The Role of Mesenchymal Stem Cells on Regulating PDGF and Islet Cells in Diabetes

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ABSTRACT

Diabetes is a group of metabolic disease characterized by increased level of blood glucose as a result of defect in β Cells destruction and/or insulin resistance. Clinical studies showed that transplantation of islet is sufficient cure to relief from the diabetes-related symptom. However, the lack of applicable donor is becoming a serious problem. Recent study has shown that Mesenchymal Stem Cells (MSCs) has high-potential to regenerate pancreatic tissue damage including islet Cells by involving various growth factors such as PDGF. The aim of this study is to determine the effect of umbilical cord MSCs in increasing the number of islet Cells and PDGF level appearance on the 44th day of remodeling phase in pancreatic tissue damage. This study used 20 streptozotocin-induced balb-c mice (Mus musculus) to diabetes divided into four group (n=5/group) consisting control (K) and treatment (P). The control group was treated PBS while P1, P2, and P3 treated different MSCs dose intraperitoneally (P1=1.5x10⁵, P2=3x10⁵, dan P3=6x10⁵ Cells). Day 44, ELISA essay were performed using blood samples to identify PDGF level appearance and tissue histopathology for counting pancreatic islet Cells. The result of this study presented the increased of islet sells significantly (p<0.05) and PDGF level appearance similar to the control. Here, our experimental in model of diabetic-induced balb-c mice (Mus musculus) suggested that MSCs is able to increase the number of islet cells and regulated PDGF level.

Keyword: Diabetes, islet cells, MSCs, PDGF level

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INTRODUCTION

Diabetes is a group of metabolic syndromes characterized by hyperglycemia including metabolic disorders of carbohydrates, fats, and proteins as a result of insulin secretion deficiency and insulin function disorders (1). In general, diabetes is divided into two, namely, type 1 and type 2. Type 1 diabetes is caused by pancreatic β cell damage due to autoimmune processes, while type 2 diabetes is a combination of genetic and non-genetic factors which results in insulin resistance and insulin deficiency (1). The International Diabetes Federation reports that there are 415 million people suffering from diabetes mellitus (DM) with a prevalence of 9.1% in 2015 and will be 10% in 2040 (2). Pancreatic β cell transplantation shows good results in treating diabetes, but donor limitations and rejection possibility by immune cells are the constraints (3), whereas insulin injections have the risk of long-term dependence, difficulty in adjusting the amount of exogenous insulin and the potential for insulin resistance (4). Mesenchymal Stem Cells (MSCs) can differentiate into various types of cells including insulin-producing cells (5), thus can regenerate pancreatic β cells and improve insulin resistance among DM patients (6).

Mesenchymal stem cells (MSCs) are adult stem cells originating from stromal tissue with a characteristic of being able to express various surface markers in the forms of CD73, CD90 and CD105, and expressing a little of CD45, CD34, CD14 or CD11 CD79/CD19, and HLA-DR (7). Mesenchymal stem cells (MSCs) are obtained from various tissues such as the spinal cord, adipose, placenta, and umbilical cord (8). Mesenchymal stem cells (MSCs) from the umbilical cord are thought to have a stronger stemness potential than those from bone marrow, besides that the technique of extracting MSCs from the umbilical cord is relatively easier and non-invasive. Mesenchymal stem cells (MSCs) are capable of self-renewal and differentiate to specific cells (multilineage differentiation) including being pancreatic β cells (9). On the other hand, in a paracrine way MSCs also can secrete various bioactive molecules such as vascular endothelium growth factor (VEGF) and platelet-derived growth factor (PDGF) which play a strong role in increasing the vascular regeneration, angiogenesis, and decreasing the number of apoptotic cells (3). When a wound occurs, pro-inflammatory molecules such as TNF-α will activate MSCs to release some growth factors (10,11). Levels of growth factors, especially PDGF, are generally used as indicators of pancreatic islet cell growth (12).

Platelet-Derived Growth Factor (PDGF) acts as one of the factors of cell growth and cell division, especially during angiogenesis phase and blood vessel formation from previous vascular tissue (12,13). Platelet-Derived Growth Factor (PDGF) will send signals that can trigger the migration of various cell types during inflammation besides inducing cellular proliferation. PDGF level increases in the initial week with an aim of enhancing the neovascularization process and slowly decreases in line with the wound healing phase (14). This study aimed to determine the effect of umbilical cord MSCs on increasing the number of pancreatic islet cells in the remodeling phase which is accompanied by decreasing PDGF level.

METHOD

Experimental Diabetes Induction

Female mice (Mus musculus) Balb-C strain aged ten weeks were intraperitoneally injected with 40mg/kg streptozotocin (STZ; Sigma-Aldrich, S0130) for five consecutive days until reaching diabetic state. STZ was dissolved in sodium citrate solution, pH 4.5. All procedures were in accordance with the provision of the experimental ethics commission of the Faculty of Medicine, Sultan Agung University (Unissula). The procedure for making mice model was based on the standard protocol for obtaining a diabetes animal model. The protocol referred to the experimental research protocol book series in the Animal Modeling Study Group, Department of Physiology, Faculty of Medicine UNAIR.

