Leukocyte apoptosis as a predictor of radiosensitivity in Fanconi anemia

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Fanconi anemia (FA) is a rare cancer-prone genetic disease characterized by impaired oxygen metabolism and defects in DNA damage repair. Response of FA cells to ionizing radiation has been an issue intensively debated in the literature. To study *in vitro* radiosensitivity in patients suffering from FA and their parents (heterozygous carriers), we determined radiation-induced leukocyte apoptosis using flow cytometry. As *TP53* gene is involved in the control of apoptosis, we studied its status in FA lymphocytes using dual colour fluorescence *in situ* hybridization (FISH). FA patients and female heterozygous carriers display radiosensitive response to ionizing radiation seen as abnormal elimination of cells via apoptosis. By employment of FISH, the *TP53* allele loss in FA lymphocytes was not observed. In diseases related to oxidative stress, determination of radiation-induced apoptosis is the method of choice for testing the radiosensitivity.

Keywords: Apoptosis, Fanconi anemia, heterozygous carriers, radiosensitivity.

FANCONI ANEMIA (FA) is a rare genetic disease caused by defects in the FA–BRCA (breast cancer) pathway, a response network involved in DNA damage repair[1]. The functions of FA genes are mostly attributed to a DNA repair signaling pathway, required for protecting the genome from DNA interstrand crosslinks[2]. Another line of studies points towards a pro-oxidant state associated with mitochondrial dysfunction in FA cells[3]. FA patients are characterized by chromosomal instability, progressive bone marrow failure and predisposition to leukemia and solid tumors[4]. The hypersensitivity of FA cells to cross-linking agents (mitomycin C and diepoxybutane) is used for diagnostic purposes to confirm the clinical diagnosis of FA[5]. When exposed to these agents, cells from FA patients show prolonged cell cycle arrest in the G2/M phase, increased chromosomal aberrations and reduced survival[6]. However, an issue that has been intensively debated in the literature is the response of FA cells to ionizing radiation (IR). So far, there has been a long-standing clinical impression of increased radiosensitivity of FA patients[7]. On the other hand, *in vitro* studies with FA cells have yielded controversial results. If radiosensitive patients could be identified prior to radiotherapy, different treatment modalities could be used to avoid the late radiotoxicity[8]. Determination of radiation-induced apoptosis in peripheral blood lymphocytes by flow cytometry has been proposed as a reliable screening test for cancer-prone individuals and also for predicting normal-tissue responses following radiotherapy[9,10]. Regulation of radiation-induced apoptosis and cell cycle arrests is a p53-dependent mechanism achieved primarily through p53 phosphorylation by ATM protein[10,11]. *TP53* is a tumor suppressor gene that plays a key role in maintaining the genetic integrity of the cell[12]. Mutations and/or deletions of the *TP53* gene are the most frequent genetic alterations in cancer and are observed in a wide variety of hematological malignancies[13]. Many studies of patient-derived cells and those from FA mouse models showed that FA proteins are involved in pathways that regulate cell survival or cell death[6]. FANC proteins and p53 cooperate in apoptosis following DNA damage. Therefore, p53 may function to prevent the proliferation of damaged DNA through apoptosis. In FA patients, this leads to stem cell depletion, which may cause a bone marrow failure[14]. It has been shown that the activation of p53 leads to an increase in reactive oxygen species (ROS) that contributes to cell death, possibly by interfering with mitochondrial function[14]. Apoptosis is predominantly sensitive to the redox state of the cells, and is stringently associated with alterations in mitochondrial functionality[15]. The p53 status of cells is important in determining their sensitivity to radiation. Cells with functional p53 die by apoptosis and those cells lacking p53 function continue to proliferate, acquiring potentially oncogenic mutations[12]. Various studies have shown apoptosis to be abnormally regulated in FA cells. Some reports have shown higher levels of spontaneous apoptosis and no difference after mitomycin C, while in response to gamma irradiation controversial reports were obtained[16,17].

Therefore, the present study was designed to determine the radiosensitivity *in vitro* of FA patients and their parents (heterozygous carriers) by examination of radiation-

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induced leukocyte apoptosis, and to investigate the status of the *TP53* gene in FA cells.

