EFFECT OF SMOKING ON ATTACHMENT OF HUMAN PERIODONTAL LIGAMENT CELLS TO PERIODONTALLY INVOLVED ROOT SURFACES FOLLOWING ENAMEL MATRIX DERIVATIVE APPLICATION

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ABSTRACT
The most important aim in periodontal therapy is to regenerate the periodontal supporting tissue lost as a result of periodontitis. Several studies have demonstrated that tobacco use interferes with periodontal therapy and substantially reduces the possibility of favorable treatment outcomes. The present study was performed to determine the attachment of PDL cells to the diseased roots of a smoking patient compared to non-smoking controls with enamel matrix derivative (EMD) application. Teeth both from a patient smoking more than 20 cigarettes daily and from another non-smoking patient were extracted and PDL tissue biopsies were taken from these teeth. Fibroblasts were cultured. Each root surface was divided into six equal parts. Samples were treated with citric acid and EMD, embedded into cell culture flasks, and kept in the culture for 1 h, 3 h, 5 h and 3 days. Then, electron microscopy analysis was performed. In the smoking group, collagen fibers were spread parallel to the surface as in the non-smoking group, but in one single direction rather than in different directions. It was observed that EMD application on smoking and non-smoking periodontally-diseased patients could affect the function of PDL cells and the potential of collagen production.

Keywords: periodontal ligament fibroblasts, enamel matrix derivative, smoking

Introduction
Periodontal regeneration remains a key purpose despite the development of widely available regenerative surgical techniques. A new model for periodontal regeneration in recent years is dental tissue engineering. It is based on applying biomaterials that aid in the targeting of proteins (e.g. growth factors) and regenerative cells to bioengineer the periodontium. An example of such a biomaterial is the enamel matrix derivative (EMD). A commercial EMD preparation named EMDOGAIN (Biora Inc.) was introduced in 1997. It is made from a purified acidic extract from developing embryonal enamel of six-month-old piglets. EMDOGAIN works as a tissue healing modulator that mimics the events that occur during root development to stimulate bone and periodontium regeneration (15, 16). This preparation has found successful application in restoring fully functional periodontal ligament, cementum, and alveolar bone in patients with severe attachment loss.

EMD contains enamel matrix proteins of various molecular weights including amelogenin (4), sheathlin (also called ameloblastin or amelin) (19), tuftelin (8) and enamelin (11, 20). Enamel matrix proteins secreted by the Hertwig’s epithelial root sheath, play an important role in the development of periodontium including the cementum, periodontal ligament, and alveolar bone (15, 29). The periodontal ligament (PDL) tissue plays an important role in the process of periodontium regeneration (10). Although the PDL tissue is only 0.15 mm to 0.38 mm wide (25), it is quite complex and composed of several different cell populations. The major type of cell in the periodontal ligament is the fibroblast. Fibroblasts are located throughout the connective tissues of the body, where their role is to maintain the extracellular matrix substance. Periodontal fibroblasts can extensively synthesize proteins and collagen, and respond well to the molecular mediators during the wound healing process. During this process fibroblasts show potential to develop into different cell types depending on the molecular mediators. The precursor cells of the periodontal ligament, in this case the fibroblast, can differentiate into collagen fibers, osteoblasts, or cementoblasts, forming alveolar bone or cementum, respectively (26, 27).

EMD apparently acts through a matrix-cell interaction between the EMD amelogenin aggregate and the periodontal ligament cells (17, 18, 19, 24, 40). Notably, after application of EMD, enhanced wound healing of soft tissues has been reported (39). Many in vitro studies have focused on the mechanism of action of EMD in periodontal ligament (PDL) cells during periodontal regeneration. In vitro studies demonstrate that EMD treatment of PDL fibroblasts stimulates proliferation, protein and collagen synthesis, and also induces mineralization (8, 14, 20, 23, 24, 28, 31, 33, 38).

