CELL CYCLE ANALYSIS IN PATIENTS WITH FANCONI ANEMIA FROM SERBIA

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Fanconi anemia (FA) is a rare autosomal recessive disorder, characterized by progressive bone marrow failure, chromosomal instability and cell cycle blockage in the G2 phase. The hypersensitivity of FA cells can be additionally induced with specific alkylating agents such as diepoxybutane (DEB) and mitomycin C, which is used in differential diagnosis of FA. Among 72 patients with clinical suspicion of FA, who were diagnosed at the Mother and Child Health Care Institute of Serbia “Dr Vukan Cupic” and the University Children’s Hospital (2004 – 2011), in 10 patients the diagnosis of FA was confirmed on the basis of an increased chromosome sensitivity to DEB. Five out of 10 FA patients were available for further flow cytometric analysis of cell cycle. We examined cell cycle blockage in G2 phase in untreated and with DEB treated lymphocyte cultures from FA patients and from the healthy persons, as control group. All five patients affected with FA, showed an increased DEB induced G2-phase-blockage which was over two times higher than in controls. The percentage of FA cells arrested in G2 phase was between 4,41% and 10,45% with mean value (MV) of 7,76%, but in the control group this range was lower: 1,56% - 4,11% (MV: 2.84%), with no overlapping. FA patients showed an increased spontaneous arrest in G2 phase, as well, comparing to healthy controls (MV: 14,63% vs. 5,82%). Cell cycle assay of G2 phase blockage could be used

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as an additional diagnostic tool for confirmation of FA in patients with clinical suspicion of this disease.

Key words: Fanconi anemia, cell cycle, G2 phase, diepoxybutane

INTRODUCTION

Fanconi anemia (FA) is a rare disorder with mostly autosomal recessive trait of inheritance, which is characterized by the presence of congenital anomalies, pancytopenia and elevated predisposition for malignancy (HISAMA et al., 2003; WEGNER and STUMM, 1999). FA is in the group of diseases, named chromosome breakage syndromes, which main characteristic is the presence of increased number of spontaneous and induced chromosomal aberrations (DEB test) in cells of the affected, comparing to healthy individuals. The elevated chromosomal fragility of FA cells is caused by the mutations of the genes involved in a DNA repair process. This is the reason why FA cells cannot fix damaged DNA, so they are blocked in G2 phase of cell cycle (HISAMA et al., 2003; KUBBIES et al., 1985). Percentage of FA cells spontaneously arrested in G2 phase can be enhanced using different alkylating agents, such as mitomycin C (MMC) or diepoxybutane (DEB), which are used in differential diagnosis of FA (KUBBIES et al., 1985; MIGLIERINA et al., 1991).

In view of the above, the present study was undertaken to examine the blockage in G2 phase of cell cycle in FA patients from Serbia, in order to confirm the diagnosis of this disease, based on clinical parameters and increased sensitivity to DEB (DEB test).

MATERIALS AND METHODS

Patients

Among 72 patients with clinical suspicion of FA, who were diagnosed at the at the Mother and Child Health Care Institute of Serbia “Dr Vukan Cupic” and the University Children’s Hospital (from February 2004 to June 2011), in 10 patients the diagnosis of FA was confirmed on the basis of an increased chromosome sensitivity to DEB (CIRKOVIC et al., 2011). Five out of 10 FA patients were available for further flow cytometric analysis of cell cycle.

Methods

Flow cytometric analysis of cell cycle

Flow cytometric cell cycle analysis is a method that measures DNA content in the cell and based on that, determines cell’s phase and number (ORMEROD, 2000). The G2 phase arrest of cells from FA patients was analyzed using standard procedure (MOREIRA et al., 2008; ORMEROD, 2000; SCHINDLER and HOEHN, 1999), with minor modifications. The lymphocytes from peripheral blood of each FA patient and healthy person (control counterpart) were harvested in two separated cultures under the same conditions: one treated (24h) with DEB (0,1µg/ml) and the other remained untreated (72h). After cultivation, cells were rinsed with saline solution, fixed and put in 96% ethanol on -20°C, until they were analyzed on cytometer. The cell cycle was examined on Becton-Dickinson (BD) FACSCalibur’s cytometer and deconvolution of DNA histograms was performed using simplified method (ORMEROD, 2000). The results of analysis were presented as percentage of the cells in the G2 phase.

