ORIGINAL ARTICLE

Proliferation of epithelial rests of Malassez during experimental tooth movement

Nabeel F. Talic, BDS, MS,[a] Carla A. Evans, DDS, DMSc,[b] Jon C. Daniel, MS, PhD,[c] and A. E. Moneim Zaki, BChD, DDR, MScD, PhD[d]

Chicago, Ill

The epithelial rests of Malassez (ERM), remnants of Hertwig's epithelial root sheath, are found near the root surface in the periodontal ligament. The functional significance of the ERM is still unknown. The purpose of this study was to examine the behavior of the ERM during experimental tooth movement. Tooth movement was achieved in 12 male Sprague-Dawley rats (each, 120-200 g) by placing elastic bands between the maxillary right first and second molars. The left molars served as controls. The rats were killed after 6, 12, 18, 24, 60, and 72 hours. The mitotic activity of the ERM was assessed by injecting the animals with 5-bromo-2'-deoxyuridine (BrdU) 2 to 3 hours before killing by intracardial perfusion with 4% paraformaldehyde. The molar-bearing segments were dissected and processed for histological examination. The incorporated BrdU was detected by immunohistochemistry. The number of cells in each ERM cluster was counted in all groups. In the 18-, 24-, 60-, and 72-hour experimental groups, the cell numbers were significantly higher than in the controls. The surface areas of the ERM clusters were also measured in all groups, but only in the 18-, 24-, 60-, and 72-hour specimens were the areas significantly higher in the experimental than in the control groups. The ERM cells in the experimental specimens were labeled with anti-BrdU, while those in the controls were not. It was concluded that experimental tooth movement stimulates ERM cells to proliferate and increase in size. These increased activities of the ERM are consistent with a putative role for these cells in collagen turnover in the periodontal ligament that is accelerated during tooth movement. (Am J Orthod Dentofacial Orthop 2003;123:527-33)

Collagen is the main component of the fibrous elements in the periodontal ligament (PDL). The function of collagen fibers is to withstand tensile stresses, and the function of the proteoglycan component of the extracellular matrix is to withstand compressive forces. Degradation of old PDL fibers, synthesis of new fibers, and reorganization are required to achieve tooth movement and maintain the new tooth and bone relationships afer orthodontic procedures.[1]

The epithelial rests of Malassez (ERM) are remnants of Hertwig's root sheath. After the fragmentation of the root sheath, these cells become part of the PDL. Some cells are incorporated into the cementum, and some remain very near the root surface.[2]

The functions of the ERM in the PDL are not fully understood. It has been speculated that they have no physiological significance but only a pathological role in the PDL. It has been reported that ERM are involved in the development of radicular cysts.[3] However, more recent in vitro investigations have shown that the ERM secrete collagenase and might have a role in PDL remodeling.[4,5] Remodeling of PDL collagen is regulated by complex cell/cell and cell/matrix interactions that involve the synthesis of enzymes, specific enzyme activators, and tissue enzyme inhibitors. Collagen in the extracellular matrix is degraded into small peptides by an extracellular proteolytic enzyme—mammalian collagenase—that belongs to the metalloproteinase family.[6] Collagenase is known to degrade type I collagen molecules at specific sites by cleavage of the Gly-Ile peptide bond of the α 1 chain, and the Gly-Leu bond in the α 2 chain. Collagenase is normally synthesized as a proenzyme, which then requires extracellular activation by plasmin. Tissue inhibitors of matrix metalloproteinases are the major inhibitors of collagenase. The inhibitors bind to collagenase, irreversibly forming an enzyme inhibitor complex that cannot be reactivated.[7] Further degradation of the cleaved small collagen peptide is achieved by fibroblasts that recognize collagen fibril fragments and phagocytize them. The collagen fibril fragments are digested intracellularly by lysosomal enzymes in phagolysosomes.[1]

