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치의학석사학위논문

The role of RhoA-ROCK activator and inhibitor during chondrogenesis of periodontal derived mesenchymal stem cells

치계 줄기세포의 연골 분화에서 RhoA-ROCK 촉진제와 억제제의 역할

2015년 2월

서울대학교 대학원

치의학과 치의학전공

조 설 아

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이 논문을 치의학 석사학위논문으로 제출함

2014년 11월

서울대학교 대학원

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조 설 아

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2014년 12월

위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

Abstract

The role of RhoA-ROCK activator and inhibitor during chondrogenesis of periodontal derived mesenchymal stem cells

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(Directed by Prof. Cho, Jaejin)

Stem cells can perform important role in therapy of the cartilage disease, especially temporomandibular disease(TMD). Recent studies indicated that periodontal ligament stem cells(PDLSCs) can induce chondrogenesis under specialized conditions. And *Pasteurella multocida* toxin(PMT) has been considered a stimulator of RhoA/ROCK pathway. We demonstrate that activator and inhibitor of RhoA/ROCK pathway, which affect actin cytoskeleton, can influence PDLSCs differentiation.

From the impacted mandibular third molar of a patient, PDL cells were gained. For inducing chondrogenesis, a mechanical force by centrifugation formed 3D cell cluster. In 2-D culture stage, one group is cultured with 10 ng/mL PMT and the other group is cultured in defined media for 2 days. In initiation stage, the PDL-derived 3D clusters of PMT-not treated group were differentiated in defined media as negative control and 10 ng/mL TGF-β3-containing media as positive control. The PDL-derived 3D clusters of PMT-treated group were differentiated with 10 ng/mL PMT, 10 ng/mL TGF-β3 and 10 μM Y-27632, respectively. We maintained the chondrogenic differentiation process for 7 d and 14 d. We performed the glycosaminoglycans assay, RT-PCR assay, histology and immunohistochemistry for analyzing a chondrogenesis.

GAG contents were increased by TGF-β3 compared to negative control in 7 d and 14 d; 7d:183%, 14d:210%. GAG contents also were increased in PMT-pretreated group compared to negative control in 7 d and 14 d. In PMT/defined group, GAG contents were increased by 113% in 7 d and by 115% in 14 d. Also, in PMT/Y-27632 group, GAG contents were increased by 143% in 7 d and by 188% in 14 d. In PMT/TGF-β3 group, the results are similar;173%(7 d), 204%(14 d).

RT-PCR results showed that TGF-β3, PMT/Y-27632, PMT/TGF-β3 increased the level of chondrogenesis markers such as SOX 9, collagen II expression compared to control. Especially in 7 days of culture, the PMT decreased the expression of chondrogenic marker gene such as SOX9 and collagen type II when comparing PMT/TGF-β3 group to TGF-β3 group. But in 14 days of culture, PMT increased the expression of those genes.

And the expression of collagen type X is the largest in PMT/TGF-β3 group and was increased both 7 days and 14 days group. The expression of RUNX2 and collagen type I could be observed in almost all groups.

The histologic and immunohistochemical analysis indicated that chondrogenesis-associated genes and chondrogenic protein abundance were increased in cells with 10 ng/mL TGF-β3 compared to negative control. PMT-pretreatment groups also showed that more intense staining patterns compared to negative control.

In this study, we recognized the regulation cytoskeleton activity via RhoA/ROCK signaling pathway can control chondrogenesis of PDLSCs. We also suggest that hPDLSCs can play an significant role in tissue-engineering strategy as an excellent cell source for chondrogenesis.

Keywords : Periodontal ligament cell, Stem cell, Chondrogenesis, Activator, Inhibitor, PMT

Student number : 2011-22487

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Introduction

A field of dentistry, temporomandibular disorder(TMD) is quite common disease that affect 20~25% of the population. Temporomandibular joint(TMJ) disc problem have close relation to TMD(1, 2). TMJ disc is articular cartilage that vascularity is not enough(3). Because natural articular cartilage has the limitation of self-repairing, the disease of articular cartilage in adults usually cause severe osteoarthritic changes(4). Relieving symptoms was the first goal for the management of this disease. But recently in aspect of tissue engineering, replacement to new cartilage can be a good choice. So stem cells can perform important role in therapy of the cartilage disease, especially TMD(5).

Stem cells can proliferate to produce more stem cells as undifferentiated biological cells and differentiate into specific cells(6). These stem cells consist of embryonic stem cells and adult stem cells. Adult stem cells are found throughout the body after development and have multipotency. So they generate progeny of several distinct cell types. Adult stem cells have less ethical concerns and possibility of

unwanted differentiation(7, 8) compared to embryonic stem cells,. Therefore, adult stem cells can be useful tool for understanding tissue development and regeneration(9).

Among adult stem cells, mesenchymal stem cells(MSCs) can help to regenerate various connective tissue such as muscle, bone, cartilage and so on(7). And MSCs have their self-replication ability in vitro whereas maintaining their differentiation ability, thus they are proper for therapeutic uses for the cartilage disease(4). MSCs can be originated from several teeth-associated structures, including dental pulp, periapical follicles, periodontal ligament(PDL)(10).

The PDL is a specialized connective tissue structure, with neural and vascular components. It mainly consists of collagen fibers that connect the cementum covering the root to the alveolar bone(11). The PDL also has cell populations like fibroblasts, cementoblasts, osteoblasts and their progenitors(12). PDL-derived progenitor stem cells are abundant in capillaries of the alveolar bone and can regenerate pulp and periodontal tissues(11, 13, 14).

Cartilage does not exist in normal periodontal tissue, but recent studies indicated that periodontal ligament stem cells(PDLSCs) can induce chondrogenesis under specialized conditions. Choi *et al.* suggested

chondrogenesis of PDLSCs with TGF-β3 and BMP-6(15). Gay *et al.* also suggested that PDLSCs can be differentiated into chondrocyte-like cell and express glycosaminoglycans(GAGs) and collagen type II(11).

