A THESIS FOR THE DEGREE OF MASTER

Enhanced Anti-inflammatory Effects of TNF-α and IFN-γ Treated Canine Mesenchymal Stem Cells Through the COX-2/PGE_2 Pathway

TNF-α 및 IFN-γ로 처리된 개 중간엽줄기세포의 COX-2/PGE_2 경로를 통한 항염증 효과 향상

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수의과대학 수의내과학 전공

양 혜 미

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지도교수 윤 화 영

이 논문을 수의학석사학위논문으로 제출함

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수의과대학 수의내과학 전공

양 혜 미

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Abstract

Enhanced Anti-inflammatory Effects of TNF-α and IFN-γ Treated Canine Mesenchymal Stem Cells Through the COX-2/PGE_2 Pathway

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Mesenchymal stem cells (MSCs) have been used in studies on treatment of various diseases, and their application to immune-mediated diseases has garnered interest. Various methods for enhancing the immunomodulation effect of human MSCs have been used; however, similar approaches for canine MSCs are relatively unexplored. Accordingly, I evaluated immunomodulatory effects and mechanisms in canine MSCs treated with TNF-α and IFN-γ. Canine MSCs were stimulated with TNF-α and IFN-γ for 24 hours to produce conditioned media (CM). Lipopolysaccharide (LPS)-stimulated RAW 264.7

cells were co-cultured with the MSCs for 48 h in CM. Expression of RNA was assessed by quantitative reverse transcription PCR (qRT-PCR), and protein levels were assessed by western blot. Expression of inducible nitric oxide synthase (iNOS), IL-6 and IL-1β was significantly (one-way ANOVA) decreased in LPS-stimulated RAW 264.7 cells co-cultured with naïve canine MSCs compared to that in LPS-stimulated RAW 264.7 cells alone. Furthermore, anti-inflammatory effects of TNF-α- and IFN-γ-primed canine MSCs were significantly increased compared with those of naïve canine MSCs. Expression of cyclooxygenase 2 (COX-2) and prostaglandin E2 (PGE_2) were likewise significantly increased in primed canine MSCs. The level of iNOS protein in LPS-stimulated RAW 264.7 cells co-cultured with the primed canine MSCs was decreased, but it increased when the cells were treated with NS-398(PGE_2 inhibitor). In conclusion, compared with naïve canine MSCs, cells primed with TNF-α and IFN-γ cause a greater reduction in release of anti-inflammatory cytokines from LPS-stimulated RAW 264.7 cells; the mechanism is upregulation of the COX-2/PGE_2 pathway.

Key words: mesenchymal stem cell; inflammatory cytokines; COX-2; PGE_2; anti-inflammation

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1. Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that can be isolated from adipose tissue, bone marrow, umbilical cord blood, synovium, dental pulp, and other sources (Al-Nbaheen et al., 2013; Ribeiro et al., 2013). MSCs can differentiate into cells of connective tissue, bone, fat, cartilage, and muscle. They have been isolated from the human, equine, bovine, feline, and canine sources (Jacobs et al., 2013; Marx et al., 2015).

Previous studies reported immunomodulatory functions for MSCs (Ma et al., 2014; Plock et al., 2014; Shi et al., 2012), and MSCs have been used in studies on treatment of immune-mediated diseases (Kim et al., 2016; Le Blanc et al., 2004). For example, human MSCs have been used in research on treatment of inflammatory bowel disease, systemic lupus erythematosus, graft-host disease, ulcerative colitis, Crohn's disease, multiple sclerosis, autoimmune diabetes, and rheumatoid arthritis (Dave et al., 2015; Glenn and Whartenby, 2014). In addition, canine MSCs in particular have been used for the treatment of inflammatory bowel disease, systemic lupus erythematosus, autoimmune diabetes, and perianal fistula (Ferrer et al., 2016; Hoffman and Dow, 2016).

Those studies reported that not only do MSCs migrate directly to the site of inflammation, but they also exert indirect

anti-inflammatory effects through secretory factors (Kang et al., 2008; Matthay et al., 2010; Song et al., 2017). Human MSCs regulate inflammatory processes through various soluble factors such as indoleamine 2,3-dioxygenase (IDO), TNF-α-stimulated gene-6 (TSG-6), and prostaglandin E_2 (PGE_2) (Liu et al., 2016; Spaggiari et al., 2008). PGE_2 and TSG-6 were secreted by mouse MSCs (Ghannametal., 2010; Prockop and Oh, 2012), where as TSG-6, indoleamine 2,3-dioxygenase (IDO), TGF-β, hepatocyte growth factor (HGF), and PGE_2 were secreted by canine MSCs (Kang et al., 2008).

I have focused on making immunomodulation of MSCs more effective. Despite diverse and growing research on preconditioning strategies such as stimulation by hypoxia, inflammatory stimuli, and activation by three-dimensional culture (English et al., 2007; Hemeda et al., 2010; Madrigal et al., 2014; Yu et al., 2013), studies on immune regulatory effects of canine MSCs are few and have become an active area of investigation. Accordingly, I evaluated immunomodulatory effects of canine MSCs conditioned with tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) and I examined the mechanisms by which the immunoregulation is induced.

2. Material and Methods

2.1. Animals

Stem cells were obtained from three healthy dogs at the Seoul National University Veterinary Medicine Teaching Hospital (SNU VMTH). The dogs were one to three years old and weighed from 7 to 15 kg. The mean body-condition score was grade 5. Two dogs were castrated males, and one dog was a spayed female. Procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of SNU (IACUC approval №. SNU-170724-5), and the protocol was performed in accordance with approved guidelines.

Dogs were sedated by intravenous injection of acepromazine (0.01 mg/kg), and anesthetized with alfaxane (2 mg/kg). These dogs were intubated, then maintained on inhalation anesthesia with isoflurane for cell harvest. Skin and subcutaneous tissues were desensitized by local infiltration of 2% lidocaine (Vieira et al., 2010). A fat sample was aseptically collected from the gluteal subcutaneous fat by incising the skin, after which sharp and blunt dissection was used to remove the fat as previously described (Kang et al., 2012; Kang et al., 2008). Bone marrow aspirates were aseptically harvested from the proximal humerus in a manner similar to that described

previously (Lee et al., 2011; Singh et al., 2014; Sullivan et al., 2015). The bone was palpated, and a small incision was made into the skin and subcutaneous tissues. A 12-gage Rosenthal needle was inserted, after which a 35-㎖ heparinized syringe (1000 IU/㎖ of blood) was used to aspirate bone marrow and blood.

