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SALMON CARTILAGE PROTEOGLYCAN MODULATES CYTOKINE RESPONSES TO *ESCHERICHIA COLI* IN MOUSE MACROPHAGES

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Abstract Proteoglycans (PGs) are complex glycohydrates, which are widely distributed in connective tissues and on the cell surface of mammalian tissues. We investigated the effect of PG extracted from salmon cartilage on cytokine responses to stimulation with heat-killed *Escherichia coli* (HKEC) in a mouse macrophage cell line, RAW264.7. PG exhibited the suppression of tumor necrosis factor (TNF)-α production and enhancement of interleukin (IL)-10 production compared with chondroitin 4 sulfate (C4S) and chondroitin 6 sulfate (C6S). PG, C4S and C6S suppressed HKEC-induced Toll-like receptor 4 (TLR4) and inducible nitric oxide synthase (iNOS) expression dose-dependently and the PG showed the strongest suppressive effect among 3 compounds. Only PG dramatically up-regulated the expression of signal transducers and activators of transcription 3 (STAT3) and the phosphorylation of STAT3 in mouse macrophages. Our results showed strong suppression of PG on inflammatory response and suggested that the novel interaction might exist between the extracellular matrix and immune system.

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Key words: Proteoglycan; *E. coli*; TNF-α; IL-10; macrophage

Proteoglycans (PGs) are complex glycohydrates, which are composed of core proteins and glycosaminoglycans (GAGs) that bind to core proteins. PGs are one of main components of extracellular matrix (ECM). ECM is composed from PGs, collagen, fibronectin, laminin, hyaluronic acid and other glycoproteins and they form complex in ECM[1-3]. PGs and other components exist in connecting tissue, such as skin, cartilage, bone, and vascular wall. PGs are involved in cellular proliferation and adhesion cooperated with collagen, fibronectin, and laminin[4]. Chondroitin sulfate (CS), one of elements that form PGs, is a main component of joint cartilage and is thought to be important for prevention of joint disorder in elderly people[5,6]. Several articles showed that recruitment of CS suppresses inflammation in rheumatoid arthritis[7-10]. These findings suggest that PGs could modify inflammatory responses. There have been some reports suggesting that ECM components are involved in transduction of proinflammatory signals. Reactive oxygen intermediates and hyaluronidase from macrophages and neutrophils breaks hyaluronic acid in ECM into low molecular weight fragments, and these fragments stimulate macrophages via CD44[11]. Hyaluronan fragments also activate dendritic cells[12] via Toll-like receptor (TLR) 4[13]. The secreted ECM protein, mindin, is essential for innate immune response to bacterial infections[14]. Small leucin-rich proteoglycan (SLRP), decorin, induces expressions of inducible NO synthase (iNOS), tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 in macrophages[15] and other SLRP family, biglycan, leads to activation of p38, extracellular signal-regulated kinase and nuclear factor kappa B (NF-κB) in macrophage via TLR2 and TLR4[16]. These findings indicate that ECM components promote

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inflammatory responses.

When the host is exposed to infection with bacteria and to allow bacterial invasion, ECM becomes the site that is important for innate immunity. To recognize microbial pathogens, the innate immune system uses pattern recognition receptors (PRRs) that sense pathogen-associated molecular patterns (PAMPs). TLRs, members of PRRs, are establishment of innate immunity. Bacterial components are recognized by TLRs and lipopolysaccharide (LPS) is recognized by TLR4[17].

One of host responses involved in host defense against bacterial infections is the induction of inflammatory cytokines such as interferon (IFN)-γ and TNF-α. IFN-γ plays an important role in resistance to infections with intracellular pathogens by activating microbicidal activities of macrophages[18,19]. IFN-γ is important for host resistance against bacterial infections[20-22]. TNF-α is also important for host resistance against bacterial infections. TNF-α activates resident macrophages[23], and enhances nitric oxide (NO) synthesis that is involved in the bactericidal action[24]. On the other hand, IL-10 has important regulatory effects on the immune responses[25]. IL-10 is a well-characterized anti-inflammatory cytokine[25-27], and suppresses the production of reactive oxygen and reactive nitrogen intermediates from activated macrophages by IFN-γ[24]. IL-10 also suppresses TNF-α and IL-12 production by macrophages[28].

