SEM observations of the attachment of human periodontal ligament fibroblasts to non-demineralized dentin surface in vitro

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Purpose. The purpose of this investigation was to study in-vitro the attachment behavior of human periodontal ligament (HPDL) fibroblasts to nondemineralized dentin surface using scanning electron microscope.
Study design. Thirty root slices of freshly extracted human teeth of 4 mm thickness as well as six 5 × 5 mm glass slides used as a control were used in this study. The dentin surface of the root slices was not treated with any chemicals to remove the smear layer. The root slices and the glass slides were placed in tissue culture clusters and an amount of 1 ml of HPDL fibroblast cell suspension was placed over the dentin surface of the root slices and the glass slides. They were then placed into an incubator at 37°C and 100% humidity for 4, 24, and 72 hours. At the end of the incubation, the cells were fixed with glutaraldehyde and examined microscopically.
Results. Different shapes of fully spread cells were seen. The cells were attached firmly to the dentin surface by the cytoplasmic extension of the lamellipodia and microvilli which were seen extending inside the opening of the dentinal tubules.
Conclusion. It was concluded that the human dentin surface provided an excellent surface for attachment of periodontal ligament fibroblasts. In addition, the smear layer did not affect the cell attachment.
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Root-end resection is recommended when failed root canal filling can not be retreated.[1,2] Collagen fibers must be reattached to the exposed dentin surface if regeneration is to occur following surgical procedure. Smear layer is usually created on hard tissues such as dentin surface, whenever they are cut with hand or rotary instruments. The chemical conditioning of the dentin surface to remove the smear layer has been suggested for better attachment of the cells.[3,4,5] Such treatment is not commonly done when root-end resection is performed clinically.

Attachment of primary and established fibroblasts to dentin structures has been studied using demineralized porcine, bovine, and human teeth.[6,7] Limited information was reported on the morphological appearance of the attachment of human periodontal ligament (HPDL) fibroblasts to the exposed nondemineralized human dentin structure of root apex.

The purpose of the present investigation was to study in vitro the morphology and attachment behavior of the human HPDL fibroblasts to nondemineralized dentin surface of resected human root slices using scanning electron microscope.

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MATERIALS AND METHODS

Materials

Thirty human root slices of freshly extracted human teeth and 6 glass slides (5 × 5 mm) for control were used. The teeth had no caries or filling and were extracted for orthodontic reasons.

Culture of human HPDL fibroblasts

Human periodontal ligament tissue was obtained from a healthy 21-year-old female patient undergoing extraction of an impacted upper third molar. The tooth was surgically removed by an oral surgeon and kept in Eagles Minimum Essential Medium (MEM) supplemented with 5% fetal bovine serum and antibiotics. The antibiotics were added at a concentration of 250 iu, 250 μg, and 20 μg/ml of penicillin, streptomycin, and fungi zone, respectively (Sigma Chemical, St. Louis, Mo, USA). The tissue was gently scraped from the root with a scalpel blade, cut in very small pieces and washed twice in phosphate-buffered saline solution (PBS). The tissue pieces were digested in a mixture of 0.05% trypsin and 0.02% EDTA in Hank's Balanced Salt Solution depleted with calcium and magnesium. This procedure was done at 37°C by stirring the tissues in trypsin solution in a 50-ml Erlenmeyer flask over a magnetic stirrer. Once the solution became turbid, the mixture was passed through a nylon strainer of 70-μm mesh (Falcon, McLean, Va, USA). In addition, MEM with 10% fetal bovine serum was added to stop the proteolytic effect of trypsin. Cell suspension was washed once with PBS then suspended in complete growth medium with 100 iu penicillin and

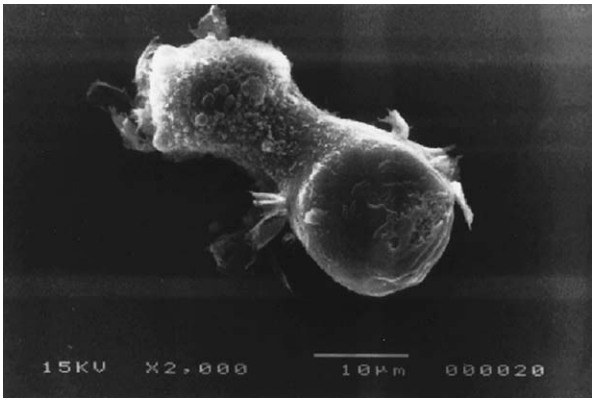

Fig 1. SEM micrograph of HPDL fibroblast attached to glass slide at 4 hours incubation, showing dividing cell roughly round in shape with irregular surface and blebs (×2000).

