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The Effects of Enamel Matrix Proteins and Dentin Collagen on the Attachment of Periodontal Ligament Cells

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SS and BHS designed the study and wrote the protocol. Authors SB and IDG managed the experimental process. Authors SB and EE managed the literature searches. Author SB wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background: The aim of this study was to evaluate the effect of enamel matrix proteins (EMP) and dentin collagen on the attachment of periodontal ligament cells to the root surfaces.
Methods: Eighty-four root slices were obtained from forty-two lower anterior teeth that had been extracted due to the periodontal disease. The root slices were subjected to one of the following treatments: 1) control group 2) EDTA demineralization + EMP, 3) dentin collagen, 4) EDTA demineralization + dentin collagen. Periodontal ligament (PDL) cells (10^5/ml) were seeded and incubated for two hours on surfaces of the roots in each group. Following the incubation the numbers of the attached cells were calculated by colorimetric assay and the morphologies of the cells were evaluated by scanning electron microscopy (SEM). One-way ANOVA was used for statistical analysis (p=0.05).

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Results: No significant difference was found among the groups regarding the number of attached cells (p>0.05). However, the mean number of the attached cells was highest in the 2nd Group (EDTA demineralization + EMP), while it was lowest in Group 3 (dentin collagen). SEM evaluation of the dentin specimens revealed that the EMPs treated specimens exhibited elongated fibroblasts with filopodial extensions while the cells in the control and dentin collagen treated groups were round with thin and short filopodia.
Conclusion: In this study, the EMPs were found effective in the attachment of cells on the root surface when compared to dentin collagen.

Keywords: Regeneration; enamel matrix proteins; dentin collagen; periodontal ligament cells.

ABBREVIATIONS

EMP : Enamel Matrix Protein
PDL : Periodontal Ligament
BMP : Bone Morphogenic Protein
SEM : Scanning Electron Microscopy
PBS : Phosphate Buffered Saline
DMEM : Dulbecco's Modified Eagle's Medium
EDTA : Ethylenediaminetetraacetic Acid
DC : Dentin Collagen
XTT :2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide

1. INTRODUCTION

The regeneration of periodontium requires restitution of periodontal attachment apparatus, new bone formation, new cementum deposition upon denuded root surface and insertion of functionally oriented new collagen fibers of periodontal ligament into new bone and new cementum [1]. The selective migration, proliferation and differentiation of the cells derived from periodontal ligament and alveolar bone are essential in achieving successful periodontal regeneration [2]. The characteristics, biological problems, and technical complications associated with periodontal wound healing and tissue regeneration have been reviewed extensively [3-6]. There are several techniques used alone or in combination to achieve periodontal regeneration, including root surface modification, bone grafts or substitutes, guided tissue regeneration, and biological mediators. The biological mediators include extracellular matrix proteins and cell attachment factors, mediators of cell metabolism and activity, and growth and differentiation factors.

Amelogenins are a family of extracellular matrix proteins that regulate the initiation and growth of hydroxyapatite crystals during mineralization of enamel. Recent studies demonstrated that amelogenins and enamel matrix proteins (EMPs) containing amelogenins as a major component, involved in formation of acellular cementum during tooth development [7,8]. In addition, enamel matrix proteins have the potential to induce regeneration of acellular cementum and collagenous fibers in animal models [9]. Clinical investigations showed that application of EMPs leads to regeneration of the acellular cementum, significant clinical attachment gain and radiographic bone filling [7,10,11]. Treatment of periodontal ligament (PDL) cells with EMPs stimulates cell proliferation, protein and collagen synthesis, and also induces mineralization in *in-vitro* studies [12-14].

Demineralized dentin was shown to have promising results *in vitro* [15] and *vivo* [16] and the presence of bone morphogenic proteins (BMPs) in dentin was strongly suggested in early studies [17] Fugazzotto et al. [18] reported 16 cases in which he used freeze-dried dentin merits and obtained osseous regeneration and suggested that freeze-dried dentin was osteoinductive.

The recruitment of PDL cells is essential for the regeneration of new attachment, several studies have focused on the migration and attachment of these cells to root surfaces [19-21]. The aim of this study was to evaluate and compare the effects of enamel matrix proteins (EMP) and dentin collagen on the attachment of periodontal ligament cells to the root surfaces by colorimetric assay and scanning electron microscopy (SEM).

