Effect of human platelet-derived growth factor-BB on attachment of periodontal ligament cells on root surfaces

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ABSTRACT

الأهداف: تقييم آثار عامل النمو للصفائح البشرية المتحولة–(PDGF–BB) (BB) على خلايا الرباط اللثية البشرية (HPLCs) على أسطح الجذور وزوال المعدن مع العوامل المختلفة.

الطريقة: أجريت هذه الدراسة خلال التفرة مابين عام 2005م وحتى عام 2006م، بجامعة إنيج – أزمير – تركيا. تم إخضاع 80 شريحة جذرية إلى أحد المعالجات بعد تسطيح الجذر: فقط إجراء تسطيح الجذر، الصفائح البشرية المتحولة PDGF–BB، نزع المعدنيات بحمض الكتريك، نزع المعدنيات بحمض الكتريك + PDGF–BB، نزع المعدنيات باستعمال عقار تيتراسيلين T–HCl، نزع المعدنيات باستعمال عقار تيتراسيلين T–HCl+ PDFG–BB، نزع المعدنيات باستعمال EDTA، نزع المعدنيات باستعمال EDTA+ PDGF-BB. تم بذر HPLCs على أسطح الجذور. تم حساب عدد الخلايا بواسطة طريقة مقياس اللون بعد فترة ساعتين. تم أخذ ثلاث شرائح من كل مجموعة لإجراء الفحص بالمجهر الإلكتروني. تم فحص عدد الخلايا المرفقة بواسطة تحليل التغير (p=0.05).

النتائج: لم يكن هنالك اختلافات ملحوظة بين المجموعات فيما يتعلق بالعدد الفعلي للخلايا المرفقة (p=0.843) والذي كان عالياً لدى المجموعة الرابعة ومنخفضاً لدى المجموعة السادسة.

خاتمة: تعتبر طريقة تسطيح الجذور أكثر طريقة معالجة أهمية لجعل أسطح الجذور المريضة متساوية حيوياً مع التصاق HPLCs. قد يؤيد تطبيق طريقة PDGF-BB لنزع المعدن عن أسطح الجذور مع الحمض الكريتيكي من أجل تعزيز تجديد اللثة.

Objectives: To evaluate the effects of platelet-derived growth factor-BB (PDGF-BB) on the attachment of human periodontal ligament cells (HPLCs) on the root surfaces demineralized with different agents.

Methods: We performed this study at Ege University, Izmir, Turkey between 2005 and 2006. Eighty root slices were subjected to one of following treatments after root planing: 1) only root planing, 2) Platelet derived growth factor-BB (PDGF-BB), 3) citric acid demineralization, 4) citric acid demineralization + PDGF-BB, 5) tetracycline hydrochloric acid (T-HCl) demineralization, 6) T-HCl demineralization + PDGF-BB, 7) ethylenediamine tetra-acetic acid (EDTA) demineralization, and 8) EDTA demineralization + PDGF-BB. Human periodontal ligament cells were seeded on the root surfaces. Following the 2-hour incubation period, the number of cells was calculated by the colorimetric assay. Three slices from each group were processed for scanning electron microscopy. The number of attached cells was tested by analysis of variance (p=0.05).

Results: There were no significant differences among the groups with regard to the mean number of attached cells (p=0.843), which was highest in the fourth group, and lowest in the sixth group.

Conclusion: Root planing is the most important treatment to make the diseased root surfaces biocompatible to HPLCs adherence. Application of PDGF-BB to root surfaces demineralized with citric acid may be advocated to enhance periodontal regeneration.

