Solid Lipid Nanoparticles Regulate Functional Assortment of Mouse Mesenchymal Stem Cells

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Introduction:

Nanotechnology brings new possibility to stem cells research and development. Measuring in nanoscale to the design, construction, and utilization of functional structures is the hallmark of the technology [1-4]. Such materials and systems are designed to exhibit novel and significantly improved physical, chemical, and biological properties, phenomena and processes as a result of the limited size of their constituent particles or molecules [5, 6].

Stem cell nanotechnology shows great attracting prospects. In recent years, application of nanotechnology in stem cells has made great advances, and is becoming an emerging interdisciplinary field [7, 8]. It requires intense current knowledge and principles to fabricate novel multifunctional or homogenous nanostructures, their processing, characterization, interface problems, high quality nanomaterials availability and nanomaterials tailoring to suit the requirements in modifying stem cell fates. It is believed that stem cell nanotechnology finds applications in treatment of various degenerative diseases [9-12]. Hence, this necessitates an understanding of basic behavior of stem cells upon its interaction with varied characters of the nanoparticles. In this regard we used the solid lipid nanoparticles and studied their interactive behavior with the stem cells of mesenchymal origin.

Mesenchymal stem cells (MSCs) are a heterogeneous population of plastic-adherent, fibroblast-like cells, which in culture are able to self-renew and differentiate into mesodermal and non mesodermal derived tissues [12-17].

Advancements in understanding tissue specific differentiation of MSCs in conjunction with global genomic and proteomic profiling of MSCs have not only provided insights into their biology but also made MSCs based clinical trials a reality for treating various debilitating diseases and genetic disorders [18-20]. The emerging evidences that MSCs are immunologically well tolerated make them even more attractive candidate for regenerative medicine [21-23].

In the present paper, we, custom prepared the solid lipid nanoparticles to study cellular characteristics of mesenchymal stem cell line (C₁₂H₁₀T₁₁S). Observations in the present study, for the first time, lend support to the notion that fate determination of mesenchymal stem cells is a function of SLNs structural assortment.

Materials and method:

CHEMICALS: DMEM (4.5gm glucose per liter, 3.7 gm sodium bicarbonate, sodium pyruvate with L-glutamine,(1X), Trypsin-EDTA solution, fetal bovine serum (FBS), Penicillin streptomycin (penstrap), isopropyl alcohol, TAE buffer, Trizol reagent, agarose were purchased from Hi-Media, India. PCR kit was purchased from invitrogen, India. The plasticware for the cell culture work viz. Falcon tubes (15 ml and 50 ml).

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Beakers(100ml and 500 ml), T-25and T-75 flasks, Six-well plates, Syringe filters, Syringes (0.5ml), PCR tubes were purchased from Tarsons Ltd., India. The Cell line C3H10T½ was provided by NCCS, Pune, India. All the other chemicals purchased from local suppliers were used in the present study.

Preparation & Characterization of lipid nanoparticles: The SLNs in different morphological assortment were prepared in the laboratory as per the method of Kakkar et al [24]. Briefly, the solid lipid nanoparticles were prepared by microemulsification method. The lipidic phase (containing lipid-8%) and the aqueous phase (polysorbate 80, soy lecithin and water) were heated ~10 degree above the lipid melting temperature of 70°C. This hot microemulsion was then transferred into cold water to obtain solid lipid nanoparticles. Excess of polysorbate 80 was removed from the particulate dispersion by washing it with distilled water using a dialysis membrane.

The mean diameter of the SLNs was determined using laser diffraction (Mastersizer 2000, Malvern Instruments, UK) and morphology was examined using a Transmission & scanning electron microscope. SLNs emulsion was lyophilized for performing scanning electron microscopy

Transmission Electron Microscopy: A drop of the SLN dispersion was spread on a 500-mesh gold grid coating and the excess droplets were removed with filter paper. After 5 min, a drop of 2% (mass fraction) phosphotungstic acid in ethanol was placed onto the gold grid. The grid was dried at room temperature and then examined under a Transmission Electron Microscope (Morgagni 268; Phillips, Holland).

Cell culture: The mouse mesenchymal stem cell line (C3H10T½) were maintained in DMEM (with high glucose content), supplemented with 10% FBS (heat inactivated), 100 U penicillin, and 100 μg/ml streptomycin. All the study was performed using third passaged cells. For the experiment purpose the cells were plated at a concentration of 1.5x10⁶ cells per well of the six well plate in the above mentioned growth medium and kept in an incubator maintained at 37 °C in the atmosphere of air and CO₂. All the experiments were performed 24 hrs post establishing the cells in culture and designated this time as 0-day of culture.

Solid Lipid Nanoparticles as Scaffold: Solid lipid nanoparticles (400ng) were added and spread uniformly in two wells of a six well plate. These SLNs were then air dried and UV irradiated overnight to form a thin film. The wells without SLNs served as control. Twenty four hours after formation of the SLN film, equal number of cells (1.5 x 10⁵ cells/well) were plated in a six well plate in the presence of 1.5 ml full growth medium. After 24 hours of incubation, the attachment of the stem cell on SLN film was observed under phase contrast microscope.

