대한치과재료학회지 44(4) : 291-298, 2017
ISSN:2384-4434 (Print); 2384-3268 (Online)
Available online at http://www.kadm.org
http://dx.doi.org/10.14815/kjdm.2017.44.4.291

포스파티딜을 함유한 리포좀으로 처리된 대식세포 조정배지의 사람치수세포 분화에 미치는 영향

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Effects of conditioned media from phosphatidylserine-liposome-treated macrophages on the differentiation of human dental pulp cells

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본 연구에서는 포스파티딜세린 (phosphatidylserine)을 함유한 리포좀 (liposome)으로 사람대식세포 (human macrophage, THP-1)를 처리하여 얻어진 조정배지가 사람치수세포 (human dental pulp cell)의 분화에 어떠한 영향을 주는지 평가하였다. 실험을 위해 포스파티딜세린, 포스파티딜콜린 (phosphatidylcholine), 콜레스테롤 (cholesterol)을 2:1:1 의 분자비로 섞은 후, 진공 증발 방법을 통해 리포좀을 제조하였다. 제조된 리포좀을 사람대식세포에 6시간과 24시간 동안 각각 처리하고 얻어진 조정배지를 사람치수세포의 분화실험에 사용하였다. 분화 확인용 실험으로 알칼리 포스파타아제 활성 (alkaline phosphatase activity) 평가, 역전사 중합효소연쇄 반응 (reverse transcriptase polymerase chain reaction, RT-PCR)을 이용한 분화능평가, 알리자린 레드 에스 염색 (alizarin red S staining)을 통한 기질세포 광화 (cell matrix mineralization) 분석을 실행하였다. 실험결과 알칼리 포스파타아제 활성은 조정배지가 전체 배양액의 10% 이상 함유되었을 때 통계적으로 유의하게 증가하였다. 조골세포 분화인자 유전자 (dentin sialophosphoprotein (DSPP), osteocalcin (OCN))들의 발현을 RT-PCR을 통해 확인한 결과 DSPP 와 OCN 모두에서 통계적으로 유의한 차이는 보이지 않았다. 알리자린 레드 에스 염색한 결과 분화조절 배지와 비교하여 조정배지는 유의한 차이를 보이지 않았다. 그러므로, 조정배지를 통한 사람치수세포 분화에 대한 영향은 미약한 것으로 판단되며, 추후 직접적인 세포간 영향 평가가 가능한 방법을 이용한 연구가 요구된다.

색인단어 : 대식세포, 분화, 사람치수세포, 포스파티딜세린

INTRODUCTION

Macrophages derived from monocytes undergo specific differentiation depending on the local tissue environment (Martinez et al., 2008). They differentiate into the following distinct functional phenotypes: M1 and M2 macrophages. The M1 macrophage phenotype is known to kill intracellular microorganisms and produce abundant pro-inflammatory cytokines such as TNF-α, interleukin (IL)-12, and IL-23, and proinflammatory mediators such as nitric oxide and reactive oxygen species (Mantovani et al., 2004). Lipopolysaccharide and interferon-γ polarize macrophages toward the M1 phenotype (Park et al., 2016). In contrast, M2 macrophages are characterized by their involvement in tissue remodeling and immune regulation (Roberts et al.,

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Received: Aug. 10, 2017; Revised: Nov. 01, 2017; Accepted: Nov. 07, 2017

1992). M2 macrophage activation is induced by various signals such as IL-4, IL-13, glucocorticoids, IL-10, and immune complexes (Branton et al., 1999). In addition, M2 macrophages function in the resolution of inflammatory response and tissue remodeling by producing transforming growth factor-β (TGF-β), IL-10, and angiogenic vascular endothelial growth factor (Hyunh et al., 2002). A shift toward the M2 macrophage phenotype and an anti-inflammatory effect are also caused by apoptotic cells *in vivo*. The mechanism underlying the activation of macrophages by apoptotic cells depends on the binding between phosphatidylserine (PS) of apoptotic cells and PS-receptors on macrophages (Fadok et al., 1992: Quan et al., 2016). Furthermore, PS- containing liposomes (PSLs) promote the activation of macrophages (Martin et al., 1995).

