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Intra-articular delivery of kartogenin-conjugated chitosan nano/microparticles for cartilage regeneration

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ABSTRACT

We developed an intra-articular (IA) drug delivery system to treat osteoarthritis (OA) that consisted of kartogenin conjugated chitosan (CHI-KGN). Kartogenin, which promotes the selective differentiation of mesenchymal stem cells (MSCs) into chondrocytes, was conjugated with low-molecular-weight chitosan (LMWCS) and medium-molecular-weight chitosan (MMWCS) by covalent coupling of kartogenin to each chitosan using an ethyl(dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS) catalyst. Nanoparticles (NPs, 150 ± 39 nm) or microparticles (MPs, 1.8 ± 0.54 μm) were fabricated from kartogenin conjugated-LMWCS and −MMWCS, respectively, by an ionic gelation using tripolyphosphate (TPP). The *in vitro* release profiles of kartogenin from the particles showed sustained release for 7 weeks. When the effects of the CHI-KGN NPs or CHI-KGN MPs were evaluated on the *in vitro* chondrogenic differentiation of human bone marrow MSCs (hBMMSCs), the CHI-KGN NPs and CHI-KGN MPs induced higher expression of chondrogenic markers from cultured hBMMSCs than unconjugated kartogenin. In particular, hBMMSCs treated with CHI-KGN NPs exhibited more distinct chondrogenic properties in the long-term pellet cultures than those treated with CHI-KGN MPs. The *in vivo* therapeutic effects of CHI-KGN NPs or CHI-KGN MPs were investigated using a surgically-induced OA model in rats. The CHI-KGN MPs showed longer retention time in the knee joint than the CHI-KGN NPs after IA injection in OA rats. The rats treated with CHI-KGN NPs or CHI-KGN MPs by IA injection showed much less degenerative changes than untreated control or rats treated with unconjugated kartogenin. In conclusion, CHI-KGN NPs or CHI-KGN MPs can be useful polymer-drug conjugates as an IA drug delivery system to treat OA.

1. Introduction

Osteoarthritis (OA), also known as degenerative arthritis or degenerative joint disease, affects millions of people around the world. Intra-articular (IA) drug delivery can be an useful modality in OA treatment, delivering a drug directly to the main focus of the disease. IA drug administration has several advantages, such as initial high local drug concentrations, lower total drug dose, avoidance of systemic side effects, and fewer drug interactions [1]. The therapeutic effect of IA drug depends mostly on the efficacy of the drug delivery system, due to the short retention time and rapid clearance of soluble drugs from the joint. Several IA drug delivery systems, including liposomes [2,3], hydrogel [4], nanoparticles [5,6], and microparticles [7–9], have been used to achieve prolonged and sustained release of drugs in the joint.

Glucocorticoids and sodium hyaluronate/hyaluronic acid (HA) are IA injection materials that have been used broadly for OA treatment. IA injection of such anti-inflammatory analgesic agents is an effective measure for alleviating the symptoms and preventing the progression of OA [10]. However, those drugs do not induce regeneration of damaged cartilage, which is crucial for obtaining 'good' long-term results in OA treatment. Only surgical treatment such microfracture, osteochondral autograft/allograft, or autologous chondrocyte implantation has provided limited regeneration of articular cartilage in OA. Recently, various studies are underway to evaluate stem cells as a regenerative medicine for OA, with some research being translated into clinical studies [11].

Kartogenin is a recently characterized material that promotes the selective differentiation of mesenchymal stem cells (MSCs) into chondrocytes, thus stimulating cartilage regeneration. Kartogenin frees core-binding factor (CBF)-β from the filament A. Freed CBF-β enters the nucleus, where it binds to the DNA-binding transcription

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factor RUNX1. The CBFβ-RUNX1 complex then activates the transcription of proteins involved in components of the cartilage matrix, such as collagen type II, aggrecan, and tissue inhibitors of metalloproteinase [12]. Kartogenin also induces type-I collagen synthesis of human dermal fibroblasts by activating the smad4/smad5 pathway [13].

