A THESIS FOR THE DEGREE OF MASTER

Immunomodulatory Effects of Soluble Factors Secreted by Feline Adipose Tissue-derived Mesenchymal Stem Cells

고양이 지방 유래 중간엽 줄기세포에서 분비되는 가용성 인자들의 면역조절능에 대한 연구

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Abstract

Immunomodulatory Effects of Soluble Factors Secreted by Feline Adipose Tissue-derived Mesenchymal Stem Cells

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Mesenchymal stem cells (MSCs) have immunomodulatory functions and differentiation capacity, and their clinical use is increasing in veterinary species. Although MSCs have been applied in the treatment in various inflammatory diseases, mechanistic research on feline MSCs is lacking. Accordingly, in this study, we aimed to investigate the immunomodulatory mechanisms of MSCs isolated from feline adipose tissue (fATMSCs).

fATMSCs from healthy cats were cultured in an appropriate manner and cocultured with transwell-separated allogeneic feline peripheral blood mononuclear cells (fPBMCs) and RAW264.7 murine macrophagess. After 48 h of coculture, RNA was extracted from RAW264.7 cells and fPBMCs. Cytokine expression in these cells was measured using quantitative real-time polymerase chain reaction (qRT-PCR) and compared according to the presence of fATMSCs. The mRNA levels of pro-inflammatory cytokines, e.g., tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase, and interleukin (IL)-1β, were significantly decreased in cocultures of mitogen-stimulated RAW264.7 cells with fATMSCs compared with that in the RAW264.7 cells control group. Additionally, changes in the expression of mRNAs extracted from fPBMCs were as follows: pro-inflammatory TNF-α, interferon-γ, and IL-6 were decreased, and anti-inflammatory IL-10 was increased during coculture of mitogen-stimulated allogeneic fPBMCs with fATMSCs. We also extracted RNA and collected supernatants from fATMSCs during transwell culture for measurement of the expression and secretion of soluble factors by qRT-PCR and enzyme-linked immunosorbent assays, respectively. The mRNA expression of immunomodulatory factors from fATMSCs, including cyclooxygenase-2 (COX-2), transforming growth factor (TGF)-β, indoleamine-2,3-dioxygenase (IDO) and hepatocyte growth factor, increased in the presence of RAW264.7 cells. Similarly, TGF-β, COX-2, and IDO mRNA expression and prostaglandin E_2 (PGE_2) secretion

from fATMSCs increased in the presence of allogeneic fPBMCs. Finally, we measured the viability of fPBMCs under various conditions. Cell viability decreased in fPBMCs suspended in fATMSC-derived conditioned medium, and this reduction was alleviated in the group supplemented with NS-398 a PGE_2 inhibitor. Our data suggested that soluble factors, including PGE_2, secreted by fATMSCs played an important role in the immunomodulatory effects of these cells. These findings may be helpful in the application of fATMSCs to feline patients with immune-related diseases.

Key words: feline mesenchymal stem cells, immunomodulation, soluble factor, macrophages, cytokines, prostaglandin E_2

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1. Introduction

According to the criteria of the International Society for Cellular Therapy (ISCT), mesenchymal stem cells (MSCs) are defined as plastic-adherent cells that proliferate in vitro, exhibit a fibroblast-like shape, express specific markers measured by flow cytometry, and have the ability to differentiate into adipocytes, osteoblasts and chondroblasts.[1-3] MSCs have been widely used in regenerative medicine due to their multipotential properties[4, 5] and have been shown to be effective in treating immune-related disorders, including arthritis, graft-versus-host disease and inflammatory bowel diseases.[6-10] Based on their immunomodulatory functions[3, 6-12], MSCs have emerged as an investigational therapy for diseases that are not responsive to current immunosuppressive therapies.

The immunomodulatory capacity of MSCs is thought to be achieved primarily through two mechanisms: 1) cell-to-cell direct contact and 2) indirect control via secreted soluble factors.[13, 14] Previous mechanistic studies related to soluble factors have mainly been carried out using human and rodent MSCs.[15, 16] Immunosuppressive soluble factors secreted from MSCs include indoleamine-2,3-dioxygenase (IDO), prostaglandin E-2 (PGE_2), tumor necrosis factor (TNF)-α-stimulated

gene/protein 6, nitric oxide (NO) and transforming growth factor-β (TGF-β).[13-15] These secreted factors vary slightly for different animals and have been investigated in various veterinary species.[15, 17, 18] Based on mechanistic studies, MSCs are used in the treatment of various inflammatory diseases in veterinary medicine, including osteoarthritis in canines and tendon injury in equines.[19-23]

In feline medicine, MSCs have been applied to the treatment in various inflammatory diseases, including asthma, gingivostomatitis and chronic kidney disease.[24-26] However, few studies have characterized the factors secreted by feline MSCs, and the functions of these cells are not well understood. Accordingly, in this study, we analyzed the immunomodulatory mechanisms of feline adipose tissue-derived MSCs (fATMSCs). Our findings provided important insights into the immunomodulatory abilities and immunosuppressive soluble factors of fATMSCs.