In the oral cavity, the promotion of differentiation of pulpal cells has beneficial effects of tooth protection. In carious lesions, odontoblasts cells have been involved in reactionary dentin formation (Njeh et al., 2016). We previously reported the effect of macrophages on the differentiation of human dental pulp cells (HDPCs) and found that conditioned media (CM) from M2 macrophages (M2CM) promoted the odontogenic differentiation of HDPCs, and TGF-β in M2CM played a primary role in enhancing the differen- tiation (Park et al., 2016). We previously reported that PSLs directly stimulated the differentiation of HDPCs (Park et al. 2017). PSLs treatment increased the activity of ALP, which is an early biomarker of differentiation and mineralization of HDPCs.

In this study, we hypothesized that PSLs-treated macrophages stimulated the odontogenic differentiation of HDPCs. To test this hypothesis, we conducted experiments to establish the effect of CM from macrophages on the differentiation activity of HDPCs.

MATERIALS AND METHODS

1. Preparation of PSLs

To obtain PSLs, PS, phosphatidylcholine and cholesterol at a 2:1:1 molar ratio were dissolved in chloroform methanol (9:1 v/v). The solvent was evaporated in the presence of nitrogen gas in a glass tube and was further removed in a vacuum chamber for 2 h. The lipid film was subsequently hydrated in phosphate-buffered saline (PBS) (pH 7.4) and vigorously vortexed to obtain a 0.25-mM suspension of total lipid. The lipid suspension was filtered through a 0.1-μm membrane filter. PS was purchased from Avanti Polar Lipids (Alabaster, AL, USA), and other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Cell culture

HDPCs for primary culture were obtained from premolars that were removed from patients for orthodontic purposes under the guidelines approved by the Institutional Review Board of Seoul National University Dental Hospital (CRI06015). The teeth were cut at the apex, and the dental pulp was removed from the pulp cavity and immersed in minimal essential medium (MEM) that contained 20% fetal bovine serum (FBS) and antibiotic solution (100 U/ml of penicillin-G and 100 mg/ml of streptomycin). The pulp was minced into pieces and incubated at 37℃ in humidified atmosphere (5% CO_2/95% air) for 20 days with media exchanges at 3-day intervals. The dental pulp cell were subcultured to the fourth passage in MEM that contained 10% FBS for use. Cell culture reagents and antibiotics were purchased from GIBCO-BRL (Carlsbad, MD, USA).

3. Preparation of CM

CM from PSL-treated macrophages was obtained after culturing treated THP-1 cells. To obtain CM, M0 macro-

phages were treated with PSLs for 6 and 24 h. After centrifuging at 10000 rpm for 5min, the supernatants were stored at −80℃ until use. The CM aliquots were labeled as CM-6 h and CM-24 h.

4. ALP activity assay

In this study, the effect of CM from PSL-treated macrophages on the differentiation of HDPCs was investigated by measuring the ALP activity. The ALP activity was assessed using the following two methods: (a) 4-nitrophenyl phosphate colorimetric assay and (b) ALP direct staining assay. For 4-nitrophenyl phosphate colorimetric assay, HDPCs were exposed to CM from macrophages for 6 days in growth media (GM, MEM containing 10% FBS) and differentiation media (DM, MEM containing 10% FBS, 50 mg/mL L-ascorbic acid, 10^{-8} M dexamethasone and 2mM β-glycerophosphate). Cells in 96-well plates were incubated in a mixture of 140 μL of alkaline buffer solution and 10 μL of 1.5 M $MgCl_2$ solution that contained 67 mM 4-nitrophenyl phosphate (Fluka, Buchs, Switzerland) for 30 min at 37℃. The reaction was discontinued by adding 0.5 M NaOH, and the optical density was measured at 405 nm. ALP enzyme activity was normalized to total protein amount. Cell protein was quantitated using a bicinchoninic acid protein assay kit (iNtRon Biotechnology; Sungnam, Korea). During the treatment, CM was exchanged by replacing the culture medium that contained CM every 3 days. For ALP direct staining assay, a culture insert (ibidi, Munich, Germany) was used. The culture insert was placed on a culture dish, and 100 μL of THP-1 cell suspension was added in one side of the insert. THP-1 cells were incubated at 37℃ for 1 day and were differentiated to non-polarized M0 state by exposure to 100 nM phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 2 days; the media was replaced with fresh media that contained PSLs for 1 day. HDPCs were incubated in the other side of the insert. Following PSL treatment, the culture insert was carefully removed, and the cells were cultured with GM and DM. After 6 days of culture, HDPCs and macrophages were stained with an ALP staining kit (SBI, California, USA) (Takahashi et al., 2006).