2.2. Harvest and isolation of canine adipose-tissue-derived mesenchymal stem cells(cAT-MSCs)

Fat samples were washed three times in Dulbecco's phosphate-buffered saline (DPBS; PAN-Biotech, Aidenbach, Germany) containing 1% penicillin-streptomycin (PS; PAN-Biotech), placed on a petri dished, and finely minced with sterile scissors. The tissues were digested with 0.1% collagenase type ⅠA (Gibco/Life Technologies, Carlsbad, CA, USA) solution, and incubated for 1 hour at 37℃ in a humidified atmosphere of filtered 5% CO_2. After digestion, high-glucose Dulbecco's modified Eagle medium (DMEM; PAN-Biotech) with 10% fetal bovine serum (FBS; PAN-Biotech) and 1% PS was added to neutralize the sample. The sample was centrifuged at 1200 xg for 5 min. The supernatant was discarded, the cell pellet was suspended in high-glucose DMEM, and the suspension was passed through a 70-㎛ Falcon cell strainer (Fisher Scientific, Waltham, MA, USA) to remove debris. The suspension was

centrifuged again at 1,200 xg for 5 min. Red blood cell lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) was added and the cell solution was incubated for 10 min at 25℃ to remove erythrocytes. Cells were washed with five volumes of DPBS and centrifuged again. The supernatant was aspirated, and then the cells were resuspended in high-glucose DMEM and seeded (3,000/㎠) onto a 100 ㎜ ϕ cell culture dish. Unattached cells were removed the next day by washing with DPBS.

2.3. Harvest and isolation of canine bone-marrow-derived mesenchymal stem cells (cBM-MSCs)

Bone marrow samples were diluted with an equal volume of DPBS, and then gently layered over Ficoll-Paque PLUS (GE Healthcare Life Sciences, Uppsala, Sweden) in a conical tube. After centrifugation at 750 xg for 30 min, the buffy coat layer was carefully collected and centrifuged at 1200 xg for 10 min. The supernatant was discarded and the pellet was treated with red blood cell lysis buffer at room temperature for 15 min. DPBS was added and the suspension was centrifuged at 800 xg for 10 min. The supernatant was discarded, the cells were resuspended in alpha modified Eagle's medium (αMEM; PAN-Biotech) as previously described (Karpov et al., 2013; Kisiel et al., 2012) then seeded as previously described for cAT-MSCs.

2.4. Cell culture and expansion

The cAT- and cBM-MSCs were incubated at 37℃ in a humidified atmosphere of 5% CO_2 in high-glucose DMEM (cAT-MSCs) or α MEM (cBM-MSCs) with 10% FBS and 1% PS. After 24 h, cultures were washed with DPBS to remove non-adherent cells and fresh medium was added. The culture medium was changed every 3 days until cells reached about 80% confluency. For all passages from P0 to P4, cultured cells were seeded at 10,000/㎠ in 100 ㎜ ϕ cell culture dishes and subcultured to 70-80% confluency using 1 ㎖ of 0.25% trypsin -EDTA (PAN-Biotech). All MSCs used in the experiment were in passage 3 or 4, as described in previous studies (Baksh et al., 2007; Kim et al., 2009; Lee et al., 2014).

The RAW 264.7 murine macrophage-like cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). RAW 264.7 cells were cultured in HDMEM containing 10% FBS and 1% PS. Medium was replaced every 2-3 days until the cells reached 70-80% confluency.

2.5. Characterization of MSCs

Canine MSCs were characterized by immunophenotyping and multilineage differentiation. For immunophenotyping, cells

were evaluated by flow cytometry for the expression of stem cell markers using antibodies conjugated with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or allophycocyanin (APC). Antibodies were against cluster of differentiation (CD) 29 (FITC), CD34 (PE), and CD73 (PE) (all from BD Biosciences, Franklin Lakes, NJ, USA); and against CD44 (FITC), CD45 (FITC), and CD90 (APC) (all from eBiosciences, San Diego, CA, USA). The cells were sorted into defined populations using fluorescent-activated cell sorting (FACS) such that a pure population with the appropriate cell marker profile was produced. Results were analyzed by FlowJo 7.6.5 software (Tree Star, Inc., Ashland, OR, USA).

For cellular differentiation, cells were plated in 24-well plates and cultured in growth medium to 90% confluency prior to differentiation. The differentiation capacity of the cells was confirmed by identifying differentiated cells using special differentiation media (PRIME-XV Adipogenic Differentiation SFM, PRIME- XV Osteogenic Differentiation SFM and PRIME- XV Chondrogenic Differentiation XSFM; all from Irvine Scientific, Santa Ana, CA, USA), followed by Oil Red O staining, Alizarin Red staining, and Alcian Blue staining, respectively.

2.6. Canine peripheral blood mononuclear cell (cPBMC) isolation

Donor blood from a healthy canine was obtained from SNU VMTH and diluted with an equal volume of PBS, and then layered over Ficoll-Paque PLUS (GE Healthcare Life Sciences, Uppsala, Sweden) in a conical tube. After centrifugation at 850 xg for 30 min, the buffy coat layer was carefully collected. The collected samples were incubated with RBC lysis buffer at 25℃ for 15 min. After PBS addition, samples were centrifuged at 750 xg for 10 min. Washing and centrifugation were repeated. cPBMCs were resuspended in Roswell Park Memorial Institute-1640 medium (Pan-Biotech, Dorset, Germany) containing 10% FBS and 1% PS.

2.7. Preparation and of conditioned medium (CM)

The cAT-/cBM-MSCs (3×10^5 cells/well) were seeded in 6-well plates and cultured in αMEM medium containing 10% FBS and 1% PS for 3 days to yield conditioned medium (Timmers et al., 2011). After 6 hours, the cAT-/cBM-MSCs were stimulated with the pro-inflammatory cytokines TNF-α (20 ng/㎖; PROSPEC Protein Specialists, NJ, USA) and IFN-γ (20 ng/㎖; Kingfisher Biotech, MN, USA) for 24 h (English et al., 2007; Prasanna et al., 2010). Concentrations and times were chosen after optimization studies (data not shown). After stimulation, cells were washed 3 times with DPBS and cultured with αMEM. After 3 days, conditioned medium was harvested

and centrifuged at 850 rpm for 5 min to remove cellular debris. The supernatant was transferred to a conical tube and stored at −80℃.

2.8. Co−culture experiments

RAW 264.7 cells or cPBMCs were co−cultured with conditioned media of cAT− or cBM−MSCs. RAW 264.7 cells or cPBMCs were stimulated with lipopolysaccharide (LPS) (200 ng/㎖; Sigma−Aldrich, St. Louis, MO, USA), and another population of unstimulated cells was used as a control. After incubation for 24 h, stimulated and unstimulated RAW 264.7 cells or cPBMCs were washed with DPBS and seeded in 6−well plates (1×10^6 cells/well) in triplicate. After the cells had adhered to the plate, the medium was suctioned off and replaced with the conditioned media from cAT− or cBM−MSCs, after which incubation continued for 3 days.

