Therapeutic Efficacy of Differentiated Versus Undifferentiated Mesenchymal Stem Cells in Experimental Type I Diabetes in Rat

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Abstract

Background: The purpose of this study was to investigate the effect of differentiated mesenchymal stem cells (MSCs) in treatment of type 1 diabetes mellitus (DM) in rats and to compare between the effect of the differentiated versus the undifferentiated MSCs in treatment of experimental type 1 diabetes mellitus (DM).

Methods: This work included: in vitro study and in vivo study. MSCs were derived from the bone marrow of rats. The MSCs were characterized morphologically and by RT-PCR for CD29 expression. MSCs were differentiated into pancreatic β cells by addition of exendin-4 and TGF-β. Insulin is measured in the medium also gene expression of Smad2 by PCR.

Ninty Rats were Divided Equally Into: Healthy control group, healthy control group that received acellular medium, diabetic group was induced by injection of streptozotocin (STZ) intraperitoneal (IP), diabetic group received acellular medium, diabetic group that received undifferentiated MSCs, diabetic group received differentiated MSCs.

Therapeutic efficacy of undifferentiated versus differentiated MSCs was evaluated via assessment of quantitative gene expressions of insulin1, Smad-2, Smad-3, Pdx-1, neuroD. Blood glucose and insulin hormone levels were also assessed.

Results: Diabetic rats that received undifferentiated or differentiated MSCs showed lower serum glucose and high plasma insulin with expression of Insulin1, Smad2, Smad3, Pdx1, and neuroD by real time, reverse transcription-polymerase chain reaction (RT-PCR) in rat pancreatic tissue.

Results were better in case of differentiated more than the undifferentiated MSCs.

Conclusion: MSCs derived from rat bone marrow may provide a treatment option for type 1 diabetes mellitus. While both differentiated and undifferentiated MSCs have the ability to become insulin producing cells capable of controlling blood glucose level in diabetic rats, differentiated MSCs are found to be better than undifferentiated MSCs in therapy of experimental type 1 diabetes mellitus.


Introduction

TYPE I diabetes mellitus (DM) is a prevalent disease affecting millions of people with serious morbid complications. Efficient glycemic control with exogenous insulin therapy is the cornerstone of treatment which imposes a great burden to most patients. Isolated beta cells or whole intact pancreas transplantation is an alternative treatment for patients with type I DM. However, the shortages of cadaveric pancreas and the need to immune-suppressive drugs are limiting factors [1].

Recent progress in regenerative therapies has focused attention on generation of surrogate β cells from mesenchymal stem cells derived adult tissues [2]. Bone marrow-derived mesenchymal stem cells (BM-MSCs) are multipotent that have efficient trans-differentiation potentials into mesodermal, endodermal, and ectodermal lineages [3,4]. BM-MSCs are good sources for generation of large numbers of autologous β cells circumventing the major limitations of cadaveric organs availability and allogetic rejection [5,6].

Exendine-4 is a novel insulinotropic peptide and a long acting analogue of glucagon-like peptide-1 (GLP-1). It interacts with endocrine pancreatic islet GLP-1 receptors, inducing a stimulatory effect on insulin secretion. Exogenous exendin-4 has shown to induce insulin-positive/endocrine differentiation in pancreatic exocrine AR42J cells [7,8].

The co-application of exogenous exendin-4 and, specifically, low-dose exogenous transforming growth factor-1 (TGF-1) led to a dramatic 20-fold increase in insulin mRNA levels, supporting a
novel synergistic and codependent relationship between exendin-4 signaling and TGF-isofrom signaling [9]. Exogenous TGF-beta1 and exendin-4 each individually enhanced both insulin and glucagon differentiation dose-dependently. However, when combined there was an additive effect to a 4.5-fold increase in insulin-positive differentiation [10]. The pancreatic lineage differentiation was evaluated via assessment of gene expressions of insulin-1, PDX-1 and NeuroD [11].

The present study was conducted to assess the therapeutic efficacy of undifferentiated versus differentiated mesenchymal stem cells in experimental type I diabetes mellitus in rats. Selective differentiation protocol using TGF-beta and exendin-4 was implemented.

Material and Methods

The experiment was conducted in the animal house of the department of biochemistry and Molecular Biology between 2013 and 2014.

Preparation of BM-derived MSCs:

Bone marrow was isolated and propagated according to the standard described method [12]. On day 14, the adherent colonies of cells were trypsinized, and counted. Cells were identified as being MSCs by their morphology, adherence, and their power to differentiate into osteocytes [13] and chondrocytes [14].

