법랑기질유도체가 인간 치주인대세포의 증식 및 성장인자 발현에 미치는 영향

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Effects of enamel matrix derivatives on the proliferation and the release of growth factors of human periodontal ligament cells

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Purpose: Stimulating the proliferation and migration of periodontal ligament cells (PDLCs) has become the main goal of periodontal regeneration. To accomplish this goal, regeneration procedures have been developed, but results have not been predictable. Recently, tissue engineering using enamel matrix derivatives (EMDs) and growth factors has been applied to periodontal regeneration; however, the mechanism of EMDs is largely unknown. The aim of this study was to investigate the effects of EMDs on the proliferation and release of growth factors from PDLCs. **Materials and methods:** Human PDLCs were removed from individually extracted 3rd molars of healthy young adults, and cultured in the media containing EMDs (Emdogain, Biora, Malmo, Sweden) at concentration of 0, 12.5, 25, 50, 100, and 200 μg/mL each. Cell proliferation and ALP (alkaline phosphatase) activity were measured. The evaluation of growth factors released by PDLCs was also performed by one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test. **Results:** Significantly increased proliferation and ALP activity were observed in PDLCs treated with over 25 μg/mL and 50 μg/mL EMDs, respectively. Additionally, treatment of PDLCs with 50 μg/mL resulted in significantly increased release of vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β after 24 h and 48 h, respectively. **Conclusion:** EMDs enhance the proliferation and ALP activity of PDLCs, and promote the release of growth factors, including VEGF and TGF-β, from PDLCs. Therefore EMDs could be one of the effective methods for periodontal regeneration. *(J Korean Acad Prosthodont 2016;54:203-9)*

Keywords: Enamel matrix derivatives; Periodontal ligament; Periodontal regeneration; Tissue engineering; Growth factor

Introduction

A fundamental goal of periodontal treatment is the functional rehabilitation of damaged periodontal tissue and the regeneration of tissue from the periodontal ligament (PDL). To accomplish these goals several procedures, such as bone grafting and guided tissue regeneration have been used.

However, the results of these therapies are not always predictable. Recently, tissue engineering technology using an extracellular matrix, such as enamel matrix derivatives (EMDs) and growth factors have been used for periodontal regeneration.[1]

The composition of an enamel matrix derivative is 90% amelogenin and 10% hydrophobic protein. Protein matrix released from ameloblast during the generation of tooth enamel forms enamel by organizing a crystal layer, amelogenin is the protein that composes the intercellular layer. Thus, amelogenin evolute and play an important role in cementogenesis.[2] Acellular cementogenesis begins at the inner epithelium of Hertwig's epithelial root sheath,[1]

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and the protein found in the acellular cementum is similar to enamel matrix derivative.[3] Amelogenin is also known not only as cell adhesion protein which has high compatibility with both hydroxyapatite and collagen, but also promoting cell spreading property.[4]

When EMDs are applied on exposed root surfaces, tissue regeneration is achieved by osteogenesis, and acellular cementogenesis, histologically.[5] According to a clinical experiment which EMD was applied, Heiji et al.[6] reported a bone gain of 2.6 mm in one- or two-wall bone defect. Froum et al.[7] reported that in case of horizontal bone loss, the periodontal ligament attachment level was retained by open flap debridement with EMDs application. Sculean et al.[8] reported a clinical result of periodontal surgery using EMDs showing a favorable long term outcome over more than 4 years. And Van der Pauw et al.[9] reported that EMDs increase the stimulation of periodontal attachment by increasing alkaline phosphate (ALP) level. However, it is not yet clear how the EMDs imitate root and supporting tissue formation.

Periodontal regeneration is associated with a serial procedure, which is proliferation of periodontal ligament cells (PDLCs), and composition and calcification of an organic substance, such as enamel matrix derivative.[10] PDLCs are thought to play an important role in maintenance, regeneration and recovery of the tooth attachment apparatus.[11] For the successful regeneration, PDLCs and probiont should interact with each other, and there may be some factors that influence productivity and growth powerfully.

Therefore, we intended to identify the effects of enamel matrix derivatives on the proliferation and the release of growth factors of human PDLCs and ALP activity, by culturing human PDLCs at different EMD concentrations.

