6. INCREASED SPONTANEOUS APOPTOSIS IN T LYMPHOCYTES IN DiGEORGE ANOMALY

INTRODUCTION

DiGeorge anomaly (formerly called DiGeorge syndrome) is a developmental defect of the pharyngeal pouches, manifested by typical facies, cardiovascular defects, thymic defects, increased susceptibility to a variety of infections, and in some cases absence of the thyroid and parathyroid glands (324, 325). Circulating thymic hormone levels are significantly reduced or absent (326, 327). This disorder is inherited as autosomal dominant, autosomal recessive, or sporadic. The etiology of DiGeorge anomaly is not known; however, a number of chromosomal abnormalities have been described, including monosomy 22q and 22d deletion (324, 328).

Apoptosis, or programmed cell death, is a process whereby a diverse group of developmental or environmental factors activate a genetic program that leads to the death and efficient disposal of a cell (35, 329). Apoptosis is essential for normal development and cellular homeostasis (319, 330, 331). In the immune system, apoptosis appears to play an important role in selection of the T cells in the thymus and in peripheral tolerance (319, 330). Apoptosis is tightly regulated by a number of gene products that either promote apoptosis or extend cell survival (332). Fas antigen (CD95/APO-1) is a type I transmembrane protein which belongs to the tumor necrosis factor receptor (TNF-R)/nerve growth factor receptor (NGF-R) family (44). Fas ligand (FasL) is a type II transmembrane protein that belongs to the TNF family (99). Fas transduces signal to induce apoptosis when cross-linked by specific agonistic antibodies or FasL (44, 98). In contrast, Bcl-2, the founding member of a multigene family, prolongs the survival of cells (71, 332) and inhibits Fas-mediated apoptosis.
The long form of alternatively spliced Bcl-x, the Bcl-XL, also prolongs the survival of cells (322). We hypothesized that T cell deficiency in DiGeorge anomaly may be in part due to increased apoptosis of T cells. Our data show that T cells from a patient with DiGeorge anomaly expressed increased levels of Fas and FasL and decreased levels of Bcl-2. In addition, a significant proportion of T cell subsets underwent spontaneous apoptosis \textit{in vitro}.
Results

6.1 Case Report

This 15-month-old girl was born at the 40th week of gestation to an 18-year-old mother with gravida 3 para 3. The birth weight was 2850 grams. At birth, the patient was found to have congenital heart disease diagnosed as interrupted aortic arch and ventricular septal defect. This defect was surgically corrected. The patient has the characteristic facies of patients with DiGeorge anomaly, and fluorescent in situ hybridization (FISH) analysis of chromosomes showed a deletion of 22q 11 locus D22S75, confirming the diagnosis of DiGeorge anomaly. Serum levels of parathyroid and thyroid hormones, and total and ionized calcium, were normal. Serum thymosin-α1 was absent. Since birth, the patient has had multiple hospitalizations, one almost every 4-6 weeks, for various pulmonary and systemic infections with a variety of organisms, including Actinomycetes species, Enterobacter aerogenes, parainfluenza type 3, Hemophilus influenzae, Staphylococcus aureus, Streptococcus viridans, and Moraxella catarrhalis.

6.2 Immunological profile of patient with DiGeorge anomaly

In order to determine the immunological profile of the patient with DiGeorge anomaly, the proportions of subsets were determined using dual color flow cytometry and the lymphocytic functions determined using lymphocyte transformation assays by DNA synthesis. The percent and absolute number of T and B cell subsets and their subpopulations are summarized in Table 6.1.