2. Material and Methods

2.1. Isolation and characterization of MSCs

fATMSCs were isolated from the abdominal cavity of a healthy donor cat (domestic short hair, 1-year-old, female) during ovariohysterectomy at the Seoul National University Veterinary Medicine Teaching Hospital (SNU VMTH) with the consent of the owner. The donor cat was determined not to have any infectious diseases and showed normal blood analysis and imaging findings. The isolated tissue was washed three times with phosphate-buffered saline (PBS; PAN Biotech) containing 1% penicillin and streptomycin (PS; PAN Biotech). Mechanical dissociation was performed with a sterile blade and scissors. Tissue was incubated for 1 h at 37°C in 5% CO_2 with filtered 0.1% collagenase type IA (1 mg/mL; Sigma-Aldrich) solution. After incubation, tissue was transferred to a conical tube and neutralized with Dulbecco's modified Eagle's medium (DMEM; PAN Biotech) containing 20% fetal bovine serum (FBS; PAN Biotech) and 1% PS. After centrifugation at 1200 × *g* for 5 min, the supernatant was removed, and remaining pellets were suspended with DMEM containing 20% FBS and 1% PS. Suspended solutions were passed

through a 70-μm Falcon cell strainer (Fisher Scientific) to remove debris. We repeated the centrifugation and removed the supernatant. Remaining pellets were resuspended in RBC lysis buffer (Sigma-Aldrich) and incubated at 25°C for 5 min. Pellets were resuspended in DMEM containing 20% FBS and 1% PS and transferred to 100-mm dishes at a density of 3,000/cm^2. Transferred cells were incubated at 37°C in a humidified atmosphere of 5% CO_2, and the medium was replaced every 2–3 days until the adhered cells showed a fibroblast-like morphology. After adhering to plastic culture plates and obtaining a spindle-shaped morphology, 1×10^6 cells were characterized by flow cytometry using antibodies against the following proteins, as described previously [2], [27] : cluster of differentiation (CD) 9, CD44 (GeneTex), CD34-phycoerythrin (PE) and CD45-fluorescein isothiocyanate (FITC; eBiosciences). For CD9 and CD44, indirect immunofluorescence was performed using goat anti-mouse IgG-FITC and goat anti-rat IgG-PE (Santa Cruz Biotechnology), respectively. Characterization results were analyzed using FlowJo 7.6.5 software (Tree Star, Inc.). The differentiation capacity of the cells was confirmed by identifying differentiated cells using special differentiation media (StemPro Adipogenesis Differentiation, StemPro Osteogenesis Differentiation and StemPro Chondrogenesis Differentiation kits; Gibco/Life Technologies) according to the manufacturer's instructions. Oil Red O staining, 1%

Alizarin Red staining and Alcian Blue staining (all from Sigma-Aldrich) were used to confirm the presence of adipocytes, osteoblasts and chondroblasts, respectively.

2.2. Isolation of feline peripheral blood mononuclear cells (fPBMCs)

The blood of healthy feline donors was obtained from SNU VMTH with the consent of the owner, diluted with an equal volume of PBS, and then layered over Ficoll-Paque PLUS (GE Healthcare Life Sciences) in a conical tube. Donor cats (domestic short hair, 3–6 years old, castrated males) did not have infectious diseases, and the process for collecting 10 mL blood was approved by the Institutional Animal Care and Use Committee (IACUC) in SNU (IACUC approval no. SNU-161026-4). After centrifugation at 400 × *g* for 30 min, the buffy coat layer was carefully collected. The collected samples were resuspended with RBC lysis buffer and incubated at 25°C for 15 min. After PBS addition, samples were centrifuged at 3000 rpm for 10 min. Washing and centrifugation were repeated once more, and fPBMCs were resuspended in Roswell Park Memorial Institute (RPMI)-1640 medium (PAN Biotech) containing 20% FBS and 1% PS.

2.3. Cell culture

RAW264.7 murine macrophages were purchased from the Korean Cell Line Bank. RAW264.7 cells were cultured in RPMI medium containing 10% FBS and 1% PS. fATMSCs were cultured in DMEM containing 20% FBS and 1% PS, as described previously [2]. Medium was replaced every 2–3 days until the cells reached 70–80% confluence. After reaching 70–80% confluence, cells were banked or subcultured. All fATMSCs used in the experiment were in passage 3 or 4, as described in previous studies, suggesting that cells in the early passages are effective [28, 29]. fPBMCs were cultured in RPMI medium containing 20% FBS and 1% PS. All cells used in this study were incubated at 37°C in a humidified atmosphere containing 5% CO_2 until use.

2.4. Preparation of conditioned medium (CM)

fATMSCs (5×10^5 cells/well) were seeded in 6-well plates and cultured in RPMI medium containing 20% FBS and 1% PS for 3 days, as previously described, yielding conditioned medium [30]. After 3 days, conditioned medium was harvested and centrifuged at 850 rpm for 5 min to

remove cellular debris. After centrifugation, the supernatant was transferred to a conical tube and stored at -80°C until use.

2.5. Analysis of cell viability

Isolated fPBMCs was stimulated with 5 μg/mL concanavalin A (ConA; Sigma-Aldrich) and 1 μg/mL lipopolysaccharide (LPS; Sigma-Aldrich) for 4 h. The stimulated fPBMCs were transferred to a conical tube and centrifuged at 3000 rpm for 10 min. The supernatant was removed, and the remaining fPBMCs were resuspended in RPMI containing 20% FBS and 1% PS or CM from fATMSCs. fPBMCs from each group (1×10^5 cells/well) were seeded in 96-well plates (SPL Life Sciences). After incubation for 48 h, 5 mg/mL 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich) solution was added at 10% of the total volume of sample in each well and mixed. The cells were incubated for 4 h, and the supernatant was removed carefully. One hundred microliters of dimethyl sulphoxide (Sigma-Aldrich) was added to each well, and the plate was mixed well for 15 min. The absorbance was measured at 540 nm and corrected at 650 nm using a spectrophotometer (680 Microplate Reader). Additionally, in other experiments, the PGE_2 inhibitor NS-398 (5 μM; Enzo Life Sciences) was added to some CM groups obtained from fATMSCs to

determine whether PGE_2 secreted by fATMSCs had a significant effect on the proliferation of fPBMCs. The viability of fPBMCs from each group was measured using a D-Plus CCK Cell Viability Assay Kit (DonginLS) according to the manufacturer's instructions.