5. Gene expression assay

To observe the effect of PSL-treated macrophages on the odontogenic differentiation of HDPCs, mRNA expressions of dentin sialophosphoprotein (DSPP) and osteocalcin (OCN) were investigated. HDPC was exposed to CM for 10 days in DM to measure DSPP and OCN mRNA expressions. Total RNA was then extracted from cells using an RNA extraction reagent (WelPrep Total RNA Isolation Reagent; Welgene, Daegu, Korea). cDNA was then prepared using a cDNA synthesis kit (Power cDNA Synthesis kit, iNtRON Biotechnology). Real-time polymerase chain reaction was performed with a 20-μL mixture that contained 10 μL of SYBR Premix Ex Taq (Takara Bio, Otsu, Japan), 0.4 μL of ROX Reference Dye II (Takara Bio), cDNA, and primers. The reaction was performed using the ABI PRISM 7500 Sequence Detection System Thermal Cycler (Applied Biosystems, Foster City, CA). The primer sequences were as follows: DSPP,
forward 5'-TGACTCAAAAGGAGAGAAGATGAT-3',
reverse 5'-ATTTACCTTTGCCACTGTCTGATTT-3'; OCN,
forward 5'-CTGACGAGTTGGCTGACC-3',
reverse 5'-CAAGGGGAAGAGGAAAGAAGG-3'; GAPDH,
forward 5'-GTCGGAGTCAACGGATTTGG-3',
reverse 5'-GGGTGGAATCATATTGGAACATG-3'.

PCR conditions were 95℃ for 30 s followed by 40 cycles of denaturation at 95℃ for 15 s and annealing at 60℃ for 34 s. Gene expressions were evaluated using the CT (threshold cycle) value and were reported as the average expression ratio of the target gene to GAPDH to normalize the expression.

6. Cell matrix mineralization

Alizarin red S staining was used to determine the extent of cell matrix mineralization. HDPCs were exposed to CM for 20 days in DM. To stain calcified nodules, cell cultures were fixed with 3.7% formaldehyde for 24 h, washed with PBS, and then stained with 20 mg/mL alizarin red S for 10 min. The amount of alizarin red S that bound to the cell matrix was quantified by extraction with 10% cetylpyridinium chloride, as previously described (Lee DH et al., 2006).

7. Statistical analysis

One-way ANOVA was performed to determine the significance of the effects of CM PSL-treated macrophages on ALP activity, DSPP and OCN mRNA expressions, and ECM mineralization of HDPCs. p values of < 0.05 were considered to be statistically significant.

RESULT

1. ALP activity of HDPCs

ALP is an early marker for cell differentiation (Stucki et al., 2001). The ALP activity was assessed using two methods: (a) 4-nitrophenyl phosphate colorimetric assay and (b) ALP direct staining assay. To investigate the effects of CM from PSL-treated macrophages on the ALP activity of HDPCs, 4-nitrophenyl phosphate colorimetric assay was used. HDPCs used in this experiment had an ALP value of 31.9 (units/mg protein) in GM and 83.4 (units/mg protein) in DM, which confirmed sufficient differentiation potency of these cells (Figure 1). HDPC was cultured in diluted CM for 6 days, and the amount of CM in total media was increased from 5% to 50%. In GM, no statistically significant differences were observed after measuring the

