Carboxymethyl Chitosan Promotes Migration and Inhibits Lipopolysaccharide-Induced Inflammatory Response in Canine Bone Marrow-Derived Mesenchymal Stem Cells

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Abstract The study was conducted to evaluate the effects of carboxymethyl chitosan (CMC) on proliferation, migration, and lipopolysaccharide (LPS)-induced inflammatory response in canine bone marrow-derived mesenchymal stem cells (BMSCs). The proliferation and migration of BMSCs were examined after treatment with CMC. The effect of CMC on the mRNA expression of inflammatory cytokines, such as interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α , IL-10, and transforming growth factor (TGF)- β , was also evaluated by reverse transcription polymerase chain reaction (RT-PCR). In the proliferation assay, no significant changes were found at all CMC concentrations compared with controls. The migration assay showed that CMC dose-dependently stimulated the migration of BMSCs in normal and LPS-treated conditions. RT-PCR showed that TNF- α and IL-10 expressions were suppressed in the BMSCs after CMC treatment. However, other genes were not affected. Taken together, CMC promoted BMSC migration and inhibited TNF- α and IL-10. Therefore, CMC may be possible to regulate wound healing when mesenchymal stem cells are applied in inflammatory diseases.

Key words chitosan, migration, inflammatory response, stem cells.

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Introduction

Mesenchymal stem cells (MSCs) have the ability to self-renew and exhibit multilineage differentiation. Due to these features, bone marrow-derived MSCs (BMSCs) can differentiate into osteoblasts, chondrocytes, adipocyte, and connective tissue (9,28). Previous studies have shown that BMSCs have a beneficial effect in vitro in bone tissue regeneration (17,18).

Recently, BMSCs have been used with a biodegradable scaffold, allowing cells to attach and proliferate (1,22). In previous studies, biomaterials, such as chitosan, have been used with MSCs to evaluate its potential applications in bone tissue engineering (3,11,24,27).

Chitosan is insoluble at neutral and alkaline conditions, but carboxymethyl chitosan (CMC) demonstrates a higher hydrophilicity and better degradation rate because of the carboxymethyl group. The range of properties of CMC has already been demonstrated, including its biocompatibility; biodegradability; nontoxicity; water retention; and anti-inflammatory, antitumor, and antifungal effects (6,8,13,15,16,19,20,25). For these reasons, CMC has been widely used for biomedical applications, such as tissue engineering scaffolds, wound dressings, and antibacterial coatings (13).

This study aimed to investigate the effects of CMC on proliferation, migration, and lipopolysaccharide (LPS)-induced inflammatory response in canine BMSCs.

Materials and Methods

Bone marrow-derived mesenchymal stem cell culture

Canine bone marrow-derived mesenchymal stem cells (BMSCs) were provided by the Animal and Plant Quarantine Agency (Korea). The general procedure has been described here subsequently. The bone marrow was rinsed thrice with phosphate-buffered saline (PBS) and then centrifuged at 1200 rpm for 5 min. The supernatant was discarded. Further, low-glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) with 10% fetal bovine serum (FBS) (Gibco), 100 IU/mL penicillin, and 100 µg/mL streptomycin was added and used as a culture medium. The cells were incubated for 24 h at 37°C and 5.0% CO₂. Flow cytometry analysis with established BMSCs was performed, and obtained BMSCs were negative for CD34 (1.0%) and CD45 (0.7%) and positive for CD29 (98.0%) and CD44 (94.5%). All assays were performed using passage 3 of canine BMSCs.

Preparation of carboxymethyl chitosan

CMC was provided by Chembio Co. (Chitopol[®], Chembio Co., Korea). The powder was mixed with distilled water, and the suspension was heated in a heating plate at 100°C for 10 min to obtain 1% (w/v) aqueous solution. After cooling, the obtained aqueous solution was filtered with a 100 µL nylon mesh.

Proliferation assay

Canine BMSCs were seeded at a density of 1.0×10^3 cells/well in 96-well culture plates and maintained with a serum-free medium for 24 h. The culture medium was changed to a serum-free medium added with control, 0.0001% CMC, 0.001% CMC, 0.01% CMC, or 0.1% CMC. The cells were cultured for 18 h. Proliferation assay was performed using the cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Gaithersburg, MD, USA) according to the manufacturer's protocol. The optical density was measured at 450 nm using a microplate spectrophotometer (Epoch, Biotek Instruments, USA). A second assay was performed under the same conditions but with added 100 ng/mL LPS stimulation.