Especially cell signaling of actin cytoskeleton have effect on MSCs differentiation(16, 17). That is, actin cytoskeleton organization is controlled by RhoA/ROCK pathway and cell shape is also affected by actin cytoskeleton. Because cell shape is a strong determinant of cell physiology and growth, cell shape was a major determinant for stem cell lineage. Activation of RhoA/Rock pathway induced formation of spread, flattened cells and increased osteogenesis of MSCs(16, 18). On the contrary, inhibition of RhoA/Rock pathway induced formation of unspread, round cells and increased chondrogenesis of MSCs. For instance, in human articular chondrocytes, inhibition of RhoA/ROCK pathway increased chondrogenic gene expression such as collagen type II and accumulation of proteoglycans(19).

In recent studies, it was suggested that *Pasteurella multocida* toxin(PMT) stimulated RhoA/ROCK pathway via p63RhoGEF and transactivation of MAPK cascade(20). So we wonder how PMT affect chondrogenesis of PDLSCs and design this study. In this study, we demonstrate that agonist and antagonists, which affect actin

cytoskeleton, can influence PDLSCs differentiation. We used human PDLSCs from the third molar teeth. The cells were cultured with PMT, RhoA activator or TGF-β3, chondrogenic growth factor or Y-27632, ROCK inhibitor. The agents were treated in pre-treatment stage, initiation stage and after that time. So pre-treatment or initiation effect of control of RhoA/ROCK pathway in chondrogenesis could be evaluated. Also our results suggested a future strategy to articular cartilage production.

Materials and Methods

Cell isolation and cell culture

From the normal third molar of a patient at Seoul National University Dental Hospital(SNUDH), human PDL cells were collected; this protocol was permitted by the Institutional Review Boards(IRB) of Seoul National University Medical Ethics Committee. First, tooth was washed with fresh Hank's balanced salt solution(HBSS) supplemented with a 3% antibiotic-antimycotic solution(GIBCO, Grand Island, NY, USA). And then from the root surface, PDL tissue was collected. This tissue was enzymatically degraded in 2.4 mg/mL dispase(GIBCO, Grand Island, NY, USA) and 1 mg/mL collagenase type I for 1 hour at 37 ℃. And then the degraded solution was filtered with 100 μm mesh. Sieved solution was centrifuged at 400 G for 4 min at 4 ℃ and the PDL cell-derived pellet was resuspended in Dulbecco's modified Eagle's Medium(DMEM; Welgene, Daegu, Korea) with 20% Fetal Bovine Serum(FBS, HyClone Laboratories, Vancouver, Canada). Cells were cultured in DMEM solution with 20% FBS and 1% antibiotic-antimycotic solution at 37 ℃ in a 5 % CO_2 humidified atmosphere. Cells at the sixth passage were used for experiments.

Chondrogenic differentiation

To induce chondrogenesis, 2.5×10^5 PDL-derived stem cells per cluster counted by hemocytometer were centrifuged at 500 G for 5 min at 4 ℃. Defined medium include 40 ㎍/mL L-proline, 50 ㎍/mL ascorbate-2-phosphate, 100 nM dexamethasone, 100 ㎍/mL sodium pyruvate and 1% ITS+Premix (all Sigma-Aldrich, St. Louis, MO, USA) based on high-glucose DMEM; this media served as a negative control.

To evaluate the pre-treatment effect of PMT for chondrogenesis on hPDLSCs, a defined media containing 10 ng/mL PMT(Sigma-Aldrich, St. Louis, MO, USA) was used. PMT is known as agonist of RhoA-Rock pathway. In 2-D culture stage, one group is cultured with PMT, the other group is cultured in defined media for 2 days.

In initiation stage, the PDL-derived 3D clusters of PMT-not treated group were differentiated in defined media as negative control and TGF-β3-containing media as positive control. The PDL-derived 3D clusters of PMT-treated group were differentiated with PMT, TGF-β3 and Y-27632, respectively.

TGF-β3 is known as chondrogenic growth factors for MSCs. Y-27632 is known as antagonist of actin cytoskeleton and an inhibitor of ROCK1

so that RhoA/Rock signaling pathway is inhibited by this material.

For chondrogenesis, the defined media was supplemented with 10 ng/mL TGF-β3 (R&D Systems, Minneapolis, MN, USA); this media served as a positive control. Also, to evaluate the antagonist effects of Y-27632 for chondrogenesis on hPDLSCs, a defined media containing 10 μM Y-27632 (Sigma-Aldrich, St.Louis, MO, USA)was used. We maintained the chondrogenic differentiation process for 7 d and 14 d.

Glycosaminoglycans(GAGs) assay

The sulfated GAGs contents of hPDLSC pellets cultured on day 7 and 14 were measured and normalized versus the total amount of DNA. The pellets were digested in 1 mL Papain buffer (10 nM ethylene diamine tetraacetic acid (EDTA), 100 mL of 0.2 M sodium phosphate buffer, 5 mM L-cysteine and HCl, pH 6.4) with 7.6 μL/mL of papain for 18 h in a 65 ℃ water bath. The amount of GAGs was measured by using a Blyscan Sulfate Glycosaminoglycan Assay (Biocolor Ltd, Belfast, Ireland). After centrifuging at 10000 rpm for 10 min, absorbance of the samples was determined by an enzyme-linked immunosorbent assay (ELISA) reader (S500; BIO-RAD, Hercules, CA, USA) at 656 nm.

Chondroitin-4-sulfate solution was used as standard. Total cellular DNA content was calculated using a pico-green dsDNA assay kit (Invitrogen, Camarillo, CA, USA).