Materials and Methods

Cell isolation and culture

PDLCs were prepared from extracted third molars that were removed for orthodontic reason in young healthy volunteers. The study protocol was approved by the Institutional Review Board of Ewha Womans University Mokdong Hospital (No. 2015-02-022). Using sharp blades or curettes, PDLCs were obtained from the middle one-third of individual extracted teeth and placed in Dulbecco's modified minimum essential medium F-12 (DMEM/F12; GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY, USA), 100 U/mL penicillin, 100 μg/mL streptomycin and were overlaid with cover slips. Cultures were performed in an incubator with an environment of 37℃, 95% air, and 5% CO_2 for 24 hours. While waiting for the release of PDLCs from the tissue for about 5 days, culture medium

was replaced once in two days. Outgrowing cells were sub-cultured in fresh culture medium. Cells of the fourth to sixth passage were used for the experiments described below.

Preparation of EMDs

Emdogain (Biora, Malmo, Sweden), a mixture of freeze-dried enamel matrix proteins harvested from the developing crowns of 6-month-old swine, is a unique material that came into the market as a device promoting regeneration of PDLCs4, used as EMDs in this study. Gel-type Emdogain was completely dissolved in 10 mM glacial acetic acid, then was added to culture media.

Effect of EMDs on the proliferation of PDLCs

The PDLCs were seeded in 96-well plate at a density of 1×10^4 cells/well and incubated in 100 μL DMEM containing 10% FBS. After 24 hours, the medium was replaced with serum-free DMEM. After 24 hours, they were subcultured into two groups. The medium was replaced with serum-free DMEM containing EMD in the test group, and serum-free DMEM in the control group. EMDs were present in each test media at concentration of 0, 12.5, 25, 50, 100, and 200 μg/mL.[2] After 3 days of incubation, analysis was performed using cell proliferation assay kit (Cell counting kit-8, Donjindo Lab., Kumamoto, Japan). All samples were prepared every 3 units.

Effect of EMDs on the ALP activity of PDLCs

The PDLCs were seeded in 96-well plate at a density of 1×10^4 cells/well and incubated in 100 μL DMEM containing 10% FBS. After 24 hours, the medium was replaced with serum-free DMEM. After 24 hours, they were subcultured into two groups and the medium was replaced with DMEM containing EMDs in test group, and serum-free DMEM in the control group. Five subgroups were set in the EMD group by concentrations of 0%, 2.5%, 10%, 20%, and 40% EMD.

ALP activity in the cell layer was determined by ALP analyzing reagent (BioAssay Sys., Hayward, CA, USA). The cells from test and control groups were washed twice with Dulbecco's phosphate buffered saline (DPBS) and 50 μL of 0.2% Triton X-100 was added and shaked on a plate-shaker for 20 minutes. 150 μL of ALP analyzing reagent (1 M p-nitrophenyl phosphate and 0.2 M Mg acetate) was added and incubated at 37℃ for 30 minutes.

The ALP disintegrated p-nitrophenyl phosphateto p-nitrophenol and phosphate forming yellow color. After stopping the reaction by adding 100 μL of 1 M NaOH, p-nitrophenol formed was measured

at 405 nm using a microplate reader (Synergie HT, Bio-Tek, Winooski, VT, USA). The more it appears brown, the higher activity it shows.

Effect of EMDs on the release of growth factors from PDLCs

3×10^5 cells of PDLCs were added to each 35 mm culture dishes and incubated in DMEM medium supplemented with 10% FBS. After 24 hours, culture medium was replaced with serum-free DMEM. Two groups were set after 24 hours. The test group was incubated in DMEM medium supplemented with EMD of minimum concentration that increase cell proliferation and ALP activity at former experiment and the control group was incubated in serum-free DMEM medium. The conditioned media was harvested after 0, 8, 24, 48, and 72 hour and kept in -70℃. Then, platelet derived growth factor (PDGF)-AB, PDGF-BB, transforming growth factor (TGF)-β1, vascular endothelial growth factor (VEGF) level of each conditioned media according to time were measured by ELISA method. Average data of 3 unit samples were used.