For lymphocyte transformation assays, response to mitogens (including PHA, ConA, and PWM) and response to antigens (including Candida albicans and Tetanus toxoid) was determined. Freshly isolated lymphocytes (5x10⁵) from the
patient and age-matched control were incubated with or without PHA (1 μg/ml), ConA (1 μg/ml) and PWM (1 μg/ml) for 72 hours. For lymphocyte transformation assays for antigens, cells (5x10⁵) were incubated with Candida albicans (1 μg/ml) and Tetanus toxoid (1 μg/ml) for six days. Following incubation, the cells were incubated in the presence of (³H) thymidine for another 16 hours. The cells were harvested and the tritiated thymidine incorporated determined in cpm. The results obtained from the patient and control are summarized in Table 6.2.

**Table 6.1: Immunological Profile of patient with DiGeorge anomaly**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Patient</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3⁺ T cells (#)</td>
<td>39% (393)</td>
<td>62-69 (1800-3000)</td>
</tr>
<tr>
<td>CD4⁺ T cells (#)</td>
<td>32% (323)</td>
<td>30-40 (1000-1800)</td>
</tr>
<tr>
<td>CD8⁺ T cells (#)</td>
<td>7% (71)</td>
<td>25-32 (800-1500)</td>
</tr>
<tr>
<td>CD19⁺ T cells (#)</td>
<td>34% (343)</td>
<td>21-28 (700-1300)</td>
</tr>
<tr>
<td>CD16⁻CD16⁺(#)</td>
<td>24% (242)</td>
<td>8-15 (200-600)</td>
</tr>
<tr>
<td>CD4⁺CD45RA⁺/CD45RO⁺</td>
<td>0.7</td>
<td>2.6-7.8</td>
</tr>
<tr>
<td>CD8⁺CD45RA⁺/CD45RO⁺</td>
<td>2.4</td>
<td>5.0-10.0</td>
</tr>
</tbody>
</table>
Table 6.2: Immunological Profile of patient with DiGeorge anomaly

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Patient</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Response to mitogens (cpm)</td>
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<tr>
<td>PHA</td>
<td>23890</td>
<td>147,433-213,091</td>
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<tr>
<td>ConA</td>
<td>39410</td>
<td>158,715-261,277</td>
</tr>
<tr>
<td>PWM</td>
<td>40901</td>
<td>37,199-71,178</td>
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<tr>
<td></td>
<td>Response to antigens (cpm)</td>
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<tr>
<td>Candida</td>
<td>7791</td>
<td>30,332-87,196</td>
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<tr>
<td>Tetanus</td>
<td>1584</td>
<td>12,864-95,480</td>
</tr>
</tbody>
</table>

6.3 Expression of Fas, FasL, and Bcl-2

Expression of Fas, FasL, and Bcl-2 was examined in freshly isolated MNC with specific monoclonal antibodies and isotype controls by dual color analysis, using FACScan. Data for Fas expression are shown in Figure 6.1. A higher proportion of both CD4⁺ (85%) and CD8⁺ (80%) T cells from the patient expressed Fas as compared to age-matched healthy control (CD4⁺:9% and CD8⁺:4%). FasL is generally expressed on activated T cells (334); however, a significantly increased (P<0.001) proportion of freshly isolated CD4⁺ (12%) and CD8⁺ (14%) T cells from the patient expressed FasL as compared to CD4⁺ (1.2%) and CD8⁺ (0.8%) T cells from age-matched control (Figure 6.2). Data on Bcl-2 expression are shown in Figure 6.3. Decreased expression of Bcl-2 was observed in both CD4⁺ (MFC# 42) and CD8⁺ (MFC# 48) T cells from the patient as compared to CD4⁺ (MFC# 280) and CD8⁺ (MFC# 270) T cells from age-matched control.
Figure 6.1 Expression of Fas in CD4⁺ and CD8⁺ T cells
Freshly isolated lymphocytes from patient with DiGeorge anomaly and an age-matched control were stained for Fas using antibodies against Fas, CD4 and CD8. G1 is isotype control. Numbers in inset represent percent Fas⁺ cells in each subset.
Figure 6.2 Expression of FasL in CD4+ and CD8+ T cells
Freshly isolated T cells from patient with DiGeorge's anomaly and an age-matched control were activated as described in Materials and Methods and stained using antibodies to FasL, CD4 and CD8. Dotted lines represent isotype controls. Numbers in inset represent percent FasL+ cells.
Figure 6.3 Expression of Bcl-2 in CD4$^+$ and CD8$^+$ T cells
Freshly isolated lymphocytes from patient with DiGeorge's anomaly and an age-matched control were permeabilized and stained using antibodies to Bcl-2, CD4 and CD8. Data in inset represent mean fluorescence channel numbers (MFC#).
6.4 Expression of fas, bcl-2, and bcl-xL mRNA

