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Fanconi Anemia Is Characterized by Delayed Repair Kinetics of DNA Double-Strand Breaks

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Among patients with bone marrow failure (BMF) syndrome, some are happened to have underlying Fanconi anemia (FA), a genetically heterogeneous disease, which is characterized by progressive pancytopenia and cancer susceptibility. Due to heterogeneous nature of the disease, a single genetic test, as in vitro response to DNA cross-linking agents, usually is not enough to make correct diagnosis. The aim of this study was to evaluate whether measuring repair kinetics of radiation-induced DNA double-strand breaks (DSBs) can distinguish Fanconi anemia from other BMF patients. An early step in repair of DSBs is phosphorylation of the histone H2AX, generating γ-H2AX histone, which extends over mega base-pair regions of DNA from the break site and is visualised as foci (γ-H2AX foci) with specific antibodies. The primary fibroblasts, established from FA patients, were exposed to γ-rays, a dose of 2 Gy (^{60}Co), incubated for up to 24 hours under repair-permissive conditions, and assayed for the level of γ-H2AX foci and apoptosis at different recovery times after the treatment. Cell lines originating from FA patients displayed a significant delay in the repair of radiation-induced DNA DSBs relative to non-FA bone marrow failure (non-FA BMF) and control cell lines. The delay is especially evident at recovery time of 24 hours, and is seen as about 8-fold increase of residual γ-H2AX foci compared to self-state before irradiation. The delay in repair kinetics of FA cells represents the unique feature of FA cellular phenotype, which should be exploited to distinguish FA cellular phenotype.

Keywords: double-strand breaks; repair kinetics; γ-H2AX foci; apoptosis; irradiation
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Fanconi anemia (FA) is a rare recessive disorder characterized by diverse developmental abnormalities, progressive bone marrow failure (BMF) and predisposition to both hematological malignancies and solid tumors (Okuyama and Mishina 1987). The hallmark of the FA cellular phenotype is sensitivity to DNA cross-linking agents such as mitomycin C (Sasaki and Tonomura 1973) and diepoxybutane (DEB) (Auerbach and Wolman 1976; Auerbach et al. 2003). However, results obtained after exposure of FA cells to ionizing radiation are not uniform. For example, cells from patients that belong to complementation groups FANC-A, FANC-C and FANC-F show defects in rejoining DNA double-strand breaks (DSBs), but surprisingly the cellular radiosensitivity is normal (Sasaki and Tonomura 1973; Natarajan et al. 1984; Casado et al. 2005). By contrast, some reports claim that FA cells are radiosensitive, and these claims are supported by observations of clinical radiosensitivity after preconditioning for bone marrow transplantation (Gluckman et al. 1983; Gluckman et al. 1990; Marcou et al. 2001).

Although laboratory analysis of chromosomal aberrations induced by DEB or other crosslinking agents provides a unique diagnostic marker, accurate diagnosis of FA can be difficult. As a result, diagnostic strategies combining different tests, in addition to the above chromosomal aberration test, are needed. A recent study by Pinto and coworkers (Pinto et al. 2009) suggests the flow cytometry–based mytomycin C sensitivity test as a reliable alternative method to evaluate FA phenotype in fibroblasts. Assuming that Fanconi anemia belongs to human disorders that are conferred by defects in proteins that function in response to DNA DSBs, a newly developed method, gamma phosphorylation assay is used to study response and sensitivity to ionizing radiation (Kinner et al. 2008). Phosphorylation of histone H2AX is one of the first steps in DNA DSBs repair. Within minutes of the induction of DNA DSBs, histones H2AX become phosphorylated at serine 139 and form γ-H2AX, which extend over mega base-pair regions and can be readily visualised as foci (γ-H2AX foci) using antibodies specific for phosphorylated H2AX. The γ-H2AX-foci are

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an indication of unrepaired DNA double-strand breaks (Rogakou et al. 1998; Fernandez-Capetillo et al. 2003) γ-H2AX foci are essential for the efficient evaluation of the DNA repair kinetics.