2.6. Transwell cultures and RNA extraction

fPBMCs and RAW264.7 cells were cocultured with fATMSCs. Cells were separated by a transwell membrane (0.4 μm pore size; Costar). Some RAW264.7 cells were stimulated with 2.5 μg/mL LPS for 4 h. Stimulated and unstimulated RAW264.7 cells (1×10^6 cells/well) were seeded in triplicate in the lower chamber of 6-well plates as a control. Stimulated RAW264.7 cells (1×10^6 /well) were seeded in the lower chamber of 6-well plates, and fATMSCs (2×10^5 cells/well) were seeded in 0.4-μm pore-sized transwell inserts (SPL Life Sciences). fPBMCs were stimulated with 5 μg/mL ConA and 1 μg/mL LPS (1×10^6 cells/well) and seeded in the lower chambers of 6-well plates. Some of the stimulated fPBMCs were cocultured with transwell-separated fATMSCs (2×10^5 cells/well). fPBMCs without fATMSCs and fATMSCs without fPBMCs were cultured as controls. After incubation for 48 h, total RNA from all cell groups was isolated using an Easy-BLUE Total RNA Extraction Kit (Intron

Biotechnology) following the manufacturer's instructions. Briefly, 200 μL chloroform was added to the isolated RNA with 1 mL Easy-BLUE reagent, followed by vortexing. After centrifugation at 13,000 rpm for 10 min at 4°C, 400 μL of the upper fluid was transferred to another Eppendorf tube and an equal volume of isopropanol solution was added. The mixture was left at 25°C for 10 min and centrifuged at 13,000 rpm for 5 min at 4°C. After removal of the supernatant, remaining pellets were washed with 75% ethanol and air-dried. Pellets were resuspended in 20 μL diethylpyrocarbonate (DEPC)-treated distilled water. RNA purity and concentration were measured using a nanophotometer (Implen).

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

Extracted mRNA was converted into cDNA using LaboPass M-MuLV Reverse Transcriptase (Cosmo Genetech) following the manufacturer's instructions. The cytokine RNA levels were measured by qRT-PCR. A master mix was prepared by adding 7.4 μL PCR-grade dH_2O, 10 μL AMPIGENE qRT-PCR Green Mix Hi-RO with SYBR Green dye (Enzo Life Sciences), 0.8 μL forward and reverse primers (Cosmo Genetech; Table 1) and 1 μL template cDNA. Samples were dispensed in duplicate, and the instrument was programmed using following conditions:

polymerase activation (2 min at 95°C), denaturation and annealing/extension (40 cycles at 95°C for 5 s and 62.5°C for 25 s) and melt analysis (according to the manufacturer's instructions). Quantification of cytokine mRNA levels was determined by comparison with glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.8. Enzyme-linked immunosorbent assay (ELISA)

During transwell culture, all supernatants from fPBMCs and fATMSCs were obtained after 48 h of incubation. After 5 min of centrifugation at 850 rpm to remove debris, the supernatant was used for protein analysis. PGE_2 amount was measured using PGE_2 ELISA (Enzo Life Sciences) according to the manufacturer's instructions.

2.9. Statistical analysis

All experimental data were analyzed using GraphPad Prism v.6.01 software (GraphPad Software Inc.). One-way analysis of variance (ANOVA) and unpaired Student's *t*-tests were used to analyze the data. Differences with *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Characterization of MSCs

Isolated feline adipose tissue-derived cells were proven to be MSCs based on the criteria of the ISCT. The cultured cells had a fibroblast-like morphology and the ability to adhere to plastic culture plates (data not shown). The phenotypes of the cells were analyzed by flow cytometry. The cells were positive for CD9 and CD44 and negative for CD34 and CD45 (Figure 1a). When specific differentiation media were used, fATMSCs showed the ability to differentiate into adipocytes, osteoblasts and chondroblasts (Figure 1b–d).

3.2. Anti-inflammatory effects of fATMSCs on RAW264.7 cells

In order to ascertain the immunomodulatory capacity of fATMSCs, several pro-inflammatory cytokines secreted by RAW264.7 cells were measured at the RNA level by qRT-PCR. The mRNA levels of *TNF-α*, inducible nitric oxide synthase (*iNOS*) and interleukin-1β (*IL-1β*) increased significantly in RAW264.7 cells stimulated with LPS compared with that in unstimulated RAW264.7 cells. When stimulated RAW264.7 cells and

fATMSCs were cocultured using transwells, TNF-α, iNOS and IL-1β expression levels were significantly reduced ($P< 0.01$; Figure 2a).

