치의학석사 학위논문

Effects of RhoA/ROCK inhibitors on chondrogenesis of human periodontal ligament-derived mesenchymal stem cells

사람 치주인대 유래 간엽줄기세포에서 RhoA/ROCK 억제에 의한 연골분화과정

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Abstract

Effects of RhoA/ROCK inhibitors on chondrogenesis of human periodontal ligament-derived mesenchymal stem cells

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Several researches reported that the control of cytoskeleton activity via RhoA-ROCK may promote the chondrogenic differentiation of stem cells. Therefore, we determined whether the inhibition of RhoA-ROCK pathway by ROCK inhibitor Y-27632 and actin polymerization inhibitor cytochalasin D can promote chondrogenesis in PDLSCs in the presence or absence of TGF-β3. PDLSCs were isolated and purified from the periodontal ligament of the human third molar teeth. After initiating

chondrogenesis with 3D cell cluster formation, the clusters were maintained under 10 ng/mL TGF-β3, 3 μM cytochalasin D, 10 μM Y-27632, TGF-β3+cytochalasin D, TGF-β3+Y-27632 and without treatment as negative control. We analyzed the chondro-clusters by glycosaminoglycan assay (GAG), histology evaluation, safranin O and von Kossa staining and PCR. Immunohistochemistry were performed to measeure the expression for Collagen I, II as well as aggrecan. Clusters treated with TGF-β3 and Y-27632 had the highest level of GAG synthesis. Unlike cytochalasin D, Y-27632 was found to exert a synergistic effect as well. Combination treatment of TGF-β3 and Y-27632 increased the expression of the chodro-related genes, collagen type II and sox9 while decreasing expression of the osteogenic gene, Runx2. On the other hand, treatment with TGF-β3 and cytochalasin D let do the greatest expression of Runx2 and collagen X, a hypertrophic chondrocyte marker gene. Calcium deposits were visualized by von Kossa staining. The least amount of calcium deposition was found in clusters exposed to TGF-β3 with Y-27632. In conclusion, our data suggest that the treatment of TGF-β3 along with RhoA/ROCK inhibition using Y-27632 induced more selective chondrogenesis. Furthermore, PDLSCs and control of the RhoA/ROCK pathway have the potential to be for cartilage tissue repair including the temporomandibular joint disc regeneration.

Key words : periodontal ligament, mesenchymal stem cell, RhoA/ROCK, Y-27632, chondrogenesis

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Introduction

The periodontal ligament (PDL) connects the cementum of teeth and alveolar bone. This ligament maintains the tooth position within the alveolar bone and helps to bear the chewing load. The PDL is composed of is known to including several types of cells such as fibroblasts, osteoblasts and cementoblasts.(1, 2) Cells in PDL may contribute the periodontal wound healing and regeneration of alveolar bone.(3, 4) This indicates that PDL contains stem cells. Recently, PDL-derived mesenchymal stem cells (PDLSCs) were found to have characters similar to those of stem cells from other sources.(3, 5) However, the multi-potential capacity of the PDLSCs is still not well known.

Cartilage is an avascular tissue. Recovery of this tissue after injuries is slow and limited. Spontaneous damage to cartilage can lead to osteoarthritis (OA). Spontaneous damage to cartilage can lead to osteoarthritis (OA). More than 30% of elderly individuals suffer from OA which is painful disease in daily bases.(6) In dental field, Temporomandibular disorder (TMD) is similar to OA. TMD is common, 20~25% of the population in general. Symptoms of TMD include temporomandibular joint (TMJ) pain or masticatory muscle pain, limited mouth opening, deviation during mandible movement, and TMJ noise.(7, 8) TMJ disc is composed of articular cartilage. Cartilage is incapable of regeneration due to insufficient vascularity and the low replicable potential of chondrocytes.(9) If degenerative changes are too severe to

be treated with non-invasive management modalities, invasive surgical treatments are considered.(10) These surgical techniques include the use of autologous grafts or alloplastic materials such as acrylic resin, synthetic fibers and full titanium joints. However, autologous graft can damage on the donor site and alloplastic materials are associated with a risk of foreign body reaction or implant displacement/fracture.(11, 12) An interesting progress has been achieved in cartilage tissue repairing using mesenchymal stem cells (MSCs) in dental research field recently. It was reported that autologous MSCs could be used for the in vivo replacement of a rabbit temporomandibular joint disc.(17) The next TMJ disc tissue regeneration studies need the evaluation of available stem cell sources.(14) Additionally, various cell sources include dental stem cells and biomaterials should be studied for clinical application in dentistry.

Previously, we demonstrated human PDLSCS possess stem cell properties using FACS analysis.(13) This study proved that TGF-β3 and BMP-6 alone or in combination can induce PDLSCs chondrogenesis biochemical and molecular biological analysis. These findings demonstrated the human PDLSC could be a potential source for cell therapy of cartilage repair includes TMD.

Recently, a number of investigations that RhoA/ROCK signal might be a crucial role during chondrogenesis.(14, 16, 30, 37) Since chondrocytes are rounded or polygonal, these cells may de-differentiate into fibroblast-like cells with the activation of RhoA/ROCK. Therefore, the inhibition of RhoA or its downstream effectors could activate

chondrocyte maturation. Y-27632, a ROCK inhibitor, could up-regulate the collagen II and aggrecan, major components of cartilage.(15, 16) Furthermore, the inhibition of ROCK by Y-27632 increased Sox9 expression, a major transcription factor for chondrogenesis.(17) Although RhoA/ROCK signaling may be important during chondrogenesis, the role of the pathway and the effectiveness of controlling its signaling in various cell types are still scarce, especially regarding the impact on the differentiation of stem cells into cartilage.