Migration assay

To assess the migratory capacity of canine BMSCs, 70 µL (5.0×10^5 cells/mL) were seeded onto culture inserts (Ibidi, GmbH, Germany). The bottoms of the dish films were removed carefully 24 h after cell seeding. The cells were washed with PBS and incubated for 24 h with the following treatments: DMEM, 0.0001% CMC, 0.001% CMC, 0.01% CMC, and 0.1% CMC. To quantify the area size occupied by migratory cells, phase-contrast photographs of the center of the created gap were captured pretreatment and posttreatment with a digital camera coupled to an inverted microscope (CKX41; Olympus, Japan). The gap size was calculated, and the area occupied by migratory cells was measured using ImageJ software (National Institutes of Health, Maryland, USA). The results are shown as the area of migration (%) 18 h after treatments. A second assay was performed under the same conditions but with 100 ng/mL LPS stimulation.

Reverse transcription polymerase chain reaction analysis

BMSCs were seeded and grown until confluence with DMEM containing 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Subsequently, cells were maintained in a serum-free medium for 24 h. In the first assay, 0.1% CMC was added for comparison with the control group. Secondly, 100 ng/mL LPS was added to stimulate inflammation in both

the control and 0.1% CMC groups. The cells were harvested, and total RNA was isolated using the TRIzol[®] Reagent (Life Technologies, USA) according to the manufacturer's instructions. The expression profiles of interleukin (IL) 6 and tumor necrosis factor (TNF) α , transforming growth factor (TGF) β , IL-1 β , and IL-10 were examined. For PCR amplification, the following conditions were used: 94°C for 30 s (denaturation), 55°C for 1 min (annealing), and 72°C for 1 min (extension) for one cycle and 72°C for 1 min for 30 cycles. The amplified PCR products were separated with 2% agarose gel and then stained with ethidium bromide. The sequences of primers used for RT-PCR are shown in Table 1.

The relative mRNA expression was examined in canine BMSCs assessed by reverse transcriptase polymerase chain reaction after 24 h treatment (control and 0.1% CMC) under normal conditions as well as after 24 h treatment with 100 ng/mL LPS and LPS + 0.1% CMC.

Statistical analysis

All data are expressed as mean $\pm$ standard deviation. For the statistical calculation of significant differences between groups, non-parametric Mann–Whitney U tests were used using IBM SPSS Statistics Version 22 (IBM SPSS Inc., Chicago, IL, USA). *p*-values < 0.05 were considered statistically significant.

Results

Proliferation assay

The CCK assay showed that the cell proliferation of canine BMSCs was slightly increased by CMC treatment up to 18 h

(Fig. 1A). However, no significant changes in BMSC growth was observed at all CMC concentrations compared with the control group under LPS treatment (Fig. 1B).

Migration assay

The culture-insert assay demonstrated that CMC dose-dependently promoted and significantly increased cell migration at 0.1% CMC concentration under both normal and LPS conditions (*p* < 0.05) (Fig. 2A, B). When compared with the control group, CMC stimulated the migration of canine BMSCs into cell free area in a dose dependent manner in normal condition (Fig. 2C) and in LPS induced condition (Fig. 2D).

Fig. 1. Effect of carboxymethyl chitosan (CMC) on the proliferation of canine bone marrow-derived stem cells (BMSCs) under normal and lipopolysaccharide (LPS)-induced conditions. Canine BMSCs were exposed to control, 0.0001% CMC, 0.001% CMC, 0.01% CMC, and 0.1% CMC for 18 h (A). Canine BMSCs were exposed to control, 0.0001% CMC, 0.001% CMC, 0.01% CMC, and 0.1% CMC for 18 h under 100 ng/mL LPS stimulation (B).