In this study we wanted to find out whether measuring repair kinetics of DNA DSBs using γ-H2AX assay (Kinner et al. 2008) can distinguish FA from other BMF patients, and to investigate possibility of its employment as predictive test of cellular radiosensitivity *in vitro*. We used ionizing radiation and DEB to induce DNA damage and monitored DNA DSBs repair kinetics. Interestingly, our results revealed that cells from FA patients show delayed repair kinetics after exposure to ionizing radiation, but not DEB, suggesting that repair kinetics of ionizing radiation induced DSBs can be used to distinguish FA from non-FA BMF patients and should be considered as a specific characteristic of FA cellular phenotype.

Methods

Subjects

From January 2006 to January 2008, 27 consecutive patients and their parents visited Institute for Mother and Child Care in Belgrade. At least one medical appointment with complete history and physical examination were performed by FA-experienced physician and data recorded. Patients included in study were children with progressive bone marrow failure, without full clinical picture of FA or any evidence of underlying etiology and diagnosis was based on commonly seen findings and subjective impression of the physician. The children (10 boys and 17 girls) were aged 8 ± 6 years on the average. Various hematological parameters including Hb [median (range) 8.6 (2.1-14.9) g/dL], WBC [median (range) 3.0 (0.2-9.2) × 10^9/L] and neutrophils [median (range) 0.6 (0.03-5.8) ×10^9/L] clearly indicated BMF patients. Only one patient was presented with growth retardation, radial ray aplasia and renal abnormalities. All the others had no major congenital anomalies.

Peripheral bloods from 27 patients and their parents (mainly mothers) were collected. To confirm FA cellular phenotype, baseline incidence of chromosomal aberrations and classic chromosomal breakage DEB test on peripheral blood lymphocytes have been used as first tests (Auerbach et al. 2003) in both, children and their parents. The results of DEB sensitivity showed that 10 patients out of 27 were DEB positive (four boys and six girls) whereas only in one subject in parent group chromosomal aberrations were found. From DEB positive patients fragments of skin were obtained with a minimally invasive 3mm punch using standard technique, and skin fibroblasts were cultured. Harvested fibroblasts were also DEB tested and chromosome aberrations were recorded according to criteria of Auerbach et al. (2003). Control cell lines were obtained from skin biopsies of healthy volunteers undergoing plastic surgery. Methods that are further employed (γ-H2AX and apoptosis assays) were approved by Ethical Committee, and parents signed an informed consent regarding this investigation.

Tissue culture and chromosomal aberrations analysis

For all experiments described here, exclusively cell lines in early passages (passage number 4) were used. The DEB test in fibroblasts confirmed 6 DEB positive patients, who were further considered as FA patients, while 4 were DEB negative and were further considered as non-FA BMF patients. The complementation group analysis (Universität Würzburg, Institut für Humangenetik, Germany) showed that one of the FA patients belonged to the FANC-D2 complementation group and the remaining five patients belonged to the FANC-A complementation group. Control primary fibroblasts were taken from six healthy volunteers undergoing plastic surgery. Each fibroblast culture grew successfully and a total of 16 primary fibroblast cell lines have been established. All cell lines were grown under standard tissue culture conditions in DMEM (Gibco, Invitrogen Ltd., Paisley, UK) supplemented with 10% of fetal bovine serum (Gibco, Invitrogen Ltd., Paisley, UK) at 37°C and in the atmosphere of 10% CO_2. For immunostaining, exponentially growing cells were seeded on polylysine glass slides (Sigma-Aldrich Co., Steinheim, Germany) and allowed to attach to the slide surface for 24 h before treatment with ionizing radiation or DEB.

Irradiation and DEB treatment

Cells were irradiated using ^{60}Co γ-ray source (dose of 2.0 Gy at a dose-rate 0.45 Gy/min). The dimensions of the radiation field were 20 x 20 cm and the distance from the source was 74 cm. After irradiation, cells were returned to the tissue culture incubator. The repair kinetics of DNA double-strand breaks were measured before (untreated) and at different times after exposure to ionizing radiation (30 minutes, 2 hours, 5 hours and 24 hours) using γ-H2AX assay (see below).

Duplicate seeded polylysine coated glass-slides were treated with DEB (0.1 μg/ml) according to method of Auerbach (Auerbach et al. 2003) and examined at the same recovery periods as irradiated cells using γ-H2AX assay.

