Detection of VipAlbumin® Effect in CD34 and SDF-1 Mobilization in Streptozotocin-induced Diabetes Mellitus Mice

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Abstract

Diabetes mellitus is a disease in which the body loses its ability to provide tight regulation and maintain a dynamic interaction between the tissue sensitivity and insulin secretion by β cells. The impact of this dysfunctional mechanism is uncontrolled blood glucose levels that lead to hyperglycemia condition. Highly reactive free radicals have a strong involvement in the pathogenesis of diabetes mellitus, where one of its forming process may be triggered by hyperglycemia condition. Patients with diabetes mellitus itself vulnerable to endothelial dysfunction, which is caused by a decrease in circulating endothelial progenitor cells, and also a decrease in chemokines which play a role in affecting the activities of these cells. Hyperglycemia condition and free radical activity is a major cause of these endothelial progenitor cells dysfunction. The purpose of this study was to determine the role of VipAlbumin®, a supplement derived from *Channa striatus* albumin extracts in inhibiting the action of free radicals that are formed due to hyperglycemia condition, which can affect the increase in endothelial progenitor cells relative amount. This study used BALB/C mice that induced to undergo diabetes mellitus through streptozotocin injection intraperitoneally at 5-day old. Mice who have reached 4 week old and positive to diabetes mellitus (blood glucose levels $> 200 \text{ mg.dl}^{-1}$) will be administered with VipAlbumin® orally for 14 days. VipAlbumin® dosage was divided into 4 groups: positive control (without VipAlbumin®); 1st dose ($0.01664 \text{ mg.gr}^{-1} \text{ BW}$); 2nd dose ($0.416 \text{ mg.gr}^{-1} \text{ BW}$); 3rd dose ($10.4 \text{ mg.gr}^{-1} \text{ BW}$). The last step was flow cytometric analysis to determine the development of endothelial progenitor cells relative amount, which isolated from bone marrow. The variables measured in this study were the relative amount of CD34⁺ and SDF-1. Based to flow cytometric analysis, mice with VipAlbumin® administration did not show any significant improvement in CD34 relative amount when compared to the positive control. Relative amount of Chemokine SDF-1 itself, although only occur at the 3rd dose of VipAlbumin® treatment, has increased and significantly different from the positive control.

Keywords: CD34, diabetes mellitus, free radicals, hyperglycemia, SDF-1, streptozotocin, VipAlbumin®

INTRODUCTION

CD34 is a transmembrane glycoprotein sized 115-kD which is strongly expressed on progenitor /hematopoietic stem cell (HSPC), and progressively decreased when HSPCs differentiate [1,2]. CD34 is a wide used marker to detect hematopoietic progenitor in human. Several researches show that antigen CD34 has signal with transduction capacity and is involved in cell adhesion, which resulting polymerization of actin and homotypic adhesion on cells of CD34⁺ and KG1a [3-7]. Circulation of immature cells derived from bone marrow, one of which is CD34⁺ cells, contributes in holding vascular homeostasis and repair, and play an important role in maintaining vascular endothelial function [8].

The amount of CD34⁺ cells, as the part of endothelial progenitor cells, are lower in diabetic

patients compare to those with normal glucose tolerance [9]. This leads to the reinforcement of the disease in triggering the endothelial dysfunction and the other forms of complication.

The high-level of blood glucose and free radicals activity on diabetic patients also can influence the reduction of SDF-1, a chemokine functioned to stimulate the mobilization of endothelial progenitor cells from bone marrow [10]. Stromal cell-derived factor-1 (SDF-1) or CXCL12 is a chemokine for CXC subfamily which is generally characterized as the pre-B cell stimulation factor and cloned from bone marrow cells supernatant [11]. SDF-1 is chemotactic factor for T cells, monocyte, pre-B cell, dendritic, and hematopoietic progenitor cells, and also functioned to support proliferation of B and CD34⁺ progenitor cells. SDF-1 gave its effect by binding with CXCR, a member of G protein-coupled receptor superfamily [14,15].

Responding to ischemia, SDF-1 regulation is usually increased, and through its binding with CXCR4, will stimulate the bone marrow to release EPC (one of them is CD34⁺ cells) which will later

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be recruited in ischemic area [10]. In diabetic animal, the deformed response of SDF-1 to ischemia correlates with the decline of progenitor cells release from bone marrow and will cause post-ischemic angiogenesis deformation [16].

