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Biocompatibility and safety of a hybrid core–shell nanoparticulate OP-1 delivery system intramuscularly administered in rats

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ABSTRACT

A hybrid, localized and release-controlled delivery system for bone growth factors consisting of a liposomal core incorporated into a shell of alternating layer-by-layer self-assembled natural polyelectrolytes has been formulated. Hydrophilic, monodisperse, spherical and stable cationic nanoparticles (≤350 nm) with an extended shelf-life resulted. Cytocompatibility was previously assayed with MC3T3-E1.4 mouse preosteoblasts showing no adverse effects on cell viability. In this study, the in vivo biocompatibility of unloaded and loaded nanoparticles with osteogenic protein-1 or OP-1 was investigated. Young male Wistar rats were injected intramuscularly and monitored over a period of 10 weeks for signs of inflammation and/or adverse reactions. Blood samples (600 μL/collection) were withdrawn followed by hematological and biochemical analysis. Body weight changes over the treatment period were noted. Major organs were harvested, weighed and examined histologically for any pathological changes. Finally, the injection site was identified and examined immunohistochemically. Overall, all animals showed no obvious toxic health effects, immune responses and/or change in organ functions. This hybrid core–shell nanoparticulate delivery system localizes the effect of the released bioactive load within the site of injection in muscle with no significant tissue distress. Hence, a safe and promising carrier for therapeutic growth factors and possibly other biomolecules is presented.

1. Introduction

Bone morphogenetic proteins (BMPs) are cytokines that are able to induce new bone formation in vitro and in vivo [1]. BMP-7 (also known as osteogenic protein-1 or OP-1) has been shown to accelerate the formation of new bone in numerous pre-clinical and clinical studies [2]. Nonetheless, the clinical efficacy of OP-1 still depends on the carrier or delivery system used to ensure a sustained, multi-step, and prolonged delivery of adequate protein concentrations to the desired site of tissue repair or restoration [3,4]. The foremost limitations include the rapid diffusion of OP-1 away from the site of application and loss of its bioactivity, resulting in orthotopic/heterotopic bone formation or suboptimal local induction and hence failure of bone regeneration. Consequently, supra-physiological, unsafe and expensive dosages of OP-1 in the milligram range for satisfactory bone healing continue to be required [5]. Liposomes are the commonly investigated vehicles for delivery of therapeutic compounds, such as enzymes and proteins [6,7] because of their biocompatibility and appealing ability to carry hydrophobic and hydrophilic drugs. Nonetheless, stability in vivo remains a setback, mainly due to their high tendency to degrade or aggregate leading to leakage of the entrapped drug during storage or after administration with considerable toxic effects. Additionally, they are rapidly cleared from circulation via uptake by the reticulo-endothelial system [8]. To overcome such problems, modifying the surface by means of coating the liposome with a single layer of hydrophilic polymers has been investigated [8–10]. In an initial in vitro study [10], we formulated monodisperse nanoparticles (NPs) constituting a core of cationic liposomes (L) and a shell constructed through the layer-by-layer (l-b-l) self-assembly of alternating layers of naturally-available anionic alginate (AL) and cationic chitosan (CH). The NPs had a cumulative

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size of 383 ± 11.5 nm and a Zeta (ζ) potential surface charge of 44.61 ± 3.31 mV, suitable for complex formation with anionic proteins such as OP-1 [11]. The choice of reducing particle size of biomaterials from the microscale to the nanoscale is mainly to improve the bioavailability of the encapsulated drug once administered in situ and hence allowing for the effective use of much lower and safer dosages [12]. In a subsequent work [11], the cytobiocompatibility (effect on MC3T3-E1.4 preosteoblast cell viability) and capability of the hybrid core–shell NPs to encapsulate a range of concentrations of bioactive OP-1 for their potential administration via a parental injection was investigated. The system exhibited high physical stability in simulated physiological media allowing for immediate protein loading prior to administration, thus preventing degradation or loss of the entrapped growth factor. A sustained tri-phasic linear release of the water-soluble and readily diffusible positively-charged OP-1 was evident for an extended period of 45 days with the bioactivity of the protein maintained via promoting preosteoblast differentiation with no evident cytotoxic effects (96% cell viability). In vivo, the localized and release-controlled potential of the delivery system were then demonstrated in rabbits where enhanced new bone regeneration resulted following a single injection of the NPs loaded with doses as low as 1.0 μg OP-1 [13]. This established the efficiency as well as the potential biocompatibility and safety of the delivery system where no adverse effects or behavioral changes were noted in all animals over a period of ~21 days. Besides the known advantages including the size property, longer shelf-life, favorable preparation methods and subsequent ability to entrap more bioactive drugs [14], nano-sized delivery systems have the advantage of residing longer in circulation when compared to microparticles [15], raising safety concerns where acidic by-products, foreign body responses and heterotopic bone formation in undesirable tissues have been reported; especially with injectable nanoparticulate delivery systems [16,17].