In this study, we investigated the effect of PG and CS extracted from salmon cartilage on response of macrophages to bacteria, and we here show that PG suppresses inflammatory response of macrophage by up-regulation of IL-10 production and down-regulation of TNF-α production mediated by TLR down-regulation. All data in this study was expressed as means ± standard deviations, and Student's t test was used to determine the significance of the differences in the bacterial counts of the specimens by the control and experimental groups. Each experiment was repeated at least twice. Mouse macrophage cell line RAW264.7 was purchased from Dainippon Pharmaceutical Co. Ltd., Osaka, Japan. Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% of fetal bovine serum (JRH Biosciences, Lenexa, KS), 3% of L-glutamine (Wako Pure Chemical Industries Ltd., Osaka, Japan). *E. coli* strain IFO3806 was provided by Institution of Fermentation, Osaka, Japan. *E. coli* was grown in LB broth (Invitrogen Co., Carlsbad, CA) and heat-killed *E. coli* (HKEC) were obtained by heating the bacteria in boiling water for 1 h. PG extracted from salmon cartilage was prepared as described previously[29]. Chondroitin-4-sulfate (C4S) and chondroitin-6-sulfate (C6S) were purchased from Seikagaku Co. Ltd., Tokyo, Japan. RAW264.7 cells were prepared to 2×10^6/well in a 24-well culture plate and stimulated with 2×10^7/well of HKEC for 48 h in the absence or presence of various doses of PG, C4S or C6S. At first, to assess the response of mouse macrophages to bacteria, the titers of TNF-α as one of inflammatory cytokines and IL-10 as a member of anti-inflammatory cytokines in the culture supernatants were determined (Figure 1).

Titers of TNF-α and IL-10 were determined by ELISAs as described previously[30]. Stimulation with HKEC up-regulated the production of TNF-α from RAW264.7 cells (Figure 1). Treatment with PG decreased the up-regulation of TNF-α induced by HKEC in a dose-dependent manner (Figure 1A). Neither C4S nor C6S affected the up-regulation TNF-α production in response to HKEC stimulation (Figure 1A). IL-10 production from cells stimulated with HKEC was up-regulated by treatment with PG, C4S, and C6S

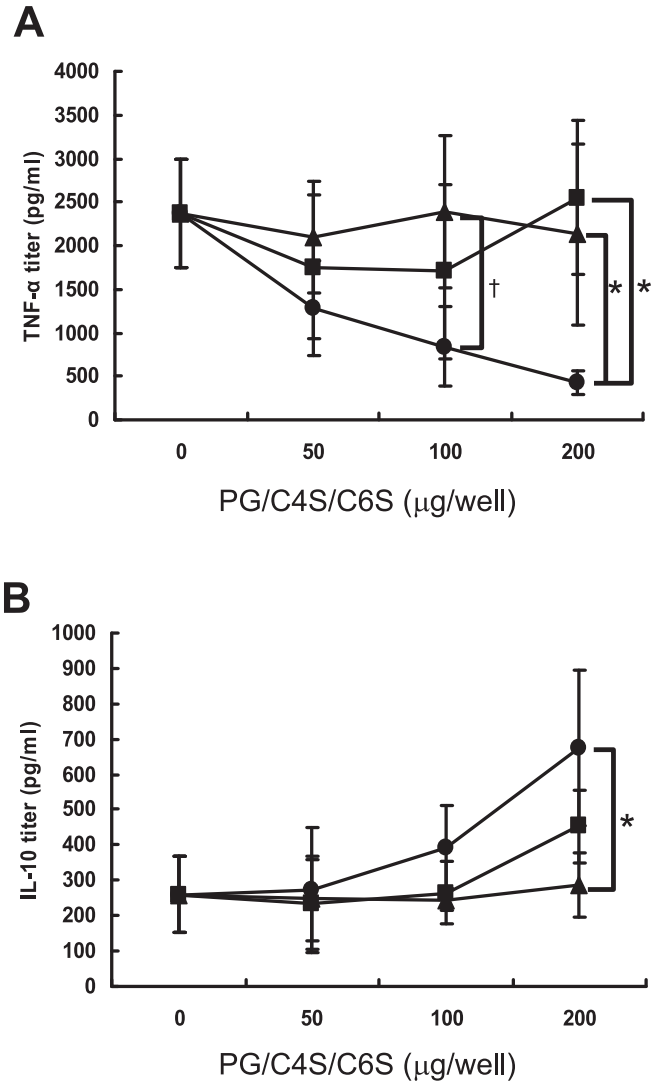

Figure 1 TNF-α and IL-10 production from mouse macrophage cell line RAW264.7 stimulated with heat-killed bacteria. RAW264.7 cells were prepared to 2×10^6 /well in DMEM and stimulated with 2×10^7 /well of HKEC for 48 h supplemented with 50, 100 or 200 ìg/well of PG (circle), C4S (square) or C6S (triangle). Then culture supernatants were collected and titers of TNF-α (A) and IL-10 (B) were determined by ELISAs. Each result represents the mean and SD for the group of four samples. An asterisk ($P<0.01$) and a cross ($P<0.05$) indicate that values are significantly different between two groups.