In the present study, we used human PDL-derived MSCs from the third molars. The teeth were obtained from three patients, so that individual variations could be compared. After initiation forming 3D clusters, the clusters were cultured with TGF-β (a chondrogenic growth factor), cytochalasin D (an actin polymerization inhibitor) or Y-27632 (a ROCK inhibitor). The three agents were administered alone or combination, and the synergistic effect on PDLSCs differentiation into chondrocytes was evaluated. Our goal was to elucidate the effects governing chondrogenic regeneration using PDLSCs and understand how these cells could contribute to TMD treatment.

Materials and methods

PDLSCs isolation

The following procedure was approved by the Institutional Review Boards (IRB) of Medical Ethics Committee of School of Dentistry, Seoul National University (No.20120016). Impacted normal healthy third molars were extracted from three patients at Seoul National University Dental Hospital (Seoul, South Korea). The teeth were washed with fresh Hank's balanced salt solution (HBSS) containing antibiotic-antimycotic solution (GIBCO, Grand Island, NY, USA). PDL tissue from the surface of the each tooth was enzymatically dissociated with 1 g/L collagenase type I and 2.4 g/L dispase (GIBCO, Grand Island, NY, USA) for 1 h at 37℃. The cell suspension was filtered and centrifuged at 400g for 4 min at 4℃. The resulting cell PDL-derived cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM; Welgene, Daegu, South Korea) containing 20% fetal bovine serum (FBS; HyClone Laboratories, Vancouver, Canada).

Cell culture and experimental design

The cultures were incubated at 37 ℃ in a 5% CO_2 humidified atmosphere with DMEM solution containing 20% FBS and antibiotic-antimycotic solution. The used cells used for experiments at the sixth passage. To initiate chondrogenesis, the 3D clusters were formed by centrifugation. 2.5×10^5 PDLSCs counted with a hemocytometer were

centrifuged at 500g for 5 min at 4℃. Differentiation of the PDLSCs-derived 3D clusters was induced with 10ng/mL TGF-β3 (R&D Systems, Minneapolis, MN, USA), 3μM cytochalasin D (Sigma-Aldrich, St. Louis, MO, USA) and 10μM Y-27632 (Sigma-Aldrich). To evaluate the synergistic effects of these compounds, the clusters were treatment with combination of 10ng/mL TGF-β3 and 3μM cytochalasin D, or 10ng/mL TGF-β3 and 10μM Y-27632. Defined medium, consisted of high-glucose DMEM supplemented with 50μg/mL ascorbate-2-phosphate, 100μg/mL sodium pyruvate, 40μg/mL L-proline and 1% ITS+Premix (all Sigma-Aldrich, St. Louis, MO, USA). 3D clusters incubated with this medium served as a control. For chondrogenesis, the defined medium was supplemented with either 10ng/mL TGF-β3 (R&DSystems, Minneapolis, MN,USA), 3μM cytochalasin D (Sigma-Aldrich, St. Louis, MO, USA) and 10μM Y-27632 (Sigma-Aldrich) alone or combinations. The medium was replaced every other day and chondrogenic differentiation was allowed to proceed at 37 ℃ in a 5% CO_2 humidified atmosphere until 7d and 14d.

Glycosaminoglycan (GAG) assay

Cellular sulfated GAG was harvested from the hPDLSCs-derived 3D clusters on 7d and 14d, quantified, and normalized based on the total amount of DNA. PDLSCs-derived 3D clusters were digested in 1 mL Papain buffer [100mL of 0.2M sodium phosphate buffer, 0.1M sodium acetate, 0.01 M ethylene diaminetetraacetin acid (EDTA) and 5mM L-

cysteine, pH 6.4] containing 7.6 μL/mL of papain for 18h in a 65℃ water bath. The amount of GAGs was measured using a Blyscan Sulfate Glycosaminoglycan Assay (Biocolor Ltd, Belfast, Ireland). After centrifuging at 10,000g for 10 min, absorbance of the samples was measured with an enzyme-linked immunosorbent assay (ELISA) reader (S500; BIO-RAD, Hercules, CA, USA) at 656 nm using chondroitin-4-sulfate as standard. Total cellular DNA concentrations were measured using a Pico-green dsDNA assay kit (Invitrogen, Camarillo, CA, USA).

Total RNA extraction and reverse transcription-polymerase chain reaction

Total RNA was extracted from PDLSCs after 7 and 14d of culture using Trizol reagent (Invitrogen, Carlsbad, CA, USA). To eliminate possible genomic DNA contamination, the RNA was treated with DNase I (Roche, Mannheim, Germany). cDNA was synthesized with M-MLV reverse transcriptase (Invitrogen, Camarillo, CA, USA) from 2 μg of total RNA according to the manufacturer' s protocols. The cDNA (diluted 1:10) was used for PCR analysis using AccuPower PCR PreMix (Bioneer, Daejeon, Korea) to measure the expression of type I, II, and X collagen (ColI, ColII and ColX); SOX9, and RUNX2. PCR was performed for 28~37 cycles under the following conditions: denaturation at 94℃ for 5 min, amplification at 94℃ for 30s, annealing at specific temperatures (ColI: 54℃, ColII: 58℃, ColX: 58℃, Sox9: 60℃, RUNX2: 64℃) for 30s, and extension at 72℃ for 20 s, based on reported previously.(13) Gene

expression was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Histology, special staining and immunohistochemistry

PDLSCs-derived 3D clusters cultured for 14 d were fixed in 4% phosphate buffered formalin and embedded in paraffin. Sections 4 μm-thick were deparaffinized and stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA), alcian blue (Wako, Osaka, Japan) and safranin O/fast green (Fisher, Fair Lawn, NJ, USA).(13) Von Kossa staining was also conducted to visualize the extent of mineralization of the samples.

