11. In-Vitro Differentiation Adipose-Derived Mesenchymal Stem Cells into Pancreatic Progenitor Cells

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In-Vitro Differentiation Adipose-Derived Mesenchymal Stem Cells into Pancreatic Progenitor Cells

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Abstract

Background: Adult stem cells are currently reliable sources of mesenchymal stem cells for regenerative therapy, include diabetes mellitus. The aim of this study is to develop endocrine pancreatic progenitor cells characterized by Pdx1 and insulin expression from rat adipose-derived mesenchymal stem cells using two steps in-vitro differentiation.

Methods: In this experimental study, ADMSCs were isolated from rat adipose tissue and exposed to insulinogenic differentiation medium containing nicotinamide, activin A and glucagon-like peptide-1 (GLP-1). After induction, the existence of pancreatic progenitor cells (PPCs) was confirmed by immune-staining assay of Pdx1 and insulin.

Results: After three weeks of in-vitro differentiation, expression of Pdx1 and insulin proteins showed up as green in the immunofluorescence assay. Immunofluorescence intensity of Pdx1 was higher in PPCs than in ADMSCs control ($p<0.05$). Immunofluorescence intensity of insulin was also higher in PPCs than in ADMSCs control ($p<0.05$). Therefore, in-vitro differentiation was successful to develop PPCs from rat ADMSCs

Conclusion: This study has demonstrated the in-vitro differentiation of ADMSCs into PPCs that expressed Pdx1 and insulin

Keywords: *diabetes, adipose-derived mesenchymal stem cells, Pdx1, insulin*

Introduction

According to a report by the WHO, 442 million people suffer from diabetes worldwide[1] and more than 10 million people have diabetes in Indonesia [2]. Both type 1 and type 2 diabetes mellitus are characterized by destruction or dysfunction of pancreatic beta cells, regardless of the type of diabetes, the main focus of DM therapy is insulin therapy [3]. Currently, regenerative therapy is growing rapidly, including various studies that investigate the formation of insulin-producing cells (IPCs) from embryonic stem cells, umbilical cord, and various adult tissues [4].

Adult stem cells are currently reliable sources of mesenchymal stem cells for regenerative therapy. Among the various sources of adult stem cells, adipose tissue is an ideal population of stem cells. Adipose tissue contains an abundance of stem cells, which can be obtained by a procedure that is relatively easy, inexpensive and minimally invasive[5,6]. Several studies were conducted to differentiate MSCs into cells that were similar to pancreatic islets and which, when transplanted, proved to be functional and producing insulin in diabetic rats[6]. Pancreatic progenitor cells (PPCs) give rise to all cell

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types in the pancreas during development [7]. Pancreatic progenitors cells expressing the key transcription factor Pdx1 are recognized as the indispensable precursors of functional pancreatic β cells [7,8].

There are several main mechanisms of pancreatic regeneration such as replication of mature beta cells, differentiation of stem cells, cell fusion, and trans-differentiation of one type of stem cells to another type[9]. Adipose-derived mesenchymal stem cells (ADMSCs) are mesenchymal stem cells (MSCs) which obtained from adipose tissue and had unique characteristics making them suitable for differentiation into endocrine pancreatic progenitor[10,11]. Differentiation MSCs into endocrine progenitor pancreas can be carried out using many extrinsic factors such as nicotinamide, exendin-4, and GLP-1 receptor agonist[6].

The aim of this study is to develop endocrine pancreatic progenitor cells characterized by Pdx1 and insulin expression from rat adipose-derived mesenchymal stem cells using two steps in-vitro differentiation.

Materials and Methods

Isolation, culture and characterization of cells

Rats were obtained from Stem Cells Research and Development Center Universitas Airlangga, and this study was approved by the Ethics Committee of the same University. Subcutaneous adipose tissue from male Wistar rats was removed under sterile conditions, cut into small pieces and incubated to liberate the cells in 25 cm2 flasks containing Dulbecco's Modified Eagle's Medium (DMEM) and 1.0 mg/ml of collagenase. The cell suspension was centrifuged at 3500 rpm for 5 minutes at room temperature to obtain pellets containing cells from the stroma. Furthermore, erythrocyte lysis was carried out with ammonium chloride lysis buffer then washed with phosphate-buffered saline. Cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) which had been supplemented with 20% Fetal Bovine Serum (FBS; Gibco, USA), 1% penicillin-streptomycin (Invitrogen) and 1% Amphotericin B (Gibco, USA). The cultures of ADMSCs were inspected every three days and passaged when the ADMSCs had reached approximately 80% confluence. The mesenchymal stem cells were isolated based on their ability to adhere to the bottom of the flask. The ADMSCs appeared spindle-shaped in the culture. They were harvested in passage 3 and characterized as mesenchymal stem cells using CD73 (1 mg/mL; BD Pharmingen), CD90 (1 mg/mL; BD Pharmingen, San Diego, CA, USA), CD105 (1mg/mL; BD Pharmingen), and CD34 (1mg/mL; BD Pharmingen).

