Research Article

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SUPPLEMENTATION OF ZINC INDUCES RETINOIC ACID-MEDIATED DIFFERENTIATION OF RAT BONE MARROW MESENCHYMAL STEM CELLS (MSCS) IN TO INSULIN PRODUCING CELLS (IPCS) *IN-VITRO*

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ABSTRACT

Mesenchymal stem cells (MSCs) from bone marrow of adult Wistar albino rats were harvested and cultured in a basic cell culture medium. Retinoic acid was used to induce the differentiation of MSCs in to pancreatic beta cell lineage. Differentiation media tested with and without zinc supplementation. It was observed in this study that zinc supplementation induces differentiation of MSCs into insulin producing cells (IPCs). The result was confirmed by morphological changes in the cells following differentiation and enhanced production of insulin in differentiated cells by dithizone staining. This finding may help researchers to further optimize their protocols to increase the yield of differentiated cells of beta cell lineage from bone marrow MSCs.

Keywords: Mesenchymal stem cells, insulin producing cells, Wistar albino rats, diabetes mellitus, stem cell differentiation, zinc, beta cell.

INTRODUCTION

Most of the diabetes mellitus (DM) cases without insulin resistance are degenerative diseases, in which the pancreatic beta cells fail to produce enough insulin required by the body,[1] which leads to hyperglycemia and related complications.[2] Regeneration of the degenerated tissues / organs or transplantation of healthy tissue / organ is the most direct and convincing treatment for this. There are lots of hurdles for tissue / organ transplantation like immunological reactions and subsequent graft rejection due to incompatibility of the donor and recipient at the immunological level. Tissues derived from the stem cells of the same person can solve these issues considerably. Hopefully in any adult body, there are adult stem cells, which has the capacity to form any tissue type in case of damage / repair.[3,4] The study area is matured enough to enable us to collect and direct these stem cells to differentiate in to the desired tissue types.

Because of their multiple differentiation potential, stem cells can be used to generate insulin producing cells (IPCs).[5-9] Adult stem cells especially mesenchymal stem cells are a good choice in this regard due to its easiness to handle, better availability and broad differentiation ability.[10-12] In this study, we could successfully isolate bone marrow mesenchymal stem cells from adult Wistar albino rats and induce them to differentiate into insulin-producing cells by supplementing with zinc. The induction was mediated by nicotinamide and retinoic acid as detailed by Kramer.[13,14] These differentiated cells expressed insulin, confirming its potential use as a therapeutic method.

MATERIALS AND METHODS

Experimental animals and collection of mesenchymal stem cells

Adult Wistar albino rats maintained in the Department of Life Sciences, University of Calicut were used in this study. The rats were euthanized following the ethical guidelines in place. Tibia bones were removed aseptically following standard surgical procedures. Both ends of the bones were cut aseptically and the marrow was extruded using a syringe. The inside of the bones was washed with phosphate buffered saline. The extrudate was gently pipetted multiple times to make single cell suspension. The cells were counted and seeded in T-25 flasks at 1 x 10^6 /ml cell density. Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 20% fetal bovine serum (Gibco, USA) and 1% antibiotic-antimycotic solution (Gibco, USA) was added sufficiently and the cells were incubated at 37 °C with 5 % CO_2 in a humidified chamber (Heal Force, Shanghai).

Expansion culture of stem cells

Unlike other marrow cells, mesenchymal stem cells adhere readily to the walls of the culture flasks. Making use of this property, after 48 hours of initial culture, suspended cells were discarded and the adherent cells were further cultured for 2 weeks with media change once in every three days. Upon attaining 90% confluence, the cells were washed with PBS and passaged twice with a gap of 1 week. The morphology of the cells was observed regularly through an inverted microscope.

