The effects of enamel matrix derivatives on the proliferation and gene expression of PDL fibroblast, $SaOs_2$ cells and Cementum derived cells

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I. Introduction

The ultimate goals of periodontal therapy are to control inflammation of periodontal tissue and to regain supporting tissue lost as a consequence of periodontal disease. So many regenerative procedures to restore lost attachment apparatus have been proposed. Treatment for lost periodontal tissue according to the principles of guided tissue regeneration has gained increasing acceptance in recent years, but the true periodontal regeneration remains elusive.

An alternative approach to obtain periodontal regeneration is to try to mimic the events that take place during the development of the dental root. According to Slavkins'[1] and Lindskogs'[2-4] studies, Hertwig's epithelial root sheath(HERS) breaks during root embryogenesis following dentin deposition and the inner layer of this sheath secretes enamel matrix proteins. These proteins stimulate the differentiation of cementoblasts from the mesenchymal cells of the dental follicle with the formation for acellular cementum. Recently, a purified enamel matrix protein product-Emdogain(Enamel matrix derivatives(EMD; BIORA AB, Malmo, Sweden) was introduced. EMD is prepared from developing porcine teeth.[6] This protein mixture consists primarily of amelogenin(>90%), and contains no detectable growth factors.[7]

However, there are many reports which show that the origin of cementoblasts is the mesenchymal cells of the dental follicle and the roles of enamel matrix derivatives in cementogenesis is minimal.[8,9] According to Diekwisch,[8] mesenchymal cells from the dental follicle penetrate the HERS bilayer

*This work was supported by the S.N.U foundation and Overhead Research Fund.
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and deposit initial cementum matrix while immediately adjacent epithelial cells are separated from the root surface by a basal lamina and do not secrete any cementum matrix. So, we tried to determine whether EMD had an effect on gene expression and cell proliferation in PDL fibroblasts, osteoblasts and cementoblasts in vitro.

Although EMD is commonly used in clinics, little is known regarding its effects on periodontal regeneration in cellular level. A major rationale for using EMD clinically is based on the hypothesis that epithelial- mesenchymal interactions are required for the maturation of the developing periodontium and hence for the regeneration of periodontal tissues.

The purpose of this study is to investigate the differential effects of EMD on proliferation and gene expression of PDL fibroblasts, osteoblast-like $SaOs_2$ cells and human cementum-derived cells in vitro.

II. Materials and methods

Materials

The Emdogain®(Enamel Matrix Derivative, EMD) was supplied by BIORA AB, Malmo, Sweden. EMD is insoluble at physiologic pH and at body temperature due to its protein composition. Its solubility increases at acid or alkaline pH and low temperature.[6)]

Therefore, 5 mM acetic acid was used for study purposes. This concentration of acetic acid was enough to dissolve the compound without negatively interfering with the experimental conditions.[11)]

Cell isolation & Expansion

Methods used to isolate and culture PDL fibroblasts and cementum-derived cells have been described in previous report.[12)] The brief methods will be described below. Healthy human premolar teeth extracted for orthodontic reasons were used.

PDL fibroblast culture

PDL from the middle portion of the root was scraped with a curet and then placed in 100 mm tissue culture dishes, containing 5 ㎖ of growth medium(DMED supplemented with 10% FBS and 1% antibiotics). Cells were incubated in a humidified atmosphere, 95% air, 5% CO_2, at 37℃. Media were changed every third days.

Cementum-derived cells culture

After PDL was manually scraped from the root, the tooth was washed with DMEM medium and incubated in 4 ㎖ of medium containing 100mU/㎖ of collagenase P for 1h at 37℃. Released cells from the medium were discarded and the teeth were washed three times with fresh medium. Using a sterile surgical scalpel, cementum and a thin layer of underlying dentin were dissected and collected. Cementum/dentin fragments were thoroughly washed with medium (five times) and then minced with scissors until small fragments were obtained. The small fragments were washed with medium one more time and digested again with collagenase P for 1h at 37℃. The medium with

released material was discarded. The final fragments were washed thoroughly with medium and then placed in 100㎜ tissue culture dishes containing 5㎖ of growth medium.

