• BASIC RESEARCH •

Differentiation of rat marrow mesenchymal stem cells into pancreatic islet beta-cells

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Abstract

AIM: To explore the possibility of marrow mesenchymal stem cells (MSC) *in vitro* differentiating into functional islet-like cells and to test the diabetes therapeutic potency of Islet-like cells.

METHODS: Rat MSCs were isolated from Wistar rats and cultured. Passaged MSCs were induced to differentiate into islet-like cells under following conditions: pre-induction with L-DMEM including 10 mmol/L nicotinamide+1 mmol/L β-mercaptoethanol+200 mL/L fetal calf serum (FSC) for 24 h, followed by induction with serum free H-DMEM solution including 10 mmol/L nicotinamide+1 mmol/L,β-mercaptoethanol for 10 h. Differentiated cells were observed under inverse microscopy, insulin and nestin expressed in differentiated cells were detected with immunocytochemistry. Insulin excreted from differentiated cells was tested with radioimmunoassay. Rat diabetic models were made to test *in vivo* function of differentiated MSCs.

RESULTS: Typical islet –like clustered cells were observed. Insulin mRNA and protein expressions were positive in differentiated cells, and nestin could be detected in pre-differentiated cells. Insulin excreted from differentiated MSCs (446.93±102.28 IU/L) was much higher than that from pre-differentiated MSCs (2.45±0.81 IU/L (P<0.01). Injected differentiated MSCs cells could down-regulate glucose level in diabetic rats.

CONCLUSION: Islet-like functional cells can be differentiated from marrow mesenchymal stem cells, which may be a new procedure for clinical diabetes stem -cell therapy, these cells can control blood glucose level in diabetic rats. MSCs may play an important role in diabetes therapy by islet differentiation and transplantation.

INTRODUCTION

Diabetic mellitus (DM), one of the leading causes of morbidity and mortality in many countries, is caused by an absolute insulin deficiency due to the destruction of insulin secreting pancreatic cells (type 1 DM) or by a relative insulin deficiency due to decreased insulin sensitivity, usually observed in overweight individuals (type 2 DM). In both types of the disease, an inadequate mass of functional islet cells is the major determinant for the onset of hyperglycemia and the development of overt diabetes. Islet transplantation has recently been shown to restore normoglycemia in type 1 DM[1]. However, a limited supply of human islet tissues prevents this therapy from being used in patients with type 1 DM. Alternatively, much effort has been made to increase β cell mass by stimulating endogenous regeneration of islets or *in vitro* differentiated islet-like cells[2-5]. Multipotent stem cells have been described within pancreatic islets and in nonendocrine compartments of the pancreas[6-12], and these cells have the capacity of differentiating into pancreatic islet-like structures. Furthermore, cells that do not reside within the pancreas, such as embryonic stem cells(ESC), hepatic oval cells, cells within spleen, have been differentiated into pancreatic endocrine hormone-producing cells *in vitro* and *in vivo*[13-21]. However, despite their differentiating potency, differentiation of various stem cells into islet cells has two major obstacles preventing clinical application: One is that these stem cells do not originate from DM patients, transplanting them would unavoidably be rejected by DM recipients. The other is that the source is not enough to provide abundant stem cells. The current article reports a potential means to generate insulin-producing cells, islet differentiation from bone marrow-derived stem cells. We suggest that cells within the adult bone marrow (mesenchymal stem cells MSC) are capable of differentiating into functional pancreatic β cell phenotypes.

MATERIALS AND METHODS

Materials

Wistar rats were bought from Animal Center, Tongji Medical College. All procedure was accordant with animal experiment guideline of the university. Cell culture medium L-DMEM (4.5 mmol/L glucose), H-DMEM (23 mmol/L glucose) and fetal calf serum (FCS) were bought from GIBCO Co. Nicotinamide, β-mercaptoethanol, B27 were from Sigma Co. Anti-nestin, anti-insulin monoclonal antibodies were bought from Santa Cruz Co. RT-PCR kit and primers were purchased from GIBCO Co. Radioimmunoassay (RIA) kit was purchased from Beijing North Biotechnology Co.