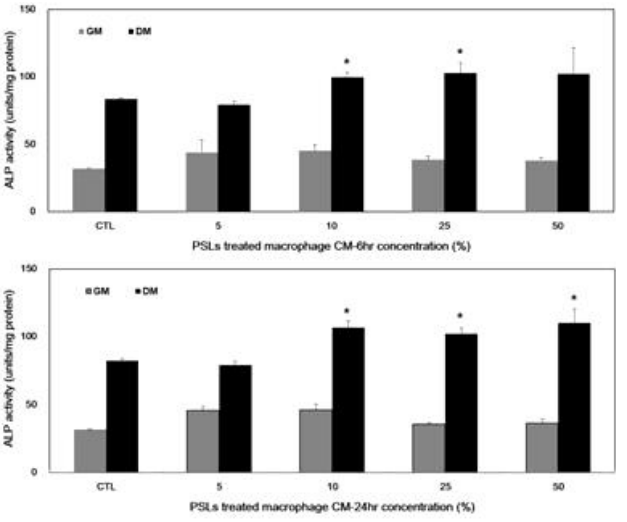

Figure 1. The ALP activity of HDPCs cultured with CM from PSL-treated macrophages. HDPCs were cultured with CM from PSL-treated macrophages in GM and DM for 6 days for the ALP activity. Each value represents the mean ± SD of the triplicate experiments. *p 〈 0.05 vs. untreated control group.

ALP activity of HDPC using CM-6 h, but in DM, a statistically significant increase by 10% was observed, with similar values until 50%. The experiment conducted with CM-24h showed similar results using CM-6h.

The ALP activity of HDPCs co-cultured with PSL-treated macrophages is shown in Figure 2. There was no difference in the intensity of ALP staining between PSL-treated and non-treated groups.

2. Effects of CM on gene expression

DSPP and OCN mRNA expressions were observed with GM, DM, CM-6h, and CM-24h. DSPP mRNA expressions increased in DM compared with those in GM. Average values in DM, CM-6h, and CM-24h decreased but were not statistically significant. In contrast, OCN mRNA expressions did not increase in GM and DM. There was no change in the average value of OCN expression, no statistically significant differences were observed in the correlation with

to the results obtained for DSPP (Figure 3).

3. Effects of CM on the mineralization of HDPC

The effect of CM on mineralization was investigated by alizarin red S staining after 20 days of incubation in GM, DM, CM-6h, and CM-24h. When mineralization was quantified, DM showed a 4-fold increase than GM, whereas no significant changes were noted in CM-6h and CM-24h (Figure 4).

DISCUSSION

The normal dental pulp contains heterogeneous cell populations that comprise mostly of fibroblast-like cells, inflammatory and immune cells, and pulpal stem cells, which are mostly involved in self-renewal (Goldberg et al., 2008). The importance of inflammation in the healing of the pulp in dental caries has been underestimated and is often considered to be an undesirable effect, leading to, in most cases, pulp necrosis and other adverse effects. In view of recent results, the inflammatory process should be reexamined to understand the potential and beneficial effects of this process. The effects of calcium hydroxide [$Ca(OH)_2$] that contains pulp-capping agents on pulp cell migration, proliferation, and differentiation have been previously described (Schröder et al., 1985; Goldberg et al., 2015). $Ca(OH)_2$ induces beneficial effects owing to chemical injury caused by hydroxyl ions. A limited necrosis is induced against the vital pulp tissue (Hoffmann et al., 2005).

In this study, CM from PSL-treated macrophage stimulated the ALP activity of HDPCs (Figure 1), potentially indicating that CM from PSL-treated macrophage enhances

