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# Attachment and Morphological Behavior of Human Periodontal Ligament Fibroblasts to Mineral Trioxide Aggregate: A Scanning Electron Microscope Study

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**The attachment and morphology of human periodontal ligament fibroblasts to mineral trioxide aggregate (MTA) was evaluated using a scanning electron microscope. The material was placed at an apical cavity of 30 single-rooted slices of extracted human teeth. The specimens were divided into two groups of 15 root slices each (freshly mixed and set state). For each experimental group, five root slices were used per observation period (4, 8, and 24 h). A set of two glass slides was used per observation period for the control group. The experiments were performed in tissue-culture cluster 96-well plates in which 1 ml of human periodontal ligament fibroblast cell suspension was placed over the MTA filling and the control glass slides. For the positive-control group, 0.5 ml of methyl methacrylate 2% (vol/vol) was added to the cell suspensions before being dispensed into the wells. Results showed the normal cell morphology in the negative controls. Few round cells with less smooth surfaces and many rough blebs were seen in the positive control, and most of these cells did not show any attachment to the substratum. Similar observations were seen with the freshly prepared-MTA group. In the set-MTA group, cells were round and flattened, displayed smooth surfaces, and appeared to be tightly attached to MTA. It was concluded that the quality and quantity of cell attachment to the retrofilling material could be used as a criterion to evaluate material's toxicity.**

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The main objective of a root–end-filling material is to provide an apical seal that prevents the movement of bacteria and the bacterial products from the root-canal system into the periradicular tissues. According to Gartner and Drone (1), an ideal root–end-filling material should have these properties: easy to manipulate, radiopaque, dimensionally stable, nonabsorbable, insensitive to moisture, nontoxic, and biocompatible. In addition, the retrofilling materials should permit healing of the periradicular tissue in an ideal manner via regeneration of cementum, periodontal ligament, and alveolar bone across the resected root-end surface.

Materials that have been advocated for use as retrofilling include amalgam, composite resin, super-EBA cement, glass-ionomer cement, polycarboxylate cements, Cavit, and gutta-percha (2). These materials are usually placed in direct contact with the periodontium for a prolonged period of time; therefore, their biocompatibility is of primary importance.

In vitro cytotoxic screening as a primary factor of biocompatibility is determined by cell culture of different types using primary or transformed cells (3). The transformed cells have different biological properties from those of the primary human diploid cells (4). Recently, mineral trioxide aggregate (MTA) has been proposed for use as a retrofilling material. Its ability to seal a resected canal system was assessed in vitro (5), and when used as a root–end-filling material in dogs, cementum was reported to have formed over its exposed surface (6). Several investigators using agar-overlay, radiochromium method, MTT, and crystal violet (CV) assays (7–9) also evaluated its cytotoxicity. Although the MTA seems to cause no harm to the periradicular tissue, limited information is available regarding the attachment and morphological behavior of the periodontal ligament cells when contacting MTA. The purpose of this study was to evaluate, at scanning electron microscope level, the attachment and morphological behavior of human periodontal ligament fibroblast (HPLF) to MTA when used as retrograde-filling material.



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## MATERIAL AND METHODS

### Preparation of the Specimens

Thirty, single-rooted, human teeth with completely formed apices were used in this study. The selected teeth were cleaned of any attached bone or soft-tissue tags and stored in 1% sodium hypochlorite until used. The apical 3-mm of each root was resected perpendicular to the long axis of the root using a sterilized plain tapered carbide-fissure bur ISO size 169 in a high-speed handpiece under saline irrigation. The patency of the root-canal space at the apical third was evaluated with an endodontic explorer to assist in selecting teeth with no calcification. Class I cavities (3 mm in deep) were prepared on the apical side of the root slices using #34 inverted cone carbide bur in a high-speed handpiece and saline irrigation. The cavity depth was determined by marking the pre-adjusted length of the bur shank with permanent ink pen. The cavities were generally circular and were approximately 1.5 to 2 mm in diameter. The root was cut off with a diamond disc, 4-mm coronal and parallel to the apically prepared surface; as a result 4-mm thick slice was made from each root.