Statistical analysis

Independent t-test was used for evaluation of statistically significant difference between the groups: patients affected with FA (FA group) and healthy controls (control group).
RESULTS

Cell cycle analysis of DEB-treated lymphocyte cultures showed that all five FA patients had increased G2 phase blockage comparing to controls (Fig. 1). Percentage of the cells blocked in G2 phase in DEB treated cultures of FA group ranged from: 4.41% - 10.45%, with mean value (MV) of 7.76%, what was more than two and half times higher than in the control group (range: 1.56-4.11, with MV of 2.84) (Tab. 1). Patient No. FA-1 had maximal value of DEB-treated cells in G2 phase (10.45%), while patient No. FA-2 had minimal value of 4.41% for the same parameter (Tab. 2). Intervals of values found in FA and control groups were not overlapped (Tab. 1) with statistically significant difference (t-test: p < 0.05).

Cell cycle analysis in untreated cultures of FA patients showed an increased spontaneous arrest of cells in G2 phase, which was about two and half times higher comparing to spontaneous arrest in controls (Tab. 1). The mean value of percentage of arrested cells in G2 phase in untreated cultures of FA patients was 14.63% (range: 11.20% – 18.48%), while in control group this value was much lower: 5.82% (range: 2.84% – 7.68%), without overlapping (Tab. 1).

![Fig. 1. DNA histograms of cell cycle in FA patients and healthy persons: cell arrest in G2 phase (peak M3).](image)

Tab. 1. Spontaneous and DEB induced blockage of lymphocytes (percentage of cells) in G2 cell cycle phase in FA and control group.
Tab. 2. Spontaneous and DEB induced blockage of lymphocytes (percentage of cells) in G2 cell cycle phase of five FA patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Spontaneous arrest</th>
<th>Induced arrest</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA-1</td>
<td>15,02</td>
<td>10,45</td>
</tr>
<tr>
<td>FA-2</td>
<td>11,20</td>
<td>4,41</td>
</tr>
<tr>
<td>FA-3</td>
<td>18,48</td>
<td>7,71</td>
</tr>
<tr>
<td>FA-4</td>
<td>15,38</td>
<td>8,92</td>
</tr>
<tr>
<td>FA-5</td>
<td>13,08</td>
<td>7,32</td>
</tr>
<tr>
<td>MV</td>
<td>14,63</td>
<td>7,76</td>
</tr>
<tr>
<td>SD</td>
<td>2,73</td>
<td>2,24</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Literature data showed that patients affected with FA have prolonged G2 phase of cell cycle (KUBBIES et al., 1985), and that this spontaneous arrest can be additionally enhanced, in the same way as in chromosomal breakage analysis (AUERBACH, 1988; AUERBACH et al., 1989), by an action of specific mutagens (FABIO et al., 2000; HEINRICH et al., 1998; KUBBIES et al., 1985). For that reason cell cycle analysis nowadays is used as additional tool in differential diagnosis of FA (FABIO et al., 2000; SEYSCHAB et al., 1995).

In this study we presented the results of cell cycle analysis in five patients, who were diagnosed with FA, on the basis of clinical data and increased number of DEB-induced chromosomal breaks (CIRKOVIC et al., 2011). Cell cycle analysis showed that there was spontaneous and DEB-induced G2 phase arrest of lymphocytes in the affected persons, over two times higher than in control group, while the mean value of the cell blockage in the control group was in the range of previously reported data by SEYSCHAB et al. (1995). When the values of the induced blockage in the FA group of Serbian patients (4,41% - 10,45%) were compared to those revealed in FA patients from Brazil (48.29% - 62,23%) (MOREIRA et al., 2008), it was obvious that percentage of arrested lymphocytes of Brazilian FA patients was much higher. These results may be due to different methods used in cell cycle analysis, or the fact that in our sample of suspicious FA patients, mosaic FA patients, usually less sensitive to DEB, were present (somatic...
mosaicism). Somatic mosaicism represents a phenomenon when in some cells during haematopoiesis in the FA gene, previously inactivated with mutation, reversion is occurred, which results in normal function of the gene. The final result of such haematopoiesis is the presence of two cell clones in the blood of these patients: one clone sensitive to DEB and the other clone without response to this mutagen because of reverting mutation (GREGORY et al., 2001; GROSS et al., 2002). Mosaic form of FA complicates establishing diagnosis of FA, and for that reason in such patients, beside chromosomal breakage analysis, it is necessary to perform other diagnostic tests, such as cell cycle analysis for instance, or to conduct additional research in other tissues (skin fibroblasts) (SCHINDLER et al., 2007; SHIMAMURA et al., 2002; SOULIER et al., 2007).

