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Attachment of human periodontal ligament fibroblasts to 3 different root-end filling materials: Scanning electron microscope observation

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Objective. The attachment behavior of the human periodontal ligament (PDL) fibroblasts to root-end filling materials (amalgam and Super-EBA) was compared in vitro to gutta-percha by means of scanning electron microscope.

Study design. Amalgam and Super-EBA were placed in a prepared cavity of root slices of freshly extracted human teeth and evaluated freshly prepared. Root slices of teeth with cold-burnished gutta-percha filling with AH26 sealer were used for comparison. The root slices were placed in tissue culture cluster, and 1 mL of cell suspension was added carefully over the root slice. They were incubated at 37°C and 100% humidity for 4, 24, and 72 hours.

Results. Results showed that the cold-burnished gutta-percha provides a better substrate than amalgam and Super-EBA for cell growth and attachment. Amalgam was the most toxic material, showing early manifestation of cell injury.

Conclusion. It was concluded that the composition and surface texture of the substrate have an influence on the morphology and the attachment of the PDL fibroblasts. It is suggested that cell attachment and morphology might reflect the biocompatibility of the substratum.

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Root-end filling material has been recommended by a number of investigators to be placed in almost all teeth undergoing root-end resection.^{1,2} The aim of placing a root-end filling material is to develop an apical seal, which reduces the leakage of residual irritants from the root canal into the periradicular tissues. Several materials have been suggested as root-end filling materials including amalgam, Super-EBA, and mineral trioxide aggregate. Safavi et al³ evaluated in vitro the attachment of L929 mouse fibroblasts to the cut dentin surface and root-end materials. They showed that the cell densities on the surface of the tested material tend to increase by incubation time. Recently, Zhu et al⁴ observed the adhesion of human osteoblasts on root-end filling materials. They found that the attachment and

spreading of the osteoblasts are different for the material tested. Human osteoblasts and established L929 mouse fibroblasts are not the appropriate cells to be used when evaluating root-end filling materials. In addition, behavior of established cell lines could be different than primary cells of human source. Furthermore, periodontal ligament (PDL) fibroblasts are usually formed around the root-end filling materials and not the osteoblasts. Limited information was reported on the ability of human PDL fibroblast attachment to the surgically exposed root apex and root-end filling material.^{5,6} The purpose of the present investigation was to study in vitro at scanning electron microscope level the attachment behavior of the human PDL fibroblasts to amalgam and Super-EBA and compare this to cold-burnished gutta-percha. Furthermore, the morphologic changes of the cells were observed.

MATERIAL AND METHODS

Test materials

Sixty-nine human root slices were divided into 3 experimental groups of 21 root slices each and 6 for positive control. The root-end filling materials used in this study were amalgam (regular set, Dispersalloy;

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Dentsply International Inc, Milford, Del) for group 1, Super-EBA (regular set; Harry J. Bosworth Co, Skokie, Ill) for group 2, and gutta-percha and AH26 cement for group 3.

Isolation and culture of human PDL fibroblasts

The PDL tissue was obtained from impacted upper third molar of a 19-year-old woman with an unremarkable medical history. The tooth was surgically removed and kept in Eagles minimum essential medium (MEM) supplemented with 5% fetal bovine serum and antibiotics. Antibiotics were added at a concentration of 250 IU/mL penicillin, 250 µg/mL streptomycin, and 20 µg/mL fungi zone. The tissue was tweezed from the tooth, 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 calcium and magnesium-free Hank's balanced salt solution. 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, B&D, NJ). MEM with 10% fetal bovine serum was added to stop the proteolytic effect of trypsin. The cell suspension was washed once with PBS and then suspended in complete growth medium with 100 IU/mL penicillin and 100 µg/mL streptomycin. A 25-cm² tissue culture flask (Nunc, Roskilde, Denmark) was seeded with the cell suspension and incubated at 37°C in an environment of 5% CO₂ and 95% relative humidity. The culture medium was changed after 1 week and then every 2 days until a confluent monolayer was formed. The fifth subculture was used for the experiment. All tissue manipulations were done under a sterile laminar flow Biohazard Class II cabinet (Baker Co, Sanford, Me).