Cyclooxygenase-2 (COX-2), TGF-β, IDO and hepatocyte growth factor (HGF) are immunomodulatory factors secreted by MSCs.[17], [18]To determine whether the expression levels of these factors were increased in fATMSCs cocultured with RAW264.7 cells, RNA was extracted from fATMSCs in transwell inserts and measured by qRT-PCR. The expression levels of these factors in fATMSCs cocultured with RAW264.7 cells was significantly increased compared with that obtained from fATMSCs cultured in the absence of RAW264.7 cells ($P< 0.05$; Figure 2b).

3.3. Suppression of fPBMCs proliferation by CM from fATMSCs

To evaluate the effect of fATMSCs conditioned media on the proliferation of the fPBMCs, isolated fPBMCs stimulated with mitogens were suspended in RPMI containing 20% FBS, 1% PS or fATMSCs-derived CM and seeded in 96-well. After 48h incubation, proliferation of the fPBMCs was evaluated by MTT assay. As a result, the group suspended in fATMSC-derived CM showed less cell viability on both fPBMCs stimulated with ConA and LPS ($P< 0.001$) (Figure 3).

3.4. Anti-inflammatory effects of fATMSCs on fPBMCs

Next, the expression levels of several fPBMC-derived cytokines, including TNF-α, IFN-γ, IL-6 and IL-10, known to play important roles in immunomodulation[13, 31-33], were measured by qRT-PCR. The levels of TNF-α, IFN-γ and IL-6 markedly increased after mitogenic stimulation, whereas that of IL-10, which has immunosuppressive properties[33], decreased after mitogenic stimulation. Following treatment with ConA, the levels of TNF-α, IFN-γ and IL-6 significantly decreased when fPBMCs were cocultured with fATMSCs ($P< 0.05$). In contrast, the level of IL-10 tended to increase in the coculture; however, this difference was not significant (Figure 4a). When fATMSCs were cocultured with LPS-stimulated fPBMCs, the levels of TNF-α, IFN-γ, and IL-6 decreased significantly, whereas the level of IL-10 increased significantly ($P< 0.05$; Figure 4b).

3.5. Increased expression of immunomodulatory factors in fATMSCs cocultured with allogeneic fPBMCs

To determine whether immunomodulatory factors containing COX-2, TGF-β and IDO were increased in fATMSCs cocultured with fPBMCs at the RNA level, we measured the expression levels of these factors using

qRT-PCR after isolation of mRNA from fATMSCs. The expression levels of COX-2, TGF-β and IDO were significantly increased in the presence of fPBMCs stimulated with both mitogens compared with that in the absence of fPBMCs ($P< 0.01$; Figure 5).

3.6. Changes in PGE_2 secreted by fATMSCs and the effects of PGE_2 on the proliferation of fPBMCs

As previously described, PGE_2 is a major effector molecule having immunoregulatory capacity.[34],[35] Based on the increased expression of COX-2 in RNA extracted from cocultures of MSCs with fPBMCs, we measured PGE_2 production by ELISA. Higher levels of PGE_2 production were confirmed in transwell insert CM from fATMSCs in the presence of fPBMCs stimulated with both mitogens compared with that in CM from fATMSCs and fPBMCs alone ($P< 0.001$; Figure 6a). Increased production of PGE_2 was significantly higher than the sum of control group production in fATMSCs and fPBMCs (data not shown). When measuring the viability of mitogen-treated fPBMCs using D-Plus CCK Cell Viability Assays, fPBMC proliferation was inhibited by fATMSC-derived CM, as shown in Figure 3, and restoration of the inhibitory effect was observed in the cells cultured in fATMSC-derived CM supplemented with NS398 ($P < 0.001$; Figure 6b).

4. Discussion

MSCs exhibit immunomodulatory effects in various species.[15, 36-39] Here, we report the immunomodulatory functions of fATMSCs at the in vitro level. We also identified a potential immunomodulatory pathway in felines by comparing PGE_2 production from fATMSCs according to the presence of fPBMCs and changes in cell viability using PGE_2 inhibitor.

In this study, we used RAW264.7 cells to demonstrate the immunomodulatory effects of fATMSCs in vitro because macrophages play key roles in the immune response.[16, 40-42] TNF-α and IL-1β are mainly secreted by macrophages and monocytes and function to recruit inflammatory cells.[31] iNOS is the principal regulator of NO produced by LPS-stimulated macrophages, and excess production of NO can lead to immune-mediated complications.[42] Our results showed that the levels of pro-inflammatory cytokines (TNF-α, IL-1β and iNOS) were significantly decreased in cocultured with transwell-separated fATMSCs and LPS-stimulated RAW264.7 cells, suggesting that soluble factors from fATMSCs pass through the transwell and exhibit anti-inflammatory effects. When we examined changes in the factors expressed in fATMSCs, we found that the expression levels of COX-2, TGF-β, IDO and HGF from fATMSCs were

significantly increased in the presence of transwell-separated RAW 264.7 cells; these cytokines act as immunomodulatory factors in a variety of species.[17, 18] Thus, changes in expression at mRNA level are expected to affect secretion, and these increased soluble factors secreted from fATMSCs may play a major role in mediating the anti-inflammatory effects of MSCs.