2.9. RNA extraction, cDNA synthesis, and qRT−PCR

Total RNA from Raw 264.7 cell was extracted using the Easy−BLUE Total RNA Extraction kit (Intron Biotechnology, Sungnam, Korea). cDNAs were synthesized from 1 ㎍ of total RNA using the CellScript All−in−One 5X First Strand cDNA Synthesis Master Mix (CellSafe, Seoul, Korea). Samples were

evaluated in duplicate in 10 ㎕ AMPIGENE qPCR Green Mix Hi-ROX with SYBR Green dye (Enzo Life Sciences, Farmingdale, NY, USA) using 1 ㎕ cDNA and 400 nM each of forward and reverse primers (BIONICS, Seoul, Korea). Expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.10. Western blot

Total protein from the MSCs was extracted using PRO-PREP Protein Extraction Solution (Intron Biotechnology). Protein concentration was measured using the Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Proteins were separated by SDS PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked (5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20), then incubated with primary antibodies against COX-2 (1:500; Santa Cruz Biotechnology, Dallas, Texas, USA) at 4℃ overnight, followed by incubation with secondary antibodies for 1 h at room temperature. Immunoreactive bands were normalized to β-actin (1:1000; Santa Cruz) and visualized using enhanced chemiluminescence (Advansta, Menlo Park, CA, USA).

2.11. Enzyme-linked immunosorbent assay (ELISA)

PGE_2 was measured in cleared supernatants (centrifugation for 5 min at 850rpm) from treated or non-treated cAT-/cBM-MSCs using the canine PGE_2 ELISA Kit (Cusabio Biotech, MD, USA).

2.12. Statistical Analysis

Data are expressed as mean ± SD. The statistical significance of the mean values was compared by one-way ANOVA and Student's t test using the GraphPad Prism v.6.01 software (GraphPad Software Inc., CA, USA). p-values < 0.05 were considered to indicate statistical significance.

3. Results

3.1. Characterization of cAT- and cBM-MSCs

Cells obtained from canine adipose tissue and from bone marrow were characterized by immunophenotyping and tri-lineage differentiation. Three to five days after seeding, spindle-shaped cells were adhered to the culture plates. The cells were positive for expression of CD29, CD44, CD73, CD90 but did not express CD34 and CD45 (Fig. 1A and B). cAT- and cBM-MSCs were able to differentiate into adipocytes, osteocytes, and chondrocytes (Fig. 1C and D).

3.2. Enhanced anti-inflammatory effects of TNF-α- and IFN-γ- primed cAT- and cBM-MSCs

In order to determine the enhanced anti-inflammatory capacity of primed cAT- and cBM-MSCs, iNOS and several pro-inflammatory cytokines such as IL-1β and IL-6 were measured at the RNA level in LPS-stimulated RAW 264.7 cells.

IL-1β, IL-6 and iNOS expression levels of LPS-stimulated RAW 264.7 cells cultured with conditioned media (CM) of cAT-MSCs were significantly reduced compared with those of LPS-stimulated RAW 264.7 cells alone. Notably, expression levels of the inflammatory markers were reduced in the LPS-stimulated RAW 264.7 cells cultured with TNF-α/IFN-γ-primed cAT-MSCs compared with those in LPS-stimulated RAW 264.7 cells cultured with naïve cAT-MSCs (Fig. 2). Furthermore, in the case of cBM-MSCs, all expression levels in LPS-stimulated RAW 264.7 cells were shown to be further reduced when cultured with CM of TNF-α/IFN-γ primed cBM-MSCs compared to co-culture with naïve cBM-MSCs (Fig. 2).

I also measured pro-inflammatory cytokine levels in cPBMCs. iNOS and IL-6 expression levels of LPS-stimulated cPBMCs cultured with TNF-α/IFN-γ-primed CM of cAT-MSCs were both reduced compared to those in LPS-stimulated cPBMCs cultured with naïve cAT-MSCs. In addition, for cBM-MSCs, all expression levels in LPS-stimulated cPBMCs were further reduced when cultured with CM of TNF-α/IFN-γ primed cBM-MSCs compared to in co-culture with naïve cBM-MSCs, and the difference for iNOS was significant. Although IL-6 showed a decreasing tendency, the difference was not significant (Fig. 3).

3.3. Increased expression of COX-2/PGE_2 in TNF-α/IFN-γ-primed cAT- and cBM-MSCs

Next, I measured the protein level of COX-2 from TNF-α/IFN-γ-primed cAT- and cBM-MSCs, and from naïve cAT- and cBM-MSCs. The protein level of COX-2 in TNF-α/IFN-γ-primed cAT-MSCs was 7.4 times higher than in naïve cAT-MSCs (Fig. 4A). Likewise, the protein level of COX-2 in TNF-α/IFN-γ-primed cBM-MSCs was 1.7 times greater than in naïve cBM-MSCs (Fig. 4B). Thus, a significant difference occurs in COX-2 levels between the TNF-α/IFN-γ-primed groups and the naïve groups in cAT- and in cBM-MSCs.

Based on the increased COX-2 level in TNF-α/IFN-γ-primed cAT- and cBM-MSCs, I measured PGE_2 production in these cells. Higher levels of PGE_2 production were measured insupernatant of TNF-α/IFN-γ-primed cAT-MSCs than in supernatant of naïve cAT-MSCs (Fig. 5A). Likewise, higher levels of PGE_2 production were measured in supernatant of TNF-α/IFN-γ-primed cBM-MSCs than in that of naïve cBM-MSCs (Fig. 5B).

3.4. Decreased anti-inflammatory effects of CM

with PGE_2 inhibitor, NS-398

Next, I examined how the PGE_2 inhibitor NS-398 (Enzo Life Sciences, Farmingdale, NY, USA) affected the immunoregulatory capacity of CM by measuring the level of iNOS in LPS-stimulated RAW 264.7 cells co-cultured with cAT-MSCs. The decrease in iNOS level seen in LPS-stimulated RAW 264.7 cells co-cultured with TNF-α/IFN-γ-primed cAT-MSCs was negated by NS-398 treatment (Fig. 6).

4. Discussion

MSCs have been applied to treatment of various diseases. Recently, attention has focused on the application of MSCs to immune-mediated diseases (Aggarwal and Pittenger, 2005; Shi et al., 2010; Shi et al., 2012; Uccelli et al., 2007; Yagi et al., 2010), and numerous attempts have been made to increase immunomodulation effects of MSCs (Barrachina et al., 2017; Chiesa et al., 2011; Das et al., 2009; Yu et al., 2013). It has been shown that MSCs primed with inflammatory cytokines enhance immune reactions in humans (Hemeda et al., 2010) and in mice (English et al., 2007). However, studies of canine MSCs are few. Accordingly, I evaluated immunomodulatory effects and mechanisms of canine MSCs treated with TNF-α and IFN-γ. I used mesenchymal stem cells derived from two different sources: adipose tissue and bone marrow.