BM-derived MSCs differentiation into islet like cell clusters (ICC):

MSCs at passage 2 was induced with L-DMEM (4.5mmol/L), glucose 5%, FBS, nicotinamide (10 mmol/L) and β-mercaptoethanol (2.5mmol/L) for 24 hrs. Re-induction was conducted for 10 hrs with the same culture medium without FBS and with L-DMEM (25mmol/L). Maintenance of ICC was conducted for 3 weeks with L-DMEM (25 mmol/L), glucose 5%, without FBS, nicotinamide (10mmol/L), β-mercaptoethanol (2.5mmol/L), exendin-4 (10pmol/L) and TGFβ-1 (100pmol/L). Smad2 gene expression was assessed in the undifferentiated and in the differentiated MSCs in vitro.

Labeling stem cells with PKH26:

Undifferentiated and differentiated MSCs cells were labeled with PKH26 according to the manufacturer’s recommendations, (Sigma, Saint Louis, Missouri, USA). Cells were injected intravenously into rat tail vein. After one month, pancreatic tissue was examined with a fluorescence microscope to detect the cells stained with PKH26.

Preparation of animal groups:

The present study was conducted on ninety female rats inbred strain (Cux 1: HEL 1) of matched age and weight (6 months-1 year & 120-150gm). Animals were inbred in the experimental animal unit, Faculty of Medicine, Cairo University. Rats were maintained according to the standard guidelines of Institutional Animal Care and Use Committee and after Institutional Review Board approval. Animals were fed a semi-purified diet that contained (gm/kg): 200 casein, 555 sucrose, 100 cellulose, 100 fat blends, 35 vitamin mix, and 35 mineral. Diabetes type I was induced in the relevant rat groups using a single intra-peritoneal injection of streptozotocin dissolved in 0.1M sodium citrate buffer, pH 4.5, at a dose of 50mg/kg. Induction of diabetes was confirmed by assessment of blood glucose after 72 hr of streptozotocin injection. Rats with blood glucose above 200mg/dL were considered to be diabetic.

Animals were divided equally into the following groups:

Group 1: Included 15 normal healthy rats that received the standard diet i.e. negative control group.

Group 2: Included normal healthy rats 15 rats that received the standard diet and acellular tissue culture medium.

Group 3: Included 15 rats that received streptozotosin to induce type I diabetes mellitus. i.e. diabetic control (positive control group).

Group 4: Included 15 diabetic rats received acellular tissue culture media.

Group 5: Included 15 diabetic rats that received undifferentiated MSCs stained with PKH26 red florescence (3 x 10^6 cells intravenously, once) [8].

Group 6: Included 15 diabetic rats that received selectively differentiated MSCs into pancreatic progenitor cells stained with PKH26 red florescence (10^7-10^3 cells intravenously, once) [4].

Three rats died in the control diabetic group and 5 rats died in the control diabetic injected with acellular group. All rats were sacrificed at the end of the third month. Blood samples were collected at the first, second and the third month to assess blood glucose and insulin levels by ELISA.

Real-time quantitative analysis for, Real-time quantitative analysis for insulin1, Smad-2, Smad-3, PDX-1, neuroD and GAPDH genes in pancreatic tissues.
Total RNA was extracted from pancreatic tissue homogenate using RNeasy purification reagent (Qiagen, Valencia, CA). cDNA was generated from 5 µg of total RNA using RT reagent kit (Applied biosystems, catalogue number 4306736). Real-time qPCR amplification and analysis were performed using SYBR® Green PCR Master Mix Reagents Kit (Catalog Number 4309155) and Applied Biosystem Instrument with software version 3.1 (StepOneTM, USA). The qPCR assay with the primer sets were optimized at the annealing temperature.

**Analysis of pancreatic tissue pathology:**

Pancreatic tissue samples were collected into PBS and fixed overnight in 40g/L paraformaldehyde in PBS at 4°C. Serial 5-µm sections of the pancreatic tissues were stained with hematoxylin and eosin (HE) and were examined histopathologically.

**Statistical analysis:**

Data were coded and entered using the statistical package Spass version 16. Data was summarized using mean and standard deviation. Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc test in normally distributed quantitative variables while nonparametric Kruscal-Wallis test and Mann-Whitney test were used for non-normally distributed quantitative variables by pearson correlation. *p*-values less than 0.05 were considered as statistically significant.

**Results**

Isolated and cultured MSCs were identified by their characteristic fibroblast shape after 1 and 2 weeks (Fig. 1A,B). Mesenchymal stem cells labeled with PKH26 fluorescent dye detected in the pancreatic tissue, confirming homing into the injured pancreatic tissues (Fig. 1C,D).

