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Induction of osteoblast differentiation in human adipose derived stem cells by lanthanum ions

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Abstract: Adipose derived stem cells represent a readily available source of adult stem cells for various biomedical applications. In this study, the proliferation and osteogenic differentiation potential of lanthanum nitrate (La^{3+}) on human adipose derived mesenchymal stem cells (hADSCs) were investigated for the first time and compared with that of dexamethasone (Dex). Our results provided evidence that La^{3+} at 50 μmol/L concentration promoted proliferation of hADSCs upto 2.4 fold when treated for 21 d in DMEM medium. Treatment of hADSCs with La^{3+} containing osteogenic induction medium (α-MEM with ascorbic acid and β-glycerophosphate) for 7 d resulted in higher calcium deposition than that in the presence of Dex (0.1 μmol/L) as shown by Alizarin red S and von Kossa staining. Scanning electron micrographs also showed more extracellular matrix mineralization in the presence of La^{3+}. After 7 d of treatment with La^{3+} (10 μmol/L) the expression of RunX2, osteopontin (OP) and osteocalcin (OC) increased 3.4, 5.5 and 2.7 fold respectively. Our results provided evidence that in the presence of La^{3+} osteogenic differentiation occurred earlier than that in the presence of Dex.

Keywords: hADSCs; lanthanum; osteoblast differentiation genes; calcium deposition; SEM; RunX2; rare earths

Adipose tissue has been identified as a potential source of mesenchymal stem cells (MSCs) and adipose derived stem cells (ADSCs) have attracted great interest recently due to their multilineage potential, easy availability, low levels of senescence[1] and possible use in treating bone defects. Investigations regarding the use of ADSCs have revealed that culture expanded ADSCs when implanted into immunocompromised animals are replaced by normal tissue. Moreover, they do not exhibit any toxicity including the development of malignancy[2]. Several osteoinductive materials have been reported to induce proliferation of osteoblasts as well as differentiation of MSCs and ADSCs into osteoblast lineage. This includes BMPs[3], ascorbic acid[4], dexamethasone[5], 1,25-dihydroxy vitamin D_3[6], hyaluronic acid[7,8] etc., all of which increase the levels of osteogenic markers such as collagen, alkaline phosphatase activity, osteocalcin (OC), bone sialoprotein (BSP) and calcium deposition. Inorganic compounds have also been shown to differentiate rat osteoblasts and a content of 5%–10% strontium in hydroxyapatite enhances differentiation and mineralization[9].

Beneficial effects of rare earth compounds on bone metabolism and renal metabolism are being realized in recent times[10]. Various rare earth elements like Ce^{3+}, Y^{3+} and Gd^{3+} have been shown to promote differentiation of primary mouse osteoblasts in a concentration and time dependent manner *in vitro*[11–13]. Amongst the rare earth compounds, $LaCO_3$ is in use for hyperphosphatemia treatment[14]. Since La^{3+} resembles Ca^{2+} in many of its physical properties, the possibility of using it for bone defect repair has been attempted and localization of lanthanum in the bone of rats does not elicit toxicity[15]. La^{3+} suppresses oxysterol induced apoptosis of HUVE cell lines (ECV-304) by reducing oxidative stress[16]. Another beneficial effect observed is the suppression of calcification of vascular smooth muscle cells by lanthanum in the presence of H_2O_2[17].

Though the osteogenic differentiation potential of La^{3+} on rat osteoblasts and murine primary bone marrow stromal cells has been reported[18,19], the effect of La^{3+} on human ADSCs (hADSCs) has not been investigated. In the present study the concentration range over which La^{3+} proliferates the hADSCs and does not elicit any cytotoxicity was investigated and the differentiation into osteoblasts under these concentrations was worked out.

1 Materials and methods

1.1 Materials

DMEM, α-MEM, phosphate buffered saline (PBS), collagenase, ammonium chloride, streptomycin, gentamicin, penicillin, amphotericin B, trypsin, MTT (3-

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(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), β-glycerophosphate (β-GP), dexamethasone (Dex), lanthanum nitrate, silver nitrate, BCIP-NBT substrate, pNPP substrate and Tween-20 were purchased from Sigma, USA. CD markers were purchased from Beckton Dickinson, San Jose, USA. Fetal Bovine Serum (FBS) and TRIzol® were purchased from GIBCO, USA. Protoscript m-mulv first strand cDNA synthesis kit was purchased from New England Biolabs and Mastermix for PCR was from Eppendorf, Germany. Primers for RT-PCR were procured from Synergy Scientific services, India. Alizarin red S was purchased from SD Fine chemicals, India. Glutaraldehyde, osmium tetroxide and hexa methyl disilazane (HMS) were purchased from Electron Microscopy Sciences, USA. All other chemicals were of analytical grade.