Table 1. Sequences of PCR primers

Gene	Primer	Sequence
GAPDH	Forward	TCC CCA CCC CCA ATG TAT C
	Reverse	TGC CTG CTT CAC TAC CTT CTT G
IL-1 β	Forward	GTG ATG CAG CCA TGC AAT CG
	Reverse	TGG AGA GCC CGA AGC TCA TA
IL-6	Forward	CTC CTG ACC CAA CCA CAG AC
	Reverse	GTG TGC TTC ACG CAC TCA TC
IL-10	Forward	CAA GCC CTG TCG GAG ATG AT
	Reverse	CTT GAT GTC TGG GTC GTG GTT
TNF- α	Forward	GAG CCG ACG TGC CAA TG
	Reverse	CAA CCC ATC TGA CGG CAC TA
TGF- β	Forward	CAG AAT GGC TGT CCT TTG ATG TC
	Reverse	AGG CGA AAG CCC TCG ACT T

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; IL-10, interleukin-10; TNF- α , tumor necrosis factor- α ; TGF- β , transforming growth factor- β .

Fig. 2. Effect of carboxymethyl chitosan (CMC) on the migration of canine bone marrow-derived stem cells (BMSCs) under normal and lipopolysaccharide (LPS)-induced conditions. Canine BMSC migration was compared under exposure to control, 0.0001% CMC, 0.001% CMC, 0.01% CMC, and 0.1% CMC (A). Canine BMSC migration was compared under exposure to control, 0.0001% CMC, 0.001% CMC, 0.01% CMC, and 0.1% CMC under 100 ng/mL LPS stimulation (B). Phase-contrast microscopic images of canine BMSCs exposed to control (a), 0.0001% CMC (b), 0.001% CMC (c), 0.01% CMC (d), and 0.1% CMC (e) for 18 h of treatment under normal conditions (C). Phase-contrast microscopic images of canine BMSCs exposed to control (a), 0.0001% CMC (b), 0.001% CMC (c), 0.01% CMC (d), and 0.1% CMC (e) for 18 h of treatment under 100 ng/mL LPS-induced inflammatory conditions (D). * $p < 0.05$ compared with the control.

RT-PCR assay of inflammatory cytokines

To examine the effect of CMC on the mRNA expression of inflammatory cytokines in BMSCs, RT-PCR was performed to examine the expressions of IL-1 β , IL-6, TNF- α , IL-10, and TGF- β under normal and inflammatory conditions.

First, IL-1 β expression was slightly upregulated, whereas IL-6 was not affected by the CMC treatment. Furthermore, CMC significantly suppressed TNF- α and IL-10 expression under normal conditions ($p < 0.05$, Fig. 3).

Under inflammatory conditions, the CMC-treated groups showed higher expressions of IL-1 β and IL-6 mRNA compared with the LPS-treated groups. However, the mRNA expressions of TNF- α and IL-10 were significantly suppressed by CMC treatment under LPS-induced inflammatory conditions ($p < 0.05$; Fig. 4).

DISCUSSION

This study aimed to examine the effect of wound healing for inflammatory disease with BMSCs and CMC. The demand for new treatments for diseases affecting musculoskeletal tissues is continuously increasing, especially considering the high number of canines suffering from degenerative inflammatory diseases.

For tissue regeneration, cell proliferation and migration into tissue lesions is essential (1,22). Various studies have been performed to study the effects of CMC on proliferation (10,29). To the best of our knowledge, no studies on canine BMSC proliferation under CMC treatment have been published. In the present study, no effects were observed on canine BMSC proliferation due to CMC. However, previous studies have shown the effect of CMC on the proliferation of skin fibroblast cells and human normal liver cells and gastric

Fig. 3. The relative mRNA expression in canine bone marrow-derived stem cells as assessed by reverse transcriptase polymerase chain reaction after 24-h treatment (control and 0.1% CMC) under normal conditions. Data are indicated as mean $\pm$ standard deviation.

cancer cells (2,33). In all of these experiments, no significant increase or decrease in proliferation was found. We thus concluded that CMC is not cytotoxic, which is supported by the present study.

In the wound healing process, activated fibroblasts must transfer from connective tissues around the wound edge and transverse through the temporary matrix. Accordingly, cell migration is critical for wound healing. In the migration assay conducted, almost all CMC concentrations had no visible effects on canine BMSCs. Cell migration was more effective with increasing CMC concentration; however, the effect de-

creased from 0.001% to 0.1% concentration.

However, in particular, a significant increase in BMSC migration was observed at over 0.001% CMC concentration. Our results show that a higher CMC concentration does not indicate a better BMSC migration. The effect of CMC on the migration of BMSCs was not dependent on concentration.