The expression of fas, bcl-2, and bcl-xL was measured by quantitative RT-PCR. Graded amounts of cDNA were used. Mean±SD data (in cpm) obtained from three experiments are shown in Figure 6.4. A significantly higher (P<0.001) level of fas mRNA and a lower level of bcl-2 mRNA were observed in the patient’s MNC as compared to age-matched control. No significant difference was observed in bcl-xL mRNA between patient and control. Representative gel photograph following quantitative PCR using 2 µl cDNA is shown in Figure 6.5.

6.5 Spontaneous Apoptosis

In vitro spontaneous apoptosis was examined quantitatively by PI staining, using FACScan and DNA fragmentation by gel electrophoresis. Freshly isolated lymphocytes from the patient and age-matched control were i) stained with anti-CD4, anti-CD8, and propidium iodide and percent dead cells determined by dual color flow cytometry and ii) DNA was extracted DNA fragmentation determined using gel electrophoresis. Data for PI staining are shown in Figure 6.6. A significant proportion of both CD4+ (12%) and CD8+ (10%) T cells from the patient underwent apoptosis as compared to none in age-matched control. Increased DNA fragmentation was observed in the patient’s MNC but not in the cells from control (Figure 6.7).
**fas, bcl-2 and bcl-xL expression in Digilorge's syndrome**

![Graph showing expression levels of fas, bcl-2, and bcl-xL](image)

**Figure 6.4 Expression for fas, bcl-2 and bcl-xL mRNA**

Total cellular RNA from lymphocytes from DiGeorge's anomaly and an age-matched control was prepared. Quantitative PCR analysis was performed using 2 μl cDNA from RT. Mean±SD cpm ratio for fas/β-actin, bcl-2/β-actin, and bcl-xL/β-actin are shown.
Figure 6.5 Gel electrophoresis of quantitative PCR
Representative gel photograph following quantitative PCR using fas, bcl-2 and bcl-x_L primers in patient (P) and age-matched control (C) is shown. **β-actin** was used as an internal control.
Figure 6.6 *In vitro* spontaneous apoptosis of CD4+ and CD8+ T cells
The percent dead cells from freshly isolated lymphocytes from patient with DiGeorge's anomaly and an age-matched control were determined using propidium iodide staining method.
Figure 6.7 DNA fragmentation in mononuclear cells
DNA was extracted from freshly isolated lymphocytes from the patient with DiGeorge’s anomaly (P) and an age-matched control (C) and electrophoresed on a 2% agarose gel. M is the DNA marker.
DISCUSSION

The present patient with DiGeorge anomaly has an immunological profile that is consistent with what would be termed “partial DiGeorge syndrome”. Similar to other patients with DiGeorge anomaly, the present patient has a history of recurrent and severe infections necessitating multiple hospitalizations.