From the University of Illinois at Chicago.
[a]PhD candidate, Department of Anatomy and Cell Biology.
[b]Professor and head, Department of Orthodontics.
[c]Associate professor, Department of Oral Biology.
[d]Professor, Departments of Oral Biology, and Anatomy and Cell Biology.
Reprint requests to: Dr Nabeel Talic, University of Illinois at Chicago, College of Dentistry, Department of Orthodontics, 801 S Paulina, M/C 841, Chicago, IL 60612; e-mail, ntalic@aol.com.
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It has been shown that ERM synthesize and secrete latent collagenase, while periodontal fibroblasts synthesize collagen and a collagenase inhibitor.[4,5] Furthermore, the fibroblasts do not produce collagenase after isolation and separation of each cell type in vitro.[4,5] This study led to proposing the following model system for the regulation of collagen degradation in the PDL. The ERM in the PDL secrete latent collagenase into the extracellular matrix for transformation to active collagenase by enzymatic cleavage. After degradation of the collagen molecule into three-fourth and one-fourth fragments by collagenase, fibroblasts then phagocytize the small peptide fragments. A feedback regulatory mechanism initiates synthesis and secretion of collagenase inhibitor by periodontal fibroblasts; it binds to the active collagenase enzyme to form an inactive enzyme-inhibitor complex.[4,5]

The aim of this study is to evaluate the mitotic activity and behavior of the ERM during experimental tooth movement. An increase in activity during tooth movement could clarify whether the ERM have a physiological function.

MATERIAL AND METHODS

Tooth movement was achieved in 12 male Sprague-Dawley rats (each, 120-200 g) with elastic bands placed between the maxillary right first and second molars.[8] The elastic band produced a wedging effect, causing the maxillary first molars to migrate mesially, while the second molars moved distally. This created areas of compression and tension around the roots of the first and second molars. The contralateral sides served as controls. The rats were divided into 6 experimental groups of 2 rats each. The elastic bands were left in place for 6, 12, 18, 24, 60, and 72 hours. The rats were killed by intracardial perfusion with a fixative solution of 4% paraformaldehyde, pH 7.4. All procedures were approved by the UIC Animal Care Committee and were performed under anesthesia by mixture of ketamine (90 mg/kg) (Abbott Laboratories, North Chicago, Ill) and xylazine (5 mg/kg) (Burns Veterinary Supply, Rockville, NY), which also have an analgesic effect. After perfusion, the maxillae were dissected free, and a cut was made along the midline to separate the experimental and the control sides. Molar-bearing segments of alveolar bone were cut from each side and further fixed in separate jars containing 50 mL of 4% paraformaldehyde overnight at 4°C.

The specimens were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) (Sigma, St Louis, Mo), pH 7.2, for 6 to 8 weeks. The endpoint of decalcification was determined by periodic (every 2 weeks) radiographic examination. The decalcified specimens were dehydrated in ascending concentrations of ethanol and then marked and embedded in paraffin. Oblique serial 4 to 6-μm thick sections, including the crestal areas mesial and distal to the maxillary molars, were cut with a microtome. The oblique plane of sectioning was chosen to maximize the number of the ERM cells included in the sections because these cells are known to form a network near the cementum. The sections were mounted on L-poly-lysine coated glass slides (Corning, Acton, Mass), and every other slide was stained with hematoxylin and eosin.