Patients and methods

Patients and controls

The studied groups included five FA patients (four girls belonging to complementation group FA-D2 and one boy belonging to FA-A complementation group, 8 ± 4 years old) and their parents who visited Mother and Child Health Care Institute of Serbia from January 2006 to January 2008. The Ethical Committee of Mother and Child Health Care Institute of Serbia approved the study and parents signed an informed consent regarding this investigation. A total of 30 unrelated, healthy, age-matched individuals (10 children and 20 adults – 10 females and 10 males) with normal blood profiles and not suffering from any chronic disease were taken as control groups. Peripheral blood was collected from all individuals into heparinized vacutainer tubes in accordance with the current Health and Ethical Regulations in Serbia[18].

Irradiation

Heparinized whole blood was aliquoted into sterile plastic test tubes, placed in a 15×15 cm Plexiglas container and irradiated using a ^{60}Co γ-ray source at room temperature. The radiation dose employed was 2 Gy, the dimensions of the radiation field were 20×20 cm and the distance from the radiation source was 71.1 cm.

Apoptosis of leukocytes

For apoptosis assay, irradiated blood aliquots (0.5 ml) of patients, their parents and the respective controls were incubated in a RPMI-1640 medium (Invitrogen-Gibco, Paisley, United Kingdom) supplemented with 15% calf serum (Invitrogen-Gibco, Paisley, UK) without phytohaemagglutininin a CO_2 incubator for 24 h. Cells were collected by centrifugation, gently washed with physiological saline (0.9% NaCl) at 37°C, and fixed in methanol : acetic acid (3 : 1)[19]. Next, the pellet was 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 was calculated using the CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).

TP53 fluorescence in situ hybridization analysis

The unirradiated peripheral blood samples (0.5 ml) of FA patients were cultured for 72 h in PBmax-karyotyping medium (Invitrogen-Gibco, Paisley, UK) at 37°C. Cells were collected by centrifugation and treated with hypotonic solution. Cell suspension was fixed in methanol/acetic acid (3 : 1), washed three times with fixative, and dropped onto a clean slide. The p53 fluorescence *in situ* hybridization (FISH) was performed according to the manufacturer's protocol with minor modifications (MP Biomedicals, France). The p53 (17p13)-specific DNA probe was direct-labelled with Rhodamine (red) and the chromosome 17 alphasatellite probe was direct-labelled with Fluorescein (green). Analysis was performed by enumeration of hybridization signals in at least 200 interphase lymphocytes. Cells with two red and two green signals were scored as normal. Slides were evaluated using an AxioImager A1 microscope (Carl Zeiss, Jena, Germany) and the computer software ImageJ version 1.44.

Statistical analysis

Statistical analysis was performed using the statistical software package Statistica 8.0 for Microsoft Windows. Student's *t* test was used; *P* values less than 0.05 were considered significant and those less than 0.01 were considered highly significant.

Results

This study analyses leukocyte apoptosis induced by gamma-irradiation in FA patients, their parents and healthy controls (Figures 1 and 2). In addition, the status of *TP53* gene was determined in lymphocytes of FA patients (Figure 3).

Determination of radiation-induced leukocyte apoptosis

FA patients are characterized by the highest percentage of apoptotic cells (13.77 ± 4.27), 2.63-fold higher compared to control (5.24 ± 0.70), statistically highly significant ($P = 0.000024$). A typical example of flow cytometric analysis of radiation-induced apoptosis in one of the FA patients is presented in Figure 2.

Similar to the results obtained in FA patients, in female heterozygous carriers (mothers of FA patients), percentage of apoptotic cells (12.52 ± 3.32) was 2.12-fold higher compared to control group of women (5.90 ± 2.13; $P = 0.0004$).

On the other hand, male heterozygous carriers (fathers of FA patients) displayed a percentage of apoptotic cells (5.36 ± 0.20) similar to that obtained in control group of men (5.03 ± 0.76), with no statistical significance ($P > 0.05$).

It is worth noting that FA patients exhibited significantly ($P = 0.0023$) higher percentage of apoptotic cells