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Periodontal regeneration is one of the major goals in periodontal therapy. The biocompatibility of the root surface is important for achieving periodontal healing. The repopulation of the pathologically exposed and altered root surface by cells from the periodontal ligament, is a prerequisite for new attachment formation.[1-3] In the past decade, several growth factors (GFs) have received attention as they can regulate migration, attachment, proliferation and differentiation of periodontal ligament cells, and enhance periodontal regeneration in *in-vivo* experiments.[4,5] Platelet derived growth factor-BB (PDGF-BB), is a well-characterized GF that was shown to have important roles in wound healing.[4] Platelet derived growth factor (PDGF) family consists of PDGF -A, -B, -C and -D. It has been found to exist in different isoforms; PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD homodimer forms, and PDGF-AB heterodimer form.[6,7] It was shown that PDGF has the capacity to negate, and reverse the inhibitory effects of lipopolysaccharides on human gingival fibroblast proliferation.[8] Platelet derived growth factor is a potent stimulator, and act as a strong mitogenic agent[9] for human periodontal ligament cells (HPLCs), and promotes collagen and total protein synthesis of these cells.[10] Gamal and Mailhot[11] reported that PDGF caused an increase in the number of adherent HPLCs on periodontally involved root surfaces. There are different methods with regard to the delivery of the GFs to periodontium, some carrier materials such as methyl cellulose gel,[6] dexamethasone, and type I collagen[12] have been used for this purpose. These application modes of GFs may cause some potential problems. Carriers may be cleared early, and GFs can be available in the wound shorter than needed for repair, or carriers may remain in place for a considerable period, and hinder migration of HPLCs. In addition, the carriers may lead to inflammatory and immunological responses, and interfere with cell-surface interaction, which is essential for early cell repopulation and fiber development.[6] Root conditioning is a therapeutic method used for achieving periodontal regeneration since 1980. Demineralization of the root surfaces with agents such as citric acid,[13] tetracyclin hydrochloric acid (T-HCl),[14] and ethylenediamine tetra-acetic acid (EDTA)[15] removes surface smear layer, increases the total surface area, and exposes collagen matrix.[16] The use of these agents improves the surface favorably for cell migration. It was suggested that the ability of PDGF to bind to matrix components[17] may permit the applied GF to attach to the demineralized root surface from which the sustained release of the growth factor can be achieved.[18] Zaman et al[19] reported that recombinant human platelet-derived growth factor-BB (rhPDGF-BB) and rhBMP-2 application to EDTA demineralized dentin surfaces promoted the early HPLC responses by increasing cell proliferation and differentiation. Gamal et al[20] studied the effect of PDGF-BB on the adherence of HPLCs to T-HCl conditioned and non-conditioned periodontally involved root surfaces by scanning electron microscopy (SEM), and reported that T-HCl conditioned root surfaces showed a statistically significant increase of cellular adherence in the PDGF group on T-HCl conditioned surface, when compared to the only T-HCl conditioned group, but similar to non-T-HCl conditioned group (PDGF-BB group). However, the effects of using various demineralization agents before application of PDGF-BB on the attachment of HPLCs have not been studied previously. Therefore, the purpose of this study was to evaluate the effects of PDGF-BB application on the attachment of HPLCs on the root surfaces etched with various agents by colorimetric assay and SEM.

Methods. This study was performed in 2005 and 2006 in Ege University, Izmir, Turkey and an ethical approval was obtained from the Research Ethics Committee of the University. All patients were informed for the nature and extent of the study, and their consent forms were obtained according to the Helsinki Declaration. Human periodontal ligament cells were obtained from healthy premolar teeth extracted for orthodontic reasons, from systemically and periodontally healthy patients, according to the method of Somerman et al[21] with slight modification. Briefly, following the extraction, the teeth were immediately placed in transfer medium containing Dulbecco's modified Eagle medium/F-12 (DMEM/F-12) (Biochrom, Germany) with 0.1% sodium bicarbonate ($NaHCO_3$), 400 U/ml penicillin, 400 µg/ml streptomycin, and 0.5% amphotericin B. Under sterile conditions, the periodontal ligament tissue was collected from the mid-third portion of the roots of the teeth with a scalpel, transferred into 35-mm culture dishes, and then cultured with DMEM/F-12 supplemented with 20% fetal bovine serum (FBS) (Biochrom, Germany) 0.1% $NaHCO_3$, 400 U/ml penicillin, 400 µg/ml streptomycin and 0.5% amphotericin B, and incubated in humidified atmosphere of 95% air, and 5% carbon dioxide (CO_2) at 37 °C. Within 30 days, the outgrowth became confluent. These confluent cells were detached and transferred to 25 cm^2 culture flasks. The fifth passages were used in this study. Forty lower anterior teeth were obtained from systematically healthy patients (30-65 years of age) who were nonsmokers. The teeth were extracted as part of the dental treatment and selected using the following criteria; had lost 2/3 of their periodontal attachment, no history of scaling or root planing in the previous 6 months, no signs of caries, fractures or anatomic abnormalities on the root surfaces.