Confirmation of MSCs

Apart from the adherent properties, the passaged cells were confirmed to be of MSCs by immunofluorescence. Rat MSCs are known to be CD 44(+) and CD 45(-) [15,16]. Rat specific CD 44 and CD 45 antibodies were purchased from Biorad, USA and used following the manufacturer's recommendations. Briefly, the cells were washed twice with phosphate buffered saline for 30 seconds each and scraped using a cell scraper, and the cells were collected by centrifugation at 1500 rpm for 3 minutes. The pellets were then re-suspended in PBS/BSA (1% bovine serum albumin fraction V in PBS) at a density of 1x10^6 cells/ml. To 20 µl of cell suspension taken in two tubes were added 6 µl of

CD 44 and CD 45 antibodies, mixed well and incubated at 37 °C for 30 minutes. After incubation, cells were collected by centrifugation at 1500 rpm for 5 minutes, washed twice in 0.2 ml of PBS/BSA. Cells were finally re-suspended in 40 µl of PBS/BSA containing 50 % v/v glycerol (20 µl of glycerol in 20 µl of PBS/BSA) and a drop of which was analyzed under a fluorescent microscope fitted with a blue filter.

Induction of differentiation with retinoic acid

After second passage, the cells were collected and re-suspended in differentiation medium (serum free medium) for 48 hours in two cell culture flasks at a concentration of 2000-2500 cells/cm^2 each. This was followed by maintaining the cells in base differentiation medium for 4 days. The base medium contained DMEM with FBS, antibiotic-antimycotic solution, 10 mM nicotinamide (Sigma, U.S.A) and 2 mM L-glutamine (Sigma, U.S.A) in the first flask while the second had 0.2 mM $ZnCl_2$ as an additional supplement. The first flask was labelled as 'no-zinc' control. On 5th day, 10 µM retinoic acid (Sigma, U.S.A) in dimethyl sulfoxide was added to the medium (1% of the total volume) of both flasks for 3 days. This 4/3 days' cycle was repeated till morphological changes were visible to the cells. To confirm differentiation of the MSC cells into pancreatic beta cell lineage dithizone (DTZ) staining was performed.

Dithizone (DTZ) staining

Insulin producing cells are characteristic to have high concentration of zinc. In IPCs, insulin monomers assemble into dimers and in the presence of Zinc, to hexameric crystals with 2 zinc atoms per hexameric unit. Dithizone being a zinc-chelating agent, binds to this insulin hexameric crystals and provides a tool to identify insulin-producing beta cells from a mixed population of cells.

Dithizone staining experiments were conducted with DTZ staining kit (Millipore, U.S.A) following the manufacturer's protocol. Briefly, the DTZ enzymatic reaction was prepared by vortexing 30 µl of 100X DTZ stain solution with 3 ml of DMEM. The DTZ enzymatic reaction was filtered using the 0.2 µm syringe filter unit. Differentiated cells were prepared for analysis by aspirating the medium fully from the flasks to be analyzed. Cells were washed with PBS and the prepared DTZ enzymatic reaction was overlaid the cells followed by incubation at 37°C for 30 minutes. After the incubation period, applied DTZ enzymatic reaction was removed and the flasks were washed three times with 1X DTZ rinse solution provided. The stained cells were visualized under an inverted light microscope. Insulin producing pancreatic beta cells were expected to be selectively stained red.

Reverse transcription PCR

RNA isolation was carried out using TRIzol Reagent (Invitrogen, U.S.A) according to the manufactures procedure. Briefly, approximately 5x106/ml each of MSCs, differentiated MSCs supplemented with zinc and differentiated MSCs without zinc supplementation were pelleted by centrifugation, washed with phosphate buffered saline, lysed and homogenised in TRIzol reagent. Chloroform was added for phase separation. From the aqueous phase, RNA was pelleted with ice cold isopropanol and washed with 70% ethanol. The pellet was air dried and reconstituted in 100 µL RNase free water.

First strand cDNA synthesis of the isolated RNA was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo scientific, U.S.A) according to the manufactures protocol. Oligo (d T) 18 primer was used for reverse transcription. The reaction mixture containing reaction buffer, RiboLock RNase Inhibitor, dNTP Mix, primer, RevertAid™ M-MuLV RT enzyme and RNA was incubated at 42°C for 1 hour. Reaction was terminated by heating the reaction mix at 70°C for 5 min. 1 µL of the generated cDNA was directly used for PCR.

