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A study of PDLF-related gene expression according to FGF and/or PDLF-conditioned media on bone marrow stromal cell of dog

조선대학교 대학원

치 의 학 과

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개의 골수기질세포에 FGF와 PDLF-conditioned media 처리시
치주인대섬유모세포 관련 유전자의 발현에 관한 연구

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치   의   학   과

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유현주의 석사학위 논문을 인준함.

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유 현 주

지도교수 : 장 현 선

조선대학교 대학원 치의학과

발거가 예상되는 치아들에서는 치주인대가 상실되어 있어 치아의 재식이 현실적으로 어려워, 고비용의 임플란트 치료가 증가하고 있어서 그로인한 환자의 부담이 가중되고, 임프란트 주변에는 치주인대가 없기 때문에 향후 재생시 한계점을 드러낸다. 치주조직 재생에 필수적인 치주인대섬유모세포가 상실된 심한 치주염환자들에서 치주재생에 필수적인 요소를 획득할 수 있도록 줄기세포를 이용한 조직공학의 연구가 필요한 시점이다. 그러나, 다양한 분화가 가능한 줄기세포에서의 치주인대섬유모세포로의 분화에 관한 연구는 미비한 실정이다. 본 연구의 목적은 섬유모세포 성장인자와 치주인대섬유모세포 배양액이 줄기세포에서 치주인대섬유모세포로의 분화에 끼치는 영향을 평가하기 위하여 치주인대 관련유전자들의 발현을 평가하고자 한다.

줄기세포는 Dog의 골수기질세포를 실험에 이용하였다. 줄기세포에 섬유모세포 성장인자와 치주인대섬유모세포 조건배지을 첨가한 것을 실험군으로 하였다. 실험군을 세분하였는데, 섬유모세포성장인자을 적용한 것을 실험1군, 치주인대섬유모세포-조건배지를 적용한 것을 실험 2군, 그리고 섬유모세포성장인자와 치주인대섬유모세포-조건배지를 함께 적용한 것을 실험 3군으로 세분하였다. 대조군은 아무런 처치를 하지 않고 단순히 골수기질세포만을 배양한 것으로 하였다. 치주인대섬유모세포 조건배지는 개의 치주인대섬유모세포를 배양하여 그 배양액을 조건배지로 이용하였다. 세포는 60mm 배양접시에서 10% Fetal Bovine Serum(FBS, Gibco BRL)이 함유된 Dulbecco's Modified Eagles Medium(DMEM, Gibco BRL)을 이용하여 5% CO_2, 37℃, 100% 습도 조건에서 배양하였다. 섬유모세포성장인자는 3ng/ml로 적용하였고, 치주인대섬유모세포 조건배지는 치주인대섬유모세포만 배양하여 얻은 그 배양액을 줄기세포 배양액과 1:1 비율로 적용하였다. 배지는 이틀에 한번씩 교체하고 7일동안 배양한 후, 치주인대관련 유전자 발현을 평가하기 위하여 Trizol reagents (Gibco, BRL, Rockville, USA)를 이용하여 각각의 세포에서 total RNA를 추출하였다. 줄기세포에서

치주인대섬유모세포로의 분화의 영향을 평가하기 위하여 Nestin, UNCL, S100A2 mRNA의 발현유무를 확인하기 위하여 RT-PCR를 시행하였다. 본 연구 결과, 줄기세포에 FGF와 치주인대섬유모세포-조건배지를 적용시 치주인대섬유모세포 관련유전자들의 발현이 더 두드러졌다. 또한 FGF 적용시 전반적인 유전자의 발현이 증가하는 경향을 나타내었다. 본 연구결과 줄기세포의 치주인대섬유모세포로의 분화에 FGF와 치주인대섬유모세포-조건배지가 기여할 수 있을 것으로 사료되며, 향후 치주인대공학에 응용할 수 있을 것으로 사료된다.

주요어: 줄기세포, 치주인대섬유모세포, 유전자, 섬유모세포성장인자, 조건배지

Introduction

The periodontal tissue is composed of cementum, bone, gingiva, and periodontal ligament. Periodontitis can be caused by infection, mechanical stress and aging. Once the periodontal tissue is impaired, the tissue has a limited capacity for regeneration.(1) Since cementoblasts, osteoblasts, and periodontal ligament fibroblasts are derived from periodontal ligament(11), regeneration of periodontal tissue can be limited in severe periodontitis patients with periodontal ligament loss.