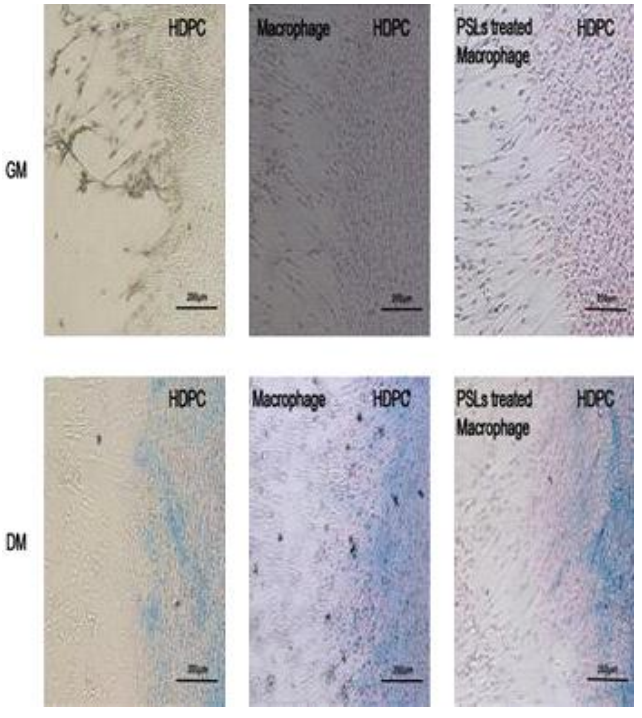

Figure 2. The ALP staining of HDPC cultured with PSL-treated macrophage. HDPCs were co-cultured with PSL-treated macrophages in GM and DM. The blue color shows the staining for ALP of HDPCs.

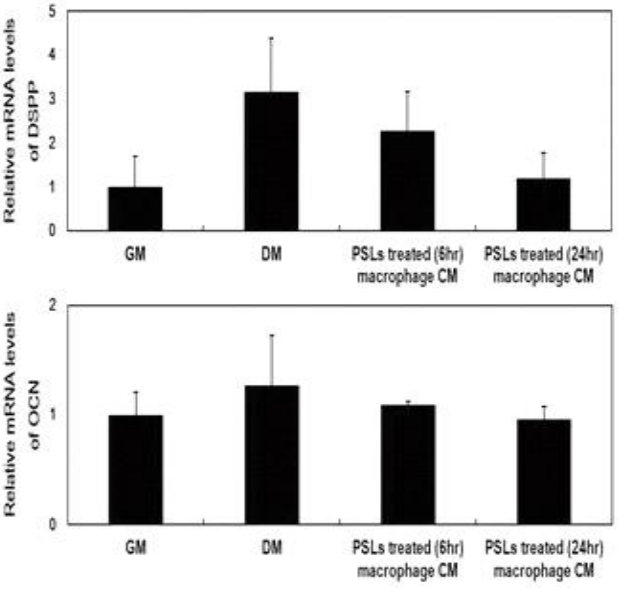

Figure 3. Effects of CM from PSL-treated macrophages on DSPP and OCN mRNA expressions in HDPCs. HDPCs were cultured in GM, DM, and DM that contained 10% CM from PSL-treated macrophages for 10 days to assess DSPP and OCN mRNA expressions. Each value represents the mean ± SD of triplicate experiments.