ColI and aggrecan contents were evaluated by immunohistochemical staining using an ABC kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer' s instructions. After treating the specimens with serum-blocking solution (Invitrogen, Camarillo, CA, USA) for 30 m to minimized nonspecific binding, the specimens were then incubated with antibodies. To detect ColI, polyclonal rabbit anti-human ColI (Abcam, Cambridge, UK) was used as the primary antibody and anti-rabbit IgG (Invitrogen, Camarillo, CA, USA) served as the secondary antibody. To stain for of ColII and aggrecan, monoclonal mouse anti-human ColII (Calbiochem, Darmstadt, Germany) and anti-aggrecan (Thermo, Rockford, IL, USA) antibody were used as primary antibody and anti-mouse IgG (Invitrogen, Camarillo, CA, USA) was used as the secondary antibody. Antibody binding was visualized with streptavidin-HRP-

conjugated tertiary antibody(Invitrogen, Camarillo, CA USA), followed by treatment with diaminobenzidine (DAB kit; Invitrogen, Camarillo, CA, USA).

Results

Effects of TGF-β3, Y-27632 and cytochalacin D on GAG synthesis

The amount of GAG produced by differentiated 3D clusters from PDLSC was measured on 7 and 14d after treatment with TGF-β3, Y-27632 and cytochalacin D alone or in combination. The results were normalized according to cellular DNA contents. The GAG/DNA ratios for the PDLSCs-derived 3D clusters from the three patients were similar on days 7 and 14. On day 7, GAG/DNA contents of TGF-β3 treated 3D clusters from the three patients (P1, 2 and 3) were higher than those of the control by 194% (P1), 162% (P2) and 163% (P3), Compared to TGF-β3, single treatment with either cytochalasin D and Y-27632 alone did not increase GAG/DNA contents while higher than control. Combined treatment with TGF-β3 and cytochalasin D resulted in similar levels of GAG/DNA contents compared to 3D clusters treated with only TGF-β3. However, co-treatment with TGF-β3 and Y-27632 increased GAG/DNA synthesis by 137% (P1), 109% (P2) and 126% (P3) compared to 3D clusters treated with TGF-β3 alone. Both combined treatments increased

GAG/DNA contents between 2- to 3- fold relative to the control. (Fig. 1) Total cellular DNA contents of all treated PDLSCs-derived 3D clusters from three patients were similar relative to the control.

On day 14, the GAG/DNA ratio and total cellular DNA contents were generally increased compared to those observed on day 7, and the tendency was maintained in all PDLSCs from three patients. TGF-β3 enhanced GAG synthesis by 196% (P1), 169% (P2), and 187% (P3) compared to the control. Compared to TGF-β3, treatment with cytochalasin D alone resulted in decreased GAG synthesis [77.0% (P1), 65.6% (P2) and 62.4% (P3)]. Furthermore, treatment with cytochalasin D alone resulted in GAG/DNA contents similar to those found in the control. In contrast, PDLSCs-derived 3D clusters from P1 and P2 that were treated with Y-27632 had almost similar production levels of GAG/DNA compared to ones exposed to TGF-β3 [92.8% (P1), 92.3% (P2)]. This was not observed for cells from P3 (70.6%). Combined treatment with TGF-β3 and cytochalasin D lowered the GAG/DNA contents in 3D clusters from P2 and P3 compared to cells treated with TGF-β3 alone. This was not observed for PDLSCs-derived 3D clusters from P1 in which the combined treatment resulted in similar production levels of GAG/DNA compared to exposure to TGF-β3 alone. Combined treatment with TGF-β3 and Y-27632 increased GAG/DNA synthesis compared to administration of TGF-β3 alone by 148% (P1), 114% (P2) and 114% (P3). (Fig. 2)

The total cellular DNA contents measured on 14d showed more distinct

variations among the group. In PDLSCs-derived 3D clusters from P1, DNA contents were slightly increased in all treatment groups by about 10% compared to the control except for cells treated with Y-27632 alone. However, variation of the DNA contents in cells from P1 and P2 depended on the type of treatment. In cells from P2, TGF-β3 and cytochalasin D alone as well as those combined treatments. In contrast, the treatment with Y-27632 alone or in its combination treatment with TGF-β3 did not increase DNA synthesis. PDLSCs from P3 showed similar patterns. (Fig. 2)

RT-PCR

The mRNA expressions in the PDLSCs were quantified by RT-PCR. On day 7, the control cells expressed ColI, ColX and RUNX2 but not ColII and Sox9. When TGF-β3 was administered, the 3D clusters expressed ColII and Sox9 while ColI and RUNX2 expressions were decreased compared to the control. ColX was increased by the treatment with TGF-β3. ColI expressions in the presence of cytochalasin D or Y-27632 was relatively high but was decreased in the treatments of TGF-β3 with cytochalasin D or Y-27632. Cytochalasin D did not affect ColII, ColX or Sox9 expression. With Y-27632 treatment, ColX expression was faint while Sox9 level was relatively high. Combined treatment with TGF-β3 and cytochalasin D decreased ColI levels compared to treatment with TGF-β3 alone but Sox9 expression was increased compared to treatment with cytochalasin D alone. This combination also decreased

ColII and RUNX2 expression.

Co-treatment with TGF-β3 and Y-27632 produced synergistic effects on mRNA expression observed on day 7. ColI expression was decreased by the combined treatment compared to both the control (45.9%) and exposure to TGF-β3 alone (38.8%). This treatment also increased the expression of ColII and Sox9 compared to the control while ColII levels were similar to those found with TGF-β3 alone. On the other hand, ColX mRNA level observed with the combined treatment was in between those found in the control and cells treated with TGF-β3 alone. Runx2 expression was decreased to a low level compared to the control (61.5%) and cells treated with only TGF-β3 (51.5%), and Y-27632 only (66.5%).