Statistical analysis

Data were analyzed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

In cell proliferation assay according to EMD concentration and ALP activity analysis, statistical analysis was performed by one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test ($P < .05$).
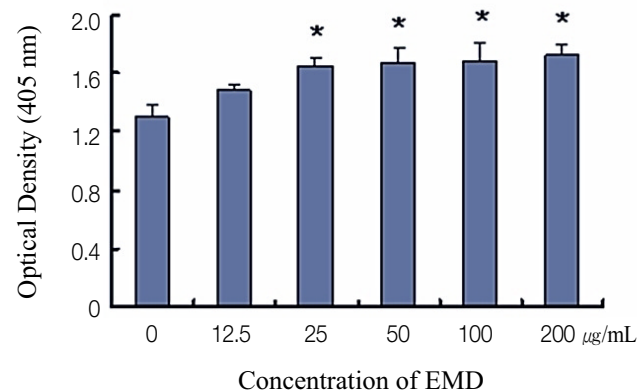
Results

A. Effect of EMDs on the proliferation of PDLCs

The effect of different EMD concentrations on the proliferation of PDLCs was examined. The results represent that cell proliferation of PDLCs cultured in more than 25 μg/mL EMD was significantly higher than control group ($P < .05$) (Fig. 1).

B. Effect of EMD on the ALP activity of PDLCs

Effect of different EMD concentration on the ALP activity of PDLCs was examined. The results represent that ALP activity of PDLCs cultured in 50 μg/mL EMDs was significantly higher than control group ($P < .05$) (Fig. 2).

C. Effect of EMDs on the release of growth factors of PDLCs

According to the result about an effect of different EMD concentrations on the ALP activity and proliferation of PDLCs, the concentration of 50 μg/mL EMD was selected to evaluate the release of VEGF and TGF-β. The growth factors of PDLCs were measured and evaluated over time for 72 hours (Fig. 3, Fig. 4). VEGF and TGF-β released from PDLCs cultured in 50 μg/mL EMD were significantly higher than control group ($P < .05$).

Fig. 1. The effect of different enamel matrix derivatives (EMDs) concentrations on the proliferation of PDLCs. The results represent the means $\pm$ SD of three replicates. * Significantly different from the control group ($P < .05$).
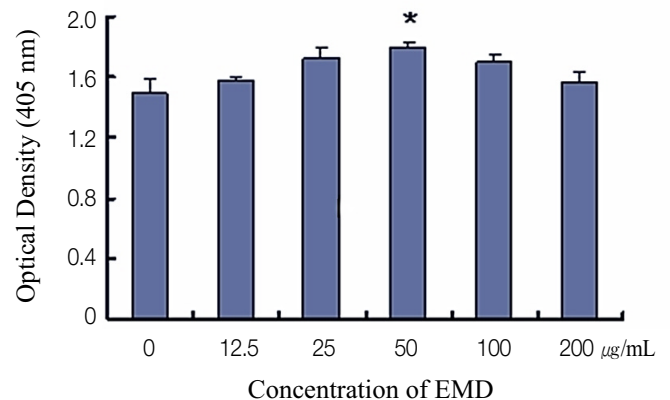

Fig. 2. The effects of different EMDs concentrations on the ALP activity of PDLCs. The results represent the means $\pm$ SD of three replicates. * Significantly different from the control group ($P < .05$).
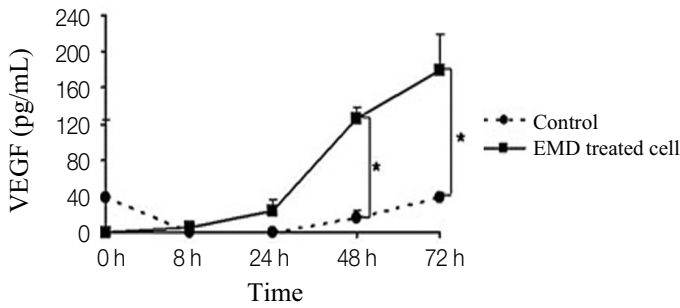
Fig. 3. Time-dependent effect of EMDs (50 μg/mL) on the release of VEGF from PDLCs. The results represent the means ± SD of three replicates. * Significantly different from the control group (*P* < .05).

