displayed cell death above control levels compared to 57% of Bax low (Fig. 4b). However, no correlation was found among the different types of cell death and chromosome breakage (data not shown).

Figure 3. Percentage of cell death in 24 h lymphocyte cultures from 26 FA patients and 14 control samples. a, c, e and g – Spontaneous cell death. b, d, f and h – activation-induced cell death. Scatter plots represent: (a) and (b) percentage of total cell death; (c) and (d) percentage of ‘pure’ apoptotic cells; (e) and (f) percentage of ‘pure’ necrotic cells; (g) and (h) the percentage of cells in secondary necrosis (double positive cells PI⁺ and Annexin⁺).

Figure 4. Percentage of secondary necrosis in the Bax subgroups (high and low). Scatter plots represent: (a) spontaneous cell death; (b) activation-induced cell death. Ctr = control samples ($n = 14$); Bax^{high} ($n = 15$), Bax^{low} ($n = 7$).

DISCUSSION

Despite suggestions that deregulated apoptosis might be involved in some of the features of FA patients, and that a variant of Bax (18 kDa) is increased and involved in cell death caused by cisplatin hypersensitivity in FA lymphoblastoid cells transfected by Epstein-Barr virus-episomal vectors expressing Fanconi anaemia complementation group A and Fanconi anaemia complementation group C (Ferrer *et al.* 2003), expression of the pro-apoptotic Bax protein had not been studied in samples of patients with the disease. In the present study, Bax expression was examined in 26 lymphocyte samples of FA patients and found to be elevated in 69% of them. These results differ from those described by Ferrer *et al.* (2003) in lymphoblasts where no increased expression of the 21 kDa Bax isoform was observed.

A clear difference in the susceptibility to apoptosis could also be observed between control and FA cell samples, independent of them being spontaneous apoptosis or mitogen-induced apoptosis. However, a mixture of necrotic and apoptotic features involving cell death triggered by cisplatin in FA lymphoblasts has been previously reported (Ferrer *et al.* 2003). In the present study, cell death was analysed as the percentage of Annexin⁺ cells – ‘pure’ apoptosis, the percentage of cells incorporating PI – ‘pure’ necrosis, and the percentage of cells presenting both features, that is cells that at the same time were Annexin⁺ and incorporated PI. A correlation between increased apoptosis of FA samples was seen when both features were considered. Similarly, a correlation between high levels of Bax and cell death was observed in cells with both characteristics when mitogen-induced apoptosis was analysed. It has been described that the incorporation of PI during AICD is time-dependent reaching a plateau at 18 h (Wesselborg & Kabelitz 1993). Our analyses were performed 20 h after AICD induction, and this was probably the reason that cells presenting both features (Annexin⁺ and PI⁺) were more representative than cells presenting Annexin⁺ alone.

No inverse correlation was seen between Bcl-2 and Bax expression, nor between cell death and Bcl-2 expression. Furthermore, none of the features studied correlated with chromosome breakage tests.

When FA haematopoietic cells were studied they differed from normal haematopoietic cells in relation to their apoptotic phenotype showing a reduced apoptotic threshold and blunted

survival signalling pathways (Fagerlie *et al.* 2001). It has been proposed (Fagerlie & Bagby 2006) that lymphocytes from FA patients may affect the pathogenesis of the disease in at least two manners: hyperactive CD8 lymphocytes from FA patients produce increased levels of tumor necrosis factor and interferon leading to a suppressive microenvironment for FA haematopoietic cells that are more sensitive to apoptotic cues, leading to bone marrow failure. Conversely, some FA patients develop tumours and this may be the result of hypoactive CD4 lymphocytes or natural killer cells from FA patients that fail in their surveillance role, allowing cells with chromosomal instability to survive leading to leukaemogenesis and carcinogenesis.

It is not clear whether this diminished activity is a result of activation-induced cell death in FA lymphocytes. Our results, obtained with samples from 26 FA patients, confirm that lymphocytes of the majority of FA patients are more susceptible to cell death, especially activation-induced, that the death process has features of both necrosis and apoptosis, and that this susceptibility is associated with increased 21 kDa Bax expression. Two apoptotic pathways are clearly defined: the intrinsic pathway, involving the disruption of mitochondrial membrane potential, where Bax plays a pro-apoptotic role, and the extrinsic pathway that depends on the activation of death receptors (Green & Reed 1998; Parone *et al.* 2002). The results presented here suggest that the mitochondrial pathway is involved in the majority of FA samples, which is in agreement with what has been suggested before in relation to cisplatin susceptibility (Ferrer *et al.* 2003). Furthermore, our previous work (Baruque *et al.* 2005) showed that Bax was a better indicator than the Fas receptor in FA patients. Despite this apparent increased susceptibility of peripheral lymphocytes to apoptotic induction, no correlation could be observed between Bax levels and the various haematological parameters or androgen therapy. The importance of apoptosis and Bax expression in the clinical development of Fanconi anaemia is still unclear.

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