Flowcytometric characterization revealed that MSCs were uniformly positive for CD29, and CD44 and negative for CD34 and CD45 (Fig. 2).

Fig. (1): (A): Spindle shaped MSCs at one week culture, (B): MSCs at 2 weeks culture. (C): Homing of PKH26 fluorescent labeled undifferentiated MSCs in rat pancreas. (D): PKH26 fluorescent labeled differentiated MSCs into pancreatic like cells in rat pancreas.

Fig. (2): Flow-cytometric characterization analysis of bone marrow-derived MSCs. Cells were uniformly positive for CD29, and CD44 and negative for CD34 and CD45.
Morphological change of unstained and stained (dithizone stain; DTZ) rat bone marrow MSCs during differentiation showed that MSCs started to form cell clusters after 10 days of differentiation, collected cell clusters after 20 days and well defined cell clusters with spheroid configuration were formed after 25 days of differentiation (Figs. 3,4).

Fig. (3): Unstained rat bone marrow MSCs during differentiation. (A): MSCs started to form cell clusters after 10 days of differentiation (X200). (B): Collected cell clusters after 20 days of differentiation (X200). (C): Well defined cell clusters with spheroid configuration were formed after 25 days of differentiation (X200).

Fig. (4): Rat bone marrow MSCs during differentiation stained with DTZ: (A): MSCs started to form cell clusters after 10 days of differentiation (X200). (B): Collected cell clusters after 20 days of differentiation (X200). (C,D,E): Well defined cell clusters with spheroid configuration were formed after 25 days of differentiation (X200).
Smad-2 gene expression in cultured MSCs showed significant increase in the undifferentiated MSCs versus the differentiated MSCs in culture (Fig. 5).

![SMAD-2 relative gene expression](image)

**Fig. (5):** Smad2 relative gene expression in the undifferentiated and the differentiated MSCs in vitro.

As regards blood glucose levels, use of either undifferentiated or differentiated MSCs exhibited significant decrease in blood glucose levels as compared to diabetic rats, whereas, the levels were still higher than healthy control animals except three months after injection of differentiated MSCs where blood glucose levels were normalized (Fig. 6).

![Blood glucose levels](image)

**Fig. (6):** Blood glucose levels (mg/dL) in the studied rat groups after 1st, 2nd and 3rd months.

Insulin levels were significantly decreased in diabetic rat groups. Use of either undifferentiated or differentiated MSCs led to a significant increase and normalization in insulin levels except after the 1st month of injection of undifferentiated MSCs (Fig. 7).

![Insulin hormone levels](image)

**Fig. (7):** Insulin hormone levels (IU/mL) in the studied rat groups after 1st, 2nd and 3rd months.

Insulin 2 gene expressions showed significant decrease in diabetic rat groups. Use of undifferentiated MSCs led to a significant elevation in insulin genes expression but their levels were still lower than control group. Use of differentiated MSCs led to a significant elevation and normalization of insulin genes expression as compared in control rat group (Fig. 8).

![Relative gene expression of insulin-1](image)

**Fig. (8):** Relative gene expression of insulin-1 in the pancreatic tissues of the studied rat groups.
As regards SMAD-2, SMAD-3, PDX-1 and NeuroD gene expressions, their levels showed significant decrease in diabetic rat groups. Use of undifferentiated and differentiated MSCs led to a significant elevation of expression levels of all genes with more superior effect with differentiated MSCs (Fig. 9).

![Graph showing gene expression levels for smad2, smad3, PAX4, and neuroD in different conditions.](image)

**Fig. (9):** Relative gene expression of smad2, smad3, PAX4, and neuroD in the pancreatic tissues of the studied rat groups.

**Discussion**

As a paradigm for stem cell therapy, we have studied the combined use of exendin-4 and TGFβ1 to induce selective lineage differentiation of MSCs into pancreatic progenitor beta cells. Results showed that gene expression levels of smad2 were significantly elevated in the undifferentiated MSCs with subsequent significant down-regulation of smad2 expression and upregulation of smad3 in the differentiated MSCs in the in vitro study. The same pattern of gene expression was observed in the in vivo study for smad2 and smad3.

Yew et al., (2004) [15] stated that the acquisition of a β-cell phenotype, including insulin mRNA and protein expression, islet amyloid polypeptide (IAPP) expression, and pdx-1 expression, was dependent on smad2 pro-endocrine differentiation in the initial stages of differentiation followed by smad3 induced maturation and expression of β-cell-specific markers such as PDX1 [15,16]. Based on these data, it appears that smad3 may support maturation of β-cells and inhibition of proliferation but only after smad2 has initiated a pro-endocrine differentiation program.