Notably, fPBMC proliferation in response to both ConA and LPS was inhibited by CM from fATMSCs, suggesting that soluble factors of fATMSCs-derived CM may play an important role in suppressing both T- and B-cell proliferation, similar to previous studies in other species.[18, 43, 44]Furthermore, analysis of cytokines obtained from fPBMCs revealed the anti-inflammatory effects of fATMSCs on allogeneic fPBMCs. As described earlier, TNF-α has pro-inflammatory properties and plays key roles in innate and adaptive immunity.[31] Additionally, IFN-γ is a pro-inflammatory cytokine produced by various immune system cells, including T-helper (T_H) 1 cells,[32] and functions in the induction of immune regulation.[15, 45] IFN-γ may also be used as a marker for various diseases, including autoimmune diseases, infection, transplant rejection and allergy.[46, 47] IL-6 is a cytokine with pro-inflammatory properties and has a significant role in the recruiting neutrophils and triggering the humoral immune response.[13] The expression of these pro-inflammatory cytokines decreased when fPBMCs were cocultured with fATMSCs, indicating that

soluble factors from fATMSCs can effectively suppress the expression of these cytokines. IL-10 is an anti-inflammatory T_H2 cytokine and is known to suppress the functions of neutrophils and macrophages.[13] Our results showed that IL-10 expression increased in the presence of fATMSCs, consistent with previous MSC studies.[16, 48, 49] These changes in immunomodulatory cytokine expression may be associated with suppression of fPBMC proliferation and the immunomodulatory effects of fATMSCs on allogeneic fPBMCs.

In this study, we found that the expression of immunomodulatory factors from fATMSCs, including TGF-β, IDO and COX-2, increased in the presence of fPBMCs. TGF-β is an immunoregulatory factor that plays critical roles in T-cell proliferation, suppression of B cells and macrophages and induction of regulatory T cells.[13] This cytokine has also been shown to modulate MSC secretion in many veterinary species, including dogs, horses, pigs and rabbits.[17] IDO is an immunoregulatory factor secreted by MSCs; this factor is actively secreted from human and canine MSCs, whereas secretion from murine and equine MSCs is limited.[13, 17] COX-2 is an important enzyme involved in the synthesis of PGE_2 from arachidonic acid and has been shown to be expressed in canine MSCs.[17] Increased expression of these factors from fATMSCs in the presence of stimulated fPBMCs supported the increased occurrence of these signals from feline

MSCs in an inflammatory environment. In fATMSCs, overexpression of these factors may be associated with immunomodulatory effects in allogeneic fPBMCs.

Finally, we evaluated the immunomodulatory pathway in feline MSCs by measuring PGE_2 production using ELISA and the restoration of reduced cell viability when treated with PGE_2 inhibitor. PGE_2 is a major immunoregulatory molecule secreted from MSCs,[13] and PGE_2 production has been confirmed in variety of species, including human, murine, canine and equine MSCs.[14-18, 34] The amount of PGE_2 in the supernatants of transwell inserts with fATMSCs was significantly higher in the presence of stimulated fPBMCs than in both the fATMSC and fPBMC control groups, indicating that secretion of this molecule from fATMSCs was enhanced in the inflammatory environment. Additionally, suppression of PBMC proliferation was alleviated in fATMSC derived-CM supplemented with the PGE_2 inhibitor NS-398, suggesting that PGE_2 may play an important role in inflammatory cell suppression. In other species, elevated PGE_2 production from MSCs increases IL-10 secretion in macrophages and phenotype conversion of macrophages to the M2 phenotype.[15, 16] These PGE_2-dependent changes reduce the secretion of inflammatory cytokines, such as TNF-α, and inhibit lymphocyte proliferation.[13, 15-17] Although additional experiments with PGE_2 secreted from cat-derived stem cells may

be necessary , as is shown in other species, PGE_2 secreted from fATMSCs may be a key factor that plays an important role in immunomodulation.

5. Conclusion

We confirmed that fATMSCs exhibited anti-inflammatory effects in vitro. Additional studies of other immunomodulatory factors and in vivo analyses are needed to elucidate the detailed mechanisms of fATMSCs. Our findings provide important insights into the application of fATMSCs in feline patients.

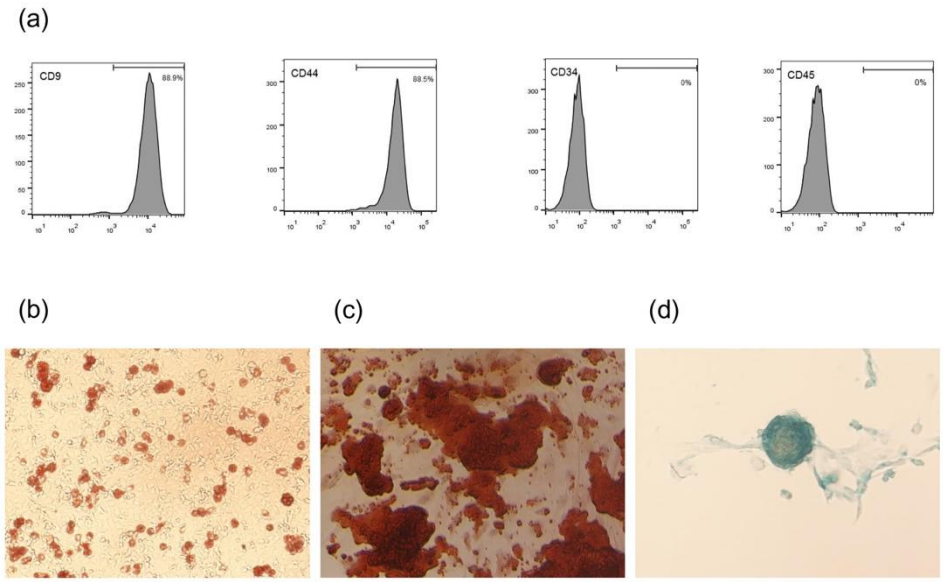

Figure 1. Characteristics of feline adipose tissue-derived mesenchymal stem cells (fATMSCs). (a) Immunophenotype of fATMSCs. (b) Adipogenic differentiation. Intracellular lipid vacuoles were stained pink with Oil Red O. (c) Osteogenic differentiation. fATMSCs stained positive for calcium deposits with 1% alizarin red. (d) Chondrogenic differentiation. Proteoglycans were stained with Alcian blue.