γ-H2AX assay

At various time points after the treatment, the cells were fixed in a 4% formaldehyde (AppliChem GmbH, Darmstadt, Germany), permeabilised with 0.2% triton X-100 (Sigma-Aldrich Co., Steinheim, Germany) and stained with the H2AX primary antibody (Upstate, Cell Signalling Solutions, Lake Placid, NY, USA) and a FITC-labeled secondary antibody (DAKO Ltd., Cambridgeshire, UK). The slides were mounted with a 4´, 6´-diamidino-2-phenylindole (DAPI)-containing antifade solution (Vector Laboratories Inc., Burlingame, CA, USA), covered with coverslips and sealed. Foci positive for γ-H2AX were counted using an epifluorescent Axiomager A1 microscope (Carl Zeiss, Germany) and the computer software Image J.

Apoptosis and cell cycle analysis

For apoptosis assay, at each time point after irradiation and/or DEB treatment, cells were detached from flasks surface with 0.025M trypsin-EDTA (Gibco, Invitrogen Ltd., Paisley, UK), washed with pre-warmed PBS (Bioatlas, Tartu, Estonia) at 37°C and fixed in 96% ethanol. Apoptosis was assessed by flow cytometric (Becton Dickinson, Heidelberg, Germany) identification of cells displaying apoptosis associated DNA condensation. DNA content was assessed by measuring the UV fluorescence of propidium iodide-stained DNA. Apoptotic population and cell cycle analysis was performed using CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).

Results

Baseline incidence of chromosome aberration in blood samples from all patients ranged from 0 to 0.27 breakages per cell. The results of DEB sensitivity revealed that only

Table 1. Spontaneous and DEB induced chromosomal aberrations (CAs) in lymphocyte cultures established from 10 BMF patients.

Patients	Spontaneous CAs/cell	Frequencies of DEB induced CAs/cell
1	0.07	0.34
2	0.05	0.45
3	0.23	0.47
4	0.08	0.37
5	0.07	0.34
6	0.18	0.39
7	0.06	0.38
8	0.06	0.35
9	0.08	0.38
10	0.05	0.37

Table 2. Spontaneous and DEB induced chromosomal aberrations (CAs) in patients´fibroblasts; subjects 1-6 were clearly sensitive to DEB relative to the remaining.

Cell line	Spontaneous CAs/cell	Frequencies of DEB induced CAs/cell
1	0.08	0.32
2	0.05	0.49
3	0.18	0.41
4	0.06	0.38
5	0.05	0.36
6	0.11	0.31
7	0.01	0.01
8	0.00	0.00
9	0.00	0.01
10	0.01	0.01

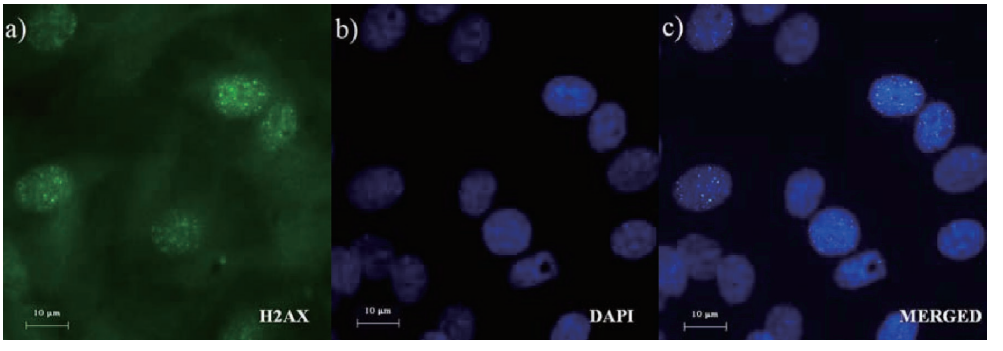

Fig. 1. Fibroblasts established from FA patients (a) immunofluorescently labeled against phospho-H2AX, (b) counterstained blue with DAPI and (c) shown merged.

10 subjects, out of 27, were DEB positive (Table 1). Interestingly, the response to DEB in fibroblasts from those 10 patients revealed 6 subjects with typical FA cellular phenotype, whereas remaining 4 were DEB negative and were further considered as non FA BMF patients (Table 2). It was confirmed that one of the DEB positive cell lines belonged to the FANC-D2 complementation group and the remaining five patients belonged to the FANC-A complementation group.