Twelve 5- × 5-mm glass slides were used as control group (six positive and six negative). The root slices and the glass slides were autoclaved at 250°F for 15 min. After sterilization, all further procedures were performed under aseptic conditions using sterile instruments. The apical cavities of the specimens were filled with MTA (Dentsply, Tulsa Dental Products, Tulsa, OK, patent #5415547). The material was mixed according to manufacturer's instructions, carried into the apical cavities with Endo Gun, packed with small condenser, and the excess material was removed from the cut dentin surface with a spoon excavator. The root slices were divided into two groups of 15 root slices each. The first group included the tested material in a freshly mixed state, whereas the material in the second group set for 24 h at 37°C with approximately 75% relative humidity. For each experimental group, five root slices and two glass slides for the control group were used per observation period of 4, 8, and 24 h.

### Culture of Human Periodontal Ligament Fibroblast

HPLF were obtained from previously established stocks that had been stored in liquid nitrogen (10). The periodontal ligament fibroblasts used in this study were between their fourth and fifth passage in culture. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum supplemented with 2 mM L-glutamine, 100 IU $ml^{-1}$ of penicillin, and 100 $\mu g$ $ml^{-1}$ of streptomycin.

### Experimental Procedure

The growth of HPLF cells was examined with a light microscope before beginning 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, Oxnard, California). One root slice was placed, with the apical surface up, in each well. One milliliter of cell suspension was added carefully over the root slice. In addition, 1 ml of cell suspension was dispensed in wells that contained glass slides to act as a negative control. For the positive-control group, 0.5 ml of methyl methacrylate 2% (vol/vol) was added to the cell suspensions before being dispensed into the wells. This material caused absolute inhibition in the cell attachment to the plastic surface. The tissue-culture clusters were incubated at 37°C in an environment of 75% relative humidity for 4, 8, and 24 h. On completion of incubation, the specimens were prefixed with a few drops of 0.1% glutaraldehyde in cacodylate buffer (pH 7.2) for 5 min and then in 2% glutaraldehyde for a period of 30 min. The specimens were dehydrated and washed sequentially in a series of 50%, 70%, 90%, and 95% ethyl alcohol and twice in absolute ethyl alcohol for 30 min before they were critical point dried with $CO_2$ (Samdri - PVT - 3B, Tousimis Research Corp., Rockville, MD). Specimens were mounted on copper grid and coated with gold (Jeol Fine Coat, Ion Sputter JFC-1100) for 3 to 5 min. They were viewed carefully under a scanning electron microscope (Jeol, JSM, T330A, Electron Optical Laboratory, Japan) at an accelerating voltage of 25 kV. Several photomicrographs were taken at various magnifications to ensure that representative data of micrographs were collected.

The cytoplasmic surface extensions formed by cultured fibroblasts were given these terms (11):

- Filopodia: a cylindrical or conical process of smaller diameter; these processes were often long (up to 10–20 $\mu m$).
- Microvilli: the process of smallest diameter (0.1–0.2 $\mu m$).
- Lamellipodia: the flattened extensions usually approximately 0.1- to 0.5-$\mu m$ thick.
- Blebs: spherical or hemispherical and usually 1 to 2 $\mu m$ in diameter.

## RESULTS

### Control

The 4-h incubation of the negative control showed large number of fibroblasts. These cells were round to discoid in shape with microvilli or small blebs covering their surfaces. They were attached to the glass slides with filopodia. As the incubation period increased, the cells appeared flat with smooth surface and firmly attached to the substratum with lamellipodia [Fig. 1 (A and B)]. The positive control showed few round cells at 4-h incubation. They have less smooth surfaces with many rough blebs and microvilli. Most cells lack attachment to the substratum. The size of the cells increased as the incubation period increased and very few were attached to the glass slides via microvilli and filopodia [Fig. 2 (A and B)].

### MTA Material

In the freshly mixed samples and during the three observation periods, the cells were round, less in density, and lacked attachment to MTA. The cell surface appeared less smooth with many vacuoles and few blebs [Fig. 3 (A and B)]. Flattened and spread cells were seen occasionally. The 24–h-set samples showed round and flattened cells with smooth surfaces and appeared to be tightly attached to MTA with filopodia and lamellipodia [Fig. 4 (A and B)]. Round cells with vacuoles and small blebs were seen rarely. As the incubation period increased, fibroblast cells increased in number and became tightly attached to the substratum.