In-vitro differentiation ADMSCs into PPCs

We performed the two-step differentiation protocol in this study for three weeks[12]. The cells that had been placed on multiwell plate M 24, each $5x10^3$ cells per well then cultured on insulinogenic differentiation medium containing 10 mmol nicotinamide (Sigma), 4 nmol activin A (Peprotech, Rocky Hill, NJ, USA), and 10 nmol glucagon-like peptide-1 (GLP-1, Sigma). During the first week of culture, cells were cultured in DMEM-high glucose (DMEM-HG) medium containing 10% FBS. Then two weeks later, culture in DMEM-low glucose (DMEM-LG) medium containing 10% FBS.

Characterization of Pdx1 and Insulin

The progenitor cells were placed on a multiwell plate M24 containing slides that were coated with poly-L-lysine with 6 slides of replication slides for each marker. The slides were fixed in 4% paraformaldehyde for 20 minutes, then added with Triton X-100 0.2% for 30 minutes and blocked with 5% normal goat serum (Sigma) for 20 minutes. Incubation of primary antibodies has been labelled with Pdx1 or insulin. It was continued with incubation of antibodies labelled FITC for 1 hour at room temperature, then washed with PBS-Tween 0.5% 2 times before reading it under a microscope. Images were captured using an Olympus CKX53 fluorescent microscope (Olympus, Japan). From each group, a minimum of ten slides were examined. Immunostaining intensity was estimated using quantitative score using digital software program ImageJ.

Statistical Analysis

Data distribution was analyzed using the Kolmogorov-Smirnov test. The comparisons of two group means of intensity were performed using Mann-Whitney tests, and $P<0.05$ was considered statistically significant.

Results

Cell surface markers of ADMSCs detected by

immune-staining revealed that ADMSCs expressed CD73, CD90 and CD105, whereas expression of CD34 was negative (Figure 1).

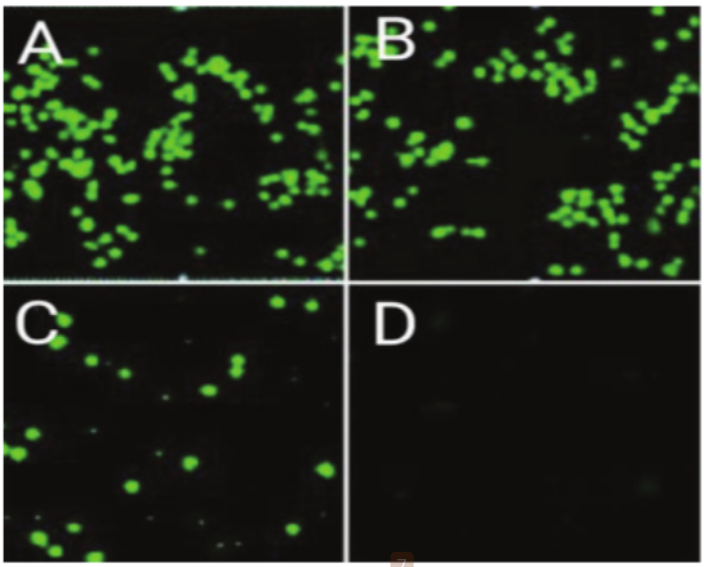

Figure 1. Immunofluorescence of cell surface markers. A. Expression of CD73, B. Expression of CD90, C. Expression of CD105, D. Expression of CD34 (magnifications: ×200).

After three weeks of in-vitro differentiation, expression of Pdx1 and insulin proteins showed up as green in the immunofluorescence assay (figure 2C and 2E). The ADMSCs cultured in non-differentiation inducing media (control group) showed very low expression of Pdx1 and insulin proteins (figure 2D and 2F).

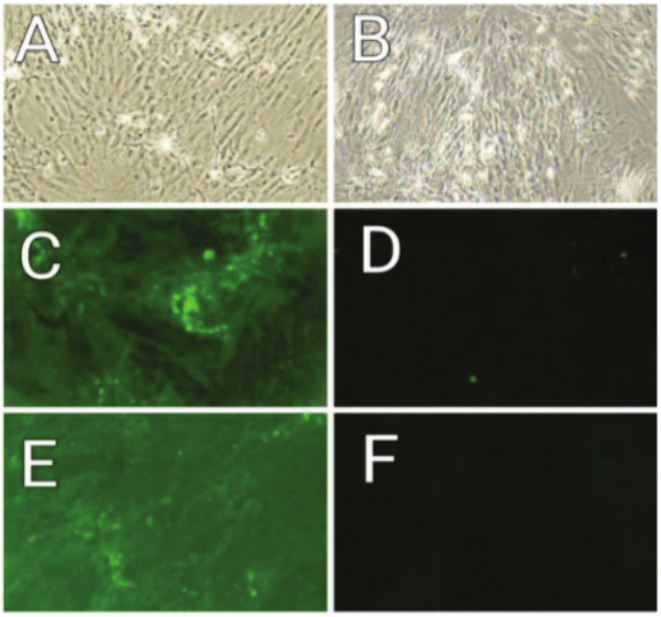

Figure 2. Progenitor cell identification and comparison with control. A. Progenitor cell proliferation. B. Control cell proliferation. C. Expression of Pdx1 on progenitor cells. D. Expression of Pdx1 on control cells. E. Expression of insulin on progenitor cells. F. Expression of insulin on control cells (magnifications: ×200).