Smoking is the primary behavioural risk factor for periodontitis, resulting in loss of the periodontal apparatus (5, 13, 21, 36, 37). Nicotine is one of more than 4000 chemicals
present in smoke and acts as a key modulator of cellular processes such as cell growth, motility, and differentiation. Nicotine has a negative biological effect on PDL cells in vitro by decreasing their proliferation, migration, attachment, alkaline phosphatase activity, and chemotaxis in a concentration-dependent manner (15, 32). It is now known that smokers tend to respond less favorably to periodontal treatment procedures (1, 3). However, to the best of our knowledge, no study has been performed to investigate the effect of smoking on the attachment of human cultivated PDL fibroblasts derived from patients with periodontal disease to EMD treated periodontally diseased roots. Martinez et al. (25) studied in vitro fibroblast cultures in standard environments and evaluated nicotine-kotinine applications in specific rates. In our experimental design, PDL cells are evaluated in an environment that mimics EMD application in the clinic to a non-smoking and a smoking patient’s root surfaces. The present study was performed to determine the attachment of PDL cells to the diseased root surfaces of a smoker compared to a non-smoking control patient.

Materials and Methods

Three periodontally diseased teeth with the following criteria were selected in this study as described by Babay (2): 1) no history of scaling or root planing in the previous 6 months, 2) proximal attachment loss of 5 mm or more, and 3) no history of acute pain or swelling. Cigarette smoking patients were excluded. The ethical principles of the Helsinki Declaration were followed and the study protocol was approved by the Ethics Committee of the Faculty of Dentistry, University of Erciyes.

The teeth were individually placed in sterile capped tubes containing saline and were processed immediately after the extraction. The teeth were cleaned of blood, saliva and irrigating solutions with a sterile soft bristle toothbrush and deionized water. Afterwards, the periodontal attachment level of the teeth was marked with a notch. Following an initial phase of scaling, root planing with gracy 7/8 curets, biopsies were taken from the periodontal ligament of the three teeth compromised by periodontitis and the other two teeth belonged to a 45-year-old female patient smoking more than 20 cigarettes daily. The teeth were placed in Petri dishes containing saline and were processed immediately after the extraction. The teeth were cleaned of blood, saliva and irrigating solutions with a sterile soft bristle toothbrush and deionized water. Afterwards, the periodontal attachment level of the teeth was marked with a notch. Following an initial phase of scaling, root planing with gracy 7/8 curets, biopsies were taken from the periodontal ligament of the three teeth compromised by periodontal disease. After removing the periodontal ligament, the portions of the remaining teeth between the periodontal attachment and free gingival margin were divided into six pieces approximately 1 mm³ in size. Samples were kept within physiological saline solution (0.9 % NaCl) until analysis.

Fig. 1 shows the scanning electron micrographs of the root surface obtained from non-smoking and smoking patients treated with EMD (× 5000). Fig. 2 demonstrates the SEM micrograph of PDL cells attached to the cut-dentin surface at 1 h incubation of the non-smoking patient’s tooth surface obtained from the smoking patient and embedded into a fibroblast culture for 3 h after treatment with EMD (× 2000).

When the examination of the root surface obtained from the non-smoking patient was performed with a scanning electron microscope, collagen fibers were arranged, the fiber networks expanded to the confluence. PDL cells between the fourth and fifth passage were used for this study. In order to mimic the clinical situation, four of the six samples were kept in 24 % ethylenediamine-tetraacetic acid (EDTA) gel (PrefGel®, Biora AB, Malmö, Sweden) for 2 min and then, thoroughly washed with saline. Then, these samples were treated with EMD and were placed in Petri dish cultures of fibroblasts. These samples were kept for 2 h, 3 h, 5 h and 3 days. To determine the effect of EMD on the tooth surface, one of the two samples was kept in EDTA solution for 2 min and was treated with fibroblast culture. The other sample was kept waiting in the culture to which EMD was applied. The same procedure was applied to samples taken from both the smoking and the non-smoking patient.

Specimens for SEM were rinsed two times in phosphate buffer saline (PBS) at 37 °C and fixed in 0.1 M cacodylate buffer containing 2.5 % glutaraldehyde in room temperature for 45 min. This was followed by post-fixation in 1 % osmium tetraoxide in 0.1 M cacodylate buffer for 15 min, rinsing in water. The specimens were dehydrated through a graded series of acetone (50 % to 100 %) and then critical point dried. Each specimen was coated with gold palladium and examined by a scanning electron microscope (LEO 440), at 15 Kv with a tilt angle of 0° to 40°.