In a preliminary experiment I found the optimal concentration of inflammatory cytokines for priming canine MSCs; the combination of TNF-α (20 ng/㎖) and IFN-γ (20 ng/㎖) was most suitable (data not shown). IL-1β, IL-6 and iNOS expression levels of LPS-stimulated RAW 264.7 cells were significantly reduced in the TNF-α/IFN-γ-primed cAT and BM-MSC group compared to those in the naïve cAT- and

BM-MSC group (Fig. 2). Next, I used canine PBMCs to evaluate the anti-inflammatory effects of canine MSCs. iNOS expression levels in LPS-stimulated cPBMCs co-cultured with TNF-α/IFN-γ-primed CM of cAT and BM-MSCs were significantly reduced compared to those in LPS-stimulated cPBMCs co-cultured with naïve cAT- and BM-MSCs. IL-6 levels tended to decrease in cPBMCs co-cultured with TNF-α/IFN-γ-primed CM of cAT- and BM-MSCs compared to in cPBMCs co-cultured with naïve cAT- and BM-MSCs (Fig. 3). Taken together, I found that priming with TNF-α and IFN-γ enhanced the anti-inflammatory effects of cAT-MSCs and of cBM-MSCs.

It has been shown that MSCs regulate the inflammatory process through various soluble factors such as TSG-6 (Liu et al., 2016; Song et al., 2017), IDO (Spaggiari et al., 2008), TGF-β (Liu et al., 2015), HGF (Puissant et al., 2005), and PGE_2 (Spaggiari et al., 2008). Among these, PGE_2 has been identified as pivotal for the anti-inflammatory effects of MSCs (Solchaga and Zale, 2012; Manferdini et al., 2013). Similarly, COX-2 is an immunomodulatory factor secreted by MSCs (Kang et al., 2008; Carrade and Borjesson, 2013). Based on these previous findings, it is tempting to speculate that the COX-2/PGE_2 pathway from cAT- and cBM-MSCs plays a key role in enhancing anti-inflammatory action. Indeed, our experiments showed the protein level of COX-2 from TNF-α/IFN-γ-primed cAT- and cBM-

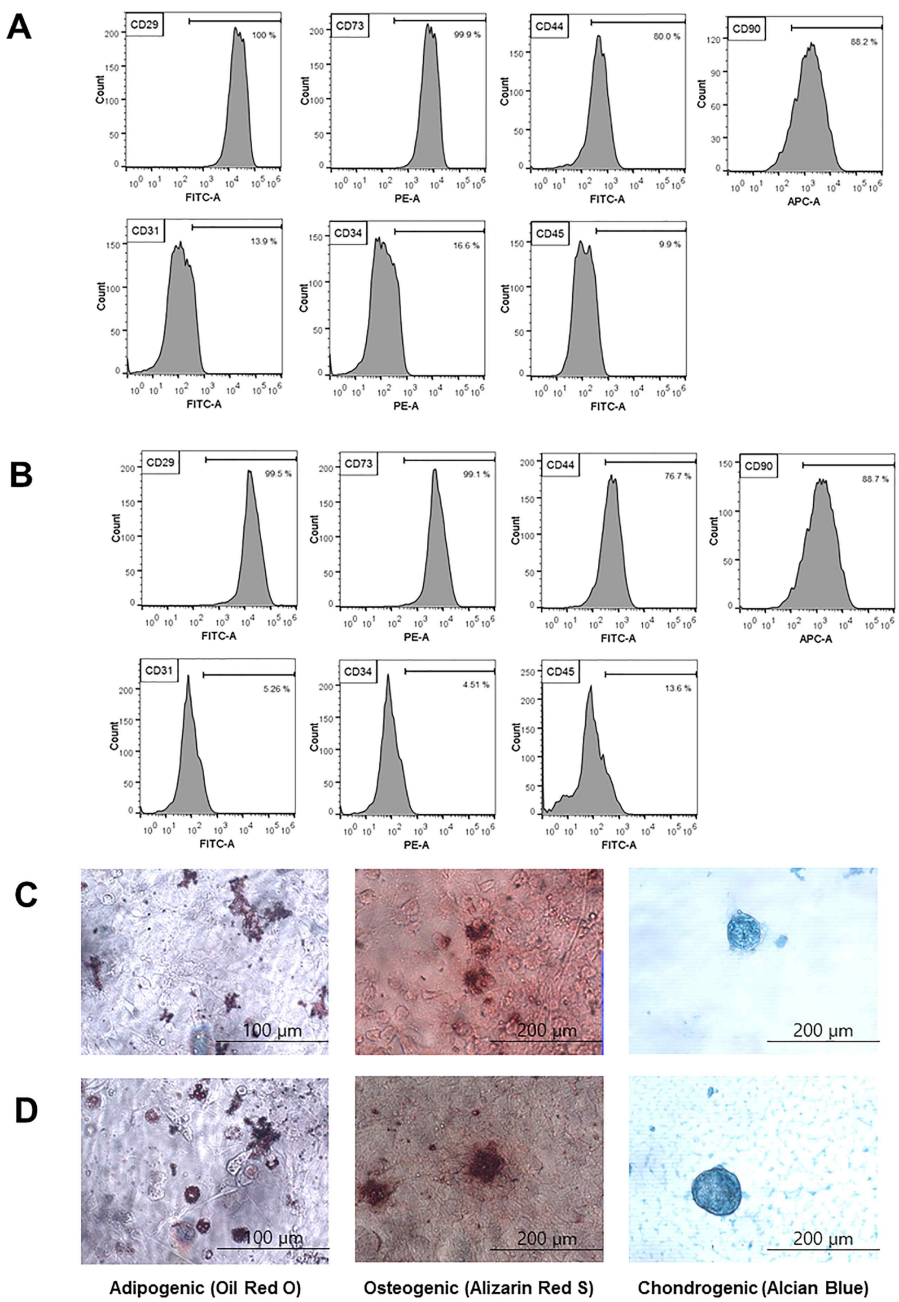
MSCs to be significantly higher than that from a naïve population. In addition, PGE_2 in TNF-α/IFN-γ-primed cAT- and cBM-MSCs was markedly higher than that in naïve cells. Therefore, I have shown that the increased the anti-inflammatory effects in TNF-α/IFN-γ-primed cAT- and cBM-MSCs are related to increased activation of the COX-2/PGE_2 pathway.

I next asked whether PGE_2 is involved in enhancing the anti-inflammatory effect of MSCs pretreated with TNF-α and IFN-γ by examining the effect of the PGE_2 inhibitor NS-398 on iNOS protein levels. The decreased iNOS level in LPS-stimulated RAW 264.7 cells co-cultured with TNF-α/IFN-γ-primed cAT-MSCs was absent after NS-398 treatment. Thus, the increase in PGE_2 secretion of TNF-α/IFN-γ-primed cAT-MSCs directly affected the anti-inflammatory action.

