Protective effect of controlled release of cytokine response modifier A from chitosan microspheres on rat chondrocytes from interleukin-1β induced inflammation and apoptosis

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Abstract. The aim of the present study was to investigate the protective effect of cytokine response modifier A (CrmA) released from chitosan (CS) microspheres in a controlled manner on interleukin (IL)-1β-induced inflammation and apoptosis in chondrocytes. The CrmA release kinetics were characterized by an initial burst release, which was reduced to a linear release over 8 days. Furthermore, chondrocytes were isolated from 1-week-old Sprague Dawley rats. The cell culture was established by stimulation with 10 ng/ml IL-1β and subsequent incubation with CS-CrmA microspheres. Following stimulation with IL-1β, the viability of chondrocytes was decreased. However, the cell viability was attenuated by CS-CrmA microspheres as revealed by a cell counting kit-8 assay. CS-CrmA microspheres significantly inhibited IL-1β-induced inflammation in chondrocytes by attenuating increases in the gene expression levels of inducible nitric oxide synthase and cyclooxygenase-2, as well as the concentrations of nitric oxide and prostaglandin E2. CS-CrmA microspheres significantly decreased the number of apoptotic chondrocytes induced by IL-1β as indicated by a terminal deoxyribonucleotide transferase deoxyuridine triphosphate nick-end labeling assay. In addition, CS-CrmA microspheres blocked IL-1β-induced chondrocyte apoptosis by increasing B-cell lymphoma 2 (Bcl-2) and decreasing Bcl-2-associated X protein, caspase-3 and poly adenosine diphosphate-ribose polymerase expression at the mRNA and protein levels, as indicated by reverse-transcription quantitative polymerase chain reaction and western blot analysis, respectively. The results of the present study revealed that CS-CrmA microspheres, as a controlled release system of CrmA, may protect rat chondrocytes from IL-1β-induced inflammation and apoptosis via regulating inflammatory and apoptosis-associated genes.

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Introduction

Osteoarthritis (OA) is an age-associated joint disease that is characterized by the degeneration of articular chondrocytes, leading to osteophyte formation and subchondral bone sclerosis and producing pain and loss of function (1). Chondrocytes are only one cell type, which are responsible for the maintenance of the extracellular matrix (2). Interleukin (IL)-1β has been reported to have important roles in OA and stimulation with IL-1β is able to upregulate the expression of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in chondrocytes, which promotes the production of nitric oxide (NO) and prostaglandin E2 (PGE_2) (3,4). Apoptosis is known to be important in the pathophysiological process of OA and IL-1β has been reported to accelerate aging and increase the apoptotic index of chondrocytes (5,6). Therefore, inhibition of the IL-1β pathway that activates inflammation and apoptosis is of great importance for cell apoptosis inhibition in chondrocytes during OA pathogenesis. One of the major endogenous inhibitors of the IL-1β pathway is the cytokine response modifier A (CrmA), which is able to bond with the IL-1β converting enzyme (ICE) as a pseudosubstrate (7). This serpin can prevent the proteolytic activation of IL-1β and subsequently block the cleavage of pro-IL-1β by ICE, thereby suppressing an IL-1β response to infection and decreasing the secretion of IL-1β (8,9).

Chitosan (CS) is obtained by deacetylation of its parent polymer chitin, a polysaccharide that is widely distributed in nature (10). It has been reported to be non-toxic and bioabsorbable, and is known to promote wound healing and contribute to the maintenance of the chondrogenic phenotype, particularly in its morphology (11-13). Additionally, CS was widely used to elaborate different nanocarriers attributed to the capacity of the polymer to interact with negatively charged cell surfaces (14,15). It has been widely used in drug, DNA delivery and tissue engineering due to its non-toxicity, biocompatibility and biodegradability (16-18). Furthermore, it promotes attachment, proliferation and viability of mesenchymal stem cells, and due to these features, CS and its derivatives are considered as promising biomaterials (19).

The present study attempted to use CS to develop CrmA-carrying microspheres and to investigate their effect against IL-1β-induced inflammation and apoptosis in rat chondrocytes by establishing an *in vitro* model of OA. The results indicated that CS-CrmA microspheres efficiently released CrmA to attenuate chondrocyte inflammation and apoptosis and may be suitable for the treatment of OA.

Materials and methods

Reagents. CS (molecular weight, 150 kDa; deacetylation, 98%) and sodium tripolyphosphate (STPP) were provided by Sigma-Aldrich. (Merck KGaA, Darmstadt, Germany). Recombinant rat IL-1β and CrmA were purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). Trypsinase, collagenase II, Dulbecco's modified Eagle's medium (DMEM)/F12, fetal bovine serum (FBS), 6-diamidino-2-phenylindole dihydrochloride (DAPI) and penicillin/streptomycin were all obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan; cat. no. CK04). An *in situ* cell apoptosis detection kit was purchased from Roche Diagnostics (Basel, Switzerland; cat no. 11684795910). Rabbit monoclonal antibodies for B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax; cat no. 14796) and β-actin (cat no. 3700), and rabbit polyclonal antibodies for Bcl-2 (cat no. 2876), caspase-3 (cat no. 9662) and polyadenosine diphosphate-ribose polymerase (PARP; cat no. 9542) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). All other reagents used in the present study were of the highest available commercial grade and obtained from Sigma-Aldrich (Merck KGaA) unless stated otherwise.