On day 14, ColI and ColX mRNA expressions in all treated groups along with the control were up-regulated compared to the levels observed on day 7. Co-treatment with TGF-β3 and Y-27632 resulted in similar expression levels of ColII compared to those found with TGF-β3 alone as the positive control. Furthermore, this co-treatment maintained Sox9 expression similar to TGF-β3 alone while the expression of RUNX2 was decreased.

Histological and immunohistochemical analysis

The control group treated with defined medium expressed ColI (Fig. 6a) at a level similar to that observe in cells treated with only TGF-β3 (Fig. 6b). In the group treated with TGF-β3 and Y-27632, relatively low

expression of ColI was found (Fig. 6c). Administration of TGF-β3 alone or co-treatment with TGF-β3 and Y-27632 resulted in greater expression of ColII as well as aggrecan compared to the control. The combined treatment produced greater aggrecan expression than TGF-β3 alone. Control cells were primarily spindle-shaped and had a pale cytoplasm, especially ones in the outer layer of the 3D clusters (Fig. 5a). Treatment with either TGF-β3 alone or TGF-β3 with Y-27632 resulted in a more round cell shape and larger nucleus not only in the inner but also outer layer (Fig. 5b, c). Compared to the control, cells in 3D clusters treated with TGF-β3 alone were strongly positive for safranin O staining (Fig. 5d). Furthermore, this staining was stronger in cells subjected to the combined treatment with TGF-β3 and Y-27632 than ones treated with TGF-β3 alone (Fig. 5e). von Kossa staining for osteogenesis (mineralization) revealed that the control cells and ones treated with only TGF-β3 had random focal positive regions (Fig. 5g, h) while PDLSCs treated with TGF-β3 and Y-27632 were negative (Fig. 5i).

Discussion

Previously, we showed that PDLSCs have the ability to differentiate into chondrocytes after exposure to growth factors including TGF-β3 and BMP-6.(13) Thus, PDLSCs could serve as a cell source for therpaies in dental research such as ones for treating TMD. In the present study, we optimized the condition that control cell cytoskeleton activity by inhibiting ROCK (Y-27632) or cytoskeleton assembly (cytochalasin D) during chondrogenesis in PDLSCs. By performing biochemical analysis such a GAG assay along with genetic and histological/immunohistological assays, we measured the synergistic effect of the growth factor and monitored the control of cell shape. Our results showed the combined treatment with a growth factor (TGF-β3) and an inhibitor of cytoskeleton activity (Y-27632) produced a synergistic effect that influenced PDLSCs chondrogenesis.

To strengthen our findings, we used cells from three individual patients in this investigation. All analyses were performed in triplicate. After initiation by mechanical force (centrifugation), we treated PDLSCs-derived 3D clusters with TGF-β3 as positive control. This compound is known as a potent inducer of chondrogenesis.(13, 18, 19) Inhibitors of cytoskeleton activity such as cytochalasin D or Y-27632 were used to treat the PDLSCs-derived 3D clusters alone or in combination with TGF-β3.

Treatment with Y-27632 resulted in more GAG synthesis than

administration of cytochalasin D. Chondrogenesis-related mRNA expression and histological/immunohistochemical analyses also confirmed this finding (Fig. 1 and 2). These results indicated that Y-27632 has a stronger effector on PDLSCs chondrogenesis than cytochalasin D. Combined treatment with TGF-β3 produced similar results. While cytochalasin D with TGF-β3 did not produce a synergistic effect on GAG production, the combination of Y-27632 with TGF-β3 produced more GAG than TGF-β3 alone as positive control in all three units and on day 7 as well as day 14. Interestingly, TGF-β3 and cytochalasin D, but not Y-27632, increased total DNA contents on day 14. These results indicate that cytochalasin D increases the proliferation ability of PDLSCs similar to TGF-β3 as we reported previously reported.(13) This may suggests that cytochalasin D can be a potential proliferative and DNA replicative agent for PDLSCs.

Basically, our trial is the adding cell shape changing from spiral to round by the control of cytoskeleton activity via controlling RhoA/ROCK pathway to help the chondrogenic differentiation of PDLSCs. Recent investigations have produced a theory stating that changes of cell shape and tension may help to define the lineage commitment.(14, 16, 30, 37) Several reports have also shown that flatten cells may represent osteogenic cells while that are round are chondrocytes.(20, 21) Although the mechanism is still unclear, cell shape is determined by dynamic organization of the cytoskeleton and RhoA/ROCK signaling pathway is important for this process because the pathway regulates actin

cytoskeleton activity.(20, 21)

Cytochalasin D is recognized in majority reports that prevent actin filament polymerization and cause impairment in cell division and function leading to cell death.(25) However, opposing observations have been reported as well. Cytochalasin D activates the survival signaling cascade following disruption of the actin filament.(25) Destruction of the cytoskeleton is thought to be involved in transducing growth promotion signals.(26) In addition, cytochalasin D inhibits cytoplasmic cleavage following nuclear division that increases the possibility of polyploid nuclei formation, and may increase the total DNA contents.(27). However, this compound did not affect the production of GAG in the present study while Y-27632 did. The result indicates that because of our cells are affected more blocking ROCK activity (Y-27632) than cytoskeleton polymerization (cytochalasin D). Changes in chondrogenesis-related mRNA synthesis were also dramatic. Y-27632 induced the expression of ColII and Sox9 levels similar to TGF-β3 and unlike cytochalasin D. Type II collagen is the predominant building material of cartilage and a biomarker of chondrogenesis. Collagen type X collagen is a hypertrophic chondrocyte-specific marker. RUNX2 is essential for osteoblastic lineage formation. Sox9 is an essential transcription factor for the induction of chondrogenesis, particularly during the early steps.(31, 37, 38) In the current investigation, combined treatment with TGF-β3 and Y-27632 produced a synergistic effect on PDLSCs-chondrogenesis. During the early stage, ColII and Sox9 levels were increased while ColI,