Fig. 4. Time-dependent effect of EMDs (50 μg/mL) on the release of TGF-*β* from PDLCs. The results represent the means ± SD of three replicates. * Significantly different from the control group (*P* < .05).

1. VEGF

The release of VEGF relevant to angiogenesis, increased over time. The release of VEGF was significantly higher than control group after 48 hours (*P* < .05) (Fig. 3).

2. TGF-*β*

The release of TGF-*β* increased over time. TGF-*β* released from PDLCs cultured in 50 μg/mL EMD were significantly higher than the control group after 24 hours (*P* < .05) (Fig. 4).

Discussion

PDLCs are essential in supporting and healing the tooth attachment apparatus. Thus, the ultimate goal of periodontal regeneration is to promote PDLC productivity and calcified tissue by stimulating growth of PDLC. As PDLC can proliferate and migrate, they promote periodontal tissue regeneration by differentiating to osteoblast-like and cementoblast-like cells.[1] During this procedure, EMDs are thought to play a considerable role in promoting PDLC attachment and growth.[4] There have been reports that EMD control intracellular cAMP signals which are associated with PDLC proliferation and metabolism activity and secretion of numerous self releasing growth factor.[12]

EMD is composed of 2 protein families, 90% of amelogenins which are thought to form the periodontal attachment during tooth development, and the non-amelogenins such as ameloblastin, enamelin, and tuftelin.[13] The theory that EMDs play a considerable role in tissue regeneration to promote PDLC proliferation and repair has been applied clinically. There have been reports that EMDs promote the proliferation of PDLC and stimulate osteogenesis by production of collagen and reinforcement of calcification,[14] but there has been no definite answer about the mechanism how EMDs influence PDLC. It was identfied that EMD didn't contain the growth factor includ-
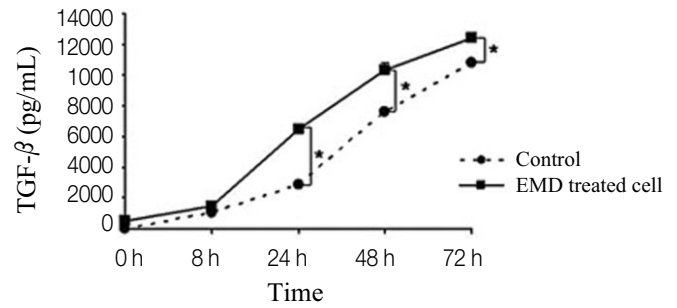
ing PDGF, TGF-*β*, and insulin like growth factor.[4] The previous study demonstrated that EMDs enhanced proliferation, increased protein production, and promoted mineral nodule formation.[4] Thus, we observed ALP activity which reflects the change of cell proliferation rate and osteogenetic ability when EMD was applied to PDLC, and examined optimum concentration of EMD.

As a result, cell proliferation of PDLCs cultured in >25 μg/mL EMDs were significantly higher than the control group, and ALP activity of PDLCs cultured in 50 μg/mL EMDs was significantly higher than the control group (*P* < .05). Accordingly, 50 μg/mL EMD was used in the next experiment which evaluated the effect of EMDs on release of growth factor from PDLCs over time.

In this experiment, >25 μg/mL EMDs promoted cell proliferation of PDLCs, 50 μg/mL EMDs increased ALP activity of PDLCs. As ALP activity is the marker of early osteogenesis, this result shows validity of the theory that EMDs play a primary role in periodontal regeneration by PDLCs. Van der Pauw *et al.* announced that EMDs increase ALP which is not proliferating PDLCs, but stimulating attachment of PDLCs.[9] According to *in vitro* experiment of Chong *et al.* about which area among damaged site is most effective when EMDs was applied, EMDs did not reveal significant wound closure at center of wounds, and showed obvious proliferation of PDLCs and increased wound closure only when PDGF-BB was added.[2] One of the problems of regeneration of PDLCs is to promote proliferation of PDLCs and to suppress grouping epithelial cells at the same time, in this procedure PDGF is effective to heal wound.

