

Original Article

Chitosan-p CrmA nanoparticles protect against cartilage degradation of osteoarthritis in a rabbit model

Hua-Jie Li, Xiong-Feng Xu, Ming Gong, Bo Qiu

Department of Orthopedics, Renmin Hospital of Wuhan University, Wuhan, Hubei, China

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Abstract: Some reports have shown that CrmA could ameliorate the interleukin-1 β induced osteoarthritis. In this study we investigated the effects of chitosan (CS)-CrmA nanoparticles (NPs) on metalloproteases expression (MMPs) in chondrocytes in vitro/in vivo and the underlying mechanism. CS-pDNA NPs were prepared by gel complex method and characterized. The expression of GFP and CrmA was detected by fluorescence microscope or western blot after the NPs were added into the culture medium of primary rabbit chondrocytes. Primary rabbit chondrocytes were treated with PBS, CS/pCDNA3.1 (+) or CS/pCDNA3.1 (+) CrmA NPs to analyze the changes of MMPs expression and apoptosis. Rabbit osteoarthritis (OA) models were established using anterior cruciate ligament transaction (ACLT) method. Nanoparticles including CS/pCrmA were injected into the rabbit joint once a week for one month after operation. All joint tissues were obtained to analyze the pathogenic changes, the expression levels of IL-1 β , MMP-1, -3, -9, -13 by real-time RT-PCR, and cell apoptosis by TUNEL. The binding of CS and pDNA were verified by agarose gel electrophoresis. The in vitro release of pDNA in NPs at pH 2.0 and pH 7.0 presented a bi-phase kinetic release-curve. GFP and CrmA in CS/pDNA NPs were confirmed in rabbit primary chondrocytes. CS/pCrmA NPs can not only significantly inhibit apoptosis of the primary chondrocytes induced by IL-1 β and the expression levels of MMP-1, -3, -9, and -13 compared with controls ($P < 0.05$) but ameliorate the progression of osteoarthritis by inhibiting the primary chondrocytes apoptosis, which was further confirmed by histopathologic analysis. This study demonstrated that CS and CrmA in CS/pCrmA NPs synergistically inhibit the expression of MMPs, reduce chondrocyte apoptosis, and enhance the repair response of cartilage following cartilage insults. Altogether, CS/pCrmA NPs presented therapeutic potential for patients with osteoarthritis.

Keywords: Osteoarthritis (OA), chondrocyte, chitosan (CS), cytokine response modifier A (CrmA)

Introduction

Several studies have investigated the important role of metalloproteases (MMPs) in degrading cartilage in OA [1-4]. Collagenases MMP-1 and MMP-13 play a key role in collagen degradation in OA. Collagenase MMP-1 has a high activity of cleaving proteoglycan and can breakdown type I, II and III collagens [5]; however collagenase MMP-13 play a much more important role compared with other collagenases due to its primary expression in chondrocytes and a stronger catalytic ability for type II collagen [6, 7]. MMP-3 which is mainly generated by chondrocytes belongs to the stromelysins group. In addition to its ability to degrade proteoglycan, MMP-3 can activate MMP-1 to promote the breakdown of collagen in a pathological condition [8, 9]. MMP-9 is an important member of

gelatinases which can further degrade the type II collagen that has been firstly cleaved by collagenases [4, 10].

In addition, cytokines, especially IL-1 β , may have an important role during the pathogenesis of OA. IL-1 β converting enzyme (ICE) can cleave the precursor of IL-1 β (pro-IL-1 β) into matured and functional form of IL-1 β . Although some studies have shown the inhibition of ICE can prevent apoptosis and the inflammation process [11], the role of ICE in initiating and promoting OA has not been reported. Interestingly, the ICE expression level increases in the cartilage of OA [12]. Cytokine response modifier A (CrmA) encoded by poxvirus is a natural inhibitor of ICE. CrmA has LVAD tetrapeptide amino acid sequence which is similar with the substrate peptide sequence (Tyr-Val-Ala-Asp, YVAD)

Table 1. Gene and primer sequence

Gene	Primer Sequence (5'-3')	Size (bp)	Annealing Temp
MMP-1	GGGAGATCATCGGGACAACTC GGGCCTGGTTGAAAAGCAT	72	58
MMP-3	GCCAAGAGATGCTGTTGATG AGGTCTGTGAAGGCGTTGTA	363	58
MMP-9	GCCCTGTGGAGCGCGGTGAC GAATGCCGGGGCCAGGAGGG	160	62
MMP-13	CCTGGCCGCCTGCCTCCTCT GAG CCTGTCAACCATGGAGC	210	61
β -actin	TTCTACAATGAGCTGCGTGTGGCT GCTTCTCCTTAATGTACGCACGA	373	60

of ICE. Therefore, CrmA can bind to ICE as a mimic substrate and block ICE cleaving the pro-IL-1 into IL-1. Chitosan (CS) is a kind of polysaccharide widely existed in nature. CS has been considered as an ideal gene carrier for gene therapy, attributed to its high biocompatibility, non-immunogenicity, and non-toxicity [13, 14]. Chitosan possesses a positive charge in normal physiological condition, allowing it to effectively bind DNA or siRNA with negative charge to form nanoparticles [13-15].

We applied a novel method to deliver CrmA gene carried by chitosan (CS)-DNA NPs into primary cultured articular chondrocytes and the joints of OA rabbit models to investigate the effect of CrmA on MMPs expression in articular chondrocytes in vitro and in vivo and to reveal the possible mechanism.

Materials and methods

Isolation and culture of primary rabbit chondrocytes

Articular cartilages from the knees of New Zealand rabbits were dissected and subjected to digestion with 1 mg/mL pronase (Roche Applied Science, Mannheim, Germany) for 30 min. The sample was then centrifuged and the pellet was subjected to digest with 1 mg/mL collagenase P (Roche Applied Science, Mannheim, Germany) for 3 h at 37°C. Articular cartilage chondrocytes were counted and checked for viability using trypan blue staining. More than 95% cells were viable after isolation. Chondrocytes were cultured with Dulbecco's Modified Eagles Medium/Hams' F-12 (DM-EM/F-12) (GIBCO, BRL, UK) plus 10% fetal bovine serum (FBS, GIBCO, BRL, UK) and 100 U/ml penicillin-streptomycin, and were incubated

at 37°C under a humidified 5% CO₂ atmosphere until reaching confluence.