Before extracting the teeth, periodontal attachment loss was measured from the cemento-enamel junction to the base of the periodontal pocket by the Williams probe. Scaling was performed ultrasonically after extraction of the teeth and root planing was carried out with Gracey 5/6 periodontal curette (Hu Friedy, Chicago, IL, USA) for 15-20 strokes for every root surface of the extracted teeth. No effort was applied in order to expose dentin. Two horizontal cuts were made; one from a plane of the most coronal part of the attachment level of the teeth according to the measurements taken before extraction, and the other from the plane 1 mm apical to cemento-enamel junction. A 2 standard round root slices with a diameter of 5 millimeters, and thickness of 1 mm were obtained from each tooth by using diamond fissure bar and a special cutting mill with an inner diameter of 5 mm, which were developed by the present workgroup. A groove was made on the pulpal surface of each block to identify the pulpal surface. The root slices were divided into 8 groups, and the roots in each group divided into 3 subgroups, and used for experiments on 3 different days. The roots were subjected to one of the following treatments: group 1: only root planing (RP), group 2: RP + PDGF-BB application, group 3: RP + citric acid demineralization, group 4: RP + citric acid demineralization + PDGF-BB application, group 5: RP + T-HCl demineralization, group 6: RP + T-HCl demineralization + PDGF-BB application, group 7: RP + EDTA demineralization, group 8: RP + EDTA demineralization + PDGF-BB application.

The demineralization of the root surfaces in groups 3 and 4 was carried out by citric acid solution[22] at a pH of 1, in groups 5 and 6 by 125 mg/ml T-HCl,[23] and in groups 7 and 8 by 24% EDTA[15] solution at a pH of 7.2. All solutions were freshly prepared, and applied to the root surfaces for 4 minutes by burnishing technique with cotton pellets, and the pellets were changed every 30 seconds. All root slices were washed with phosphate buffered saline (PBS) for 30 seconds, and stored at 1000 U/ml penicillin and 1000 µg/ml streptomycin solution overnight, and then washed with DMEM with 100 U/ml penicillin, and 100 µg/ml streptomycin.

Recombinant human platelet-derived growth factor-BB (Sigma-Aldrich,USA) was reconstituted freshly according to the manufacturer's instructions; 0.5 µg/ml solution of rhPDGF-BB was prepared with minimum essential medium (MEM) containing 0.1% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin.[19] The root slices in groups 2, 4, 6, 8 were stored in rhPDGF-BB solution for 10 minutes while the rest of the root slices were placed in PBS solution for also 10 minutes. After 10 minutes, the root slices were picked up and dried with air in sterile condition. The air-dried root slices were embedded into sterile paraffin, exposing only the periodontally involved surfaces in 96-well culture plate, one root slice for each well. A hundred microliter of PBS containing 106 HPLCs /ml were added into the wells, and onto the root surfaces in each group. Following the 2 hour incubation, the unattached cells removed gently by washing with 100 µl PBS. The XTT assay, reduction of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl) -5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide to a yellow formazan product was used for the detection of the numbers of the attached cells. One hundred micro liter (µl) of PBS containing 0.4 mg/ml (XTT) (Appli Chem, Germany), and 0.04 mg/ml co-enzyme Q10 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 the medium obtained from each well was determined at 450 nm in a ultraviolet visible spectrophotometer multiplate reader (Versa Max, Molecular Device, USA). All measurements were repeated 3 times. Following XTT assay, 3 root slices from each group were processed for SEM viewing, and photographed at different magnifications. All SEM procedures were performed at 4°C. 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 and 2) was added to each well, and incubated for 30 minutes. 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 minutes, respectively. Following this procedure, the root slices were dehydrated in 35%, 70%, 85%, 95%, and 100% ethanol solutions for 5 minutes at room temperature. They were immersed in hexamethyldisilazane solution for 5 minutes, and air-dried for 30 minutes. The root slices were mounted on SEM carriers, transferred into a glass desiccator containing phosphorus pentoxide for 48 hours, and then sputter coated by gold to a thickness of 200 Angstrom. Finally, the specimens were examined using a SEM (JEOL-5200, Tokyo, Japan), and micrographs were obtained at different magnifications.

Statistical analysis. The number of attached cells per 1 mm^2 of root surfaces in different groups was tested by analysis of variance according to 4x2 factorial randomized designs (surface modifications x PDGF-BB application) (p=0.05).