Microsphere preparation and characterization. CS-CrmA microspheres were prepared according to an emulsion ionic cross-linking method modified from previously described methods (20). Briefly, 2 g CS was dispersed into 100 ml acetic acid under vigorous stirring for 3 h at ambient temperature (<20˚C) to obtain transparent CS solution (2% w/v). Subsequently, microspheres were obtained via inotropic gelation between the positively charged amino groups of CS and the negatively charged amino groups of STPP and CrmA proteins. Under magnetic stirring with a thermostatic magnetic stirrer (MYP11-2; Shanghai Mei Yingpu Instrument Manufacturing Co., Ltd., Shanghai, China) at room temperature, 3.5 ml of an aqueous solution of STPP (0.06 mg/ml) and CrmA (0.05 mg/ml) was added to 3.5 ml CS solution [1%, w/v, (pH 5.0)]. The reaction mixture was continuously stirred at room temperature for 10 min for complete stabilization of the system. Subsequently, the microspheres were transferred into Eppendorf tubes and isolated by centrifugation in a glycerol bed at 16,000 x g at 25˚C for 30 min. The supernatant was collected and the microspheres were then resuspended in ultrapure water by shaking on a vortex mixer (Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China). Subsequently, the microspheres were centrifuged from the fixed volume of microsphere suspension under identical conditions without a glycerol bed. Following removal of the supernatant, CS-CrmA microspheres at the bottom of the vessel were collected. The CS microspheres were prepared using an identical method using a 0.9% NaCl solution to replace CrmA. Finally, the microspheres were fixed with 2% glutaraldehyde at room temperature for 30 min and freeze-dried, and then the Au-coated sizes and shapes of the microspheres were examined under a scanning electronic microscope (SEM; S-800; Hitachi, Ltd., Tokyo, Japan).

In vitro release profiles. Microspheres (~30 mg) containing CrmA were placed in 20 1.5-ml microcentrifuge tubes containing 1 ml phosphate buffered saline (PBS). The microsphere suspension of four microcentrifuge tubes containing CS-CrmA was agitated in a 37˚C water bath at 60 x g for various time periods of up to 10 days (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days) and the remaining 16 microcentrifuge tubes were stored at 4˚C until they were agitated in order to perform the same procedure. Periodically, the microsphere suspension was centrifuged at 10,000 x g at 37˚C for 15 min to harvest the supernatant for analysis of release CrmA following agitation, followed by resuspension of the microspheres in fresh PBS containing lysozyme (Thermo Fisher Scientific, Inc.; catalogue no. 89833). The CrmA concentration in the supernatant was assessed using an ELISA kit (cat no. CSB-EL004543RA; Cusabio Biotech Co., Ltd., Wuhan, China) according to the manufacturer's instructions.

Chondrocyte isolation and culture. A total of 20 7-day-old male Sprague-Dawley rats weighing ~50 g were obtained from the Experimental Animal Center of Wuhan University (Wuhan, China). All experimental animals used in the experiments were individually maintained under standard conditions of controlled temperature (22±1˚C), lighting (12-h light/dark cycle) and humidity (50±10%) with *ad libitum* access to food and water. Rats were euthanized by cervical dislocation following anesthesia with isoflurane (3 ml/kg; Shenzhen Reward Life Technology Co., Ltd., Shenzhen, China; catalogue no. R510-22; http://www.rwdmall.com/Shop/dongwumazui/mazuipeijian/mazuiji/186.html). The protocols for the animal experiment of the present study were in accordance with the recommendations and guidelines of the National Institutes of Health (Bethesda, MD, USA) and were approved by the Wuhan University Animal Care and Use Committee (Wuhan, China).

For the isolation of chondrocytes, cartilage was obtained from the knee joints of the rats and placed into PBS. Briefly, harvested cartilage was cut into small pieces, incubated in a 0.2% trypsin-containing solution and agitated at 80 x g for 2 h at 37˚C. Following digestion, the isolated chondrocytes were washed with DMEM and PBS twice, and then suspended in DMEM/F12 medium supplemented with 10% FBS and 1% penicillin/streptomycin antibiotics (1% v/v) at 37˚C in a humidified atmosphere with 5% CO_2. Cells were used at passage 0 or 1 to avoid dedifferentiation and maintained in a monolayer culture throughout the study. Additionally, cell viability was determined using a cell viability analyzer. Briefly, chondrocytes were seeded in 6-cm dishes at a density of $1x10^6$ cells/well with a total volume of 2 ml growth medium and cultured at 37˚C in a humidified atmosphere with 5% CO_2 to 80% confluence. After washing with PBS, the cells were detached with 0.125% EDTA-trypsin and collected. The cell numbers were detected by the Vi-CELL cell viability analyzer (viability, >90%; Beckman Coulter, Inc., Miami, FL, USA).

Treatment. First-generation rat chondrocytes were maintained in a monolayer culture throughout the present study. After the cells were cultured in DMEM/F12 containing 10% FBS and 1% penicillin/streptomycin and reached 70-80% confluence, the culture medium was removed and the cells were cultured in DMEM/F12 containing 2% FBS and 1% antibiotics at 37˚C for 24 h after washing three times with PBS to avoid the influence of other cytokines. At the end of incubation, the FBS medium was removed, and the cells were incubated at 37˚C with serum-free medium for 1 h before the onset of experimental treatments. The culture medium was replaced with DMEM/F12 supplemented with 10% FBS. Subsequently, IL-1β (10 ng/ml) was added to the culture medium followed by incubation at 37˚C for an additional 48 h. Finally, chondrocytes were co-cultured with CS and CS-CrmA microspheres for a period of 4 h. A negative control group was kept untreated except for replacement of the medium. A positive control group consisted of cells treated with 10 ng/ml IL-1β alone. Each group consisted of five independent samples from different rats, and each experiment was repeated 10 times.

