Impact of treatment with methimazole on the Bcl-2 expression in CD8+ peripheral blood lymphocytes in children with Graves' disease

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Abstract

Introduction and objectives: The protein product of the proto-oncogene Bcl-2 is a physiological inhibitor of programmed cell death. The results obtained in our previous researches suggest that apoptosis may be involved in the regulation of an immune response in hyperthyroidism. The most common cause of hyperthyroidism is Graves' disease. The aim of this study was evaluation of expression of Bcl-2 protein in peripheral blood T lymphocytes in hyperthyroid children due to Graves' disease (GD) before and after therapy with methimazole (MMI), in comparison with healthy controls.
Material and methods: Thirty-two children with newly diagnosed hyperthyreosis due to GD before and after 4–6 weeks treatment with MMI, and 20 healthy controls were included into the study. The staining with monoclonal antibodies against CD8 and Bcl-2 was performed within 2 hours after collection, and followed with flow cytometry acquisition and analysis.
Results: Our study revealed that the expression of Bcl-2 protein in circulating CD8+ T lymphocytes of patients with hyperthyreosis was significantly lower than in healthy controls (p<0.03). The expression of Bcl-2 after 4–6 week therapy with MMI returned to normal level. The difference in Bcl-2 expression between patients after treatment and control group was insignificant.
Conclusions: The use of MMI in the treatment of hyperthyroidism due to GD leads to the normalization of the Bcl-2 expression on the CD8+ lymphocytes in peripheral blood. Our findings suggest that the changes in the expression of Bcl-2 on the CD8+ cells indicate the involvement of these cells and Bcl-2-regulated apoptotic pathway in the pathogenesis of GD.

Key words

Bcl-2, Graves' disease, methimazole, T lymphocytes

INTRODUCTION

The pathogenesis of hyperthyroidism in the course of Graves' disease (GD), which is an autoimmune disorder, is not fully understood. Immune disturbances, genetic predisposition and environmental factors affecting the thyroid gland, appear to play the main role in the contribution and development of the disease.

The most common cause of hyperthyroidism is GD, followed by toxic multinodular goitre, or thyroiditis. The prevalence of hyperthyroidism in females between 0.5 – 2%, and is 10 times more common in females than in males in iodine-replete communities. In research called NHANES III, in those subjects who were neither taking thyroid medications nor reported histories of thyroid disease, 2 per 1000 had 'clinically significant' hyperthyroidism, defined as a serum TSH concentration 0.1 mIU/l and a serum total T4 concentration 170 nmol/l [1]. The prevalence data in elderly persons show a wide range between 0.4 – 2.0% [2], and a higher prevalence is seen in iodine-deficient areas [3]. GD is by far the most common cause of hyperthyroidism in children and adolescents. In a national population-based study of thyrotoxicosis from the United Kingdom and Ireland in the year 2004, the annual incidence was 0.9 per 100,000 children <15 years of age, with Graves' disease

accounting for 96% of cases [4]. Overall, the prevalence of Graves' hyperthyroidism in children is approximately 0.02% (1:5,000), mostly in the age group 11–15 [5]. In a report of 143 children with GD, 38% were prepubertal at diagnosis [6]. Girls are affected more commonly than boys, at a ratio of about 5:1. The ratio is considerably lower among younger children, suggesting that estrogen secretion in some way affects the occurrence of GD. The incidence data available for overt hyperthyroidism in males and females from large population studies are comparable, at 0.4 per 1,000 females and 0.1 per 1000 males, but the age-specific incidence varies considerably. The peak age-specific incidence of GD was between 20 – 49 years in two studies, but increased with age in Iceland and peaked at 60–69 years in Malmo, Sweden [7]. Other cohort studies provide comparable incidence data, which suggests that many cases of hyperthyroidism remain undiagnosed in the community unless routine testing is undertaken [8]. In the large population study in Tayside, Scotland, 620 incident cases of hyperthyroidism were identified with an incidence rate of 0.77/1,000 per year (95% CI): 0.70–0.84) in females and 0.14/1,000 per year (95% CI: 0.12–0.18) in males [9]. The incidence increased with age, and females were affected 2 – 8 times more than males across the age range. Recent further analysis suggested that the incidence of thyrotoxicosis was increasing in females but not in males between 1997 – 2001 [10].