Results of the present study showed that use of either undifferentiated or differentiated MSCs led to a significant increase in insulin 1, insulin hormone levels and genetic markers of beta cell maturation as PDX-1 and neurogenin D. Differentiated MSCs was superior to undifferentiated MSCs regarding all of the studied parameters. Similar findings were reported by other studies. Yew et al., (2004, 2005) [9,15] reported that exendin-4 induce insulin-positive/endocrine differentiation in rat AR42J pancreatic epithelial cells. Insulin-positive differentiation was initiated by exendin-4-induced down-regulation of smad2 and up-regulation of smad3 gene expression. The latter appears to be dependent on endogenous transforming growth factor (TGF) -β release by the AR42J cells and could be a mechanism to promote β-cell maturation. The co-application of exogenous exendin-4 and low-dose exogenous TGF -β1 led to a dramatic 20-fold increase in insulin gene expression, supporting a novel codependent synergistic relationship between exendin-4 signaling and TGF-β isoform signaling.

Our findings agreed with previous studies that showed the efficient protocol used in this study for trans-differentiation of bone marrow-derived MSCs into beta cells. The differentiated cells expressed nestin, pancreatic duodenal homeobox-1 (PDX-1), Neurogenin3, insulin and glucagon [17,18,19].

PDX-1 gene is a transcriptional activator of several genes, including insulin, somatostatin, glucokinase, islet amyloid polypeptide, and glucose
transporter type 2. The encoded nuclear protein is involved in the early development of the pancreas and plays a major role in glucose-dependent regulation of insulin gene expression. Defects in this gene are a cause of pancreatic agenesis, which can lead to early-onset insulin-dependent diabetes mellitus (NIDDM), as well as maturity onset diabetes of the young type 4 (MODY4) [20]. PDX-1 is a master switch gene that control gene expression cascade of all other transcription factors responsible for endocrine neogenesis [21]. These facts could explain our results that demonstrated significant elevation of PDX-1 gene expression and other pancreatic genes: Insulin-1 and neuroD with the use of MSCs in diabetic rats [22].

Conclusion:

Use of undifferentiated or differentiated MSCs showed marked therapeutic potentials in experimental type 1 diabetes in rats with more significant therapeutic value with the use of differentiated MSCs. Further studies are recommended for use of differentiated MSCs in experimental diabetes with long term follow-up to ensure safety and efficacy for justification of clinical trials.

References

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المجموعة الخامسة: تشمل خمسة عشر فارا وتمثل المجموعة الضابطة المصابة بمرض البوال السكري وتتم إعطائهم الخلايا الجذعية غير المتحورة عن طريق الوريد.

المجموعة السادسة: تشمل خمسة عشر فارا وتمثل المجموعة الضابطة المصابة بمرض البوال السكري وتتم إعطائهم الخلايا الجذعية المتحورة عن طريق الوريد.

وقد تم صح عبوات الدم وتم ذبح الفئران بعد 12 أسبوعًا و أخذ البنكرياس لقياس:

- التعبير الجيني الكمي لجينات Insulin1, Smad2, Smad3, Pdx1، and neuro D.
- مستويات الجلوكوز والأنسولين في الدم.

بالإضافة إلى الفحص البيئولوجي للبنكرياس.

وقد وُجد تحسن كبير في مستوى الجلوكوز وارتفاع كبير في مستوى هرمون الأنسولين في الدم في الفئران المصابة بالبوال السكري عند حقنها بالخلايا الجذعية غير المتحورة أو الخلايا الجذعية المتحورة.

وقد وُجد أن هذا التحسن أفضل عند حقن الفئران المصابة بالبوال السكري بالخلايا الجذعية المتحورة.

المصادر:

ما سبق يمكن أن نستنتج أن النخاع الطبيعي يحتوي على الخلايا الجذعية القادرة على التحول إلى خلايا مثيرة للهرمون الأنسولين وبالتالي يتم تنظيم نسبة الجلوكوز في الدم وذلك مؤشر على امكانية استخدام هذه الخلايا غير المتحورة أو المتحورة في علاج مرض البوال السكري.

ونوصي بمزيد من الدراسات للخلايا الجذعية غير المتحورة أو المتحورة في علاج مرض البوال السكري واتاحة المزيد من وقت المتابعة في حالة حقن الفئران بالخلايا الجذعية غير المتحورة.