The analysis of repair kinetics of DNA DSBs after irradiation is shown in Fig. 1. The maximal frequency of radiation-induced γ-H2AX foci was observed in FA cell lines 30 minutes after irradiation and was significantly higher compared to non-FA BMF cell lines ($p < 0.05$). Furthermore, the frequency of γ-H2AX foci 24 hours after irradiation was significantly higher in FA cell lines relative to both non-FA BMF ($p < 0.01$) and control cell lines ($p < 0.001$). The results indicate that FA cell lines display significant delay in repair kinetics 24 hours after irradiation seen as 8-fold increase of unrepaired DNA DSBs (Fig. 2a) compared to self-state before irradiation ($p < 0.01$).

The analysis of apoptotic response and cell cycle progression by flow cytometry (Fig. 3) revealed that both cell types, FA and non-FA BMF, displayed statistically significant difference in the apoptotic response compared to control only 30 minutes after irradiation ($p < 0.05$), whereas at all other examined recovery times no significant differences between groups were found (Table 4 and Fig. 2c). The cell cycle analysis has shown difficulties of FA cells in progressing through the cell cycle. In untreated FANCD2 cell line, significant portion of cells has been arrested in G2/M, whereas after irradiation majority of cells are permanently arrested in G2/M. All non-FA cell lines are found to be in the S-phase of the cell cycle in untreated samples, whereas in irradiated samples high number of cells dying via apoptosis follows each decline of S-phase cells. Controls behave in similar manner: most of the irradiated cells are in S-phase of the cell cycle, and this number correlates negatively with numbers of cells dying via apoptosis ($p < 0.05$).

The response of FA cells to DEB treatment is seen as statistically insignificant when analyzing frequencies of γ-H2AX foci (Table 3 and Fig. 2b). Interestingly, the yield of DEB-induced foci in neither FA cells nor non-FA BMF cells is significantly different when compared to the controls. The analysis of apoptosis shows similar patterns (Table 4 and Fig. 2d).