Plasmid construction

CrmA gene was synthesized (Generay Biotech, Shanghai, China) according to the sequence published on GenBank (No. M14217.1). CrmA gene and a Kozak sequence was inserted into the pCDNA3.1 (+) vector digested by EcoRI and XhoI to generate the recombinant

plasmid of pCDNA3.1 (+) CrmA. pCDNA3.1 (+) CrmA plasmid was digested by EcoRI and XhoI to retrieve CrmA gene fragment, which was cloned into pET-28a or pFlag-CMV2 to generate the recombinant plasmids pET-28a-CrmA or pFlag-CMV2-CrmA. All these plasmids were verified by DNA sequencing (GBH gene biotech Company, China). The recombinant protein containing His-Tag was expressed upon induction with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG, Sigma, USA) and purified on Ni²⁺-NTA Resin (Qiagen, Hilden, Germany). CrmA protein was confirmed by Western blot analysis either with mouse monoclonal anti-CrmA antibody (Zymed, San Francisco, CA, USA) or mouse anti-His polyclonal antibody. Immune complexes were detected with goat HRP-conjugated anti-mouse immunoglobulins (Chemicon, Temecula, CA, USA) and the result was revealed via electrochemical luminescence (ECL, Pierceces).

Identification of plasmid pFlag-CMV2-CrmA

Plasmid pCDNA3.1 (+) CrmA was transfected into cultured chondrocytes by Polyethylenimine (PEI, Sigma) or chitosan (MW: 2000-5000, water soluble, Wako Pure Chemical Industries Ltd., Osaka, Japan). 48 h after transfection, cells were collected and lysed in lysis buffer (0.3% NP40, 1 mM EDTA, 50 mM Tris-Cl (pH 7.4), 2 mM EGTA, 1% Triton X-100, 150 mM NaCl, 25 mM NaF, 1 mM Na₃VO₃, 10 μ g/mL PMSF). The expression of Flag-CrmA fusion protein was detected by western blot with anti-Flag monoclonal antibody (Santa Cruz, USA).

Preparation of chitosan-pDNA nanoparticles

A 0.02% chitosan solution was prepared by dissolving chitosan in acetate buffer and adjusted pH to 5.5 by 2M NaOH, which was then filtered

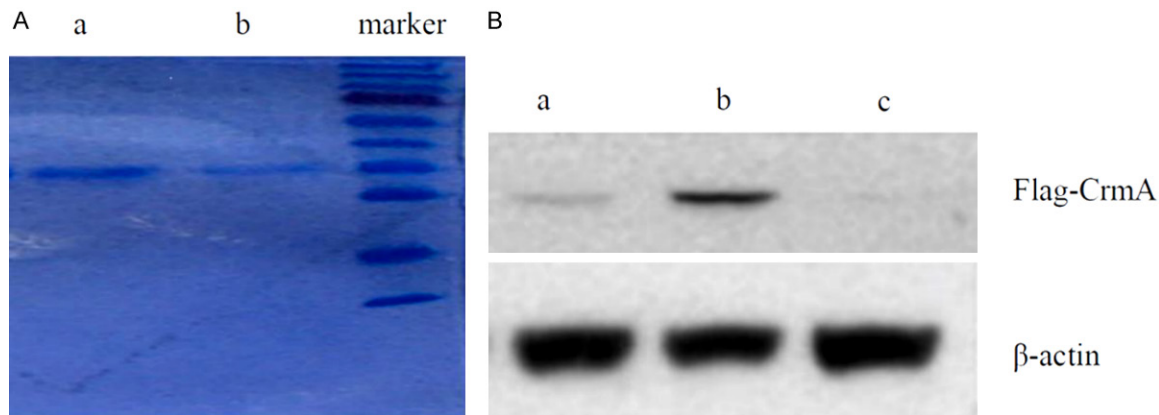


Figure 1. A, B. Electrophoresis on SDS-PAGE gel. A. CrmA protein was expressed in *E. coli* from pET-28a-CrmA plasmid by 0.5 mmol/L IPTG induction. After purification and electrophoresis, a 38 kD band can be observed as expected. Lane A: protein purified with 100 mmol/L imidazole, Lane B: protein purified with 200 mmol/L imidazole. B. Western blot analysis of protein expression by CS-pFlag-CMV2-CrmA and PEI-pFlag-CMV2-CrmA nanoparticles in rabbit chondrocytes. Lane 1: CS-pFlag-CMV2-CrmA; Lane 2: PEI-pFlag-CMV2-CrmA; Lane 3: CS-pFlag-CMV2. The ratio of plasmid to chitosan is 1:1 (2 mg/ml of plasmid and 2 mg/ml of PEI).

using a 0.22 μ m filter. Both chitosan solution and pDNA solution were preheated to 55°C. An equal volume of both solutions was quickly mixed and vortexed (2500 r/min) for 30 s. The mixed solution was kept at room temperature for 15-30 min without disturbance to form CS-pDNA NPs.

Morphology of chitosan-pDNA nanoparticles

The particle morphology was examined by a field emission scanning electron microscopy (FESEM) using a JEOL JSM-6700F system operated at a 5.0 kV accelerating voltage. To prepare samples for FESEM, the particles were fixed on the stub by a double-sided sticky tape and then coated with platinum layer by JFC-1300 automatic fine platinum coater (JEOL, Tokyo, Japan) for 40 s.

Determination of pDNA integrity with chitosan by agarose gel electrophoresis

The different chitosan-pDNA nanoparticles containing 0.2 μ g of pDNA were loaded on a 0.7% agarose gel containing ethidium-bromide. Electrophoresis was carried out with a current of 100 V for 2 h in TAE running buffer. A DNA 2000 marker was used as a molecular mass marker, and naked pDNA was used as a control.

