CD40 Ligand Expression on Stimulated T-Helper Lymphocytes in Patients with Common Variable Immunodeficiency

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ABSTRACT

Common variable immunodeficiency (CVID) is the most common symptomatic primary antibody deficiency, characterized by reduced serum immunoglobulins levels and increased susceptibility to recurrent pyogenic infections.

In this study, we evaluated CD40 ligand expression on stimulated versus unstimulated T-helper lymphocytes of nine Common variable immunodeficient patients in comparison with fifteen normal controls. Phorbol myristate acetate (PMA) and Ionomycin were used to stimulate cells in vitro. After six hours stimulation, the cells were subjected to surface staining with three-color staining procedure. Events were analyzed by flow cytometer, using FloMax software.

Results were reported as the percentage of lymphocytes expressing CD markers. We did not find any significant statistical difference in CD40 ligand expression between patients and controls (p>0.05), despite having stimulation documented by CD69 expression as activation marker in each run.

The results of this study are in agreement with some other studies, indicating that CD40 ligand expression on stimulated T-helper lymphocytes of Common variable immunodeficiency patients is similar to normal controls.

Key words: Common Variable Immunodeficiency (CVID); CD40 Ligand (CD154)

INTRODUCTION

Common variable immunodeficiency (CVID) is one of the most common forms of primary immunodeficiency disorders, characterized by hypogammaglobulinemia and recurrent bacterial
infections. The first case of CVID was described in 1953 by Janeway et al. CVID patients showed an increased susceptibility to infections of the respiratory and gastrointestinal tracts with encapsulated bacteria. A subgroup of patients show increased risks of splenomegaly, granulomatous lesions, autoimmune diseases and malignancies. The incidence of CVID varies from 1 in 10,000 to 1 in 2,000,000 people in different regions. The disease is very rare among Asians.1-4 However CVID is the most frequent PID in Iran, with cumulative incidence of 2.5 per 1,000,000 population over the past 10 years.5 CVID may present at any age, from childhood through post-retirement, although the peak of presentation is in childhood and early adult life.

Previous studies indicated that T-cell dependent isotype switching depends on interaction between CD40 and its ligand and the presence of various cytokines such as IL-4 and IL-10. On the other hand, defective expression of the CD40 ligand has been detected in X chromosome-linked hyper-IgM syndrome.6-11 Other studies suggested that most CVID patients have normal numbers of circulating T cells and surface Immunoglobulin-positive B cells; however, B cells of CVID patients fail to differentiate into immunoglobulin-secreting plasma cells in vivo.12,13 Some studies showed that B cells of CVID can proliferate and produce IgE after in vitro stimulation with anti-CD40 and interleukin-4.14 Although B cell defects are responsible for a significant proportion of CVID cases, some other cells which are involved in the orchestration of the immune response (e.g. T cells) can be the cause of the immunodeficiency. Based on these findings, investigators evaluated CD40L (gp39) mRNA expression and T cell surface expression of functional gp39 protein by activated lymphocytes from CVID patients and observed significantly depressed gp39 expression and low cell surface expression of gp39 protein in a number of these patients. In order to demonstrate that the defective expression is due to intrinsic functional abnormalities of CD4+ lymphocytes, other researchers evaluated CD40 ligand expression in purified CD4+ positive cells of some CVID patients after stimulation with PMA+Ionomycin and reported that CD40 ligand expression is defective in a number these of patients. The defect was variable in different patients and was marginal in some cases.

T cell defects in CVID include decreased proliferative responses to mitogens, impaired cytokine production, and defective CD40 ligand expression.13, 15-17 Despite extensive investigation in various ethnic groups in several countries, little is known about cell surface expression of CD40 ligand in Iranian CVID patients. Considering significant variability observed in CD40 ligand expression in different patients even in a single ethnic group, we were encouraged to conduct such study in Iran.

In fact, this study is the first serious evaluation of CD40 ligand expression in heterogeneous Iranian CVID patients.

MATERIALS AND METHODS

Patients and Controls

A total number of 9 well-documented CVID patients (6 males and 3 females, age range: 9-46 years old, median = 15 years), who were referred to Children’s Medical Center, the main referral center for primary immunodeficiency disorders, were included in this study. The diagnosis of CVID was made according to standard diagnostic criteria.5 Informed consent was taken from all subjects before specimen collection. The characteristics and immunological findings of our CVID patients are summarized in Table 1.