Total RNA extraction and RT-PCR

On day 7 and 14 from hPDLSC pellets, total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Roche, Mannheim, Germany) to eliminate possible DNA contaminants. The complementary DNA was gained by reverse-transcribing of the RNA by M-MLV reverse transcriptase (Invitrogen, Camarillo, CA, USA). The amount of cDNA was determined by PCR using AccuPower PCR PreMix (Bioneer, Korea). Thermal profile was as follows: denaturation at 94 ℃ for 5 min, amplification at 94 ℃ for 30 sec, annealing at specific temperature for 30 sec, extension at 72 ℃ for 20 sec, and total 28~37 cycles. In this way, the gene expression level of chondrogenesis-related markers including collagen type II, X and SOX9 and osteogenic markers including RUNX2, collagen type I and RhoA/ROCK signaling makers including RhoA, ROCK1, CDC42, RAC1 in the pellets were measured and normalized to their glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level.

Histological and immunohistochemical analysis

Three-dimensional hPDLSC pellets incubated after 7, 14 days of culture were fixed in 4% phosphate buffered formalin and embedded in paraffin. And then paraffin block were sectioned as 4-μm-thick slices. With Histo-clear II (Natural Diagnostics, USA), the paraffin sections were deparaffinized and hydrated using alcohol. The specimens were stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA) to examine cell morphology. Also the specimens were stained with and safranin-O/fast green (Fisher, Fair Lawn, NJ, USA) to visualize GAGs.

Type II collagen and aggrecan contents in the pellets were measured by immunohistochemical staining using the ABC kit (Invitrogen, Camarillo, CA, USA).

Briefly, deparaffinized sections were hydrated with step-by-step concentrations of ethanol and cultured with serum blocking solution (Invitrogen, Camarillo, CA, USA) for 30 min to prevent non-specific background on the slides. The specimens were then stained with monoclonal mouse anti-human type II collagen (Calbiochem, Darmstadt, Germany) as a primary antibody and incubated for 60 min at room temperature. After washing the specimens with Dulbecco's Phosphate Buffered Saline (DPBS; GIBCO, Grand Island, NY, USA), they were

incubated again with anti-mouse IgG (Invitrogen, Camarillo, CA, USA), a secondary antibody for 30 min. The same procedures were followed for staining of aggrecan. But for the staining of that, aggrecan (Thermo, Rockford, IL, USA) were used as primary antibody.

Immuno-located antibodies were visualized by applying streptavidin-HRP-conjugated tertiary antibody (Invitrogen, Camarillo, CA USA), followed by diaminobenzidine (DAB kit; Invitrogen, Camarillo, CA, USA).

Statistical analysis

Five independent experiments were performed in triplicate and the data are presented as means ± SDs. Data analysis was performed using Student's t-test at a significance level of $p < 0.05$.

Results

Glycosaminoglycans(GAGs) assay

After 7 days of culture, we measured the amount of sulfated GAG contents in each hPDLSC pellets and normalized using the total cellular DNA contents. Synthesized GAGs were increased by 183% in 10 ng/mL TGF-β3 containing media and by 113% in a series of 10 ng/mL PMT and defined media compared to negative control. The serial treatments of 10 ng/mL PMT and 10 μM Y-27632, 10 ng/mL PMT and 10 ng/ml TGF-β3 enhanced GAG synthesis by 143%, 173% respectively compared to negative control.

After 14 days of culture, we also measured the amount of sulfated GAG contents in each hPDLSC pellets and normalized using the total cellular DNA contents. They have also similar trend but a little difference of each amount in culture of 14 days. Synthesized GAGs were increased by 210% in 10 ng/mL TGF-β3 containing media and by 115% in a serial treatment of 10 ng/mL PMT and defined media. The serial treatment of 10 ng/mL PMT and 10 μM Y-27632, 10 ng/mL PMT and 10 ng/ml TGF-β3 induced GAG synthesis by 188%, 204% respectively compared to negative control.

The increases in GAG levels were all statistically significant. ($p<0.01$) In both 7, 14 days results, total amount of cellular DNA which reflected the proliferation and viability of cells in five different conditions, was similar indicating that these results are reliable.

Total RNA extraction and RT-PCR

The expression of chondrogenic gene was measured by RT-PCR. The assayed genes were SOX9, collagen type II (COL II), collagen type I (COL I), collagen type X (COL X), RUNX2, RhoA, ROCK1, CDC42, and RAC1. We used the glyceraldehyde-3-phosphate dehydrogenase(GAPDH) mRNA level to normalize chondrogenic markers mRNA level.

We can show the expression of SOX9 which is a critical chondrogenic transcription factor and collagen type II which is a chondrogenic differentiation marker, except negative control and PMT/defined group in 7 days and 14 days of cultures. The expression of collagen type X, which is a chondrocyte hypertrophy marker, is the largest in PMT/TGF-β3 group. It was 409% in 7 days of culture and 724% in 14 days of culture compared to negative control. And the expression of RUNX2 and collagen type I, which are an osteogenic differentiation marker,

could be observed in almost all groups.

The TGF-β3, which is chondrogenic growth factor, enhanced the expression of chondrocyte-associated genes like SOX9 (288% in 7 d, 134% in 14 d), collagen type II (956% in 7 d, 441% in 14 d) compared to negative control.

In 7 days of culture, the PMT, which is RhoA inhibitor, decreased the expression of chondrocyte-associated genes like SOX9 (203%), collagen type II (362%) when comparing PMT/TGF-β3 group to TGF-β3 group. But in 14 days of culture, PMT increased the expression of SOX9 (111%) and collagen type II (134%). And expression of collagen type X, which is known as a chondrocyte hypertrophy marker, was also increased by 718% in 7 days of culture and 226% in 14 days comparing PMT/TGF-β3 group to TGF-β3 group.

Histological and immunohistochemical analysis

Hematoxylin and eosin(H&E) staining showed that the cells grown in TGF-β3 and PMT/TGF-β3 were more round-shaped compared to others, and had bigger nuclei than others. Also cells cultured with TGF-β3 arranged in two layers; an inner layer and an outer layer.