Results. The mean number of the attached HPLCs was highest in group 4 in which citric acid demineralization, and PDGF-BB applied following root planing (32030 ± 30288 cells/mm^2), while it was lowest in group 6 (RP + T-HCl + PDGF-BB) (7423 ± 3765 cells/mm^2) (**Table 1**). The other groups demonstrated a

cellular attachment between 10225-23678 cells/mm^2. However, there was no statistical difference in the number of the attached cells among the 8 groups (p=0.843). The T-HCl application (group 5) demonstrated the highest number of attached cells (23678±28324 cells/mm^2), when PDGF-BB application was not considered. On the other hand, citric acid application (group 3) gave the lowest number of attached cells in this respect (11401 ±11977 cells/mm^2). However, this difference was not statistically significant (p=0.843) (**Table 1**). After PDGF-BB application, there was a dramatic decrease in the T-HCl groups (groups 5 & 6) (23678-7423 cells/mm), while there was a considerable increase in citric acid groups (groups 3 & 4) (11401-32030 cells/mm^2). However, there was no significant difference between the groups (p=0.407). In addition, regardless of the type of demineralization, PDGF-BB application did not cause a statistically significant increase in the number of the attached cells (p=0.975) (**Table 1**).

In the SEM evaluation, all specimens of 8 different groups, which HPLCs were seeded showed cell attachment (**Figure 1**). Most of the adherent cells were flat (spindle or stellate-shaped) with prominent microvilli, forming a multilayer on the specimen surfaces. There were also numerous rounded cells on top of the flat cells. Due to the presence of these layers of HPLCs, the dentin surface was not visible in most instances. More areas devoid of cells were observed in group 6, which was treated with PDGF-BB in addition to root planning and T-HCl treatment (**Figure 1f**). There was no morphological difference between cells in the different study groups.

Discussion. The potential therapeutic use of PDGF-BB, which is a potent chemoattractant for fibroblasts, has been considerably focused to achieve periodontal regeneration. Previous studies have demonstrated the potential for PDGF-BB to increase periodontal regeneration,[18,24] but there is a problem regarding the delivery method of the GFs to the periodontium. Platelet derived growth factor-BB has been applied to the demineralized root surface, and it may have some advantages over the use of foreign carrier[18] such as methyl cellulose gel,[5] dexamethasone, and type I collagen.[12] In this *in vitro* study, the effects of different etching modalities and PDGF-BB application on the attachment of periodontal ligament cell, on periodontally exposed root surfaces of lower anterior teeth were evaluated. Whithlin and Hancock[25] found that fibroblasts did not attach to the root surfaces of periodontally diseased teeth, unless they were root planned. Garrett et al[26] showed that the application of citric acid had no beneficial effects on root surfaces, which were diseased and untreated. Hou et al[27] also reported that cementum might facilitate the migration and attachment of HPLCs, and periodontal regeneration. For these reasons, untreated root surfaces were not included in the present study, and complete removal of cementum to expose dentin was not performed as carried out in some previous studies.[20,28] In the present study, ultrasonic scaler was used and root planing was accomplished with Gracey 5/6 for 15 strokes, as usually carried out in the clinical treatment of periodontal disease. Tetrazolium salts are used for colorimetric procedures, they are reduced to light yellow or orange tetrazolium formazan products, by mitochondrial dehydrogenase of metabolically active cells. Xml tunneling technology (XTT) procedure has the advantages of allowing rapid quantitative measurement of only metabolically active cells in the whole root surface, not a limited area like in electron microscopy studies.[29] It is not necessary to remove cells from the root surface for cell counting, thus, it is not associated with cell loss. The present study is the first study where XTT method has been used for studying attachment of HPLCs on root surfaces. According to the results, there was no significant difference between the control (only root planing) and the experimental groups (root planed and demineralized with citric acid, T-HCl and EDTA). A study by Boyko et al[30] examined the attachment of porcine periodontal ligament fibroblasts to healthy porcine root surfaces that were demineralized with EDTA, T-HCl and citric acid. The cell attachment to the various demineralized and non-demineralized root surfaces was determined after 2 hours by removing cells with trypsin, and cells were counted with a hemocytometer. The results showed that more cells were attached to demineralized root surfaces with no difference between the various demineralizing agents in enhancing cell attachment. The different results of the current study as compared to the study of Boyko et al,[30] may rise from the different techniques used, to count the attached cells. In addition, Boyko et

Table 1 - The mean±SD of attached cells in different groups.