PCR was performed with insulin specific primers [17] and GAPDH primers. GAPDH served as a positive control. The reaction mixture contained 1X Reaction Buffer, 2 µL 10 Mm d NTP Mix, 1 µL each (10 mM) of forward and reverse primers (Insulin-1/GAPDH), 0.5 µL Taq DNA polymerase (2U/ µL) and 1 µL cDNA. The forward and reverse primers for insulin were 5'-GGGGAACGTGGTTTCTTCTA-3' and 5'-TAGACGAGGGAGATGGTTGACC-3' respectively and the amplicon size was 186 bp; while that of GAPDH were 5'-TGGTATCGTGGAAGGACTCATGA-3' and 5'-ATGCCAGTGAGCTTCCCGTTCAGC-3' generating an amplicon of 496 bp. The thermocycler program for insulin comprised of initial denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C×30 s, 55 °C ×30 s, 72 °C ×30 s followed by a final extension at 72°C for 7 min in personal thermal cycler (Applied Biosystems). The thermocycler program for GAPDH comprised of initial denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 45 sec followed by a final extension at 72 °C for 7 min. PCR amplica were analysed by 1.5 % agarose gel electrophoresis in Tris-Boric acid-EDTA (TBE) buffer. The gel was stained by ethidium bromide (EtBr) by incorporation at a final concentration of 0.5 µg /ml. The gel was visualized under U.V and images were acquired and documented for further analysis.

Ethical consideration

The study was approved by institutional ethical committee. Study conducted according to guidelines of CPCSEA.

RESULTS

After 24 hours of initial culture, a portion of the cells were found attached to the surface of the culture flask. The non-adherent cells were discarded after 48 hours of incubation. The adherent cells formed a monolayer within 2 weeks. Cell morphology varied from round to spindle shape.

Immunofluorescence

Bright green fluorescence was observed to the cells stained with CD 44 antibody. No fluorescence was observed in cells stained with CD 45. The cells behaved as expected in immunofluorescence. They were CD 44(+) and CD 45(-).

Dithizone (DTZ) staining

Cells in the 'no-zinc' control flask was not stained by dithizone by the end of 4th week as indicated by no development of red colour. At the same time the cells received zinc as supplement was strongly stained with dithizone even by 2nd week of culture.

Reverse transcription PCR

In the PCR, differentiated MSCs produced intense band corresponding to high production of insulin. The insulin specific bands were absent in the case of undifferentiated MSCs and in the cells that cultured in differentiation media without zinc supplementation. The GAPDH gene mRNA used as positive control in the PCR was present in both undifferentiated and differentiated MSCs.

DISCUSSION

Stem cell based treatment strategies for degenerative diseases are gaining popularity even though the field is still in its infancy. In this study, we have attempted to isolate mesenchymal stem cells from adult wistar albino rats and its directed differentiation in to insulin producing cells (IPCs). We could succeed in isolating mesenchymal stem cells from the tibia bone marrow of the experimental wistar albino rats. Stem cell nature of the isolated cells were confirmed by its adherent nature, cell morphology and by immunofluorescence. The cells were positive for CD 44 and negative for CD 45 surface antigens as established already.[15,16] Hence they are unlikely to be hematopoietic stem cells and should be able to generate insulin producing cells.[8,9]

Induction of retinoic acid was successful as the cell morphology changed with time. However, following published protocols for in-vitro differentiation of MSCs, we advanced little unless the culture media was supplemented with zinc. Without supplementing with zinc, the cells showed signs of differentiation by changes in morphology from that of MSCs, but insulin was not produced. Zinc supplementation helped the differentiated cells (IPCs) to produce insulin at a very early stage.

The present study opens a new window for inducing differentiation of bone marrow MSCs in to IPCs effectively. Our protocol provides an economical and feasible solution in the treatment of diabetes mellitus by directed differentiation of bone marrow MSCs in to functional IPCs.