Cell viability assay. The effect of microspheres on the cell viability of chondrocytes was assessed using a CCK-8 assay kit in accordance with the manufacturer's protocol. Chondrocytes were cultured at 37˚C in 96-well plates at a density of $1x10^4$ cells/well in a total volume of 200 μl growth medium (DMEM-F12 containing 2% FBS). Following 48 h of co-culture with IL-1β (10 ng/ml) and then 4 h of co-culture with CS and CS-CrmA microspheres, the culture medium was discarded and chondrocytes were washed twice with PBS. Next, 10 μl CCK-8 reagent was added to each well and incubated at 37˚C for 2 h. The WST-8 Tetrazolium salt in the reagent may be reduced to orange-yellow formazan by dehydrogenase, which is proportional to the number of viable cells. Furthermore, the absorbance at 450 nm was recorded using a microplate reader (Bio-Rad 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA). A standard curve was designed using chondrocyte suspension with different dilution rates to calculate the viable cell numbers in each sample.

Quantification of NO. Nitrite levels in culture medium were assessed using Griess reaction, as previously described (21). To measure nitrite levels in the medium, 50 μl sample aliquots were mixed with 50 μl Griess reagent (catalogue no. G4410; Sigma-Aldrich; Merck KGaA) and then incubated at 37˚C for 10 min. After incubation, the absorbance was spectrophotometrically determined at the wavelength of 550 nm using a microplate reader (Bio-Rad 680; Bio-Rad Laboratories, Inc.). Nitrite concentrations were determined relative to a standard curve derived from increasing concentrations of sodium nitrite. Finally, the results were expressed as nmol/ml and were normalized against the control concentration.

Assay of PGE_2 concentrations. PGE_2 levels were investigated using a commercially available ELISA kit (cat no. KGE004B; R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. Furthermore, PGE_2 concentrations were determined relative to a standard curve, and the results were expressed as ng/ml and normalized against the control concentration.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. An *in situ* cell apoptosis detection kit (catalogue no. 40306ES50; Shanghai Yeasen Biotechnology Co., Ltd., Shanghai, China) was used to detect cell apoptosis according to the manufacturer's instructions. Chondrocytes were seeded on cover slips in 24-well plates at a density of $1.25x10^5$ cells/well in a total volume of 1 ml growth medium (DMEM-F12 containing 2% FBS). After implementing the above-described experimental design, the culture medium was removed from each well, and the chondrocytes were washed with PBS. Chondrocytes were stained with DAPI at 37˚C for 30 min. Additionally, apoptotic chondrocytes were recognized with dual TUNEL and DAPI staining. After the final wash with PBS, cells were observed under an inverted fluorescence microscope, and apoptosis signals were counted manually. Furthermore, images were randomly selected from three fields of each specimen and the stained cells were counted under x200 magnification. A control group was kept untreated with the exception of replacing the medium without IL-1β, CS and CS-CrmA microspheres. The rate of TUNEL-positive cells in each field was calculated.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol and chloroform reagents (Sigma-Aldrich, Merck KGaA) were used to extract the total RNA from chondrocytes according to the manufacturer's instructions. Briefly, 2 ml TRIzol reagent was added to split the chondrocytes for 20 min, and the sample was then transferred into a 2 ml Eppendorf tube. Subsequently, 400 μl chloroform was added. The tube was vigorously agitated for 30 sec and allowed to stand for 15 min. The sample was then centrifuged at 13,000 x g for 15 min at 4˚C. The supernatant from the final extraction step was transferred to a clean 2 ml Eppendorf tube and the RNA precipitated with 500 μl isopropanol at -20˚C for 2 h. Precipitated RNA was collected by centrifugation at 13,000 x g for 15 min at 4˚C, and the pellet was washed with 1 ml 75% ice-cold ethanol. The RNA pellet was then resuspended in 20 μl nuclease-free water and the two duplicate tubes were combined. The RNA concentration was determined using a spectrophotometer (Biolab ND-1000; Thermo Fisher Scientific, Inc.) at 260 nm, and the purity was assessed by measuring the A260/A280 ratio. Purified RNA with an A260/A280 ratio between 1.8 and 2.0 was used in the present study.

cDNA was synthesized from RNA using a PrimeScript reverse transcriptase kit (cat. no. AB-1455/A; Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The total extracted RNA was diluted to the final concentration of 1 μg/μl. RT-qPCR was performed using a 20 μl reaction volume containing 10 μl SYBRpremix Ex Taq II (Takara Bio, Inc., Otsu, Japan), 1 μl RNase-free DNase I (catalogue no. EN0521; Fermentas; Thermo Fisher Scientific, Inc.), 0.4 μl ROX Reference Dye II, 0.8 μl each forward and reverse primer, 2 μl cDNA and 6 μl nuclease-free water. Reactions were run on a 7500 Real Time PCR system (Thermo Fisher Scientific, Inc.) for 45 consisting of the following steps: Initial denaturation at 95˚C for 5 min, followed by a set cycle of denaturation at 94˚C for 10 sec, and different annealing temperatures for each pair of primers (ranging between 53 and 62˚C) for 10 sec, extension at 72˚C for 28 sec, and a

Table I. Sequences of primers for the reverse transcription quantitative polymerase chain reaction experiments.

Gene	Direction	Sequence 5'→3'	Size (bp)
iNOS	F	ACCAGTACGTTTGGCAATGG	70
	R	TCAGCATGAAGAGCGATTTCT	
COX-2	F	CTTACAATGCTGACTATGGCTAC	242
	R	AAACTGATGCGTGAAGTGCTG	
Bcl-2	F	CCACCAAGAAAGCAGGAAACC	177
	R	GGCAGGATAGCAGCACAGG	
Bax	F	CAGATGTGGTCTATAATGC	110
	R	CTAATCAAGTCAAGGTCAC	
Caspase-3	F	CATGGAAGCGAATCAATGGACT	139
	R	CTGTACCAGACCGAGATGTCA	
PARP	F	TCTTTGATGTGGAAAGTATGAAGAA	64
	R	GGCATCTTCTGAAGGTCGAT	
β-actin	F	CGTTGACATCCGTAAAGAC	201
	R	TGGAAGGTGGACAGTGAG	

iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; PARP, poly ADP ribose polymerase; F, forward; R, reverse.

final elongation at 72˚C for 5 min. The generation of specific PCR products were subjected to melting curve analysis, and gene expression was normalized to the expression of the housekeeping gene, β-actin, and expressed as the fold ratio compared with the control according to previously described method by Cheng *et al* (2). Sequences of primers used (Takara Bio, Inc.) are presented in Table I.