To figure out the effect of blood glucose level increase towards cells development which expresses CD34 and SDF-1, mice models of diabetes mellitus are used. The mice are injected by streptozotocin, a molecule of 2-Deoxy-2-([methyl (nitroso) amino] carbonyl) amino) - β -D glucopyranose, that produce selective toxic toward β cells and inducing diabetes mellitus in most of laboratory animals. High dose of β cells toxin such as streptozotocin and alloxan inducing insulin deficiency and type 1 diabetes with ketosis. But a precise dose calculation would partially destruct β cells mass and conduct mild insulin resistance that characterize type 2 diabetes [17].

The increase of blood sugar level through the injection of streptozotocin would trigger highly reactive radicals formation. In this research, VipAlbumin®, a supplement which extracted from *Channa striatus*, is used to obstruct free radicals impact on the endothelial progenitor cells activity. Several researches show that the snakehead murrell (*Channa striatus*) has positive effect as anti-inflammatory agent [18] reviewed from the high arachidonic acids level and important amino acids such as aspartic, glycine, and glutamic acid [19]. It also becomes the key factor in polypeptide formation which takes role in growth and wound healing [20,21]. The other functions are due to substance conductor, osmotic pressure regulator, platelets and anti-thrombotic formation hindrance, increase cells permeability, and as antioxidant [22,23].

MATERIALS AND METHODS

This research was conducted from November 2014 to May 2015 in Laboratory of Animal Physiology, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya.

Research design

The research was categorized into experimental research, in which the researcher intentionally manipulating the treatment to see the emerging effect. This research used *in vivo* procedure. There were 5 different treatment groups, i.e. negative control (healthy mice without streptozotocin injection and

VipAlbumin® administration), positive control (diabetes mellitus model mice without VipAlbumin® administration), and diabetes mellitus model mice with VipAlbumin® administration on 3 different doses. Each treatment were divided into 5 repetition.

In vivo Procedure

Diabetes mellitus model-mice induction through streptozotocin injection

Healthy mice were induced to have diabetes mellitus through streptozotocin injection intraperitoneally at a dose of 100 mg.kg⁻¹ BW in the age of 5 days. Each mice in this research was assumed to weigh 2.5 g, thus, the proper dose of streptozotocin is 50 μ l. This amount derived from the mixture of 0.005 g of streptozotocin powder with 1 ml of citrate buffer. Successfully injected mice were then nurtured in sterile environment. The measurement of blood glucose level was conducted when the mice were reaching 4 week-old, by using glucometer. Mice was diagnosed undergo diabetes mellitus if the blood glucose levels were more than 200 mg.dl⁻¹.

VipAlbumin® administration on various doses

Oral administration of VipAlbumin® was done daily for 14 days to the diabetic mice. This administration were divided into 3 treatment doses: 0.01664 mg.g⁻¹ BW (dose 1); 0.416 mg.g⁻¹ BW (dose 2); 10.4 mg.g⁻¹ BW (dose 3). These three doses were derived from the albumin dose conversion for 1 kg of human BW i.e. 33.3 mg.kg⁻¹ with the assumption that the normal weight of adults was 60 kg. In order to figure out VipAlbumin® effect in decreasing blood glucose level, the measurement was done regularly in every 3 days.

Cell isolation from bone marrow

Mice were dislocated and dissected. Afterward, the femur and tibia part of the mice were flushed with PBS by using 1 ml spuit at one end. The obtained suspension is later put into propylene tube and centrifuged on 2500 rpm, 10°C temperature, for 5 minutes. The pellet formed was then resuspended with 1 ml PBS. The resuspension then taken 5 μ l, put in microtube, and added with 95 μ l evans blue. Pipetting is done for homogenization. The amount of successfully isolated cells was later counted with haemocytometer under microscope.

Antibody staining and Flowcytometric analysis

The staining process were divided into two phases based on the antibody type used. The first phase was extracellular antibody staining. 250 µl cell suspension isolated from bone marrow was put into microtube and centrifuged on 2500 rpm speed, 10°C temperature, for 5 minutes. Supernatant was eliminated and the pellet was added with 1 µl antibody which has being liquified with 50µl PBS dan 10% FBS. The suspension was later incubated in 4°C ice box for 20 minutes. The staining combination used was FITC-conjugated rat anti-mouse CD34.