In vitro drug release

To investigate the in vitro release of pDNA from CS-pDNA NPs, 5 mg of CS/pIRES2-EGFP (n=6)

were incubated in 1 ml of DPBS buffer (pH 7.4) in a micro-centrifuge tube in shaking incubator at 37°C. After being incubated for 24 h, half of the samples (n=3) were transferred into a 25 mM sodium acetate buffer (pH 2.0). Samples were taken periodically and were centrifuged at 14,000 rpm for 10 min to obtain pellet NPs. The supernatants were removed and replaced with fresh buffer, and NPs were resuspended by vigorous pipetting. The supernatants were analyzed by UV measurement.

In vitro transfection of chondrocytes with chitosan-pIRES2-EGFP

To observe the GFP expression in CS-pIRES2-EGFP NPs, 2×10^5 rabbit primary chondrocytes/well were inoculated into 6-well culture plate with cover slip laid on bottom. 24 h after inoculation, 2 μ l CS-pIRES2-EGFP NPs was added to culture medium and GFP fluorescence was observed after 3 days.

Cell viability

The cytotoxicity of the NPs was determined using MTT assay. The culture medium was removed and replaced by 20 μ l/well MTT (5 mg/mL) solution in each well, followed by 4 h incubation at 37°C in a fully humidified atmosphere with 5% CO₂. MTT was taken up by active cells and transformed into insoluble purple formazan granules in the mitochondria. The medium was subsequently discarded, the precipitated formazan was dissolved in DMSO (150 μ l/well), and optical density of the solution

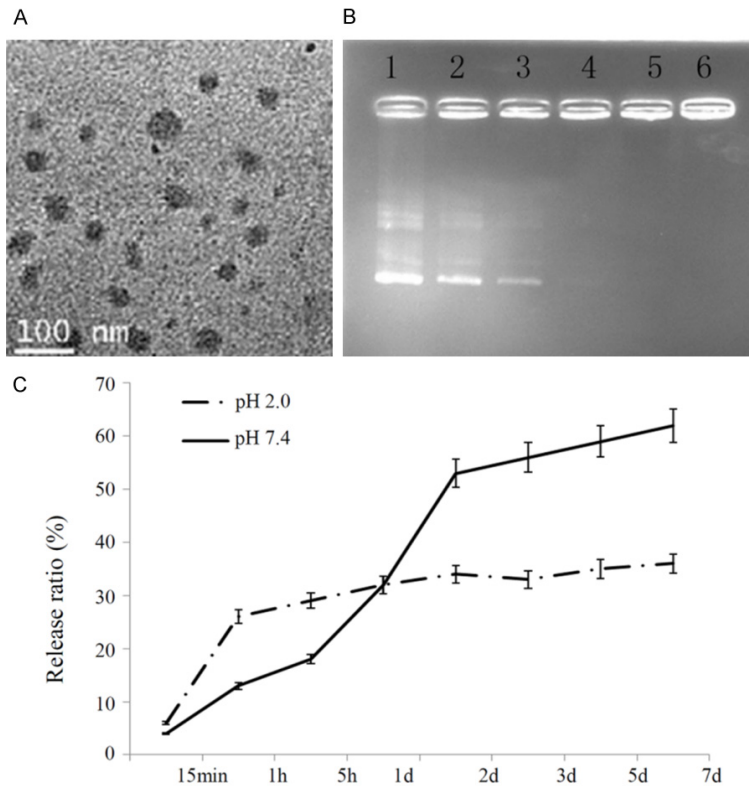


Figure 2. A. The morphology of chitosan-pDNA nanoparticles was examined by FESEM. B. The binding of CS and plasmids was analyzed using agarose gel electrophoresis. To test the binding of chitosan with plasmid at different concentration, different amount of 0.2% of chitosan was mixed with 2.5 μ L of 2 μ g/ μ L plasmid. Lane 1-6: amounts of chitosan on each well were 0, 0.5, 1, 2, 4, 8 μ L. C. The kinetic curve of the CS-pDNA in pH 2.0 and pH 7.0 buffers. CS-pDNA NPs have a slow release rate at pH 2.0 buffer but a rapid release in pH 7.0 PBS buffer during the first 2 days. The release rate of DNA was decreased after 2 days at both pH 2.0 and 7.0 buffers.

was evaluated using a microplate spectrophotometer at a wavelength of 570 nm. The analytical assays were performed on Day 2 and Day 3, and at least 4 wells were randomly taken into examination each time. The determination of cell viability depends on these physical and biochemical properties of primary chondrocytes.

Apoptosis detection

Cells were seeded at a density of 2×10^5 cells/well in 12-well plates and incubated overnight. Cells were then exposed to IL-1 β (10 ng) alone or plus CS/pCDNA or CS/pCrmA for 48 h. After treatment, all cells (i.e., both floating and detached) were subjected to Annexin V-PI staining (Kengentec, China) assay following the manufacturer's instructions. The apoptosis rate was then analyzed by flow cytometry.

Examination of the expression of MMP-1, -3, -9 and -13

Rabbit primary chondrocytes were inoculated in 6-well culture plate. 24 h after inoculation, these cells were treated with IL-1 β (10 ng/mL) for 24 h, then 2 μ L CS/pCDNA3.1 (+) (5 μ g/mL) NPs, CS/pCDNA3.1 (+)-CrmA (5 μ g/mL) NPs were added to culture medium respectively for another 24 h. After washing the cells twice with PBS, the total RNAs were extracted by Trizol reagent (Invitrogen, US). 1 μ g of total RNAs were used as template to be reverse-transcribed into cDNAs by random hexamer primer using SuperScript first synthesis stand kit (Invitrogen, US). Real-time PCR was performed in an ABI7900HT system (Applied Biosystems, U.S.) with a 5 ng of initial RNAs in 20 μ L reaction volumes. Each sample was repeated three times and the results were normalized to the expression level of β -actin. Primer sequence, size and annealing temperature of each gene were listed in **Table 1**. To analyze the protein levels of MMP-1, -3, -9 and -13,

cells were lysed in lysis buffer (0.3% NP40, 1 mM EDTA, 50 mM Tris-Cl (pH 7.4), 2 mM EGTA, 1% Triton X-100, 150 mM NaCl, 25 mM NaF, 1 mM Na₃VO₃, 10 μ g/mL PMSF). Protein samples were resolved on SDS-PAGE and analyzed by Western blot with mouse monoclonal first antibody for MMP-1, MMP-3, MMP-9, MMP-13 and β -actin (Santa Cruz, USA). The bands were scanned and quantified by densitometry.