Groups	PDGF-BB	Mean±SD
1. Root planing	-	11821 ± 9728
2. Root planing	+	10225 ± 5894
3. Root planing + citric acid	-	11401 ± 11977
4. Root planing + citric acid	+	32030 ± 30288
5. Root planing + T-HCl	-	23678 ± 28324
6. Root planing + T-HCl	+	423 ± 3765
7. Root planing + EDTA	-	11679 ± 5284
8. Root planing + EDTA	+	11984 ± 10165

PDGF-BB - platelet-derived growth factor-BB, T-HC1 - tetracyclin hydrochloric acid, EDTA - ethylenediamine tetra-acetic acid

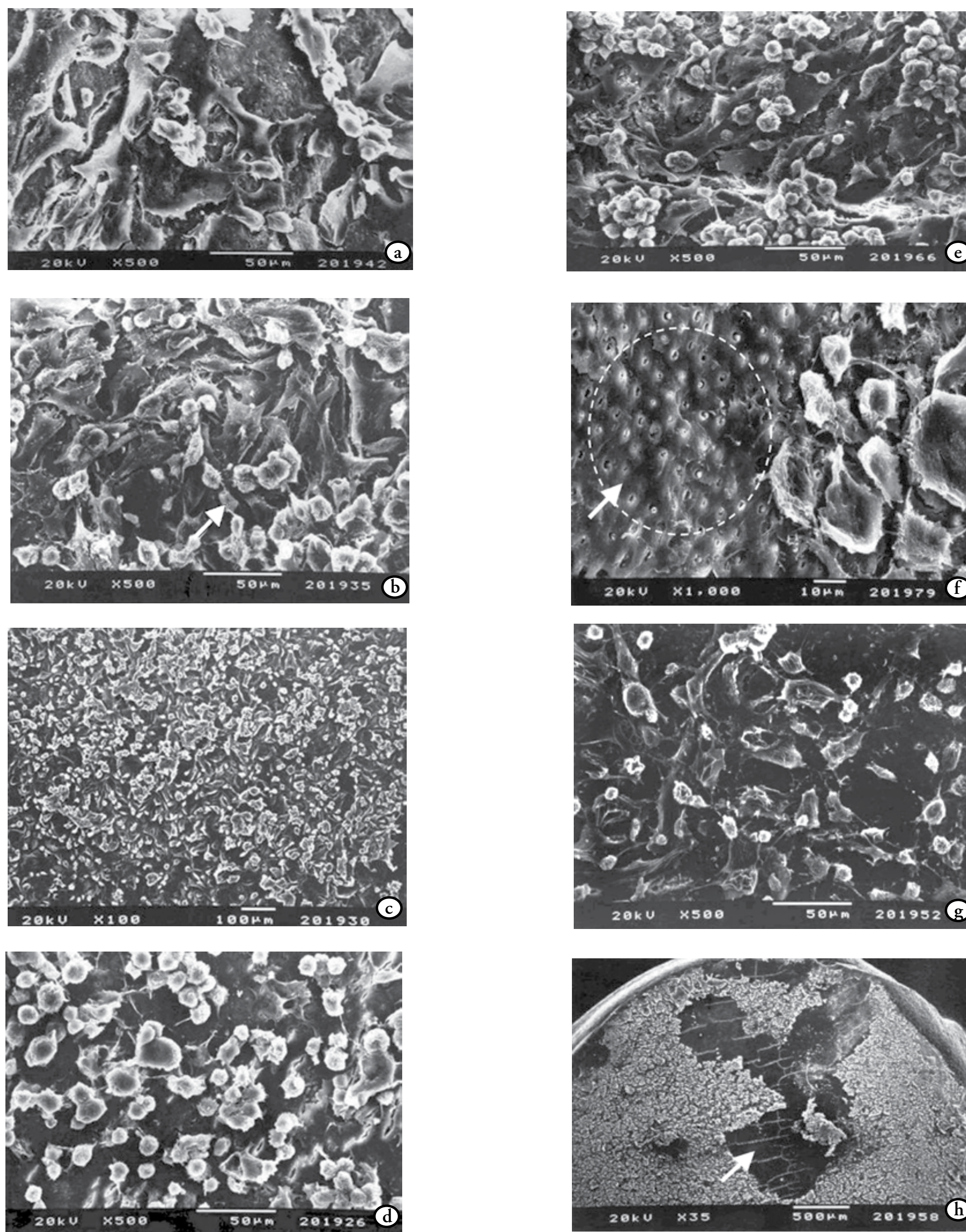

Figure 1 - Scanning electron microscopy showing **a**) a specimen received only root planing (x500). There are numerous flat cells and few rounded cells. The surface is covered by a layer of fibroblasts. **b**) Electron micrograph of a specimen treated with root planing and Platelet derived growth factor-BB (PDGF-BB) (x500). Please note multilayer of spindle and stellate human periodontal ligament cells (HPLCs). There are also numerous rounded cells on top of the flat cells. **c**) A low magnification electron micrograph of a specimen treated with root planing and citric acid (x100). There are a dense mass of flat and rounded cells on the surface. **d**) Electron micrograph of a specimen treated with PDGF-BB in addition to root planing and citric acid treatment (x500). There are numerous rounded cells over the multilayered flat cells. **e**) Electron micrograph of a specimen treated with root planing and tetracyclin hydrochloric acid (T-HCl) (x500). A multilayer of spindle and stellate cells are masking the root surface. There are also a dense mass of rounded cells. **f**) Electron micrograph of a specimen treated with PDGF-BB in addition to root planning and T-HCl treatment (x1000). More than half of the surface is devoid of cells. However, the present cells are strongly attached to the surface with numerous microvilli. **g**) Electron micrograph of a specimen treated with root planing and ethylenediamine tetra-acetic acid (EDTA) (x500). There are numerous flat spindle and stellate HPLCs on the surface with minor unoccupied areas. **h**) A low magnification electron micrograph of a specimen treated with root planing + EDTA + PDGF-BB (x35). There is an area in the center of the surface devoid of cells.

al[30] used healthy root surfaces. Periodontally involved root surfaces were preferred in the present study, since they have many differences from the healthy teeth such as mineral content. Similar to our results, Cogen et al[31] demonstrated that root planing, regardless of acid treatment, promoted the growth of cultured gingival fibroblasts. The results of Cogen et al[31] and our study have shown, that root planing is an important procedure for the cell attachment on periodontally exposed root surfaces. Gamal et al[20] also reported that the T-HCl demineralization of human dentin, insignificantly increased human periodontal ligament fibroblast adherence, compared to scaled and root planed dentin alone. Demineralization of the planed root surface was performed to expose collagen matrix, as PDGF-BB has the ability to bind the collagen matrix. In the present study, there was no significant difference among all groups. According to the mean numbers of the attached cells in different groups, the fourth group (root planing + citric acid + PDGF-BB) demonstrated highest