Acta Biomaterialia 58 (2017) 214–224

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Simultaneous delivery of hydrophobic small molecules and siRNA using Sterosomes to direct mesenchymal stem cell differentiation for bone repair

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ARTICLE INFO

Article history:
Received 18 January 2017
Received in revised form 22 May 2017
Accepted 30 May 2017
Available online 31 May 2017

Keywords:
Co-delivery
Sterosomes
Hydrophobic small molecular drugs
siRNA
Osteogenic differentiation

ABSTRACT

The use of small molecular drugs with gene manipulation offers synergistic therapeutic efficacy by targeting multiple signaling pathways for combined treatment. Stimulation of mesenchymal stem cells (MSCs) with osteoinductive small molecule phenamil combined with suppression of noggin is a promising therapeutic strategy that increases bone morphogenetic protein (BMP) signaling and bone repair. Our cationic Sterosome formulated with stearylamine (SA) and cholesterol (Chol) is an attractive co-delivery system that not only forms stable complexes with small interfering RNA (siRNA) molecules but also solubilizes hydrophobic small molecules in a single vehicle, for directing stem cell differentiation. Herein, we demonstrate the ability of SA/Chol Sterosomes to simultaneously deliver hydrophobic small molecule phenamil and noggin-directed siRNA to enhance osteogenic differentiation of MSCs both in *in vitro* two- and three-dimensional settings as well as in a mouse calvarial defect model. These results suggest a novel liposomal platform to simultaneously deliver therapeutic genes and small molecules for combined therapy.

Statement of Significance

Application of phenamil, a small molecular bone morphogenetic protein (BMP) stimulator, combined with suppression of natural BMP antagonists such as noggin is a promising therapeutic strategy to enhance bone regeneration. Here, we present a novel strategy to co-deliver hydrophobic small molecule phenamil and noggin-targeted siRNA *via* cationic Sterosomes formed with stearylamine (SA) and high content of cholesterol (Chol) to enhance osteogenesis and bone repair. SA/Chol Sterosomes demonstrated high phenamil encapsulation efficiency, supported sustained release of encapsulated drugs, and significantly reduced drug dose requirements to induce osteogenic differentiation of mesenchymal stem cells (MSCs). Simultaneous deliver of phenamil and noggin siRNA in a single vehicle synergistically enhanced MSC osteogenesis and calvarial bone repair. This study suggests a new non-phospholipid liposomal formulation to simultaneously deliver small molecules and therapeutic genes for combined treatment.

1. Introduction

Dr. Alec Bangham et al. first reported their discovery of liposomes in 1964 [1]. Over the past half century, liposomal nanotechnology has significantly advanced and provides medical and pharmaceutical benefits [2,3]. Liposomes have already been commercialized for various therapies: Doxil®, Caelyx® and Myocet® are used to treat Kaposi's sarcoma, ovarian and metastatic breast cancer, multiple myeloma; and DaunoXome® for Kaposi's sarcoma. Visudyne® is used for age-related macular degeneration, myopia and ocular histoplasmosis; Ambisome® against fungal infection. DepoDur® is applied for pain relief following surgery.

Combined treatment *via* simultaneous delivery of drugs and genetic materials in a single delivery vehicle presents potential

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http://dx.doi.org/10.1016/j.actbio.2017.05.057
1742-7061/

advantages compared with individual therapy: synergistic effects of multiple medications [4], suppressed drug resistance [5], fine-tune of the dosages of multiple agents, etc. However, the natures of macromolecular nucleic acids and small molecular drugs are intrinsically different, presenting challenges in the development of co-delivery vehicles. Liposomes are essentially used as nanocarriers for protecting, transporting and targeting solutes. Liposomes can encapsulate both hydrophilic drugs in the aqueous core and hydrophobic drugs in the lipid bilayers. In gene therapy, cationic liposomes are reported to interact and complex with DNA, RNA or oligonucleotides, markedly prevent nuclease degradation, facilitate the intracellular uptake and further endosomal escape of oligonucleotide and result in a better intracellular distribution [6–8]. In light of these properties, liposomes are great candidates for co-delivery of small molecular drugs and nucleic acids.

Recently, it has been reviewed that mixture of single-chain amphiphiles and high content sterols can form fluid lamellar phases. Even though these single-chain amphiphiles or sterols do not form fluid lamellar phases once hydrated individually, their mixtures lead to stable liquid-ordered bilayers, and the resulting liposomes were named Sterosomes because of the high sterol content [9]. Compared to the commercially available lipofectamine 2000, cationic Sterosomes formulated with stearylamine (SA) and cholesterol (Chol) showed significantly increased nanoparticle stability and cellular uptake efficiency in our previous study.

The use of mesenchymal stem cells (MSCs) is an attractive option to enhance bone tissue engineering [10]. However, MSCs alone had very limited success on bone repair in challenging healing environments [11,12]. Bone morphogenetic protein-2 (BMP-2) is believed to be the most potent cytokine to promote osteogenic differentiation of MSCs and bone regeneration with extensive clinical use [13,14]. Supraphysiologic BMP-2 dosage is, however, required for clinical treatment, leading to worrisome side effects including ectopic bone formation, life-threatening tissue swelling and cancer [13,15]. Thus, recent promising alternative strategies are toward complementing BMP activity in MSC osteogenesis.