In vivo animal experiments

New Zealand white rabbits (weight 4-5 kg) were obtained from Hubei Medical Laboratory Animal Center (Wuhan, China). Animal experiments were performed according to the protocols approved by the Laboratory Animal Welfare and Ethics Committee of Wuhan University (Wuhan, China) and the methods were carried out in accordance with the approved guide-

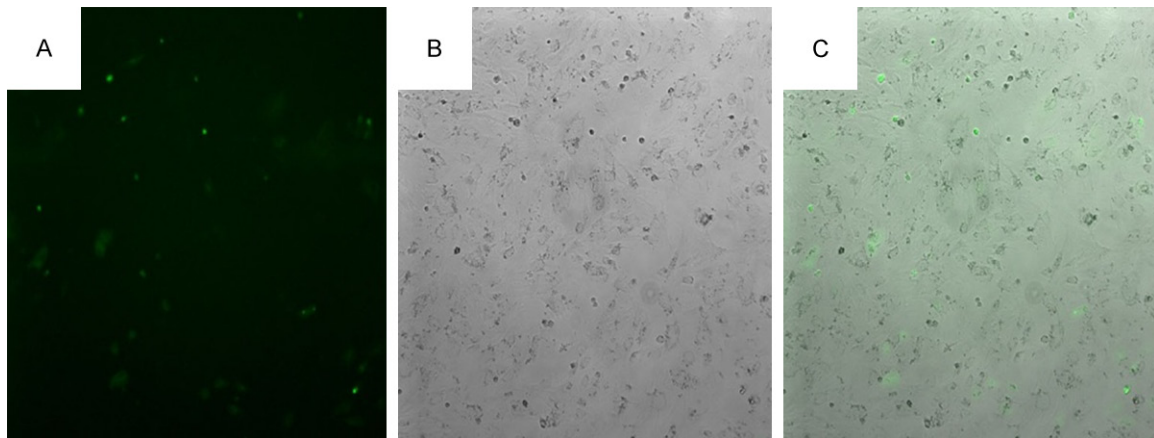


Figure 3. GFP expression was analyzed in primary chondrocytes transfected with CS-pIRES2-EGFP plasmids. (A) Image at fluorescence light source. (B) Image at white light source. (C) Superimpose of image (A and B).

lines. The animals were housed in metal cages and the room was maintained at a temperature of approximately 20°C with 30-70% relative humidity and a light/dark cycle of 12 h/12 h. Rabbit chow and tap water were provided ad libitum to the animals for the period of the study. OA was induced in the knee joints of rabbits using anterior cruciate ligament transaction (ACLT) method [16]. 1 week post operation, OA was assessed and verified after sacrificing 2 rabbits. Then the animals were divided into four groups of eight rabbits including untreated (Group A), CS/pEGFP nanoparticles (Group B), CS/pCrmA NPs (Group C) and normal controls (Group D). The joints of animals except Group A and D were directly injected NPs CS/pDNA or CS/pCrmA NPs at 1 mg/5 mL once a week for 4 weeks after ACLT surgery. At the fifth week of post-operation, all animals were sacrificed and the knee joints were harvested. The severity of the cartilage destruction was valued according to The Scoring principles of Mankin's. A small part of the cartilage tissue in 4% paraformaldehyde, and the other part were put into Trizol RNA exaction solution for real-time RT-PCR for IL-1 β , MMPs. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed to quantitatively assess cell apoptosis with an in situ Cell Death Detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The number of apoptotic cells was quantified by determining the percentage of positively stained cells for all nuclei from six randomly chosen fields/sections at $\times 200$ magnification.

Statistical analysis

All data were expressed as means $\pm$ SE. SPSS 16.0 and one-way ANOVA and post-hoc study were used to analysis the difference between multiple groups. $P < 0.05$ was indicated as significant difference.

Results

Expression of CrmA protein

A 38 kD band as expected was observed after pET-28a-CrmA was transformed into *E. coli* and induced to express (Figure 1A). Then CrmA could also be expressed in primary chondrocytes after pFlag-CMV-CrmA plasmids were transfected into primary chondrocytes (Figure 1B). All these results indicate that the plasmids containing CrmA gene can be expressed in either prokaryotic or eukaryotic cells.

Preparation and characteristics of chitosan/pDNA NPs

The size of chitosan-pDNA was about 50 nm with a globe shape and acoarse surface (Figure 2A). The binding ability of chitosan with plasmid was verified by agarose gel electrophoresis (Figure 2B). We also examined the free pDNA level in chitosan: pDNA (2:1 ratio) particles at pH 2.0 and pH 7.0 PBS buffer by 260 nm wavelength UV light, and the result showed that the pDNA released with a bi-phase release pattern. During the first two days, the DNA released rapidly up to 50%. The speed of pDNA release is faster at pH 2.0 buffer than at pH 7.0 during the

CS/pCrmA NPs protect against cartilage degradation

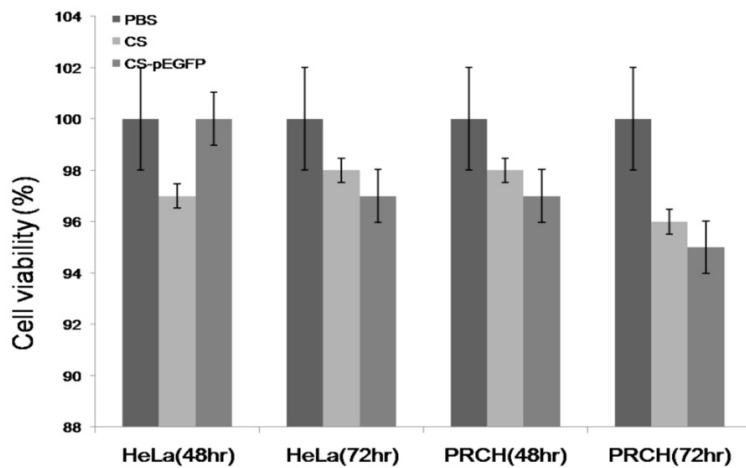


Figure 4. The cytotoxicity of CS and CS/pCDNA NPs on HeLa cells and chondrocytes was measured using MTT assay. PRCH represents primary rabbit chondrocytes.

first day but slower than in pH 7.0 buffer several days later (**Figure 2C**).