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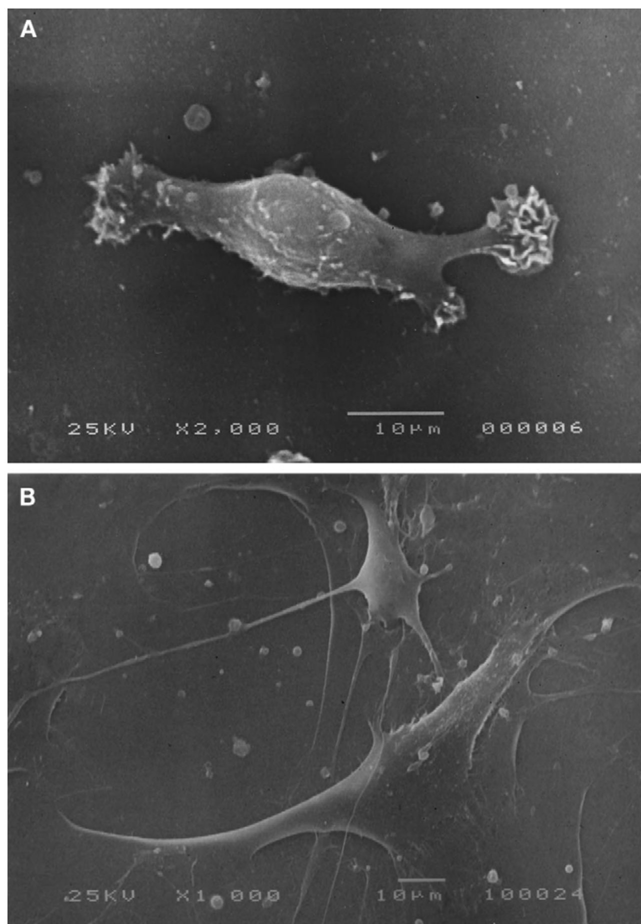


FIG 1. Scanning electron micrograph of HPLF attached to the glass slide acted as negative control. (*A*) 4-h observation: spindle-shaped cell with a smooth surface and ruffles was attached with lamellipodia (original magnification ×2000). (*B*) 24-h observation: spread cells with a smooth surface and ruffles attached firmly to the substratum with filopodia and lamellipodia (original magnification ×1000).

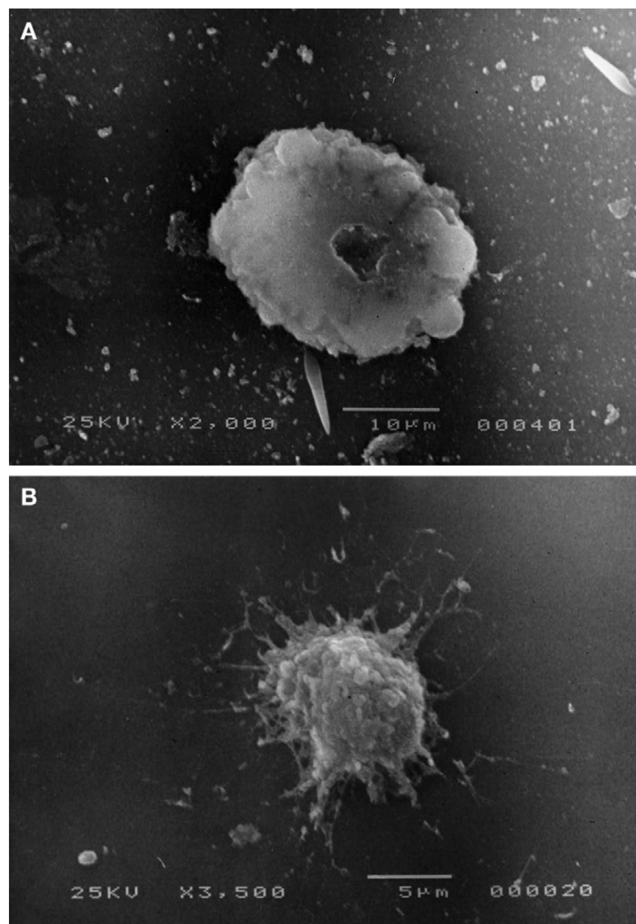


FIG 2. Scanning electron micrograph of HPLF attached to the glass slide acted as positive control. (*A*) 4-h observation: roughly discoid-shaped cell covered with blebs (original magnification ×2000). (*B*) 24-h observation: discoid-shaped cell covered with blebs and attached to the substratum with filopodia (original magnification ×3500).

