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ORIGINAL ARTICLE

Telomerase activity and apoptosis genes as parameters of lymphocyte aging in Down syndrome patients

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Abstract It is hypothesized that Down syndrome (DS) patients are associated with abnormalities of the immune system. Accordingly, this study was conducted to measure replicative aging and apoptosis in lymphocytes, which play an important role in the immune system, before and after being biostimulated with He:Ne laser. Replicative aging was measured in terms of telomerase activity, and ETS-2 gene relative expression. Apoptosis was measured in terms of DNA fragmentation and apoptosis genes (Fas, FasL and Bax) and antiapoptotic Bcl-2 protein. Results showed that Telomerase activity, ETS-2 mRNA expression, plasma DNA fragmentation, Fas and FasL were significantly higher among DS patients compared to controls: Telomerase activity (1.5 ± 0.5 vs. 0.9 ± 0.4, $p < 0.001$); ETS2 mRNA expression (0.6 ± 0.1 vs. 0.43 ± 0.04, $p < 0.0001$); plasma DNA fragmentation ($0.45\% \pm 0.12$ vs. $0.2\% \pm 0.1$, $p < 0.0001$); Fas protein (5.3 ± 1.2 vs. 2.3 ± 0.2, $p < 0.0001$); FasL mRNA relative expression (0.37 ± 0.05 vs. 0.24 ± 0.01, $p < 0.001$); Bax mRNA relative expression (0.9 ± 0.1 vs. 0.5 ± 0.1, $p < 0.00001$). Bcl-2 protein was significantly low in DS patients compared to controls (8.6 ± 1.3 vs. 10 ± 2.1, $p < 0.01$). He:Ne laser biostimulation applied to evaluate lymphocytes' response significantly increased the former parameters in DS patients compared to their level before irradiation, except for Bcl-2, which was significantly decreased. In conclusion: increased telomerase activity associated with increased activity and overexpression of ETS-2 on chromosome 21 in DS patients may contribute to the increased rate of early senescence in circulating lymphocytes, which consequently contributes to the abnormalities of the immune system observed in DS. Increased apoptosis is due to increased oxidative stress, which induces an increase in the apoptotic genes Bax, Fas and FasL accompanied by a decrease in the antiapoptotic gene Bcl-2.

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1. Introduction

Down syndrome (DS), or trisomy 21, is the most frequent genetic cause of mental retardation in man; the incidence is approximately one in 750 live births [1]. Autoimmune phenomena manifested by the increased frequency of hematological

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malignancies, autoimmune diseases and infections in DS, had already led to the hypothesis that DS is associated with abnormalities of the immune system [2–6].

Immunosenescence is the global term used to describe the observed age-associated decline in immune competence characterized by functional and phenotypic alterations to the immune system as a whole [7]. Immunosenescence in the elderly human population is associated with increased susceptibility to infectious diseases and cancer, reduced effectiveness of vaccinations, increased autoimmunity, and damage to various organ systems through dysregulated inflammation [8–12].

Cellular senescence is defined as the irreversible loss of division potential in somatic cells and is marked by a number of distinct phenotypic changes [13]. It is believed that senescence has an important role *in vivo*, protecting organisms on one hand against a build-up of cells containing DNA damage and on the other hand contributing to age-dependent tissue dysfunction [13]. Evidence is mounting that cells bearing senescent markers accumulate in several tissues with age [14,15], as well as in several age-related diseases [16].

At least a few hundred nucleotides of telomere repeats must ''cap'' each chromosome end in order to suppress DNA damage pathways. Critically short or ''uncapped'' telomeres may be repaired by the enzyme telomerase or by recombination [17]. However, the capacity of these telomere repair processes appears limited in most human somatic cells [18]. Apoptosis or cellular senescence is triggered when too many ''uncapped'' telomeres accumulate [19], not only posing a barrier to tumor growth, but also contributing to loss of cells with age [20]. However, human T lymphocytes are able to upregulate telomerase in concert with activation and thereby retarding telomere loss [21].

