Journal of Oral Science, Vol. 42, No. 1, 33-38, 2000

The effects of attachment factors on initial attachment of human periodontal ligament fibroblasts on different root surfaces: A light and scanning electron microscopic study

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(Received 9 August 1999 and accepted 18 February 2000)

Abstract: This study was aimed to test the effect of fibronectin (FN), vitronectin (VN) and a fibronectin analog (fibronectin-like engineered protein) on the attachment of periodontal ligament cells to mechanically-treated and mechanically non-treated periodontally involved and non-diseased root surfaces in vitro. Periodontal ligament fibroblasts were incubated with a total of 44 periodontally diseased and non-diseased root slices which had been treated in the following manner: 1) FN applied to mechanically-treated and non-treated root slices, 2) VN applied to mechanically-treated and non-treated root slices, 3) FN-like engineered protein applied to mechanically-treated and non-treated root slices, and 4) mechanically-treated and non-treated root slices. After the 1 hour incubation period in a humidified atmosphere of 95% air and 5% CO_2 at 37°C, the adherence of the fibroblasts was determined using light microscopy with an ocular grid system and orientation was evaluated by scanning electron microscopy. The results indicated that the number of attached cells to non-diseased cementum sites was significantly greater than the number of attached cells to diseased cementum sites for all of the groups ($p < 0.05$). Likewise, the number of attached cells to mechanically-treated diseased cementum sites was significantly greater than the number of attached cells to mechanically-non-treated diseased cementum sites ($p < 0.05$). Our findings suggest that these attachment factors cannot promote cell attachment to different cementum sites. (J. Oral Sci. 42, 33-38, 2000)

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Key words: human periodontal ligament fibroblast; initial attachment; attachment factors; root surface.

Introduction

Periodontitis causes pathological alterations on the cementum surface that is exposed to the periodontal pocket. The cementum surface that is contaminated by bacterial products loses its collagen fiber attachments. Finally, the mineral content and composition of the cemental surface is changed (1-3). Therapeutic modalities should aim not only at eliminating the gingival inflammatory process and preventing the progression of periodontal disease, but also at reestablishing and regenerating the periodontal tissues previously lost to the disease (1,2). In order to attain a biologically acceptable cemental surface for new attachment, various chemical and biological agents such as acids, attachment factors and/or growth factors were used in additional to mechanical removal of cytotoxic factors (3-9). Attachment factors such as fibronectin (FN), which acts in wound healing to facilitate the attachment of cells to other cells and to the extracellular matrix, have been studied as potential enhancers of periodontal regeneration (2,10). Fibronectin is a high-molecular-weight glycoprotein (440,000 Da/molecule) found on the surface of cells, in plasma, in the extracellular matrix and in the basement membrane. Fibronectin plays a major role in wound healing and also plays a significant role in promoting the attachment of cells to one another and to extracellular substrates. The authors suggested that FN was a primary matrix in the organization of collagenous tissue during the repair process. Vitronectin (VN) is a cell adhesion protein and it has been implicated in several biological functions, including cell

attachment, blood coagulation, fibrinolysis and immune defence mechanisms (1,11-13). Fibronectin-like engineered protein is a synthetic attachment glycoprotein with a molecular weight of 72.738 Da/molecule. Recombinant polymer incorporates multiple copies of the attachment ligand of human FN interspaced between repeated structural peptide units. There is no data about FN-like engineered protein confirming this attachment protein should be applied for new attachment procedures. The purpose of this study was to evaluate the initial attachment of human periodontal ligament (PDL) fibroblasts to different root surfaces after FN, VN and FN-like engineered protein application *in vitro*.

Materials and Methods

Preparation of Root Surfaces

Twenty-two caries-free periodontally-involved single-rooted teeth with attachment loss of 6 mm or more were obtained from patients 42 to 50 years old with no history of systematic diseases and with no periodontal treatment within 6 months prior to extraction. Following an atraumatic extraction, the crown of each tooth was excised slightly below the cemento-enamel junction. Then a 1 mm wide horizontal groove was cut with a diamond bur at the attachment level. Another groove was cut perpendicular to this groove, dividing the diseased portion into two parts. The roots were sectioned along the sagittal axis. Experiments were performed on one half and the other half was used as a control. The diseased cementum sites were scaled and root planed using a Gracey curette 1-2, and the other half of the diseased cementum sites were not instrumented. Instrumentations were done on every cementum site for 10 seconds. On the non-diseased cementum sites, the remnants of periodontal ligaments were carefully removed using a Gracey curette 1-2 (Fig. 1). Then FN, VN and FN-like engineered protein (Sigma, St Louis, MO, USA) were applied to the root surfaces and allowed to stand at room temperature for 30 minutes according to the manufacturer's instructions (0.1% solution). Each group included 11 root specimens.