There were some limitations in our study. I obtained cAT- and cBM-MSCs from two dogs. Although these two MSC populations showed similar immunomodulatory effects, larger populations should be investigated. Studies on other immunomodulatory factors, together with *in vivo* applications, are needed to elucidate the role of TNF-α/IFN-γ-primed cAT- and cBM-MSCs.

5. Conclusions

In conclusion, this study revealed that TNF-α/IFN-γ-primed canine MSCs more effectively reduced inflammation of activated RAW 264.7 cells than did naïve canine MSCs. In addition, I suggest that an increased COX-2/PGE_2 pathway is one mechanism of enhanced immunomodulatory effects in TNF-α/IFN-γ-primed canine MSCs. Furthermore, our study might provide insight into the application of TNF-α/IFN-γ-primed cAT- and cBM-MSCs for canine patients.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Adipogenic (Oil Red O)
Osteogenic (Alizarin Red S)
Chondrogenic (Alcian Blue)

Figure 1. Characteristics of MSCs isolated from canine adipose tissue and bone marrow. Immunophenotypic analysis by flow cytometry of canine-adipose-tissue-derived mesenchymal stem cells (A), and canine-bone-marrow-derived mesenchymal stem cells (B). Adipogenic (Oil Red O staining), osteogenic (Alizarin Red S staining), and chondrogenic (Alcian Blue staining) differentiation of cAT-MSCs (C), and cBM-MSCs (D).

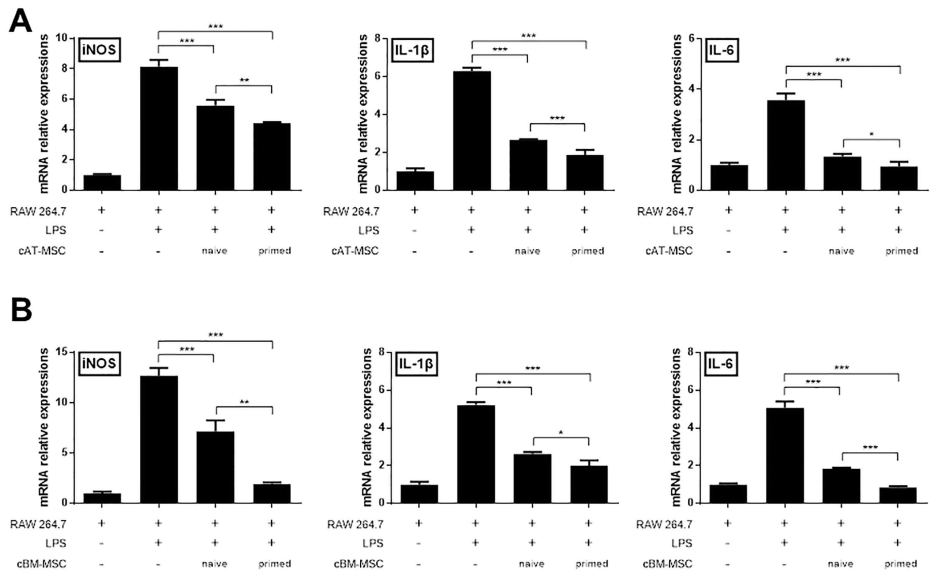

Figure 2. Anti-inflammatory effects of primed cAT- and cBM-MSCs on LPS-stimulated RAW 264.7 cells. Effect of cAT-MSCs on anti-inflammatory cytokine levels. mRNA expression levels of iNOS, IL-1β, and IL-6 in RAW 264.7 cells (A). Effect of cBM-MSCs on anti-inflammatory cytokine levels. mRNA expression levels of iNOS, IL-1β, and IL-6 in RAW 264.7 cells (B). Data are shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001. (RAW 264.7 +: exist, LPS -: non-treated, LPS +: treated, cAT- and cBM-MSCs -: absence)

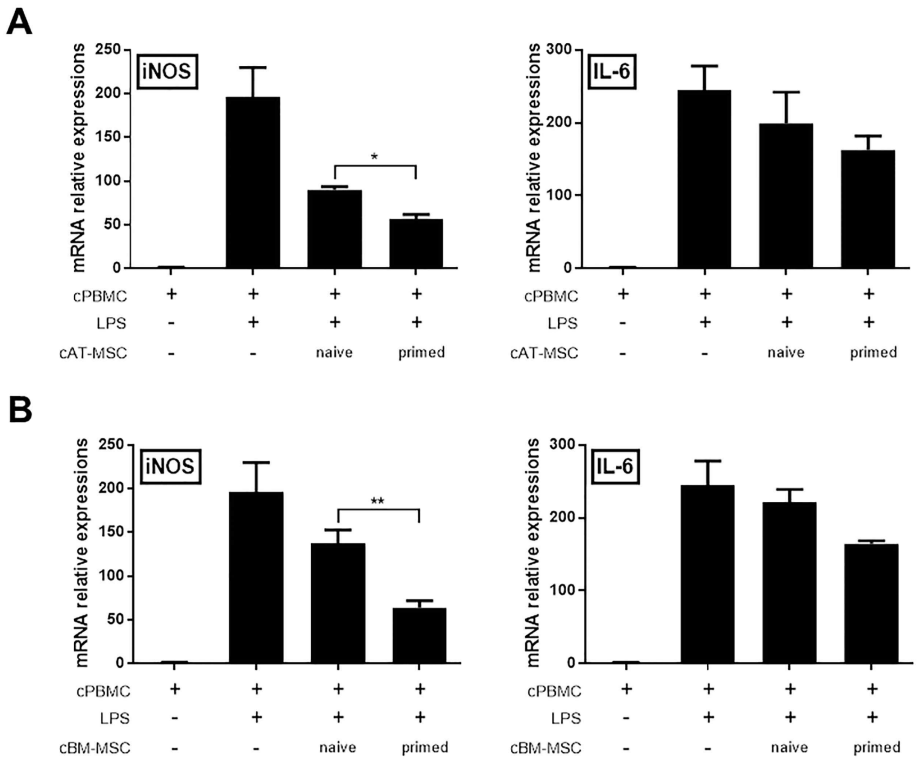

Figure 3. Anti-inflammatory effects of primed cAT- and cBM-MSCs on LPS-stimulated cPBMCs. Effect of cAT-MSCs on anti-inflammatory cytokine levels. mRNA expression levels of iNOS and IL-6 in cPBMCs (A). Effect of cBM-MSCs on anti-inflammatory cytokine levels. mRNA expression levels of iNOS and IL-6 in cPBMCs (B). Data are shown as mean ± SD. *P < 0.05, **P < 0.01. (cPBMC +: exist, LPS -: non-treated, LPS +: treated, cAT- and cBM-MSCs -: absence)