Cytogenetic and molecular studies of individuals with partial duplications of chromosome 21 and features of DS have sought a ''critical region'' on this chromosome, whose duplication is postulated to be both necessary and sufficient to produce the DS phenotype [22]. Located on the critical region is the transcription factor Erythroblastosis virus oncogene homolog 2 (ETS-2), which is a pro-oncogene, located in human chromosomal region 21q22.3 [23]. ETS-2 may be involved in the regulation of cellular proliferation and differentiation and may play a critical role in T-cell activation and cytokine production [24]. However, increased levels of ETS-2 have been shown to predispose to apoptosis [25]. An *in vitro* study indicated that ETS-2 promotes degeneration of DS neurons by activation of a mitochondrial pathway [26].

The apoptosis cascade can be triggered by 2 main pathways, via an intrinsic, endogenous system such as the mitochondrial Bax/Bcl-2 or via an extrinsic system involving transmembrane receptors of the Fas and FasL [27]. FasL is a 40-kDa type II membrane protein that belongs to the tumor necrosis factor superfamily and induces apoptosis through cognate interaction with its receptor Fas (CD95/Apo-1), which includes recognition of a distinctive element on the target cell by a corresponding lymphocyte receptor, formation of a contact zone between the two cells, and induction of cytolysis [28]. Activation of the Fas receptor through an interaction with transmembrane FasL leads to the recruitment and activation of numerous signaling molecules, including apoptosis-inducing transcription factors. This mechanism is crucial for the suicide of activated T cells and macrophages [29]. However this mechanism has not been previously measured in blood of DS.

The aim of the present study is to investigate whether lymphocytes in DS patients are subjected to early senescence. This is achieved by measuring replicative aging and apoptosis. Replicative aging was measured in terms of telomerase activity, and ETS-2 mRNA gene relative expression. Apoptosis was measured in terms of DNA fragmentation and apoptosis gene mRNA expression (Bax and FasL and fas protein) and antiapoptotic Bcl-2 protein. Stimulation of lymphocytes was carried out with He:Ne laser at laser fluency known to induce biostimulation in blood cells [30]. Markers of aging and apoptosis were measured in both DS and controls before and after laser irradiation.

Patients and methods

Patients

Patients were 62 Down syndrome cases (34 males and 28 females). Age ranged from 2 to 9 years (mean 4.2 ± 1 year). Controls were 30 matched healthy normal children (18 males and 12 females), age ranged from 2 to 9 years (mean 4.5 ± 1.1 years). DS patients were selected from cases already diagnosed by chromosomal karyotyping in the genetics unit, pediatric department, Cairo University. The selective criteria of the patients and controls were to be free from any infection and in good nutritive status. Blood was drawn after an informed consent of the parents in accordance with the current revision of the 1975 Helsinki Declaration.

Methods

Blood irradiation

Blood was distributed over 96 well tissue culture plates and were incubated for 48 h with and without irradiation with 2 J/cm^2 He:Ne laser at a wave length of 632.8 nm, which induces lymphocyte division.

Telomerase assay

Telomerase activity was determined using the telomerase repeat amplification protocol (TRAP). PCR ELISA protocol was carried according to the manufacturer's protocol (Boehringer Mannheim Biochemicals, Mannheim, Germany) [31].

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for ETS-2, FASL and Bax

Total RNA was extracted from lymphocytes using a QIAGEN RNA extraction Kit (QIAGEN Inc, USA). The RNA samples were reverse transcribed using superscript reverse transcriptase, using a QIAGEN One Step RT-PCR kit (QIAGEN Inc USA, Clini Lab). EST-2 primer sequences, forward: 5′-GTAGTG CAGAAAGClCCCGCAG-CGGCAGGATGAAT-3′ reverse 5′ GTCGGAGAAITCATGCCGAACCTCTGCAG-3′, FasL primer sequences, (forward: 5′-CAA GTC CAA CTC AAG GTC CAT GCC-3′; reverse: 5′-CAG AGA GAG CTC AGA TAC GTT- TGAC-3′). Primers for β-actin were synthesized simultaneously as an internal reference for all samples (forward: 5′-GTG GGG CGC CCC AGG CAC CA-3′; reverse: 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′). Bax sequences [32], (forward: 5′-CAC CAG CTC TGA- GCA GAT G-3′; reverse: 5′-GCG AGG CGG TGA- GCA CTC C-

3′). [33] 5 µl of RT reaction of each of the formed cDNA was processed for PCR. 10 µL from each PCR reaction product was separated on a 2% agarose gel then stained with ethidium bromide. The appearance of specific bands (ETS-2 470 bp, Bax 516 bp, β-actin 540 and FasL 345 bp) was evaluated under ultraviolet light and photographed.