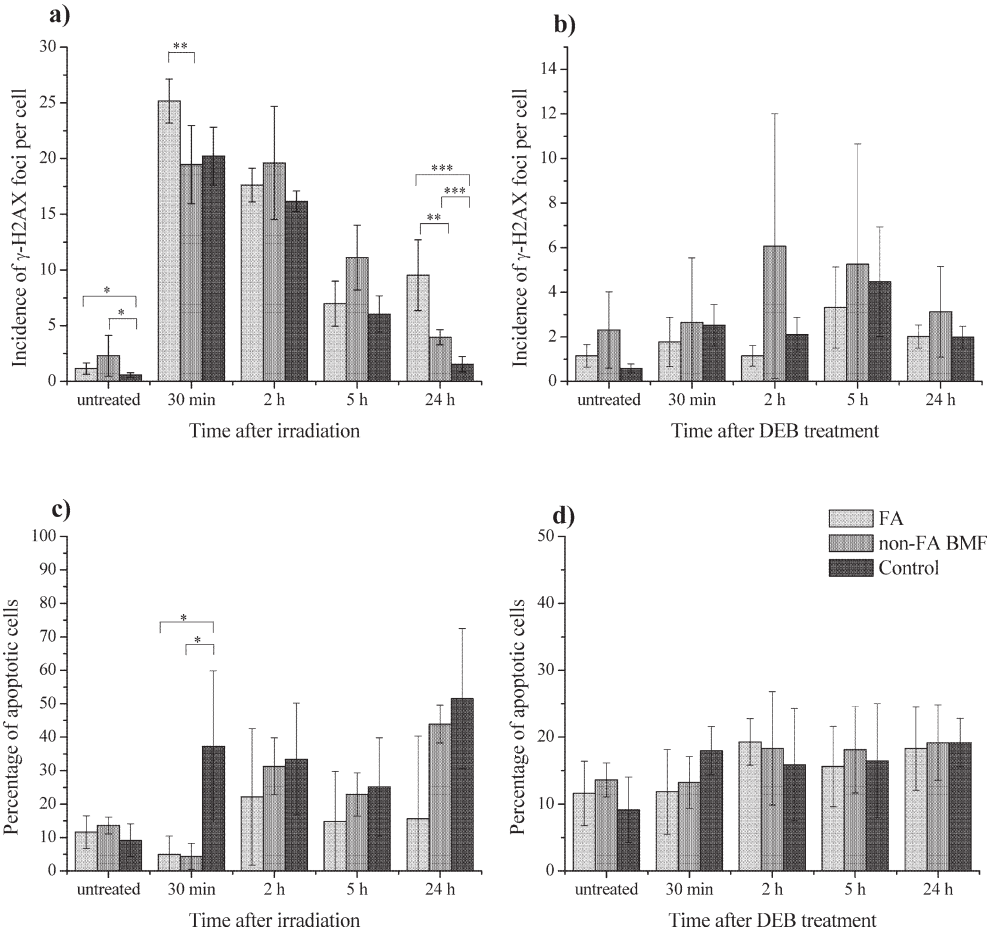

Fig. 2. Frequencies of γ-H2AX positive foci in FA, non-FA BMF and control cell lines (a) after irradiation and (b) DEB treatment. Percentages of apoptotic cells in FA, BMF and control cell lines (c) after irradiation and (d) DEB treatment. Results are presented as
mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. studied groups by *t* test.

Discussion

Although FA has been known to clinicians for decades, a correct diagnosis can be difficult due to variations in phenotype severity, or well known problems of cell mosaicism. As a result, recent diagnostic strategies combine different assays (Pinto et al. 2009) to make diagnosis easier. Moreover, patients with BMF underlying Fanconi anemia may experience major toxicity if given standard dose-conditioning regimens for hematopoetic stem cell transplant. There is also a clinically justified need for quick discrimination between FA and the rest of BMF patients. The classical chromosomal aberration test after DEB exposure is time consuming, requires highly trained personnel and overlap results with other chromosomal instability syndromes. Several earlier studies have reported defects in rejoining DSBs in FA patients (Sasaki and Tonomura 1973; Natarajan et al. 1984; Casado et al. 2005). Since quantification of γ-H2AX foci has emerged as a gold standard for monitoring DSBs levels in human cells (Fernandez-Capetillo et al. 2004), we reasoned that monitoring of DSB repair kinetics could serve as a quick test for identifying FA in a larger group of BMF patients, enabling at the same time estimation of individual DSB rejoining capabilities, i.e radiosensitivity.

Quantification of γH2AX foci allows for an accurate and sensitive detection of DSBs, since phosphorylation rapidly occurs at the site of the DSBs (within 3 min after induction) and specificity of this reaction provides a reliable marker for DSB formation (Rogakou et al. 1998, 2000; Sedelnikova et al. 2002, 2003).

The results obtained in our study have shown that FA patients can be distinguished from the rest of BMF patients, based on γ-H2AX assay, 24 h after exposure of cells to ionizing radiation. Each of our FA patients showed enhanced level of unrepaired DSBs when compared to self-state before irradiation, suggesting that residual γ-H2AX foci, observed 24 hours after irradiation, are reliable measure of DSB rejoining capability. FA cell lines exhibit the highest incidence of radiation-induced foci 30 minutes after irradiation when plateau of foci formation has been reached. However, neither FA nor non-FA cells showed differences in the extent of γ-H2AX formation from those in controls at the time when plateau is reached (30 minutes). When com-