number of cells attached to root surfaces. The cells were less likely to attach to the root surfaces in group 6 (root planing + T-HCl +PDGF-BB). This finding was in contrast to the result of Gamal et al,[20] who reported that cells in the T-HCl conditioned and PDGF-BB applied group were significantly greater than in the non-T-HCl and T-HCl-conditioned control groups. There are 2 main differences: first, Gamal et al[20] removed all the cement from the root surface by using a diamond bur, however in the present study, only root planing was performed in order to mimic the clinical conditions. Second, Gamal et al[20] used SEM for counting the attached cells, where XTT was used in our study. Sterrett et al[32] have found that higher concentrations of T-HCl demineralize dentin more effectively, which tend to leave more tetracycline on the root surface, and released from the root surface. Gamal et al[20] also reported that T-HCl might retain on the demineralized root surface after irrigation of root surfaces, and that might cause changes in the physical or chemical properties of growth factors. In the present study, capsule form of T-HCl was used as a demineralizing agent. The capsules provided by the pharmacy for oral use introduce a significant amount of filler substances into the solution, and the effects of these materials on the root surface need to be determined.[33] The retention and release of T-HCl from the root surface may affect PDGF-BB function negatively, and the fillers in the capsule T-HCl may have reduced the attachment of HPLCs in the sixth group, in which T-HCl demineralization and PDGF-BB were applied. The SEM results revealed that the HPLCs attached to all root surfaces in each group, which is in agreement with Cogen et al.[31] The cells did not present any morphological difference among the groups after 2 hours incubation time, which is a short period for morphological changes. There were some areas, whether demineralized or not, where the HPLCs did not prefer to attach. This may strongly result from the pathological changes, such as mineral changes of the periodontally exposed root surfaces. The multilayer alignment of cells with different morphologies, made the cell count impossible. Since there were also cell-free areas, it was considered that the cell counting in a chosen area during SEM observation would not be much reliable to represent the number of all cells attached on the whole root surface. In a recent study, Belal et al[34] showed a positive effect of PDGF-BB on adhesion and growth of cultured fibroblasts to periodontally diseased root surfaces, which were only root planed, not demineralized. The use of PDGF-BB in combination with erbium-doped: yttrium, aluminum, and garnet (Er:YAG) laser application, was studied and found to offer a promising periodontal therapy, although the combined application seemed to be slightly more effective than only Er:YAG laser application.[35] The current methodology and experimental design in the present study could not discriminate the relative superiority of root conditioning agents, and prove the effective potential of PDGF-BB on promoting cell attachment, other more sensitive strategy, such as biochemical binding assay of PDGF-BB on treated root surfaces, may be considered in future studies.