Because conventional periodontal regeneration methods remain insufficient to obtain a complete regeneration in periodontitis patients, the concept of periodontal tissue engineering has resently been needed to improve the healing of periodontal tissues.(2)

Stem cells are pluripotent cells with unlimited proliferation potential and differentiation capacity to all types of somatic cells. Periodontal tissue engineering based on in vitro expanded cells holds the promise to overcome the limitations associated with contemporary regenerative techniques. Inanc B, et al. (5) reported that stem cells may become a cell source with unlimited supply for periodontal tissue engineering applications.

Fibroblastic differentiation from stem cells is of widespread significance in the engineering of virtually all tissues including tendons, ligaments, periodontal ligament, cranical sutures and as interstitial filler of all organs.(9)

Lee CH, et al. (9) observed that connective tissue growth factor (CTGF)-treated hMSCs failed to show osteogenic or chondrogenic differentiation, and that CTGF is an effective induction factor for fibroblastic differentiation of human human mesenchymal stem cells. Ogiso B, et al. (14) reported that co-culture of human periodontal ligament fibroblast or rat skin fibroblast with bone marrow stromal cells(BMSCs) resulted in a large reduction of bone nodule formation, and that fibroblasts may inhibit osteoblast differentiation.

MSCs generally are positive for nestin, which is an early marker of neuronal progenitors.(4, 16) Bone marrow stromal cells can be expanded rapidly in vitro and have the potential to be differentiated into neuronal cell types. (16)

Hermann A, et al. (4) observed that their data provide an impetus for differentiating human bone marrow stromal cells in vitro into mature neuroectodermal cells. MSCs can become a valuable cell source as an autograft for clinical application involving regeneration.

The aim of this study was to investigate the periodontal ligament differentiation possibility of BMSCs under the influence of fibroblast growth factor and/or periodontal ligament-conditioned media in vitro.

Materials and Methods

Cell Culture

Then bone marros stromal cells (BMSCs) and periodontal ligament fibroblasts (PDLF) of dog were used for our study. Fresh media obtained from culturing human periodontal ligament fibroblasts of dog were used for conditioned media. 8 passage BMSC and 6 passage PDLF were used for this study. The BMSC and PDLF were cultured in Dulbecco's Modified Eagles Medium (DMEM, Gibco BRL, USA) containing 10% fetal bovine serum (FBS) at 5% CO_2, 37℃, and 100% humidity.

Conditioned medium experiments

The cells were cultured at 5% CO_2, 37℃, and 100% humidity in a 60 ㎜ petri dish until 7 days. The media change was done in a humid incubator in a 2-day interval by exchanging DMEM medium containing 10% FBS. The control group was BMSC without treatment. The experimant groups were BMSC treated with FGF, FGF plus fresh media of hPDLF, fresh media of hPDLF, fresh media of dPDLF, FGF plus fresh media of dPDLF, respectively. The 3 ng/㎖ fibroblast growth factor (FGF) was applied in a 2-day interval when the experimental cell culturing media was changed. The FGF applied media was named by FGF-treated media.

Fresh media obtained from culturing periodontal ligament fibroblasts of human and dog was applied in a 2-day interval when the experimental cell culturing media was changed. It is named by PDLF-conditioned media. The PDLF-conditioned media was obtained in a 2-day interval when the PDLFs culturing media was changed. The PDLF-conditioned media was made by mixture of 50:50 ratio with fresh media obtained from PDLF and culture media.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The total RNA was extracted according to the manufacturer's instruction using Trizol Reagent (Invitrogen, USA). The first-strand cDNA was

synthesized using 25U of oligo-d(T) primer and premix (Bioneer, Korea) per 1 μg of total RNA from cultured gingival fibroblast. The sense and anti-sense oligonucleotide primers for the base sequences of nestin, UNCL, S100A2 and GAPDH were ordered (Table 1). Using the template of cDNA processed from the reverse transcription (RT) process, 20 $\mu\ell$ of PCR mixture was prepared by adding 20 pmols of primer, 5 $\mu\ell$ of cDNA, AccuPower Premix (Bioneer, Korea), and distilled water.

PCR was done by 30-35 cycles of denaturation, annealing, and extension (Table 2) using PTC-200 (MJ Research Inc., Watertown, MA, U.S.A). PCR products were analyzed by eletrophoresing in 1.5% agarose gel to confirm gene expressions.

Table 1. Nucleotide sequences of the primers used for RT-PCR.