(Figure 1B). The treatment with PG strongly up-regulated IL-10 production from HKEC-stimulated cells compared with C4S and C6S (Figure 1B). TNF-α is a major mediator of inflammation induced by macrophages during bacterial infections[32]. TNF-α shows various functions, such as induction of apoptosis[33], induction of IL-1, IL-6 and IL-10 secretion, activation of T cell and other cells involved in inflammation[32]. Our results showed that PG down-regulated TNF-α production induced by stimulation with HKEC (Figure 1A) and up-regulated IL-10 production (Figure 1B) from macrophages. IL-10 is anti-inflammatory cytokine, and mediates immunosuppression induced by $CD4^+CD25^+$ T cells in autoimmune or inflammatory diseases[34,35]. IL-10 also suppresses the production of TNF-α and IL-6

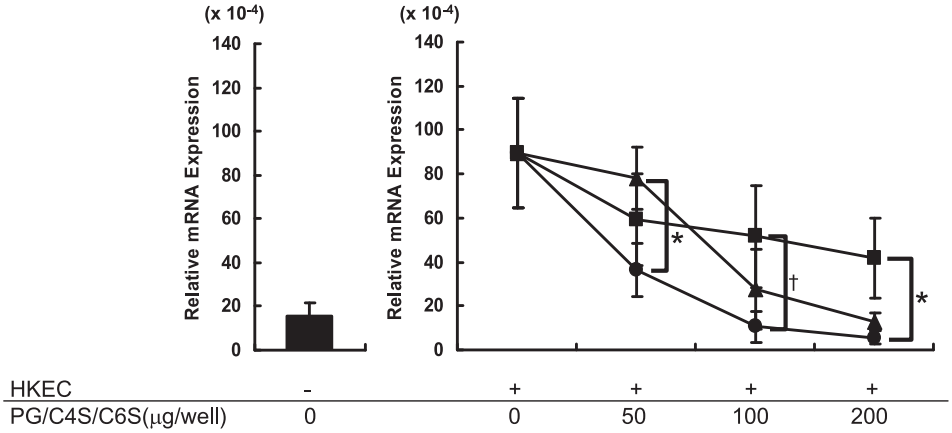

Figure 2 Expression of TLR4 mRNA in RAW264.7 cells stimulated with heat-killed bacteria. RAW264.7 cells were prepared to 2×10^6 /well in DMEM and stimulated with 2×10^7 /well of HKEC for 48 h supplemented with 50, 100 or 200 ìg/well of PG (circle), C4S (square) or C6S (triangle). Then cells were collected and mRNAs were prepared. Expression of TLR4 was determined by quantitative real-time RT-PCR. Each result represents the mean and SD for the group of four samples. An asterisk ($P<0.01$) and a cross ($P<0.05$) indicate that values are significantly different between two groups.

from macrophages, synoviocytes, and T cells in an experimental inflammation model[36]. This result suggests that PG has a suppressive effect on inflammatory responses during bacterial infections.

Next, we investigated the expressions of TLR4 in mouse macrophages in response to HKEC by quantitative real time RT-PCR. For quantitative real time RT-PCR, total RNA was isolated from cultured cells by a guanidium thiocyanate-phenol-chloroform single-step method[31]. First-strand cDNAs were synthesized by reverse transcription of 1 μg total RNA using random primers (Takara, Shiga, Japan) and reverse transcriptase Moloney murine leukemia virus (Invitrogen). For detecting TLR4, these primers were used; forward, 5'-AG TGGGTCAAGGAACAGAAGCA-3', and reverse, 5'-CTTTACCAGCTCATTTCACACC-3', and for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as internal control, forward, 5' -TGAAGGTCGGTGTGAACGGATTTGG-3', and reverse, 5'-ACGACATACTCAGCACCAG CATCAC-3'. SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA) was used as a PCR solution. PCR was run following protocol: initial activation of Taq DNA polymerase at 95 ℃ for 5 min, 30 sec at 95 ℃ for denaturing, 30 sec at 55 ℃ for annealing, 30 sec at 72 ℃ for elongation and 40 PCR cycles were performed. All experiments were run in duplicate and non-template controls and dissociation curves were used to detect primer-dimer conformation and nonspecific amplification. The threshold cycle (C_T) of each target product was determined and set in relation to the amplification plot of GAPDH. The detection threshold is set to the log linear range of the amplification curve and kept constant (0.05) for all data analysis. Difference in CT values (ΔC_T) of two genes was used to calculate the relative expression [relative expression=$2^{-(C_T \text{ of STATs-}C_T \text{ of GADPH})}=2^{-\Delta C_T}$]. The TLR4 expression was up-regulated by HKEC stimulation compared with unstimulated controls (Figure 2). PG, C4S, and C6S suppressed TLR4 up-regulation by HKEC stimulation (Figure 2). TLR4 recognizes LPS[37,38] and TLR4 triggers TNF-α secretion in macrophages through activation of NF-κB[39,40]. Therefore we examined the effect of PG on