In the limitation of the present study, the results had confirmed that root planing of the periodontally affected root surfaces is important for the attachment of periodontal ligament cells on root surfaces.[25] Citric acid demineralization of root planed surfaces before the application of PDGF-BB, may positively affect the attachment of HPLCs on the root surface. Using T-HCl demineralized root surfaces, as an application site for PDGF-BB, needs to be studied for possible negative effects on HPLCs attachment.

References

1. Karring T, Nyman S, Lindhe J. Healing following implantation of periodontitis affected roots into bone tissue. *J Clin Periodontol* 1980; 7: 96-105.
2. Nyman S, Karring T., Lindhe J, Planten S. Healing following implantation of periodontitis affected roots into gingival connective tissue. *J Clin Periodontol* 1980; 7: 394-401.
3. Selvig KA. Ultra structural changes in cementum and adjacent connective tissue in periodontal disease. *Acta Odontol Scand* 1966; 24: 459-500.
4. Lynch SE, Ruiz DE, Castella G, Williams RC. The effect of short-term application of a combination of platelet derived growth factor and insulin like growth factor on periodontal wound healing. *J Periodontol* 1991; 62: 458-467.
5. Caffesse RG, Quinones CR. Polypeptide growth factors and attachment proteins in periodontal wound healing and regeneration. *Periodontology* 2000; 1: 69-79.