	Primer	Sequences 5′→3′	Predicted size (base pairs)
GAPDH	Sense	CCATGGAGAAGGCTGGG	200
	Anti-sense	CAAAGTTGTCATGGATGACC	
Nestin	Sense	GCCCTGACCACTCCAGTTTA	98
	Anti-sense	GGAGTCCTGGATTTCCTTCC	
S100A2	Sense	AAGAGGGCGACAAGTTCAAGC	278
	Anti-sense	GAATGTTGCAGGAAACAGCCA	
UNCL	Sense	ACCAGTGGGCCAGAGATGACC	493
	Anti-sense	GAAAGCCCGTAGAGCAGAATCAGA	

Table 2. Conditions for RT-PCR.

	Temperature (℃)			Time (min.)
	GAPDH	Nestin, S100A2	UNCL	
Predenaturation	94	94	94	5
denaturation	94	94	94	1
Annealing	55	55	45	1
Polymerization	72	72	72	*

*Polymerization time: GAPDH, UNCL (1.5min), S100A2, nestin(0.5), min.: minute

Results

Morphological changes according to the FGF-treated media and/or PDLF-conditioned media of BMSC

Morphological changes of the cells were observed under a phase contrast microscope. At 7 days, more proliferation was observed under the FGF treated, PDLF-conditioned, FGF plus PDLF-conditioned groups than the control group.
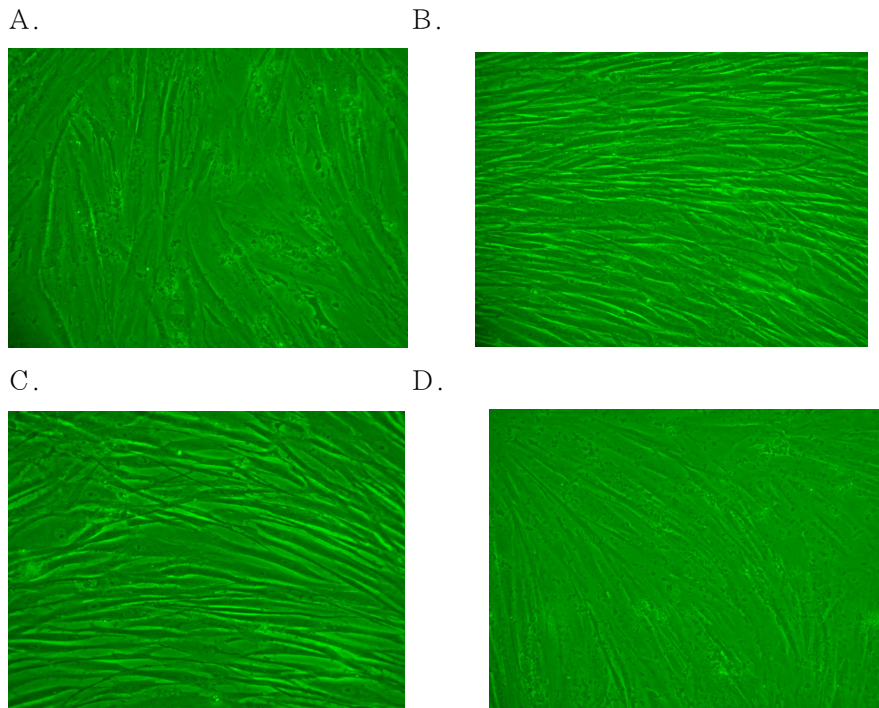
A. B.

C. D.

Figure 1. Effect of FGF treated and/or PDLF-conditioned media on bone marrow stromal cell. At 7 days, cell proliferation was more observed in FGF and/or PDLF-conditioned groups than the control group. A. Untreated BMSC cultures, B. FGF-treated BMSC, C, PDLF-conditioned medium, D. FGF-treated plus PDLF-conditioned medium.

Pattern of gene expression using RT-PCR

The PDLF related genes was increasing tendency in the experimental groups. The expression of UNCL mRNA genes were increased with FGF treated and FGF plus PDLF-conditioned media than the control group. S100A2 mRNA was more expressed in the PDLF-conditioned media than the control group. The nestin, UNCL, and S100A2 mRNA genes was expressed in the control group. The expression of nestin mRNA was similar between FGF treated and the control group. The expressions of nestin mRNA showed reducing tendency

in the PDLF-conditioned media and FGF-treated plus PDLF-conditioned media of the experimental groups.