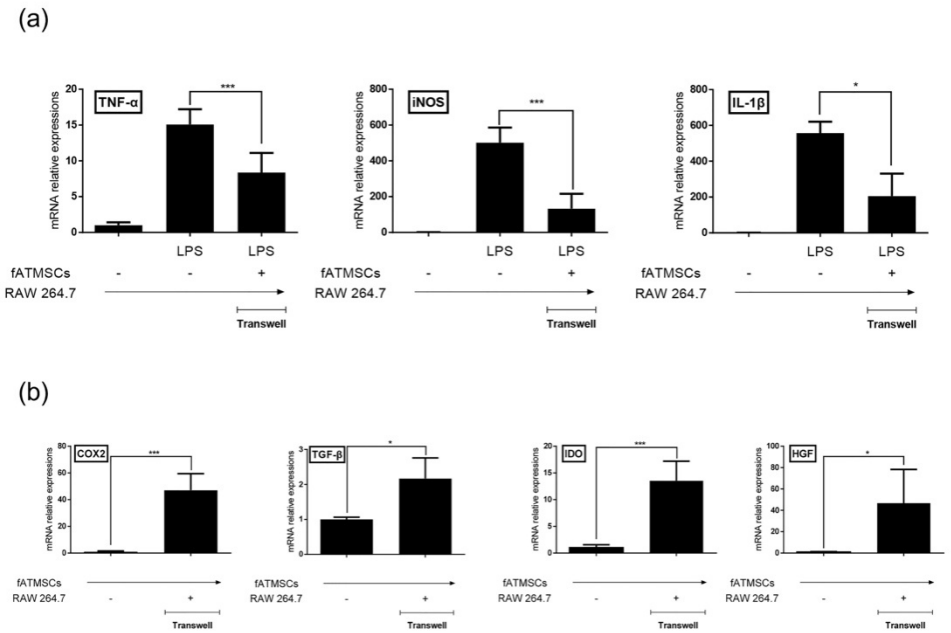

Figure 2. Anti-inflammatory effects of feline adipose tissue-derived mesenchymal stem cells (fATMSCs) on RAW264.7 cells. (a) Expression of mRNAs encoding pro-inflammatory cytokines in RAW264.7 cells in the presence or absence of fATMSCs using qRT-PCR. (b) Expression of immunomodulatory factors from fATMSCs in the presence or absence of RAW264.7 cells. All experiments were conducted in triplicate independently. *P < 0.05, **P < 0.01, ***P < 0.001.

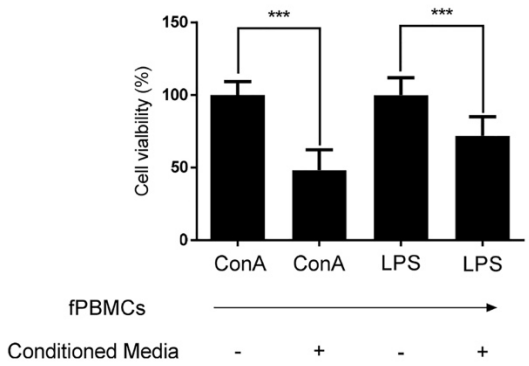

Figure 3. Suppressive effects of conditioned medium (CM) from feline adipose tissue-derived mesenchymal stem cells (fATMSCs) on the proliferation of allogeneic feline peripheral blood mononuclear cells (fPBMCs). fPBMCs were stimulated with mitogens and suspended in RPMI medium or CM from fATMSCs. Cell viability in each group was evaluated by MTT assay. Concanavalin A: ConA; lipopolysaccharide: LPS. All experiments were conducted in triplicate independently. ***$P < 0.001$.