Solid Lipid Nanoparticles as adiopogenic differentiation medium: Varying amounts of SLNs (0, 1, 4, 8, 16, 20,100and 200 ng were used to study the effects of SLNs on differentiation of C3H10T½ cell line. For this, 24 hrs post establishment the cell (1.5 x 10⁵ cells/well of a six well plate) were treated at the above mentioned concentrations and observed under phase contrast microscope for any morphological characterization.

Oil-Red-O (ORO) staining: To determine the adiopogenic potential of SLNs, oil red O staining of intracytoplasmically accumulated lipid was carried out at varying doses of SLNs. Briefly, the cells were fixed for a period of 5 min with 10% formalin followed by washing with 60% isopropanol. The cells were then incubated with Oil-Red-O solution for 10 min at room temperature and then were rinsed with water. Stained cells were visualized microscopically and observations recorded.

RT- PCR Analysis: Total RNA of C3H10T½ cells under different experimental conditions was extracted by Trizol method (invitrogen) according to the manufacturer’s instructions. Specific PCR amplification procedures were carried employing forward and reverse primers of Adipsin (forward -5’ATG GTA TGA TGT GCA GAG TG 3’; REVERSE- 5’CAC ACA TCG TGT TAA TGG TGA C 3’), β-Catenin (forward 5’AAG GAA GCT TCC AGA CAT GC 3’; Reverse 5’AGC TTG CTC TCT TGA TTG CC 3’), PPAR gamma (forward 5’TTC CGG GCC GGC CAT GCT TTA CG 3’), RIGS15 (forward 5’TTC CGG GCC GGC CAT GCT TTA CG 3’) and PPAR gamma (forward-5’ ATG GCC ATT GAG TGC CGA GTG TG 3’, reverse –5’ACT GGT CCC TCA AGG AGT TTG CGG 3’). The amplified products were analysed on 0.6 % agarose gels.

Results & Discussion:

We prepared the solid lipid nanoparticles with average particle size of approximately 120 nm (10% particles were >188 nm), having a zeta potential -1.45 mV. The scanning electron microscopic (SEM) images revealed their morphological characteristics which were mainly round in shape (Figure 1a). The shape and surface morphology of these synthetic made solid lipid nanoparticles were studied with TEM (Figure 1b, c). Transmission electron microscopy (TEM) was performed with the aid of a negative-staining method.
Concentrations of SLNs ranging from 1-200 ng per ml remained refractory towards any alterations in the morphology of SLNs and these remained rounded as seen in Figure-1a however at higher concentrations (400 ng per ml) these SLNs arranged themselves as sheet like structures on cell culture plastic ware. This sheet like arrangements is shown in photomicrograph (Figure-2 a, & 2b). In spite of the amphiphatic nature of the lipids used during SLN preparation, the sheet like arrangement of SLNs in the aqueous milieu remained very sensitive to touch and handling. These sheets of SLN start to break and eventually fall apart with in 36 hrs. Hence, broken sheets with cell attached are shown in these photomicrographs at different magnifications (Figure-2a, 4x & 2b, 10x).

Parallel to this however, the effect of varied concentration of SLNs in aqueous milieu demonstrated a variable effect on their interaction with the stem cells. As shown in photomicrographs (Figure-3 panels b to f), the concentrations SLNs varying from 1-20 ng adopted a physical arrangements that enabled these particles to behave more like a cell differentiation molecules and triggered the differentiation of the C3H10T½ mesenchymal stem cells to adipogenic lineages as demonstrated by positive staining for the oil O red (Figure-3b-f) compared to untreated cells (Figure 3a). Oil O red is an important marker for the adipocytic cells because of its ability to stain the triglyceride component of the cells.

The concentrations of SLNs beyond 20 ng per ml specifically the amount used in the study i.e. 100 and 200 ng severely affected the cell attachment ability and morphology. At these concentrations SLNs were found to be inhibitory to cell establishment. As shown in Figure-3g (100 ng, SLN conc.) & 3h (200 ng, SLN conc.) the cells attained rounded morphology and were found to be rather floating in suspension. Despite the repeated attempts of washing these floating cells could never got attached to the plate surface. Surprisingly none of these cells (100ng SLN conc.) took up trypan blue suggesting these cells were still live but lost the ability to establish.