6. Li X, Ponten A, Aase K, Karlsson L, Abramsson A, Uutela M, et al. PDGF-C is a new protease-activated ligand for the PDGF alpha-receptor. ***Nat Cell Biol*** 2000; 2: 302-309.
7. LaRochelle WJ, Jeffers M, McDonald WF, Chillakuru RA, Giese NA, Lokker NA, et al. PDGF-D, a new protease-activated growth factor. ***Nat Cell Biol*** 2001; 3: 517-521.
8. Bartold PM, Narayanan AS, Page RC. Platelet-derived growth factor reduces the inhibitory effects of lipopolysaccharide on gingival fibroblast proliferation. ***J Periodontal Res*** 1992; 27: 499-505.
9. Marcopoulou CE, Vavouraki HN, Dereka XE, Vrotsos IA. Proliferative effect of growth factors TGF-beta1, PDGF-BB and rhBMP-2 on human gingival fibroblasts and periodontal ligament cells. ***J Int Acad Periodontol*** 2003; 5: 63-70.
10. Blom S, Holmstrup P, Dabelsteen E. A comparison of the effect of epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor on rat periodontal ligament fibroblast like cells'DNA synthesis and morphology. ***J Periodontol*** 1994; 65: 373-378.
11. Gamal AY, Mailhot JM. The effect of local delivery of PDGF-BB on attachment of human periodontal ligament fibroblasts to periodontitis-affected root surfaces-in vitro. ***J Clin Periodontol*** 2000; 27: 347-353.
12. Rutherford RB, Ryan ME, Kennedy JE. Platelet derived factor dexamethasone combined with collagen matrix induce regenertion of the periodontium in monkeys. ***J Clin Periodontol*** 1993; 20: 537-544.
13. Register AA, Burdick FA. Accelerated reattachment with cementtogenesis to dentin, demineralised in situ. II. Defect repair. ***J Periodontol*** 1976; 47: 497-505.
14. Bjorvatn K, Skaug N, Selvig KA. Tetracycline-impregnated enamel and dentin: duration of antimicrobial capacity. ***Scand J Dent Res*** 1985; 93: 192-197.
15. Pitaru S, Gray A, Aubin JE, Melcher AH. The influence of the morphological and chemical nature of dental surfaces on the migration, attachment, and orientation of human gingival fibroblasts in vitro. ***J Periodontal Res*** 1984;19: 408-418.
16. Rompen EH, Goffinet GH, Nusgens B. Human periodontal ligament fibroblast behavior on chemically conditioned dentine: an in vitro study. ***J Periodontol*** 1999; 70: 1144-1152.
17. Kelly JL, Sanchez A, Brown GS, Chesterman CN, Sleigh MJ. Accumulation of PDGF-B and cell binding forms of PDGF-A in the extracellular matrix. J Cell Biol 1993; 121: 1153-1163l.
18. Cho M, Lin WL, Genco RJ. Platelet derived growth factor modulated guided tissue regenerative therapy. ***J Periodontol*** 1995; 66: 522-530.
19. Zaman KU, Sugaya T, Kato H. Effect of recombinant human platelet-derived growth factor-BB and bone morphogenetic protein-2 application to demineralized dentin on early periodontal ligament cell response. J Periodont Res 1999; 34: 244-250.
20. Gamal AY, Mailhot JM, Garnick JJ, Newhouse R, Sharawy MM. Human periodontal ligament fibroblasts response to PDGF-BB and IGF-1 application on tetracycline HCl conditioned root surfaces. ***J Clin Periodontol*** 1998; 25: 404-412.
21. Somerman MJ, Archer SY, Imm GR, Foster RA. A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro. ***J Dent Res*** 1988; 67: 66-70.
22. Sterrett JD, Bankey T, Murphy HJ. Dentin demineralization, the effects of citric acid concentration and application time. ***J Clin Periodontol*** 1993; 20: 366-370.
23. Trombelli L, Scabbia N, Calura GC. Nondiseased cementum and dentin root surface following tetracycline hydrochloride conditioning: SEM study of the effects of solution concentration and application time. ***Int J Periodont Rest Dent*** 1994; 14: 461-470.
24. Nevins M, Camelo M, Nevins ML, Schenk RK, Lynch SE. Periodontal regeneration in humans using recombinant human platelet derived growth factor BB (rh PDGF-BB) and allogenic bone. J Periodontol 2003; 74: 1282-1292. 25.Wirthlin MR, Hancock EB. Biologic preparation of diseased root surfaces. ***J Periodontol*** 1980; 51: 291-297.
25. Wirthlin MR, Hancock EB. Biologic preparation of diseased root surfaces. ***J Periodontol*** 1980; 51: 291-297.
26. Garrett JS, Crigger M, Egelberg J. Effects of citric acid on diseased root surfaces. ***J Periodont Res*** 1978; 13: 155-163.
27. Hou LT, Liu CM, Lei JY, Wong MY, Chen JK. Biological effects of cementum and bone extracts on human periodontal fibroblasts. ***J Periodontol*** 2000; 71: 1100-1109.
28. Zaman KU, Sugaya T, Hongo O, Kato H. A study of attached and oriented human periodontal ligament cells to periodontally diseased cementum and dentin after demineralizing with neutral and low pH etching solution. ***J Periodont Res*** 2000; 71: 1094-1099.
29. Sen BH, Chugal NM, Liu H, Fleischmann J. A new method for studying the adhesion of Candida albicans to dentin in the presence or absence of smear layer. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2003; 96: 201-206.
30. Boyko GA, Brunette DM, Melcher AH. Cell attachment to demineralized root surfaces in vitro. ***J Periodontal Res*** 1980; 15: 297-303.
31. Cogen RB, Al-Joburi W, Gantt DG, Denys FR. Effects of various root surface treatments on the attachment and growth of human gingival fibroblasts: histologic and scaning electron microscopic evaluation. ***J Clin Periodontol*** 1984; 11: 531-539.
32. Sterrett JD, Simmons J, Whitford G, Russell CM. Tetracycline demineralization of dentin the effects of concentration and application time. ***J Clin Periodontol*** 1997; 24: 457-463.
33. Isik AG, Tarim B, Hafez AA, Yalçin FS, Onan U, Cox CF. A comparative scanning electron microscopic study on the characteristics of demineralized dentin root surface using different tetracycline HCL concentrations and application times. ***J Periodontol*** 2000; 71: 219-225.
34. Belal MH, Watanabe H, Ichinose S, Ishikawa I. A time-dependent effect of PDGF-BB on adhesion and growth of cultured fibroblasts to root surfaces. ***Oral Dis*** 2006; 12: 543-552.
35. Belal MH, Watanabe H, Ichinose S, Ishikawa I. Effect of Er: YAG laser combined with rhPDGF-BB on attachment of cultured fibroblasts to periodontally involved root surfaces. ***J Periodontol*** 2007; 78: 1329-1341.