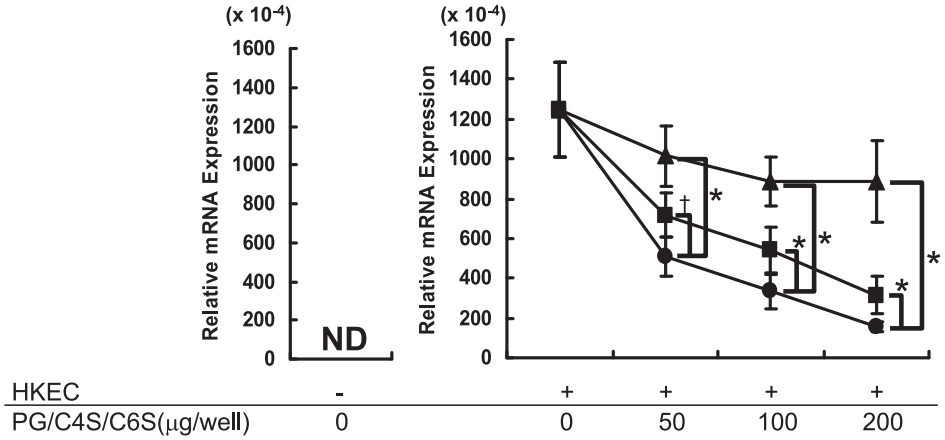

Figure 3 Expression of iNOS mRNA in RAW264.7 cells stimulated with heat-killed bacteria. RAW264.7 cells were prepared to 2×10^6 /well in DMEM and stimulated with 2×10^7 /well of HKEC for 48 h supplemented with 50, 100 or 200 ig/well of PG (circle), C4S (square) or C6S (triangle). Then cells were collected and mRNAs were prepared. Expression of iNOS was determined by quantitative real-time RT-PCR. Each result represents the mean and SD for the group of four samples. ND indicates that iNOS expression is under detectable level. An asterisk ($P<0.01$) and a cross ($P<0.05$) indicate that values are significantly different between two groups.

TLR4 expression during HKEC stimulation. PG treatment suppressed the enhancement of TLR4 expression induced by stimulation with HKEC (Figure 2), suggesting that the suppressive effect of PG on inflammatory responses during HKEC stimulation may be due to suppression of enhancement of TLR4 expression.

TNF-α induces inducible NO synthase (iNOS) expression. Therefore we examined the expression of iNOS in mouse macrophages stimulated by HKEC (Figure 3). For detecting iNOS, these primers were used; forward, 5'- ATGGCTTGCCCCTGGAAGTTTC-3', and reverse, 5'- GGACTTGCAAGTGAAATCCGATG-3'. Stimulation with HKEC induced iNOS expression, and the treatment with PG, C4S and C6S suppressed iNOS expression in a dose-dependent manner (Figure 3). In HKEC stimulation, PG suppressed iNOS expression compared with other groups (Figure 3). Activated macrophages up-regulate the synthesis of NO that is involved in bactericidal activity[41]. Therefore we investigated the expression of iNOS in mouse macrophages during stimulation with HKEC. Treatment with PG, C4S and C6S suppressed iNOS induction by stimulation with HKEC (Figure 3). This result indicates that these compounds may show the suppressive effect on the bactericidal function of macrophages and PG reveals the strongest effect among 3 compounds.