Cell Culture and Cell Attachment

Briefly, PDL tissues were obtained from PDL premolar teeth extracted for orthodontic reasons. After extraction, the teeth were placed in biopsy media (Dulbecco's modified Eagles media [DMEM] with 10% fetal calf serum [FCS], 250 μg/ml gentamicin sulfate, 5 μg/ml amphotericin B, 100 units/ml penicillin, and 100 μg/ml streptomycin) (Sigma, St Louis, MO, USA). Only PDL attached to the middle third of the root was removed with a curette to avoid contamination with gingival and apical tissues. The PDL tissues were cut into small pieces, and placed in tissue culture dishes. The PDL tissues were incubated in culture medium (DMEM with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. After reaching confluence, cells were passaged with 0.25% trypsin (5,14). The PDL fibroblasts were used to 5th passage for all experiments.

The fragments from each group were placed in separate culture wells and incubated with 2.8×10^5 cells in 2 ml of DMEM in a humidified atmosphere of 95% air and 5% CO_2 for 1 hour at 37°C. After a 1 hour incubation period, the root segments were transferred to fresh wells and stained with Giemsa. The adherence of PDL fibroblast on the root surfaces was evaluated by using an Olympus Vanox Light microscope. The number of attached cells per unit area were determined by using an ocular grid (10 × 10 mm) system at magnification × 100. Quantitative results for each root surface of the groups were obtained from the counts of 4 different randomly chosen areas. Two fragments from each group were processed for scanning electron microscopic (SEM) observations. They were fixed in 2% gluteraldehyde and coated with gold. Then the fragments were viewed using a JEOL-JSM 6400 Scanning Electron Microscope and were photographed at magnification × 300.

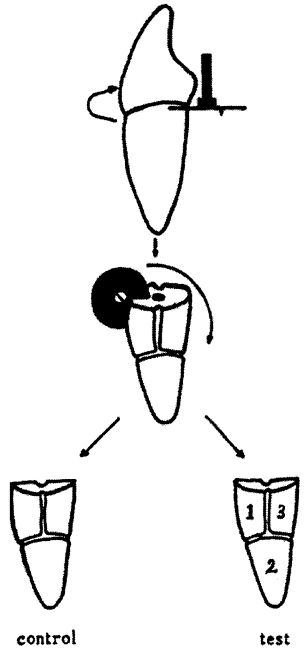


Fig. 1 Schematic drawing presenting the preparation of a root fragment.
1: Instrumented diseased cementum site.
2: Non-diseased cementum site.
3: Non-instrumented diseased cementum site.

Statistical analyses were made by independent sample *t*-tests. A level of $p < 0.05$ was accepted for statistical significance.

Results

Quantitative Results of Light Microscopic Observations

The results indicated that human PDL fibroblasts attached to all root surfaces regardless of the attachment proteins (Fig. 2). The mean value of initially adhered cells in both control and test groups, the number of cells attached to non-diseased cementum sites were significantly greater than the number of cells attached to the instrumented diseased cementum sites and the non-instrumented diseased cementum sites ($p < 0.05$). Likewise, the mean value of initially adhered cells in both control and test groups and the number of cells attached to instrumented diseased cementum sites were significantly greater than the number of cells attached to the non-instrumented diseased cementum sites ($p < 0.05$).

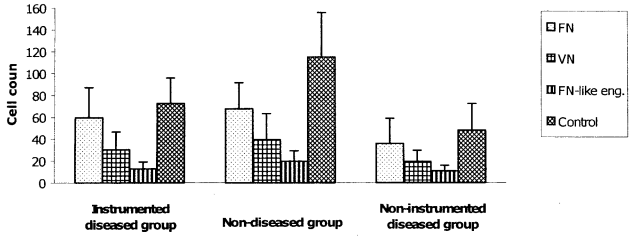


Fig. 2 Comparison of the mean number of attached cells on different root surfaces.