A number of genes and their encoded proteins have been shown to regulate apoptosis (reviewed in 330). The Fas/FasL system appears to play an important role in the induction of apoptosis and in activation-induced T cell death (44, 250, 331, 335). In contrast, Bcl-2 and Bcl-xL inhibit apoptosis and prolong cell survival (71, 322, 332). In the present patient with DiGeorge anomaly, expression of Fas and FasL was increased and Bcl-2 expression was decreased in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Furthermore, a significant proportion of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells underwent spontaneous apoptosis. *In vitro* activation of T cells is associated with up-regulation of Fas and down-regulation of Bcl-2 (264). Furthermore, FasL is predominantly expressed on activated T cells (267, 336). Therefore, it is likely that T cells in the present patients are activated *in vivo*. Although the precise mechanism of spontaneous apoptosis of T cells in the patient is unclear, Fas/FasL interaction may play a major role. The deletion of activated T cells in the periphery depends critically on Fas/FasL interactions (250, 335). In this context, it is interesting to note that in the present patient, the proportions of CD4<sup>+</sup> and CD8<sup>+</sup> T cells that underwent spontaneous apoptosis corresponded to the proportions of CD4<sup>+</sup> and CD8<sup>+</sup> T cells that expressed FasL. A role of interaction between cell surface Fas and soluble FasL in spontaneous apoptosis cannot be excluded. Soluble FasL is produced by activated T cells (156, 336) and shown to induce apoptosis by interacting with cell surface Fas in an autocrine
fashion (248). Recently, it has been observed that both mouse (337) and human (338) naive T cells are sensitive and human memory (CD45RO+) subsets of both CD4+ and CD8+ T cells are resistant to apoptosis by soluble FasL (338). This could explain increased proportions of CD45RO+CD4+ and CD45RO+CD8+ T cells in patient.

It has been demonstrated that immature double positive (CD4+CD8+) thymocytes (which are the potential target cells for apoptosis mediated by negative selection or neglect) express a high level of Fas (339) and are selectively killed by agonistic Fas antibodies (340) or by soluble FasL (337). However, in the present patient less than 1% T cells were double positive and no γ/δ T cell receptor positive T cells were present in the peripheral blood (data not shown).

Bcl-2 and Bcl-xL proteins differentially regulate lymphoid apoptosis (71, 332). Both proteins inhibit Fas-mediated apoptosis (71, 333). In the present patient Bcl-2 expression was decreased in both CD4+ and CD8+ T cells; however, no significant difference was observed in Bcl-xL expression. Since Bcl-2 is an anti-apoptotic protein and inhibits Fas-mediated apoptosis, it is likely that, in the present patient, decreased Bcl-2 expression may contribute to Fas/FasL-induced spontaneous apoptosis of T cell subsets.

In summary, increased expression of Fas/FasL and decreased expression of Bcl-2 appear to play a role in spontaneous apoptosis of T cells and T cell deficiency in DiGeorge anomaly. Although apoptosis was analyzed at a time when the patient had no evidence of overt clinical infection, a role of sub-clinical infection cannot be excluded. McCormack et al. (341) have reported profound apoptosis of mature T cells in vivo by chronic exposure to exogenous superantigen.
SUMMARY

The purpose of the study was to determine whether increased apoptosis in the peripheral blood lymphocytes plays a role in T cell deficiency associated with DiGeorge anomaly. T cell subsets from a patient with DiGeorge anomaly were examined for the expression of Fas, FasL, Bcl-2, and Bcl-XL at the protein level with monoclonal antibodies, using dual color flow cytometry, and at the mRNA level in mononuclear cells by quantitative reverse transcriptase polymerase chain reaction. In vitro spontaneous apoptosis was examined by propidium iodide staining and DNA fragmentation was examined using gel electrophoresis respectively. It was seen that Fas and FasL expression, both at the level of protein and mRNA, was increased, whereas Bcl-2 expression was decreased both at the level of protein and mRNA. However, no difference in bcl-xL expression was observed between the patient and an age-matched control. A significant proportion of both CD4^+ and CD8^+ T cells from the patients underwent spontaneous apoptosis, whereas almost no spontaneous apoptosis was observed in age-matched control. These data suggest that spontaneous apoptosis in T lymphocytes, at least in part, may be responsible for T cell deficiency in DiGeorge anomaly.