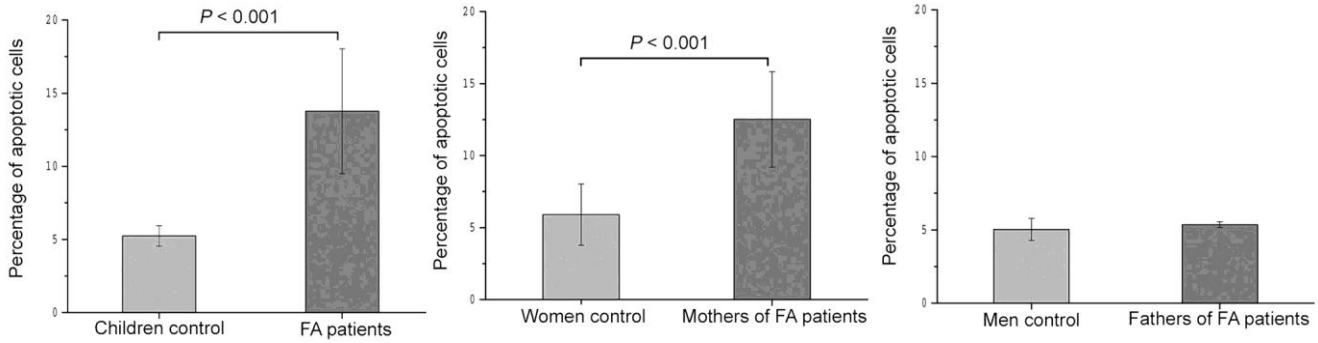

Figure 1. Radiation-induced apoptosis of leukocytes (mean ± SD) in Fanconi anemia (FA) patients, their parents and healthy controls.

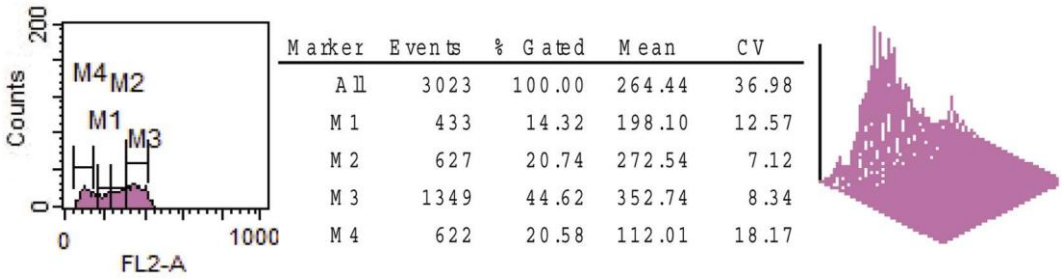

Figure 2. A typical example of flow cytometric analysis of radiation-induced apoptosis in one of the FA patients. M4, Percentage of apoptotic cells.

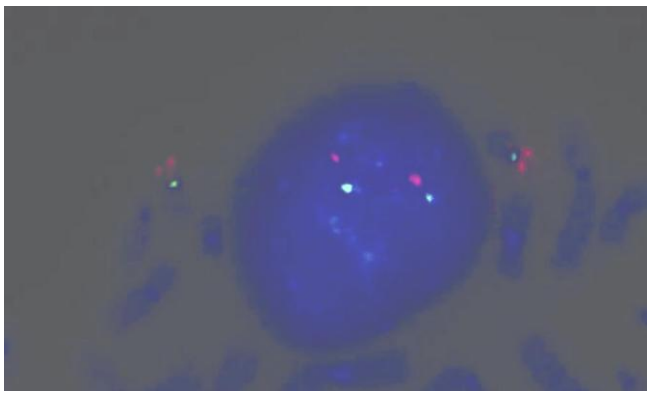

Figure 3. Example of interphase FISH showing normal hybridization pattern. There was no allele loss of *TP53* gene in interphase nucleus.

relative to their fathers, whereas no significant difference relative to their mothers was found.

TP53 FISH analysis

FISH is performed to detect possible deletion of *TP53* gene in lymphocytes of FA patients. A fluorescently labeled DNA probe for *TP53* gene, at chromosome band 17p13, is hybridized to interphase nuclei on a slide. Two hundred cells are evaluated to determine whether two signals are present (no deletion) or fewer than two signals are present (deletion). As shown in Figure 3, deletions of *TP53* gene in lymphocytes of FA patients were not observed.

Discussion

The complex phenotype of FA suggests that FA genes may be involved in the cell cycle progression, oxidative metabolism, transcriptional regulation and control of cellular homeostasis, playing a key role in mechanisms involved in response to stress conditions. The *FANCD2* gene has a key role in the FA-BRCA pathway[20]. After exposure to ionizing radiation, the FANCD2 protein is phosphorylated on Ser 222 by ATM, which allows normal activation of the S-phase checkpoint. The loss of FANCD2 results in both mitomycin C and IR sensitivity[21]. Another mechanism of action, proposed a many years ago, is the role of oxygen and oxidative damage, which could explain part of the sensitivity of FA cells to both mitomycin C and IR[22,23]. A body of literature links FA with oxidative stress and mitochondrial dysfunction[3,4]. The *in vivo* prooxidant state of FA cells has been determined in earlier studies[24–27]. The increased production of ROS caused by compromised mitochondrial respiratory function could influence both mitochondrial and nuclear DNA integrity. Cells with impaired mitochondrial function have a weakened ability to cope with oxidative and genotoxic stress (e.g. ionizing radiation). Ionizing radiation increases the production of ROS and affects the activity of antioxidant enzymes[28]. Studies of intrinsic radiosensitivity in a healthy population showed the crucial role of cellular antioxidant enzymes, particularly of MnSOD in radiation response[28,29]. In our earlier experi-