In the instrumented diseased sites of control group, the number of cells attached to the root surfaces was greater than the number of cells attached to the instrumented diseased sites for all of the test groups ($p < 0.05$). In the non-instrumented diseased sites of control group, the number of cells attached to the root surfaces was greater than the number of cells attached to the non-instrumented diseased sites for all of the test groups ($p < 0.05$). In the non-diseased sites of control group, the number of cells attached to the root surfaces was greater than the number of cells attached to the non-diseased sites for all of the test groups ($p < 0.05$). In the instrumented diseased sites, non-instrumented diseased sites and non-diseased sites of the FN group, the number of cells attached to the root surfaces was greater than the number of cells attached to the instrumented diseased, non-instrumented diseased and non-diseased sites of the VN group and the FN-like engineered protein group ($p < 0.05$). Similarly, in the instrumented diseased, non-instrumented diseased and non-diseased sites of the VN group, the number of cells attached to the root surfaces was greater than the number of cells attached to the instrumented diseased, non-instrumented and non-diseased sites of the FN-like engineered group ($p < 0.05$).

Scanning Electron Microscopic Observations

The electron micrographs revealed an initial attachment and orientation of the periodontal ligament fibroblasts to the root surfaces in the marked sites (Fig. 3: A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2, D3). The cells which adhered to all the root surfaces showed variations in shape and size from a slightly rounded to a slightly flattened appearance.

Discussion

This study was designed to evaluate the initial attachment of human PDL fibroblasts to different root surfaces after FN, VN and FN-like engineered protein application in vitro. Several studies have previously examined the effect on cell adhesion of coating root surfaces with FN and other proteins (5,7,15). This study differs from previous studies in that as close to clinical conditions of the root surfaces as possible were preserved. Therefore, in the present study, root fragments were not sterilized and cells were added as quickly as possible. Also, the root planing which we performed was similar to the root planing one could make during an operation.

Various alterations occur on the periodontally involved root surfaces as a result of the absorption of endotoxins and antigens (16,17). Scaling and root planing seem to effectively remove the endotoxin-contaminated root surface (18). However, animal experimentation (2) and human block sections (2,13,19) indicate that this does not lead to regeneration of a new connective tissue attachment. Instead, healing is generally dominated by the long junctional epithelium. Thus, many experimental studies have been directed at altering this diseased root surface in a manner that will promote new connective tissue attachment (2,19,15). In those studies, demineralizing agents, and growth and attachment factors have been used widely (6,19-22). Fibronectin was used in combination with demineralizing agents and/or the Guided Tissue Regeneration (GTR) technique in many studies. The results of the animal studies have histologically shown an increased in new connective tissue attachment, following the use of acid demineralization and FN (2,23,24). Ripamonti et al. (25) have shown that this potential may be enhanced by the use of specific attachment glycoproteins and plasma

factors. Human studies have shown a statistically significant, but clinically insignificant, difference between sites treated with modified Widman flaps versus modified Widman flaps treated with citric acid and FN (26). Human histologic studies using tetracycline as a demineralizing agent plus FN failed to show any evidence of regeneration (27). Caffesse et al. (9) have investigated the effects of GTR with and without citric acid conditioning and autologous