Western blot analysis. Protein was extracted from harvested chondrocytes using a cell extraction buffer (cat no. FNN0011; Fermentas, Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The protein concentrations were determined using the bicinchoninic acid protein assay kit (cat no. 23225; Fermentas, Thermo Fisher Scientific, Inc.). After chondrocytes were treated according to the experimental design, the culture medium was discarded and chondrocytes were washed twice with precooled PBS. The chondrocytes were then collected using a cell scraper and transferred into a 1.5 ml Eppendorf tube, a cell extraction buffer (catalogue no. FNN0011; Fermentas, Thermo Fisher Scientific, Inc.) containing protease and phosphatase inhibitors (catalogue no. 78440; Fermentas, Thermo Fisher Scientific, Inc.) was added and placed on ice for 30 min. The lysate was collected and centrifuged at 12,000 x g for 20 min at 4˚C. Each well in the 10% SDS-PAGE was loaded with 20 μl protein. After adjusting for equal amounts of total protein, protein mixtures were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Fermentas, Thermo Fisher Scientific, Inc.). Following the transfer, non-specific binding sites of the membranes were blocked for 1 h at room temperature in PBS (pH 7.4) containing 5% non-fat dry milk, and then incubated overnight at 4˚C with the following antibodies (all dilutions, 1:1,000): anti-Bcl-2, anti-Bax, anti-caspase-3 and anti-PARP. Anti-Bcl-2 did not cross-react with Bcl-2β or other Bcl-2 family members, Bax recognized endogenous levels of total Bax protein in rodent samples, caspase-3 detected endogenous levels of caspase-3 resulting from cleavage and PARP did not cross-react with related proteins or other PARP isoforms.

In order to control protein loading, the membranes were probed with an anti-β-actin antibody (dilution, 1:1,000; catalogue no. 3700; Cell Signaling Technology, Inc.). Subsequently, the membranes were washed three times with TBST [10 mM Tris-HCl, (pH 7.4), 100 mM NaCl and 0.2% Tween-20] and incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit immunoglobulin G; catalogue no. 7075; dilution, 1:1,000; Cell Signaling Technology, Inc.), followed by visualization using the enhanced chemiluminescence kit (cat. no. 35085; Fermentas; Thermo Fisher Scientific, Inc.). The results were scanned using a gel imaging system (Geldoc-It 310; UVP, LLC., Upland, CA, USA), and densitometry measurements were performed with Image Lab version 4.1 software (Bio-Rad Laboratories, Inc.).

Statistical analysis. Values were expressed as the mean ± standard deviation and statistical analyses were performed using SPSS software, version 19.0 (IBM Corp., Armonk, NY, USA). Each experimental condition was performed in triplicate wells, and the mean from each culture replicate was calculated and combined as one value for analysis. Significant differences among the mean values of multiple groups were evaluated with analysis of variance followed by Student-Newman-Keuls' method. A two-sided $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characterization of microspheres. Microsphere morphology was observed via SEM (Fig. 1). The CS-CrmA microspheres, which were fabricated using the emulsification method in the

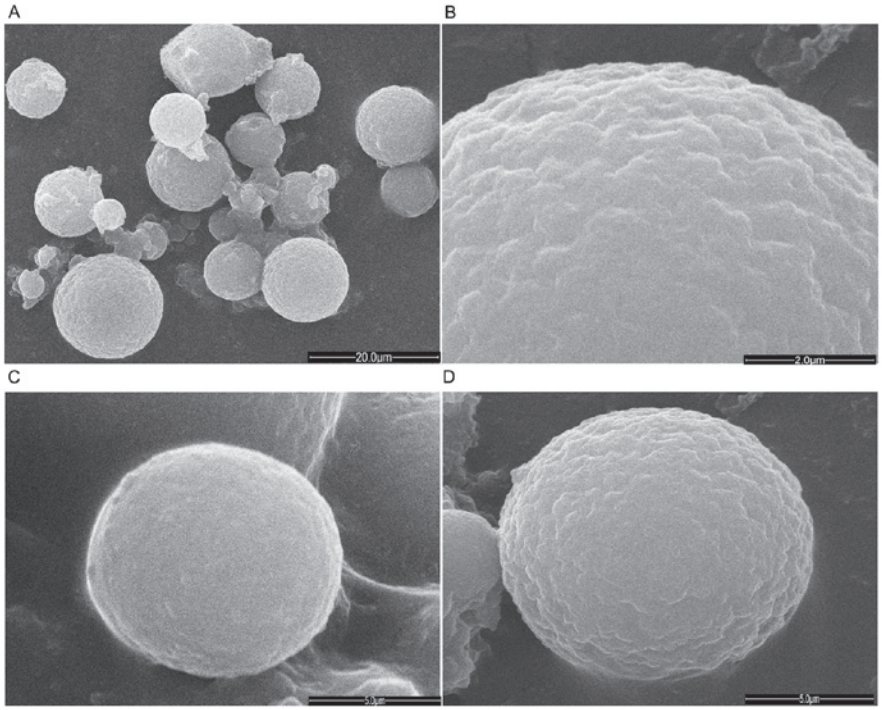

Figure 1. Characterization of microspheres by scanning electron microscopy. (A) The microspheres were uniformly distributed and ranged in size from 8-17 μm (magnification, x2,000). (B) The microspheres were spherical and the surfaces were almost smooth (magnification, x2,000). With regard to the variation in composition and structure, there was a slight decrease in the microsphere size in (C) chitosan (magnification, x12,000) compared with (D) chitosan-cytokine response modifier A microspheres (magnification x12,000).

