Isolation of Oct4+, Nanog+ and SOX2-mesenchymal cells from peripheral blood of a diabetes mellitus patient
Abstract  Diabetes mellitus is a chronic metabolic disorder that affects millions of people worldwide. The most common form is type 2 diabetes mellitus, which results in impaired beta cell function combined with insulin resistance in peripheral organs. One recently proposed treatment approach is the use of adult stem cells derived from bone marrow in autologous stem cell transplantation. Alternatively, peripheral blood can be obtained in a more non-invasive manner. In this study, we isolated and cultured mesenchymal cells (MCs) from the peripheral blood of a diabetes mellitus patient. The cultured cells were large and elongated and had an in vitro migratory capacity in the culture dish. They expressed embryonic stem cell pluripotency markers Nanog and Oct4 as well as mesenchymal markers CD105 and CD13, and they lacked expression of hematopoietic marker CD45. These characteristics suggest that these cells have a mesenchymal phenotype similar to that obtained from bone marrow cells. The SOX2 gene was downregulated in both the peripheral blood cells and the isolated mesenchymal cell line, indicating a defective mechanism of SOX2 in diabetes mellitus. The overall results of study demonstrate that peripheral blood can be used as a source of MCs from diabetes mellitus patients for use in future regenerative stem cell therapy and that this particular model system may be useful to study the mechanism of diabetes mellitus involving downregulation of the SOX2 cascade.

Keywords  Circulating progenitor cells · Mesenchymal stem cells · Diabetes mellitus · Stem cell markers · Stem cell therapy

Introduction

Diabetes mellitus is a syndrome involving an etiologically and clinically heterogeneous group of pathological conditions. It mainly involves the lack of functional beta cells, leading to complications associated with the development of hyperglycemia [1]. Recent research efforts have highlighted the use of adult stem cells due to their multilineage differentiation potential and their compatibility for use in autologous transplantation [2].

The presence of multiple progenitor cells in the peripheral blood of adult humans has relevant clinical implications for the treatment of multiple diseases in several organs. Cesseli et al. identified two main populations of peripheral blood progenitor cells, namely, fibrocytes and mesenchymal stromal cells (MSCs), in human peripheral blood. They found that the MSCs displayed the morphology and surface phenotype of bone marrow resident mesenchymal cells (MCs) and could be proliferated in vitro [3]. Tondreau et al. recently isolated MSCs from mobilized peripheral blood that are CD133+ and Oct4+ which were able to differentiate into adipocytes, osteocytes, chondrocytes, and neuronal/glial cells. Based on their results, these authors stated that peripheral blood is of major clinical importance due to easy accessibility and that the MSCs derived from peripheral blood have multilineage potential and can be used for cellular and gene therapies [4]. Stem cells can be characterized by specific molecular markers to determine their phenotype. CD105 and CD13 are considered to be putative markers for the mesenchymal stem cell.
phenotype, whereas CD34 and CD45 markers are mainly expressed in hematopoietic stem cells. Nanog, Oct-4, and SOX2 are transcription factors which are expressed at high levels in embryonic stem cells, and the downregulation of these transcription factors correlates with the loss of pluripotency and self-renewal [5]. A unique tag-single nucleotide polymorphism located in the 3′-untranslated region of the SOX2 gene has recently been implicated in the development of diabetic nephropathy [6].

The underlying hypothesis of the study reported here is that due to the high blood sugar level with insulin resistance found in diabetes mellitus patients, MCs would be recruited to the peripheral blood of these patients, from which they can be isolated and identified by specific molecular markers. As the SOX2 gene is involved in diabetic nephropathy, this study focused on the expression of the SOX2 gene in vitro and in vivo in order to gain an understanding of the mechanism of diabetes mellitus.

Materials and methods

Sample collection

Patients attending the diabetic clinic at Jaslok hospital who had a high sugar level of 600 mg/dl and insulin resistance were recruited for this study under the ethical guidelines of the Jaslok Hospital and Research Center. Firstly, a venous blood sample was collected in a sterile EDTA vacutainer tube. The blood sample was processed using the standard Ficoll–Hypaque method, and the middle buffy coat layer containing mononuclear cells [peripheral blood (PB) MNCs] was used for both in vivo and in vitro studies.

Isolation of the mesenchymal cell line from peripheral blood of diabetes mellitus patient

The isolated PBMCs were washed with 1× phosphate buffered saline (PBS) solution and centrifuged at 2000 rpm for 5 min. The cell pellet obtained was then suspended in freshly prepared growth medium, RPMI 1640 (HiMedia, Mumbai, India) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Grand Island, NY) and 1% PenStrep (Hi Media, India). The cells were counted and viability determined using Neubauer’s hemocytometer and 0.1% Erythrosin B, and the cell suspension was seeded at density of 1 × 10^4 cells/ml in a 65-mm Nunc dish and was incubated in a CO2 incubator at 37°C with 5% CO2.