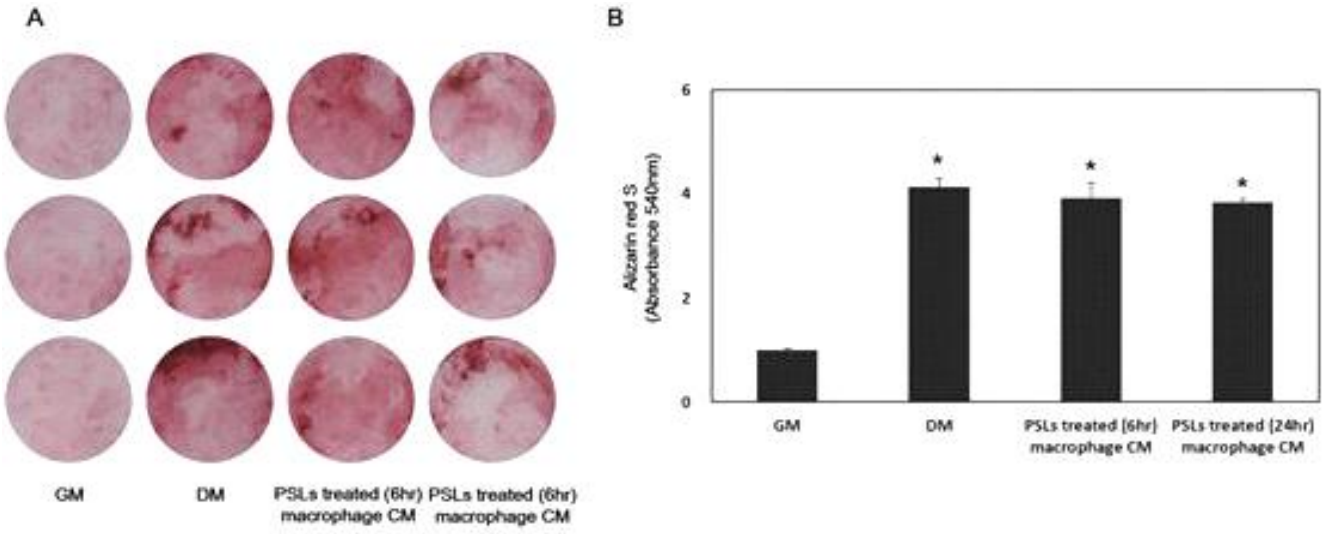

Figure 4. The matrix mineralization of HDPC cultured with CM from PSL-treated macrophages. HDPCs were cultured with DM that contained 10% CM from PSL-treated macrophage for 20 days and were then stained with alizarin red S (A). The mineralization was quantified by measuring the amount of alizarin red S (B). Each value represents the mean ± SD of triplicate experiments. *p < 0.05 vs. control group (GM).

the ability of HDPCs to differentiate; it can be safely assumed that the main factor involved in the osteogenic activity of CM is TGF-β. However, the enhancement of ALP activity of HDPCs was not dose dependent. These results suggest that the differentiating substances in CM are not sufficient to affect the ALP activity of HDPCs in a dose-dependent manner.

Because CM from PSL-treated macrophages increased the ALP activity of HDPCs, we expected that CM could enhance mRNA expressions. However, CM did not affect DSPP and OCN mRNA the expression. Moreover, CM slightly attenuated DSPP mRNA expressions in HDPCs (Figure 3). Various signaling pathways such as Wnt, TGF-β, Hedgehog, and FGF signaling pathways have been involved in regulating osteoblast differentiation. Accordingly, the contradiction between the ALP activity and mRNA expressions imply that CM inhibited the signaling pathway associated with osteogenic differentiation. In line with the results of mRNA expressions, CM did not induce the mineralization of HDPCs (Figure 4), suggesting that calcium binding by CM is not sufficient to enhance calcium deposition in HDPCs.

CONCLUSION

In conclusion, this study showed that CM from PSL-treated macrophages enhanced the early phase of HDPC differentiation.

ACKNOWLEDGMENTS

This study was supported by a grant from the Korea Health Technology R&D project, Ministry of Health & Welfare, Republic of Korea (A120822).

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Effects of conditioned media from phosphatidylserine-liposome-treated macrophages on the differentiation of human dental pulp cells

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This study aimed to examine the effect of phosphatidylserine-treated macrophages (THP-1) on the differentiation of human dental pulp cells. Phosphatidylserine, phosphatidylcholine, and cholesterol were mixed at a molar ratio of 2:1:1, and liposomes were produced via the vacuum evaporation method. Conditioned media (CM) were collected after treating macrophages, with liposomes for 6 and 24h, and were used for differentiating human dental pulp cells. To confirm the differentiation, we performed an evaluation of alkaline phosphatase (ALP) activity, reverse transcriptase polymerase chain reaction (RT-PCR) of dentin sialophosphoprotein (DSPP) and osteocalcin (OCN) gene expressions, and extracellular matrix mineralization assay via alizarin red S staining. There was a statistically significant increase in alkaline phosphatase activity with the conditioned medium containing 10% in the overall culture medium. RT-PCR assay revealed that conditioned media did not increase the mRNA expressions of DSPP and OCN genes. The degree of matrix mineralization was not affected by the conditioned medium. These results confirm a slight effect of the conditioned medium on the differentiation of human dental pulp cells. Future studies may adopt a method in which macrophages and human dental pulp cells are closely co-existed, to evaluate the effect of phosphatidylserine on the interaction between these cell types.

Key Words : Differentiation, Human dental pulp cell, Macrophage, Phosphatidylserine