Results and Discussion

To the best of our knowledge, this study is the first one to compare the attachment of PDL cells to the EMD-treated diseased roots of smoking and non-smoking patients in vitro. Cigarette smoking has been described as one of the most significant risk factors for the development and progression of periodontal diseases. In vitro studies have demonstrated nicotine to alter the cellular morphology and viability of cells derived from human periodontal tissue, as well as influence the ability of these cells to attach to a substrate and to synthesize extracellular matrix molecules (6, 15, 25, 27, 41).

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Fig. 1 shows the scanning electron micrographs of the root surface and EMD granules on the tooth surface of the non-smoking patient (× 5000). Fig. 2 demonstrates the SEM micrograph of PDL cells attached to the cut-dentin surface at 1 h incubation of the non-smoking patient’s tooth surface (× 3500). Fig. 3 shows the SEM appearance of PDL cells on the smoking root surface obtained from the smoking patient and embedded into a fibroblast culture for 3 h after treatment with EMD (× 2000).

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EMD was intensively adhered to the root surface in the form of granules of various sizes in accordance with the arrangement of collagen fibers.

Fig. 1. Scanning electron micrographs of the tooth surface (A) and EMD granules (B) on the tooth surface of the non-smoking patient (× 5000).

Fig. 2. Scanning electron micrograph of PDL cells attached to the cut-dentin surface following 1 h incubation of the tooth surface of the non-smoking patient (A) and the smoking patient (B) (× 3500).

Fig. 3. The appearance of PDL cells on the smoking root surface obtained from the smoking patient and embedded into fibroblast culture for 3 h after treatment with EMD (× 2000).

Fig. 4. Scanning electron microscopy of collagen fibers on the tooth surface obtained from the non-smoking patient and embedded into fibroblast culture for 5 h after treatment with EMD (×10,000).

Fig. 5. The collagen fibers on the tooth surface obtained from the smoking patient and embedded into fibroblast culture for 3 h after treatment with EMD (×10,000).

The direction of collagen fibers may have an affect on periodontal tissue resistance due to affecting the adhesiveness to root surface (22, 34). Zhou et al. (42) reported nicotine and P. gingivalis had an additive effect on human gingival fibroblast-mediated collagen degradation and these findings help to explain why smoking is a major factor contributing to the initiation and/or exacerbation of periodontal diseases. In the current study, before being incubated in the culture of the smoking patient, EMD granules were seen in their globular structure but the rareness of the web structure and the globules were less than in the non-smoking patient, and the collagen fibers gave a tight packaged thick fiber view. Also, collagen...
fiber degradation and typical appearance of cellular structure corruption due to smoking were observed. The direction of fibers to be parallel to each other is consistent with recent studies (12, 15) attributed to increased fibroblast activity resulting in early fibroblast degradation due to nicotine and suggesting collagen structure formed more mature against the chronic effects of nicotine.

When samples embedded into PDL cell culture for 1 h after EMD treatment were examined, PDL cells containing numerous and long filopodia in samples from the non-smoking patient (Fig. 2A) were fastened tightly on the tooth surface. EMD granules were located on the PDL cells and surface of teeth. As for the smoking patient (Fig. 2B), PDL cells had less filopodia and the EMD granules were arranged scarcely.

When the samples embedded into PDL cell culture for 3 h after EMD treatment were examined, EMD granules were seen scarcely arranged to some degree and regular on the tooth surface of the non-smoking patient. The cell bodies of PDL cells were shorter and oval in comparison with the samples kept in the culture medium for 1 h. EMD granules were seen abundantly on the filopodia of these cells. In the samples from the smoking patient, collagen fibers were generally arranged individually and parallel to each other. However, the collagen fibers formed thick bundles running parallel to each other in some regions of the tooth surface and oval shaped PDL cells with less and shorter filopodia were located on these bundles.

The PDL cells present on the tooth surface obtained from the non-smoking patient and embedded into fibroblast culture for 5 h after treatment with EMD, were long and spindle shaped and the fiber networks were preserved. As for samples belonging to the smoking patient, the findings resembled those in the 3 h sample.