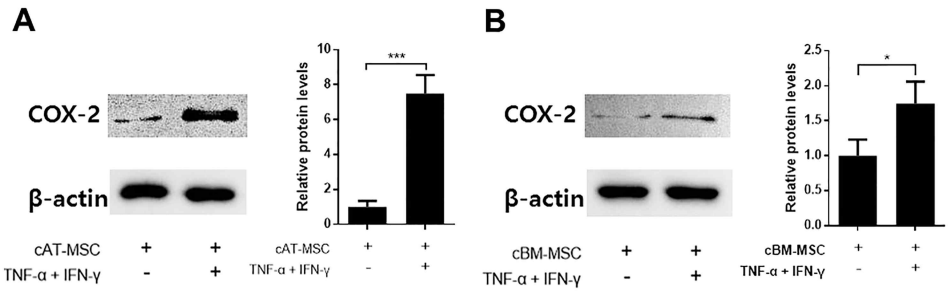

Figure 4. COX-2 protein level in TNF-α- and IFN-γ-primed cMSCs. The COX-2 level in TNF-α/IFN-γ-primed cAT-MSCs was 7.4 times higher than in naïve cAT-MSCs (A). Likewise, the COX-2 level in TNF-α/IFN-γ-primed cBM-MSCs was 1.7 times greater than in naïve cBM-MSCs (B). *P < 0.05, ***P < 0.001. (cAT- and cBM-MSCs +: exist, TNF-α+IFN-γ -: non-treated, TNF-α+IFN-γ +: treated)

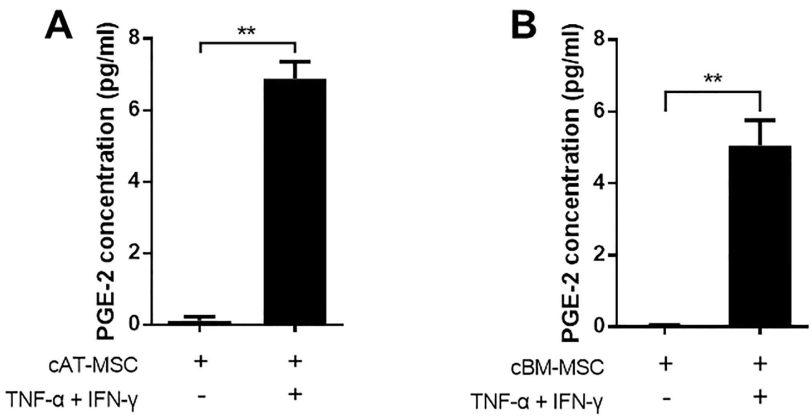

Figure 5. Protein level of PGE_2 in supernatant of TNF-α- and IFN-γ-primed cMSCs. Higher levels of PGE_2 production were measured in supernatant of TNF-α/IFN-γ-primed cAT- (A) and cBM-MSCs (B) than in supernatants of naïve cAT- and cBM-MSCs. **P < 0.01. (cAT- and cBM-MSCs +: exist, TNF-α+IFN-γ -: non-treated, TNF-α+IFN-γ +: treated)

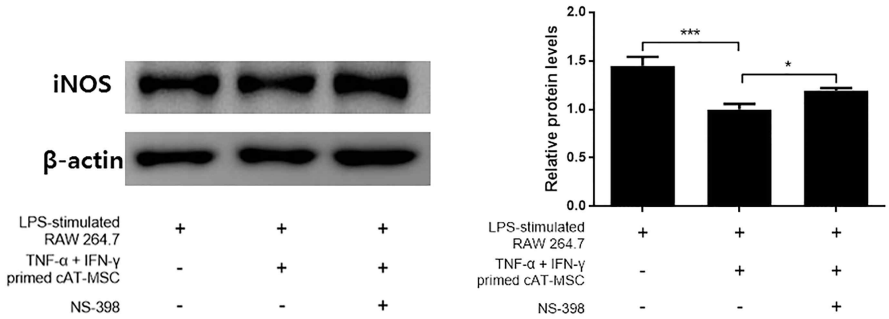

Figure 6. Changes in the secretion of PGE_2 in NS-398-treated CM. Decreased the protein level of iNOS in LPS-stimulated RAW 264.7 cells co-culture with TNF-α/IFN-γ-primed canine AT-MSCs was increased again in the NS-398 treated group. *P < 0.05, ***P < 0.001. (LPS-stimulated RAW 264.7 +: exist, TNF-α+IFN-γ primed cAT-MSC -: absence, TNF-α+IFN-γ primed cAT-MSC +: presence, NS-398 -: non-treated, NS-398 +: treated)

6. References

Aggarwal, S., Pittenger, M.F., 2005. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 105, 1815–1822.

Al–Nbaheen, M., Ali, D., Bouslimi, A., Al–Jassir, F., Megges, M., Prigione, A., Adjaye, J., Kassem, M., Aldahmash, A., 2013. Human stromal (mesenchymal) stem cells from bone marrow, adipose tissue and skin exhibit differences in molecular phenotype and differentiation potential. Stem Cell Reviews and Reports 9, 32–43.

Baksh, D., Yao, R., Tuan, R.S., 2007. Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. Stem Cells 25, 1384–1392.

Barrachina, L., Remacha, A.R., Romero, A., Vázquez, F.J., Albareda, J., Prades, M., Gosálvez, J., Roy, R., Zaragoza, P., Martín–Burriel, I., 2017. Priming equine bone marrow–derived mesenchymal stem cells with proinflammatory cytokines: implications in immuno modulation-immunogenicity balance, cell viability, and differentiation potential. Stem Cells and Development 26, 15–24.

Carrade, D.D., Borjesson, D.L., 2013. Immunomodulation by mesenchymal stem cells in veterinary species.

Comparative Medicine 63, 207-217.

Chiesa, S., Morbelli, S., Morando, S., Massollo, M., Marini, C., Bertoni, A., Frassoni, F., Bartolomé, S.T., Sambuceti, G., Traggiai, E., 2011. Mesenchymal stem cells impair in vivo T-cell priming by dendritic cells. Proceedings of the National Academy of Sciences 108, 17384-17389.

Das, R., Jahr, H., van Osch, G.J., Farrell, E., 2009. The role of hypoxia in bone marrow-derived mesenchymal stem cells: considerations for regenerative medicine approaches. Tissue Engineering Part B: Reviews 16, 159-168.

Dave, M., Mehta, K., Luther, J., Baruah, A., Dietz, A.B., Faubion Jr, W.A., 2015. Mesenchymal stem cell therapy for inflammatory bowel disease: a systematic Review and Meta-analysis. Inflammatory Bowel Diseases 21(11), 2696-2707.

English, K., Barry, F.P., Field-Corbett, C.P., Mahon, B.P., 2007. IFN-γ and TNF-α differentially regulate immuno modulation by murine mesenchymal stem cells. Immunology Letters 110, 91-100.