Chitosan has been investigated extensively for drug delivery systems because of its biodegradability, biocompatibility, polycationic characteristics, good solubility at a pH value close to the physiological range, and the presence of amino groups along the chitosan chain that can be used for further functionalization [14]. Kartogenin, which is a hydrophobic and low-molecular-weight compound, has a carboxyl group that can couple covalently with the amine groups of chitosan. Drug conjugation to a hydrophilic polymer not only enhances the aqueous solubility of hydrophobic drugs, but can also change drug pharmacokinetics in the body [15,16]. Thus, the conjugation of kartogenin to chitosan may possibly enhance the solubility and permeability of kartogenin, promoting its therapeutic efficacy.

In the present study, the kartogenin conjugated chitosan (CHI-KGN) was synthesized to enhance the aqueous solubility and the biocompatibility of hydrophobic kartogenin. Two drug delivery systems according to size range, nanoparticles (CHI-KGN NPs) and microparticles (CHI-KGN MPs), were prepared by an ionic gelation of the CHI-KGN conjugate with tripolyphosphate (TPP) anion that can interact with cationic chitosan by electrostatic forces. Thus the aim of this study was to (1) characterize the CHI-KGN particles for sustained release and chondrogenic activity *in vitro*, (2) evaluate the CHI-KGN particles as IA drug delivery systems for cartilage regeneration in OA joint *in vivo*.

2. Materials and methods

2.1. Materials

2.1.1. Polymers and reagents

Low-(50–190 kDa; deacetylation degree, ~85.0%) and medium-(190–310 kDa; deacetylation degree, ~85.0%) molecular weight chitosan powders purchased from Sigma–Aldrich (St. Louis, MO, USA) were used. Kartogenin was obtained from Tocris Bioscience (Bristol, UK). TPP, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma–Aldrich. Leucine and 2,4,6-trinitrobenzene sulfonic acid (TNBSA) were obtained from Thermo Scientific (Waltham, MA, USA). All other chemicals used were of analytical reagent grade. Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12) and Alpha-Minimum Essential Medium (Alpha-MEM) purchased from Welgene (Dalseodu, Daegu, Korea) were used for pellet culture of human bone marrow mesenchymal stem cells (hBMMSCs) and chondrocyte culture, respectively. Insulin/transferrin/selenium (ITS) and bovine serum albumin (BSA) of cell culture grade were purchased from Gibco (Grand Island, NY, USA). Dexamethasone, ascorbate-2-phosphate, L-proline and sodium pyruvate were obtained from Sigma–Aldrich.

2.1.2. Cells and experimental animals

Bone marrow samples, used to isolate hBMMSCs, were obtained from three patients (mean age: 64 years, range: 54–72 years) undergoing total hip replacements due to OA. The isolated hBMMSCs were characterized and cultured according to our previous report [17]. Chondrocytes were isolated from the fragments of human articular cartilage that were obtained during total knee arthroplasties [7]. The donors were three patients (age range 59–65 years) who had advanced OA of the knee joints. Informed consent was obtained from all donors. The isolated hBMMSCs or chondrocytes from each patient were separately stored in liquid nitrogen and all the experiments were performed with cells from single individuals.

The animal experiments conducted in this study were approved by the Animal Research and Care Committee of our institution. Nine-week-old male Sprague Dawley rats (Orient Inc., Seoul, Korea) were used according to the policies and regulations for the care and use of laboratory animals (Laboratory Animal Center, Dongguk University Ilsan Hospital, Goyang, Korea).