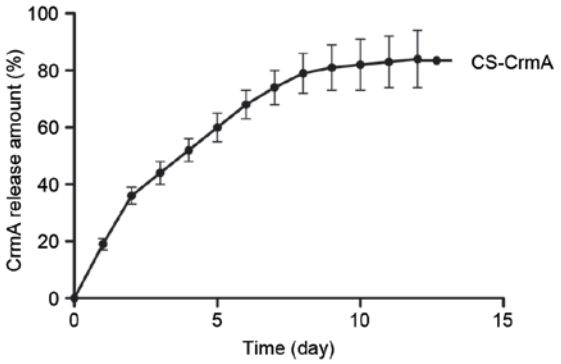

Figure 2. Release kinetics of CrmA microspheres. Values are expressed as the mean ± standard deviation. CS, chitosan; CrmA, cytokine response modifier A.

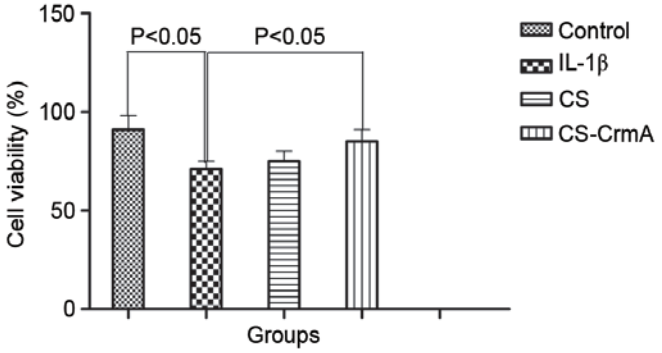

Figure 3. CS-mediated release of CrmA attenuates IL-1β-induced reduction of chondrocyte viability. Values are expressed as the mean ± standard deviation. IL-1β, interleukin-1β; CS, chitosan; CrmA, cytokine response modifier A.

presence of STPP, were spherical in shape with smooth surfaces. The resulting microspheres were spherical and ranged in size from 8-17 μm (Fig. 1A). Furthermore, the microsphere surface appeared to be porous and relatively smooth (Fig. 1B). With regard to the variation in composition and structure, a slight decrease in microsphere size was observed in CS (Fig. 1C) compared with CS-CrmA microspheres (Fig. 1D).

In vitro release profiles. The release kinetics of CrmA from CS-CrmA microspheres are presented in Fig. 2. The CrmA release kinetics were monitored for 10 days and characterized by an initial burst release, which was gradually reduced to a linear release. The CrmA from CS-CrmA microspheres was released slowly. The final release rate was ~82% in the CS-CrmA microspheres at 10 days of incubation.

CrmA-releasing microspheres attenuated IL-1β-mediated reduction in cell viability. Cell viability in the various experimental groups is presented in Fig. 3. The cell viability of the IL-1β group was 71±4%, which was significantly lower than 91±7% of the control group (P<0.05). Slight but not marked differences in cell viability were also observed between the IL-1β (71±4%) and CS (75±5%) groups. However, co-treatment with CS-CrmA significantly increased the cell viability to 85±6% (P<0.05 vs. IL-1β).

CrmA-releasing microspheres attenuate IL-1β-induced gene expression of iNOS and COX-2 and release of NO and PGE_2 by chondrocytes. The gene expression of iNOS and COX-2 and the concentrations of NO and PGE_2 in the various experimental groups are depicted in Fig. 4A-D. Stimulation