The current concept is that thyroid-specific T cells in GD primarily act as helper (CD4+ Th1) cells. When activated, these lymphocytes secrete interleukin-2 (IL-2), interferon γ, and

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tumour necrosis factor-γ, which activate cytotoxic (CD8+) cells and may induce thyroid cell apoptosis. The Th2 lymphocytes secrete IL-4 and IL-5, and activate antibody production [11, 12].

Apoptosis, also referred to as 'cell suicide' or 'programmed cell death', is an active mode of cell death. The control of cell survival is of vital importance in tissues with high cell turnover, such as the lymphoid system. Activation and proliferation of lymphocytes due to antigen stimulation leading to the increase in their number should be efficiently controlled. Just after ending the immunological response, the increased number of activated lymphocytes should return to the primary condition preserving the necessary number of long-living memory lymphocytes (CD45RA-/CD45RO+). These memory cells are necessary for their quick response to the same antigen. When their task is performed the majority of activated T and B lymphocyte undergo apoptosis and their amount returns to normal [13].

One of the most important findings of the studies on autoimmune diseases' biology is the discovery that apoptosis plays an essential role in the pathogenesis of several disorders belonging to this group. The results from our previous study indicated that the spontaneous apoptosis measured using chloromethyl-X-rosamine (CMXRos) to detect disruptions in mitochondrial membrane potential ($\Delta\psi_m$), one of the earliest events in the apoptotic pathway, in GD patients before treatment, was significantly lower in comparison with the same group of children with methimazole (MMI)-induced euthyroidism [14].

Genes homologous to Bcl-2, play a key role in regulating programmed cell death (apoptosis). The protein encoded by the Bcl-2 (B cell lymphoma/leukemia-2) proto-oncogene has been involved in the regulation of lymphocyte cell survival and has been shown to interfere with apoptosis. The Bcl-2 gene product regulates programmed cell death, and a number of experiments suggest that Bcl-2 is involved in the selection and maintenance of long-lived memory T cells. Up-regulation of Bcl-2 and its continued expression appears to be the normal mechanism for the survival of mature peripheral T cells [15].

It seems interesting to find answers to the questions: how does this mechanism work in GD adolescent patients in comparison with healthy controls, and how does the MMI influence Bcl-2 expression?

The aim of this study was evaluation of the expression Bcl-2 protein in peripheral blood T lymphocytes in hyperthyroid children due to GD before and after therapy with methimazole, in comparison with healthy controls.

MATERIALS AND METHODS

Study group and control group
Peripheral blood (PB) was obtained from 32 previously untreated adolescent patients with hyperthyroidism due to GD (median age: 13.20±3.62 years; 25 females, 7 males) from rural areas in Lublin province, hospitalized in the Department of Paediatrics, Division of Paediatric Endocrinology in Lublin, Poland. Children with any other health problems or using other medicaments at the time of examination or in the past were excluded from the examined group. The patients were diagnosed on the basis of clinical examination as well as morphological and immunological criteria. The patients were treated with MMI at initial doses

of 0.50±0.05 mg/kg body weight/day during approximately 4–6 weeks, and after that time, when in euthyroidism, blood samples were collected again to assess apoptosis. Later, the patients received maintenance doses of approximately 0.1 mg/kg body weight/day (mainly 5 mg/day) MMI in combination with a low dose of L-thyroxin. Table I presents patient baseline characteristics.

Control group consisted of 20 healthy volunteers (median age: 12.52±1.83 years; 14 females, 6 males).

The study was approved by the Local Ethical Committee, and informed consent was signed by all the tested individuals.

Table 1. Patients' characteristics.

Number of patients		32
Gender (Female / Male)		25 / 7
Age [years]	mean ± SD median (min.- max.)	13.20 ± 3.62 13.5 (11 – 17)
Body weight [kg]	mean ± SD median (min.- max.)	45.48 ± 12.52 47.55 (34–71)
Dosage of methimazole [mg/kg/day]	mean ± SD median (min.- max.)	0.50 ± 0.05 0.5 (0.42–0.65)
Total daily dose of methimazole [mg/day]	mean ± SD median (min.- max.)	24 ± 5.63 22.5 (15–40)
Treatment duration of hyperthyroidism (from diagnosis to methimazole-induced euthyrosis) [days]	mean ± SD median (min.- max.)	2 ± 10.08 30 (24 – 49)