## DISCUSSION

Retrograde root-filling material, when placed in contact with periradicular tissue, should possess the highest possible biocompatibility. Safavi et al. (12) described the quality and quantity of cell attachment to the retrofilling materials as an indication of the materials biocompatibility. Zhu et al. (13) suggested that cell adhesion and spreading on root–end-filling materials could be used as a criterion for evaluation of root–end-filling materials. A disadvantage of commonly used in vitro biocompatibility testing system is the fact that in such assays, only the material's cytotoxicity is studied. Other factors such as the material's physical structure and surface characteristics, known to influence the tissue response to the materials, are rarely considered (14, 15). HPLFs were used in this study to simulate the clinical environment. Studies evaluating the cytotoxicity of MTA have used primary and established cell lines (7–9). Established cell lines have the advantage of enhanced reproducibility of results and are recommended by the ISO for preliminary cytotoxicity screening. For specific sensitivity testing to simulate the in vivo situation, primary cell strain derived from living tissues are necessary (16) and also are recommended by the ISO.

Adhesion and spreading of the cells on a material surface are the initial phase of cellular function. The major events in this phase are the attachment of the cell to the substratum, radial growth of filopodia, cytoplasmic webbing, and the resultant flattening of the cell (17). Similar observation was noticed with the set samples of MTA and the negative control during the three observation periods. In general, the positive control and fresh sample of MTA showed changes in the cell morphology at 4 h in which cells appeared round, almost detached from the substrate, and had vacuoles in their surface. The extent of vacuolization was proportional to the incubation period. The persistence of rounded cells with little or no spreading suggested that the surface material might be toxic (18). Lettre (19) and Berliner et al. (20) using phase contrast microscopy, studied the action of some drugs on fibroblasts in culture and reported that as the cells become round in shape, cell processes shorten, vacuoles appear, and bubbling occurs at the cell surface. Observations made in this study confirmed these findings using scanning electron microscope.

The cytotoxicity of fresh samples of MTA during the three observation periods could be caused by the presence of leachable and toxic components in this material. Such toxic products affect both the morphology and the attachment behavior of the cells.

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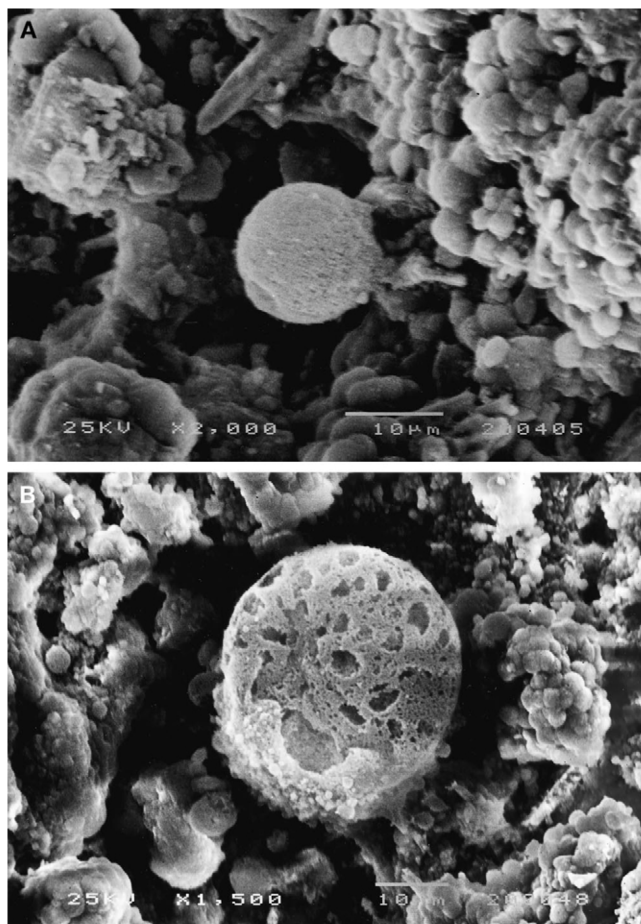


FIG 3. Scanning electron micrograph of HPLF attached to the freshly mixed MTA. (*A*) 4-h observation: round-shaped cell had small vacuoles on its surface and attached lightly to the substratum (original magnification ×2000). (*B*) 24-h observation: round-shaped cell had large vacuoles and small blebs on its surface (original magnification ×1500).

