Assessment of genotoxicity associated with Behcet's disease using sister-chromatid exchange assay: vitamin E versus mitomycin C

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Abstract Behcet's disease (BD) is a multisystemic chronic inflammatory disorder that presents throughout the world with high frequency in Turkey and Middle East. BD has been shown to be associated with genotoxicity as patients with the disease have demonstrated high rates of sister chromatid exchange (SCE) and oxidative DNA damage. In this study, we examined the effect of vitamin E, which is known for its strong antioxidant activity, on the rate of SCE in cultured lymphocytes obtained from BD patients. In addition, the susceptibility of patient lymphocytes to the mutagenic agent mitomycin C (MMC) was also investigated. The results showed significant elevation in the rate of SCE in lymphocytes obtained from patients compared to those from healthy subjects ($P < 0.01$). Treatment with vitamin E normalized the elevated rate of SCE to a comparable level observed in the control group ($P < 0.01$). Finally, treatment of cultures with MMC significantly increased the rate of SCE in the lymphocytes of both patients and controls ($P < 0.001$). The magnitude of change in the rate of SCE induced by MMC was equivalent in both groups.

This result suggests similar sensitivity of BD lymphocytes and control ones to MMC. In conclusion, genotoxicity associated with BD can be overcome by treatment with vitamin E. Lymphocytes of BD have normal sensitivity to the mutagenic agent MMC.

Keywords Behcet's disease · Genotoxicity · Sister chromatid exchange · DNA damage · Mitomycin C · Oxidative stress

Abbreviations

BD Behcet's disease
MMC Mitomycin C
SCE Sister chromatid exchange

Introduction

Behcet's disease (BD) is a multisystemic chronic inflammatory disorder characterized by frequent episodes of oral and genital aphthae, skin lesions and retinal vasculitis (Hatemi et al. 2013). In some severe cases of BD other systems may be involved including cardiovascular, gastrointestinal, skeletal and central nervous systems (Verity et al. 2003). The disease is more common in males and in countries of Mediterranean basin (Evereklioglu 2005) with a prevalence of about 8 per 10,000 in Turkey (Khairallah et al. 2012). The causes of BD are not known, however, the disease is probably mediated by combinations of genetic and environmental factors (Khairallah et al. 2012).

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Behcet's disease has been shown to be associated with genotoxicity as measured by cytogenetic and oxidative DNA damage assays. For example, elevation in sister chromatid exchange (SCE) in BD lymphocytes has been shown by several reports (Ikbal et al. 2006; Karaman et al. 2009; Oztas et al. 2006; Sonmez et al. 1998). The increase in the SCE rate was evident in patients with active and inactive episodes (Karaman et al. 2009) and in patients typed positive or negative for HLA-B51 allele (Ikbal et al. 2006). Similar findings were reported using micronucleus assay on blood lymphocytes and oral mucosa cells obtained from patients (Hamurcu et al. 2005; Karaman et al. 2009). The mechanism for this genotoxicity is still not clear, however, significant elevation in the oxidative damage biomarkers such as 8-hydroxy deoxyguanosine, plasma malondialdehyde and protein carbonyl was detected in BD patient's (Sezer et al. 2012) (Bashir et al. 1993). In addition, levels of ascorbic acid and antioxidant enzymes that include catalase, glutathione peroxidase and superoxide dismutase were relatively lower in BD patients than in healthy controls (Gulbahar et al. 2007; Harzallah et al. 2008; Taysi et al. 2007). Thus, imbalance of antioxidant/oxidant system in BD could account for the genotoxicity observed in patients.