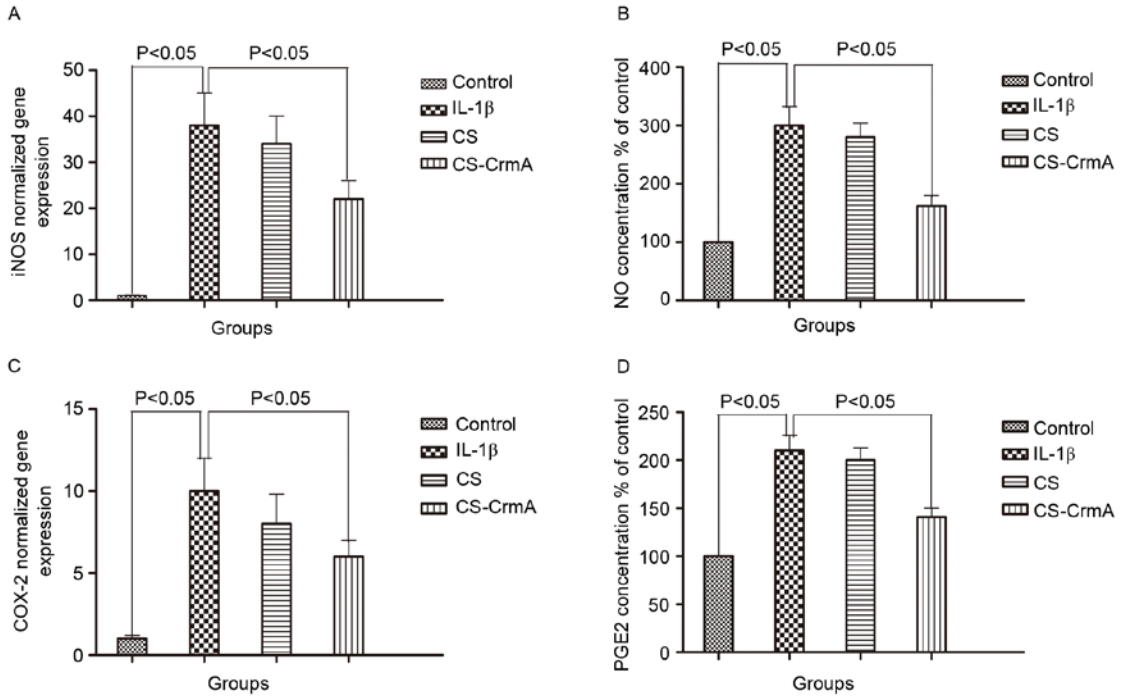

Figure 4. Effect of microspheres on IL-1β-induced gene expression of (A) iNOS and (B) COX-2 and the production of (C) NO and (D) PGE_2. The normalized levels of gene expression were expressed as the ratios of the copy number of the mRNA of the targeted genes and of β-actin cDNA. Culture media were analyzed for nitrite concentration on behalf of NO production. Values were expressed as the mean ± standard deviation. iNOS, inducible nitric oxide synthase; IL-1β, interleukin-1β; CS, chitosan; CrmA, cytokine response modifier A; NO, nitric oxide; COX-2, cyclooxygenase-2; PGE_2, prostaglandin E2.

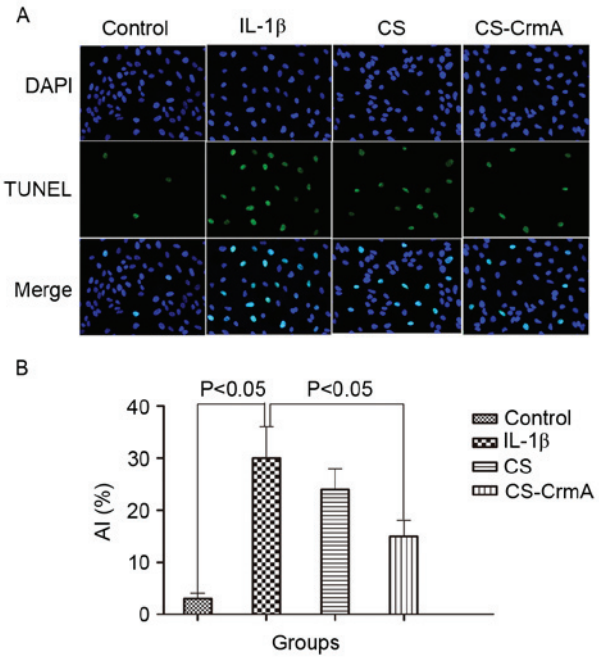

Figure 5. CS-mediated release of CrmA reduces IL-1β-induced chondrocyte apoptosis. (A) Apoptosis in various treatment groups was assessed by TUNEL staining and fluorescence microscopic analysis (magnification, x200). Nuclei were counter-stained with DAPI. (B) AIs were obtained by quantification of apoptosis. Values were expressed as the mean ± standard deviation. DAPI, 4',6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; IL-1β, interleukin-1β; CS, chitosan; CrmA, cytokine response modifier A; AI, apoptotic index.

with IL-1β (10 ng/ml) for 48 h led to a 38-fold increase in iNOS expression and a 3.0-fold increase in NO production in the supernatant, compared with controls (both P<0.05). Furthermore, CS microspheres slightly but not markedly decreased this iNOS expression and NO production, whereas CS-CrmA microspheres significantly decreased the iNOS expression and NO production, compared with the IL-1β group (both P<0.05). Similar patterns were observed for the COX-2 expression and PGE_2 concentration in the supernatant.

CrmA-releasing microspheres reduce IL-1β-induced chondrocyte apoptosis. TUNEL staining of chondrocytes in the different treatment groups for the detection of apoptosis and apoptotic index is shown in Fig. 5. The percentage of TUNEL-positive cells in the IL-1β group was 30±6%, whereas that in the untreated control group was significantly lower at only 3±1% (P<0.05). Compared with the IL-1β group, co-treatment with CS slightly but not markedly decreased the apoptotic rate of the chondrocytes (24±4 vs. 30±6%). However, following co-culture with CS-CrmA microspheres, the percentage of TUNEL-positive cells was 15±3%, which was significantly lower than that in the IL-1β group (P<0.05).

CrmA-releasing microspheres attenuate IL-1β-induced apoptosis signaling. The gene expressions of Bcl-2, Bax, caspase-3 and PARP in various experimental groups are presented in Fig. 6A-D, respectively. Stimulation with IL-1β (10 ng/ml) led to a significant decrease of Bcl-2 expression and a significant increase of Bax, caspase-3 and PARP expression compared with those in the control group (all P<0.05). Slight but not marked differences in the gene expression of Bcl-2, Bax, caspase-3 and PARP were observed between the IL-1β and CS groups (P>0.05). However, the expression of Bcl-2 was significantly increased following co-treatment with