Proliferation study

Cultured three different types of cells were plated on 35㎜ Petri dishes at 5×103 cells/Petri dish in Dulbecco's modified eagle medium(DMEM) supplemented with 10% fetal bovine serum(FBS) and 1% antibiotics. Cells were incubated for 24h at 37℃ in a 5% CO_2 atmosphere. The dishes were then washed with culture medium to remove floating cells. Two control groups were cultured in 10% FBS and 1% antibiotics with or without 5 mM acetic acid, respectively and the other two test groups were incubated in DMEM with 10% FBS and 1% antibiotics 100 ㎍/㎖ EMD with or without acetic acid, respectively. For each group (control without and with acetic acid, EMD without and with acetic acid), three Petri dishes were used for each day(at 1, 3 and 8 days). From every groups, we took three samples and counted the cells with the Burker's chamber. Thus for each cell type we made a total of twelve counts at day 1, 3 and 8. Data were statistically analyzed with repeated measures of ANOVA by comparing total cell numbers.

Detection of mRNA by RT-PCR

Three different types of cells were plated on 35㎜ Petri dishes at 5×10^4 cells/Petri dish in DMEM supplemented with 10% FBS and 1% antibiotics. Cells were incubated for 24h at 37℃ in a 5% CO_2 atmosphere. The dishes were then washed with culture medium to remove floating cells. Cells were divided into three different groups; control, 5mM acetic acid control, 100㎍/㎖ Emdogain containing 5mM acetic acid. Cells were cultured at day 1, 3 and 8.

Total RNA from cultured cells was isolated with the use of high pure RNA Isolation Kit(Roche Molecular Bio chemicals, Manheim, Germany) according to manufacturer's instruction. RT-PCR assays were carried out with thermal cycler(Effendorf AG, Hamburg, Germany).

1㎍ of total RNA were reverse transcribed into complementary DNA(cDNA) with unit/㎕ RT(AMV reverse transcriptase, Roche Molecular Biochemicals), 2.0㎕ of 1× reaction buffer(100mM Tris, 500mM KCl; pH 8.3), 4.0㎕ of 5mM $MgCl_2$, 2.0㎕ of deoxynucleotide mix primer, 1.0㎕ of RNAase inhibitor at 25℃ for 10 minutes for annealing and then at 42℃ for 60 minutes for reverse transcription resulting in cDNA synthesis. Following the 42℃ incubation, the AMV reverse transcriptase is denaturated by incubating the reaction at 99℃ for 5 minutes and then cooled down to 4℃ for 5 minutes.

The resulting single stranded DNA is amplified using the reverse transcribed mixture containing 250 uM dNTP, 2 mM $MgCl_2$, 1× volume of reaction buffer, and 0.5 unit of Tag polymerase(Roche Molecular Biochemicals, Manheim, Germany) as a template with the specific oligonucleotide primers for human which were derived from known sequences(table1). The primers of human col-

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

		Sequence	Expected size of PCR product
18s rRNA	(s)	5′-GCGAATTCCTGCCAGTAGCATATGCTTG-3′	126 bp
	(as)	5′-GGAAGCTTAGAGGAGCGAGCGACCAAGG-3′	
Human ALP	(s)	5′-ACGTGGCTAAGAATGTCATG-3′	475 bp
	(as)	5′-CTGTAGGCGATGTCCTTA-3′	
Human COL I	(s)	5′-TATGGCGGCCAGGGCTCCGACCTG-3′	325 bp
	(as)	5′-CCAAGGGGGCCACATCGATGATGGG-3′	
Human OC	(s)	5′-CATGAGAGCCCTCACA-3′	310 bp
	(as)	5′-AGAGCGACACCCTACAC-3′	
Human OP	(s)	5′-CCAAGTAAGTCCAAAG-3′	347 bp
	(as)	5′-GGTGATGTCCTCGTCTGTA-3′	
Human BSP	(s)	5′-TCAGCATTTTGGGAATGGCC-3′	615 bp
	(as)	5′-GAGGTTGTTGTCTTCGAGGT-3′	

* s: sense, as: antisense
* ALP: alkaline phosphatase, COL I: collagen type I, OC: osteocalcin, OP: osteopontin, BSP: bone sialoprotein

lagen type 1(COL 1), human osteopontin (OP), human osteocalcin(OC), human alkaline phosphatase(ALP) and human bone sialoprotein(BSP) were used for RT-PCR to detect the presence of mRNA.