FGF-treated and PDLF-conditioned media showed differentiation trend of BMSCs into PDLF in vitro. Differentiated cells strongly expressed PDLF related genes(e.g. UNCL, S100A2) detected by RT-PCR method.
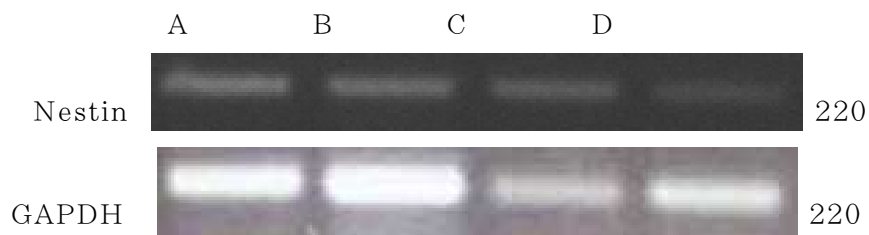
Figure 2. Gene (Nestin mRNA) expression pattern according FGF-treated and/or PDLF-conditioned media. A. Untreated BMSC cultures, B. FGF-treated BMSC, C, PDLF-conditioned medium, D. FGF-treated plus PDLF-conditioned medium. at 7 days.

Figure 3. Gene (UNCL mRNA) expression pattern according FGF-treated and/or PDLF-conditioned media. A. Untreated BMSC cultures, B. FGF-treated BMSC, C, PDLF-conditioned medium, D. FGF-treated plus PDLF-conditioned medium. at 7 days.

Figure 4. Gene (S100A2 mRNA) expression pattern according FGF-treated and/or PDLF-conditioned media. A. Untreated BMSC cultures, B. FGF-treated BMSC, C. PDLF-conditioned medium, D. FGF-treated plus PDLF-conditioned medium. at 7 days.
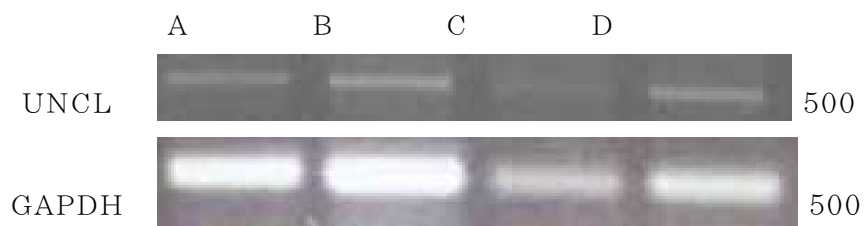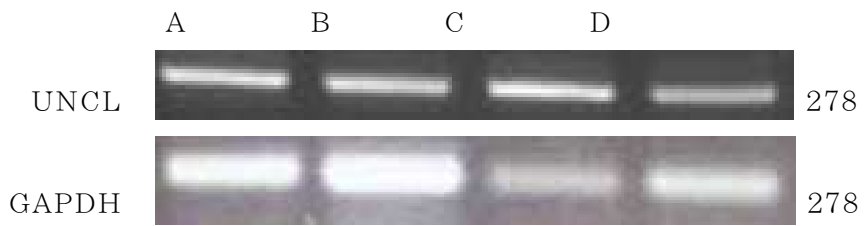
Discussion

Bone marrow stromal cells (BMSCs) reside in bone marrow and provide a lifelong source of new cells for various tissues. A BMSC-specific genetic catalog may facilitate future studies on molecular mechanisms governing core properties of these cells.(7)

The dental follicle is an ectomesenchymal tissue surrounding the developing tooth germ. It is believed that this tissue contains stem cells and lineage committed progenitor cells or precursor cells (PCs) for cementoblasts, periodontal ligament cells, and osteoblasts. Morsczeck C, et al. (12) reported the isolation of PCs derived from dental follicle of human third molar teeth. These fibroblast-like, colony forming and plastic adherent cells expressed putative stem cell markers Notch-1 and nestin. They compared gene expressions of PCs, human mesenchymal stem cells (hMSCs), periodontal ligament cells (PDL-cells) and osteoblasts (MG63) for delimination of PCS. Morsczeck C, et al. (12) demonstrated that cultured PCS are unique undifferentiated lineage committed cells residing in the periodontium prior or during tooth eruption.