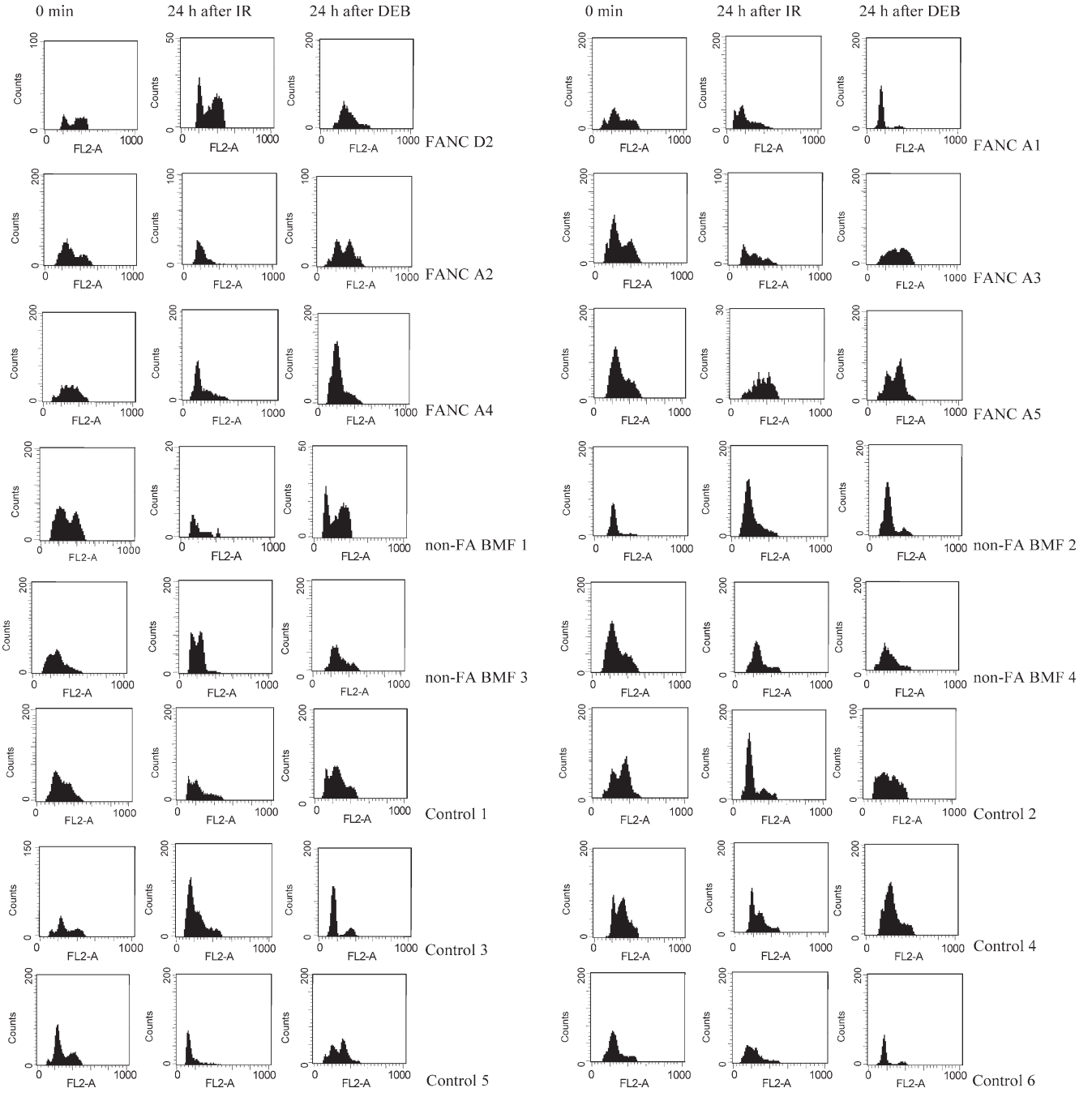

Fig. 3. Apoptosis and cell cycle flow cytometry data before (untreated) and 24 h after irradiation/DEB treatment in FA, non-FA BMF and control cell lines.

pared to the controls both FA and non-FA cell lines show statistically significant difference in frequencies of spontaneous γ-H2AX foci, and particularly 24 hours after irradiation. In FA cell lines frequency of residual γ-H2AX foci is approximately 2.5-fold higher compared to that in non-FA BMF patients and 8-fold higher on the average, when compared to self-state before irradiation. Since it is generally accepted that frequencies of γ-H2AX positive foci at various intervals after treatment with DNA damaging agents reflect kinetics of DSB repair (Rogakou et al. 1999; Redon et al. 2002) it can be concluded that FA cell lines repair radiation induced DSBs slowly, with a significant delay relative to both non-FA cell lines and control cell lines. Whereas 10.7% of cells in untreated FA cultures before irradiation were focus positive, showing 1.15 ± 0.41 foci per cell, the percentage of focus positive cells 24 hours after irradiation was 45.68, showing 9.53 ± 4.27 foci per cell. It is well known that cell undergoing DNA damage signaling induces cell cycle arrest in order to provide sufficient time for repair. During this time cell either activates or recruits a cascade of proteins needed for a complete repair or commits suicide by apoptosis. Delayed repair kinetics of FA cells

Table 3. Incidence of γ-H2AX foci per cell at different times after irradiation/DEB treatment.