The nuclei and cells in the inner layer appeared round but, those in the outer layer had a spindle-morphology. In the cells grown in a series of PMT and other materials, the staining were much less intense in the outer layer of the cell clusters than the inner layer.

Safranin O stains appears brilliant red. The cells grown in a series of PMT and other chondrogenic factors were stained to more deep red than control. The nuclei were more round and prominent with safranin-O staining.

Aggrecan and type II collagen were detected by immunohistochemical approach. A pale brownish color appeared generally. Also, there was same trend that the staining was more strong in the outer layer of the cell clusters compared to the inner layer in both immunohistochemical staining. Same as above, the cells grown with a series of PMT and other chondrogenic factors were stained more prominent than the cells in defined media.

Discussion

In this study, we demonstrated that activator and inhibitor which affect actin cytoskeleton can regulate human periodontal ligament derived stem cells(hPDLSCs) differentiation, especially chondrogenesis. We induced chondrogenesis of hPDLSCs with TGF-β3, Y-27632 and PMT in single or serial treatment. The degree of chondrogenesis was investigated with respect to sulfated glycosaminoglycan synthesis, expression of chondrogenesis-associated genes, and chondrogenic protein deposition by IHC. We compared the effects of RhoA/ROCK activator and inhibitor to TGF-β3 which increase chondrogenic differentiation when PMT is pre-treated or not. TGF-β3 may facilitate early stage in chondrogenesis and keep their control through the differentiation of chondrocytes(21). And Y-27632 increase chondrogenesis-related gene expressions but inhibit actin cytoskeleton polymerization and decrease the adhesive force(22).

TGF-β3 and Y-27632 induced more GAG synthesis than defined media regardless of PMT pre-treatment. And they have also similar trends but a little difference of each amount between 7 days of culture and 14 days of culture. It was similar result in our lab's previous study. There were studies about effects of TGF-β3 and Y-

27632 to chondrogenesis of hPDLSCs in our lab. In previous studies, we found that cells with treated 10 ng/mL TGF-β3 or 10 μM Y-27632 enhanced GAG synthesis while keeping a constant level of cell viability and proliferation and expressed more chondrogenic markers compared to negative media. The inhibition of RhoA/ROCK signaling and actin cytoskeleton aggregation can affect chondrogenesis of MSCs(15) and increases chondrogenic markers such as SOX9, collagen type II(Col II) and glycosaminoglycans(GAGs)(17, 23).

In figure 1, 2, 3 and 4, synthesized sulfated glycosaminoglycan, expression of chondrogenesis-associated genes, and chondrogenic protein abundance are increased in cells with treated 10 ng/mL TGF-β3 and this results are similar with previous studies. Also, all chondrogenic markers are up-regulated in cells with 10 ng/mL TGF-β3, and 10 μM Y-27632 although PMT pre-treatment. Especially, the serial addition of PMT and TGF-β3 showed the decreased expression of collagen type II compared to TGF-β3 treatment group in 7 days of culture. So we can suggest that the treatment of PMT showed an antagonistic effect on chondrogenesis. But in 14 days of culture, the result was reverse. So we can suppose that PMT have an effect on initial stage of chondrogenesis.

The addition of Y-27632 increase chondrogenic gene expression in previous study(22). The serial treatment of PMT and Y-27632 in 14 days of culture showed a considerable expression of GAGs compared to positive control, TGF-β3 group. We supposed that addition of Y-27632 in 14 days counterbalance the effect of PMT pre-treatment. Y-27632 is known as an inhibitor of ROCK1 so that can inhibit RhoA/ROCK signaling pathway. As a result, Y-27632 act as an antagonist of skeleton of actin filament. So it can influence cell shape and increase hPDLSCs differentiation to chondrocyte(22).

Mesenchymal aggregation is the first stage of chondrogenesis regulated first by paracrine factors like bone morphogenetic protein(BMP) , transforming growth factor-β(TGF-β) and subsequently by SOX9(24-26). SOX9 is related to the expression of a few key genes in chondrogenesis, in other words SOX5, SOX6, and collagen type II α1(Col2α1)(21). Thus, the increased expression of SOX9 in our results suggests the stimulation of chondrogenesis.

However, the expression of both collagen type X, a chondrocyte hypertrophy marker, was increased in cells with serial treatment of PMT and TGF-β3. And Runx2, an osteogenesis-related factor, was also expressed in almost all groups. We can suggest TGF-β3 and PMT have

an important role to chondrocyte hypertrophy and osteogenic differentiation as well as chondrogenesis.

RhoA, CDC42 and RAC1 are part of Rho family and ROCK1 is the signaling marker of RhoA/ROCK pathway. Rho family GTPases have relations with cytoskeletal reorganization(27, 28). Cdc42 and Rac1 have a role as positive regulators of chondrogenesis, but they use different molecular and cellular mechanisms(29). For interpretation meaning of expression of unexpected markers in RT-PCR assay, we need to conduct further studies.

Proteoglycans are rich proteins in cartilage matrix, and chondroitin sulfate is the major glycosaminoglycan in cartilage(30). Aggrecan, known as chondroitin sulfate proteoglycan 1, is the most rich proteoglycan in cartilage(31). Accumulation of aggrecan is a trademark of chondrogenesis(32). Immunohistochemical staining expressed synthesis of type I and type II collagens in the extracellular matrix. Cells grown in PMT/TGF-β3 and PMT/Y-27632 as well as TGF-β3 aranged in two layers; an inner layer and an outer layer.

According to a previous study, cell shape was known as a main determining factor for stem cell lineage. Cell shape is controlled by RhoA/ROCK pathway in the cytoplasm and this pathway can regulate

actin arrangement and lineage differentiations in MSCs(33). HPDLSCs, which are a kind of MSCs, also differentiate depends on RhoA/ROCK pathway. The concept that cell shape induce stem cell lineage has been explained using the cell attachment, which is regulated by micropattern of extracellular matrix(ECM)(29, 34). Cells adhered to small ECM micropatterned islands showed round morphology, while on large ECM islands, cells showed flattened morphology. The change of cell shape from round to flattened forms results from the structure of the actin cytoskeleton and the focal adhesions(34).