PCR of the cDNA products was carried out in a final volume of 20㎕ containing of 2 ㎕ cDNA, 1㎕ of 20pmol/㎕ of each of the forward and reverse primers, and 16㎕ of autoclaved DDW. PCR protocol was 30 cycles of denaturing, annealing and primer extension, which was controlled by the Thermal Cycler(Mastercyler Gradient, Eppendorf, Hamburg, Germany).

III. Results

Proliferation study

In all cell types, cell numbers increased significantly over the time during examination periods, regardless of addition of EMD or acetic acid($p<0.05$). However, there were no significant differences in the total cell number between two control groups and two test groups and also between acetic acid groups and non acetic acid groups($p>0.05$). Neither 5mM acetic acid nor 100㎍/㎖ Emdogain did change cell proliferation. At day 1, in PDL cells, EMD and EMD plus acetic acid groups had more cell numbers than control and acetic acid control groups, but, these differences were not statistically significant($p<0.05$).

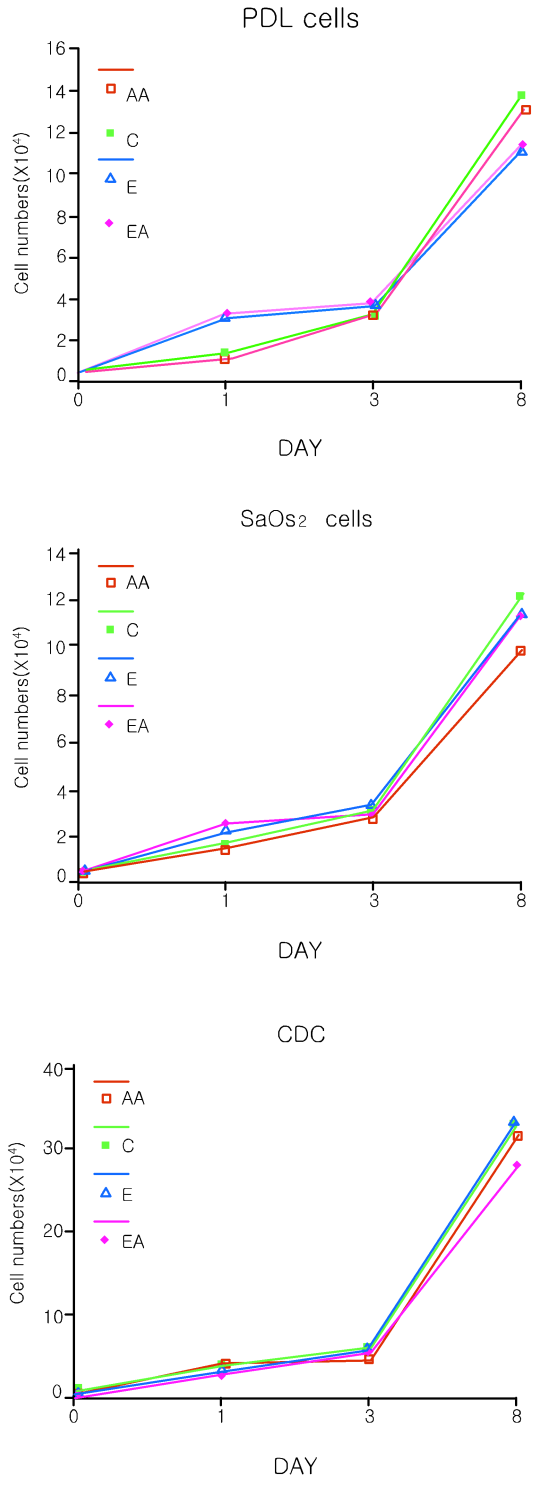

Figure 1. Mean cell numbers of PDL fibroblasts, $SaOs_2$ cells and CDC at day 1, 3, 8.

Table 2. Mean cell numbers (x10^4) of PDL fibroblasts, $SaOs_2$ cells and CDC at day 1, 3, 8.