Determination of Soluble Fas was in duplicate plasma samples. Soluble Fas protein was measured using commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits .This kit uses the competitive binding enzyme immunoassay format [34].

ELISA Bcl-2

The amounts of Bcl_2 in circulating lymphocytes were determined by a sandwich enzyme linked immunosorbent assay (ELISA) purchased from Clinical, using two anti-human BCL2 monoclonal murine antibodies [35].

DNA fragmentation assay

This is done according to the method of Ioannou and Chen 1996 [36]. In apoptosis DNA is broken down into fragments ranging from 20 to 200 base pairs. Separation of both fragmented and total DNA was carried out using DNA separating kit. DNA fragments were gradiently separated from the intact DNA using polyethylene glycol (5% in Ethyl ether) and then quantified spectrophotometrically using Hoechst 33258 (0.2 µg/ml) as a chromophore.

Statistical analysis

Each experimental condition was performed in triplicate. Data are expressed as mean ± SD. Comparisons were made by Student's *t*-test.

Results

Markers of cell proliferation in DS patients are presented in Table 1. Telomerase activity was significantly higher among DS patients compared to controls (1.5 ± 0.5 vs. 0.9 ± 0.4, $p < 0.001$). He: Ne laser irradiation significantly increased telomerase activity among DS patients compared to their levels before irradiation (20.4 ± 2 vs. 1.5 ± 0.5 $p < 0.00001$) and compared to controls post irradiation (20.4 ± 2 vs. 16.5 ± 4, $p < 0.001$). ETS-2 mRNA expression (Fig. 1) was significantly increased in DS patients compared to controls (0.6 ± 0.1 vs. 0.43 ± 0.04, $p < 0.0001$). Exposure to He:Ne laser induced a significant increase in ETS-2 mRNA expression in DS patients compared to itself before laser irradiation (0.8 ± 0.1 vs. 0.6 ± 0.1, $p < 0.01$) and compared to controls after irradiation (0.8 ± 0.1 vs. 0.5 ± 0.03, $p < 0.001$).

Figure 1 ETS-2 mRNA expression in DS patients compared to controls Lanes 1–3 before He:Ne laser and lanes 4–6 post HeNe laser.

Markers for apoptosis are presented in Table 2. Plasma DNA fragmentation was significantly higher among DS patients compared to controls (0.45% ± 0.12 vs. 0.2 ± 0.1%, $p < 0.0001$). Exposure to He:Ne laser induced a significant increase in DNA fragmentation in DS patients compared to itself before laser irradiation (0.87 ± 0.5% vs. 0.45 ± 0.12%, $p < 0.0001$) and compared to controls (0.87 ± 0.5% vs. 0.16 ± 0.02%, $p < 0.0001$).

Fas was significantly higher in DS patients compared to controls (5.3 ± 1.2 vs. 2.3 ± 0.2, $p < 0.0001$). Exposure to He:Ne laser induced a significant increase in Fas among DS patients compared to itself before laser irradiation (8.7 ± 2.8 vs. 5.3 ± 1.2, $p < 0.0001$) and compared to controls (8.7 ± 2.8 vs. 4.5 ± 1.3, $p < 0.001$). FasL mRNA expression (Fig. 2) in circulating lymphocytes was also significantly higher in DS patients compared to controls (0.37 ± 0.05 vs. 0.24 ± .04, $p < 0.01$) and became significantly increased after exposure to He:Ne laser compared to itself before laser irradiation (0.6 ± 0.03 vs. 0.37 ± 0.05, $p < 0.001$) and compared to controls (0.6 ± 0.03 vs. 0.13 ± 0.02, $p < 0.00001$).