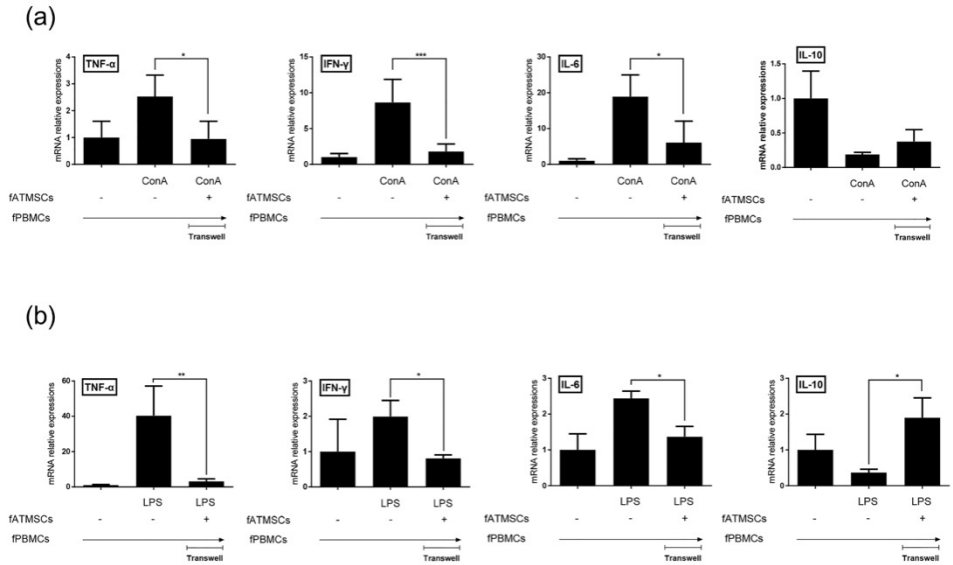

Figure 4. Changes in the expression of mRNAs encoding several feline peripheral blood mononuclear cell (fPBMC)-derived cytokines, including TNF-α, IFN-γ, IL-6 and IL-10, in the presence of feline adipose tissue-derived mesenchymal stem cells (fATMSCs). (a) Changes in cytokine expression following treatment with concanavalin A (ConA). (b) Changes in cytokine expression following treatment with lipopolysaccharide (LPS). All experiments were conducted in triplicate independently. *P < 0.05, **P < 0.01, ***P < 0.001.

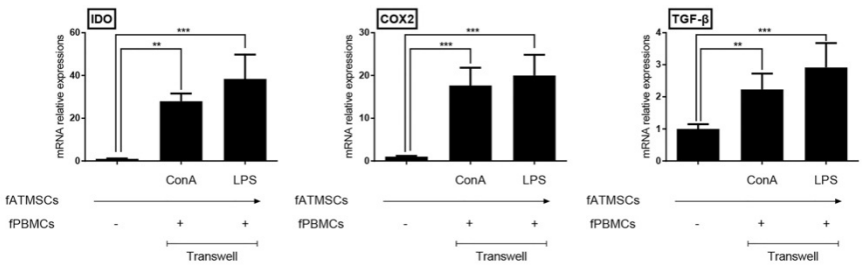

Figure 5. Changes in expression of mRNAs encoding immunomodulatory factors in feline adipose tissue derived mesenchymal stem cells (fATMSCs) cocultured with mitogen-treated allogeneic feline peripheral blood mononuclear cells (fPBMCs). Expression of immunomodulatory factors from fATMSCs was measured in the presence or absence of mitogen-treated allogeneic fPBMCs. All experiments were conducted in triplicate independently. **P < 0.01, ***P < 0.001.

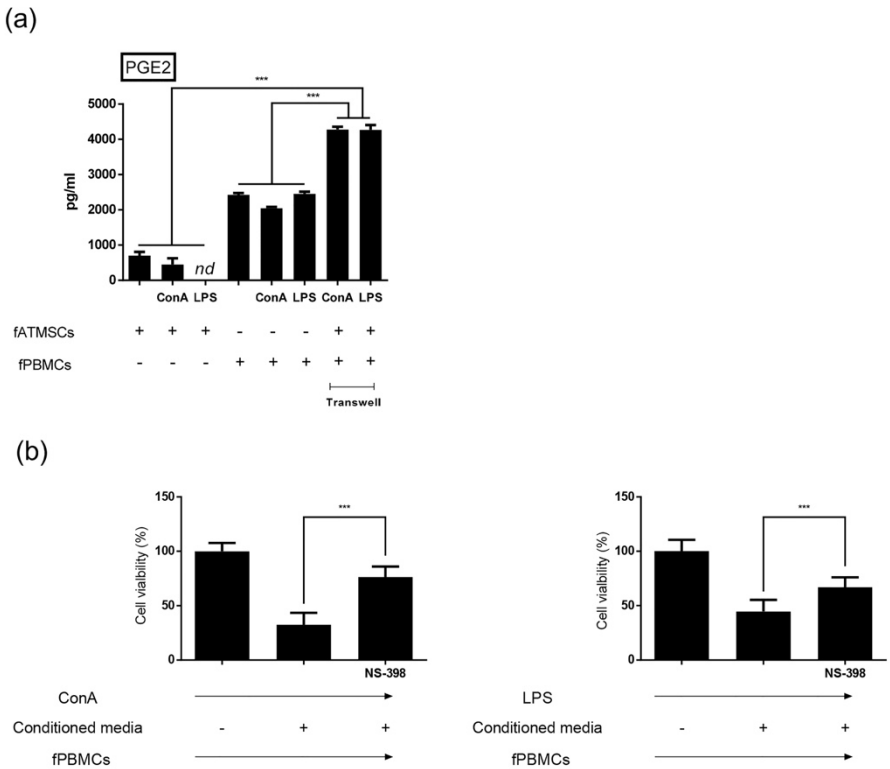

Figure 6. Changes in the secretion of prostaglandin E_2 (PGE_2) in feline adipose tissue-derived mesenchymal stem cells (fATMSCs) cocultured with mitogen-treated allogeneic feline peripheral blood mononuclear cells (fPBMCs) and alleviation of reduced cell viability upon addition of PGE_2 inhibitor. (a) PGE_2 production under various conditions was measured by ELISA. (b) fPBMCs were stimulated with mitogens and suspended in various conditions. The PGE_2 inhibitor, NS-398 (5 μM; Enzo Life Sciences) was added to the fATMSC-derived CM. Cell viability in each group was measured using a D-Plus CCK Cell Viability Assay Kit.

Concanavalin A: ConA; lipopolysaccharide: LPS. All experiments were conducted in triplicate independently. ***P < 0.001.