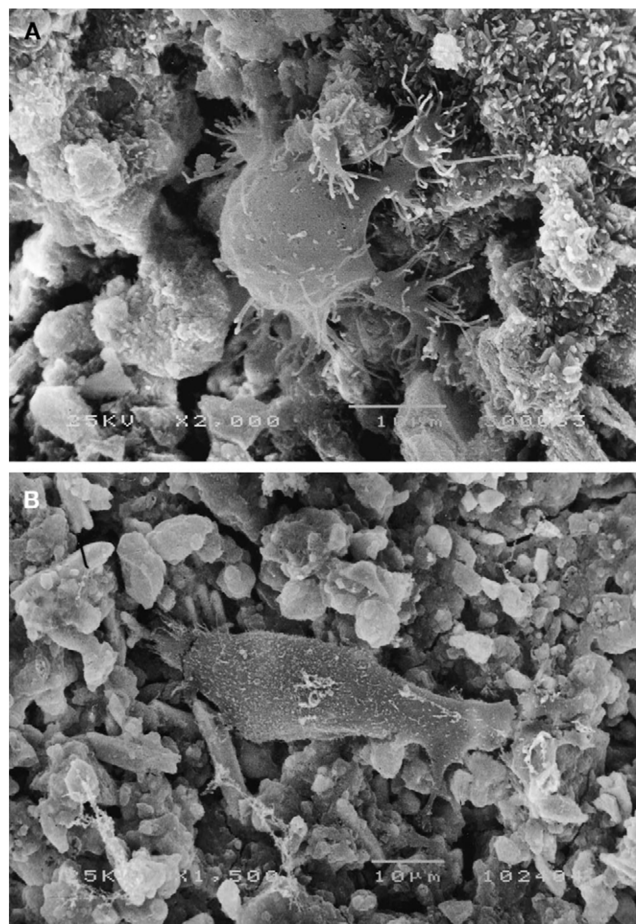


FIG 4. Scanning electron micrograph of HPLF attached to 24-h set sample of MTA. (*A*) 4-h observation: discoid-shaped cell covered partially with ruffles and was attached to the MTA with lamellipodia and filopodia (original magnification ×2000). (*B*) 24-h observation: roughly spindle-shaped cell with a smooth surface and ruffles attached firmly with lamellipodia (original magnification ×1500).

Vacuolization of the cytoplasm is a common finding in injured cells and could be caused by the uptake and storage of early toxic products by the fibroblast. The presence of blebs on the surface of injured cells may be caused by cytoplasmic shrinkage. Breaks of the cell membranes occurred in these blebs resulting in excretion of cell content including organelles (16).

The result of this study was in general agreement with Torabinejad et al. (7), who found that freshly mixed and set MTA was less toxic than amalgam, Super-EBA, and Intermediate Restorative Material when the radiochromium method was used. When the agar–overlay-assay method was used, the degree of cytotoxicity of fresh and set amalgam was significantly less than the materials used. The agar-overlay method prevents direct contact between the tested materials and the cultured cells. In addition, only material that contains liquid might diffuse through the agar and touch the cells. MTA, Super-EBA, and Intermediate Restorative Material are materials mixed with liquid component that is different than amalgam. The radiochromium-release method provides direct contact between the tested materials and the cultured cells (16). It is more accurate than the agar-overlay method. In this study direct contact between the material and the cells was achieved. Similar results were reported by Osorio et al. (8) and Keiser et al. (9), who showed that MTA was less toxic to human periodontal ligament cells than Super-EBA at all concentrations in both the freshly mixed and 24–h-set states. This may be due to differences in experimental procedures and cell lines. This study supports the view that the quality and quantity of cell attachment to the retrofilling materials could be used as a criterion for evaluation of the toxicity of retrofilling materials.

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