In this study, we further evaluate the biocompatibility and safety of the injectable nanoparticulate OP-1 delivery system in healthy rats to investigate any potential hazards and/or adverse effects. The commonly-measured blood markers, clinical signs and major organs (and site of injection in quadriceps muscle) were examined for any pathological abnormalities.

2. Materials and methods

2.1. Formulation of hybrid nanoparticles and evaluation of OP-1 encapsulation

The formulation and characterization of the hybrid core–shell nanoparticulate protein delivery system have been previously described [10,11]. Briefly, a lipid phase was prepared via thin-film hydration by dissolving 1, 2-Dipalmitoyl-sn-glycero-3-phosphocholine (Genzyme Pharmaceuticals, Switzerland), cholesterol and a cationic surfactant; dimethyldioctadecyl-ammonium bromide (DDAB) obtained from Sigma–Aldrich Chemical in a chloroform–methanol (Fisher Scientific) mixture (4:1, v/v). DDAB was used in a 4% molar concentration to tailor the surface charge of the phospholipids. The solvent mixture was then removed from the lipid phase by rotary evaporation under vacuum resulting in the deposition of a homogenous dry lipid film. The film was hydrated with 18.2 MΩ cm^{-1} highly-pure water (HPW), vortexed to obtain a suspension of positively-charged multi-lamellar vesicles and transferred into a mini extruder (Avanti® Polar Lipids, Inc.) for filtration through double 200 nm pore size 19 mm polycarbonate filters (GE Osmonics). For the l-b-l shell coating, fresh AL (sodium salt; 2% viscosity) and CH (85% deacetylated with molecular weight of 91.11 kDa) obtained from Sigma–Aldrich Chemical were prepared in HPW (1 mg/mL). The final pH of the CH solution was then adjusted with 1 M NaOH to 5.5. The cationic liposomes (L) were coated with alternating layers of anionic AL and cationic CH until the desired number of polyelectrolyte layers was achieved; 6 layers: $L(AL\text{-}CH)_3$, herein and after denoted as NPs. With the deposition of each polymeric layer, the solution was incubated at room temperature for 60 min and centrifuged at 1600*g* for 15 min for washing. Prior to protein loading, aliquots of nanoparticle suspensions were freeze-dried in the presence of 10% w/w sucrose serving as a cryoprotectant at −54 °C for 48 h (Modulyo D-115, Thermo Savant, MA). The recombinant human (rh) osteogenic protein-1/rhOP-1 (15.7 kDa molecular weight, lyophilized) was purchased from bio-WORLD, OH and stored at −20 °C until use, according to the manufacturer's instructions. Lyophilized NPs were rehydrated back to the original volume with different concentrations of OP-1 solution (0.0–1.0 μg/mL in 0.3 mL HPW). Average size and surface charge changes upon loading were then determined using low-angle laser light-scattering (DLS-HPPS, Malvern Instruments, UK) and laser Doppler anemometry (Zeta-Plus, Brookhaven Instruments, NY), respectively.

2.2. Animals

A total of 22 young and healthy (10–15 weeks old, 350–400 g at the start of the acclimatization period) male normal Wistar rats (Harlan Sprague Dawley®) were included in this study. The animals were housed individually in type III Macrolon cages under conventional hygienic conditions, at 20–24 °C and 30–70% relative humidity and with natural day/night light rhythm. Rats were fed a pelleted diet, allowed access to tap water ad libitum and inspected daily by fit animal health care personnel. The housing, care and experimental protocol were approved by the McGill University Animal Care and Ethics Committee.