CD4/CD8 ratio was depressed in some patients and was normal in others. All patients were under regular intravenous immunoglobulin (IVIG) therapy at 3 to 4 weeks intervals. Each patient was studied at the maximum time interval after the last IVIG infusion to avoid possible effects of IVIG therapy on lymphocyte function. A total number of 15 normal volunteers (11 males and 4 females, age range: 25-38 years old, median = 27 years) served as controls and studied in parallel with patients.

PBMC Isolation

4 mL heparinized whole blood was collected and peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation method. Viability test showed 96.6 to 99 percent viability. Isolated cells were resuspended in RPMI 1640 culture medium containing 1 mM L-glutamine, 25 mM HEPES (Sigma, USA), and 1% penicillin and streptomycin commercial solution (PenStrep, Gibco, UK). Cell suspensions were stored at 4°C till the next day.
CD40L Expression in CVID Patients

Table 1. Characteristics and immunological variables of CVID patients

<table>
<thead>
<tr>
<th>Patients Number</th>
<th>Age</th>
<th>Sex</th>
<th>WBC (/μl)</th>
<th>IgM (mg/dl)</th>
<th>IgG (mg/dl)</th>
<th>IgA (mg/dl)</th>
<th>IgE (mg/dl)</th>
<th>CD3 (%)</th>
<th>CD4 (%)</th>
<th>CD8 (%)</th>
<th>CD19 (%)</th>
<th>CD4/CD8 (ratio)</th>
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<tr>
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<td>F</td>
<td>5600</td>
<td>220</td>
<td>200</td>
<td>210</td>
<td>ND</td>
<td>85.2</td>
<td>23.4</td>
<td>49.9</td>
<td>8.3</td>
<td>0.47</td>
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<tr>
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<td>30</td>
<td>F</td>
<td>6800</td>
<td>65</td>
<td>290</td>
<td>58</td>
<td>3</td>
<td>79.25</td>
<td>40.4</td>
<td>35.2</td>
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<tr>
<td>P3</td>
<td>19</td>
<td>M</td>
<td>10800</td>
<td>220</td>
<td>240</td>
<td>210</td>
<td>ND</td>
<td>ND</td>
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<tr>
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<td>382</td>
<td>320</td>
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<td>1.2</td>
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<td>150</td>
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<td>32</td>
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<td>1.37</td>
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<tr>
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<td>F</td>
<td>3000</td>
<td>&lt;20</td>
<td>&lt;100</td>
<td>&lt;10</td>
<td>1</td>
<td>66.3</td>
<td>35.2</td>
<td>31.2</td>
<td>24.9</td>
<td>1.13</td>
</tr>
</tbody>
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ND: not determined; F: female; M: male

Cell Culture

Peripheral blood mononuclear cells were washed with RPMI and cultured at a final concentration of 1-1.5 × 10^6 cells per mL in RPMI 1640 culture medium containing 10% heat-inactivated fetal bovine serum (Biowest, South America) under standard conditions (37°C, 5% CO2, and Humidity) in the presence and absence of 15 ng PMA (Sigma, USA) and 750 ng Ionomycin (Sigma, USA) for 6 hours.7

After incubation, cells were washed with ice-cold RPMI 1640 culture medium and incubated for 5 minutes with 250 µg/mL human IgG (Bayer Corporation, USA). Then, Cells were washed with ice-cold sterile Ca^2+Mg^2+ free PBS.

Flow cytometry

Immunophenotyping of cultured cells was performed by flow cytometry. The percentage of cells expressing CD markers was used as measuring unit. Surface staining was performed with anti-CD3-FITC, anti-CD8-RPE-CY5, anti-CD40L-RPE, anti-CD69-RPE (clones UCHT1, DK25, TRAP1 and FN50, respectively; all from Dako, Denmark), and anti-CD69-RPE (BD, USA) monoclonal antibodies. IgG1-FITC, IgG1-PE, and IgG1-PE-cy5 (Dako, Denmark) were used as negative controls in parallel with other monoclonal antibodies to discriminate between positive and negative fluorescence. In other words, percentage of CD40L-positive and CD69-positive cells was determined as the fraction of cells showing fluorescence greater than the isotype-matched negative control. Events were analyzed by Partec PAS flow cytometer using FloMax software. The flow cytometer was calibrated twice a week for fluorescence sensitivity and spectral overlap (compensation) using standard fluorescent beads (Dako, Denmark). In order to measure CD40 ligand expression only on activated CD4-positive T lymphocytes, a negative gating strategy was adopted, because the concentrations of PMA and Ionomycin used in the stimulation protocol significantly reduce the level of CD4 expression on T cells, preventing its use as a positive selection marker.18