Flow cytometric analysis
The cell surface antigens and Bcl-2 mitochondrial oncoprotein in each case were determined on fresh cells immediately after collection. Mononuclear cells were isolated by density centrifugation on Lymphoprep (Nycomed, Norway) and washed twice in phosphate buffered saline (PBS) containing 1% bovine serum albumin. Double colour immunofluorescence studies were performed using combinations of phycoerythrin (PE) and fluorescein isothyocyanate (FITC) conjugated monoclonal antibodies. Monoclonal antibodies were obtained from Ortho Diagnostic Systems (Germany), Becton Dickinson (Germany), or Dako (Denmark). The following antibody combinations were used: FITC IgG1/IgG2a PE negative control antibody, and CD8 PE. 10^6 cells were incubated with antibodies for 30 min at 4°C and washed twice with PBS afterwards. For the immunodetection of the intracellular Bcl-2 protein, 10^6 cells were fixed and permeabilized with 0.25% paraformaldehyde (15 minutes at room temperature) and cooled to 4°C in 70% methanol for 60 minutes before incubation with FITC conjugated Bcl-2 mouse monoclonal antibody or IgG1 FITC negative control. All samples were measured on a FACSCalibur flow cytometer (Becton Dickinson). 10,000 cells per test were analyzed. In order to estimate the levels of fluorescence, the mean fluorescence intensity and fluorescence signal strength of the Bcl-2+ cells were calculated. The mean fluorescence intensity and fluorescence signal strength of the Bcl-2 histogram were measured from the upper limit of the negative control. Figure 1 presents a sample flow cytometric analysis of Bcl-2 expression in CD8+ lymphocytes.
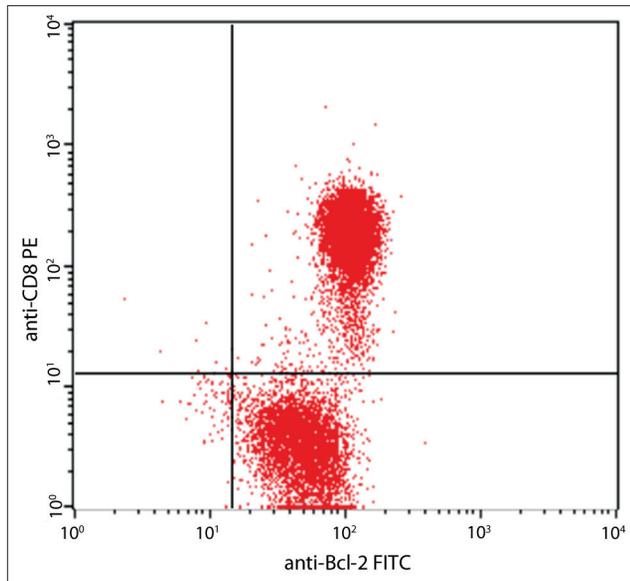
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Figure 1. Sample flow cytometric analysis of Bcl-2 expression in CD8+ lymphocytes.

Statistical analysis

Data processing and analysis were performed using Statistica 7.0 PL software, StatSoft Inc. The results are expressed as mean±SD, minimum, maximum and median values. Continuous variables were compared using a nonparametric test (*U* Mann-Whitney test). Statistical significance was set at p<0.05.

RESULTS

The presented study revealed that the expression of Bcl-2 protein in circulating CD8+ T lymphocytes of patients with hyperthyreosis measured as the mean fluorescence intensity was 116±10.02, min. 108, max. 124, median 114, and was significantly lower than in healthy controls: 124.65±10.47, min. 114.5, max. 135, median 126 (p=0.0018) (Fig. 2). Expression of Bcl-2 after approximately 4–6 weeks therapy with MMI returned to normal level 122.4±8.92), and this increase was statistically significant (p=0.0021) (Fig. 3). The difference of Bcl-2 expression between patients after treatment and control group was insignificant (p>0.05) (Fig. 4).

DISCUSSION

The Bcl-2 protein group plays an important role in the regulation of apoptosis connected with the absence of signals originating in cytokines. This group consists of antiapoptotic (Bcl-2, Bcl-XL) and proapoptotic (Bcl-2 associated protein X, Bax; B-cell homologous antagonist/killer, bak) molecules [16]. A decreased expression of the anti-apoptotic Bcl-2 protein, is observed in apoptotic cells. The increased expression of some members of this group (e.g. Bcl-2 and Bcl-x) check apoptosis in case of deficiency of cytokines. Over-expression of other subset from this group (e.g. Bax) suppresses signals originating from cytokine receptors and induces apoptosis [17].