The PBMCNs were observed daily under a phase contrast microscope for changes in morphology. After 2 days, many of the cells in culture had adhered to the bottom of the petri dish. Thereafter, the suspension cells were removed, and the adherent cells remaining were cultured in growth medium comprising Dulbecco’s modified Eagle’s medium (DMEM) + 10% FBS + 1% PenStrep. The adherent cells were observed each day for changes in morphology and growth rate. After 7–10 days, a few colonies of fibroblast-like cells were observed growing in the culture, which attained confluency within 30–45 days of culture. All of these cultures were fed twice a week. After attaining confluency, the cells were passaged using 0.25% trypsin–ethylenediaminetetraacetic acid (HiMedia) and plated in new 65-mm petri dishes. The same process was repeated, and the cells were finally grown in 50-mm tissue culture flasks. At the time of writing, the cells were at passage 4.

In vitro cell movement study

The trafficking and homing properties of MSCs are of particular interest for various clinical applications. Our cell movement study was mainly carried out to determine the capacity of the cells to migrate in vivo in the receiving tissue when used for a therapeutical purpose. For the in vitro study of cell movement, we marked a specific area of the plate and observed that part of the plate daily by phase contrast microscopy to monitor the position of the cells. Cell movement was photographed using a camera attached to the phase contrast microscope equipped with TSVview Software (Tucsen Imaging, Fuzhou, PR China) to capture the images.

Molecular marker analysis

Total RNA was extracted from whole plasma sample of the diabetes mellitus patient (in vivo study) and the MCs at passage 1 (in vitro study) using TRIZOL reagent (Invitrogen, Grand Island, NY). cDNA was prepared from the extracted RNA using the High Capacity Reverse Transcriptase kit (Applied Biosystems, Foster City, CA). The molecular marker study was carried out for the genes CD105, CD13, CD34, CD45, LIF, Keratin 18, Oct 4, Nanog, SOX2, and β-actin using the following PCR reaction conditions and primer sequences described by Potdar and Sutar [5]. The PCR products were analyzed by electrophoresis on a 2% agarose gel and photographed under UV light.

Results

Establishment of the mesenchymal cell line from the peripheral blood of a diabetes mellitus patient

The peripheral blood sample was processed for culture, and the PBMCNs were separated by the Ficoll–Hypaque method as previously described. The viability of the cells
was 80%, and the cells were plated at a density of $1 \times 10^5$ cells/ml in 65-mm Nunc plates, suspended in growth medium containing RPMI 1640 (HiMedia) + 10% FBS + 1% PenStrep. The cultured cells were incubated at 37°C in CO$_2$ incubator. These cells were monitored daily for changes in morphology and growth by phase contrast microscopy. Within 24 h, the cells had adhered to the plate without any substratum. After 7 days in culture, a few elongated adherent cells were observed in some areas of the plate. The cells were flat and had a fibroblast-like morphology, as shown in Fig. 1a. These adherent cells were then cultured in growth medium comprising DMEM + 10% FBS + 1% PenStrep, instead of RPMI growth medium.

After 10 days of culture, these fibroblast-like cells were large, with a prominent nucleus and nucleolus (Fig. 1b). These cells attained confluency within 4 weeks of culture (Fig. 1c). After attaining confluency, the cells were subcultured by trypsinization and transferred to 50-mm tissue culture flasks for in vitro expansion. These cells were monitored each day, and within a period of 1 month, the cells were large, with a clear nucleus, nucleolus, scanty cytoplasm, and a filamentous structure. The cells were routinely passaged, and this cell line was designated as the DM2010 cell line.

These cells show spontaneous differentiation in culture into adipocytes and chondrocyte-like cells (Fig. 2a, b). In passage 2, they showed a high growth rate and colony-forming ability. After the fourth passage, they had large morphological aspects, with a clear nucleus, nucleolus, scanty cytoplasm, and a filamentous structure. At this passage the cells showed a deterioration in their growth rate and remained viable in culture without any further multiplication.

In vitro cell movement study

The stem cells showed constant in vitro movement in the culture dish without any stimuli, suggesting that these cells have the capacity to mobilize to the target tissue or injured tissue and, therefore, can be used for therapy (figure not shown).