Preparation of the specimens

Sixty-nine single roots of freshly extracted human teeth with completely formed roots and no curvature, 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. Forty-eight single roots were used for amalgam, Super-EBA, and positive control (roots going through the retrocavity preparation only). The apical 3 mm of these roots were resected perpendicular to the long axis of the root by using a sterilized plain tapered carbide fissure bur, ISO size 169, in a high-speed handpiece under saline irrigation. Class I cavities (3 mm in depth) were prepared on the apical side of the root slices by using a #34 inverted cone carbide bur in a high-speed handpiece and saline irrigation. The depth was determined

by using permanent marking pen on the preadjusted length of the bur shank. The cavities were generally circular and were approximately 1.5 to 2 mm in diameter. Each root was cut off with diamond disk 4 mm coronal and parallel to the apically prepared surface. The 4-mm root slices were cleaned in ultrasonic bath with distilled water for 10 minutes and then were thoroughly rinsed under running water for 15 minutes to remove dentin debris. All root slices used for the experiment including the negative control were steam autoclaved at 250°F for 15 minutes. After sterilization, all further procedures were carried out under aseptic condition with sterile instruments. The test materials were mixed and condensed according to manufacturer's instructions and evaluated while they were fresh. The root slices were washed with saline for 2 to 3 minutes before and after insertion of the tested materials to mimic the clinical situation. For each experimental material, 7 root slices were used per observation periods (4, 24, and 72 hours).

The crowns of the remaining 21 teeth were removed by using a water-cooled 170L carbide bur attached to a high-speed handpiece. Working length was determined by placing a #15K file into the canal until it was visible at the apical foramen, and then 1 mm was subtracted. The canals were enlarged to size 50K with step-back technique and 1% sodium hypochlorite irrigating solution. The prepared canals were dried with paper points and filled with gutta-percha and AH26 silver-free sealer cement (De Trey Dentsply, Milford, Del) by lateral cold condensation technique. Radiographs were taken to ensure complete filling of the root canal. The specimens were kept in a sealed jar that contained 2 × 2-inch sterile gauze pad soaked with physiologic saline solution to ensure 100% humidity. The jar was kept in an incubator at 37°C for 1 week. After that, the apical 3 mm was cut off perpendicular to the long axis of the root with a sterilized plain tapered carbide fissure bur ISO size 169 in a high-speed handpiece under saline irrigation. The exposed gutta-percha was compressed back into the canal space and burnished with a No. 1 ball burnisher. The root was cut off with diamond disk 4 mm coronal and parallel to the apically prepared surface. The apical surface of all the samples was examined under light stereomicroscope (Wild MPS 55 Stereomicroscope, Heerbrugg, Switzerland) at 12× power for crack detection. Root slices that had a crack were excluded from the study. Six root slices without filling materials were used as positive controls. One glass slide was used per 1 observation period as a negative control to evaluate the attachment and the normal morphology of the PDL fibroblasts.



Fig 1. Scanning electron micrograph of PDL fibroblasts attached to glass slide at 4-hour incubation. Roughly stellate-shaped cell covered with ruffles and small blebs attached with microvilli and lamellipodia (original magnification $\times 3500$).

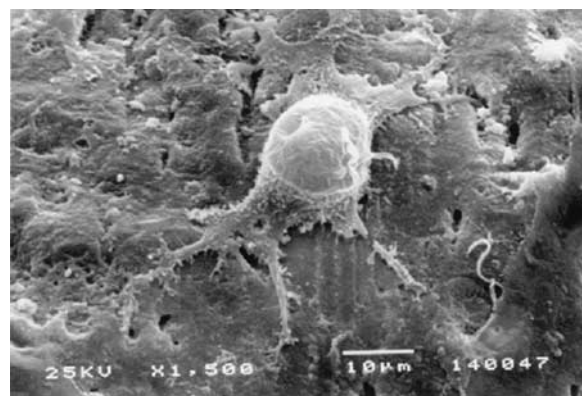


Fig 2. Scanning electron micrograph of PDL fibroblasts attached to cut dentin surface at 4-hour incubation. The spread cell has smooth surface with few ruffles at the cell periphery. The cell is attached with filopodia and lamellipodia (original magnification $\times 1500$).

Experimental procedure

The growth of the PDL fibroblasts was examined with light microscope before starting the experiment. The experiment was performed in a tissue culture cluster containing 96 wells, each with an inner diameter of 6 mm (Linbro; Flow Laboratories, McLean, Va). One root slice was placed in each well with the apical surface up. One milliliter 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 cluster was placed into an incubator at 37°C and 100% humidity. The specimens were incubated for 4, 24, and 72 hours. The medium was not replaced during the observation periods. On completion of incubation, the specimens were prefixed 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 PBS 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 30 minutes. The specimens then were critical point dried with CO₂ (Samdri-PVT-3B; Tousimis Research Corp, Rockville, Md), mounted on copper stubs, and coated with gold. The specimens were tilted at 45 degrees, examined, and photographed by using a scanning electron microscope (Jeol, JSM, T330A; Electron Optical Laboratory, Tokyo, Japan) at an accelerating voltage of 25 kV.