ColX and RUNX2 levels were decreased treated with TGF-β3 and Y-27632 on day 7. On day 14, Co-treatment with TGF-β3 and Y-27632 increased ColI and ColX levels. In contrast, the treatment induced Sox9 level higher than TGF-β3 alone and maintained in low level of RUNX2. Based on these results, we do not know whether TGF-β3 and Y-27632 influence the mechanism underlying chondrogenesis or not. However, we can assume that the two agents may exert a synergistic effect not only during early period of chondrogenesis but also for a relatively long period of time in vitro. Meanwhile, expression of ColX and Sox9 were less increased in cells co-treated with TGF-β3 and Y-27632. Even on day 14, the expression level of RUNX2 was much lower than that the control. This implies Y-27632 may delay chondrocyte hypertrophy and osteogenesis. However, contradictory evidence demonstrating that RhoA/ROCK signaling suppresses hypertrophic chondrocyte differentiation and ROCK inhibition causes hypertrophic gene expression has also been reported.(30)

Results of our histological and immunohistochemical analysis demonstrated the synergistic effects of the co-treatment with TGF-β3 and Y-27632 as well. The cells became more round similar to chondrocytes after exposure to the combined treatment with TGF-β3 and Y-27632 and the the special staining Safranin O showed more positive result than TGF-β3 alone (Fig. 5). Interestingly, von Kossa staining specific for calcium deposition was reduced after the co-treatment with TGF-β3 and Y-27632 compared to TGF-β3 alone.

These findings indicate that TGF-β3 may induce osteogenesis while TGF-β3 with Y-27632 inhibits these processes. Cho et al. also previously observed non-specific chondrogenic differentiation of human bone marrow-derived MSCs initiated by TGF-β3.(31) The cells incubated with this compound had higher levels of calcium deposition and significantly increased expression of hypertrophic chondrocyte and osteogenic marker genes. Thus, an agent with more directed chondrogenic inductive potential was suggested based on in vitro analysis. There is a report showing that TGF-βs signaling promotes osteoprogenitor proliferation and osteoblastic differentiation.(32) Further, the signaling is associated with normal bone development and bone healing.(24, 33) These data indicate the non-selective chondrogenic activity by TGF-βs.

Based on the findings from our study, we suggest that inhibition of the RhoA/ROCK pathway may be useful for cartilage tissue regeneration including restoration of the TMJ disc. In this investigation, PDLSCs were obtained from third molar teeth. The third molar is easily available because it is frequently extracted prophylactically, and can serve as a good stem cell source.

When these stem cells are isolated and differentiate into the intended tissue type, they can have advantages of autologous cells. Therapies based on autologous stem cells have a significantly reduced potential for foreign body reaction and involve less invasive technique. We expect that our data will contribute to understanding stem cell chondrogenesis and

can be applied to discovering novel techniques for TMJ disc regeneration to treat TMD.

Acknowledgements

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Figure 1.

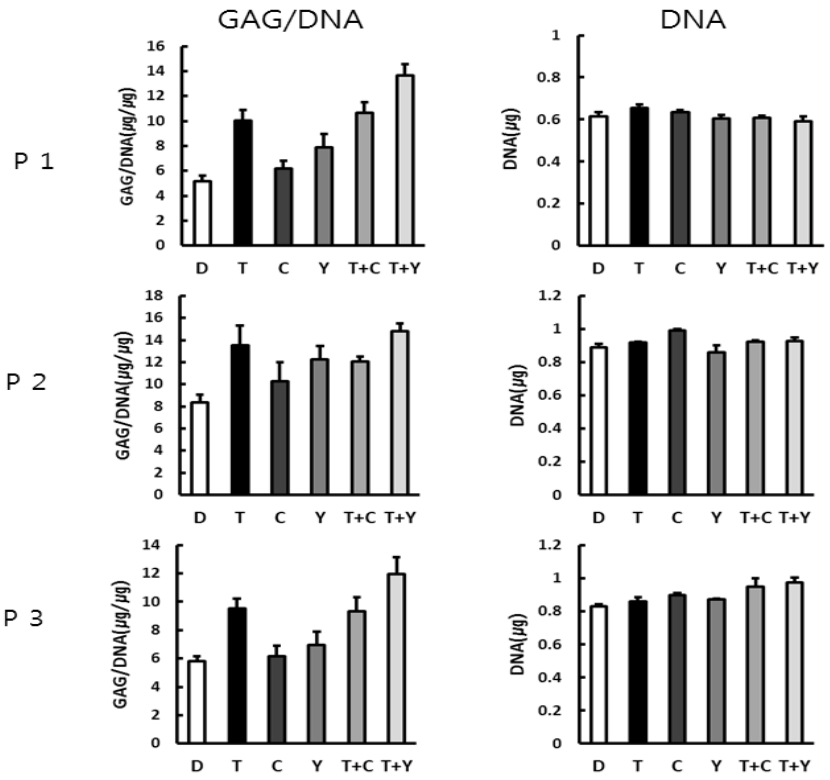

Figure 1. Relative amount of glycosaminoglycan (GAG)/DNA on day 7 in each chondro-cluster from three patients after six different treatments. In the cluster from patient 1 (P1), the amount of GAG/DNA was increased by treatment with T, C, Y, T+C, and T+Y compared to the control by 194%, 119%, 152%, 207%, and 265%, respectively. In the cluster from patient 2 (P2), the amount of GAG/DNA was increased by treatment with T, C, Y, T+C, and T+Y compared to the control by 162%, 123%, 146%, 144%, and 177%, respectively. Total amount of cellular DNA was similar with the six different treatments. In the cluster from patient 3 (P3), the amount of GAG/DNA was increased with T, C, Y, T+C, and T+Y compared to the control by 163%, 106%, 119%, 160%, and 205%, respectively. D, control; T, 10 ng/mL TGF-β3; C, 3 mM cytochalasin D; Y, 10 mM Y-27632; T+C, 10 ng/mL TGF-β3 plus 3 mM cytochalasin D; T+Y, 10 ng/mL TGF-β3 plus 10 mM Y-27632; GAG, glycosaminoglycan; TGF, transforming growth factor.