Differentiation of rat marrow mesenchymal stem cells into functional islet β cells

Bone marrow was isolated from femoral bone under aseptic condition and dispersed into single cell suspension, L-DMEM cells were cultured in a density of 1×10^9/L at 37 ℃, 50 mL/L CO_2 for 48 h. Suspended cells were disposed and adherent cells were cultured in L-DMEM with 200 mL/L FCS for about 10 d, culture medium was changed at 3-d intervals. These cells were digested with 2.5 g/L trypse and passed for 2-3 generations when the confluence reached 70-90%. Then, cells with 70-80% confluence were induced to differentiate into functional pancreatic cells. Cells were pre-induced with 10 mmol/L nicotinamide and 1 mmol/L

β-mercaptoethanol in L-DMEM for 24 h, and re-induced with 10 mmol/L nicatinomide and 1 mmol/L Mercaptoethanol in serum-free H-DMEM for another 10 h. Cells induced without nicotinamide or β-mercaptoethanol were used as controls.

Function assessment of differentiated cells

Cell morphology changes were investigated under converted microscope. Insulin-1 mRNA or protein expression was detected with immunocyto-chemical procedure and reverse transcription polymerase chain reaction (RT-PCR), and insulin level in culture suspension secreted from differentiated cells was detected with radio-immunological assay (RIA).

RT-PCR

Total RNA from 5×10^6 pre-treated or post-treated MSC cells was isolated according to a Qiagen protocol including DNase treatment. Reverse transcription was carried out using the Superscript protocol. Taq-man RT-PCR was performed using the Master Mix (Applied Biosystems). Insulin-1 primers were designed using the primer express program (Applied Biosystems) according to gene bank sequences. The following primers were used for Insulin-1: forward: 5'-GGGGAACGTGGT TTCTTCTA-3', backward: 5'-TAGACGAGGGAGATGGTTGA CC-3'. 35 cycles of 94 ℃×30 s, 55 ℃×30 s, 72 ℃×30 s were performed and the PCR product for Insulin-1 was 187 bp. GAPDH was used as internal control with the following primers: forward: TGGTATCGTGGAAGGACTCATGA. backward: ATGCCAGTGAGCTTCCCGTTCAGC. Products were tested with 15 g/L gel electrophoresis.

Immunohistochemistry

Cells adherent to slides were fixed with 40 g/L para-formaldehyde. After washed, the slides were incubated with a biotin-goat anti- rat insulin or nestin monoclonal antibodies (Santa Cruz Co, USA) diluted 1:200 in 50 mL/L normal goat serum for 20 min at room temperature. Immuno-reactive cells were visualized using the Vectastain Elite ABC Kit (Vector Labs, USA) with 3'3 diaminobenzidine tetrachloride (DAB) (Boehringer-Mannheim) as the chromogen. All sections were counterstained with hematoxylin.

Radioimmunoassay

The amounts of immunoreactive insulin in supernatants secreted from differentiated cells 48 h after treatment and cells 24 h before treatment were determined by RIA using a commercially available RIA kit according to the manufacturer's instructions. Briefly, to each polypropylene RIA tube 100 μL each of anti-Insulin, ^{125}I- insulin, and insulin or the samples were added. Immune complexes were precipitated 24 h later with 1 mL of 160 mL/L polyethylene glycol solution, and a gamma counter was used to determine the radioactivity in the precipitates. There was no nonspecific interference of the assay with the components of the samples. Determinations were carried out in triplicate and the means and standard deviations were obtained.

Primitive glucose controling role of differentiated MSCs on STZ-diabetic rats

Diabetic animal models were made according to the standard procedure with modifications. Briefly, 10 Wistar rats (weighting about 200 grams) were intravenously injected with 50 mg/L streptozotocin (STZ) from caudal veins, and glucose levels were tested 1 week later with Roche ACCU-CHEK glucose tester. Two rats died and were excluded. After stable hyperglycemia level was achieved, 3 animals were subcutaneously injected with 5×10^6 differentiated cells, while 2 others received the same amount of un-differentiated cells, the remaining 1 did not receive any cells. One week after cell injection, animal glucose level

was recorded.

Statistical analysis

Data were analyzed with Student's *t* test, $P < 0.05$ was considered statistically significant.

RESULTS

Morphological changes of MSC differentiation

Under inversed microscope, undifferentiated MSCs were typical of adherent spindle and fibrocyte- like. However, under differentiation, these spindle-like cells changed rapidly into round or oval types with confluence. These cells were abundant in endocrinal granules, similar to those differentiated islet cells from ES cells. These grape-like cells lasted for at least 2 wk. Some cells changed into neuron-like cells with typical processes.

Figure 1 Islet-like grape-shaped cells isolated from marrow mesenchymal stem cells.