Distribution of data was analysed using the Kolmogorov-Smirnov test and revealed that the distribution was not normal. Then the data were analysed using the Mann-Whitney test to compare immunofluorescence intensities of Pdx1 and insulin between PPCs and ADMSCs (control). Immunofluorescence intensity of Pdx1 was higher in PPCs than in ADMSCs control (p=0.0001). Immunofluorescence intensity of insulin was also higher in PPCs than in ADMSCs control (p=0.0001). Therefore, in-vitro differentiation was successful to develop PPCs from rat ADMSCs (table 1).

Table 1. Immunofluorescence intensities of Pdx1 and Insulin

Markers	PPCs		ADMSCs (Control)		Mann-Whitney Test
	Median	Range	Median	Range	
Expression of Pdx1 (/mm2)	12.455	8.479 - 15.547	5.486	4.263 - 6.187	P = 0.0001*
Expression of Insulin (/ mm2)	31.785	24.407 - 35.918	4.015	3.163 - 5.873	P = 0.0001*

*statistically significant (p<0.005)

Discussion

Stem cells are currently becoming the focus of beta cell replacement, due to flexibility and abundant sources. Stem cells derived from pluripotent embryonic stem cells (ESCs) have great differentiation potential, but ethical issues and malignant transformation are still the main problems[13]. Mesenchymal stem cells (MSCs) are found in various body tissues such as bone marrow, umbilical cord and adipose tissue and are immune-naive. MSCs derived from adipose tissue or called adipose-derived mesenchymal stem cells (ADMSCs) have the advantage of being easily obtained in abundance and have the potential to differentiate into various pathways, both mesoderm, ectoderm and endoderm[14]. Approximately 1-10% of the stromal fraction of adipose tissue are MSCs and they are capable to differentiate into multiple pathway[15]. Standard criteria for MSCs are positive phenotypes for CD105, CD73 and CD90, and negative phenotype for CD45, CD34, CD14 or CD11b, CD79 alpha or CD19[13,16]. The phenotypes characterization of MSCs in this study showed positive staining results for CD73, CD90 and CD105, as well as negative staining for CD34, according to the existing theory.

Pancreatic progenitor cells are multipotent cells derived from the endoderm that have the ability to differentiate into specific progenitor pathways that are responsible for pancreatic development[17,18]. In this study, ADMSCs were differentiated into pancreatic progenitor cells which later became insulin-producing cells. The induction of stem cells to become insulin-producing cells requires a multi-stage protocol. Most protocols require at least a two-stage protocol, while others can go up to 5-6 stages. It can last from several days to several months. Finally, the addition of combination extrinsic factors can stimulate the proliferation and differentiation of beta cells [19,20].

In this study, the differentiation of ADMSCs into progenitor cells was performed in two stages. The first stage used insulinogenic differentiation medium containing nicotinamide, activin A and glucagon-like peptide-1 (GLP-1) with DMEM-high glucose (DMEM-HG) medium containing 10% FBS for one week. Meanwhile, the second stage of the insulinogenic medium was cultured in DMEM-low glucose (DMEM-LG) medium containing 10% FBS for two weeks. Therefore, it needed a total of three weeks for the

differentiation of ADMSCs into insulin-producing cells[13,21]. The glucose medium had a function to increase beta-cell replication and hypertrophy. Nicotinamide is associated with the development of beta-cell outgrowth from the undifferentiated epithelial cell cluster. The growth factor activin A triggered beta cell regeneration by increasing the mass of beta cells. Meanwhile, GLP-1 accelerated the functional maturation of beta cells by secreting insulin [22,23].

Pancreatic and duodenal homeobox1 (Pdx1) is the first transcription factor produced by the pancreas. Loss of expression of this factor in humans and mice will lead to pancreatic agenesis due to the absence of endocrine, exocrine or pancreatic duct cells [24]. However, Pdx1 expression became limited to pancreatic beta cells only. If Pdx1 is removed from pancreatic beta cells, hyperglycaemia will occur due to reduced insulin-producing cells and an increase in glucagon-producing cells [25–27]. Pancreatic progenitor cells were characterized by expression of Pdx1, Myc and Ptf1A. Furthermore, cells expressing Ptf1A will differentiate into exocrine pancreatic cells [28]. In this study, the expression of Pdx1 and insulin were marker that showed ADMSCs differentiated into pancreatic progenitor cells. Expression of Pdx1 is a key factor that determines the fate and development of the pancreas, meanwhile, insulin was the target of progenitor cell treatment.

Conclusion

This study has demonstrated the in-vitro differentiation of ADMSCs into pancreatic progenitor cells that expressed Pdx1 and insulin. This method may help the formation of an unlimited source of cells for pancreas transplantation.

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