In this experiment we examined growth factors released from cultured PDLCs. Results showed that VEGF and TGF-*β* were detected significantly, but PDGF-AB and PDGF-BB were not detected significantly. According to the experiment of Hoang *et al.* which is about wounds, PDLCs showed significant proliferation when both EMDs and PDGF-BB were applied.[1] In the experiment of Boyan *et al.* about rats, EMDs took part in the proliferation and differentiation of

osteoblasts, and when EMDs and demineralized freeze-dried bone allografts (DFDBA) were used together, EMDs strengthened the release of TGF-β and IL-6.[11]

Mirastschiljski reported that EMDs promote the production of VEGF, which is an angiogenic growth factor that forms blood vessels by stimulating fibroblasts in adult skin.[15] That is, EMDs directly promote angiogenesis by stimulating endothelial cells, and indirectly promotes release of growth factor which participate in angiogenesis.

It has been known that TGF-β which is powerful growth factor associated with platelet and osteogenesis, stimulates synthesis and accumulation of extracellular matrix. Especially, TGF-β1 influences proliferation, chemotaxis, and differentiation of osteoblast.[16] TGF-β is an additional factor used in the regenerative treatment of periodontal tissue for recovering and regenerating calcified tissue, but the expression effect of TGF-β, which is a self-releasing growth factor, is not obvious.[17,18]

In the experiment of Lyngstadaas *et al*., production and release of growth factor, such as TGF-β began in medium on which PDLCs were cultured for several days, TGF-β was released faster and showed more abundant concentration in medium which EMDs were added.[12]

In this experiment the release of VEGF and TGF-β was detected significantly, the amount of each growth factor detected was different according to time. VEGF relevant to angiogenesis, was released significantly after 48 hours culturing, and detected amount of TGF-β was significantly higher than that in the control after 24 hours culturing.

Therefore, the positive effect of EMDs on periodontal regeneration is achieved by promoting the release of growth factors like VEGF and TGF-β of PDLCs.

Our results suggest that EMDs are important for the success of periodontal tissue regeneration, but we can not apply these *in vitro* results to *in vivo* experiments. Thus, more exact cytological experiments and clinical studies will be needed in the future.

Conclusion

Conclusively, EMDs enhanced the proliferation of PDLCs, the ALP activity of PDLCs, and the release of growth factors such as VEGF and TGF-β from PDLCs. Therefore, EMDs could be one of the effective methods for periodontal regeneration.

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References

1. Hoang AM, Oates TW, Cochran DL. In vitro wound healing responses to enamel matrix derivative. J Periodontol 2000;71:1270-7.
2. Chong CH, Carnes DL, Moritz AJ, Oates T, Ryu OH, Simmer J, Cochran DL. Human periodontal fibroblast response to enamel matrix derivative, amelogenin, and platelet-derived growth factor-BB. J Periodontol 2006;77:1242-52.
3. Slavkin HC. Towards a cellular and molecular understanding of periodontics. Cementogenesis revisited. J Periodontol 1976;47:249-55.
4. Gestrelius S, Andersson C, Lidström D, Hammarström L, Somerman M. In vitro studies on periodontal ligament cells and enamel matrix derivative. J Clin Periodontol 1997;24:685-92.
5. Hammarström L. Enamel matrix, cementum development and regeneration. J Clin Periodontol 1997;24:658-68.
6. Heijl L, Heden G, Svärdström G, Ostgren A. Enamel matrix derivative (EMDOGAIN) in the treatment of intrabony periodontal defects. J Clin Periodontol 1997;24:705-14.
7. Froum SJ, Weinberg MA, Rosenberg E, Tarnow D. A comparative study utilizing open flap debridement with and without enamel matrix derivative in the treatment of periodontal intrabony defects: a 12-month re-entry study. J Periodontol 2001;72:25-34.
8. Sculean A, Donos N, Miliauskaite A, Arweiler N, Brecx M. Treatment of intrabony defects with enamel matrix proteins or bioabsorbable membranes. A 4-year follow-up split-mouth study. J Periodontol 2001;72:1695-701.
9. Van der Pauw MT, Van den Bos T, Everts V, Beertsen W. Enamel matrix-derived protein stimulates attachment of periodontal ligament fibroblasts and enhances alkaline phosphatase activity and transforming growth factor beta1 release of periodontal ligament and gingival fibroblasts. J Periodontol 2000;71:31-43.
10. Melcher AH. On the repair potential of periodontal tissues. J Periodontol 1976;47:256-60.
11. Boyan LA, Bhargava G, Nishimura F, Orman R, Price R, Terranova VP. Mitogenic and chemotactic responses of human periodontal ligament cells to the different isoforms of platelet-derived growth factor. J Dent Res 1994;73:1593-600.
12. Lyngstadaas SP, Lundberg E, Ekdahl H, Andersson C, Gestrelius S. Autocrine growth factors in human periodontal ligament cells cultured on enamel matrix derivative. J Clin Periodontol 2001;28:181-8.
13. Grandin HM, Gemperli AC, Dard M. Enamel matrix derivative: a review of cellular effects in vitro and a model of molecular arrangement and functioning. Tissue Eng Part B Rev 2012;18:181-202.
14. Schlueter SR, Carnes DL, Cochran DL. In vitro effects of enamel matrix derivative on microvascular cells. J Periodontol 2007;78:141-51.
15. Mirastschijski U, Konrad D, Lundberg E, Lyngstadaas SP, Jorgensen LN, Agren MS. Effects of a topical enamel matrix derivative on skin wound healing. Wound Repair Regen 2004;12:100-8.
16. Pfeilschifter J, Oechsner M, Naumann A, Gronwald RG, Minne HW, Ziegler R. Stimulation of bone matrix apposition in vitro by local growth factors: a comparison between insulin-like growth