2. MATERIALS AND METHODS

This study was conducted in Ege University, School of Dentistry, Department of Periodontology and School of Engineering, Department of Bioengineering. In this study, forty-two lower anterior periodontally-involved human teeth were used for the preparation of root slices and PDL cells were isolated from eleven healthy premolar teeth extracted for orthodontic reasons. The protocol of the study was approved by Research Ethics Committee of Ege University School of Medicine (02-5/14) and all patients gave informed consent before providing the samples.

2.1 Periodontal Ligament Cell Isolation and Culture

Human PDL cells were isolated and cultured following previously published procedures [21,22]. Briefly, clinically healthy premolar teeth that were extracted for orthodontic reasons were washed several times with phosphate buffered saline (PBS). The periodontal ligament fragments were curetted from the middle third of the roots using a scalpel. After washing, the fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 23 mM NaHCO3 (Gibco Biocult, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco Biocult, Paisley, UK) and antibiotics (50 lg/mL of streptomycin, 100 U/mL of penicillin). When the cells were confluent, they were harvested with 0.25% trypsin (Gibco BRL, California, USA) and 0.1% ethylenediaminetetraacetic acid (EDTA, Gibco BRL, California, USA) in PBS and used for the sub-culture.

All the cells were incubated at 37℃ in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was changed every 2 days. In this study, fibroblasts between the 3rd and 5th passage were used for all the experiments.

2.2 Preparation of Root Slices

Forty-two lower anterior teeth with no caries and restoration were extracted due to the periodontal disease from patients without any systemic disease. The root cementum was removed and eighty-four round dentin slices with 5 mm diameter and a thickness of 1-1.5 mm were prepared by using cutting mills.

Finally 84 dentin slices were obtained and randomly divided into four groups (n=21), UV sterilization for 30 minutes were applied and the slices were subjected to one of the following procedures.

2.2.1 Control group

This group served as control and no additional application was applied to the dentin surfaces. The root slices were carefully embedded into sterile parafilm exposing only the periodontally involved surfaces in 96-well culture plate, 1 root slice for each well.

2.2.2 EDTA/EMP group

Dentin slices in this group were treated with EDTA solution and EMP was applied. Briefly, 24% -EDTA solution was applied for 3 minutes and then the dentin slices were washed with distilled water for 30 seconds. The air dried root slices were carefully embedded into sterile parafilm exposing only the periodontally involved surfaces in 96-well culture plate, 1 root slice for each well. EMPs (Biora AB, Malmo, Sweeden) were dissolved in 0.1% acetic acid and 10 mg/ml stock solution was obtained. This stock solution was diluted to 100 µg/ml concentration with PBS just prior to initiating experiments. 50 µl of this solution was added into the wells in EMP group.

2.2.3 Dentin Collagen (DC) group

DC was applied to the root slices which were embedded into sterile parafilm exposing only the periodontally involved surfaces in 96-well culture plate. Eight periodontally healthy teeth with no caries and restoration were obtained from patients without systemic disease and used for the preparation of the DC. All cementum, enamel and the pulpal tissues were removed. The remaining dentin bulk was grounded and dentin powder was obtained. The dentin powder was kept in 24%-EDTA solution for five days by changing the EDTA solution every day in order to remove the mineralized component of dentin. At the end of the 5 days, the remaining dentin collagen was dried, frozen and lyophilized. Twenty five kgrey gamma radiation was applied for 16 hours for sterilization of the obtained dentin collagen.

Dentin collagen was dissolved in 0.1% acetic acid and 10 mg/ml stock solution was obtained. This stock solution was diluted to 100 µg/ml concentration with PBS just prior to initiating

experiments. 50 µl of this solution was added into the wells in DC group.

2.2.4 EDTA/DC group

These slices were first treated with EDTA and then, DC was applied to the surfaces as explained.

The experiments were repeated in 3 separate days and 28 root slices (7 slices per group) were used in each experiment day.