PDLSCs differentiate to chondrocyte with similar pattern compared with other mesenchymal stem cells, synthesizing ECM proteins chondrogenesis-associated genes and expressing in the presence of growth factors and inhibitors of RhoA/ROCK pathway. In short, we investigated the stem cell characteristics of PDL cells by inducing chondrogenesis using growth factor TGF-β3 and inhibitors of RhoA/ROCK pathway such as Y-27632 and PMT. Especially, we found that PMT is also the factor that affect to chondrogenesis of hPDLSCs.

TGF-β3 is known as good growth factor for chondrogenesis, but it has several side effect such as cancer induction, metastasis and kidney fibrosis. So TGF-β3 has a limitation to clinical application of cartilage

regeneration. Therefore, we need to study about another factors such as PMT that affect chondrogenesis of hPDLSCs.

This study indicated the potential of hPDLSCs as an excellent origin for chondrogenesis, by extension cartilage regeneration. These results also proved that hPDLSCs can play an important role in tissue-engineering strategy for the management of temporomandibular joint disease and osteoarthritis.

Figures

Figure 1.

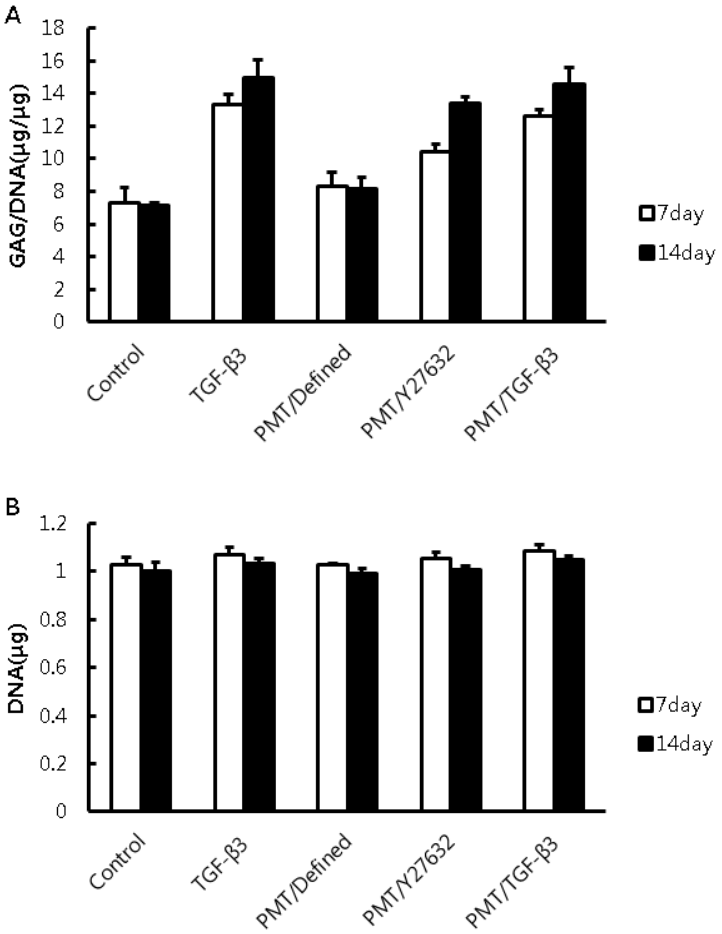
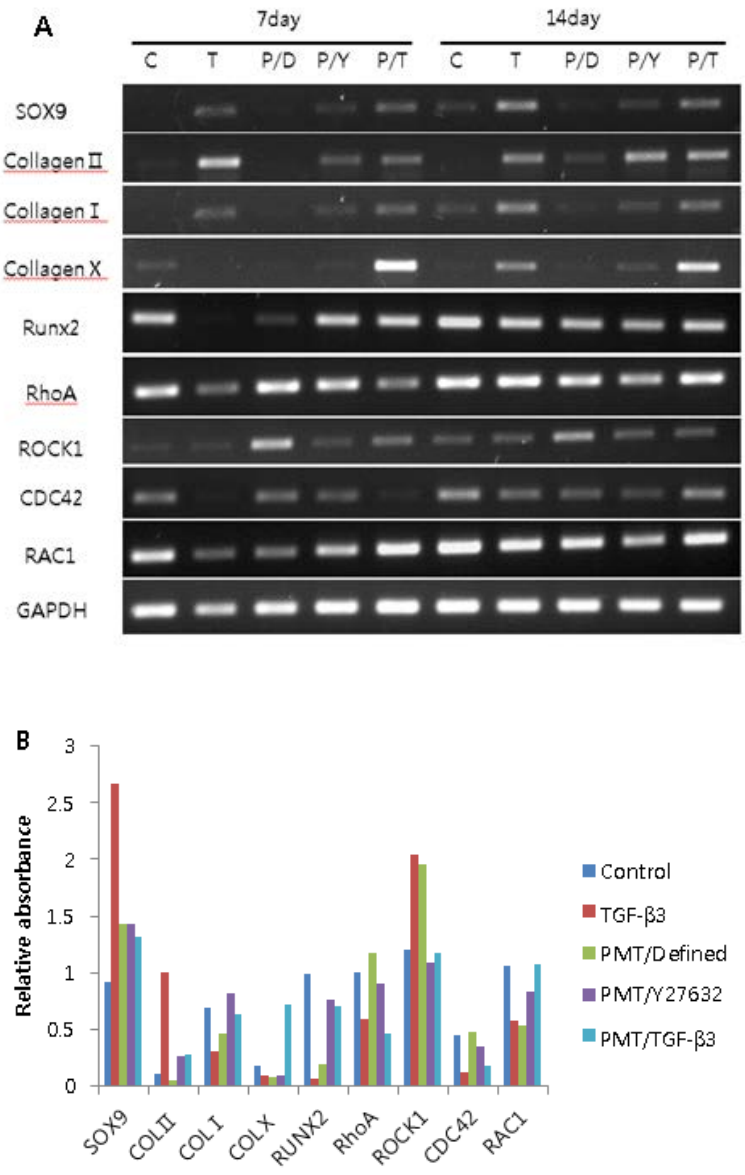