Vitamin E includes fat-soluble tocopherols and tocotrienols compounds that have strong antioxidant activity (Hyman et al. 2005). Vitamin E mediates several functions in the body such as cell signaling, immune response and regulation of certain kinases (Zingg and Azzi 2004). Recently, vitamin E has been shown to overcome oxidative damage caused by many conditions such as sleep deprivation, high fat diet, aerobic exercises, aging and hypercholesterolemia (Abbas and Sakr 2013; Alzoubi et al. 2012b; Prasad et al. 2012; Sun et al. 2013). Moreover, vitamin E has been shown to protect from DNA damage induced by oxidative stress in pesticides-treated PC12 cells (Wang et al. 2012), human hepatocellular carcinoma cell lines (Fantappie et al. 2004), human keratinocytes (Huang et al. 2004), and carbofuran-treated human lymphocytes (Sharma et al. 2012; Sharma and Sharma 2012). Thus, vitamin E has a potential to be used as a protective drug against genotoxicity caused by oxidative stress associated with diseases and chemical agents.

In this project, we examined the effect of vitamin E on genotoxicity associated with BD using SCE assay.

In addition, the sensitivity of the blood lymphocytes obtained from BD patients to the genotoxic agent mitomycin C (MMC) was also examined. The results might have clinical applications in the management of BD.

Materials and methods

Subjects

Six males with BD were recruited to participate in the study from King Abdullah University Hospital, Irbid, Jordan. The patients were diagnosed to have BD by Dr. Alawneh according to the criteria of the International Study Group (1990). All patients were either newly diagnosed or were not taken any medications at least 3 months prior to blood sampling. As a control group, 6 healthy male subjects were selected to match BD patients for age and geographical area. All subjects were non-alcoholic, non-smokers and free of chronic diseases except BD in the patients group. In addition, subjects were excluded if they were taking supplementations or medications during 3 months period prior to blood withdraw. The study was approved by the Institutional Review Boards at Jordan University of Science and Technology. The study procedures and goals were explained to all participants prior to taken their informed consent.

Blood sampling and culture initiation

Blood samples were obtained from subjects via venipuncture in sterile heparinized tubes. Blood cultures were initiated by adding 1 mL of fresh blood to 9 mL of karyotyping medium obtained from Gibco-Invitrogen (Paisley, UK). For differential staining of sister-chromatides, 5-bromodeoxyuridine (final concentration: 10 µg/mL, Sigma-Aldrich, St. Louis, MO, USA) was added to cultures immediately after initiation. Similarly, vitamin E (D-alpha-tocopherol; final concentration of 1 µg/mL; Acros Organics (EKIN Kimya Ticaret Ltd., Istanbul, Turkey)) was added to the vitamin E groups at the beginning of the culture period (Khabour et al. 2013b). The mutagenic agent MMC (final concentration: 0.01 µg/mL, Sigma-Aldrich) was added to the MMC groups 48 h after culture initiation (M'Bemba-Meka et al. 2007). Cultures were incubated in the dark at 37 °C for 72 h in CO₂ incubator/appropriate humidity.

Sister-chromatid exchange assay

In the last 2 h of blood cultures, colcemid (final concentration: 0.1 $\mu\text{g/mL}$, Sigma-Aldrich) was added to arrest lymphocytes at the metaphase stage. Lymphocytes were harvested and fixed as previously described (Al-Sweedan et al. 2012; Azab et al. 2009). Metaphase spreads were obtained by dropping the fixed cellular suspension on cold microscope slides. Slides were air dried in the dark prior to the fluorescence-plus-giemsa staining, which was performed as previously described (Khabour et al. 2011, 2013a). For each treatment, 150 (25 per subject) well-spread second metaphases (contained between 42 and 46 chromosomes) were included in the analysis of SCE (Alzoubi et al. 2012a).

Cell kinetics analysis

About 1,000 cells per treatment/per subject were used to determine the mitotic index (MI), which was calculated by dividing cells that were in the metaphase stage/total cells examined (Sadiq et al. 2000). For the proliferation index (PI), 100 metaphase cells from each donor/treatment were included in the analysis. Calculation of PI was as previously described (Khabour et al. 2013a).

Statistical analysis

Graphpad Prism Statistical Software (version 4, La Jolla, CA, USA) was used for statistical analysis. Two group comparisons were performed using Student *t* test. The effects of vitamin E and MMC on SCE rates were analyzed using one way ANOVA followed by Newman–Keuls Multiple Comparison test. Differences were regarded significant at $P < 0.05$.