Phenamil, a small molecular derivative of diuretic amiloride, is found to promote BMP activity through upregulating tribbles homolog 3 (Trb3), a positive regulator of BMP signaling [16]. Phenamil enhanced osteogenesis and calvarial bone formation induced by BMP-2 while significantly lowering total BMP-2 dose [17]. The use of small molecules presents several advantages over growth factor therapies, including high stability, non-immunogenicity and relatively low cost. However, hydrophobic nature of such small molecules including phenamil requires the use of toxic organic solvents and often limits their clinical potential [18,19].

Upon BMP stimulation, BMP efficacy is greatly reduced due to the enhanced expression of natural BMP antagonists such as noggin to auto-regulate endogenous BMP-2 levels. Noggin is a secreted polypeptide that binds BMPs and inhibits their function by preventing BMP from binding their receptors on the cell surface. Small interfering RNA molecules have been used to downregulate expression of noggin, therefore to enhance osteogenesis *in vitro* as well as bone formation *in vivo* [20–22]. Our previous studies of viral mediated noggin knockdown showed that suppression of noggin enhanced both osteogenic differentiation of MSCs *in vitro* [23] and bone formation *in vivo* with both calvarial [24] and mandibular [25] defect models. However, delivery of noggin-directed RNAi molecules *via* non-viral modalities will be more advantageous by avoiding viral insertional mutagenesis.

In this work, we demonstrated the ability of the novel Sterosomes formulated with a single-chain amphiphile SA and high content of Chol to simultaneously deliver phenamil and noggin siRNA to enhance osteogenic differentiation of MSCs. Synergistic osteogenic effects were achieved by stimulating BMP signaling (phenamil) and simultaneously knocking down BMP antagonist noggin (siRNA) in a single vehicle. First, we evaluated the encapsulation and release of phenamil with SA/Chol vesicles, and assessed the bioactivity of the encapsulated phenamil compared with its free form dissolved in DMSO. We further investigated the ability of SA/Chol Sterosomes loaded with phenamil and noggin siRNA to induce osteogenic differentiation of MSCs in two-dimensional (2D) monolayer cell culture as well as in three-dimensional (3D) settings using hydrogels *in vitro*. Finally, the *in vivo* osteogenic ability of phenamil + noggin siRNA co-delivered with SA/Chol Sterosomes was evaluated in a mouse calvarial defect model. This present work demonstrated a novel and efficient co-delivery liposomal system containing small molecular drugs and nucleic acids in a single vehicle to guide stem cell differentiation, and this liposomal platform may provide new therapeutic biomaterial systems to improve current bone repair strategies.

2. Materials and methods

2.1. Materials

1-Aminooctadecane (SA, 99.0%), 3β-hydroxy-5-cholestene (Chol, >99%), phenamil methanesulfonate salt (Phe), benzene (high purity), methanol (spectrograde), dimethyl sulfoxide (DMSO, >99%), tris(hydroxymethyl)-aminomethane (TRIS, 99%), NaCl (>99%), β-glycerophosphate, L-ascorbic acid, dexamethasone, nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP), p-nitrophenol phosphate, alkaline buffer solution, *p*-nitrophenylphosphate (pNPP), alizarin red S, ethylenediaminetetraacetic acid (EDTA) and Tween 20 were supplied by Millipore Sigma (St. Louis, MO). RNeasy Mini Kit was obtained from Qiagen (Valencia, CA). Trizol, SuperScript® III First-Strand Synthesis System, Dulbecco's Modified Eagle's Medium (DMEM, 1 g/L glucose), Penicillin/Streptomycin (100 U mL^{-1}, P/S) were obtained from Life Technologies (Grand Island, NY). Fetal Bovine Serum (FBS) was purchased from Mediatech Inc. (Manassas, VA). siRNA was provided by Santa Cruz Biotechnology (Santa Cruz, CA). All reagents were used as received. C57BL/6 and nude mice were ordered from Charles River Laboratories (Wilmington, MA).

2.2. Preparation of Sterosomes

First, dissolve stearylamine, cholesterol and phenamil proportionally in benzene/methanol (90/10 v/v). Freeze the above solutions in liquid nitrogen, and lyophilize them overnight to completely eliminate the organic solvent [26]. Then, hydrate the freeze-dried mixtures in a pH 7.4 TRIS buffer (TRIS 50 mM, NaCl 140 mM). Carry out five cycles from liquid nitrogen temperature to ~70 °C, and vortex between successive cycles to obtain well-hydrated samples. Finally, liposomes were obtained with probe sonicator (20 s on and 5 s off) for 20 min.

A Sephadex G-50 spin column (diameter 0.4 cm, length 7 cm) was centrifuged at 3500 rpm for 1 min to separate free phenamil from phenamil-containing liposomes [27]. Phenamil and cholesterol content of the collected fraction were quantified using UV–Vis spectrometer and cholesterol fluorometric assay kit (Cayman Chemical, Ann Arbor, MI).

siRNA and Sterosomes complexation was prepared and validated according to our previous work [24]. Briefly, Sterosomes and siRNA were diluted separately in Tris-NaCl buffer in Eppendorf tubes and kept at room temperature for 5 min. The contents of two tubes were combined and incubated at room temperature for 20 min.

A Malvern Zetasizer was employed to determine the hydrodynamic diameters and the zeta potential of obtained Sterosomes at 25 °C.