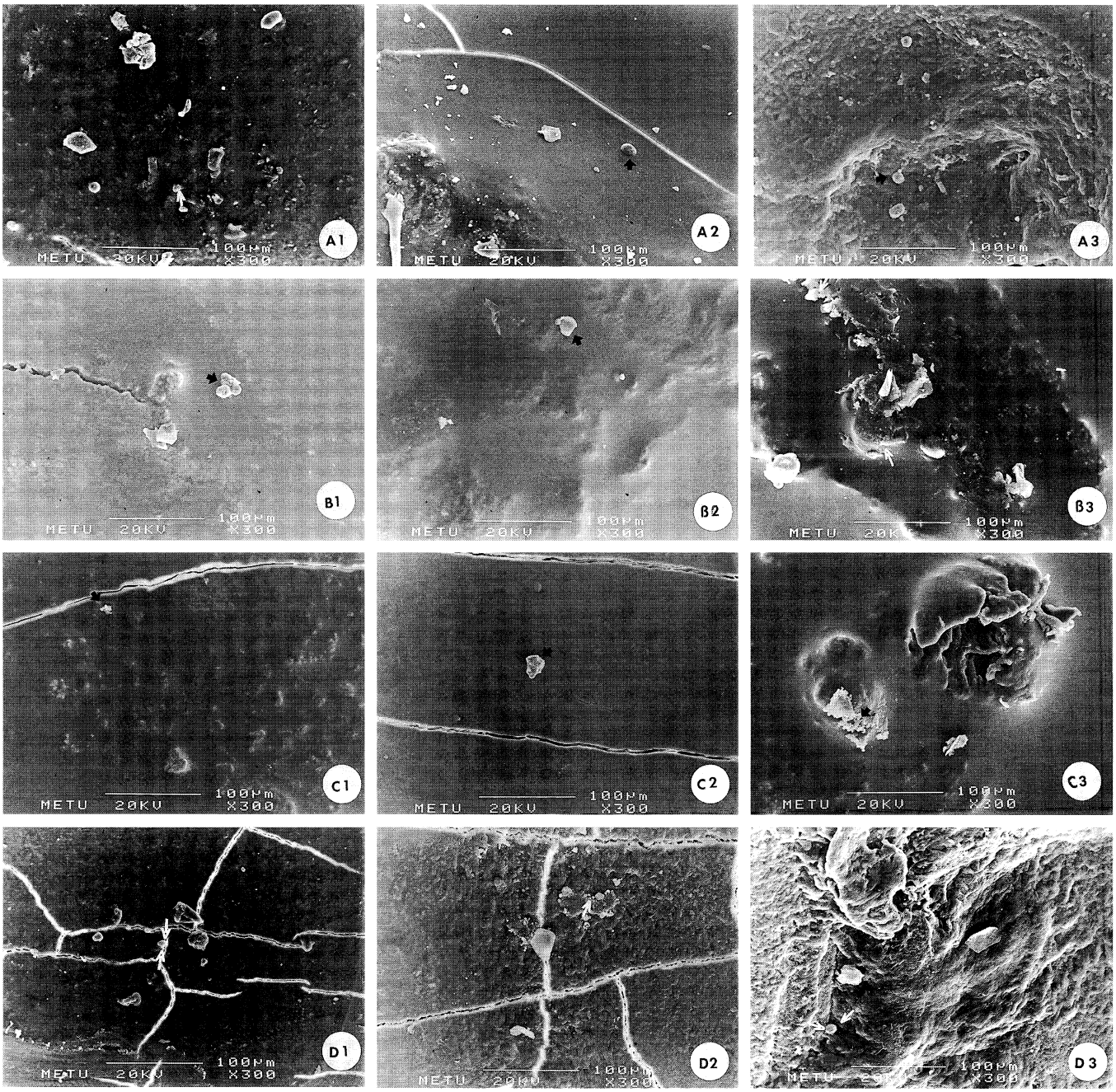


Fig. 3 Electron micrographs showing the presence of attached periodontal ligament fibroblasts to root surfaces. A1: FN- Instrumented diseased root surface, original magnification × 300. A2: FN- Non-instrumented diseased root surface, original magnification × 300. A3: FN- Non-diseased root surface, original magnification × 300. B1: VN- Instrumented diseased root surface, original magnification × 300. B2: VN- Non-instrumented diseased root surface, original magnification × 300. B3: VN- Non-diseased root surface, original magnification × 300. C1: FN-like engineered protein- Instrumented diseased root surface, original magnification × 300. C2: FN-like engineered protein- Non-instrumented diseased root surface, original magnification × 300. C3: FN-like engineered protein- Non-diseased root surface, original magnification × 300. D1: Control- Instrumented diseased root surface, original magnification × 300. D2: Control- Non-instrumented diseased root surface, original magnification × 300. D3: Control- Non-diseased root surface, original magnification × 300.

FN application. Their results showed that adjunctive citric acid plus autologous FN produced slightly better results, but these differences were not statistically significant. Trombelli et al. (8) have shown that there was no additional benefit with tetracycline demineralization and topical fibrin-fibronectin sealing system application in conjunction with flap debridement surgery. Smith et al. (6) reported there was no obvious advantage of increasing the concentration of FN above the plasma level. Results of our study showed that FN, VN and FN-like engineered protein application on the root surfaces did not promote cell attachment. The results of our study for FN group are consistent with previous findings (1,8,11,28-30). In spite of the role of FN in promoting periodontal regeneration being evaluated in previous studies, VN and FN-like engineered protein were not evaluated.

The electron micrographs showed only initial attachment of cells on the root surfaces. The cells showed variations in shape and size from a slightly rounded to a slightly flattened appearance. There were no apparent differences in the cells morphology for the different groups. In the comparison of attachment proteins, the number of attached cells in the FN group was higher than the VN and FN-like engineered protein groups ($p < 0.05$). The protein characteristics may have caused these different results. In light of our findings; we suggest that the role of these attachment proteins in wound healing must be evaluated in an in vivo manner. Also, further studies using these proteins in an in vivo manner are needed to assess long-term cellular reorganization. According to our results; it may be stated that it is more important to remove the pathologically altered cementum than altering root surfaces with various chemical agents.

Acknowledgments

This project was financially supported by the University of Gazi Research Foundation, project no. DF/ 95-1.

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