Association of sCD40 Level in Serum with Risk for Relapse in Graves' Disease

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Keywords: sCD40, Graves' Disease, Relapse, Remission, ELISA.

Abstract: CD40 is a transmembrane protein that influences the pathological of autoimmune disease, like Graves' Disease (GD). Interaction with its ligand (CD40L) on the surface of $CD4^+$ T cells can enhance the humoral immune response. Some studies indicated that GD patients have lower apoptosis in follicular cell. This condition related to sCD40 level, which also can increase autoantibodies in GD patients. The aim of this research was to find out the role of sCD40 as a marker on GD activity. This research was a case-control study comparing 30 relapse patients and 30 non-relapse patients (remission) after treatment with an anti-thyroid drug was terminated. The subject of this research was serum from both relapse and remission patients, analyzed by ELISA method. Statistical analyzes was independent t-test, a two-tailed p-value less than 0.05 was considered significant. The results, we found that sCD40 levels in serum (pg/ml) increased on relapse patients (1.62± 0.17) compared to remission patients (1.46±0.14) and showed that sCD40 level in serum was significantly different between relapse and remission ($p<0.001$). These results demonstrated that elevated levels of sCD40 serum, associated with risk for relapse. So, sCD40 may be used as a marker of the active stage of GD.

1 INTRODUCTION

Graves' disease (GD) is one of Autoimmune Thyroid Disease (AITD), characterized by hyperthyroidism and commonly found in women than men (Abbas, 2010 and Weetman, 2000). Based on Indonesia Basic Health Survey (Riskesdas) 2013, the prevalence of hyperthyroidism in Indonesia is 0.4% with the highest in Jakarta as much as 0.7% (Kemenkes, 2015). America is about 1% and Caucasian population 0.5-2% (McLeod, 2012).

GD causes thyrotoxicosis, which increases the thyroid hormone as the impact of the body's mechanisms failure to self-tolerance, then autoimmune reactions occur. Thyroid Stimulating Hormone Receptor (TSHR) is an antigen that plays a role in stimulating the formation of antibodies in the body. These antibodies can bind and activate the thyrotropin receptor and cause hypertrophy and hyperplasia of the thyroid which is characterized by enlargement of the thyroid gland and increased hormone thyroid (Płoski, 2011; Baratawijaya, 2012 and Tanwar, 2010).

The most common of GD treatment is an anti-thyroid drug. The other treatments are radioactive iodine or thyroidectomy. These therapies aim to treat the symptoms caused by hyperthyroidism and reducing the enlargement of the thyroid gland, but all kinds of these therapies have side effects and the possibility to show the symptoms of hyperthyroidism again, called a relapse (Aggarwal, 2014). Anti-thyroid drugs are considered as the first-line treatment since it is easily implemented, but it takes 1-2 years to achieve remission and even 30-50% of patients with GD take>2 Tahun (American Thyroid Association, 2014). GD patients are determined as remission when the levels of FT4, FT3 and TSH in the serum are normal without medication. In fact, more than 50 percent of patients who had remission GD may experience repeated symptoms of hyperthyroidism or may have relapse (McKenna T Joseph, 2001 and Jacobson, et al., 2007).

Amir, T., Suryandari, D., Eliana, F., Yunaini, L. and Yanti, D.
Association of sCD40 Level in Serum with Risk for Relapse in Graves' Disease.
DOI: 10.5220/0009589402240227
In *Proceedings of the 1st International Conference on Health (ICOH 2019)*, pages 224-227
ISBN: 978-989-758-454-1

GD is also known as a multifactorial disease, which is this disease caused by environmental and genetic factors. CD40 gene is one of the genes that regulate immune responses on the follicular cells of the thyroid gland (Huber, et al., 2012). CD40 signals are required for immune response and pathogenesis of the autoimmune disease (Peters, 2011). The increase of CD40 expression leads to the formation of antibodies that specific to the thyroid and produce cytokines or chemokines in follicular cells thyroid (Huber, et al., 2012).