2.3 XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) Assay

One hundred µl of PBS containing 10^6 human PDL cells/ml were added into the wells and onto the 5 dentin slices in each group and incubated for 2 hours at 37℃ in a humidified atmosphere of 95% air and 5% CO_2 for cell attachment. The remaining 2 roots were subjected to the same procedures except seeding of PDL cells on and considered as the control group for the spectrophotometric readings. Following 2 hour incubation, the unattached cells removed gently by washing with 100 µl of PBS. Then, 100 µl of PBS containing 0,4 mg/ml XTT (Applichem, Germany) and 0,04 mg/ml Co-enzyme Q_0 (Appli Chem) was added to each well containing a root slice and incubated for 30 minutes. After the incubation period, the entire content of the wells, except the root slices, were transferred into another 96-well culture plate. The absorbance of medium obtained from each well was determined by using a microplate reader UV-visible spectrophotometer at 450 nm wave length. The number of metabolically active cells attached to the root surfaces was calculated from the standard curve obtained by measuring the serial absorbance of PBS containing $5x10^2$, 10^3, $5x10^3$, 10^4, $5x10^4$, 10^5, $5x10^5$, 10^6 and $5x10^6$ cells/ml respectively.

2.4 Scanning Electron Microcopy

Following XTT assay, three root slices with attached cells from each group were processed for SEM viewing and photographed at different magnifications. Briefly, the root slices were gently washed with PBS for 30 seconds and all SEM procedures were performed on ice at 4℃. The root slices were grouped and placed into wells of 24-well culture plate. Buffer A containing 0,1 M cacodylate and 5% glutaraldehyde (pH 7,2) was added to each well and incubated for 30 min. The root slices were incubated in buffer B containing 0,1 M cacodylate and 7% sucrose, and in buffer C containing 0,1 M cacodylate and % 2 osmium tetroxide for 30 min, respectively. Following this procedure, the root slices were dehydrated in 35%, 70%, 85%, 95%, 100% ethanol solutions for 5 min at room temperature. They were immersed in hexamethyldisilazane solution for 5 min and air-dried for 30 min. The root slices were mounted on brass SEM carrier, transferred into a desiccator containing phosphorus pentoxide for 48 hours and then sputter coated by gold to a thickness of 200 Å. Finally, the specimens were examined using a SEM (JEOL-5200, Tokyo, Japan) and micrographs were obtained at different magnifications.

2.5 Statistical Analysis

All experiments were performed three times and 7 root slices were used in each group for each experiment. The mean number of attached cells per mm^2 of root surfaces and standard deviations in each group were calculated. The statistical significance of differences among each group was examined by one-way ANOVA.

3. RESULTS

3.1 XTT Assay Results

The mean number of the attached cells per mm^2 of dentin surface in each group is given in Fig. 1. Cell attachment was observed in all experimental groups. The number of the attached cells was higher in EDTA+EMPs group, which was followed by EDTA+DC group. DC group presented the lowest number of the attached cells when compared to the other three groups. However, the statistical difference between the groups was not significant ($p>0.05$).

3.2 SEM Results

All specimens showed cell attachment; however, there were some areas on the root surfaces that the cells did not attach. In the control group, the smear layer could be seen on these empty areas between the cells. The PDL cells in the control group were mostly round in shape and had short and thin microvilli and filopodia (Fig. 2a). The demineralized areas could be seen between the cells on the slices treated with EDTA+EMP.