Ferrer, L., Kimbrel, E.A., Lam, A., Falk, E.B., Zewe, C., Juopperi, T., Lanza, R., Hoffman, A., 2016. Treatment of perianal fistulas with human embryonic stem cell-derived mesenchymal stem cells: a canine model of human fistulizing Crohn's disease. Regenerative Medicine 11, 33-43.

Ghannam, S., Bouffi, C., Djouad, F., Jorgensen, C., Noël, D., 2010. Immunosuppression by mesenchymal stem cells: mechanisms and clinical applications. Stem Cell Research & Therapy 1, 2.

Glenn, J.D., Whartenby, K.A., 2014. Mesenchymal stem cells: emerging mechanisms of immunomodulation and therapy. World Journal of Stem Cells 6, 526.

Hemeda, H., Jakob, M., Ludwig, A.-K., Giebel, B., Lang, S., Brandau, S., 2010. Interferon-γ and tumor necrosis factor-α differentially affect cytokine expression and migration properties of mesenchymal stem cells. Stem Cells and Development 19, 693-706.

Hoffman, A.M., Dow, S.W., 2016. Concise review: stem cell trials using companion animal disease models. Stem Cells 34, 1709-1729.

Jacobs, S.A., Roobrouck, V.D., Verfaillie, C.M., Van Gool, S.W., 2013. Immunological characteristics of human mesenchymal stem cells and multipotent adult progenitor cells. Immunology and Cell Biology 91, 32.

Kang, B.-J., Ryu, H.-H., Park, S.S., Koyama, Y., Kikuchi, M., Woo, H.-M., Kim, W.H., Kweon, O.-K., 2012. Comparing the osteogenic potential of canine mesenchymal stem cells derived from adipose tissues, bone marrow, umbilical cord blood, and Wharton's jelly for treating bone defects. Journal of Veterinary Science 13, 299.

Kang, J.W., Kang, K.S., Koo, H.C., Park, J.R., Choi, E.W., Park, Y.H., 2008. Soluble factors-mediated immunomodulatory effects of canine adipose tissue-derived mesenchymal stem cells. Stem Cells Dev 17, 681-693.

Karpov, A.A., Uspenskaya, Y.K., Minasian, S.M., Puzanov, M.V., Dmitrieva, R.I., Bilibina, A.A., Anisimov, S.V., Galagudza, M.M., 2013. The effect of bone marrow-and adipose tissue-derived mesenchymal stem cell transplantation on myocardial remodelling in the rat model of ischaemic heart failure. International Journal of Experimental Pathology 94, 169-177.

Kim, H.-W., Song, W.-J., Li, Q., Han, S.-M., Jeon, K.-O., Park, S.-C., Ryu, M.-O., Chae, H.-K., Kyeong, K., Youn, H.-Y., 2016. Canine adipose tissue-derived mesenchymal stem cells ameliorate severe acute pancreatitis by regulating T cells in rats. Journal of Veterinary Science 17, 539-548.

Kim, J., Kang, J.W., Park, J.H., Choi, Y., Choi, K.S., Park, K.D., Baek, D.H., Seong, S.K., Min, H.-K., Kim, H.S., 2009. Biological characterization of long-term cultured human mesenchymal stem cells. Archives of Pharmacal Research 32, 117-126.

Kisiel, A.H., McDuffee, L.A., Masaoud, E., Bailey, T.R., Gonzalez, B.P.E., Nino-Fong, R., 2012. Isolation, characterization, and in vitro proliferation of canine mesenchymal stem

cells derived from bone marrow, adipose tissue, muscle, and periosteum. American Journal of Veterinary Research 73, 1305-1317.

Le Blanc, K., Rasmusson, I., Sundberg, B., Götherström, C., Hassan, M., Uzunel, M., Ringdén, O., 2004. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. The Lancet 363, 1439-1441.

Lee, K.S., Kang, H.W., Lee, H.T., Kim, H.-J., Kim, C.-L., Song, J.-Y., Lee, K.W., Cha, S.-H., 2014. Sequential sub-passage decreases the differentiation potential of canine adipose-derived mesenchymal stem cells. Research in Veterinary Science 96, 267-275.

Lee, W.S., Suzuki, Y., Graves, S.S., Iwata, M., Venkataraman, G., Mielcarek, M., Peterson, L.J., Ikehara, S., Torok-Storb, B., Storb, R., 2011. Canine bone marrow-derived mesenchymal stromal cells suppress alloreactive lymphocyte proliferation in vitro but fail to enhance engraftment in canine bone marrow transplantation. Biology of Blood and Marrow Transplantation 17, 465-475.

Liu, L., Song, H., Duan, H., Chai, J., Yang, J., Li, X., Yu, Y., Zhang, X., Hu, X., Xiao, M., 2016. TSG-6 secreted by human umbilical cord-MSCs attenuates severe burn-induced excessive inflammation via inhibiting activations

of P38 and JNK signaling. Scientific Reports 6, 30121.

Liu, W., Zhang, S., Gu, S., Sang, L., Dai, C., 2015. Mesenchymal stem cells recruit macrophages to alleviate experimental colitis through TGFβ1. Cellular Physiology and Biochemistry 35, 858-865.

Ma, S., Xie, N., Li, W., Yuan, B., Shi, Y., Wang, Y., 2014. Immunobiology of mesenchymal stem cells. Cell Death and Differentiation 21, 216-225.

Madrigal, M., Rao, K.S., Riordan, N.H., 2014. A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods. Journal of Translational Medicine 12, 260.

Manferdini, C., Maumus, M., Gabusi, E., Piacentini, A., Filardo, G., Peyrafitte, J.A., Jorgensen, C., Bourin, P., Fleury-Cappellesso, S., Facchini, A., 2013. Adipose-derived mesenchymal stem cells exert antiinflammatory effects on chondrocytes and synoviocytes from osteoarthritis patients through prostaglandin E2. Arthritis & Rheumatology 65, 1271-1281.

Marx, C., Silveira, M.D., Beyer Nardi, N., 2015. Adipose-derived stem cells in veterinary medicine: characterization and therapeutic applications. Stem Cells and Development 24, 803-813.

Matthay, M.A., Goolaerts, A., Howard, J.P., Lee, J.W., 2010.

Mesenchymal stem cells for acute lung injury: preclinical evidence. Critical Care Medicine 38, S569-573.

Plock, J., Schnider, J., Zhang, W., Tsuji, W., Schweizer, R., Kostereva, N., Fanzio, P., Ravuri, S., Solari, M., Marra, K., Gorantla, V., 2014. Immunomodulation with adipose and bone marrow-derived mesenchymal stem cells in vascularized composite allotransplantation. Vascularized Composite Allotransplantation 1, 64-64.

Prasanna, S.J., Gopalakrishnan, D., Shankar, S.R., Vasandan, A.B., 2010. Pro-inflammatory cytokines, IFNγ and TNFα, influence immune properties of human bone marrow and Wharton jelly mesenchymal stem cells differentially. PLoS One 5, e9016.