1.2 Isolation and culture of adipose derived mesenchymal stem cells

Lipoaspirates from human volunteers undergoing (Gynaecomastia) elective surgery were collected after obtaining informed consent and processed using the procedure described by Zuk et al.[1]. The experimental protocols were approved by the Institutional Review Board of Central Leather Research Institute, Chennai. Briefly, the lipoaspirates were washed with PBS and the tissue was digested with 0.075% type I collagenase. The enzyme activity was neutralized with DMEM medium with 10% FBS. RBCs were lysed with 160 mmol/L of NH_4Cl and cells obtained after centrifugation at 1200 g for 10 min were suspended in DMEM medium containing 10% FBS, 100 μg/mL streptomycin, 100 μg/mL gentamicin, 100 U/mL penicillin and 2.5 μg/mL amphotericin B and passed through 100 μm nylon mesh. The cells were seeded in 25 cm^2 tissue culture flasks and cultured at 37 °C with 5% CO_2 in a CO_2 incubator (Binder, Germany). Cells were trypsinized at about 85% confluency using 0.25% trypsin and 0.02% EDTA and serially passaged.

1.3 Characterization of hADSCs

The cultured hADSCs from passage 3 to 7 were used for the experiments. Expression of surface antigens was analysed by Flow cytometry using CELL Quest Pro software (BD FACS Calibur). The trypsinized cells were washed with DMEM containing 10% FBS and were subsequently suspended in sterile PBS. CD marker antibodies were added and incubated for 20 min in the dark and 10000 cells were acquired. The positive CD marker antibodies used were CD 44, CD 90 (FITC labelled), CD 73 and CD 105 (PE labelled). CD 34 (FITC labelled) and CD 45 (FITC labelled) antibodies were used as negative markers.

1.4 MTT assay

Effect of La^{3+} on the proliferation of hADSCs was measured using MTT assay[20]. Cells (1×10^3) seeded onto 12 well multidish (Nunclon) were allowed to grow for 48 h in DMEM medium without phenol red (Sigma) and then treated with 1, 10 and 50 μmol/L of La^{3+}. After washing the cells with sterile PBS, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (0.5 mg/mL) was added and the cells were incubated for 3 h in the CO_2 incubator at 37 °C. The formazan product was dissolved in DMSO and read at 570 nm using a multimode plate reader (Tecan Infinite M 200).

1.5 Osteogenic differentiation in the presence of La^{3+}

For osteogenic differentiation, hADSCs (1×10^4 cells) were cultured in DMEM medium for 48 h in 12 well multidish and osteogenesis was induced using α-MEM medium supplemented with 15% FBS, 50 μg/mL ascorbic acid and 10 mmol/L β-glycerophosphate (MAG medium)[21]. Differentiation into osteogenic lineage was also analyzed in the presence of MAG medium containing La^{3+} at different concentrations (1, 10 and 50 μmol/L). Comparison has been made with 0.1 μmol/L of Dex in MAG medium[5,22].

1.5.1 Staining for Ca^{2+}

After fixing the cells with 70% ethanol, they were stained with 1% Alizarin red S or 2% silver nitrate solution for 10 min in the dark (von Kossa staining)[23,24]. The stained cells were viewed using a phase contrast microscope (Leica DM IRB). For quantitation of Alizarin red S staining, extraction was carried out with 10% (*w/v*) cetylpyridinium chloride in 10 mmol/L sodium phosphate buffer (pH 7.0) for 15 min and absorbance read at 562 nm in a multimode plate reader (Tecan Infinite M 200)[25].

1.5.2 Staining and quantitation of alkaline phosphatase (ALP)

Treated cells were washed with PBS and fixed with 10% formalin for 60 s. Staining with BCIP-NBT substrate for 10 min in the dark at room temperature and washing with buffer containing 0.05% Tween 20 in PBS were carried out according to the manufacturer's instructions. The cells were viewed using a phase contrast microscope (Leica DM IRB). For quantitative analysis, cells were lysed with lysis buffer containing 0.25 mol/L NaCl, 0.05 mol/L HEPES (pH-7.4), 25 μg/mL leupeptin and 1% NP 40. The protein content was measured using Bradford assay and 10 μg of protein was taken for ALP assay. Amount of para nitrophenol (pNP) formed (nanomoles)/min/mg protein was calculated using para nitrophenyl phosphate (pNPP) as substrate[26].

1.5.3 Scanning electron microscopy (SEM) and energy dispersive X-ray diffraction (EDX)

For SEM, cells cultured over Thermanox® cover slips and osteogenic differentiation medium containing different concentrations of La^{3+} or Dex for 7 and 14 d were fixed with 2% glutaraldehyde in 0.2 mol/L sodium