Insulin-1 transcription in differentiated cells

To assess insulin-1 mRNA expression in differentiated cells, RT-PCR was applied on MSCs shortly after bone marrow isolation (Neg), 24 h before nicotinamide and β-mercaptoethanol treatment (Pre), 48 h (Islet1) and 1week after Nicotinamide and β-mercaptoethanol treatment (Islet2). There were no pre-differentiated MSCs (Figure 2). However, 48 h after treatment, insulin-1 mRNA transcription could be detected and continued for at least 1 wk. Since we did not observe any pancreatic islet-like cells in control group, RT-PCR was not performed on this group of cells.
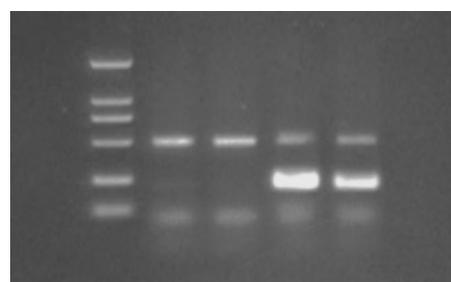
Marker Neg Pre Islet1 Islet2

Figure 2 Insulin transcription in pre-differentiated MSCs shown in RT-PCR. Neg: MSCs undifferentiated; Pre: MSCs 24 h before differentiation; Islet1: cells 24 h after differentiation; Islet2: cells 1w after differentiation.

Insulin and nestin protein expression in different stages of MSCs

Immunocytochemistry was performed to test insulin or nestin protein expressions in MSCs. Insulin could be observed obviously in those grape-like cells (Islet-like), and in unchanged spindle-like cells not positively stained. Nestin was regarded

as an important pre-marker for islet cell differentiation, and its expression was tested. Immunocytochemistry showed nestin positivity in pre-differentiated spindle-like cells (Figure 4), while no nestin positivity in differentiated islet-like cells.

To further clarify the function of these differentiated cells, RIA was used to assess the insulin excretion from these cells in 6 independent cell cultures. As shown in Table 1, pre-differentiated MSCs seldom secreted insulin into their supernatant if any. However, 48 h after differentiation, these islet-like cells produced much insulin and secreted insulin in extra-cellular medium.

Figure 3 Insulin staining for islet -like cells. Strong brown staining indicates insulin positivity in differentiatly arranged grape shape cells.

Figure 4 Positive nestin in pre-differentiated MSCs.

Table 1 Insulin excretion changes in pre- and differentiated MSCs (RIA) (IU/L)

Group	Insulin excretion in pre-treated MSCs	Insulin excretion in treated islet-like cells
1	1.67	410.79
2	2.53	383.21
3	1.53	465.81
4	3.36	308.28
5	2.20	516.45
6	3.40	597.02

In supernatant of pre-differentiated MSC cells, there was no obvious insulin excretion (2.45±0.81 IU/L). Forty-eight h after differentiation, cells excreted more insulin into supernatant, the insulin level was as high as 446.93±102.28 IU/L (t = 10.65 [b]P<0.01).

To test if these MSC-differentiated islet-like cells could exert glucose-controlling function, 6 diabetic Wistar rat models were included. Each of 3 rats was administered subcutaneously $5×10^6$ differentiated cells, 2 received similar un-differentiated MSCs injection, while the last one received none. Glucose levels of these 6 rats at different times are shown in Table 2. Although lack of statistical analysis, it could be suggested that MSC-

differentiated islet-like cells could change diabetic glucose level.

Table 2 Blood glucose level (mmol/L) changes in STZ-diabetic rats

Types of cells injected	Glucose level 24 h before injection	Glucose level 1 w after injection
Islet1	>33.3	25.4
Islet2	>33.3	21.4
Islet3	25.3	19.7
MSC1	>33.3	>33.3
MSC2	28.9	29.7
Non	>33.3	>33.3

Islet1, Islet1, Islet3: different STZ-diabetic rats received islet cell injection; MSC1, MSC2: STZ-diabetic rats received undifferentiated MSC injection; Non: STZ-diabetic rats did not receive cell injection.

DISCUSSION

Multipotent stem cells within pancreas and outside could develop into insulin-secreting islet cells[7-21]. However, differentiation of various stem cells into islet cells has two major obstacles preventing clinical application. As these stem cells do not originate from DM patients, these cells transplanted would be rejected by DM recipients. The source is not enough to provide abundant stem cells.