Several mechanisms are probably involved in determining the evolution of autoimmune thyroid disease towards
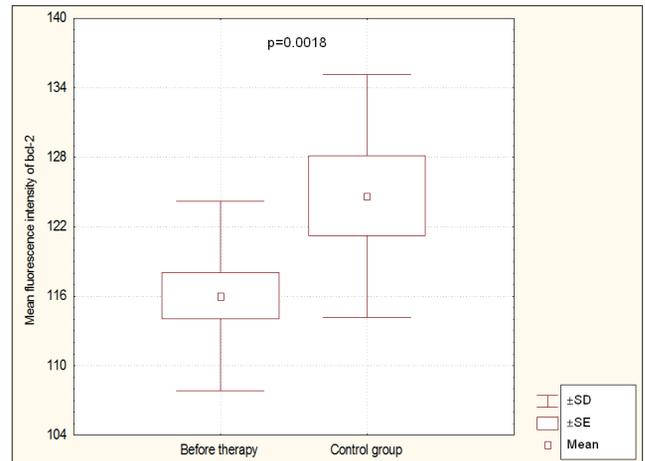

Figure 2. Expression of Bcl-2 protein in T (CD8+) lymphocytes from children with hyperthyreosis before therapy and in the control group.
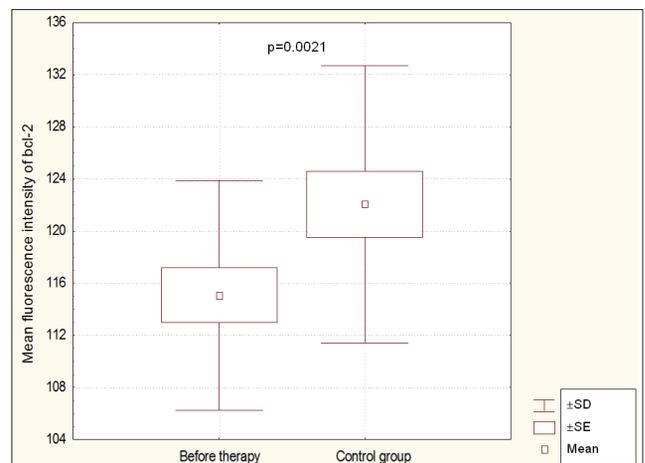

Figure 3. Expression of Bcl-2 protein in T (CD8+) lymphocytes from children with hyperthyreosis before and after therapy.
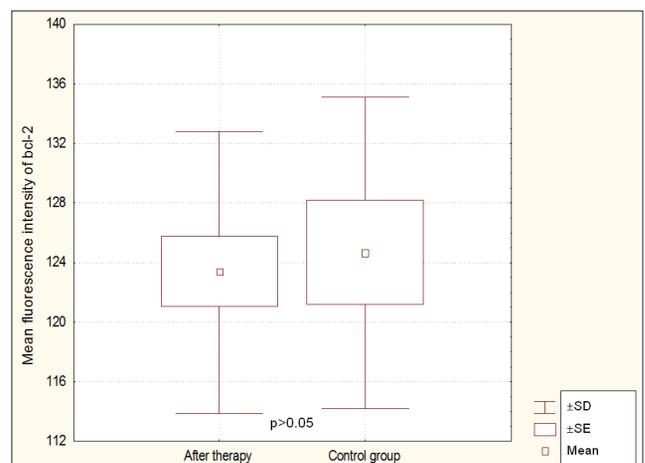

Figure 4. Expression of Bcl-2 protein in T (CD8+) lymphocytes from children with hyperthyreosis after therapy and in the control group.

either hypothyroidism and clinical syndrome known as Hashimoto's thyroiditis (HT), or toward hyperthyroidism and the symptoms of GD. The findings of Giordano et al. suggest that in GD the regulation of Fas/FasL/Bcl2 favours the apoptosis of infiltrating lymphocytes, possibly limiting their auto-reactive potential and impairing their ability

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to mediate tissue damage. Moreover, the reduced levels of Fas/FasL and increased levels of Bcl-2 favour thyrocyte survival, and favour the thyrocyte hypertrophy associated with immunoglobulins stimulating the thyrotropin receptor. In contrast, the regulation of Fas/FasL/Bcl2 expression in HT causes thyrocyte apoptosis, tissue damage, and a gradual reduction in thyrocyte numbers, leading to hypothyroidism [18]. Xu et al. revealed that the regulation of Fas/FasL/Bcl-2 in GD favours apoptosis of infiltrating lymphocytes and thyrocyte survival. The regulation of Fas/FasL/Bcl-2 in HT may promote thyrocyte apoptosis leading to hypothyroidism [19].