There was a significant increase in Bax mRNA relative concentration (Fig. 3) in DS patients compared to controls (0.9 ± 0.01 vs. 0.5 ± 0.1, $p < 0.00001$) accompanied by a significant decrease in Bcl-2 protein in circulating lymphocytes compared to controls (8.6 ± 1.3 vs. 10 ± 2.1, $p < 0.01$). Biostimulation by He:Ne laser induced a significant increase in Bax mRNA relative concentration in DS patients compared to itself before laser irradiation (1.4 ± 0.5 vs. 0.9 ± 0.1, $p < 0.00001$) and compared to controls (1.4 ± 0.5 vs. 0.07 ± 0.02, $p < 0.00001$). This was accompanied by a significant decrease in Bcl-2 protein in circulating lymphocytes of DS patients compared to its level before laser irradiation (4.2 ± 0.9 vs. 8.6 ± 1.3, $p < 0.0001$) and compared to controls (4.2 ± 0.9 vs. 11.4 ± 4, $p < 0.00001$).

Discussion

Results showed that telomerase activity was significantly higher among DS patients compared to controls and that He:Ne biostimulation significantly increased its activity both in DS patients and controls. In old men with trisomy 21 it has been

Table 1 Markers of cell proliferation and replicative aging in DS patients compared to controls.

	Before laser irradiation			Post laser irradiation			
	DS patients	Controls	P^*	DS patients	P^{**}	Controls	P^{***}
Telomerase	1.5 ± 0.5	0.9 ± 0.4	0.001	20.4 ± 2	0.00001	16.5 ± 4	0.001
ETS-2	0.6 ± 0.1	0.43 ± 0.04	0.0001	0.8 ± 0.1	0.01	0.5 ± 0.03	0.001

P^*: DS vs. controls before laser.
P^{**}: DS before laser vs. DS post laser.
P^{***}: DS vs. controls post laser.

Table 2 Percentage of DNA fragmentation, FasL, Fas, Bax and Bcl-2 in blood of DS patients.

	Before laser irradiation			Post laser irradiation			
	DS patients	Controls	P^*	DS Patients	P^{**}	Controls	P^{***}
Percentage DNA fragmentation	0.45 ± 0.12	0.2 ±. 0.1	0.0001	0.87 ± 0.5	0.0001	0.16 ± 0.02	0.0001
Fas	5.3 ± 1.2	2.3 ± 0.2	0.0001	8.7 ± 2.8	0.0001	4.5 ± 1.3	0.001
FasL	0.37 ± 0.05	0.24 ± 0.01	0.001	0.6 ± .03	0.001	0.13 ± 0.02	0.00001
Bax	0.9 ± 0.1	0.5 ± 0.1	0.0001	1.4 ± 0.5	0.00001	0.07 ± 0.02	0.00001
Bcl-2	8.6 ± 1.3	10 ± 2.1	0.01	4.2 ± 0.9	0.0001	11.4 ± 4	0.00001

P^*: DS vs. controls before laser.
P^{**}: DS before laser vs. DS post laser.
P^{***}: DS vs. controls post laser.

Figure 2 FasL mRNA expression in DS patients before and after laser exposure Lanes 1–3 before He:Ne laser and lanes 4–6 post HeNe laser.

Figure 3 Bax mRNA expression in DS patients before and after laser exposure Lanes 1–3 before He:Ne laser and lanes 4–6 post He:Ne laser.

shown that increased activity of telomerase activity is accompanied by reduced telomere length [37]. This observation is regarded as one of the cytogenetic parameters that represents a state of genetic instability and is suggested to play a role in the pathomechanism of typical features of Down syndrome, such as dementia [38]. Telomere length was found to be significantly lower in amniocytes from trisomy 21 conceptions compared to the control group [39]. In the present study DS patients, which have been previously shown to have short telomeres [39], had increased telomerase activity, which is considered a phenomenon opposite to normal.