In fact, our goal was to analyze CD40L (CD154) expression on CD3+CD4+ T cells. However, because of the mentioned drawback, we had to adopt the negative gating strategy and measure CD40L expression on CD3+CD8- T cells (Figure 1).

Culture, Passage, and Surface Staining of CD40L+ Cell Line

A cell line with fibroblastic origin, transfected in vitro with CD40L gene (Non-commercially available, Iran Cell Bank, Pasteur Institute of Iran) was purchased as suspension in RPMI 1640 culture medium. Then, cells were treated with trypsin 0.25 % (Difco, USA) and ethylene diamine tetra acetic acid (Sigma, USA) 0.02 % to avoid clumping. A total number of 2 × 10^6 cells were harvested and divided into two tubes. One part surface stained with anti-CD40L-PE mAb (clone TRAP1, Dako, Denmark). The second part was stained with IgG1-PE (Dako, Denmark) as negative control. After 30 minutes incubation in dark room, cells were fixed with 0.1 % parafomaldehyde (Merck, Germany) containing 0.1 % NaN3. Events were analyzed by Partec PAS flow cytometer using FloMax software.

Statistical Analysis

Data were entered and analyzed using SPSS software (version 11). Student t-test was used to
compare the mean percentage of CD40L-positive helper T lymphocytes of patients and controls.

**RESULTS**

**Efficacy of CD40L-PE mAb to Detect CD40L Expression**

Surface staining of a fibroblastic cell line transfected with CD40L gene was used to assess efficacy of CD40L-PE mAb to detect CD40L expression.

Immunophenotyping results showed that as many as 94.54% of cells expressed CD40L on their surfaces. This significant expression suggests that the CD40L-PE monoclonal antibody can be used optimally to detect CD40 ligand expression on stimulated versus unstimulated T cells in this study.

**CD40 L (CD154) Expression on Activated T Lymphocytes**

In our study, stimulated T-helper cells of CVID patients expressed CD154 on their surfaces in densities near to normal healthy controls. In fact, comparing the mean CD40 ligand expression levels in CVID patients with normal controls did not show significant statistical difference (33.21 ± 10.62 percent for patients versus 36.58 ± 9.16 percent for controls, P>0.05). Evaluation of the results showed a significant difference in CD40L expression on T cells of various patients. Despite success in T cells activation (more than 81.2%, from 72.91 to 93.72%, of CD3-positive cells expressed CD69 as an activation marker on their surfaces), CD40L expression on CD4-positive T cells of patients was lower than some other studies and was varied from 18.88 to 51.04 percent. Of course, CD40L expression on stimulated CD4+ cells was always more than unstimulated cells. Sometimes, this difference was small in amount but in most cases the difference was so much that it could be considered significant enough. Although CD40 ligand expression levels on stimulated T helper cells of CVID patients showed significant variance (which is attributable to variable nature of the disease itself), CD40 ligand expression in patients was similar to normal healthy controls.

**DISCUSSION**

Over the past few years, our understanding of the role of CD40 and CD40 ligand (CD40L) in disease pathobiology has expanded largely. The CD40–CD40L interaction is now well established as an immune mediator, particularly in the promotion of T-
lymphocyte dependent B-cell responses. Thus, interruption of this critical immune cell cross-talk leads to the immunodeficiency diseases like CVID. In accordance with these observations, some investigators have observed defective CD40 ligand expression in X-linked hyper-IgM syndrome. Given the role of the CD40 ligand in T-cell-directed isotype switching, they concluded that failure of CD40 ligand expression underlies the isotype switching defect in Hyper-IgM patients.