Advantage of flow cytometric cell cycle analysis in diagnosis of FA is its relatively fast and simple procedure for examination of G2 phase arrest, one of the important indicators of FA. However, it is well known that patients with aplastic anemia can show an increased number of cells blocked in G2 phase too, although they are not affected with FA (SCHINDLER and HOEHN, 1999). This analysis in patients with developed malignancy can also give false negative results (XING et al., 2007). Due to the limitation of this method, a large scale of others diagnostic procedures should be applied in FA suspicious patients in order to provide an earlier confirmation of diagnosis of this lethal disease.

An early and precise diagnosis is very important for the treatment of these patients, but also for the risk evaluation and genetic counselling in the families with affected members (ALTER and KUPFER, 2011).

CONCLUSION

Flow cytometric analysis of the cell cycle can be used as additional method, beside DEB test, for establishing the diagnosis of FA, especially in patients with mosaic form of the disease. Cytometric and cytogenetic analyses are very important in differential diagnosis of FA, and to select patients for further molecular analyses, which are needed for definitive confirmation of this lethal disease.

Establishing an early and accurate diagnosis of FA is crucial for further management of the patients and genetic counseling in their families.

ACKNOWLEDGEMENT

This study was partially financed by the Ministry of education, science and technological development of the Republic of Serbia (Grant No. 173046).

Received January 20th, 2013
Accepted June 06th, 2013
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ANALIZA ĆELIJSKOG CIKLUSA KOD PACIJENATA SA FANKONIJEVOM ANEMIJOM U SRBIJI

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Izvod
Fankonijeva anemija (FA) je retka autozorno recesivna bolest, čija je osnovna karakteristika prisustvo hromozomskih aberacija u kariotipu pacijenata, kao i zastoj čelića obolelih u G2 fazi čelijskog ciklusa. Hipersenzitivnost čelića FA može biti specifično indukovana delovanjem alkilirajućih agenasa kao što je diepoksibutan (DEB) i mitomicin C, što je primenjeno u diferencijalnoj dijagnostici FA. Od 72 pacijenata, klinički suspektnih da boluju od FA, koji su dijagnostikovani u Institutu za zdravstvenu zaštitu majke i deteta Srbije “Dr Vukan Čupić” i Univerzitetskoj dečjoj klinici (od 2004 – 2011. godine), kod 10 pacijenata je na osnovu povećane hromozomske senzitivnosti na DEB dijagnostikovana FA. Pet od 10 pacijenata obolelih od FA je bilo dostupno za dalje analize čelijskog ciklusa. Ispitivan je zastoj čelića u G2 fazi u netretiranim i DEB-om tretiranim kulturama limfocita periferne krvi bolesnika i zdravih osoba. Kod svih pet pacijenata sa FA je uočen povećan procenat čelića u G2 fazi, indukovana DEB-om, koji je bio veći nego kod zdravih kontrola. Procenat čelića FA zaustavljenih u G2 fazi se kretao od 4,41%-10,45%, sa srednjom vrednošću (SD) od 7,76%, dok se u kontrolnoj grupi taj procenat kretao od 1,56%-4,11%, sa SD od 2,84%, bez međusobnog preklapanja. Kod svih FA pacijenata je takođe uočen i povećan procenat spontano zaustavljenih čelića u G fazi u odnosu na kontrolu, koji je bio statistički značajan, bez međusobnog preklapanja (SD: 14,63% prema 5,82%). Analiza zastoj da čelića u G2 fazi se može koristiti kao dodatna metodsa u potvrđi dijagnoze kod bolesnika sa sumnjom da boluju od FA.

Odobreno 06. VI. 2013.