Transfection of chitosan-pDNA nanoparticles

We observed the GFP expression under fluorescence microscopy (**Figure 3**) after the transfection of CS-pIRES2-EGFP NPs. Western blot analysis showed that Flag-CrmA could be expressed in primary rabbit chondrocytes (**Figure 1B**). These results indicated that chitosan can mediate plasmids into primary chondrocytes.

Cell viability

Cytotoxicity of CS or CS/pCDNA NPs was determined in various concentrations using MTT assay. The cell viability was not affected by chitosan-based NPs with or without pCDNA plasmids compared with the control (treated with PBS) for 48 h/72 h (**Figure 4**). These results indicated that chitosan has little cytotoxicity and can be used as a potential gene carrier for gene therapy.

CS/pCrmA NPs inhibited chondrocyte apoptosis

In our study, primary rabbit chondrocytes were cultured and pretreated with IL-1 β (10 ng/mL), PBS or CS/pCDNA or CS/pCrmA NPs for another 48 h, and then subjected to Annexin V-PI staining apoptosis assay by flow cytometry. As shown in **Figure 5**, the apoptosis rate of cells

decreased dramatically in the IL-1 β plus CS/pCrmA NPs-treated chondrocytes than in the control groups although CS/pCDNA NPs could also inhibit cell apoptosis induced by IL-1 β . These results argued that CrmA could inhibit IL-1 β -induced apoptosis.

Chitosan/pCDNA3.1 (+) CrmA and chitosan can significantly inhibit the mRNA and protein levels of MMP-1/3/9/13 in chondrocytes

The effects of chitosan/pCDNA3.1 (+) CrmA on MMP-1, -3, -9, and -13 expression in chondrocytes were evaluated by western blot analysis. MMP-1, -3, -9, and -13 expression was significantly lower in chitosan/pCDNA3.1 (+) CrmA-treated chondrocytes than controls ($P < 0.01$) (**Figure 6**). No significant difference of MMP-3 expression was found between chitosan/pCrmA-pretreated chondrocytes and chitosan-pretreated group. Chitosan/pCrmA and chitosan can significantly inhibit the mRNA and protein levels of MMP-1, -3, -9 and -13 in primary chondrocytes. Compared to CS/pCDNA, chitosan/pCrmA has a stronger inhibitory effect. These results showed that chitosan-mediated CrmA inhibited the IL-1 β -induced expression of MMP-1, -3, -9 and -13 in chondrocytes.

Animal experiment

CS/pCrmA or CS/pDNA NPs retarded articular cartilage degradation in a rabbit model of ACLT. Surgical ACLT in rabbits is a well-established OA model, which is characterized with articular cartilage degradation including abrasion of articular surfaces, up-regulated levels of pro-inflammatory cytokines (e.g. IL-1 β), and catabolic molecules MMPs [17, 18]. This rabbit OA model is commonly used to screen biological and pharmacological agents for OA treatment [19, 20]. To examine the effect of CS/pCrmA or CS/pDNA NPs on the development of OA induced by injury, we performed ACLT surgery in the right knee joints of rabbits. After ACLT surgery, the rabbit received once weekly intra-articular injection of 1 mg/5 mL CS/pCrmA or

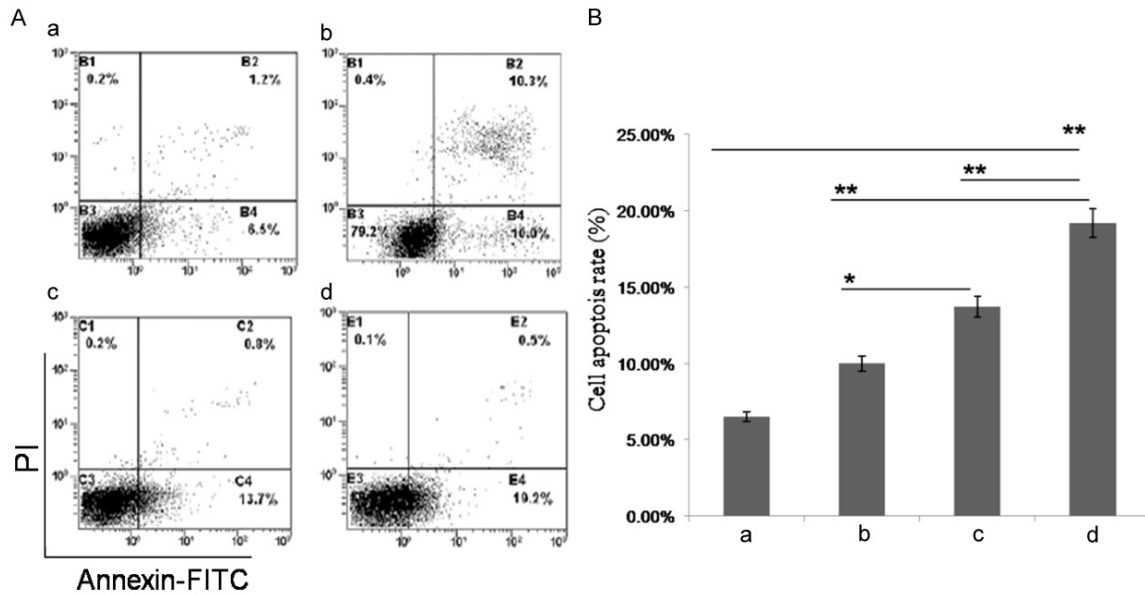


Figure 5. The analysis of apoptosis of chondrocytes treated by IL-1 β . A. Representative image of flow cytometric apoptosis detection; B. Statistical data of cell apoptosis from three experiments (* $P < 0.05$, ** $P < 0.01$). a. No IL-1 β -treated; b. IL-1 β plus CS/pCrmA; c. IL-1 β plus CS/pCDNA; d. IL-1 β .