Ligands of CD40 is CD40L/ CD154, expressed on the surface of CD^{4+} T lymphocytes. The signal of CD40-CD154 interaction indicates a significant role to maintain the process of autoimmunity in the body (Myśliwiec, 2007). Interaction between CD40 as a costimulator molecules with its ligand CD154, is necessary to activate T cells, B cells proliferation and differentiation, production of immunoglobulin (Ig), Ig class switching and also to induce the proinflammatory cytokines, such as interleukin 6 (IL-6) (Van Kooten C, 2000 and Peters, et al., 2008).

Based on the previous research, showed that sCD40 expression can affect etiology of GD. Myśliwiec, et al., 2007 found that CD40-CD154 interaction has a role in the pathogenesis of Graves' Ophthalmopathy (GO) and the serum level of CD40 (sCD40) had elevated. Other studies also demonstrated a positive correlation between sCD40 with antibody to TSHR (aTSHR) in patients GD (Mysliwiec, et al., 2007). These researchers suggested that the presence of sCD40 in serum has a role of the pathological disease. But until now, the role of sCD40 influencing the physiological function of CD40 for relapse in GD patients remains to be studied more deeply. Therefore, further research is needed to determine the role of sCD40 levels and the risk for relapse as a marker of the active stage of GD.

2 METHOD

2.1 Subjects

The selection of the case group (relapse) and control group (remission) are based on the status of patients in medical records.

2.2 Venous Blood Aspiration

A total of 2 mL venous blood sample was taken by syringe and then put into a tube not containing ethylenediaminetetraacetic acid (EDTA) to get a sera. All the sera were kept frozen at -20°C until used.

2.3 Measurement of sCD40 Level

sCD40 levels from all patients were performed using a sandwich ELISA kits (Human sCD40 ELISA Kit Platinum BMS 265, Bender Med Systems, Vienna, Austria). This ELISA commercial kits were used to determine the serum level of sCD40 concentration from 7.8 to 1.000 pg/mL.[18] First, various concentrations of standard solution was prepared by dilution (500 pg/mL, 250 pg/mL, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/mL and 7.8 pg/ m L). Plate that has been coated with a specific monoclonal antibody against of sCD40, wash with a wash buffer (1x) twice (200 mL each well) and allowed for 10-15 seconds. Next, 50 mL of each standard solution and 50 mL sample test (serum) were incorporated into different wells and then on each well added 50 mL of Biotin-Conjugated antibody (1x). Plate covered with adhesive film and incubated for 2 hours at room temperature (18º-25ºC) on a shaker machine at 400 rpm. After that, the plate was washed 10 times (each well 200 uL) with wash buffer (1x), then added 100 mL Streptavidin-Horseradish Peroxidase (HRP) on each well. The plate was resealed with adhesive film and incubated for 1 hour at room temperature (18º-25ºC) on a shaker machine at 400 rpm. When the incubation was completed, the plate is washed again as much as 10 times (each well 200 uL) with wash buffer (1x), then added 100 mL of Substrate Solution (tetramethyl-benzidine) on each well and incubated 10 min at room temperature (18º-25ºC) in darkroom (not exposed to direct light exposure). Enzyme-substrate reaction was stopped by addition of 100 mL of Stop Solution (1M phosphoric acid). A colored product is formed in proportion to the amount of human sCD40 present in the sample or standard. Then the absorbance is measured with an ELISA reader at 450 nm. A standard curve is prepared from 7 human sCD40 standard dilutions and human sCD40 sample concentration determined. sCD40 concentration is determined by comparing the OD value of the sample with a standard curve (eBioscience).

2.4 Data Analysis

Independent t-test was used to show the association between sCD40 levels with risk for relapse in GD patients at p-value less than 0.05 was considered significant.

3 RESULT AND DISCUSSION

The normality test on each group showed that data from relapse group has normal distribution (0.161), but not in the remission group (<0.001). Then we did the transformation data by log 10. The results were showed normal distribution of data (relapse= 1.49 and remission= 0.079). Further, the data is tested by an independent t-test. The results are presented in Table 1.