RESULTS

Control

Figs 1 and 2 illustrate the normal morphologic features of the PDL fibroblasts. The negative control cul-

tures demonstrated a large number of fibroblasts bearing different shapes and surface characteristics. They adhered to the substrate with microvilli and filopodia (Fig 1). As time progressed, they appeared to be fully spread and well attached to the substrate by means of lamellipodia. The cell surface was relatively smooth except for few blebs or ruffles. Similar observation were seen when the cells were grown on the cut dentin surface (Fig 2).

Amalgam

The surface texture of the amalgam was rough compared with gutta-percha and Super-EBA. Injury to the cell surface was seen at the 4-hour observation. These cells were round to discoid in shape and had small vacuoles or smooth surface. Spread cells attached to the amalgam with filopodia were seen occasionally. As the contact time was extended, the fibroblasts became few in number, were roughly round in shape, and had no microvilli or filopodia; more vacuoles were seen on the surface (Fig 3).

Super-EBA

The surface texture of Super-EBA was smooth compared with amalgam but slightly rough compared with gutta-percha. It adapted well to the dentin wall of the root-end cavity. Fibroblasts attached to Super-EBA were discoid in shape with a smooth surface and little depression. They attached to the Super-EBA with filopodia and lamellipodia (Fig 4). As time progressed, the majority of the cells were round in shape and had rough surface with depression or vacuoles.



Fig 3. Scanning electron micrograph of PDL fibroblasts incubated with amalgam for 72 hours. The cell is round in shape with different sizes of vacuoles (original magnification $\times 3500$).

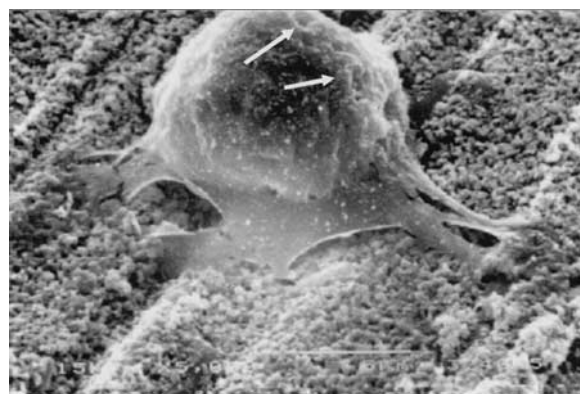


Fig 4. Scanning electron micrograph attached to Super EBA at 24-hour incubation with ruffles on its surface. The cell has a discoid shape and depressions on its surface (arrows) (original magnification $\times 5000$).

Gutta-percha

The morphology of the PDL fibroblasts observed during the 3 observation periods was not different from that seen on the control. The surface texture of the gutta-percha was smoother than the other root-end filling materials. The fibroblasts were spindle- or fan-shaped and had a smooth surface covered with ruffles. They attached with filopodia or lamellipodia (Fig 5).

The cells observed on the dentin next to the root-end filling materials had normal morphology similar to that seen in the control specimens.

DISCUSSION

Human PDL fibroblasts were successfully cultured in this study. They have been reported to be the appropriate cell line for evaluating cell attachment⁷ and for testing the cytotoxicity of endodontic filling materials.⁸ The light microscopic observation of the cultured PDL fibroblasts during examination ensured that a secure viable cell line with the typical appearance of fibroblast was present throughout the study. The cells used in this study were harvested with trypsin. Although the cells were washed before use, the surface may have been affected by trypsin. However, the effect of trypsin has been reported to be minimal, and the likelihood of morphologic changes as a result of residual trypsin is very remote.⁹

To avoid any residual infection, the root slices and the hand instruments were sterilized before the insertion of the tested materials. Contaminated root slices might compromise cellular integrity, had a negative impact on attachment, and led to inaccurate and misleading results.³

The results of the present investigation showed that the surface texture and the composition of the tested

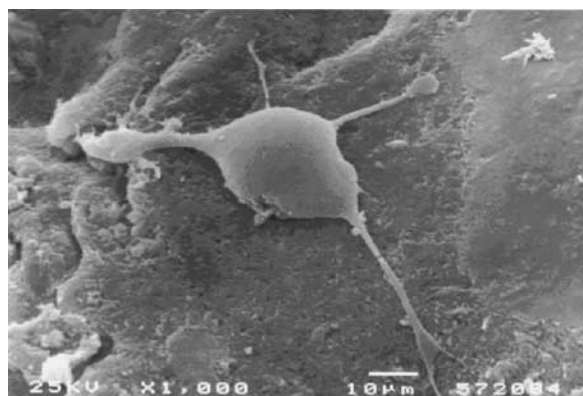


Fig 5. Scanning electron micrograph of PDL fibroblast attached to gutta-percha at 72-hour incubation. The cell is roughly spindle-shaped and has smooth surface and is attached with filopodia (original magnification $\times 1000$).