When the samples embedded into PDL cells culture for 3 days after treatment with EMD were examined, the tooth surface from the non-smoking patient showed completely regular trabecular appearance. The collagen fibers were not observed. However, when the sample of the smoking patient was examined, the trabecular appearance of the teeth surface was incomplete. Furthermore, collagen fibers and cell residues were seen at some points on the teeth surface.

Similarly to the reports of other authors (6, 15, 25, 27, 41), in our study, the morphology of PDL fibroblasts and the fibril structure in the cell culture obtained from the non-smoking patient were different from those observed in the smoking patient’s sample. Concerning the attachment of cells on a surface, Peacock et al. (29) demonstrated a positive effect of nicotine on gingival fibroblast attachment to a plastic surface and this enhanced effect was concentration-dependent. However, Raulin et al. (30) stated that exposure of such cells to nicotine resulted in structural alterations preventing them from becoming firmly attached to the plastic or root surfaces. Again discrepancies among various authors could be explained by the differences in the concentration of drug used and/or differences of fibroblast strains (26). In the present study, when the sample from the smoking patient was examined, PDL cells were slightly adhered to the tooth surfaces as reported by Raulin et al. (30). Cells binding to both dentinal and cementum surfaces and root planing alone permit diseased roots to interact with fibroblasts as well as with normal roots (1). Fardal et al. (11) revealed that incubation of periodontally-diseased teeth with human gingival fibroblasts resulted in equal attachment in the cases when the teeth were instrumented, uninstrumented or non-diseased. These authors also noted attachment of fibroblasts to calculus. Apparently it is not uncommon for fibroblasts to attach to periodontally-diseased root surfaces. Gamal et al. (12) observed significantly higher cell adherence in the healthy control group than in the periodontally-diseased treated control, suggesting that cultured human PDL fibroblasts prefer not to adhere to periodontally diseased treated root surfaces. Despite a number of reports on the role of EMD in periodontal regeneration (14, 23, 24), especially on PDL cells (8, 14, 20, 28, 38) and wound healing (31), different phenotypes of PDL fibroblasts can be isolated and may respond differently to regeneration attempts through biomimetics, which may explain the seemingly contradictory results in published studies regarding the in vitro effects of EMD (7). Chong et al. (9) showed that EMD alone has a limited effect on PDL fibroblast proliferation. Conflicting reports regarding the effect of EMD on PDL cells draw the attention of researchers in this field. In addition, there is also evidence in support of the concept that smoking influences the periodontal healing responses following periodontal therapy (1).

Research indicates that by using EMD, regeneration of lost periodontal tissues with characteristics similar to those of their natural architecture and function can be achieved. That is why EMD (a commercially available enamel matrix protein derivative) is widely used in modern clinical treatment. However, the biological characteristics and properties of EMD are still poorly known (16). In our study, the fibroblasts obtained from periodontally-diseased teeth reflected the clinical status in vitro and pointed out to the importance of smoking status to be considered as a patient selection criterion including EMD treatment of periodontal disease for regenerative periodontal surgery practices.

Conclusions
In the present study, the effect of EMD on the collagen fibers and attachment of PDL cells to periodontally involved root surfaces obtained from a smoking and a non-smoking patient were investigated in vitro. We observed that PDL fibroblasts were present on the tooth surface obtained from the non-smoking patient and embedded into fibroblast culture for 1 h after EMD treatment; they had oval and prominent cell trunks and long, plentiful filopodia indicating that the fibroblasts were active cells. In contrast to what was seen in the non-smoking healthy control samples, PDL cells from the smoking patient that were incubated for 1 h after EMD treatment had less filopodia on their surface indicating PDL cell distortion.
In the non-smoking group kept in culture medium for 3 h, PDL cells were firmly attached to the root surface and filopodia were extended between EMD granules. An amount of collagen fibers were seen around these cells. EMD granules were less than those in the 1 h sample. The collagen fibers in the smoking group were spread parallel to the root surface. Conversely, the collagen fibers in the non-smoking group formed a network attributed to the hazardous effect of smoking on collagen fibers. In the smoking patient the collagen fibers were parallel to the tooth surface and were located in an order referred to the decrement of extracellular matrix synthesis between collagen fibers.

REFERENCES