ments conducted to test the radiosensitivity of FA patients, reduced incidence of radiation-induced micronuclei in peripheral blood lymphocytes was found in the majority of patients[30]. Similarly, a mild radioresistant response to IR was observed in female carriers as well. In this study, we found extremely elevated radiation-induced apoptosis in FA patients, 2.63-fold higher than that in the control. Mothers of FA patients exhibited almost the same percentage of cells in apoptosis as FA patients, over two-fold higher relative to the women control. On the contrary, in fathers of FA patients, percentage of apoptotic cells was similar to that observed in the men control. Unfortunately, the limited number of FA patients made it impossible to examine gender-related differences in the parameter investigated.

The obtained results confirmed once again the similarity between FA patients and their mothers in response of cells to IR, and pointed out that FA patients are radiosensitive displaying an abnormal elimination of cells via apoptosis. Several studies have reported that high apoptotic frequency correlates with increased radiosensitivity[31–33].

In this context, a radioresistant response obtained by using cytokinesis-block micronucleus test could be at least in part explained by impaired mitochondrial function. The normal cells produce most of their energy in the form of ATP generated by oxidative phosphorylation, whereas cells with defective mtDNA are respiratory incompetent and rely on glycolysis for energy[34]. Knowing that micronuclei expression pathway depends on the intrinsic ATP levels[35], in cells with defective mtDNA the production of ATP is reduced, which consequently leads to the reduction of micronuclei incidence. Thus, in diseases related to oxidative stress, determination of radiation-induced apoptosis is the method of choice for testing the intrinsic radiosensitivity. Altogether, these findings shed new light on the genetics of FA implicating a possible mitochondrial involvement in the pathogenesis of the disease.

Mutations or deletions in the *TP53* gene are reported in more than 50% of human tumors, including the acute myeloid leukemia cells from FA patients[36]. In the current study, we did not detect the allele loss in *TP53* gene in FA lymphocytes. Similarly, besides the study of Abo-Elwafa *et al.*[13], who reported DNA alterations in *TP53* gene, the literature data provide no evidence for mutations of p53 itself in FA cells[37,38], suggesting that an altered apoptosis may represent an impaired physiological response to unresolved replication stress and endogenous DNA damages[39,40].

In our study, the altered apoptosis of leukocytes in FA patients and female carriers could be partly explained by redox imbalance. Results of our recent study have shown that both FA patients and female carriers displayed drastic reduction of catalase activity (fourfold and twofold respectively) accompanied with significantly enhanced level of prooxidant/antioxidant balance, which indicated the accumulation of hydrogen peroxide in blood cells. Furthermore, both patients and female carriers were characterized by drastic reduction of extracellular superoxide dismutase (SOD), whereas the decreased activity of erythrocyte SOD and enhanced process of lipid peroxidation were observed only in patients[27]. It has been shown that hydrogen peroxide plays a critical role as a physiological mediator in the onset of apoptosis that occurs in response to IR[41]. The accumulation of H_2O_2 may increase the level of phosphorylated p53 by stimulating certain protein kinases implicated in the phosphorylation of p53 (ref. 42). Thus, impaired redox homeostasis in cells of FA patients and their mothers could change the activity and expression of p53, leading to an abnormal cellular response to damage induced by ROS. It has been proposed that high levels of ROS are a part of the feedback loop that results in more p53 activity[14]. p53 results in apoptosis through a multi-step process, including the transcriptional induction of redox-related genes, the generation of ROS, and the oxidative degradation of mitochondrial components, leading to apoptosis[43,44].

In conclusion, results of the present study suggest that FA patients and female heterozygous carriers display radiosensitive response to IR seen as abnormal, massive elimination of cells via apoptosis. The elevated apoptosis is probably the consequence of impaired function of FANC proteins that leads to increased level of ROS or reduced repair of the oxidative DNA damage.

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