	PDL			$SaOs_2$			CDC		
	day1	day3	day8	day1	day3	day8	day1	day3	day8
C	1.42	3.17	13.67	1.67	3.08	12.08	4.17	6.17	32.67
AA	1.17	3.25	13.00	1.42	2.75	9.83	4.08	4.42	44.00
E	3.08	3.50	11.08	2.17	3.33	11.33	3.08	5.67	33.33
EA	3.25	3.83	11.33	2.50	2.92	11.33	2.92	5.50	28.00

C: control, AA: acetic acid control. E: EMD, EA: EMD +acetic acid
statistically different : $p < 0.05$. There were no sta
tistical differences

Detection of mRNA by RT-PCR

ALP mRNA and COL I mRNA were expressed in all three cell types from day 1 to day 8. EMD did not affect mRNA expression of ALP and COL I. OC mRNA were expressed more weakly than ALP mRNA and collagen type I mRNA in all three cell types from day 1 to day 8. EMD had negative effects on OC mRNA expression in all three different cell types at day 1, 3, 8. OP mRNA was not detected in CDC with or without EMD in all days. But, OP mRNA was expressed relatively highly in PDL fibroblasts and $SaOs_2$ cells at all test periods. The expression of BSP mRNA was higher in $SaOs_2$ cells and PDL fibroblasts at all periods and EMD did not affect on expression of BSP mRNA.

BSP mRNA was expressed weaker in CDC and was not even detected in CDC treated with EMD at day 8. Gene expressions of PDL fibroblasts were similar to those of $SaOs_2$ cells with respect to five markers. Generally, CDC reacted more weakly to five protein markers.

IV. Discussion

The present study demonstrated that EMD did not have any impact on cell growth of PDL fibroblasts, $SaOs_2$ cells and cementum-derived cells. And it was verified 5mM acetic acid did not enhance the effects of EMD and did not have harmful effects on cell proliferation. EMD had negative effects on OC mRNA expression in all three different cell types at day 1, 3, 8. But, EMD did not change mRNA expression of other marker proteins in all three different cell types.

In this study, there were no significant differences in the total cell numbers between two control groups and two test groups and also between acetic acid groups and non acetic acid groups($p<0.05$).

Neither 5mM acetic acid nor 100㎍/㎖ Emdogain did change cell proliferation. Therefore, within the limit of this study, EMD failed to show any impact on cell growth of PDL fibroblasts, $SaOs_2$ cells and cementum-derived cells. This results are comparable with several studies.[15,16)]