Available literature does not present any research on the expression of Bcl-2 protein in lymphocytes of peripheral blood in hyperthyroid children. In the presented study, a significantly lower expression of the Bcl-2 protein in circulating CD8+ T lymphocytes was found in patients with hyperthyreosis than in healthy controls. Expression of Bcl-2 after 4–6 week therapy with MMI returned to normal level. The difference in Bcl-2 expression between patients after treatment and in the control group was insignificant.

MMI is a drug of choice for GD. The typical MMI dose is 0.2–0.5 mg/kg per day, with a range from 0.1 – 1.0 mg/kg per day. MMI reduces thyroid hormone synthesis by inhibiting the oxidation and organic binding of thyroid iodide. It is 10 – 20-fold more potent than other anti-thyroid drugs and has a longer half-life. The mechanism of action of the immunosuppressive effect of anti-thyroid drugs has remained a matter of controversy. According to Volpe, the drugs act on the thyroid cells, reducing their thyroid hormone production and other activities, with a consequent reduction in thyrocyte-immunocyte signaling. The reduction in the activation of CD4+ cells, the increased number and activation of CD8+ cells, and the reduction of soluble interleukin-2 receptors are now shown to be due to amelioration of the hyperthyroidism [20]. Other authors found that treatment with thyroid hormones of T lymphocytes induced reduction of mitochondrial transmembrane potential and production of reactive oxygen species, both of which are intimately associated with apoptotic cell death. In addition, cellular expression of antiapoptotic Bcl-2 protein was clearly reduced by the treatment of lymphocytes with thyroid hormones *in vitro* [21]. Thionamides also lower the levels of thyroid autoantibodies, sometimes leading to long-term remission [22]. Fas ligand (FasL) induces apoptosis of susceptible cells by cross-linking its own receptor, Fas. While Fas is present in a wide variety of normal tissues, FasL expression is limited mainly to cells of the immune system, where it acts as an effector molecule of cell-mediated cytotoxicity, and eliminates infiltrating lymphocytes in immune-privileged organs. FasL expression is very weak in cultured thyrocytes, whereas it is strong in thionamide-treated GD glands and cultured thyrocytes. Methimazole-treated thyrocytes induce FasL-dependent apoptosis in co-cultured lymphocytes [23]. Therefore, the results of the presented study showing a correlation between MMI and the increment of apoptotic cells, confirm that this drug is able to induce lymphocyte apoptosis. MMI seems to act not only by the Fas-FasL pathway [23], but also by interaction with the Bcl-2 expression, and may contribute to the immunomodulatory effect of thionamides in this disease.

The results of this research indicate that the apoptosis in GD patients before treatment was significantly lower compared to the same group of children with methimazole-induced euthyroidism. The presented study revealed that the normalization of thyroid hormones increases lymphocyte apoptosis. Higher apoptosis of suppressor CD8+ cells can be involved in the development of autoimmune process in patients with hyperthyreosis. The results of the presented study seem to reveal that thyroid hormones also exert a negative or even no influence on cellular apoptosis *ex vivo*, as the treatment of hyperthyroidism in all patients leads to the increment of apoptotic cells. Other authors have shown that thyroid hormones inhibit apoptosis of early differentiating cerebellar granule neurons through an increase in the amounts of Bcl-2 protein [24]. Thus, increased serum Bcl-2 may be linked to accelerated apoptosis. In euthyroid Hashimoto's thyroiditis patients compared with controls and euthyroid GD, increased serum Bcl-2 has been reported [24]. In the study of Jiskra et al., a tendency towards higher Bcl-2 in Hashimoto's thyroiditis patients was found, with no difference in serum Bcl-2 between hyperthyroid GD and when the euthyroid state was achieved [25].

CONCLUSIONS

The use of methimazole in the treatment of hyperthyroidism due to GD leads to the normalization of the Bcl-2 expression on the CD8+ lymphocytes in peripheral blood. Our findings suggest that the changes in the expression of Bcl-2 on the CD8+ cells indicate the involvement of these cells and Bcl-2-regulated apoptotic pathway in the pathogenesis of GD. The methimazole seems to act by the interaction with the Bcl-2 expression and may contribute to the immunomodulatory effect of thioamides in GD.

Acknowledgements

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