2.2. Preparation of kartogenin conjugated chitosan (CHI-KGN)

2.2.1. Synthesis of kartogenin conjugated chitosan (CHI-KGN)

Carbodiimide chemistry was utilized to mediate the formation of an amide linkage between terminal carboxylic group of kartogenin and amine group of chitosan. Briefly, EDC/NHS solution at the appropriate concentration and molar ratio were prepared in ionized water according to the manufacturer's instructions. Kartogenin was immersed in an appropriate concentration mixture of EDC and NHS for 1 h at 25 °C. Low-molecular-weight chitosan (LMWCS) and medium-molecular-weight chitosan (MMWCS) in acetic acid solution (1% v/v) were reacted with the NHS-esterified kartogenin for 24 h with low-speed stirring. The amount of kartogenin used in the cross link formation was ≥95 mol% and ≥5% weight ratio to LMWCS and MMWCS, respectively. The two kinds of kartogenin conjugated chitosan (CHI-KGN) were then dialyzed against deionized water with Spectra/Por dialysis tube (molecular weight cut-off = 20 kD, Spectrum Lab., Rancho Dominguez, CA, USA) for 1 day. The content of unconjugated kartogenin in deionized water used in dialysis was monitored using isocratic reversed-phase high performance liquid chromatography (HPLC: Ultimate 3000, Thermo Dionex, Sunnyvale, CA, USA) spectrum. Inno C-18 column (150 × 4.6 mm, 5u, Youngjinbiochrom, Seoul, Korea) was used for the separation. The analysis was carried out using a flow rate of 1.0 mL/min and recorded at 274 nm with a run time of 10 min. Kartogenin was used as a standard in the range of 1–100 mg/L. The conjugates were then lyophilized for further use. The conjugation efficiency of kartogenin was calculated from the HPLC spectrum as follows:

$$\text{Conjugation efficiency}(\%) = \frac{\text{Kartogenin}_{\text{total}} - \text{Kartogenin}_{\text{unconjugated}}}{\text{Kartogenin}_{\text{total}}} \times 100\%$$

Table 1
Kartogenin formulation in synthesis of kartogenin conjugated chitosan.

	LMWCS-KGN	MMWCS-KGN
Amount of kartogenin to chitosan used in conjugation	≥95 mol% ≥5% weight ratio	≥95 mol% ≥5% weight ratio
Conjugation efficiency of kartogenin	98.1 ± 1.6%	97.9 ± 1.9%

2.2.2. Characterization of CHI-KGN conjugate

Both FTIR (Fourier transform infrared) and ^{1}H NMR (proton nuclear magnetic resonance) spectroscopy were used to characterize the surface chemistry of the synthesized CHI-KGN conjugate. The lyophilized powders of CHI-KGN conjugate were applied on the FTIR sample folder and recorded on Nicolet 6700 FTIR spectrometer (Thermo Scientific) from 650 to 4000 cm^{-1} with a resolution 8 cm^{-1} and at 32 scan. For the ^{1}H NMR studies, deuterated water (D_2O) or dimethyl sulfoxide (dDMSO) were used as the solvents. The chemical shifts were measured in parts per million (*ppm*, δ) using D_2O or dDMSO as the internal reference. The ^{1}H NMR spectra were obtained using an AVANCE 600 NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) at room temperature.

2.3. Preparation of CHI-KGN nanoparticles and CHI-KGN microparticles

2.3.1. Ionic gelation method

CHI-KGN nanoparticles (CHI-KGN NPs) and CHI-KGN microparticles (CHI-KGN MPs) were fabricated by an ionic gelation of TPP with kartogenin conjugated LMWCS and kartogenin conjugated MMWCS, respectively. Physical conditions used in the method of the CHI-KGN conjugates with TPP were as shown in Table 2. After the preparation, CHI-KGN NPs and CHI-KGN MPs were obtained by centrifugation (15,000 rpm, 20 min), and washed three times with deionized water. The particles were then lyophilized for further use.

2.3.2. Characterization of CHI-KGN NPs and CHI-KGN MPs

The morphology of the CHI-KGN NPs and CHI-KGN MPs were studied using a field-emission scanning electron microscopy (FE-SEM: ZEISS SUPRA 55VP, Carl Zeiss AG, Oberkochen, Germany). One drop of aqueous CHI-KGN NPs or CHI-KGN MPs was

Table 2
Physical conditions in production of CHI-KGN NPs and CHI-KGN MPs.

	CHI-KGN NPs	CHI-KGN MPs
Chitosan molecular weight	LMWCS: 50–190 kDa	MMWCS: 190–310 kDa
CHI-KGN concentration (w/v)	0.05%	0.85%
CHI-KGN to TPP weight ratio	2: 1	4: 1
CHI-KGN to TPP volume ratio	3.3: 1	1: 3
pH	5	5
Stirring speed (rpm)	700	700
Acetic acid concentration (v/v)	1%	1%
Elapsed time after TPP addition	10 min	10 min
Average size on 1 day	150 ± 39 nm	1.84 ± 0.54 μm
Average size on 14 day	162 ± 43 nm	1.92 ± 0.74 μm
Zeta potential (mV)	−11.84 ± 1.2	+7.80 ± 1.1