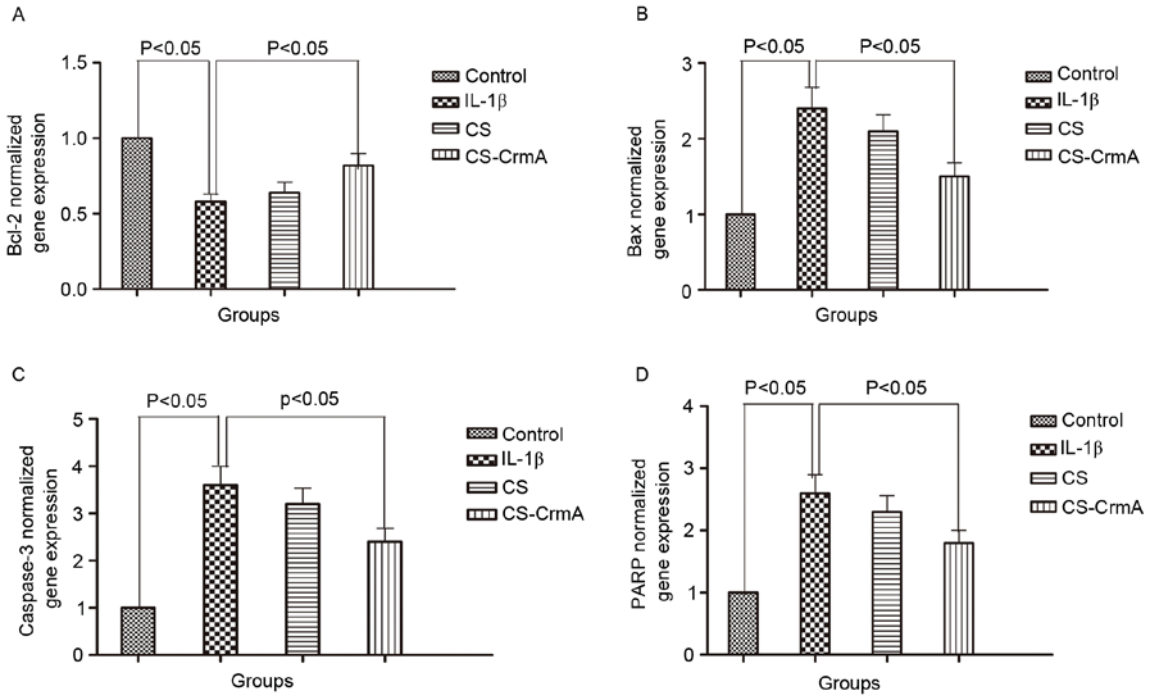

Figure 6. Effect of microspheres on the relative expression of (A) Bcl-2, (B) Bax, (C) caspase-3 and (D) PARP mRNA. The normalized gene expression levels were expressed as ratios of the copy number of the mRNA of the targeted genes and that of β-actin cDNA. Values were expressed as the mean ± standard deviation. Bcl-2, B-cell lymphoma 2; IL-1β, interleukin-1β; CS, chitosan; CrmA, cytokine response modifier A; Bax, Bcl-2-associated X protein; PARP, poly adenosine diphosphate-ribose polymerase.

CS-CrmA microspheres (P<0.05), whereas the expressions of Bax, caspase-3 and PARP were significantly decreased (all P<0.05) following co-treatment with CS-CrmA microspheres compared with those in the IL-1β group.

The protein levels and relative intensities of Bcl-2, Bax, caspase-3 and PARP in the experimental groups are presented in Fig. 7A-D, respectively. The results revealed similar trends to the mRNA levels of the respective proteins. Compared with the control group, the gene expression of Bcl-2 was significantly reduced by IL-1β, whereas the gene expressions of Bax, caspase-3 and PARP were significantly increased by IL-1β (all P<0.05). Slight but not marked differences were observed in the expression of Bcl-2, Bax, caspase-3 and PARP between the IL-1β and CS groups. However, the expression of Bcl-2 was significantly increased in the CS-CrmA group compared with that in the IL-1β group (P<0.05), whereas the expressions of Bax, caspase-3 and PARP were significantly decreased (all P<0.05).

Discussion

OA is a degenerative disease that is characterized by the progressive loss of articular cartilage and destruction of cartilage matrix (22). It is widely accepted that IL-1β is important in promoting OA lesions by leading to the production and accumulation of high levels of pro-inflammatory cytokines and by triggering apoptosis in chondrocytes (22). Therefore, using CrmA to inhibit the IL-1β pathway may prevent inflammation and apoptosis in chondrocytes.

In the present study, it was observed that CS was able to enhance the ability of the microspheres to interact with the chondrocytes and the controlled-release of the loaded drug. Additionally, the release rate of CrmA from the CS-CrmA microspheres was slow. These results were further confirmed by the microsphere structure and release kinetics of proteins from the microspheres.

In the present study, CS slightly but not markedly increased the cell viability of chondrocytes. In a previous study, administration of CS solution into the murine knee joint led to a significant increase in the density of newly formed chondrocytes, which indicated that it was able to facilitate the healing of the cartilage (19). Furthermore, it was revealed that the percentage of viable cells in the CS-CrmA group was significantly higher than that in the IL-1β group, a finding that was in accordance with that of a recent study by the present authors (7).

It has been demonstrated that iNOS is able to catalyze oxidation to generate amounts of NO within the joint fluid of OA chondrocytes, leading to cartilage destruction (23). COX-2 is a rate-limiting enzyme for the generation of PGE_2 metabolites, which are important mediators of inflammation and the anabolic/catabolic process associated with OA (24). In the present study, CS slightly but not markedly decreased the gene expression of iNOS and COX-2 and the production of NO and PGE_2. However, CS-CrmA significantly inhibited the gene expression of iNOS and COX-2 and the production of NO and PGE_2. These observations indicate that IL-1β-induced uncontrolled inflammation in chondrocytes may be attenuated by CS-CrmA microspheres. Therefore, it is suggested that CS-CrmA microspheres may act as anti-inflammatory agents similar to NSAIDs, which have been indicated to ameliorate OA symptoms by inhibiting the production of PGE_2. However, it is likely that the control-released action had a longer duration in the presence of CS. This may have been due to the