Fig. 1. (a) Relatively amount of glycosaminoglycan(GAG)/DNA contents in each differentiated chondro-ball. Each chondro-ball is grown in the five different media types for 7, 14 days. GAG/DNA levels were

increased in all stem cells in TGF-β3, PMT/Defined media, PMT/Y-27632 and PMT/TGF-β3; 7 day(in TGF-β3)=183%, 7 day(in PMT/Defined media)=113%, 7 day(in PMT/Y-27632)=143%, 7 day(in PMT/TGF-β3)=173%, 14 day(in TGF-β3)=210%, 14 day(in PMT/Defined media)=115%, 14 day(in PMT/Y-27632)=188%, 14 day(in PMT/TGF-β3)=204%.

(b) Picture showed total DNA levels. Total DNA levels in each chondro-ball, demonstrating that the proliferation of cells in the five media types were similar, which indicates that the results are reliable.

Figure 2.


A
7day
14day
C
T
P/D
P/Y
P/T
C
T
P/D
P/Y
P/T
SOX9
Collagen II
Collagen I
Collagen X
Runx2
RhoA
ROCK1
CDC42
RAC1
GAPDH
B
Relative absorbance
3
2.5
2
1.5
1
0.5
0
SOX9
COLII
COL I
COLX
RUNX2
RhoA
ROCK1
CDC42
RAC1
Control
TGF-β3
PMT/Defined
PMT/Y27632
PMT/TGF-β3

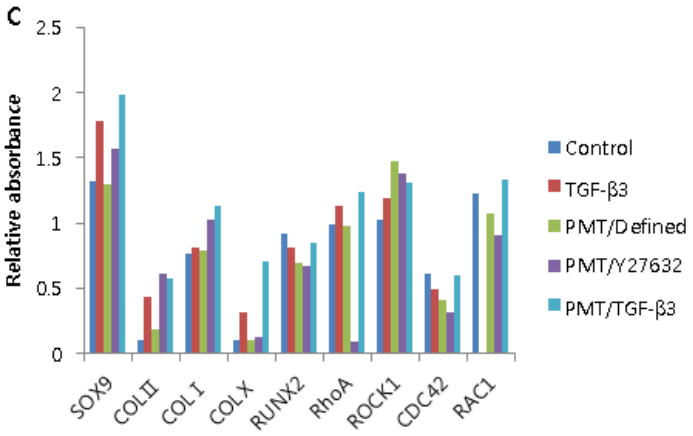


Fig. 2. (a) RT-PCR assay for chondrogenesis markers in PDLSCs. (b) The relative expression of chondrogenesis-associated genes in the RT-PCR assay in 7 d of culture. The serial addition of PMT and Y-27632 showed that greatly increased the expression of chondrogenesis markers like SOX9, COL II. Likewise, the serial addition of PMT and TGF-β3 showed similar trend. And the serial treatment of PMT and TGF-β3 showed the greatest expression of chondrocyte hypertrophy marker, COL X. (c) The relative expression of chondrogenesis-associated genes in the RT-PCR assay in 14 d of culture. The result is similar in 7 d of culture except the expression of COL II. The serial addition of PMT and TGF-β3 showed the decreased expression of COL II by 362% compared to TGF-β3 group in 7 d of culture, but in 14 d of culture, the result was reverse. The expression of COL II was increased by 134% in 14 d of culture.

Figure 3.