Figure 2.

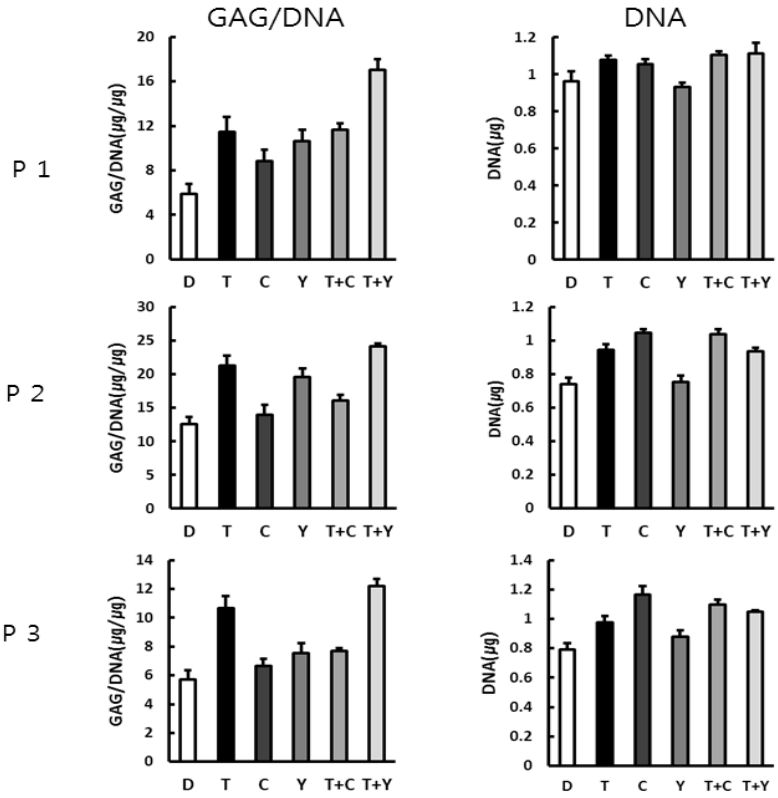

Figure 2. Relative amount of GAG/DNA on day 14 in each chondro-cluster from three patients after six different treatments. In the cluster from patient 1 (P1), the amount of GAG/DNA was increased after treatment with T, C, Y, T+C, and T+Y compared to the control by 196%, 151%, 182%, 199%, and 291%, respectively. In the cluster from patient 2 (P2), the amount of GAG/DNA was increased by treatment with T, C, Y, T+C, and T+Y compared to the control by 169%, 111%, 156%, 128%, and 193%, respectively. In the cluster from patient 3 (P3), the amount of GAG/DNA was increased by treatment with T, C, Y, T+C, and T+Y compared to the control by 187%, 117%, 132%, 135%, and 214%, respectively. The total cellular DNA contents varied under the six different culture conditions. D, control; T, 10 ng/mL TGF-β3; C, 3 mM cytochalasin D; Y, 10 mM Y-27632; T+C, 10 ng/mL TGF-β3 plus 3 mM cytochalasin D; T+Y, 10 ng/mL TGF-β3 plus 10 mM Y-27632; GAG, glycosaminoglycan; TGF: transforming growth factor.

Figure 3.

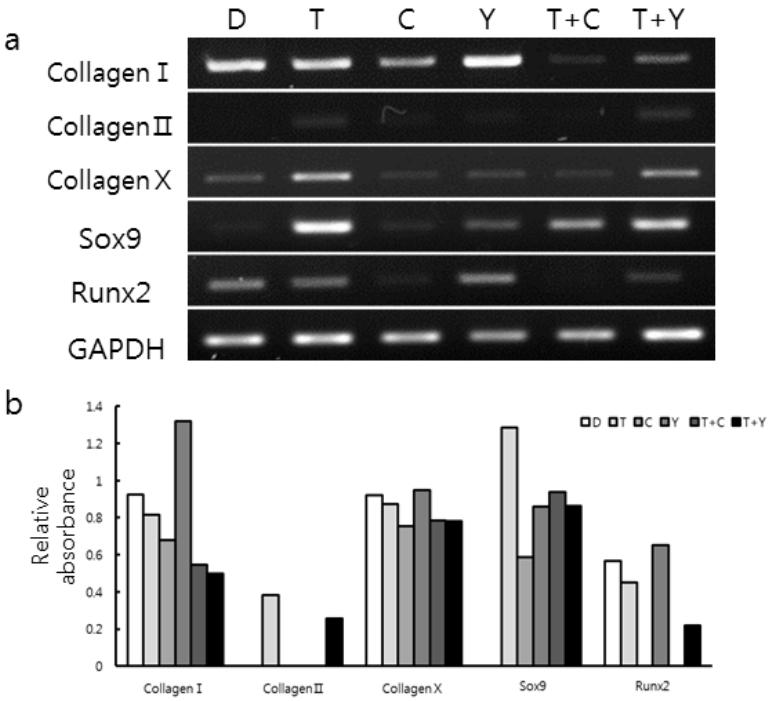

Figure 3. RT-PCR assay to evaluate the expression of chondrogenesis-associated genes in PDLSCs harvested on day 7(a). The relative absorbance corresponds to the level of gene expression in the PDLSCs cultured under six different conditions (b). D, control; T, 10 ng/mL TGF-β3; C, 3 mM cytochalasin D; Y, 10 mM Y-27632; T+C, 10 ng/mL TGF-β3 plus 3 mM cytochalasin D; T+Y: 10 ng/mL TGF-β3 plus 10 mM Y-27632.