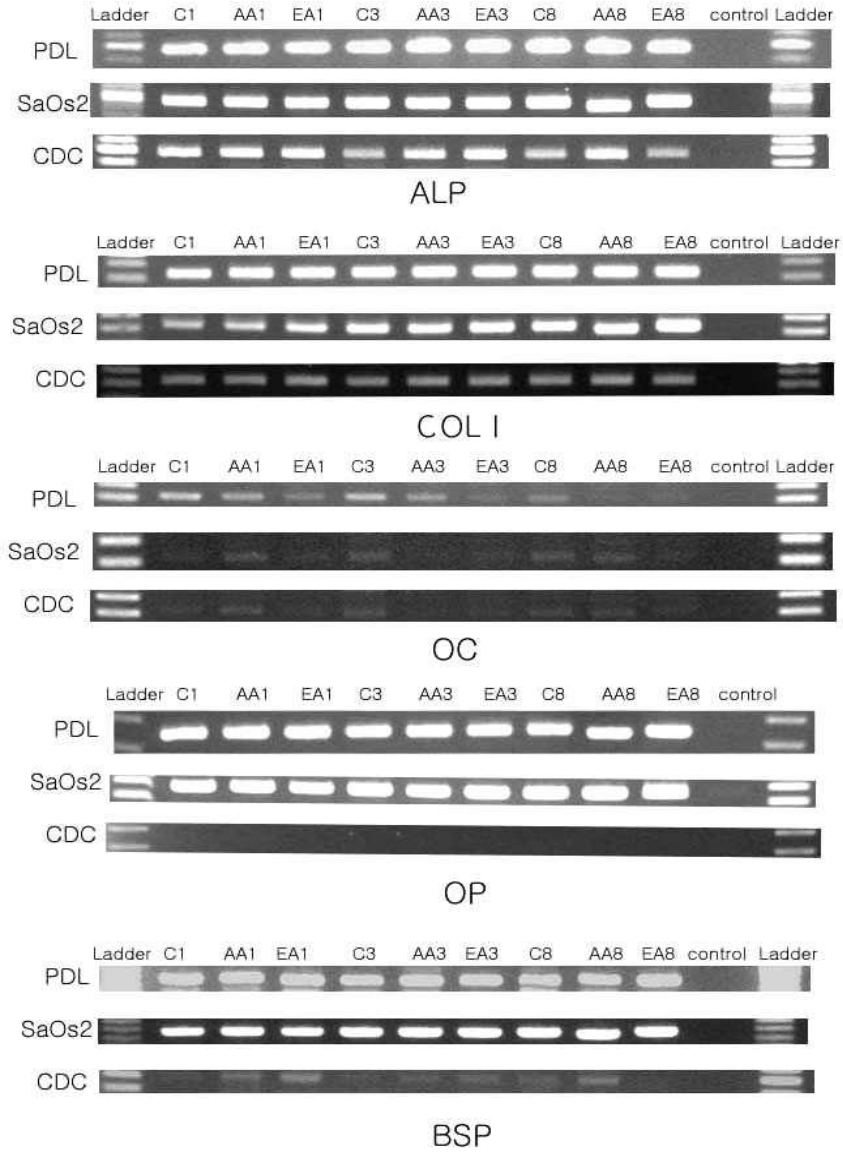

Figure 2. mRNA expression by RT-PCR

C1: control at day 1. AA1: acetic acid at day 1, EA1: EMD+acetic acid at day 1

C3: control at day 3. AA3: acetic acid at day 3, EA3: EMD+acetic acid at day 3

C8: control at day 8. AA8: acetic acid at day 8, EA8: EMD+acetic acid at day 8

PDL: periodontal ligament figroblasts, $SaOs_2$: osteosarcoma cell line,

CDC: cementum-derived cells

ALP: alkaline phosphatase, COL I: collagen type I, OC: osteocalcin, OP: osteopontin,

BSP: bone sialoprotein

In Gurpinar *et al.*,[15] showed that the EMD did not have a stimulating effect on cell growth in either mouse fibroblasts and marrow stromal osteoblasts. Jiang *et al.*[16] suggested that EMD maintained osteoblast survival without stimulating cell proliferation. But, these results contrast with other several previous studies.[11,17,18] Tokiuasu *et al.*[17] reported that EMD promoted proliferation of immortalized cementoblast(OC-CM30) over an 8-day period. He *et al.*[18] said that marked increase in cell numbers was observed in EMD-treated osteoblast groups from day 2 to day 7($p<0.01$). In Cattaneo's study,[11] a significant effect of EMD upon cellular proliferation at day 3 and day 8 was detected.

In the present study, we added EMD with 5 mM acetic acid to increase its solubility. We utilized a low degree of acid concentration in order to guarantee a stable pH level with no variation in the culture medium. However, it was verified 5 mM acetic acid did not enhance the effects of EMD and did not have harmful effects on cell proliferation.

In this study, ALP mRNA was expressed in all cell types and this phenomenon was prolonged until 8 days regardless of existence of EMD. But, He's study[18] revealed that EMD could increase mRNA expression of ALP in osteoblast after 3-week culture. Schwartz et al.[19] showed that in osteoblast-like MG63 cells, ALP mRNA expression was not changed until 7 days, peak at 14 days.

In our study, EMD did not significantly affect the level of COL I mRNA in all cell types. Viswanathan *et al.*[20] also revealed that the expression of COL I treated with EMD was not significantly different from controls. But, recent studies[18,21] had shown that the levels of collagen type I mRNA were increased with EMD treatment in both short-term(7 days) and extended culture under differentiation condition in osteoblasts.