factor I, platelet-derived growth factor, and transforming growth factor beta. Endocrinology 1990;127:69-75.

17. Gao J, Symons AL, Bartold PM. Expression of transforming growth factor-beta receptors types II and III within various cells in the rat periodontium. J Periodontal Res 1999;34:113-22.

18. Hobbs HC, Rowe DJ, Johnson PW. Periodontal ligament cells from insulin-dependent diabetics exhibit altered alkaline phosphatase activity in response to growth factors. J Periodontol 1999;70:736-42.

법랑기질유도체가 인간 치주인대세포의 증식 및 성장인자 발현에 미치는 영향

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목적: 치주조직재생을 위해서는 치주인대세포의 증식 및 이주를 촉진시키는 것이 필수적이나 현재까지 이것을 만족시키는 재생술식은 없다. 최근 법랑기질유도체(enamel matrix derivatives)가 치주조직 재생 술식에 적용되고 있으나 그 기전에 대해서는 완전히 알려지지 않았다. 따라서 본 연구의 목적은 법랑기질유도체가 치주인대세포의 증식과 성장인자 발현에 미치는 영향에 대하여 알아보고자 함이다.
재료 및 방법: 건강한 성인의 제 3 대구치로부터 치주인대세포를 추출한 후, 법랑기질유도체(Emdogain (Biora, Malmo, Sweden))의 농도가 각각 0, 12.5, 25, 50, 100, and 200 μg/mL인 배지에서 배양시켰다. 치주인대 세포증식과 알칼리성 인산분해효소(alkaline phosphatase) 활성도를 측정하고, 발현되는 성장인자를 평가하였다.
결과: 치주인대세포의 세포증식은 25 μg/mL이상의 농도의 법랑기질유도체를 첨가한 군에서, 알칼리성 인산분해효소 활성도는 50 μg/mL의 법랑기질유도체를 첨가한 군에서 각각 유의하게 증가하였고, 50 μg/mL의 법랑기질유도체를 첨가한 군에서 치주인대세포의 VEGF (vascular endothelial growth factor)와 TGF (transforming growth factor)-β의 발현이 유의하게 증가하였다.
결론: 법랑기질 유도체는 인간 치주인대세포의 세포증식과 알칼리성 인산분해효소 활성을 증진시키고, VEGF와 TGF-β 등 성장인자의 발현을 촉진함으로써 치주조직 재생에 기여할 수 있을 것이다. (대한치과보철학회지 2016;54:203-9)

주요단어: 법랑기질유도체; 치주인대; 치주조직재생; 조직공학; 성장인자

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