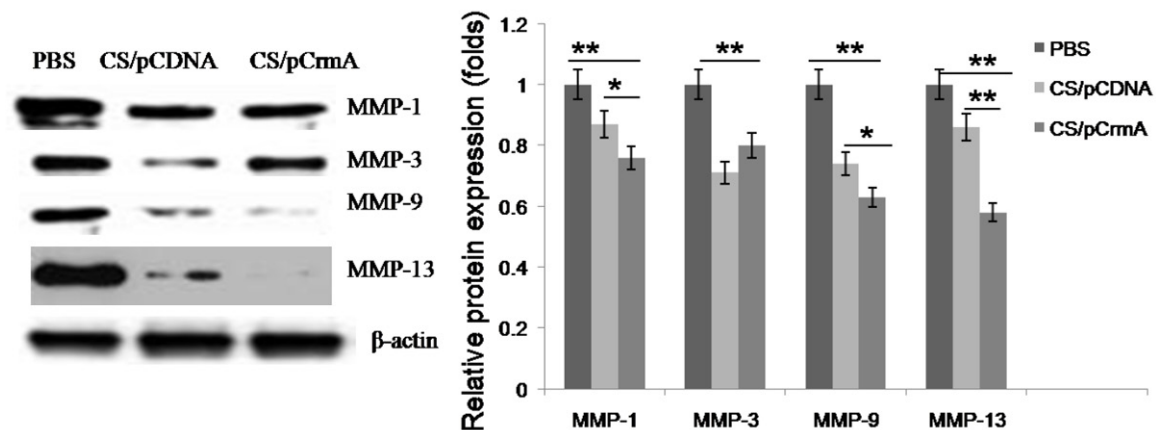


Figure 6. Effects of CS-pCrmA on the protein expression of MMP-1, -13 in IL-1 β -induced chondrocytes. 1: PBS control; 2: CS-pCDNA-treated group; 3: CS-pCrmA-treated group.

CS/pDNA NPs in NS or NS alone for 4 weeks. In view of appearance, the surface of normal joints with a few of synovial fluid was smooth and bright, whereas untreated joints displayed coarse and pale, and synovial fluid increased compared to untreated joints and CS/pDNA or CS/pCrmA-treated joints (**Figure 7A**). The score of Mankin's system for joint injury was seen in **Table 2**. The pathogenic analyses showed that cartilage cells from normal joints arranged regularly, presenting a clear four-layer-structure pattern; cartilage cells from CS/pDNA or CS/

pCrmA-treated joints demonstrated denaturalized, necrosis, and abscission, and the surface of part cartilage appeared crevice; cartilage surface from untreated joints displayed erosion, necrosis and defection of subchondral bone, and cartilage cells appeared fusiformis, paliform-like arrangement, nuclear condensation, transitional cell arrange disorder, nodular hyperplasia, and uniform distribution (**Figure 7B**). The mRNA levels of IL-1 β and MMPs were significantly increased in joints after ACLT surgery compared with that in control joints (**Figure**

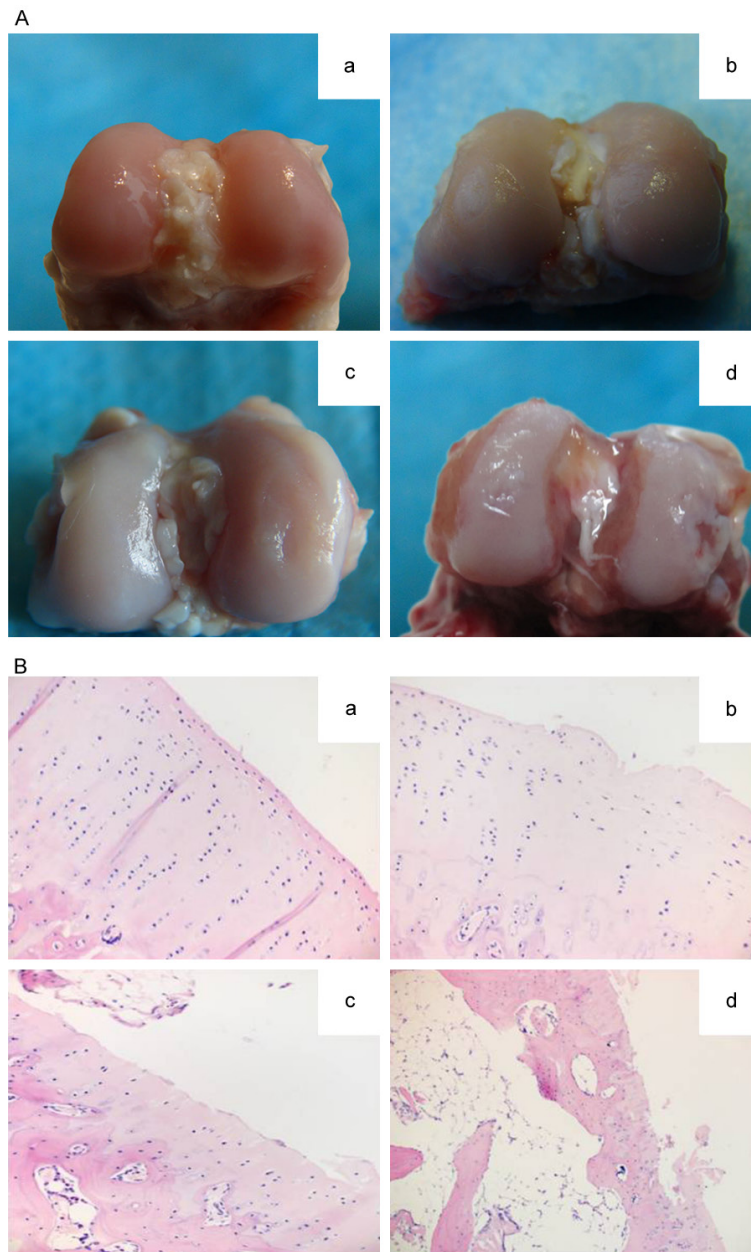


Figure 7. A. Morphologic analyses of different groups. A. Morphology of the left proximal tibial cartilage of rabbit that received ACLT operation. a. Normal; b. CS/pCrmA; c. CS/pCDNA; d. Untreated. B. Histopathogenic analysis by H&E staining of different groups. a. Normal; b. CS/pCrmA; c. CS/pCDNA; d. Untreated.