Cell line		untreated	irradiation				DEB			
			30 min	2 h	5 h	24 h	30 min	2 h	5 h	24 h
FANC D2		1.60	24.81	18.37	10.41	13.78	2.97	1.79	5.24	2.84
FANC A	1	0.80	24.31	19.49	5.04	9.54	0.81	1.21	3.51	1.42
	2	1.05	26.36	15.01	5.50	5.25	1.54	0.45	1.21	1.79
	3	0.70	25.93	17.3	6.00	12.56	3.34	1.12	5.53	2.42
	4	1.94	27.63	17.30	6.63	7.98	1.04	0.87	2.86	1.72
	5	0.83	21.93	18.22	8.29	8.05	0.91	1.44	1.56	1.91
mean ± S.D.		1.15 ± 0.50	25.16 ± 1.97	17.62 ± 1.51	6.98 ± 2.03	9.53 ± 3.17	1.77 ± 1.11	1.15 ± 0.46	3.32 ± 1.81	2.02 ± 0.52
non-FA BMF	1	1.07	19.04	13.62	11.69	4.12	1.08	6.31	4.79	2.35
	2	2.24	15.49	17.80	9.36	3.10	2.36	2.64	3.49	2.72
	3	4.92	19.28	25.45	14.97	3.90	5.86	12.78	8.33	5.91
	4	1.01	24.04	21.57	8.44	4.75	1.31	2.56	4.44	1.55
mean ± S.D.		2.31 ± 1.83	19.46 ± 3.51	19.61 ± 5.07	11.11 ± 2.91	3.97 ± 0.68	2.65 ± 2.21	6.07 ± 4.80	5.26 ± 2.12	3.13 ± 1.91
Control	1	0.60	20.01	16.50	6.83	1.11	1.50	1.37	1.61	1.49
	2	0.40	19.79	14.53	5.23	1.12	2.81	2.33	5.67	1.91
	3	0.49	17.89	16.63	7.91	1.90	1.51	1.31	1.57	1.47
	4	0.38	23.84	17.0	3.68	1.23	3.97	1.87	7.72	2.44
	5	0.76	22.62	15.54	5.15	1.14	2.59	3.42	4.55	2.68
	6	0.86	17.16	16.74	7.53	2.80	2.73	2.31	5.76	1.93
mean ± S.D.		0.58 ± 0.20	20.22 ± 2.60	16.16 ± 0.94	6.05 ± 1.63	1.55 ± 0.68	2.52 ± 0.93	2.10 ± 0.78	4.48 ± 2.46	1.99 ± 0.49

Table 4. Percentage of apoptotic cells at different times after irradiation/DEB treatment.