IL-10 is able to activate STAT3. Therefore, we investigated whether the expression of STAT3 during stimulation with HKEC in mouse macrophages is affected by PG, C4S or C6S treatment or not. For detecting STAT3, these primers were used; forward, 5'-CAAAACCCTCAAGAGCCAAGGAGAC-3', and reverse, 5'-GCCGGTGCTGCACGATAGGG-3'. The up-regulation of STAT3 expression during stimulation with HKEC was shown only PG-treated RAW cells in a dose-dependent manner (Figure 4A). Neither C4S nor C6S affected the STAT3 expression by Western blotting. For Western blotting, cultured cells were lysed in lysis buffer (0.05M Tris-HCl, 2% SDS, 6% 2-mercaptoethanol, 10% glycerol). The protein concentration of each sample was determined using the Bradford protein assay (Bio-Rad). Each amount of protein was developed by SDS-PAGE using 10% SDS-polyacrylamide gel and

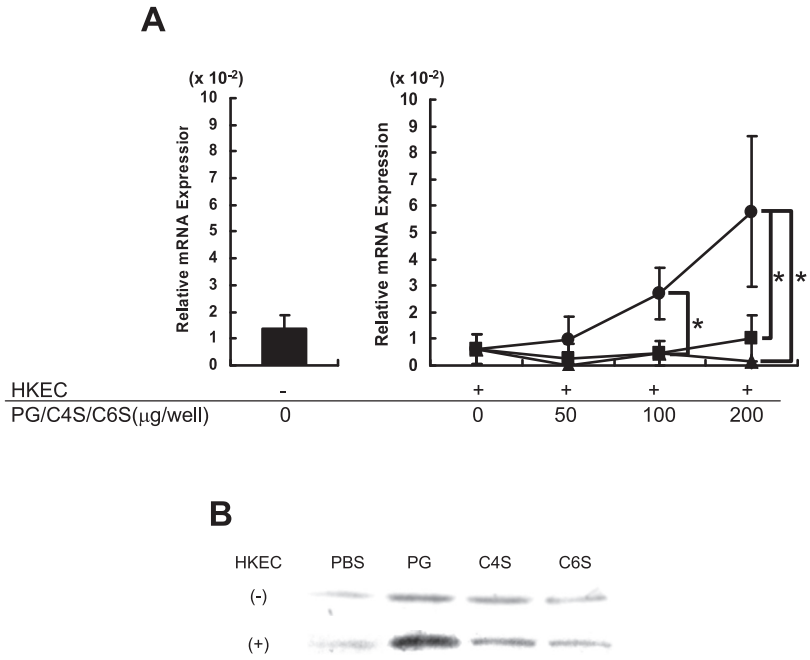

Figure 4 Expression of STAT3 mRNA in RAW264.7 cells stimulated with heat-killed bacteria. RAW264.7 cells were prepared to 2×10^6 /well in DMEM and stimulated with 2×10^7 /well of HKEC for 48 h supplemented with 50, 100 or 200 ìg/well of PG (circle), C4S (square) or C6S (triangle). Then cells were collected, and then mRNAs were prepared. (A) Expression of STAT3 was determined by quantitative real-time RT-PCR. Each result represents the mean and SD for the group of four samples. An asterisk ($P<0.01$) indicates that values are significantly different between two groups. (B) Proteins from cultured cells were prepared and phosphorylated STAT3 was detected by Western blotting.

transferred to Immobilon™-P transfer membrane (Millipore Corporation, Bedford, MA). After blocking, the membrane was incubated with a primary antibody specific for phosphorylated STAT3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After washing, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG. Immunoreactive bands were visualized ECL™ Western Blotting Analysis System (Amarsham Biosciences, UK). These results also showed that treatment with PG strongly up-regulated STAT3 phosphorylation compared with C4S- or C6S-treated group (Figure 4B). In a signaling pathway, IL-10 activates STAT3[42]. Our result showed that PG shows the strong effect on STAT3 up-regulation and phosphorylation in macrophages during stimulation with HKEC (Figure 4), suggesting that suppressive effect of PG is mediated by IL-10 up-regulation. It is still unclear why only PG exhibits the strong suppressive effect on inflammatory responses stimulated by HKEC. Previous studies showed that hyaluronan with high molecular weight inhibits NF-κB activation and with decreasing the molecular weight of hyaluronan, this inhibitory effect is reduced[43]. This report suggests that glycoprotein with high molecular weight, such as PG, might have an anti-inflammatory effect.

Finally, our present results demonstrated that PG has a potent effect on suppression of inflammatory responses induced by bacteria in mouse macrophages. These results suggest the existence of novel interaction of ECM components with macrophages in inflammatory responses during bacterial infections.

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