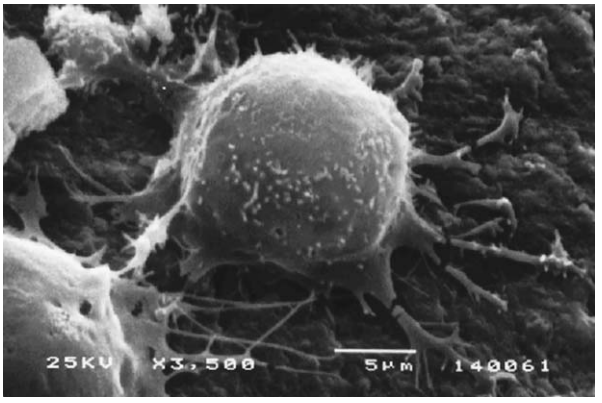

Fig 3. SEM micrograph of HPDL fibroblast attached to the cut-dentin surface at 4 hours incubation. The cell is discoid in shape with roughly smooth surface and attached with filopodia (×3500).

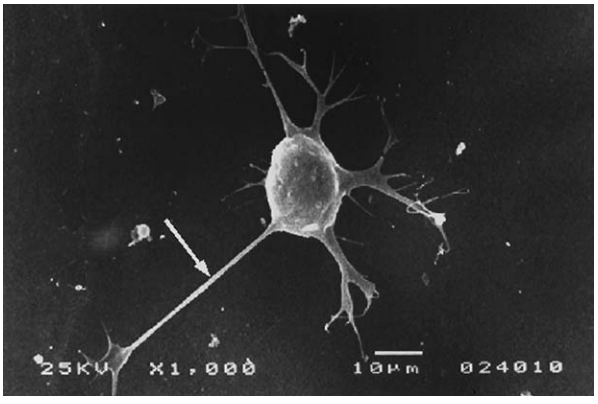

Fig 2. SEM micrograph of HPDL fibroblast attached to the glass side at 24 hours incubation. The cell has a smooth surface and attached with long filopodia (*arrow*) and microvilli (×1000).

100 µg/ml streptomycin. Twenty-five square centimeter tissue culture flask was seeded with the cell suspension and incubated at 37°C in an environment of 5% CO_2 in air and 95% humidity. The culture medium was changed after 1 week and then every 2 days until a confluent monolayer was formed. The 5th subculture was used for the experiment. All tissue manipulations were done under a sterile laminar flow Biohazard Class II cabinet (Baker NCB6, Sanford, Me, USA).

Preparation of the dentin samples

Thirty single roots of freshly extracted human teeth with completely formed roots and no caries, resorption, or cracks were collected. The selected teeth were cleaned of any attached bone or soft tissue tags and stored in 0.9% normal saline solution until used. The apical 3 mm of the roots were resected perpendicular to the long axis of the root using sterile plain tapered carbide fissure bur ISO size 169 attached to a high-speed handpiece under saline irrigation. They were discarded after resection. A 4-mm-thick dentin slice was cut off with a diamond disc parallel to the apically prepared surface. The root slices were cleaned in ultrasonic bath using distilled water for 10 minutes and then thoroughly rinsed under running water for 15 minutes to remove dentin debris. All root slices and glass slides used for the experiment were steam autoclaved at 250°F for 15 minutes. Ten root slices and 2 glass slides were used per observation period.