Lu J, et al. (10) reported the therapeutic potential of adult bone marrow stromal cells (BMSCs). BMSCs expressed neural markers nestin. Nestin and GFAP double immunofluorescence showed differentiation of BMSCs into neural stem cells in vitro. Differentiated cells express neural specific genes(e.g. 68-kDa neurofilament, β-tubulin III and nestin) detected by RT-PCR method. Bone marrow stromal cells (BMSCs) are pluripotent stem cells with self-renewal property and potential to differentiate into a variety of cell types. (6, 18)

Our study was performed to investigate the differentiation of bone marrow stromal cells (BMSC) into periodontal ligament fibroblast-like cells and to explore their potential use for periodontal ligament engineering. RT-PCR was used to examine mRNA expression of nestin, UNCL and S100A2 in the fibroblast growth factor and/or periodontal ligament fibroblast conditioned media-treated BMSCs.

Bone marrow stromal cells could express phenotypes of neurons. Yang LY, et al. (17) founded that rat bone marrow stromal cells expressed NSE and nestin

mRNA, but only weak expression of NF1 mRNA was detected. After Salvia miltiorrhiza induction for 24 hours, NSE and nestin expression maintained the same level, but NF1 expression increased apparently compared to the control. rBMSC could also differentiate into neuron-like cells and express neuron phenotype as previously described.

Cells of the periodontal attachment (cementoblast, osteoblasts, and periodontal ligament fibroblasts) are descended from a common progenitor (the cranial neural crest). Lallier TE. (8) suggested that differential expression of semaphorins and plexins may be involved in regulating cell-sorting in the formation and regeneration of the periodontal attachment structure.

Ogiso B, et al. (15) reported that fibroblasts secrete prostaglandins which can inhibit bone formation, and that this may be one mechanism whereby fibroblasts can modulate osteogenesis at the interfaces of soft and mineralizing connective tissues. Fibroblast-conditioned medium was obtained from cultures of human periodontal ligament fibroblasts that were untreated (plain) or treated with the indicated concentrations of indomethacin for the entire culture period.(15)

Moreau JE, et al. (13) reported that the sequential administration of growth factors to first proliferate and then differentiate BMSCs cultured on silk fiber matrices will support the enhanced development of ligament tissue in vitro. Confluent second passage (P2) BMSCs obtained from purified bone marrow aspirates were seeded on RGD-modified silk matrices. Seeded matrices were divided into three groups for 5 days of static culture, with medium supplement of basic fibroblast growth factor (B) (1ng/ml), epidermal growth factor (E; 1ng/ml) or growth factor-free control (C). After day 5, medium supplement was changed to transforming growth gactor-β1 (T; 5ng/ml) or C for an additional 9 days of culture. Sequential growth factor application promoted significant increases in collagen type I transcript expression from day 5 of culture to day 14, for five of six groups tested. Moreau JE, et al. (13) reported that the findings indicates significant in vitro ligament development after only 14 days of culture when using a sequential growth factor approach.

Farhadi J et al. (3) reported that human bone marrow stromal cells from six donors were expanded for two passages (expansion phase) and subsequently cultivated in osteogenic medium containing ascorbic acid, β-glycerophosphate,

and dexamethasone (differentiation phase). After each phase, cells were transferred into serum-free medium with or without FGF-2 at different concentrations and for different times, and the expression of BMP-2, TGF-β1, and VEGF was quantified at the mRNA level. In response to 5 ng/ml FGF-2 for 24 hours, the mRNA expression of VEGF increased at both culture phases (up to 6.1 fold), whereas that of BMP-2 and TGF-β1 significantly increased only after the expansion (3.1-fold) or differentiation phase (2.1-fold), respectively. FGF-2 up-regulates the expression of BMP-2, TGF-β1, and VEGF in human bone marrow stromal cells, in a pattern dependent on the cell-differentiation stage. These findings prompt for in vivo investigations on the delivery of FGF-2 for the temporally/functionally regulated enhancement of bone marrow stromal cell-based bone induction.(3)

In our study, periodontal ligament fibroblast-conditioned medium was obtained from cultures of dog periodontal ligament fibroblasts and human periodontal ligament fibroblats that were untreated (plain) or treated with the indicated concentrations (3ng/ml) of fibroblast growth factor for the 1-, 7-, 11-and 14 days culture period.

Stem cell marker gene (nestin mRNA) expression pattern was increased in the treated group with the indicated concentrations (3ng/ml) of fibroblast growth factor. PDL fibroblast marker genes (UNCL-, S100A2-mRNA) expression pattern was increased in the periodontal ligament fibroblast-conditioned medium group and the combined group than the control.

Conclusion

These findings suggest implications for engineering periodontal ligament by providing the initial evidence of a reproducible protocol for periodontal ligament fibroblastic differentiation of bone marrow stromal cells.

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저작물 이용 허락서

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본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

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