Cell line		untreated	irradiation				DEB			
			30 min	2 h	5 h	24 h	30 min	2 h	5 h	24 h
FANC D2		1.60	24.81	2.83	2.20	13.02	4.2	17.38	13.83	18.44
FANC A	1	0.80	24.31	39.68	26.09	63.13	11.16	17.64	17.99	22.57
	2	1.05	26.36	6.90	2.51	81.87	18.74	24.11	14.18	14.63
	3	0.70	25.93	54.12	7.52	28.24	4.46	14.57	26.45	12.17
	4	1.94	27.63	18.26	39.66	54.84	15.17	22.18	11.82	28.42
	5	0.83	21.93	10.90	10.88	43.03	17.22	19.83	9.53	13.56
mean ± S.D.		1.15 ± 0.50	25.16 ± 1.97	22.12 ± 20.38	14.81 ± 14.99	47.36 ± 24.74	11.82 ± 6.34	19.29 ± 3.48	15.63 ± 6.00	18.30 ± 6.23
non-FA BMF	1	1.07	19.04	23.20	41.62	78.15	14.51	28.75	21.21	18.61
	2	2.24	15.49	31.65	20.35	46.12	9.23	16.45	13.36	27.19
	3	4.92	19.28	51.89	14.34	41.25	18.05	8.23	25.74	14.24
	4	1.01	24.04	18.41	15.24	10.02	11.18	19.87	12.18	16.68
mean ± S.D.		2.31 ± 1.83	19.46 ± 3.51	31.29 ± 14.79	22.89 ± 12.77	43.89 ± 27.89	13.24 ± 3.88	18.33 ± 8.49	18.12 ± 6.47	19.18 ± 5.63
Control	1	0.60	20.01	28.97	23.14	46.91	13.11	19.70	12.96	17.83
	2	0.40	19.79	37.16	30.27	58.02	21.23	6.18	28.97	23.04
	3	0.49	17.89	60.11	26.10	55.65	18.71	13.71	9.53	20.26
	4	0.38	23.84	11.89	48.48	24.22	22.18	16.48	17.43	18.83
	5	0.76	22.62	40.76	3.83	86.08	14.36	29.88	6.41	22.19
	6	0.86	17.16	21.65	19.16	38.17	18.21	9.44	23.38	12.97
mean ± S.D.		0.58 ± 0.20	20.22 ± 2.60	33.42 ± 16.75	25.16 ± 14.60	51.51 ± 20.97	17.97 ± 3.62	15.90 ± 8.38	16.45 ± 8.56	19.19 ± 3.62

certainly confirms a nature of this disease as DNA repair disease and at the same time gives an opportunity to make diagnosis of FA easier.

Although very similar percentage of focus positive cells was observed in non-FA cells (12.44% and 46.68%, respectively), even in controls (11.63% and 40.27%, respectively) such delay in repair kinetics is not observed in non-FA cell lines or controls. This unique feature of FA cellular

phenotype can be used for diagnostic purposes for BMF patients with questionable diagnosis, a common clinical situation.

Although several studies showed that DSB arising during cellular processes, such as replication, recombination, and apoptosis, cause an efficient formation of γ-H2AX (Rogakou et al. 2000; Sedelnikova et al. 2002) the delay in DNA repair leads to accumulation of DNA damage and cellular senescence. Our results seem to confirm previous studies reporting delayed repair kinetics of DSB after irradiation in FA cells (O'Driscoll and Jeggo 2006; Thompson and Hinz 2009). The observed delay in repair kinetics of radiation induced DSBs in FA cell lines relative to non-FA cell lines could also be considered as measure of intrinsic radiosensitivity before dose-conditioning regimens for hematopoietic stem cell transplantation. This test is significantly faster than the analysis of chromosomal aberrations after treatment with cross-linking agents, and it can easily be automated using flow cytometry analysis thus significantly reducing the time between taking cells from patients and availability of results.

In contrast to ionizing radiation, DEB as cross-linking agent poorly induces γH2AX formation in all examined cell lines. Similar to our findings, no difference in DSBs formation between FA-C and wild type cells was observed by Pichierri, and coworkers (Pichierri et al. 2002), after mitomycin treatment. Moreover, no difference in DEB induced DSBs formation revealed that crosslinking agents induce different signaling for DNA repair than DSBs signaling. The majority of past literature has suggested that FA cellular phenotype can be recognized testing with DNA cross-links agents, which increase spontaneous chromosomal instability. However, recently, Gennery et al. (2004) suggested that other inherited syndromes like ataxia-telangiectasia like disorders, Nijmegan breakage syndrome and other primary immunodeficiency syndromes are positive when testing with crosslinking agents, thus indicating an overlap with the FA cellular phenotype. Similarities in clinical manifestations and cellular phenotypes observed in several syndromes highlight the need for an accurate diagnosis of chromosomal instability syndromes that can no longer be distinguished based on abnormal responses to cross-linking agents. Variants of FA have been described with more pronounced immune deficiency (Taniguchi et al. 2002), and some patients with NBS1 have been described with aplastic anemia, a complication more commonly associated with FA (Resnick et al. 2002). Overlap of clinical manifestations and cellular phenotype address the importance of precise diagnosis of chromosomal instability disorders that can no longer be diagnosed simply from abnormal responses to MMC or DEB. We propose the *in vitro* DSB repair kinetics test after ionizing radiation as a potentially rapid and sensitive test for evaluation of individual FA cellular phenotype.

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