Results

Figure 1 shows the rate of SCE in BD patients and controls. The rate of SCE was significantly higher in patients (5.63 ± 0.12) than in the control group (4.40 ± 0.13 , $P < 0.01$). The magnitude of the increase in the rate of SCE in BD patients was approximately 28 %. To assess if treatment with vitamin E can prevent the elevated rate of SCE in lymphocytes of patients, cultures were treated with

Fig. 1 Rate of SCE in lymphocytes from BD patients and healthy controls. Rate of SCE was significantly higher in cultured lymphocytes obtained from BD patients compared to those obtained from healthy controls. Six subjects were included per group. SCE rates were expressed as mean $\pm$ SEM. Asterisk indicates significant difference, $P < 0.01$

Fig. 2 Vitamin E normalized the rate of SCE in BD lymphocytes. The elevated rate of SCE in cultured lymphocytes obtained from BD patients (Pat) was normalized by treatment of cultures with 1 $\mu\text{g/mL}$ vitamin E (Vit E, $P < 0.01$). Vitamin E treatment significantly lowered the basal rate of SCE in the control (Con) group ($P < 0.05$). Six subjects were included in each group. SCE rates were expressed as mean $\pm$ SEM. Asterisk indicates significant difference from control group (ANOVA: $F = 68.39$, $P < 0.01$)

1 $\mu\text{g/mL}$ D-alpha-tocopherol (Fig. 2). Treatment with vitamin E significantly lowered the rates of SCE in both patients (5.63 ± 0.12 in BD group versus 3.83 ± 0.098 in BD + Vit E group, $P < 0.01$) and controls (4.40 ± 0.13 in control versus 3.47 ± 0.10 in Con + Vit E group, $P < 0.01$). The magnitude of change in the rate of SCE induced by vitamin E was higher in patients (-1.8) than controls (-0.93). In addition, the rate of SCE after treatment with vitamin E in the patient group was similar to that detected in the control group (3.83 ± 0.098 versus 3.47 ± 0.10 respectively, $P > 0.05$). This result suggests that

Fig. 3 Rates of SCEs after treatment of lymphocytes with MMC. The rates of SCE in lymphocytes after treatment of cultures with MMC (final concentration: 0.01 $\mu\text{g/mL}$) for 24 h. MMC significantly increased SCE in BD (Pat) and controls (Con) lymphocytes. The rates of SCE induced by MMC in lymphocytes of BD patients and controls are similar ($P > 0.05$). Six subjects were included per group. SCE rates were expressed as mean $\pm$ SEM. Asterisk and dollar symbol indicates significant difference from Con and Pat groups respectively (ANOVA: $F = 48.98$, $P < 0.001$)

vitamin E normalized elevated rates of SCE associated with BD.

To assay whether there is a difference in the susceptibility of lymphocytes obtained from patients and controls to mutagenic agents, blood cultures were treated with MMC (final concentration 0.01 $\mu\text{g/mL}$) 24 h prior to harvesting. Treatment of cultures with MMC significantly increased the rate of SCE in the lymphocytes of both patients and controls ($P < 0.001$, Fig. 3). The change in the rate of SCE is similar in patient group (5.69) and controls (5.56). Thus, lymphocytes obtained from BD patients and healthy controls showed similar levels of sensitivity to MMC.

No change was detected in the mitotic index and proliferative index due to treatment of cultured cells with MMC (0.01 $\mu\text{g/mL}$) for 24 h or with vitamin E (1 $\mu\text{g/mL}$) for 72 h (data not shown).

Discussion

In this study, we showed that the genotoxicity associated with BD can be prevented by treatment of lymphocytes with vitamin E. In addition, the susceptibility of BD lymphocytes to the mutagenic agent MMC is similar to that of healthy individuals.