root-end filling materials had an influence on the morphology and the attachment of the PDL fibroblasts. Scanning electron micrograph of the negative control showed that as time progressed, the PDL fibroblasts were moving from the stage of rounded with microvilli growth to the flattening of the cell mass with filopodia attachment. However, these different stages are not discretely separable but 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.⁹

Good attachment of PDL fibroblasts to the amalgam at 4 hours with the presence of few injured cells may be due to the short period of incubation, which was not enough to cause obvious cell changes. The vacuoles

seen on the surface of PDL fibroblasts and lack of spreading at 24 and 72 hours could be due to the release of several metals into the medium in trace amounts. Pure silver has been reported to be nontoxic,¹⁰ whereas mercury was reported to be as cytotoxic as copper and zinc.¹¹ Zinc is known to be a cytotoxic agent, and it was considered as the major contributor to the cytotoxicity of amalgam.^{12,13} It diffuses preferentially to the surface of amalgam and is easily released into tissue culture medium. The presence of the blebs on the surfaces of some injured cells may be due to the cytoplasmic shrinkage resulting from the material toxicity.¹⁴ It is also unlikely in an *in vivo* situation that a local accumulation of ions released at such a slow rate will ever accumulate to a high concentration, because tissue fluid dilution will take place.⁸ The increase in the cytotoxicity of amalgam by time, as evidenced by scanning electron micrograph, contradicts the report by Wataha et al,¹⁵ who reported that the cytotoxicity of Dispersalloy was severe initially but improved between 48 and 72 hours as the zinc release decreased. This contradiction may be due to the method of cytotoxicity measurement, sample preparation, and the type of cell line used. In their study, the Dispersalloy samples were aged for 7 days after mixing and then polished, cleaned, and disinfected. The total time between the preparation of the samples and immersion into cell culture medium was approximately 2 weeks. They used the extracts of the amalgam to measure the succinic dehydrogenase activity of the cells. Such technique will cause minimal release of the toxic component of the tested material. In our study, the amalgam was placed in the root-end cavity of root slices and incubated with the PDL fibroblasts while it was fresh to mimic the clinical situation. The 24-hour observation of this study was similar to the report of Zhu et al.⁴

The scanning electron micrograph results showed that the attachment of the fibroblasts to Super-EBA is better than to amalgam. This is related to the relatively smooth surface of Super-EBA compared with the rough surface of amalgam. On the other hand, the vacuoles seen on the surface of PDL fibroblasts at 24 and 72 hours may be due to leaked eugenol. It is the only component in the cement that showed a cytotoxic effect when the powder, eugenol, and *o*-ethoxy-benzoic acid were tested separately.¹⁶ Released free eugenol was reported to cause an inhibition of cell respiration or lysis of the cytoplasmic membrane.^{17,18} In addition, the rate of eugenol liberation is slow, and small amounts of eugenol continue to be released from zinc oxide eugenol mixtures for at least 1 year.¹⁹ Zinc released from the cement has been suggested as being partially responsible for the prolonged cytotoxic effect of this material.¹² The depression seen on the cell surface of some fibro-

blasts at 4 hours is due to the shrinkage of the cytoplasmic membrane. The increase in the cytotoxicity of Super-EBA by time as evidenced by scanning electron micrograph contradicts the results of Bruce et al,²⁰ who reported that Super-EBA displayed a minimal cytotoxicity at 0 time interval and no cytotoxicity at 7, 15, and 30 days. Bruce et al used the agar-overlay method, and the cytotoxicity was determined by measuring the zone of dead cells and the zone of cell inhibition. Such technique lacks the direct contact between the cells and the tested material. In addition, other cells can pick the stain that is released from the damaged cells. In our study, the cells were directly placed over the tested materials so the direct effect could be observed.

The PDL fibroblasts attached to the cold-burnished gutta-percha showed a good attachment, and their morphology was not different from that of the negative control in all observation periods. The normal morphology and attachment may be due to the relatively smooth surface of gutta-percha and the biocompatibility of both gutta-percha and the set AH26 sealer cement. AH26 sealer cement was reported to have decreased level of toxicity when allowed to set for 24 hours.^{8,21} In this study, AH26 cement was allowed to completely set before root resection procedure.

Thus, in summary, of the 3 materials studied here, cold-burnished gutta-percha sealed with AH26 offered the best substrate for cell attachment and growth. Although gutta-percha appears to be the best choice as biomaterial, other factors may sometimes dictate the clinical selection of root-end filling material.

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