MSCs Isolation cell

The 19-day pregnancy umbilical cords of the female rats (Rattus norvegicus) were cleaned using PBS solution. The cord blood vessels were removed, and the umbilical cord was cut 2-5 mm long using a sterile knife and placed evenly in a 66 mm culture dish. The medium used was the DMEM culture medium (Sigma-Aldrich, Louis St, MO, MFC00217342) mixed with 10% FBS, 100IU/ml penicillin/streptomycin (Gibco, Invitrogen, 15140122) and then incubated at 37°C with 5% CO₂. Medium replacement was done every 3 days. After 4-5 flush times, the cells were cultured for 8-10 days until 80% confluent and then harvested by giving 0.25% trypsin-EDTA (catalog number: 25200056) at 37°C. The cells used in this study were cells from the 5th flush.

Characteristics of MSCs Phenotype

MSCs expression from the result of isolation was assessed by using immunocytochemistry technique as indicated by the expression of CD 73 and CD 105 (MSCs marker). After MSCs were 60-80% confluent (3-4 weeks), MSCs were harvested and grown on coverslips with a density of 5x10⁴-1x10⁵ cells for 3-4 days; then they were fixated with 4% paraformaldehyde in 90% ethanol for 15 minutes at 4°C. Cells were incubated with primary antibodies CD 73 and CD 105 which were markers of MSCs (1: 100) (BD Pharmingan, San Diego, CA USA, no: 563198, 561443) for 60 minutes at room temperature. These markers were the minimum criteria that must be used in testing the MSCs phenotype in accordance with the provisions of The International Society for Cellular Therapy (15). CD 105 is a type 1 membrane glycoprotein which functions as a TGF-b receptor accessory which is a stemness factor molecule (16). On the other side, CD 73 has a special role in regulating the inflammatory process of injured tissue (17). The cells were then washed with PBS for 10 minutes, and a second antibody was added (1:2500 dilution) for 15 minutes at room temperature, and then counterstaining was carried out with 3,3’-Diaminobenzidine (DAB) (Santa Cruz Biotech, SC-24982) and observed under a microscope (18).

In Vitro Differentiation

MSCs were grown in a culture dish with a density of 5x10⁴-1x10⁵cells/well treated with osteogenic induction medium containing 10mmol/L β-glycerophosphate, 0.1µmol/L dexamethasone, 50µmol/L ascorbate-2-phosphate (Sigma-Aldrich, Louis St, MO, MFC0064136) and 10%
FBS of the total medium volume in DMEM. Osteogenic differentiation was observed by using Alizarin Red staining after 21 days of induction to determine calcium deposits. Cells were rinsed with PBS and fixed with cold ethanol 70% (v/v) for 1 hour at room temperature then rinsed three times with aquadest. As much as 1ml Alizarin Red solution 2% (w/v) (pH 4.1-4.3) was added and incubated for 30 minutes at room temperature then rinsed four times with aquadest. As much as 1 ml Alizarin Red solution 2% (w / v) (pH 4.1-4.3) was added and incubated for 30 minutes at room temperature then rinsed four times with aquadest. In vitro differentiation of MSCs into adipocyte cells was considered to represent the ability of MSCs to differentiate (19).

**MSCs Transplantation and Physiological Monitoring**

Diabetic mice (Mus musculus) (n = 5/group) were intraperitoneally injected at different doses. The control group was given PBS (Phosphate Buffer Saline) injection. P1, P2, and P3 groups were intraperitoneally injected with MSCs at doses of 1.5 x 10^7, 3 x 10^7 dan 6 x 10^7 cells in 0.5 cc PBS. After 44 days of treatment, mouse blood samples were taken through the orbital vein and then mice were sacrificed by cervical dislocation. The retrieval of pancreatic tissue was carried out for making the preparations.

**Human PDGF ELISA Testing**

PDGF Serum level test using ELISA kit (Fine test ER1240, China) was carried out according to the instructions manual at a room temperature. ELISA plates were coated with capture antibodies and incubated overnight at 4°C. Wells were washed and then blocked for 1 hour. The wells were incubated with standard PDGF mouse solution, treatment mouse blood serum, and control mouse blood serum for 2 hours, and then diluted with a ratio of 1:100 to obtain measurements in the standard curve. After washing, the wells were incubated with detection antibody for one hour and then washed over again for several times. The wells were incubated with Avidin-HRP for 30 minutes, washed, incubated with substrate solution for 15 minutes, followed by the addition of stop solution, and A$_{opt}$ results were measured using microplate reader.

**Histology Analysis**

After pancreatic tissues were obtained, they were inserted into 10% neutral-buffered formalin and implanted in paraffin or Histogel (Thermo Scientific HG-4000-012, Watham, MA, USA). The tissue that has been attached to paraffin was then cut using microtome with a thickness of 5-10µm, stained with Hematoxylin Eosin (HE), and then observed using a light microscope.

**Data analysis**

Data analysis was performed using the statistical method Kruskal-Wallis Test which then followed by the Mann-Whitney Test and correlation test using the Spearman Test.