Figure 4.

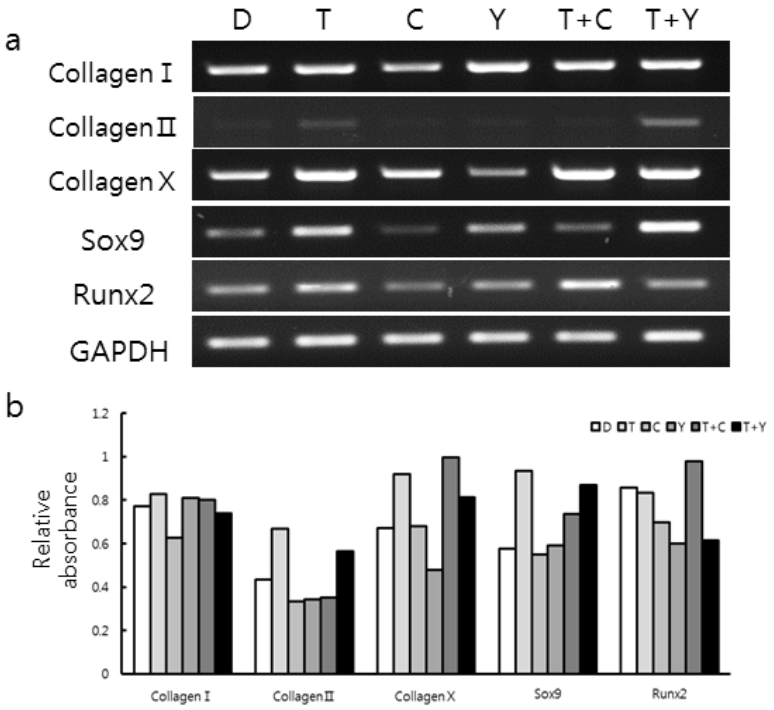

Figure 4. RT-PCR assay to measure the expression of chondrogenesis-associated genes in PDLSCs harvested on day 14 (a). The relative absorbance corresponds to the level of gene expression in the PDLSCs cultured under the six different conditions (b). D, control; T, 10 ng/mL TGF-β3; C, 3 mM cytochalasin D; Y, 10 mM Y-27632; T+C, 10 ng/mL TGF-β3 plus 3 mM cytochalasin D; T+Y: 10 ng/mL TGF-β3 plus 10 mM Y-27632.

Figure 5.

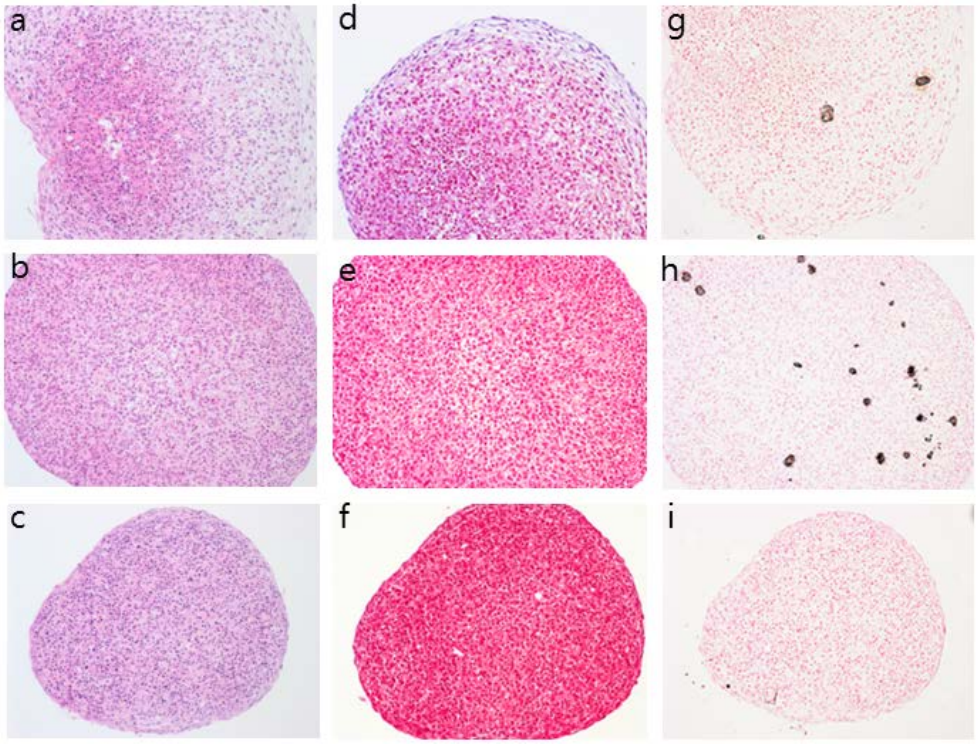

Figure 5. Special staining of PDLSCs cultured for 14 d. (a~c) Hematoxylin and eosin staining of PDLSCs cultured in defined media or in the presence of TGF-β3 and TGF-β3 with Y-27632. (d~f) Safranin O staining of PDLSCs grown in defined media or in the presence of TGF-β3 and TGF-β3 with Y-27632. (g~i) Von Kossa staining of PDLSCs cultured in defined media or in the presence of TGF-β3 and TGF-β3 with Y-27632.