Based on the results of the present study, CMC exhibited no cytotoxicity and increased the migration rate at certain concentrations. To the best of our knowledge, the effects of CMC on the proliferation and migration of canine BMSCs *in vitro* have been reported here for the first time.

Fig. 4. The relative mRNA expression in canine bone marrow-derived stem cells evaluated by reverse transcriptase polymerase chain reaction after 24-h treatment with 100 ng/mL lipopolysaccharide (LPS) and LPS + 0.1% carboxymethyl chitosan. Data are indicated as mean $\pm$ standard deviation.

LPSs are a major component of cell walls in gram-negative bacteria; they modulate the activities of most systems in infected microorganisms, inducing various pathophysiological changes (7). The molecules of LPS exhibit a variety of biological activities that may be beneficial at low concentrations but may be endotoxic at higher concentrations due to an overproduction of cytokines by immune cells, such as TNF- α and ILs (5). When LPSs as the endotoxic factor of gram-negative bacilli, are used at high concentrations, tissue necrosis, serious toxicity, and death of microorganisms can occur. The LPSs used in this experiment were extracted from *Escherichia coli*.

IL-6, IL-1 β , and TNF- α are key proinflammatory cytokines. A previous study showed that CMC stimulates macrophages to produce IL-1 β (26) and inhibits the production of proinflammatory cytokines, such as TNF- α , in human astrocytoma (21). In the present study, TNF- α showed a tendency to decrease in the CMC-treated groups, whereas IL-1 β showed an increasing tendency. Furthermore, no changes in IL-6 were observed in both normal and CMC-treated groups. Previous studies demonstrated that TNF- α and IL-6 are inhibited by CMC in macrophage cells in LPS-induced inflammatory conditions (31). Additionally, IL-1 β is produced by activated immunogenic cells and secretes proinflammatory cytokines (21).

Based on the results of previous studies, CMC can affect the inflammatory response in canine BMSCs.

TGF- β and IL-10 are anti-inflammatory cytokines. When an inflammatory reaction occurs, TGF- β makes an immunological challenge and is immediately released from the platelets in the injured area (30,32). The results of the present study showed no differences between the control and CMC-treated groups.

IL-10 may exert its anti-inflammatory action by inducing the formation of IL-1 receptor antagonist (IL-1ra), another anti-inflammatory cytokine. IL-10 showed little difference in mRNA expression between the CMC-treated and control groups. IL-10 mRNA expression is regulated by T cell regulation in the inflammatory state, and BMSC suppresses T cells. This suggests that IL-10 expression did not change significantly (4).

Since TGF- β is activated under inflammatory conditions and LPS-induced inflammatory conditions are not likely to occur (30), TGF- β would not have produced significant results in the present study. Furthermore, the cause of inflammation induction used in the experiment should be considered. First, it is about the Toll-like receptors (TLRs) of BMSCs. TLRs are innate immune receptors that mediate inflammatory signals in response to infection, stress, and damage. In a previous study, LPS (TLR4 ligand) triggered the secretion of a wide range of cytokines, including proinflammatory IL-6 and IL-1 β but impaired the induction of the anti-inflammatory cytokine TGF- β (23). In addition, it was reported that since the TLR4 ligand substance was low in the BMSCs, the inflammatory reaction caused by LPS was weak (23). For this reason, the induction of inflammation could be considered with alternative substances that stimulate other TLRs in BMSCs.

Second, the complex reaction of LPS and CMC may reduce the inflammatory response. In fact, a previous study showed that LPS has an anion and CMC has a cation; thus, when the two substances react, they form a complex (5,7). This suggests that the inflammatory response to LPS is reduced, and therefore, the reaction to chitosan cannot be clearly observed.

A previous study showed that CMC has good anti-inflammatory activity (8). Through the expressions of these cytokines, we verified that CMC could promote anti-inflammatory effects on canine BMSCs (12,14). However, further studies are needed to clarify the effect of CMC on BMSCs under inflammatory condition through more exact research techniques such as real time PCR, western blot or *in vivo* study.

Conclusions

Altogether, CMC promoted BMSC migration and inhibited

inflammatory cytokines, such as TNF- α and IL-10. Therefore, CMC may be possible to regulate wound healing when MSCs are applied in inflammatory diseases.

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No fund.

Conflicts of Interest

The authors have no conflicting interests.

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