The second phase was intracellular antibody staining. After conduct the procedure of extracellular antibody staining, cells were then given with 100 µl fixative solution cytofix/cytoperm and incubated 20 minutes in ice box. Mixed the cells with 500 µl washperm in order to clean the fixative solution remains. The suspension was re-centrifuged on 2500 rpm, 10°C temperature, for 5 minutes, whereas the pellet was then stained with intracellular antibody and incubated for 20 minutes in ice box. The staining combination used is PE/Cy5-conjugated SDF-1. The cells which were incubated by antibody staining is added with 300-500 µl PBS, and then moved into cuvette and being operated in flowcytometry machine.

Flowcytometric Data Analysis

The data obtained from flowcytometry then analyzed with BD Cellquest Pro™ software, and continued with the statistical analysis using SPSS version 16 for Windows. The statistical analysis

used was one way ANOVA parametric analysis with $p = 0,05\%$, followed by Tukey test.

RESULT AND DISCUSSION

Comparison of blood glucose levels on each treatment

The comparison of mice blood glucose levels on each treatment was measured regularly once in 3 days, for 15 days (Fig. 1). The positive control group (diabetic mice without VipAlbumin® administration) has significantly higher blood glucose levels than other treatments. Although it is not as high as the positive control, 1st dose (0.01664 mg.g⁻¹ BW) of VipAlbumin® also has an increase in blood glucose level along with the age. Both 2nd (0.416 mg.g⁻¹ BW) and 3rd dose (10.4 mg.g⁻¹ BW) of VipAlbumin® tend to have fluctuated blood glucose levels, which was able to increase or decrease depends on measurement days. However, the blood glucose levels from both treatments were significantly lower than positive control. The negative control has the lowest sugar blood level compared with the other treatments.

Flow cytometric analysis of CD34⁺ relative amount

We figured out that the relative amount of CD34⁺ cells in diabetic mice has a significant decrease compared with the normal mice (Fig. 2). The healing effort through VipAlbumin® administration has not given a notable change yet, whereas none of the three doses of VipAlbumin® treatment can increase CD34 expression and significantly different from the positive control (Fig. 3).

Figure 1. The Comparison of Blood Glucose Levels on Each Treatment

Figure 2. The Profile of CD34⁺ through flow cytometric analysis
(a) negative control, (b) positive control, (c) VipAlbumin® 1st dose, (d) VipAlbumin® 2nd dose, (e) VipAlbumin® 3rd dose

Figure 3. The comparison of CD34 relative amount on each treatment

It has been elucidated that diabetic patients experiencing the decrease of circulated progenitor. Although without complication, the amount of progenitor cells majority on diabetic patients still significantly lower than healthy control. The endothelial progenitor cells reduction can also be happened on patients with

high blood glucose level and HbA_{1c} (glycated hemoglobin) [24]. The previous research mentioned that the amount of CD34⁺, one type of the immature cell which was circulated in blood and becomes the part of endothelial progenitor cells (EPC), was lower in diabetic

patients compared with subjects having normal tolerance of blood glucose level [9].

The increase of blood glucose level (hyperglycemia) became the main factor affecting endothelial dysfunction, which was the first criterion of atherosclerosis pathogenesis [25,26]. Endothelial dysfunction takes important role in the development of atherosclerosis, and the circulation of endothelial progenitor cells derived from the bone marrow participating in the repair of vascular endothelial cells and defending the function of endothelial. On the patients with diabetes, it is reported that there was decreasing amount and dysfunction of circulated EPC, contributing to the diabetic microvascular complication [27].

Various research using animal model showed that the circulated EPC cells contribute to re-endothelialization and/or neovascularization therapy [28,29]. Several evidence indicates that various chemokine, cytokine, growth factor and their specific receptors can regulate the mobilization and recruitment of EPC from the bone marrow to the peripherals area, and in the process of proliferation and differentiation [30,31]. The chemokine (motif C-X-C) receptor 4 (CXCR4), receptor for SDF-1, play important role in the mobilization of cells from bone marrow and also in regulating the mobilization and recruitment of EPC [32-36].