Table 1: The association between sCD40 level in serum with risk for relapse on GD patients.

	Relapse	Remission	p*
sCD40 level (pg/mL)	1,62±0,17	1,46±0,14	<0,001

*independent t-test

Based on the results indicated that there is a significant association between sCD40 levels with risk for relapse on GD patients ($p<0.001$) at $\alpha=0.05$. GD patients who relapsed had an average serum sCD40 levels higher than GD patients who did not relapse.

In this study, the average levels of sCD40 in the relapse group was higher than the remission group and found a significant association between sCD40 level with risk for relapse in GD. This result indicates that high levels of sCD40 in serum on GD patients may increase the risk for relapse. A previous study by Mysliwiec et al in Poland, also demonstrated that sCD40 level was higher in GD (hyperthyroidism) compared to patients with GD (euthyroid), but did not show a significant association (Mysliwiec, et al., 2007). Other studies also measured the level of sCD40 in patients with Graves' Ophthalmopathy (GO), GD (hyperthyroidism), GD (euthyroid) and controls (people who have no history of disease GO/GD), showed that levels of sCD40 from high to low concentration were patients with GO, GD (hyperthyroidism), GD (euthyroid) and controls. The statistical analysis showed a significantly different levels of sCD40 only found on GO with control patients ($p<0.001$), GO with GD (hyperthyroidism) patients ($p<0.001$) and GD (hyperthyroidism) with controls patients ($p<0.05$), but not seen a significant difference between sCD40 level in GD (hyperthyroidism) with GD (euthyroid) group (Myśliwiec, et al., 2007).

The biological role of sCD40 in affecting physiological function on GD patients is not clear. Release of sCD40 is suspected from the transmembrane receptor. It can occur because of splicing while gene expression or proteolysis on the transmembrane protein (Van Kooten C, 1996). High levels of serum sCD40 in patients with relapsed, indicates the increase of CD40 expression on transmembrane region. Interaction of CD40, which located on the membrane with CD40L will secrete matrix metalloproteinase enzymes that act as a protease to degradation of the protein (Karimi, 2012). This condition caused by CD40 on transmembrane region has proteolysis, with the result that can be found in serum as a soluble form (sCD40).

The higher sCD40 level on GD patients indicates that sCD40 has contributed to development and risk for relapse on GD, based on research conducted by Mysliwiec et al that showed a positive correlation between sCD40 with TSHR antibody (Mysliwiec, et al., 2007). Other studies also showed that follicular cell apoptosis decreased in patients with GD and increased in HT (Kie JH, 2001).

The role of sCD40 is also suspected as an inhibitor of the CD40-CD40L interaction, so that the immune response decreases (Mysliwiec, et al., 2007). This interaction is affected by the amount of energy required for binding both sCD40 and CD40 that found on membrane with CD40L, to affect the immune response. This hypothesis would be stronger if the levels of CD40 on the membrane are known, so it can be seen clearly differences of CD40 expression contained in the transmembrane or in the form of dissolved (soluble) and its effects on the risk of relapse.

Binding of CD40-CD40L occurs in the multimerisasi form. Generally, the structure of CD40 membrane is a trimerization form, while the structure of sCD40 is not known with certainty yet. As a result, interaction CD40-CD40L on the membrane has a higher affinity than sCD40. Binding of CD40 on the membrane with CD40L occurs through binding in the form of dimerization or trimerization, but trimerization will give a strong response to intracellular signaling. CD40 and CD40L have a trimerization form. So, this interaction will create oligomerization which lead to the introduction of protein adapters such as TRAFs at the intracellular domain, improving signal cascade and activates transcription factors that play a role in producing proteins, which influence the immune response in the body either humoral immune responses or cellular and prevent apoptosis (Peters, 2011; Ellmark, 2002 and Zazzeroni, et.al., 2001).

4 CONCLUSIONS

There was a significant association between sCD40 level in serum with risk for relapse on GD patients. So, sCD40 levels in serum may be used as a marker for the determination of GD activity.

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