Figure 6.

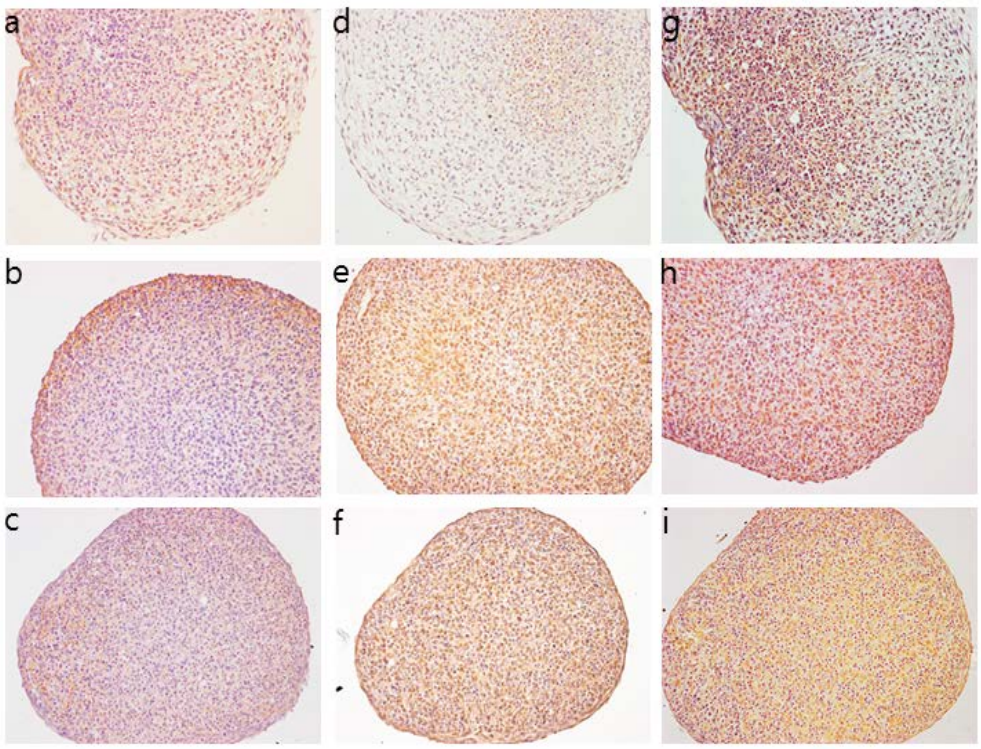

Figure 6. Immunohistochemical analysis of PDLSCs cultured for 14 d. (a~c) Type I collagen-specific staining of PDLSCs cultured in defined media or in the presence of TGF-β3 and TGF-β3 with Y-27632. (d~f) Type II collagen-specific staining of PDLSCs grown with defined media and in the presence of TGF-β3 or TGF-β3 with Y-27632. (g~i) Aggrecan-specific staining of PDLSCs cultured in defined media or in the presence of TGF-β3 and TGF-β3 with Y-27632.

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초 록

여러 연구에서 RhoA-ROCK 경로를 조절함으로써 줄기세포의 연골분화과정을 촉진시킬 수 있다는 결과가 보고되었다. 이를 바탕으로 이번 연구에서 사람 치주인대 유래 세포에 TGF-β3(연골분화유도 성장인자), Cytochalasin D(액틴중합 억제인자), Y-27632(ROCK 억제인자)를 이용하여 RhoA-ROCK 경로를 억제하였다. 치주인대유래세포는 서울대학교 치과병원에 내원한 세 명의 환자로부터 발치된 세 개의 제3대구치에서 간엽줄기세포를 분리하였다. 3D 세포군집을 형성하여 연골분화과정을 개시한 후, 10 ng/mL TGF-β3, 3 μM cytochalasin D, 10 μM Y-27632, TGF-β3+cytochalasin D, TGF-β3+Y-27632로 처리하거나 아무 처리도 이루어지지 않은 음성 대조군에서 7일 또는 14일간 배양하였다. Glycosaminoglycan assay (GAG), 세포조직학적 평가, safranin O, Von Kossa 염색, PCR을 통해 분석하였고, Collagen I, II 과 aggrecan의 발현을 확인하기 위해 면역염색을 행하였다. TGF-β3와 Y-27632를 병용처리한 경우 가장 높은 GAG 합성량을 나타내었다. Cytochalasin D와 달리, Y-27632는 시너지 효과도 나타내었다. TGF-β3와 Y-27632를 병용처리한 세포에서 collagen type II and sox9와 같은 연골분화관련 유전자의 발현이 증가하였고, Runx2와 같은 골분화 유전자는 감소하였다. 반면 TGF-β3와 cytochalasin D를 병용처리한 세포에서는 Runx2와 비대연골세포 표지 유전자인 collagen X이 가장 많이 발현되었다. 무기질 침착을 보여주는 Von Kossa 염색에서 TGF-β3와 Y-27632를 함께 처리한 세포가 가장 낮은 침착량을 나타내었다. 이 연구를 통해 TGF-β3와 RhoA/ROCK 경로를

억제하는 Y-27632를 함께 처리함으로써 선택적으로 연골분화과정을 유도할 수 있음을 확인할 수 있었다. 더 나아가 치주인대유래 줄기세포와 RhoA/ROCK 경로 조절이 연골 조직 재생에 가능성이 있다는 것을 알 수 있었고, 측두하악관절 재생에도 응용될 수 있기를 기대한다.

주요어 : 연골분화, RhoA-ROCK, 간엽줄기세포, 치주인대

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