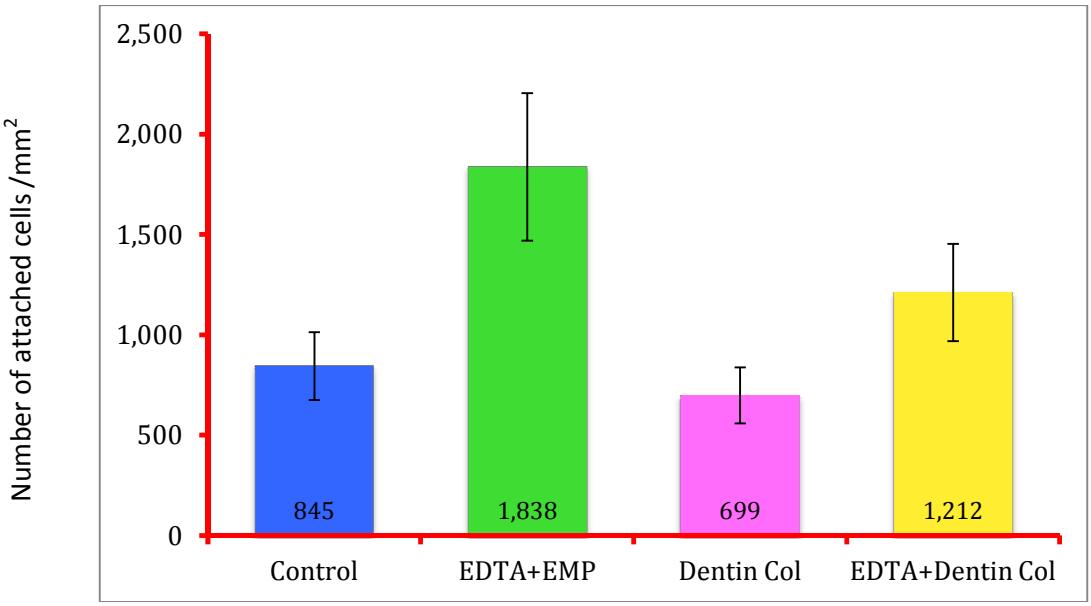

Fig. 1. The mean number of the attached cells per mm^2 of root surfaces in each study group

The PDL cells were flat and closely adapted to the dentin surface in this group and exhibited long extensions of cytoplasmic processes with numerous filopodia (Fig. 2b). The PDL cells were oval or round in the DC group and exhibited short and thin microvilli and filopodia (Fig. 2c). The PDL cells were not closely adapted to the dentin surfaces both in the DC and EDTA+DC group. The cell attachment was more limited in the EDTA+DC group with thin and little to no filopodia and the cells were oval or round (Fig. 2d).

4. DISCUSSION

Periodontal regeneration is dependent on a sequence of events including cellular proliferation, migration, and attachment to components of the extracellular matrix as well as organic matrix synthesis and mineralization [23]. EMPs were shown to control some of the critical events associated with periodontal regeneration, such as cementogenesis and development of the periodontal attachment apparatus. Allogenic, decalcified dentin has been used as a bone grafting material in early studies [16,18]. The effects of EMPs and DC on the attachment of PDL cells to dentin surfaces were evaluated quantitatively and qualitatively by XTT and SEM methods in this in vitro study. Although no significant difference was found in the number of the attached cells among the study groups, EMPs were found to enhance the number of the attached cells to previously diseased root surfaces. SEM evaluation of the dentin specimens revealed that the EMPs treated specimens exhibited elongated fibroblasts with filopodial extensions while the cells in the control and DC treated groups were round with thin and short filopodia.

In the present study EMP and DC were diluted with acetic acid to increase their solubility. Since the solubility of EMP can be strongly influenced by pH and temperature [20,12] a low degree of acid concentration was utilized in order to guarantee a stable pH level with no variation in the culture medium. In order to imitate the clinical situation, root slices were prepared from the periodontally exposed root surfaces of the human teeth extracted due to periodontal disease.

Twenty four percent EDTA was used for 2 min according to the manufacturer's instruction in the EMPs group. EDTA was shown to remove the smear layer, which could interfere with cellular adhesion and suggested to make the root more biocompatible [24]. DC was used with both EDTA-etched and non-etched surfaces in order to evaluate the possible advantages of using DC with EDTA. The results of the present study failed to show significant advantages of DC although the number of the attached cells was increased in EDTA+DC group when compared to DC and control groups. Devecioğlu et al. [15]