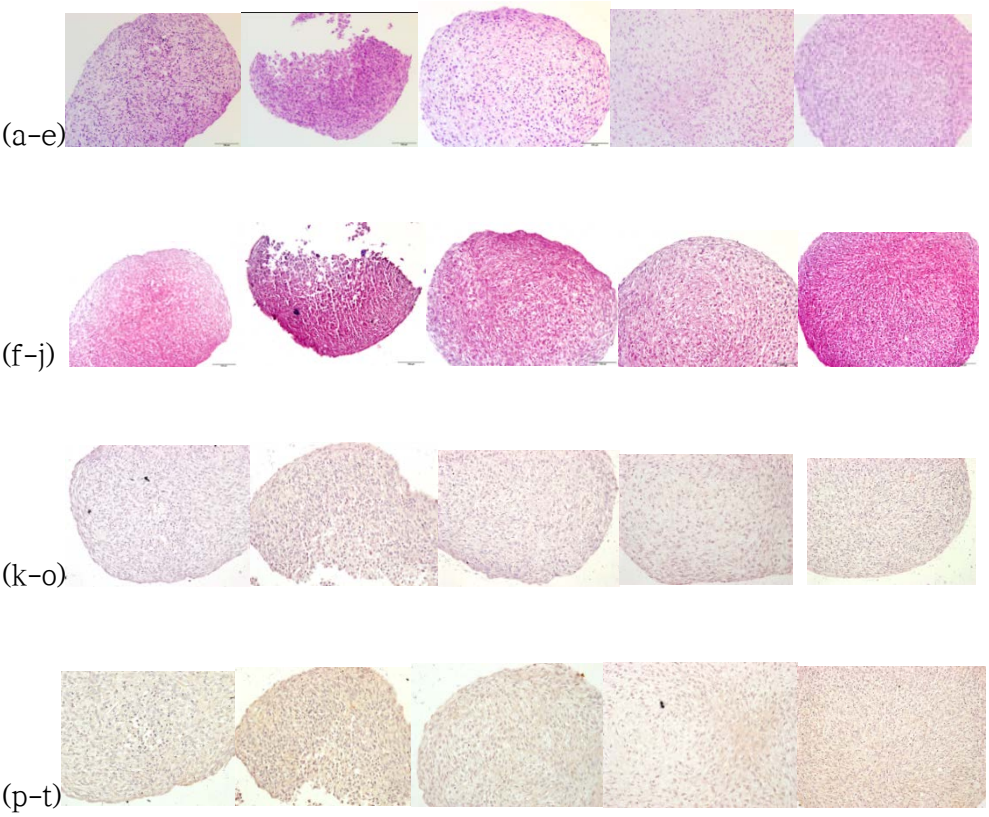


Fig. 3. Periodontal ligament stem cells(PDLSCs) differentiated in the five different media types for 7 days. (a-e) : Hematoxylin and eosin staining of the chondro-balls in defined media, 10 ng/mL TGF-β3, defined after 10 ng/mL PMT, 10 μM Y-27632 after 10 ng/mL PMT, 10 ng/mL TGF-β3 after 10 ng/mL PMT, respectively. (f-j) : Safranin-O staining of the chondro-balls in defined media, 10 ng/mL TGF-β3, defined after 10 ng/mL PMT, 10 μM Y-27632 after 10 ng/mL PMT, 10 ng/mL

TGF-β3 after 10 ng/mL PMT, respectively. (k-o) : Aggrecan immunohistochemical staining of the chondro-balls in defined media, 10 ng/mL TGF-β3, defined after 10 ng/mL PMT, 10 μM Y-27632 after 10 ng/mL PMT, 10 ng/mL TGF- β3 after 10 ng/mL PMT, respectively. (p-t) : Type II collagen immunohistochemical staining of the chondro-balls in defined media, 10 ng/mL TGF-β3, defined after 10 ng/mL PMT, 10 μM Y-27632 after 10 ng/mL PMT, 10 ng/mL TGF-β3 after 10 ng/mL PMT, respectively.

Figure 4.

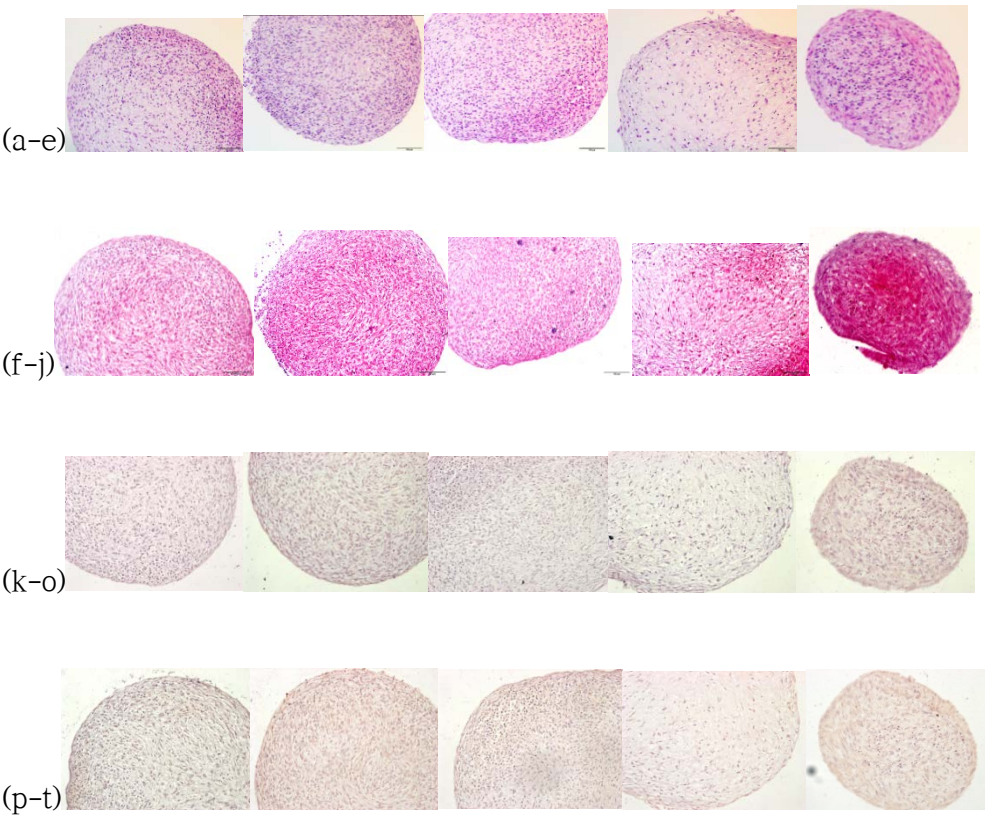


Fig. 4. Periodontal ligament stem cells(PDLSCs) differentiated in the five different media types for 14 days. (a-e) : Hematoxylin and eosin staining of the chondro-balls in defined media, 10 ng/mL TGF-β3, defined after 10 ng/mL PMT, 10 μM Y-27632 after 10 ng/mL PMT, 10 ng/mL TGF-β3 after 10 ng/mL PMT, respectively. (f-j) : Safranin-O staining of the chondro-balls in defined media, 10 ng/mL TGF-β3, defined after 10 ng/mL PMT, 10 μM Y-27632 after 10 ng/mL PMT, 10

ng/mL TGF-β3 after 10 ng/mL PMT, respectively. (k-o) : Aggrecan immunohistochemical staining of the chondro-balls in defined media, 10 ng/mL TGF-β3, defined after 10 ng/mL PMT, 10 μM Y-27632 after 10 ng/mL PMT, 10 ng/mL TGF-β3 after 10 ng/mL PMT, respectively. (p-t) : Type II collagen immunohistochemical staining of the chondro-balls in defined media, 10 ng/mL TGF-β3, defined after 10 ng/mL PMT, 10 μM Y-27632 after 10 ng/mL PMT, 10 ng/mL TGF-β3 after 10 ng/mL PMT, respectively.