2.3. Experimental study design and analyses

2.3.1. Study protocol

All animals were randomized to each receive a single injection (0.3 mL total volume) in the right quadriceps muscle using latex-free micro-fine® IM syringes (25G½ 0.36 mm × 13 mm, Becton Dickinson and Co., NJ) as follows: A. Control group 1: saline ($n = 2$); B. Control group 2: 0.5 μg OP-1 ($n = 2$); C. Control group 3: 1.0 μg OP-1 ($n = 2$); D. Experimental group 1: blank/unloaded NPs in highly-pure water ($n = 4$); E. Experimental group 2: NPs loaded with 0.5 μg OP-1 ($n = 6$); and F. Experimental group 3: NPs loaded with 1.0 μg OP-1 ($n = 6$). No anesthesia was required. Rats were examined daily for signs of infection, inflammation and adverse effects by visual observation. Body weight changes were measured and recorded over a period of 10 weeks. All animals were euthanized by carbon dioxide either on d28 ($n = 11$; 50%) or d70 ($n = 11$; 50%).

2.3.2. Serum biochemical and hematological analysis

Blood samples were collected (6 mL/kg/3 weeks: 600 μL per collection) on day 0 (baseline: pre-injections) and post-injections on days, 1, 7, 14, 28, 56 and 70. Using a biochemical autoanalyzer (VITALAB, Merck, Netherlands), serum biochemical analysis was carried out to determine the serum level of total protein, albumin, total bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), glucose, cholesterol, triglyceride, blood urea nitrogen (BUN), lactic acid dehydrogenase, creatinine, sodium, potassium, chloride, calcium, phosphorus and magnesium. Hematological parameters consisting of erythrocytes, leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils, hemoglobin, hematocrit and platelets were determined using a hematological analyzer (Coulter T540 hematology system, Fullerton, CA).

2.3.3. Histopathalogical analysis

The brain, liver, lungs, kidneys, heart, spleen and right quadriceps muscle (site of injection) were removed from each animal at time of euthanasia (50% on d28 and the other 50% on d70), weighed, sectioned and then immersed-fixed in a buffered (0.4 M phosphate buffer, pH 7.61) 4% paraformaldehyde solution for at least 24–48 h. Tissue sections (3 μm) were prepared after dehydration and embedded in paraffin. Of these, random samples were stained with hematoxylin and eosin (H&E) and processed for comparative histopathological examination under a light microscopy by a qualified and blinded veterinary pathologist to eliminate any bias.

2.3.4. Immunohistochemistry

The specimens of the right quadriceps muscle assigned for immunohistochemistry were sectioned, fixed in 4% paraformaldehyde overnight, decalcified in 20% ethylenediamine tetra-acetic acid for 3 weeks, and embedded in methyl methacrylate or MMA. Seven micrometer sections of a random selection of blocks from every experimental group were then cut. After de-paraffinization and hydration, endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min. Non-specific binding was blocked by incubation in phosphate-buffered saline containing 10% normal horse serum and Triton X-100 for 20 min. For immunostaining, commercially available polyclonal goat antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were used for the qualitative verification of the presence of genes involved in the BMP signaling pathway during bone formation. Those were categorized according to ligands (BMP-2, -3 and -7), receptors (BMPR-I, BMPR-IIA, BMPR-IIB), transcription factors (Smads 1–5 and Sox-9), differentiation marker (Collagen-II) and antagonists (BMP-3 or osteogenin and Noggin). Sections were incubated with these primary antibodies at a dilution of 1:100 in phosphate-buffered saline with 1% normal horse serum. Overnight incubation at 4 °C in a humidified chamber followed. As a secondary antibody, a biotinylated horse anti-goat antibody (Vector Labs, Burlingame, CA) at a dilution of 1:400 was used. Sections were stained using the avidin–biotin complex method along with 3,3′-diaminobenzidine tetrachloride for 30 min, followed by DAB-peroxidase revelation. Finally, the sections were counterstained with Goldner Trichrome, mounted with Permount, imaged and semi-quantified as described below under optical