One alternative way to prevent complication caused by hyperglycemia condition and the formation of free radicals is through induction of endothelial progenitor cells activation; in which this research using VipAlbumin® administration to conduct that process. Although the result shows that there was no significant increase in relative amount of CD34 compared with the positive control, it does not eliminate the potential function of albumin. Albumin functioned as the antioxidant to obstruct the free radicals ROS/NOS movement, maintaining the extracellular redox equilibrium, and playing role in the transportation process of various molecules such as fatty acid, nitric oxide, hemin, and drugs [37,38]. Some other researches show that in culture condition, albumin can be used as the inhibitor of apoptosis process for macrophages, neutrophil, lymphocyte, and endothelial cells [39-42]. In primary structure form, albumin contains 34 cysteine residues contributing with 17 disulfide bridges to form whole tertiary structure with one free cysteine residue (Cys34) which plays important role in various functions of albumin mentioned before. This very active residue that containing

80% (500 $\mu\text{mol.L}^{-1}$) total thiol in plasma is the primary scavenger of reactive oxygen and nitrogen in plasma [43].

Another strategy also proposed to improve the healthy condition from diabetic patients with hyperglycemia by using propolis treatment [44]. This material is known to contain high-level of nutrient factor such as vitamins, polyphenols, and amino acids, that expected to improve insulin sensitivity and suppress the action of inflammatory molecules. This suppression activity had a high relationship with the ability of T regulatory cells (especially on $\text{CD4}^+\text{CD25}^+$ population), while the increase number of this cells can induced by propolis intake. Highly reactive T cells could induce insulin resistance by its pro-inflammatory action, but T regulatory cells can prevent this effect by producing IL-10 and TGF- β . This cells can also prevents inflammatory condition being wide-spread, by reversing the activated memory T cells become naive type [45].

Flow cytometric analysis of SDF-1 relative amount

Flow cytometric data analysis shows that relative amount of SDF-1 on diabetic mice was significantly decrease compared to the normal control (Fig. 4). Two given doses of VipAlbumin® i.e. 1st dose (0.01664 mg.g^{-1} BW) and 2nd dose (0.416 mg.g^{-1} BW) did not show meaningful increase of SDF-1 expression. The significant increase compared with the positive control only occurred in the 3rd dose (10.4 mg.g^{-1} BW) of VipAlbumin®, although the relative amount has not yet reached the normal condition (Fig. 5).

It was reported that diabetic patients will experience the decrease of SDF-1, a chemokine that stimulates the endothelial progenitor cells mobilization (EPC) derived from the bone marrow [10]. The decreased ability of EPC mobilization from the bone marrow becomes one of the mechanism resulting the low amount of circulated EPC [28,36]. It has been known that SDF-1 and the expression of its receptor (CXCR4) play an important role in the regulation of mobilization and recruitment of progenitor cells, so that the reduction of the molecules estimated to be the cause of low amount of circulated EPC [32,34,36]. The cells expressing CXCR4 correlates positively with the progenitor cells amount on normal individual, while on diabetic patients, there is no correlation. Other research indicated that there is significant decrease of CXCR4 expression in PBMC of type 2 diabetic patients [24].

Figure 4. The Profile of SDF-1 through flow cytometric analysis; (a) negative control, (b) positive control, (c) VipAlbumin® 1st dose, (d) VipAlbumin® 2nd dose, (e) VipAlbumin® 3rd dose

Figure 5. The comparison of SDF-1 relative amount on each treatment

The decrease of circulated EPC amount is affected by the low ability of EPC cells in responding to SDF-1 [36]. It was also reported that on diabetic patients, there is decrease in ability of CD34⁺ cells in migrating, which is affected by the disability in responding the SDF-1 performance [46]. The genotype of SDF-1 can

influences insulin in mobilizing mature progenitor cells on type 2 diabetic patients [47].

CONCLUSION

In this research, the administration of three doses of VipAlbumin® were not capable to increase CD34 relative amount, one of

endothelial progenitor cells marker derived from bone marrow. 1st and 2nd dose of VipAlbumin® also has no ability to stimulate the development of SDF-1 relative amount. The development of this molecule only occur at the 3rd dose of VipAlbumin® (10.4 mg.g⁻¹ BW), and was significantly different from positive control.

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