Experimental procedure

The growth of the HPDL fibroblasts was examined with light microscope before starting the experiment. The experiment was performed in tissue culture cluster containing 96 wells, each with an inner diameter of 6 mm (Linbro, Flow Laboratories, McLean, Va). One root slice was placed, with the apical surface up, in each well. One ml of cell suspension was added carefully over the root slice. In addition, cell suspensions were dispensed in wells containing the glass slides. The tissue culture clusters were placed into an incubator at 37°C and 100% humidity. The specimens were incubated for 4, 24, and 72 hours. The medium was kept unchanged during the observation periods. Upon completion of incubation, the specimens were pre-fixed with a few drops of 0.1% glutaraldehyde for 5 minutes. The medium was aspirated out and the cells were fixed with 2% glutaraldehyde in 100 mmol/L cacodylate buffer (pH = 7.2) for 30 minutes at room temperature. The specimens were washed briefly with phosphate-buffered saline and dehydrated with sequential washes in a series of 50, 70, 90, and 95% ethyl alcohol and twice in absolute ethyl alcohol, each time for

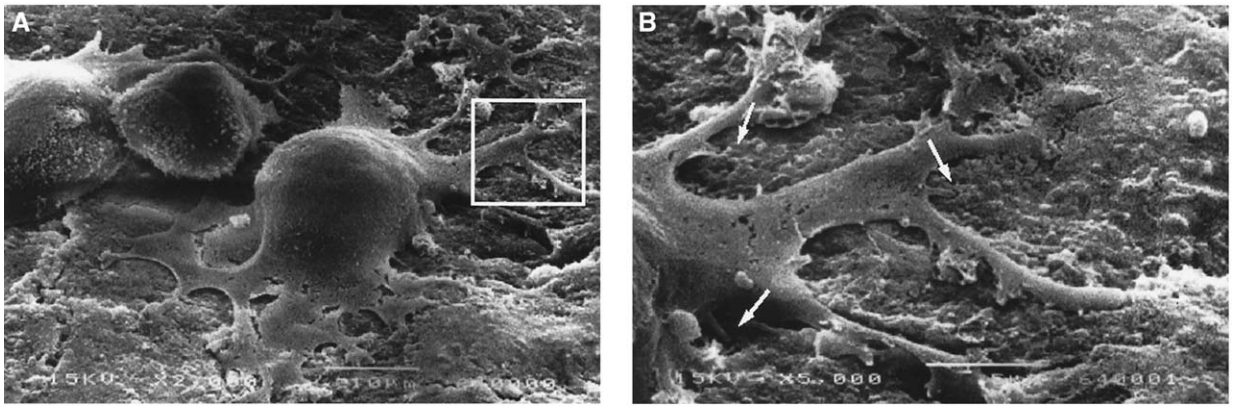

Fig 4. SEM micrograph of HPDL fibroblast attached to the cut-dentin surface at 72 hours incubation. **A**, The discoid-shaped cell has a smooth surface with few ruffles, spread well on the cut-dentin surface (×2000). **B**, High magnification shows the attachment of the filopodia to the cut-dentin surface, parts of the filopodia were inserted into the dentinal tubules (*arrows*) (×5000).

30 minutes. The specimens then were critical point dried with CO_2 (Samdri-PVT-3B; Tousimis Research Corp, Rockville, Md, USA), mounted on copper stubs, and coated with gold. The specimens were examined and photographed using scanning electron microscope (Jeol JSM T330A; Electron Optical Laboratory, Tokyo, Japan) at an accelerating voltage of 25 kV.

RESULTS

Control

Figures 1 and 2 illustrate the normal morphological features of the HPDL fibroblasts. Dividing cells were seen at 4 hours incubation (Fig 1). Large number of fibroblasts bearing different shapes and surface characteristics adhered to the substrate with microvilli and filopodia. As the time progressed they appeared to be fully spread and well attached to the substrate by means of lamellipodia (Fig 2). The cell surface was relatively smooth except for a few blebs or ruffles.

Dentin surface

The dentin surface was amorphous, presumably a collagenous or gelatin-like organic matrix. The cells grew well on the cut dentin surface similar to the control group (Figs 3 and 4, *A*). Cell spreading was already evident, and the penetration of cytoplasmic processes (microvilli and filopodia) into dentinal tubules was frequently observed after 24 hours of attachment (Fig 4, *B*). The cells were spindle- or fan-shaped and had a smooth surface covered with ruffles.