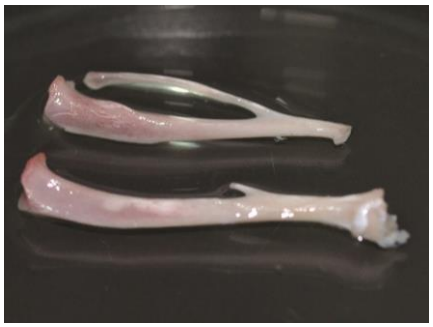

Figure 1: Dissected tibia bone of adult Wistar albino rat for isolation of MSCs

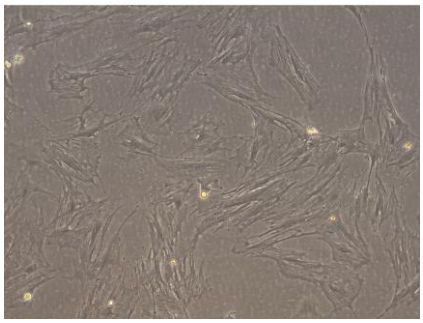

Figure 2: Culture of MSCs by the end of 2nd week

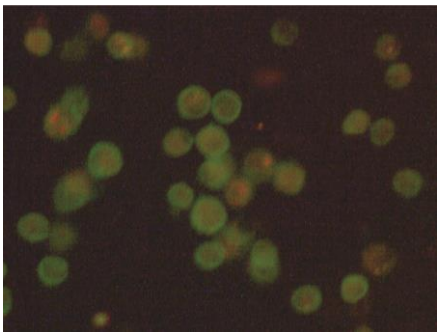

Figure 3: Immunofluorescence image of MSCs positively expressing CD44 surface antigens

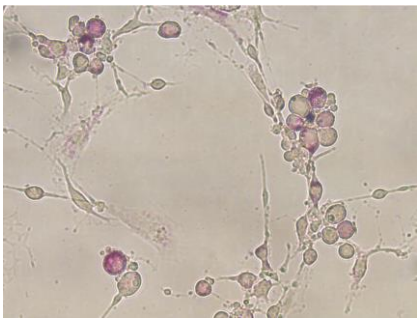

Figure 4: Image of differentiated IPCs stained with dithizone. The red color indicating a positive reaction

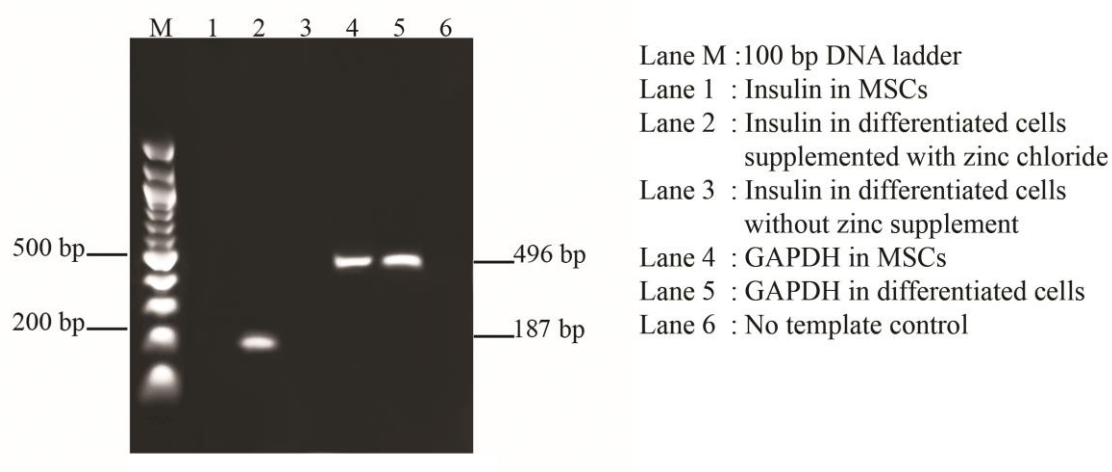

Figure 5: Ethidium bromide stained 1.5 % agarose gel image showing the insulin and GAPDH (positive control) specific PCR amplicons

CONCLUSION

We have observed that zinc supplementation induces differentiation of MSCs into insulin producing cells (IPCs). The result was confirmed by morphological changes in the cells following differentiation and enhanced production of insulin in differentiated cells by dithizone staining. This finding may help researchers to further optimize their protocols to increase the yield of differentiated cells of beta cell lineage from bone marrow MSCs.

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