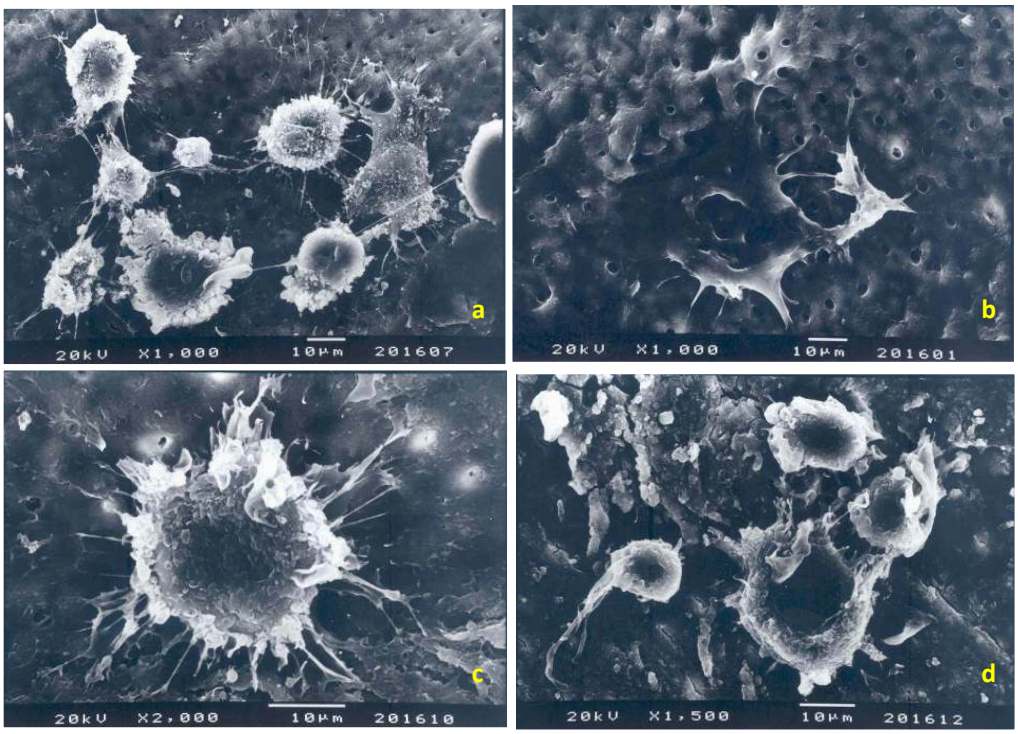

Fig. 2. a) SEM micrograph of the dentin surface in control group. PDL cells can be seen mostly round in shape with microvilli and filopodia appearing short and thin
b) SEM micrograph of the dentin surface in the EDTA +EMPs group. The etched dentin surface can be seen with the more apparent dentin tubules. The PDL cells in this group were closely adapted to the dentin surface
c) SEM micrograph of the dentin surface in the dentin collagen group. The PDL cell was round with short and thin microvilli and filopodia
d) SEM micrograph of the dentin surface in the EDTA + dentin collagen group. The PDL cells were round in shape and not adapted closely to the dentin surface

evaluated the effects of demineralized and freeze-dried dentin, bone allograft, hydroxyapatite and cementum and then, reported that dentin enhanced the proliferation of PDL cells when compared to bone allograft and cementum granules. Mineral-like nodule formation was observed significantly higher in demineralized and freeze-dried dentin compared to other materials tested for osteoblasts.

The biological mechanisms of EMPs have not been fully clarified despite the many clinical studies showing the effectiveness of EMPs in periodontal regeneration. It has been hypothesized that EMPs do not contain any growth factor and could function as a vital matrix for the cells [12]. EMD was also shown to induce matrix metalloproteinase-2 production in osteoblasts and stimulate degradation of the surrounding collagen, resulting in changes in extracellular matrix structure and promoting periodontal regeneration [25]. Lyngstadaas et al. [26] demonstrated that cultured PDL cells exposed to EMP had increased attachment rate, growth rate and metabolism, and subsequently released several growth factors into the medium. It was hypothesized that the increase in proliferation could be due to the fibroblasts' transforming growth factor B1, interleukin-6 and platelet-derived growth factor AB production in the presence of EMP [13,26].

In an *in-vitro* study, Hoang et al. [27] evaluated the attachment of PDL cells to amelogenin or EMPs coated plates and found that amelogenin and EMPs promote both cell adhesion and spreading activity. In the present study SEM examination revealed that PDL cells prefered to attach and spread on EMPs treated root surfaces and in contrast the cells on DC treated or control surfaces were round. Similar to the findings of this study Davenport et al. [19] reported that on

the EMPs treated dentin, the attached PDL cells appeared with extensive spreading on the dentin surface with numerous filopodial and lamellopodia extensions while the nontreated controls were populated with very few cells and limited filopodial processes. Cattaneo et al. [20] evaluated the attachment of PDL cells to EDTA etched and EMPs applied root surfaces by SEM, after 12 d of incubation the EMD-treated cells showed a body characterized by a flattened surface closely adherent to the substrate and an outer smooth surface rounded in shape. They concluded that EMP treated cells were more similar to cementoblasts than to fibroblasts. In the present study the incubation time was too short to observe such differences in PDL cells even though the cell morphologies were different from the control group in EMP treated group.

5. CONCLUSION

Within the limitation of the present study it can be concluded that EMPs have limited positive effects on the attachment and spreading of PDL cells to the previously periodontally exposed root surfaces. DC was shown not to aggravate the attachment of PDL cells to the EDTA etched root surfaces which let us suggest that further studies might be useful to develop new methods for the application of DC to derive benefit from its osteoinductive properties.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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