Molecular marker study

The molecular marker study was carried out to characterize the PBMNCs of the diabetes mellitus patient in vivo and the DM2010 cell line was developed from the same cells in vitro to compare the phenotypic differences of these two cell types. In vivo, these cells mildly expressed CD105 and did not express CD13, whereas in vitro, the DM2010 cell line showed a high expression of both CD105 and CD13, indicating these cells acquired the very specific...
phenotypic characteristics of MSCs. As these cells were derived from peripheral blood, we also checked the expression of CD34 and CD45, which are markers for hematopoietic lineage. In vivo, both markers were expressed; in comparison, in vitro, the expression of CD34 marker remained the same while the expression of the CD45 gene was downregulated. As these MCs were derived from peripheral blood, they were also positive for CD34, which is a hematopoietic marker. A study of the expression of differentiating markers LIF and Keratin 18 revealed that both in vivo and in vitro cells expressed both markers equally (Fig. 3). The expression of Keratin 18 by the DM2010 cell line indicates that these cells are not fibroblasts. The expression of pluripotency markers Nanog, SOX2, and Oct4 in the in vivo and in vitro cells, respectively, was also studied (Fig. 4). Nanog and Oct4 were expressed equally in both the in vivo PBMNCs and in the DM2010 cell line. However, there was complete down-regulation of SOX2 in the PBMNCs and in the DM2010 cell line, indicating the loss of SOX2 gene expression in diabetes mellitus patients.

Discussion

Diabetes mellitus is a disorder of metabolic homeostasis controlled by insulin, resulting in abnormalities in carbohydrate and lipid metabolism. The technology for isolating stem cells from bone marrow has already been established, and several studies have revealed the potential use of bone marrow-derived MSCs. Yu et al. have recently reported the isolation of MSCs from bone marrow and further induced them to differentiate into pancreatic beta cells using a three-stage differentiation protocol [7]. As the procedure for isolating stem cells from bone marrow is painful, possibly resulting in complications, an alternative method for isolating MSCs by an non-invasive procedure is essential.

Cesselli et al. have shown that multipotent progenitor cells are recruited from bone marrow to the peripheral blood in patients in various disease conditions [3]. We therefore focused on the isolation of MSCs from the peripheral blood of a diabetes mellitus patient with a high blood sugar level and a severe insulin resistant condition. Several attempts have been made to culture MCs from normal blood, but only a few MCs were obtained in these studies along with a large number of fibrocytes, which do not multiply in culture. Therefore, our isolation of MCs from diabetes mellitus peripheral blood suggests that due to the high insulin resistance disease condition, stem cell progenitors are brought into circulation and, consequently, can be isolated following the culture of blood cells. We did succeed in isolating these MCs, which showed high proliferation for up to two passages. Tondreau et al. recently isolated MSCs from peripheral blood and demonstrated their multilineage differentiation [4]. In our study, the isolated MCs were large with a prominent nucleus and nucleolus, and they also spontaneously differentiated into adipocytes and chondrocytes, as reported by Tondreau et al. [4]. These cells also showed in vitro movement in the culture dish, suggesting that the cells were constantly moving in the culture dish and that a similar effect could be expected in vivo. Thus, these cells may show the property to home to the target pancreatic tissue when they are used for regenerative stem cell therapy.

The results of our study further confirm the mesenchymal phenotype of these cells based on the expression of the CD105 and CD13 markers. There is evidence that MSCs along with CD34+ cells can be used for the treatment of several neurological disorders [8]. Although the isolated MCs showed downregulation of the CD45 hematopoietic marker, they were positive for the CD34 marker, indicating their suitability for autologous stem cell transplantation.

![Fig. 3](image1.png)  
Expression of molecular markers CD105, CD13, CD34, CD45, LIF, and Keratin 18 in isolated peripheral blood mononuclear cells (PBMNCs) (in vivo) and in the DM2010 cell line (DM MSC P-1) (in vitro)

![Fig. 4](image2.png)  
Expression of pluripotency markers, Oct4, Nanog, and SOX2 in isolated PBMNCs (in vivo) and DM2010 cell line (DM MSC P-1) (in vitro)
These cells also expressed both LIF and Keratin 18, demonstrating that these cells are quite different from fibroblast cells.

Zhang et al. confirmed that SOX2 polymorphism is associated with the development of diabetic nephropathy [6]. In previous studies in our laboratory, SOX2, which is a pluripotency marker, was found to be expressed normally in peripheral blood. We studied SOX2 expression in the peripheral blood of a diabetes mellitus patient who were insulin resistant and showed downregulation of this gene. We further studied the expression of SOX2 in MCs isolated from this patient, where we observed the complete downregulation of SOX2, clearly indicating that downregulation of the SOX2 gene may play an important role in the progression of diabetes mellitus towards insulin resistance. This is the first study that demonstrates SOX2 gene regulation in a diabetes mellitus patient having insulin resistance. The results require further study, which is currently being carried out in our laboratory.

Overall this study establishes a technology for the development of MCs from the peripheral blood of a diabetes mellitus patient. We further demonstrate that SOX2 downregulation can be used as a marker to monitor both DM nephropathy and the incidence of DM in family members of diabetes mellitus patients.

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References