8). Intra-articular injection of CS/pCrmA or CS/pDNA NPs resulted in a marked reduction of the mRNA levels of IL-1 β and MMPs in joints after ACLT surgery (**Figure 8**). These data demonstrated that CS/pCrmA or CS/pDNA NPs inhibited the activity of MMPs and IL-1 β expression in rabbit knee joints after ACLT surgery, the inhibition role of CS/pCrmA NPs on MMPs and

IL-1 β was preceded over CS/pDNA ($P < 0.05$), in according with that of in vitro inhibition of chondrocytes by CS/pCrmA NPs.

CS/pCrmA NPs also decreases chondrocyte apoptosis in knee joints of ACLT rabbits due to the CrmA inhibition on ICE activity. Since it has been reported that chondrocyte apoptosis is strongly related to OA development [21, 22], apoptosis was examined using TUNEL assay. TUNEL staining showed that CS/pCrmA treatment largely decreased chondrocyte apoptosis by 26% and 12% in the knee joints 4 weeks after ACLT surgery compared to untreated and CS/pDNA respectively (**Figure 9**). Taken together, local intra-articular injection of CS/pCrmA protected chondrocytes from apoptosis in rabbits with ACLT surgery (**Table 2**).

Discussion

The study demonstrated that chitosan can deliver CrmA into cells and CrmA can be expressed in primary chondrocytes. CS/pCrmA nanoparticles can significantly decrease the expression of MMP-1, -3, -9 and -13 at both mRNA and protein levels in vitro/in vivo to protect against the progression of rabbit OA.

It is a critical step to choose vector to deliver target gene into cells or tissues for clinical application. Generally speaking,

non-viral vectors have great advantages over viral vectors with respect to safety, convenient large-scale production, physiological stability and no immunogenicity. Up to now, a variety of particulate drug delivery system based on synthetic and natural materials including chitosan have been investigated as DNA or siRNA carriers [13, 14]. Our results demonstrat-

Table 2. A summed score of Mankin's scoring principles for each group (n=8)

Group	0	1	2	3	4	Total score
Normal	8	0	0	0	0	0
CS/pCrmA	0	3	3	2	0	15 ^{a,b}
CS/pCDNA	0	1	3	3	1	20 ^b
Untreated	0	3	1	4	3	26

^aIndicates P<0.01 (CS/pCrmA vs Untreated); ^bIndicates P<0.05 (CS/pCrmA vs CS/pCDNA and CS/pCDNA vs Untreated).

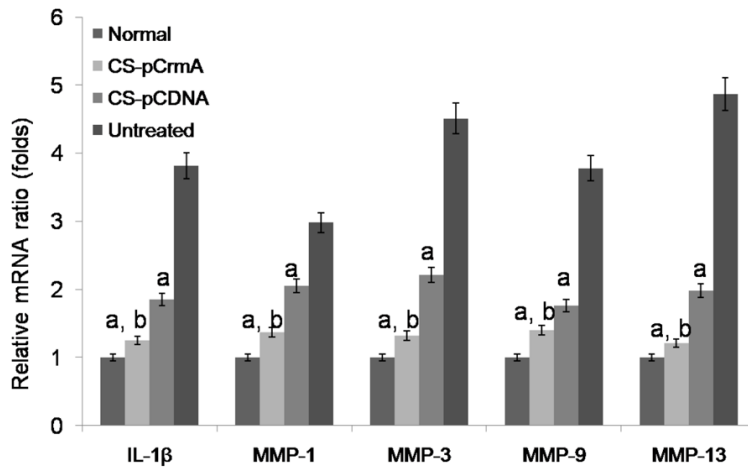


Figure 8. Relative mRNA expression levels of IL-1β, MMP-1, 3, 9 and 13. a indicates P<0.01 (CS/pCrmA or CS/pCDNA vs Untreated); b indicates P<0.05 (CS/pCrmA vs CS/pCDNA).

ed that chitosan can bind pDNA to form NPs, and the pDNA released from CS-pDNA NPs was verified in vitro, which may be related to the pH levels. CS carrier had little cytotoxicity on primary chondrocytes. Ta and colleagues reported chitosan as a biodegradable hydrogel for an anti-cancer drug and gene delivery system for successful chemo-gene therapy [23]. Another report showed that CS-interferon-β gene NP powder for inhalation treatment of lung metastasis in mice was effective [24]. These studies suggested that chitosan is a promising gene vector for gene therapy of various diseases such as cancer and OA.

Previous studies showed that IL-1β can inhibit the synthesis of proteoglycan and type II collagen, stimulate the secretion of MMPs from cells to accelerate the degradation of the proteoglycan and type-II collagen, and induce the apoptosis of chondrocytes [25-27]. Therefore, IL-1β has an important role in the OA pathogenesis and inhibition of IL-1β can be a very

promising therapeutic approach for OA [28, 29]. As a high bioactivity and multiple function cytokine, IL-1β is a classic modulator of the inflammatory response. IL-1β-induced chondrocytes has been considered as an ideal in vitro model for OA study [30]. In this study, we examined the MMPs expression in IL-1β-induced chondrocytes by real time RT-PCR and Western blot and found that MMP-1, -3, -9, and -13 was highly expressed specifically in IL-1β-induced chondrocytes, consistent with the above reports. Thus, inhibition of pro-IL-1β conversion into IL-1β has potential therapeutic role for OA.