DISCUSSION

Cell culture of HPDL fibroblasts has been reported in detail by several investigators.[8,9] They are considered to be the appropriate cell line for evaluating cell attachment[10] and for testing the cytotoxicity of endodontic filling materials.[11] The fifth subculture of the HPDL fibroblasts was used in this study. It is proved to be practical and relatively free of tissue remnants. In addition, minimal alteration of cell morphology and function due to cultivation will have occurred. They were chosen with the rationale that they may respond more like HPDL fibroblasts in vivo following root-end resection.

The glass surface has been reported to be a good substratum for normal cell growth in vitro.[12] Growth of HPDL fibroblasts on the glass slides in this study was excellent. Normal cell morphology was noticed in all observation periods.

The dentin discs, glass slides and hand instruments were sterilized prior to incubation with the tested cells. This was done to avoid residual infection. Contaminated root slices with bacteria and their endotoxins might compromise cellular integrity will cause negative impact upon attachment and lead to inaccurate and misleading results. This was clearly seen in the study of Safavi et al,[13] where variation of cell density was noticed on the dentin surfaces as well as presence of microorganisms. The addition of antibiotics to the culture media is not sufficient to control contamination when human root slices are to be used. The process of autoclaving the dentin slices did not remove the smear layer. Autoclaving might denture the collagen and other dentin protein. This did not influence cell attachment as seen in this study. These findings are in agreement with findings by Lowenberg et al.[14]

The growth of HPDL fibroblasts, L 929 mouse fibroblasts, and SV40 large T-antigen-transfected bovine pulp-derived cells on human and bovine dentin slices has

been reported to be very good.[5,7,13] Similar observation was noticed in this study, where HPDL fibroblasts were found to have a favorable response to human dentin slices. The cells grow well on the dentin surface despite the presence of the smear layer. The very similar growth characteristics of the HPDL fibroblasts on glass slide and dentin disc imply identical growth conditions and similar cell contact to the surfaces. The results of the present study are similar to findings of Rompen et al[5] and Schmalz et al.[7]

The smear layer was clearly seen microscopically in this study. Most smear layers are created under conditions that generate either high temperatures[15] or high shear forces[16] which lead to denaturation of surface collagen.[17] According to Pashley[16] this may increase the solubility and enzymatic susceptibility of the organic phase of the smear layer as well as lowering the solubility of the inorganic phase if the constituent particles of the smear layer are coated with gelatinized collagen. Lowenberg et al[6] reported no significant difference in the number of cells attaching to nondemineralized compared to demineralized dentin. The growth of the HPDL cells on the dentin surface was noticed as time progressed. The HPDL fibroblasts were moving from the stage of rounded with microvilli growth to flattening of the cell mass with filopodia attachment. However, these different stages are different phases of a continuous process. The duration of these phases and the degree of overlapping of these events may vary between different cell lines and different substrates.[18-19] Cell spreading was already evident and the microvilli sprouting from various parts of the cell body that attached to the glass and dentin surface provide good diffusion. According to Grinnell[20] cellular attachment is dependent on viability and physiological conditions of the cells. The penetration of the lamellipodial and filopodiail cellular extensions penetrating into the dentinal tubules was frequently observed on dentin. This was similar to the findings of Rompen et al.[5] Extension of the filopodia inside the dentinal tubules will stabilize the fibroblast cell and allow it to spread nicely and freely on the dentin surface.

Adhesion and spreading of cells on dentin surface are the initial phase for cellular function. Rompen et al[5] suggest that during periodontal surgery a conditioning of the root surface by citric acid or by minocycline HCl could promote the attachment, the proliferation, and the biosynthetic activity of human PDL fibroblasts, prerequisites to periodontal regeneration. Topical application of citric acid causes a superficial demineralization of the root surfaces and partially exposes collagen from radicular dentin.[21,22]

According to Wikesjö et al,[23] the sequence of healing events on dentin surfaces implanted in bone cavities of beagle dogs, conditioned with citric acid or heparin, was largely similar to the healing at native dentin surfaces. No significant difference of morphology was detected between cells attached to untreated and cells attached to conditioned periodontally exposed dentin.[5] The observations of the attached fibroblasts to nontreated dentin surface in this study assessed the biocompatibility of the human dentin surface despite the presence of the smeared layer. These data suggest that during endodontic surgery a conditioning of the dentin surface is not necessary for the attachment and the proliferation of HDPL.

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