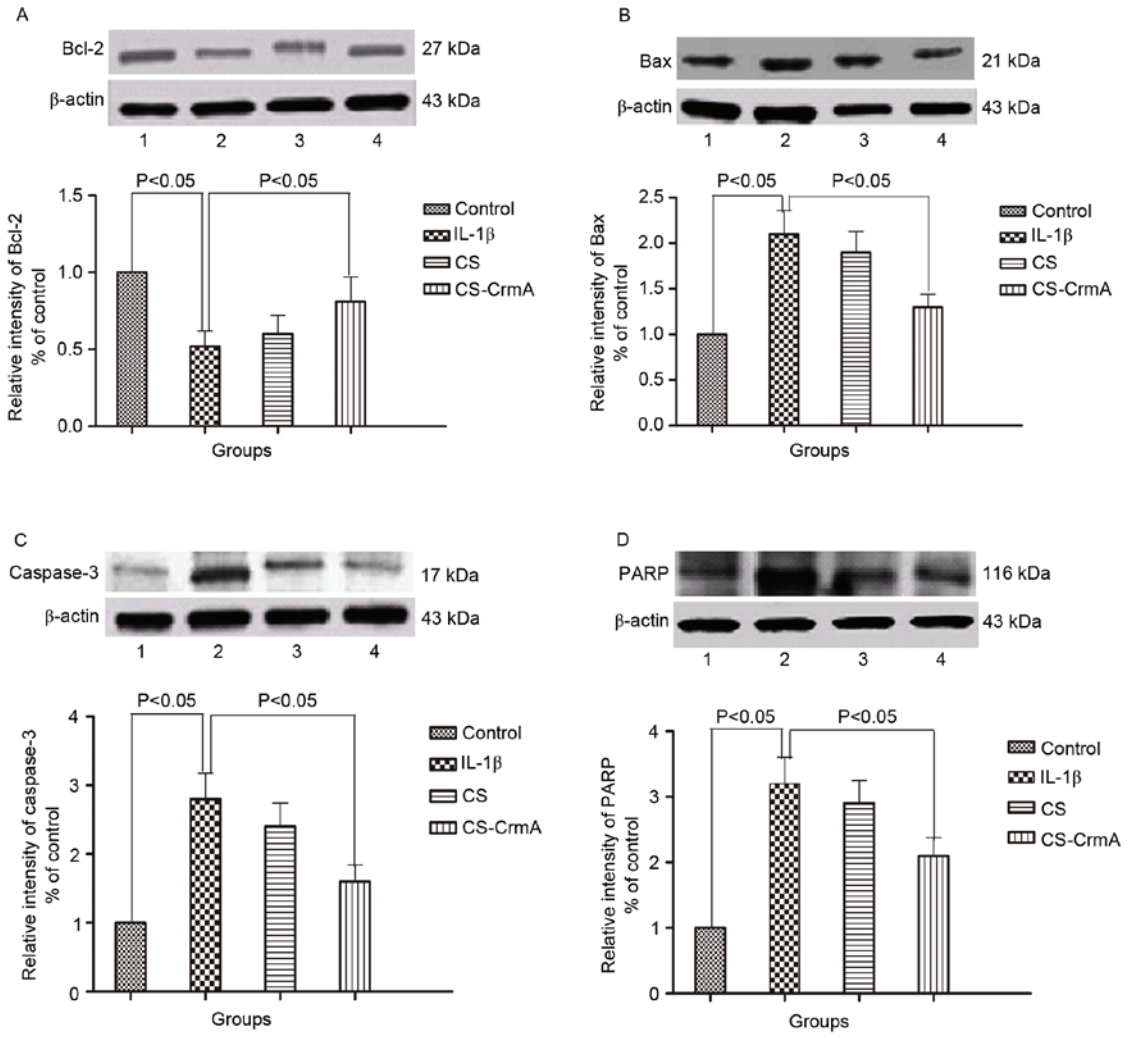

Figure 7. Western blot analysis and relative intensities of (A) Bcl-2, (B) Bax, (C) caspase-3 and (D) PARP. Lanes: 1, control; 2, IL-1β; 3, CS; 4, CS-CrmA. Protein levels were normalized to β-actin. Values were expressed as the mean ± standard deviation. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; PARP, poly adenosine diphosphate-ribose polymerase; IL-1β, interleukin-1β; CS, chitosan; CrmA, cytokine response modifier A.

capacity of the polymer to interact with the negatively charged cell surfaces.

Apoptosis is a highly-regulated, active process of cell death that is associated with the development of human and animal OA (25). In the present study, it was revealed that CS slightly but not markedly decreased the percentage of apoptotic chondrocytes. Additionally, the percentage of apoptotic cells was significantly decreased in the CS-CrmA group. It can be inferred that CS-CrmA microspheres are able to suppress chondrocyte apoptosis in an IL-1β-induced OA model.

Bcl-2 family proteins can be readily heterodimerized by protein-protein interactions between pro- and anti-apoptotic Bcl-2 family members, which determine whether cell survival or the apoptosis signal proceeds (26,27). The activation of caspase-3 is the most important pathway during the apoptotic process, which then induces hydrolysis of nucleic acids and cytoskeletal proteins (28,29). During apoptotic cell death, activated effector caspases cleave multiple cellular substrates, including the DNA repair enzyme PARP in chondrocytes (30). The present study demonstrated that CS slightly but not markedly increased the expression of Bcl-2 and decreased the expressions of Bax, caspase-3 and PARP in transcriptional and translational levels than those in the IL-1β group. Nevertheless, the CS-CrmA microspheres had significantly increased the expression of Bcl-2 and decreased the expressions of Bax, caspase-3 and PARP in the transcriptional and translational levels than compared with those in the IL-1β group. These results further confirmed the previous results of cell viability and apoptosis assay.

In conclusion, the suppression of inflammation and apoptosis within the joint may also be one important mechanism of the clinical activity of microspheres in OA treatment. Therefore, the present results suggest that the sustained release of CrmA from CS-CrmA microspheres may prevent IL-1β-induced inflammation and apoptosis in chondrocytes. This suggests that the use of CS-CrmA microspheres as CrmA carriers with controlled and prolonged release properties for the treatment of OA is promising.

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