Our observations further demonstrated that adipogenic potential of the low concentrations of SLNs towards C3H10T½ is not just a consequence of the constituent lipid accumulation rather a bonafide effect on gene expression. Hence, we sought to determine effects of SLN by using one of the adipogenic differentiation concentration of SLNs (20 ng, same dose as used in Figure-3f, (oil O red staining) on expression profile of adipsin, a marker gene (31) for adipocytic differentiation. Adipsin, a complement factor D, is expressed at high levels in adipose tissue and is a regulator of lipid accumulation in adipocytes [31-32]. To analyze this we determined expression of adipsin following RT-PCR amplification as shown in Figure-4a. The Lane one (L-1, Figure-4a) did not show adipsin expression in mesenchymal stem cells in the absence of SLNs. Upon treatment (20 ng/ml) with SLN, as shown in lane two (L-2, Figure-4a), amplification in the adipsin expression could be observed. This expression profile of adipsin goes in parallel with the positive staining for Oil Red O in the SLN treated mesenchymal cells (Figure-3 f), suggesting adipogenic potential of SLNs. Likewise SLN treatment also induced the expression of peroxisome proliferator activated receptor (gamma) (Figure4-d lane 2). Activation of PPAR induces adipocyte differentiation and lipid accumulation by adipocytes by modulating numerous genes regulating adipogenesis, lipid uptake and lipid metabolism [33]. At mechanistic level, it has been well documented that Wnt/β-catenine pathways play an important role in regulating the process of adipocyte differentiation [35]. To identify whether the SLNs adopt same putative signal transduction of a specific inhibitor of Wnt...
pathway, i.e. lithium chloride, LiCl. As shown in Figure-4a, lane three (L-3), presence of LiCl produced a significant downregulation of adipsin expression. This observation supported the fact that SLNs also exploit wnt/β-catenin signaling pathway to bring about adipogenesis. The LiCl is a well established inhibitor of glycogen synthase 3β Kinase (GSK-3 β) [34-36], that leads to stabilization/accumulation of β-catenin thus enabling β-catenine’s forced entry to nucleus to regulate the gene expression [37]. Thus downregulation of β-catenine by SLNs seems a plausible reason to induce adipogenesis. This gets support from fact that control undifferentiated mesenchymal stem cells (lane-1-4b) demonstrated a maximal relative expression of β-catenine which was found to be downregulated in the presence of SLN (L-2, Figure-4b) and started to build up again when treated with LiCl (L-3, Figure-4b). These observations thus point towards an inverse correlation between expressions of β-catenine and adipsin. Such functional character of SLNs is, probably, observed for the first time and their character changes as the concentration of SLNs was increased.

Figure 4

![Figure 4](image)

Figure 4. RT-PCR analysis of a) adipsin b) β-catenin c) RIGS/15 loading control and d) PPAR-gamma. Lane-1 represents Control (without SLN treatment), lane-2 represents SLNs treated cells, Lane-3; GSK3β inhibitor LiCl, treatment to SLN treated cells.

As shown earlier (Figure-2a & b) at concentration of 400 ng per ml these SLNs formed a rather a thick emulsion which when plated on plastic cell culture plate, acquired a sheet like appearance with in 24 hrs of plating. (Figure-5b & c). These sheet like structures were very unstable and in spite of repeated trial these could not be kept as single sheet, thus appeared as small broken sheet in culture medium. These sheet like structures, hence, were used to observe the behavior of mesenchymal stem cells. Unlike individual SLN particles, the SLNs in sheet form behaved as support scaffold for the attachment of mesenchymal stem cells (Figure-5b1 & c1). This observed arrangement of SLNs demonstrated a new character of SLNs, albeit unstable for long cell culture conditions. The studies are thus underway to develop mixed SLNs possessing components of extracellular matrix. Further as shown in this figure (Figure-5 b1 & c1), few of the cells could be seen adhered to these SLN sheets and while few cells remain suspended. All the cell populations, whether attached or suspended, however, remained rounded (Figure-5b1 & c1) and did not retain discernable structural morphology as is seen in cases when these cells are allowed to establish on uncoated cell culture plastic ware, i.e. in a fibroblast-like morphology (Figure-5a1). These observations suggested that SLNs can provide a suitable support that apparently kept the cells in undifferentiated state. This observations of ours was further supported by the fact that the cell attached to SLN sheets also remained refractory to the adipocytic and myocytic cell differentiation cocktail (Figure-5 b2 & c2), that otherwise differentiated the cells on plastic ware to respective lineages i.e. adipocytic, positive for oil Red O (Figure-5a2) and myogenic, elongated morphology (Figure-5a3). These observations thus suggest a new paradigm for maintaining the stem cell character which is the function of structural assortment of SLNs linked to its concentration in aqueous milieu.

Figure 5

![Figure 5](image)

Figure 5. SLNs as Stem Cell Scaffolds: Photomicrographs of C3H10T1/2 cells (a-1-3) plated on six well uncoated plate (control), (b-1-2), (c-1-2) when plated on SLNs coated film in a six well plate and their corresponding picture when treated with adipocytic differentiation dose (MIX) and myogenic differentiation dose (5-azacytidine), respectively. Panel a2 & a3 represents the adipogenic differentiation and myogenic differentiation of cells on uncoated plasticware, respectively. Cells plated on SLNs coated film did not differentiate into either lineage when treated with the differentiation cocktails.

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