The genotoxicity associated with BD was assessed using SCE, which is a sensitive assay used to detect genotoxicity of chemical agents both in vivo and

in vitro (Kao-Shan et al. 1987). The propose mechanism of SCE formation involves break in the DNA and the subsequent repair of the damage by homologous recombination pathway using the other intact sister-chromatid (Mateuca et al. 2012).

Several studies have shown that BD is associated with genotoxicity. This was demonstrated by SCE, micronucleus and 8-hydroxy deoxyguanosine (Ikbali et al. 2006; Karaman et al. 2009; Sezer et al. 2012). The cause of this genotoxicity was attributed to the high level of oxidative stress observed in the patients. In fact, the relationship between oxidative stress and DNA damage in human diseases is well documented (Maluf et al. 2013). Strong correlation between oxidative stress biomarkers and DNA strand breaks has been shown in type 1 and type 2 diabetes mellitus (Pacal et al. 2011). Similar correlation between DNA damage and oxidative stress has been shown in patients with rheumatoid arthritis (Altindag et al. 2007), coronary artery disease (Kaya et al. 2012), ulcerative colitis (Aslan et al. 2011), Down syndrome (Zana et al. 2006) and Graves disease (Tang et al. 2005). Moreover, oxidative stress and DNA damage have also been shown to be associated with infectious diseases such as hepatitis (Pal et al. 2010) and cutaneous leishmaniasis (Kocyigit et al. 2005). Impaired oxidant/antioxidant imbalance has been found in BD patients (Isik et al. 2007). High levels of malondialdehyde, protein carbonyl and 8-hydroxy deoxyguanosine, and decreases in the level/activity in the total sulfhydryl, super-oxide dismutase, catalase, zinc, glutathione peroxidase and glutathione have been documented in BD (Gulbahar et al. 2007; Harzallah et al. 2008; Karaman et al. 2009; Sezer et al. 2012; Taysi et al. 2007). The result “treatment of BD lymphocytes with vitamin E normalized elevated rate of SCE” provides a direct evidence that genotoxicity of BD is caused to a large extent by oxidant/antioxidant imbalance detected in the patients.

The results also showed that vitamin E not only protected lymphocytes from genotoxicity but also lowered basal rates of SCE by approximately 20 %. The anti-genotoxic property of vitamin E has been shown by several studies. Vitamin E has been shown to protect HepG2 cells and erythrocytes from genotoxic effects of Patulin and Deltamethrin, respectively (Ayed-Boussema et al. 2011; Kan et al. 2012). Moreover, pretreatment of cultured human lymphocytes with vitamin E has been shown to normalize

carbofuran-induced DNA damage as measured by the micronucleus assay (Sharma et al. 2012; Sharma and Sharma 2012).

The antimutagenic activity of vitamin E could be at least in part due to its strong anti-oxidant property. This property was demonstrated by several studies. For example, vitamin E has shown to overcome oxidative stress associated with chemical agents such as ferric nitrilotriacetate, hydrogen peroxide and pesticides (Agarwal et al. 2005; Agrawal and Sharma 2010; Bhatti et al. 2013; Saxena et al. 2011). On the other hand, Vitamin E depletion has been shown to enhance oxidative damage in stressed rats (Ohta et al. 2013). The consumption of vitamin E by BD patients has been shown to overcome oxidative stress in the patients (Gulbahar et al. 2007). Thus, vitamin E can be used clinically to relief oxidative stress and genotoxicity associated with disease.

An alternative hypothesis to explain genotoxicity associated with BH could be due to an intrinsic sensitivity of BD patients to mutagenic agents. However, the result showed that the rates of SCE induced by the strong mutagenic agent MMC in lymphocytes of BD patients and controls are similar. This suggests normal sensitivity of BD lymphocytes to mutagenic agents.

In conclusion, vitamin E can protect lymphocytes of BD patients from genotoxicity associated with the disease. BD lymphocytes showed normal sensitivity to mutagenic agents. The results might have clinical significance in protecting patients from genotoxicity associated with diseases that accompany by oxidative/antioxidative imbalance.

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Conflict of interest The authors have no conflict of interest to declare.

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