Table 1. Sequences of PCR primers used in this study

Gene	Forward (5′-3′)	Reverse (5′-3′)	Reference
fGAPDH	ACGATGACATCAAGAAGGTG	CATACCAGGAAATGAGCTTG	[50]
fTNF-α	GACACTCAGATCATCTTCTCGAACT	GACCTGGGAGTAGATGAGGTACAG	This study
fIFN-γ	TACACAAGTTTTATTTTCGCTTTCC	TGCTACATCTGGATTACTTGCATTA	This study
fIL-6	TGAAAAAGGAGATGTGTGACAACTA	CCTGAAGACCAGTAGTGATTC TTGT	This study
fIL-10	CCTTTAGTAAGCTCCAAGAGA AAGG	CAGATTTTCATCTTCATTGTCATGT	This study
fIDO	TATTGAATGCAGTAAAATGTG AGGA	TGAATTTGTTTAAACTCTTCCTTGG	This study
fTGF-β	CCAACAAAATCTATGAGAAAGTCCA	TATTGCTGTATTTCTGGTACAGCTC	This study
fCOX-2	CGATTCAGTCTCTCATCTGCAATAA	TCAGTTGAACGTTCTTTTAGCAGTA	This study
fHGF	ATTCCATGGGATTATTGTCCTATTT	TTCAAACTAACCATCCATCCTACAT	This study
*mGAPDH**	TCATTGACCTCAACTACATGGTCTA	ACACCAGTAGACTCCACGACATACT	This study
mTNF-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG	This study
miNOs	AAAGGAAATAGAAACAACAGGAACC	GCATAAAGTATGTGTCTGCAG ATGT	This study
mIL-1β	GTCTTTCCCGTGGACCTTC	TGTTCATCTCGGAGCCTGT	This study

* mGAPDH = mouse GAPDH

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국문 초록

고양이 지방유래 중간엽 줄기세포에서 분비되는 가용성 인자들의 면역조절효과에 대한 연구

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줄기세포는 분화 능력과 면역조절 기능을 가지고 있으며, 그들의 임상학적 이용은 수의학에서 증가하고 있다.다양한 염증성 질환의 치료에 중간엽 줄기세포가 적용되어 왔으나, 고양이 줄기세포의 기전 연구는 미비한 실정이다. 따라서 본 논문에서는, 건강한 고양이의 지방에서 분리한 줄기세포의 면역조절 기전에 대하여 알아보고자 하였다. 마우스의 대식세포 세포계인 RAW264.7와 동종의 고양이 말초혈단핵세포에서 RNA를 분리하였다. 이들 세포에서 발현되는 사이토카인을

고양이 지방유래 줄기세포의 유무에 따라 정량적 실시간 중합효소 연쇄반응 (qRT-PCR)을 통해 비교하였다. 또한 고양이 지방유래 줄기세포에서 분리한 RNA와 배양 시 얻은 상층액을 이용하여 줄기세포에서 발현과 분비하는 가용성 인자를 qRT-PCR과 효소결합면역흡착제 검정법으로 측정하였다. 염증성 사이토카인인 tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase, interleukin-1β (IL-1β)의 발현을 RAW264.7에서 분리한 RNA에서 분석하였을 때,트랜스웰을 이용한 줄기세포와 공동배양 시에 염증성 사이토카인이 유의적으로 감소한 것이 확인되었다.또한 줄기세포에서 분리한 RNA에서 염증조절 인자 cyclooxygenase-2 (COX-2), transforming growth factor (TGF)-β, indoleamine-2,3-dioxygenase (IDO)와 hepatocyte growth factor의 발현을 분석한 결과, 이들 인자는 RAW264.7 세포의 존재 시에 발현이 유의적으로 증가하였다. 줄기세포에서 얻은 조건 배지를 동종의 고양이 말초혈단핵세포에 분주하고 세포의 증식을 MTT assay를 통해 비교한 결과,말초혈단핵세포 증식의 억제가 확인되었다. 유사분열물질로 자극된 말초혈단핵세포와 줄기세포를 공동 배양했을 때, 염증성 사이토카인인 TNF-α, interferon-ɣ, 그리고 IL-6의 발현은 감소하였고 염증 억제성 사이토카인인 IL-10의

발현은 증가하였다. 고양이 지방유래 줄기세포에서의 TGF-β, COX-2, IDO의 발현과 프로스타글란딘 E2의 생산은 동종의 말초혈단핵세포의 존재 시에 증가하였다. 줄기세포에서 분비되는 프로스타글란딘 E2가 동종의 말초혈단핵세포의 증식에 미치는 영향을 알아보기 위해, 프로스타글란딘 억제제인 NS-398을 처리하여 증식율을 WST assay를 통해 비교한 결과, NS-398 처리군에서 줄기세포유래 조건 배지에 의해 억제된 증식율의 회복이 관찰되었다. 이러한 결과는 고양이 지방유래 줄기세포에서 분비된 프로스타글란딘 E2가 면역조절 효과에 중요한 역할을 할 것이라는 것을 시사한다. 본 학위 논문은 고양이 지방유래 줄기세포가 면역매개 질환을 가지는 고양이 환자의 치료에 효과적으로 이용될 수 있다는 과학적 근거로써 활용될 수 있을 것이다.

주요어: 고양이 지방유래 중간엽 줄기세포; 면역조절; 사이토카인; 가용성 인자; 대식세포; 프로스타글란딘 E2

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