As mentioned earlier, it is now clear that, while B cell defects are responsible for a significant proportion of CVID, other cells involved in the orchestration of the immune response can be the target of the genetic disorder. In addition, defective CD40 ligand expression is considered as the cause of T cell defects observed in at least a subset of CVID patients. In fact, decreased CD40 ligand expression by activated CD4-positive lymphocytes has been observed in one-third of the patients, while the remaining two-third have displayed normal levels of CD40L expression. Sequence analysis has shown normal CD154 sequences in all the studied patients. These findings and the finding that CVID B cells respond in vitro to stimulation with anti-CD40 and IL-10 or IL-4 encouraged us to test CD40 ligand expression defect in T helper lymphocytes of our CVID patients. In the patients included in this study, the comparison between CD40L-positive T helper lymphocytes in CVID patients and normal controls did not show any statistically significant difference. These results are consistent with some studies reported in the scientific literature. Therefore, according to the results obtained from this study and the results of some other investigations, we can conclude that CD40 ligand expression on optimally stimulated T helper lymphocytes of some CVID patients is similar to normal controls. However, in some studies (especially the studies with greater sample volumes), defective CD40 ligand expression has been observed only in a subset of CVID patients. Therefore, in future studies, inclusion of more CVID patients in similar studies may lead to better results, although defective CD40L expression has also been observed in some studies with small sample.

In addition to comparison the percentage of CD40L+ T helper lymphocytes in CVID patients with normal healthy controls, mean fluorescence intensity (mfi) was also compared between patients and controls. Interestingly, this comparison revealed statistically significant difference (p<0.05) between patients and controls. In fact, mean mfi in patients was greater than normal controls. Greater mfi in patients indicates that CD40 ligand molecule expression per cell in patients’ CD4-positive T lymphocytes is higher than controls. More expression of CD40 ligand molecule per cell in patients’ T cells probably can be due to insufficient cross-talk between immune system cells, especially T cells and B cells.

As mentioned above, a number of previous studies have shown decreased levels of CD40L in CVID patients. Because these studies were carried out in PBMC instead of purified CD4-positive T cells, thus inhibitory signals from other cells could not be excluded. In addition, removing monocytes from mononuclear cell cultures in two patients with CVID led to increased proliferative responses to T cell mitogens phytohaemagglutinin (PHA) and concanavalin A (Con-A). Therefore, probable suppression of T and B cell functions by monocytes can be to some extent the cause of depressed in vitro expression of costimulatory molecules such as CD40 ligand. On the other hand, indirect evidences suggest that CD40L(CD154) signaling is conserved in CVID patients: opportunistic infections that characterize hyper-IgM syndromes caused by mutations in CD40L(CD154) gene (HIGM1) or CD40 gene (HIGM3) are not a consistent finding in CVID patients, although a similar phenotype to hyper-IgM occurs in CVID patients. Moreover, the CD40L/CD40 axis is essential for optimal production of IL-12, and several reports point to an increased IL-12 production in CVID patients.

Although we did not find defect in the expression of the classical costimulatory molecule CD40L in CD4-positive T cells of CVID patients, we can not rule out alterations in other costimulatory molecules. As a matter of fact, some investigators have shown impaired up-regulation of B7-2 (CD86) in B cells of type A common variable immunodeficient patients. They concluded that B cell defect is the underlying mechanism leading to type A CVID in these patients.

However, a recent study showed the lack of inducible costimulator (ICOS) expression on activated T cells of a minority (4 out of 32) of well-documented CVID patients. ICOS is a costimulatory molecule closely related to CD28. CD28 and ICOS effectively costimulate all basic T cell responses, including proliferation, up-regulation of molecules mediating
cell-cell interaction, synthesis of cytokines and effective B cell help for antibody secretion.27

We conclude that, our results suggest that the CD40 ligand defect is unlikely to play a critical role in the pathogenesis of CVID in the majority, if not to say all of CVID patients studied. Of course, it should also be considered that CD40L molecule may be present, but functionally inactive in some CVID patients. Therefore, further and more comprehensive studies may be necessary to elucidate the underlying causes of CVID. However, in our opinion, this study should be taken into account as a small survey on a broad-spectrum of a subject and more success depends on conducting more complementary researches in the field of PIDs, especially CVID.

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REFERENCES


CD40L Expression in CVID Patients