The present study showed that OC mRNA was expressed more weakly than ALP mRNA and COL I mRNA in all three cell types from day 1 to day 8. EMD had negative effects on OC mRNA expression in all three cell types. The present study also showed that OP mRNA was not detected in CDC with or without EMD in all days. But, OP mRNA was expressed relatively highly in PDL fibroblast and $SaOs_2$ cells in all test periods. The expression of BSP mRNA was higher in $SaOs_2$ cells and PDL fibroblasts in all test periods and EMD did not affect expression of BSP mRNA. BSP mRNA was expressed weaker in CDC and was not even detected in CDC treated with EMD at day 8. There are many controversies on the effects of EMD on the expression of OC, OP and BSP. However, many authors reported that in osteoblasts and cementoblasts, EMD could enhance expression of OC, OP and BSP.[17,18] Jiang et al.[21] showed that EMD did not stimulate the expression of OC until 4 day in primary rat osteoblasts. Tokiyasu et al.[17] concluded that EMD down-regulated OC gene expression in cementoblasts and osteoprogenitor cells (MC3T3-E1 cells) and up-regulated OP gene expression markedly in MC3T3-E1 cells and slightly in cementoblasts at day 8. Hakkie et al.[24] reported that EMD increased OP mRNA and decreased OC mRNA in murine follicle cells. In He's short-term(7 days) and long-term culture study,[18] He revealed OP gene

expression was increased by EMD for day 1 to 3 and OC and BSP were enhanced by EMD treatment at the end of the 3-week culture period in osteoblasts. Viswanathan[20] said that 100㎍/㎖ EMD did not enhance BSP expression in immortalized murine cementoblasts, but the lowest dose of amelogenin(0.1 ㎍/㎖) slightly enhanced BSP expression for 8 days. This could be explained by that BSP are relatively later markers for osteoblastic differentiation, so they may be expressed weakly at short-term culture period.

Immunohistochemical studies[10] have shown that non-collagenous bone proteins such as bone sialoprotein(BSP), osteopontin(OP) and osteocalcin(OC) have unique distributions within the periodontium. OC is one of the extracellular matrix proteins of bone which has been implicated to have a role in calcification. According to Kagayama et al.,[22] almost all cells lining cellular cementum were positive for OC and these cementoblasts has a role for calcification of acellular cementum. OP, OC and BSP are all secreted at different stages of osteogenic cell differentiation. Studies[25] on the developmental expression of BSP have shown that BSP mRNA was expressed at high levels by osteoblast at the onset of bone formation and under steady-state conditions as well *in vitro*. Alkaline phosphatase(ALP) may play a key role in the formation and calcification of hard tissues. Collagen type I (COL I) is related with biosynthesis of extracellular matrix. Therefore, these proteins that above we mentioned may be used as markers to identify cell populations which have the potential to produce mineralized matrices and hence the ability to regenerate bone and cementum within the periodontium.

Datas from several studies[7,13,14] suggest that EMD can augment cell activities both *in vitro* and *in vivo*. Hammarström and colleagues,[13] using monkey models, reported that when they used EMD as a regenerative material they found enhanced regeneration of periodontal tissues; i.e., bone, cementum and periodontal ligament. Further studies[14] in humans have reported promising results as measured histologically and also based on clinical attachment levels and subtraction radiography. In vitro studies[7] indicate that PDL cells exposed to EMD exhibit enhanced protein production, cell proliferation, and ability to promote mineral nodule formation. But, there are so many reports contradicting the roles of EMD. On the contrary to Slavkin's study, Diekwisch showed that any amelogenin transcript was not detected in HERS in human. These findings questioned the functions of EMD. Therefore, this study was designed to determine whether EMD had an effect on gene expression, cell proliferation in PDL fibroblasts, osteoblasts and cementoblasts, respectively. Within the limit of this study, EMD seems to be regulate gene expression of several cells constituting periodontium, but EMD seems not to have major role in periodontal regeneration.

Clearly, elucidating the exact interactions between EMD and cells consisting of periodontium; PDL cells, osteoblast-like cells, cementum-derived cells still remains an area open for investigation. However, the results obtained in the present study suggest that EMD may not play a biologic role on the pro-

liferation of PDL cells, osteoblast-like cells, cementum-derived cells until 8 days. In the aspect of cell differentiation, EMD may have some effects on OC mRNA expression, thus we might regulate bone mineralization and/or cementum regeneration by using EMD. However, further studies are necessary to verify the quantitative effects of EMD on gene expression, their function and their possible different mechanism of EMD on cell differentiation.