A proposed mechanism for DS features is based on the impact of gene dosage effect [40] due to the extra-genetic material of the supernumerary chromosome 21. It is estimated that approximately 500 genes could map to chromosome 21; currently, nearly 40 are known. Among them is the ETS-2 gene, which is a transcription factor involved in embryonic development [41] sometimes dysregulated in human cancers [42]. However, in the case of DS, an increased level of ETS-2 has been shown to predispose to apoptosis [41,43]. Results of the present study showed a significant increase in ETS-2 mRNA expression in DS patients compared to controls. This was accompanied by a significant increase in plasma DNA fragmentation, indicating that increased expression of ETS-2 mRNA induces apoptosis in DS lymphocytes. Previous studies indicated that DS patients presented increased DNA damage as measured by single cell gel electrophoresis assay in relation to controls [44]. Enhanced apoptosis is a common feature in tissues and organs of DS patients [45]. Thus results of the present study indicate that apoptosis in circulating lymphocytes is a feature in DS patients.

Fas is a type I transmembrane protein and a member of the tumor necrosis factor receptor (TNFR) family whereas FasL is a type II transmembrane protein and a member of TNF family. Binding of Fas by FasL induces apoptosis of the Fas-expressing cells [28]. In the present study plasma Fas has been shown to be significantly elevated in DS patients compared to controls. It has been previously shown that neurodegeneration in DS is associated with apoptosis due to overexpression of Fas as measured in four different regions of cerebral cortex and cerebellum in nine adult DS patients [46].

There was a significant increase in mitochondrial protein Bax mRNA relative concentration, accompanied by a significant decrease in anti-apoptotic Bcl-2 protein in circulating lymphocytes in DS patients indicating the involvement of mitochondria pathway in apoptosis induced in lymphocytes. It has been previously shown that *in vitro* ETS-2 upregulation in DS neurons promotes the activation of a mitochondrial-dependent proapoptotic pathway of cell death, which is accompanied by increased bax [40]. There was also at least fivefold elevation of Bax protein together with decreased Bcl-2 values in four cortical regions and the cerebellum of one fetal Down syndrome (35 weeks' gestation) postmortem brain sample compared with a control brain sample [47].

In the present study biostimulation of leucocytes *in vitro* using He:Ne laser significantly increased DNA fragmentation in DS patients and decreased Bcl-2 protein, while in controls DNA fragmentation was decreased and Bcl-2 protein was significantly increased. It is believed that He:Ne laser exerts its action by stimulating the activity of superoxide dismutase enzyme, which has been shown, previously, to increase in the skin of animals exposed to laser [30,48]. Exposure of DS blood to He:Ne laser stimulates an oxidative stress, which consequently increased apoptosis and its markers including Fas, FasL, Bax and decreased antiapoptotic protein including Bcl-2 protein.

The decrease in Bcl-2 protein can be explained by the fact that reactive oxygen species (which are increased in DS), are products, which have the potential to modulate critical cellular components such as DNA, proteins and lipids, resulting in the activation of mechanisms that lead either to cell proliferation or to programed cell death [49].

In the present study He:Ne laser has been shown to stimulate the expression of ETS-2 gene. It is well known that the ETS-2 gene family encodes a family of transcription factors involved in a variety of biological processes, including growth

control, transformation, T cell activation and developmental programs [44,50–52]. However in the case of DS patients stimulation of ETS-2 genes induces apoptosis [28].

It is well established that genetic condition like Down's syndrome causes premature aging accompanying global senescence in various organ levels and that the rate of aging for DS patients is nearly a twofold increase as compared to healthy subjects [53]. Aging is associated with a decline in immune responses [54]. Although many aspects of the immune system demonstrate age-associated alterations, T cells show the greatest and most consistent changes [14,15]. Various immunological theories and observations to explain the predisposition of individuals with DS to infections have been published [15] and that increased apoptosis is the main cornerstone for this reduced infection suggesting that DS cells may be more sensitive to proapoptotic stimuli than normal cells [54]. Thus results of the present study provide further evidence that apoptosis is a hallmark in DS patients.

In conclusion: telomerase increased activity and overexpression of ETS-2 on chromosome 21 in DS patients may contribute to the increased rate of early senescence in circulating lymphocytes. This together with decreased antiapoptotic protein Bcl-2 promotes apoptosis, which consequently contributes to the abnormalities of the immune system observed in DS patients.

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