**RESULTS**

**Characteristics of MSCs**

Identification of MSCs characteristics was carried out using CD 73 and CD 105 markers by immunocytochemistry (Figure 1).

![Figure 1. Characteristics of cultured mesenchymal stem cells 100x magnification](image)

**Description:** Morphology of MSCs appear as spindle-shaped cells like fibroblast marked in brown color shown by arrow (a) CD 105 expression, (b) CD 73 MSCs expression

**Results of Isolation and Mesenchymal Stem Cells Differentiation Tests**

MSCs Osteogenic differentiation tests were carried out in vitro in wells. Identification of calcium deposits was using Alizarin red (Figure 2).

**PDGF levels**

![Figure 3. mean score of PDGF level on day 44](image)

**Note:** There were no significant differences in the PDGF level on day 44 among the control group and all treatments. PDGF levels in group P1: 44.04 ± 1.14pg/ml, P2: 52.46 ± 1.28pg/ml, P3: 44.26 ± 1.34pg/ml, and control: 31.17 ± 0.82pg/ml (Figure 3).

**Number of Islet Pancreatic Cells**

![Figure 4. Mean of pancreatic islet cell number on day 44](image)

**Note:** There were significant differences in the number of islet cells on day 44 among the control group and all treatments.
Calculation on the number of pancreatic islet cells was carried out in the Pathology Anatomy Laboratory of Sultan Agung Islamic Hospital. There were significant differences (p <0.05) on the number of islet cells on the 44th day. Figure 4 shows that the number of pancreatic islet cells were 155.60±47.57 cells in P3, 124.60±28.11 cells in P2, and 86.40±13.89 cells in P1 while 76.00 ± 14.27 cells in control.

Figure 5. Image of islet cells on day 44 after treatment
Note: (a) control group (b), (c), (d) group P1, P2, and P3 (black arrows point the islet islands)
On day 44 (Figure 5) islet cells of the control group showed smaller islet islands compared to those of treatments.

DISCUSSION
The growth of pancreatic tissue is determined by the differentiation process of stem cells/progenitors (neogenesis) into islet cells in addition to the islet cell proliferation itself (19). The differentiation process is also influenced by wound response (20). Differentiation occurs when MSCs migrate to the injured area due to the release of certain molecules, including hematopoietic cells E-/L-selectin ligand (HCELLS), matrix metalloproteinases (MMP), and SDF-1 (21). These molecules are able to drag MSCs into the blood vessels which are then followed by transmigration outside the endothelium (22).

The results of this study indicate that the administration of serial dosages of rat umbilical cord MSCs can increase the number of pancreatic islet cells. This is consistent with the previous studies that reported the administration of human Mesenchymal Stem Cells (hMSCs) in rat models was able to increase pancreatic β cells and insulin production (23). There were significant differences among the control and the treatment groups after a serial of MSCs dose administrations which show an increase in the number of islet cells according to the doses of MSCs. This can be seen in the microscopic picture of the number of islet cells in pancreatic tissue preparations. The mechanism of MSCs regeneration in the wound area is thought to be through the process of differentiation, vascularization by involving the release of various paracrine molecules including PDGF.

This study also obtains PDGF levels that are not significantly different on day 44 among the control and treatment groups. Apparently, there has been a decrease in PDGF levels along with a significant increase in the number of islet cells. This shows that the damaged tissue repair process has entered the remodeling phase. This fact is in accordance with the results of previous studies that PDGF experienced a significant increase in the proliferation phase, namely, day 7 and 14 after inflammation (24) because PDGF is needed by various cells in the process of proliferation and regeneration. PDGF level will decrease slowly as the healing process enters the remodeling phase. PDGF level that remains high in the remodeling phase shows that fibroblast cell activation is ongoing which has an impact on tissue fibrosis (25). This shows that the tissue repair process does not occur perfectly.

Fibroblasts activation occurs through extracellular signal-regulated kinase (ERK1/2), which is one component of MAPK in increasing the c-fos protein expression which plays a central role in tissue regeneration (26). The important role of MSCs in this study is possibly through paracrinization by secreting various bioactive molecules, especially PDGF, to activate various surrounding cells including endogenous stem cells besides differentiating into islet cells, so that the regeneration process occurs optimally. When islet cells begin to form, PDGF will decrease, thus it can provide information about the rate of pancreatic cells growth. This is in line with the results of our study, PDGF levels in all treatments were not significantly different which indicated a decrease in PDGF levels on day 44 of the remodeling phase along with the growth of islet cells that had reached the optimum. This result suggests that MSCs play a central role in various phases of tissue healing. MSCs can safely repair islet cell damage in STZ-induced hyperglycemic mice (Mus musculus). PDGF levels measured in the remodeling phase (day 44) were not significant because the wound healing process was completed. In this study, we did not analyze pancreatic β cells so the role of MSCs in differentiation into pancreatic β cells is not known.

This study proves that the administration of serial dosing of rat umbilical MSCs can increase the number of pancreatic islet cells. There are no differences in PDGF levels because it has entered the remodeling phase.

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