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법랑기질유도체가 치주인대세포, 불멸화 조골세포, 백악질 유래세포의 증식과 유전자 발현에 미치는 영향

정유지 · 김경화 · 김태일 · 설양조 · 구 영 · 이해준 · 류인철 · 정종평 · 한수부 · 이용무

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1. 목적

in vitro 상에서 법랑기질유도체가 치주인대섬유아세포, 불멸화 조골세포와 백악질 유래세포의 증식과 유전자 발현에 미치는 영향을 알아보고자 하였다.

2. 연구방법 및 재료

〈세포증식 연구〉

교정을 목적으로 발거한 치아에서 분리, 배양한 치주인대섬유아세포와 백악질유래세포, 그리고 $SaOs_2$ 세포를 이용하였다. 법랑기질유도체가 세포 증식에 미치는 영향을 알아보기 위해, 35 mm Petri dish에 dish 당 5×10^3 개의 세포를 접종하였다. 대조군은 1% 항생제와 10% FBS를 포함한 DMEM 배지를 이용했고, 5mM 초산을 첨가한 군과 첨가하지 않은 두 개의 대조군이 이용되었다. 실험군은 100 ㎍/㎖의 법랑기질유도체를 첨가한 군과 100 ㎍/㎖의 법랑기질유도체와 5 mM의 초산을 첨가한 2개의 실험군이 이용되었다. 각 군은 세 개의 배양접시에 행해졌고, 1, 3, 8일에 세포의 수를 각각 측정하였다. 결과는 repeated measures ANOVA로 통계 처리하였다.

〈유전자 발현 연구〉

각 세포의 형질 특성을 알아보기 위해 RT-PCR을 실시하여 조골세포 분화 표식자와 연관된 Human collagen type I(COL I), human osteopontin(OP), human osteocalcin(OC), human alkaline phosphatase(ALP)와 human bone sialoprotein(BSP)의 mRNA 발현을 실험 1, 3, 8일에 걸쳐, 세 군의 차이를 비교 관찰하였다.

3. 결과

〈세포증식 연구〉

치주인대세포와 백악질유래세포, 그리고 $SaOs_2$ 세포의 증식은 법랑기질유도체에 의해 영향을 받지 않았다. 대조군과 초산이 포함된 대조군 그리고 법랑기질유도체와 초산이 포함된 실험군에서 유의할 만한 세포 수의 차이가 실험 기간 1, 3, 8일에 걸쳐 나타나지 않았다($p<0.05$).

〈유전자 발현 연구〉

ALP와 COL I은 세 군의 세포에서 모두 발현되었고, 발현 정도는 EMD에 영향을 받지 않았다. OC은 세 군에서 모두 비교적 약하게 발현되었고, 특히 $SaOs_2$ cell과 백악질유래세포에서 약하게 발현되었다. EMD는 OC의 발현정도를 약하게 하였다. OP은 백악질유래세포에서 1, 3, 8일에 걸쳐 EMD 유무에 관련 없이 발현되지 않았다. 그러나 치주인대세포와 $SaOs_2$ cell에서는 강하게 발현되었다. BSP는 치주인대세포와 $SaOs_2$ cell에서 1, 3, 8일에 걸쳐 비교적 고르게 발현되었다. EMD 배지에서 배양된 백악질유래세포는 8일에는 BSP가 발현되지 않았다.

4. 결론

이번 실험 결과에 의하면 법랑기질유도체는 치주인대세포, 불멸화 조골세포와 백악질 유래세포의 증식에 있어 유의성 있는 효과를 나타내지 않았다. 그러나, 유전자 발현에 있어서는, 치주인대세포와 백악질유래세포, 그리고 $SaOs_2$ 세포 모두에서 OC mRNA의 발현을 억제하는 효과를 나타내었다. EMD는 세포의 증식에는 영향을 미치지 않지만, 유전자 발현에 있어 일부 영향을 미치는 것으로 보인다. 법랑기질유도체가 세포의 증식과 유전자 발현에 미치는 영향은 배양된 세포의 형질특성, 배양환경, 배양일수 등에 따라 달라질 수 있다. 그러므로 법랑기질유도체가 in vitro 상에서 세포에 미치는 영향은 보다 정량화된 연구가 필요하다.

주요어 : 법랑기질유도체, 치주인대세포, 백악질 유래세포, 유전자 발현, 세포증식