ICE, also named as caspase-1, can cleave the 34 KDa pro-IL-1β into 17 KDa matured IL-1β with biological functions. Compared to normal tissues, the osteoarthritic cartilage expressed more ICE [13]. CrmA is a poxvirus gene product with ICE inhibition ability. ICE can be

inhibited by CrmA with LVAD tetrapeptides that are optimal substrates for the four residues of the cleavage site of ICE. CrmA encoded by cowpox are poxviral cross-class serpins that inhibit the serine protease, granzyme B, and cysteine proteases, caspases 1 and 8 to benefit virus replication [31]. CrmA could inhibit cell apoptosis via the extrinsic death receptor pathway and the intrinsic mitochondrial pathway [12, 32]. Our study also demonstrated that CrmA inhibits primary chondrocyte apoptosis induced by IL-1β and CrmA has inhibitory effect on IL-1β expression in the joint of OA models, further confirmed our previous study [33]. Importantly, the present study showed that chitosan mediates the delivery of CrmA into cells and CrmA can be expressed in primary chondrocytes, and CS/pCrmA NPs can significantly decrease the expression of MMP-1, -3, -9 and -13 in IL-1β-induced chondrocytes.

We found that the severity of cartilage degradation in a rabbit model of surgically induced OA

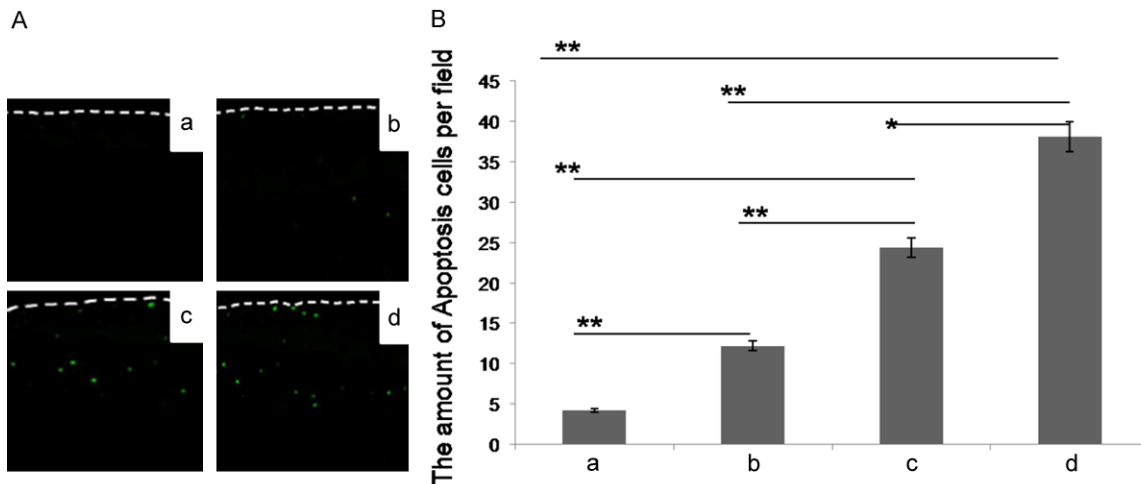


Figure 9. TUNEL assay was performed on knee joints to measure chondrocyte apoptosis. The percentage of positive cells per field were calculated from joint tissue from different groups. Values are the mean $\pm$ SEM of 3 independent trials per treatment group. **indicates $P < 0.01$ and *indicates $P < 0.05$. a. Normal; b. CS/pCrmA; c. CS/pCDNA; d. Untreated.

was attenuated by intra-articular injection of CS/pDNA or CS/pCrmA NPs, in particular, CS/pCrmA NPs. To further investigate the cellular mechanism of the effects of CS/pCrmA NPs on articular cartilage homeostasis and OA, we examined chondrocyte hypertrophy, as chondrocyte hypertrophy can result in increased metabolic activity of articular chondrocytes and trigger unbalanced cartilage homeostasis favoring degenerative changes [34]. We found that CS/pCrmA NPs treatment reduced the expressions of IL-1 β and MMP13 which are most widely used markers for identifying hypertrophic chondrocytes [20, 35], in comparison to rabbits treated with CS/pCDNA after ACLT surgery. These findings revealed that intra-articular injection of CS/pCrmA NPs prevented articular chondrocytes from hypertrophy in this surgical model of OA, which contributed to the inhibitory effect of CS/pCrmA NPs on OA development. The inhibitory effect of CS/pCrmA NPs on chondrocyte hypertrophy is consistent with Xu and coworkers' findings showing that conditional knockout of Fgfr1 in chondrocytes decreased chondrocyte hypertrophy in articular cartilage [36].

Chondrocyte apoptosis is believed to play some important roles in the pathogenesis and progression of OA [21, 22]. Inhibition of chondrocyte apoptosis is shown to alleviate the extent of OA in a rabbit model of surgically induced OA

[37]. CrmA, a poxvirus gene product with ICE inhibition ability, significantly reduced cell apoptosis. Krautwald, et al. reported that TAT-crmA reduced infarction size by 40% and preserved left ventricular function due to effective blockage of both the extrinsic and intrinsic pathways of apoptosis [32]. In this study, FACS analysis and TUNEL assays demonstrated that CS/pCrmA NPs could markedly inhibit chondrocyte apoptosis in vitro/in vivo compared with controls. Thus we postulated that CS and CrmA in CS/pCrmA NPs synergistically inhibit the expression of IL-1 β and MMPs, reduce chondrocyte apoptosis, and enhance the repair response of cartilage following cartilage insults.

In conclusion, our findings indicated that chitosan can bind pDNA to form NPs and deliver target gene into cells. This study provides the first evidence that CS/pCDNA3.1 (+) CrmA NPs can significantly decrease the expression of IL-1 β and MMPs, inhibit chondrocyte apoptosis, and enhance the repair response of cartilage, implying that CS-pCrmA NPs has potential therapeutic role for OA.

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Disclosure of conflict of interest

None.

Address correspondence to: Bo Qiu, Department of Orthopedics, Renmin Hospital of Wuhan University, 99 Zhang Zhidong Road, Wuhan 430060, China. Tel: 027-8804191-82263; E-mail: qbtg163@163.com

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