Prockop, D.J., Oh, J.Y., 2012. Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation. Molecular Therapy 20, 14-20.

Puissant, B., Barreau, C., Bourin, P., Clavel, C., Corre, J., Bousquet, C., Taureau, C., Cousin, B., Abbal, M., Laharrague, P., 2005. Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. British Journal of Haematology 129, 118-129.

Ribeiro, A., Laranjeira, P., Mendes, S., Velada, I., Leite, C., Andrade, P., Santos, F., Henriques, A., Grãos, M., Cardoso, C.M., 2013. Mesenchymal stem cells from

umbilical cord matrix, adipose tissue and bone marrow exhibit different capability to suppress peripheral blood B, natural killer and T cells. Stem Cell Research & Therapy 4, 125.

Shi, Y., Hu, G., Su, J., Li, W., Chen, Q., Shou, P., Xu, C., Chen, X., Huang, Y., Zhu, Z., 2010. Mesenchymal stem cells: a new strategy for immunosuppression and tissue repair. Cell Research 20, 510–518.

Shi, Y., Su, J., Roberts, A.I., Shou, P., Rabson, A.B., Ren, G., 2012. How mesenchymal stem cells interact with tissue immune responses. Trends in Immunology 33, 136–143.

Singh, S., Deka, D., Mulinti, R., Sood, N.K., Agrawal, R.K., Verma, R., 2014. Isolation, culture, in–vitro differentiation and characterization of canine adult mesenchymal stem cells. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences 84, 875–884.

Solchaga, L.A., Zale, E.A., 2012. Prostaglandin E2: a putative potency indicator of the immunosuppressive activity of human mesenchymal stem cells. American Journal of Stem Cells 1, 138–145.

Song, W.–J., Li, Q., Ryu, M.–O., Ahn, J.–O., Bhang, D.H., Jung, Y.C., Youn, H.–Y., 2017. TSG–6 secreted by human adipose tissue–derived mesenchymal stem cells ameliorates DSS–induced colitis by Inducing M2 macrophage polarization in mice. Scientific Reports 7,

5187.

Spaggiari, G.M., Capobianco, A., Abdelrazik, H., Becchetti, F., Mingari, M.C., Moretta, L., 2008. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2, 3-dioxygenase and prostaglandin E2. Blood 111, 1327-1333.

Sullivan, M.O., Gordon-Evans, W.J., Fredericks, L.P., Kiefer, K., Conzemius, M.G., Griffon, D.J., 2015. Comparison of mesenchymal stem cell surface markers from bone marrow aspirates and adipose stromal vascular fraction sites. Frontiers in Veterinary Science, 15.

Timmers, L., Lim, S.K., Hoefer, I.E., Arslan, F., Lai, R.C., van Oorschot, A.A., Goumans, M.J., Strijder, C., Sze, S.K., Choo, A., 2011. Human mesenchymal stem cell-conditioned medium improves cardiac function following myocardial infarction. Stem Cell Research 6, 206-214.

Uccelli, A., Pistoia, V., Moretta, L., 2007. Mesenchymal stem cells: a new strategy for immunosuppression? Trends in Immunology 28, 219-226.

Vieira, N., Brandalise, V., Zucconi, E., Secco, M., Strauss, B., Zatz, M., 2010. Isolation, characterization, and differentiation potential of canine adipose-derived stem cells. Cell Transplantation 19, 279-289.

Yagi, H., Soto-Gutierrez, A., Parekkadan, B., Kitagawa, Y.,

Tompkins, R.G., Kobayashi, N., Yarmush, M.L., 2010. Mesenchymal stem cells: mechanisms of immuno modulation and homing. Cell Transplantation 19, 667-679.

Yu, S.P., Wei, Z., Wei, L., 2013. Preconditioning strategy in stem cell transplantation therapy. Translational Stroke Research 4, 76-88.

국문 초록

TNF-α 및 IFN-γ로 처리된 개 중간엽줄기세포의 COX-2/PGE2 경로를 통한 항염증 효과 향상

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양 혜 미

중간엽줄기세포(Mesenchymal stem cells)는 다양한 질병의 치료제로 연구되어 왔으며, 면역 매개 질환에 대한 응용 또한 관심을 끌고있다. 사람의 중간엽줄기세포의 면역 조절 효과를 향상시키기위한 다양한 방법이 사용되어왔으나, 개의 중간엽줄기세포에 대한 접근법은 연구가 부족한 실정이었다. 따라서 본 학위 논문은 TNF-α 및 IFN-γ로 처리된 개의 중간엽줄기세포에서 면역 조절 효과와 그 기전을 평가하고자 하였다. TNF-α와 IFN-γ로 24시간 동안 개의 중간엽줄기세포를 자극하여 적응용 배지(conditioned media)를 만들었다. 마우스의 면

역 세포(RAW 264.7)를 지질다당질(lipopolysaccharide, LPS)로 자극하여, 적응용 배지에서 48시간 동안 개의 중간엽줄기세포와 함께 배양하였다. 개의 면역 세포(cPBMC)로 동일하게 반복 실험하였다. 역전사 중합효소 연쇄반응(qRT-PCR)에 의해 RNA의 발현을, 웨스턴 블롯(Western blot) 분석을 통하여 단백질 수준에서 평가 하였다. LPS의 자극을 받아 증가한 유도 산화질소 합성효소(iNOS), 인터루킨-6 및 인터루킨-1β의 발현이 개의 중간엽줄기세포와 함께 배양하였을 때 통계적으로(일원분산분석) 유의적으로 감소하였다($P < 0.001$). 또한, TNF-α 및 IFN-γ 전처리된 개의 중간엽줄기세포와 처리되지 않은 순수한 개의 중간엽줄기세포와 비교하였을 때 항염증 효과가 유의적으로 증가됨을 확인하였다($P < 0.05$). 다음으로, COX-2와 PGE_2의 발현이 전처리된 개의 중간엽줄기세포에서 유의하게 증가함을 확인하였다($P < 0.05$). 마지막으로, LPS에 자극 받은 RAW 264.7 세포에서 감소되었던 iNOS 단백질의 양이 NS-398 (PGE_2억제제)으로 처리하였을 때 재증가함을 확인하였다($P < 0.01$). 본 연구는 TNF-α와 IFN-γ로 전처리된 개의 중간엽줄기세포에서 처리되지 않은 순수한 개의 중간엽줄기세포와 비교하였을 때, LPS로 자극된 세포에서 방출되는 항염증성 사이토카인이 보다 더 감소하며, 그 기전 중 하나로 COX-2/PGE_2경로가 관여함을 과학적으로 알아낸 중요한 결과로 임상적으로 개 환자의 치료에 효과적으로 적용할 수 있을 것으로 기대된다.

주요어: 중간엽줄기세포; 염증 사이토카인; COX-2; PGE_2; 항염증

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