Bone marrow mesenchymal cells (MSC) reside in bone marrow and are multipotent, and can differentiate into lineages of mesenchymal tissues, such as bone, cartilage, fat, tendon, muscle, adipocytes, chondrocytes, osteocytes[22-24]. MSCs could differentiate into endodermal and epidermal cells, such as vascular endothelial cells, neurocytes, lung cells and hepatocytes[25-27]. MSCs as differentiation donors are of advantages compared with other stem cells as ESC or stem cells from organs. MSCs are of great multiplication potency. Cell-doubling time is 48-72 h, and cells could be expanded in culture for more than 60 doublings[28]. Functional cells differentiated from MSCs transplanted into MSC donors (autologous transplantation) would not cause any rejection.

Differentiation of MSCs into functional pancreatic islet cells is not yet reported. Ianus *et al.*[29] reported, using a CRE-LoxP system, bone marrow from male mice with an enhanced green fluorescent protein (GFP) replacing insulin expression was transplanted into lethally irradiated recipient female mice. After 4-6 wk, recipient mice revealed both Y chromosome and GFP positivity in pancreatic islets. These GFP positive cells expressed insulin, glucose transporter-2 and other islet β cell related markers. Cells from bone marrow were able to differentiate into islet cells. MSCs could differentiate into hepatocytes[25,27], precursor cells of hepatocytes could differentiate into pancreatic islet cells, adult hepatic stem cells could trans-differentiate into pancreatic endocrine hormone-producing cells[19,20]. These reports indicated that, MSCs had the capacity of differentiating into pancreatic islet cells.

We found that MSCs could successfully differentiate into pancreatic islet β-like cells. These cells were morphologically similar to pancreatic islet cells. More importantly, they could also transcript, translate and excrete insulin. Cells were injected subcutaneously into NOD rat models, although lack of statistical data, these MSC-derived cells could regulate NOD blood glucose level. Nestin was regarded as a marker of precursors of pancreatic islet cells[10,14]. In our study, nestin was also positive in pre-pancreatic islet MSCs, suggesting that MSCs could differentiate into islet cells. High glucose concentration was considered as a potent inducer for pancreatic islet differentiation. Nicotinamide was used to preserve islet viability and function through poly

(ADP-ribose) polymerase (PARP)[30], β-mercaptoethanol was commonly used as a neurocyte inducer. In our primary experiment, high glucose alone could not effectively induce MSC to differentiate into islet-like cells. After nicotinamide was added, they could effectively transform MSCs into islet-like cells. This may imply that nicotinamide could be an effective inducer, or it could protect differentiated cells from dying or transforming into other cell types. β-mercaptoethanol increased the potency of nicotinamide in our experiment. Considering nestin expression in pre-differentiated MSCs, MSCs might differentiate into pancreatic islet-like cells through intermediate neurocyte stage. We did not test the insulin secretion based on the number of cells, the increased insulin in supernatant might be mainly from increased insulin excretion by islet-like cells.

Bone marrow stem cells are non-endodermal cells with no immediate relationship to putative pancreatic stem cells that are resident in tissues of endodermal origin or developmental neuro-endocrine stem cells derived from the endoderm. Alternatively, stem cells in bone marrow may be derived from sites of endodermal origin. Regardless of their germ layer of origin, these cells represent multi-potent cells mediated by circulating signals, and can be recruited to neuro-endocrine compartments of the pancreas. Once homing of these cells to pancreatic islets has occurred, local cell-cell interaction as well as paracrine factors may initiate differentiation.

There was an argument[31] that Islet-like cells differentiated from ESC were falsely insulin positive from insulin-uptake. These insulin-positive cells which do not transcript insulin mRNA, are TUNEL+. Bone marrow cells could also fuse with other cells and adopt the phenotypes of these cells[32,33]. However, cell differentiation in our report was not the case. Islet cells expressed insulin at both mRNA and protein levels, the excreting insulin level was far more higher than that in culture media and that of pre-differentiated cells. Further more, these MSCs-derived cells could down-regulate glucose level in diabetic rats.

In conclusion, MSCs can differentiate into functional pancreatic islet-like cells *in vitro*. If human MSCs, especially MSCs from diabetes patients themselves can be isolated, proliferated, differentiated into functional pancreatic islet-like cells, and transplanted back into their donors (autologous transplantation), their high proliferation potency and rejection avoidance will provide one promising therapy for diabetes.

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