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요약(국문초록)

The role of RhoA-ROCK activator and inhibitor during chondrogenesis of periodontal derived mesenchymal stem cells

치계 줄기세포의 연골 분화에서 RhoA-ROCK 촉진제와 억제제의 역할

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1. 목 적

줄기세포는 연골 질환, 특히 측두하악질환의 치료에 있어 중요한 역할을 수행할 수 있다고 알려져 있다. 최근 치주인대 세포 유래 줄기세포를 연골화 분화 시킨 연구도 발표된 바 있다. 우리는 *Pasteurella multocida* toxin(PMT)을 포함하는 RhoA/ROCK pathway의 activator와 inhibitor를 사용하여 actin 세포 골격을 변화시킴으로써 궁극적으로 치주인대 세포 유래 줄기세포의 분화에 영향을 주고자 한다.

2. 방 법

(1) 연구 설계

매복 제 3대구치에서 채취한 치주인대 세포에 여러 인자를 처리하여 연골화 분화하도록 유도하였다. 줄기세포의 연골화 분화에 영향을 준다고 알려져 있는 growth factor인 TGF-β3, ROCK inhibitor인 Y-27632과 함께 RhoA/ROCK pathway의 agonist로 알려진 PMT를 추가로 사용하였다.

1) Defined media에서 7일, 14일 배양 (negative control)

2) TGF-β3를 처리한 media에서 7일, 14일 배양 (positive control)

3) 10 ng/mL PMT로 2일 전처리 후 7일, 14일 배양;

초기 1일은 PMT 처리, 나머지 6일, 13일은 defined media에서 배양

4) 10 ng/mL PMT로 2일 전처리 후 10 μM Y-27632를 처리하여 7일, 14일 배양

5) 10 ng/mL PMT로 2일 전처리 후 10 ng/mL TGF-β3를 처리하여 7일, 14일 배양

(2) 연구 방법

1) GAG assay를 통하여 연골의 대표적인 세포 외 기질인 glycosaminoglycan의 합성정도를 분석한다.

2) RT-PCR을 이용하여 연골화와 관련된 유전자의 발현 정도를 살펴본다.

3) HC, special staining 등을 이용하여 치주인대 세포가 연골화 되면서 만들어 내는 세포 외 기질을 확인한다.

3. 결 과

5개의 그룹에 대해서 연골화 분화 과정에서 만들어진 GAG와 DNA의 양을 측정하는 실험을 진행하였고, GAG/DNA(㎍/㎍)값을 비교하였다. 7일, 14일 group 모두 대조군인 defined에서 가장 작은 GAG/DNA(㎍/㎍) 값을 보였고, TGF-β3를 처리한 결과에서는 가장 큰 GAG/DNA(㎍/㎍) 값을 보였다. 본 연구에서 중요한 RhoA/ROCK pathway의 activator인 PMT를 전처리한 group에서는 TGF-β3를 처리한 결과보다는 작은 GAG/DNA(㎍/㎍) 값을 보였지만, defined의 결과보다는 증가된 것을 확인할 수 있었다; PMT/defined(7일;113%, 14일;115%), PMT/Y-27632(7일;143%, 14일;188%), PMT-TGF-β3(7일;173%, 14일;204%). 위 실험군 중에서는 PMT 전처리 후 TGF-β3를 처리한

군에서 가장 큰 GAG/DNA(μg/μg) 값을 나타내었다.

RT-PCR 결과에서도 비슷한 경향을 보였다. 전반적으로 보면 TGF-β3, PMT/Y-27632, PMT/TGF-β3을 처리했을 때 연골분화의 중요한 marker인 SOX 9과 collagen II의 발현이 증가했다. 특히 PMT/TGF-β3를 처리했을 때 TGF-β만 처리한 실험군에 비해 7일에서는 collagen type II의 발현이 감소했으나, 14일에서는 증가된 것으로 나타났다. 이 결과로 PMT가 mesenchymal condensation 같은 연골 분화의 초기 단계에 작용할 것으로 추정해 볼 수 있다. 연골세포 비대의 marker인 collagen type X의 경우에는 PMT/TGF-β3 처리 시 가장 발현이 증가되었고, 골분화 marker인 RUNX2와 collagen type I는 거의 모든 실험군에서 관찰되었다.

염색기법을 사용하여 연골분화 결과 중요한 기질인 aggrecan과 collagen type II의 생성을 비교하였고, 이는 GAG assay에서의 결과와 비슷한 양상을 보인다는 것을 확인하였다. 특히 TGF-β3를 처리한 결과에서 aggrecan과 collagen type II가 가장 많이 존재하는 것으로 생각되며, PMT를 전처리한 실험군의 경우 defined 에서보다 aggrecan, collagen type II의 양이 더 많이 존재함을 확인할 수 있었다.

결과적으로 주어진 조건에서 PMT, TGF-β3, Y-27632에 의해 치주인대 세포로부터 SOX9, aggrecan, type II collagen, RUNX2 등의 연골화 분화 관련 인자와 RhoA/ROCK pathway 관련 인자를 확인하였고, 연골의 주

세포 외 성분인 sulfated gycosaminoglycan(GAG), type Ⅱ collagen 등이 생성됨을 볼 수 있었다. 특히 PMT를 처리할 경우 initiation stage가 빠르게 일어나 negative control에 비해 GAG/DNA 값이 높게 나타난다는 사실을 확인했다. 결과적으로 치주인대 세포가 다른 성숙 세포로 분화할 수 있는 줄기세포의 특성을 가지고 있음을 증명할 수 있었으며, 이 결과는 연골의 재생 능력 결함에 의한 질환인 관절염, 턱관절 장애의 치료나 귀나 코 등의 결손부위 재건의 조직공학적인 접근에 응용될 수 있다.

주요어 : 치주인대세포, 줄기세포, 연골화분화, 촉진제, 억제제, PMT

학 번 : 2011-22487