The mitotic activity of the ERM was assessed with 5-bromo 2'-deoxyuridine (BrdU) (Boehringer Mannheim GmbH, Mannheim, Germany), a thymidine analogue, that is incorporated into newly synthesized DNA in all dividing cells, including the ERM, during the S phase (DNA synthesis) of the cell cycle. The incorporated BrdU was detected by immunohistochemical anti-BrdU monoclonal antibody (Boehringer Mannheim GmbH). The rats were injected intraperitoneally with 1 mL of BrdU per 100 g of body weight at a concentration of 10 mmol/L in phosphate buffer saline solution (PBS), pH 7.4, 2 to 3 hours before intracardial perfusion. The sections were deparaffinized in 2 changes of xylene of 5 minutes each. Then the sections were rehydrated in descending grades of ethanol. The slides were washed 3 times in PBS, pH 7.4, for 2 minutes each and then incubated with trypsin solution for 10 minutes. Subsequently, sections were incubated with 4 mol/L HCl for 15 minutes to denature the 2 DNA strands and expose the incorporated BrdU to the antibody, and then the sections were incubated in 3 changes of PBS for 2 minutes each to neutralize the sectioned tissue pH. The sections were incubated for 10 minutes with 50 to 100 μl PBS-based incubation buffer to block nonspecific binding. The sections were incubated with 1:50 anti-BrdU antibody in PBS for 45 minutes at room temperature in a dark, humid chamber. Then the slides were washed 3 times in PBS buffer and mounted with a water-soluble agent. The slides were examined under fluorescence microscopy with 488 nm excitation and a 515 long-pass filter for detection. The gingival stratified epithelium served as the positive control. One animal was not injected with BrdU before perfusion. Sections from that animal served as the negative control and were similarly processed with the experimental sections used for the detection of the anti-BrdU antibody.

The hematoxylin and eosin stained slides were examined by light microscopy to identify the ERM based on their morphology. The ERM's behavior during various times of experimental tooth movement was examined. The ERM cells in their characteristic clus-

Table I. Means and SDs of cell numbers and cluster surface areas in experimental and control groups

Group	Number of clusters	Cell number	Number of clusters	Surface area μ^2
6 hours				
Experimental	33	6.7 ± 2.2	38	312.4 ± 155.1
Control	33	5.6 ± 2.5	34	253.7 ± 110.2
12 hours				
Experimental	33	7.3 ± 1.9	32	367.5 ± 193.3
Control	33	6.1 ± 2.1	32	372.2 ± 198.5
18 hours				
Experimental	30	8.9 ± 2.8	30	505.4 ± 284.5
Control	30	6.6 ± 1.7	35	347.5 ± 149.5
24 hours				
Experimental	24	7.1 ± 2.1	37	580.9 ± 314.3
Control	24	5.5 ± 1.8	35	337.7 ± 128.3
60 hours				
Experimental	33	12.1 ± 5.1	38	505.4 ± 284.5
Control	33	7.2 ± 2.3	35	347.5 ± 149.5
72 hours				
Experimental	21	9.1 ± 4.1	31	781.1 ± 352.7
Control	21	6.1 ± 1.5	32	441.5 ± 188.8

ters were counted by using Image-pro software (MediaCybernetics, Des Moines, Iowa) with light microscopy, and the surface areas of the clusters were measured. With the independent *t* test, the mean numbers of cells in clusters and the surface areas of these clusters on the experimental side were compared with the controls at different time intervals. Also, the mean numbers of cells and surface areas of clusters in the experimental groups were compared with analysis of variance (ANOVA) and Bonferroni tests to determine the differences among the groups.

RESULTS

The means and SDs of ERM cell numbers and surface areas of clusters are shown in the Table I. The mean number of the ERM cells in a cluster on the experimental side in the 18-, 24-, 60-, and 72-hour groups was statistically different from the controls ($P < .01$) (Fig 1). The mean surface area of clusters of cells on the experimental sides was statistically different in the 18-, 24-, 60-, and 72-hour groups from the controls ($P < .01$) (Fig 2). ANOVA showed statistical differences between experimental groups in mean cell rest numbers and mean surface areas of clusters ($P < .01$). The Bonferroni corrected *t* test showed statistical differences in mean cell numbers between the 60-hour experimental group and the other groups. Furthermore, statistical differences in cluster surface area between the 6- and 12-hour experimental groups and the 24-, 60-, and 72-hour experimental groups were found ($P <$ .01). The mean surface area of clusters in the 18-hour experimental group was statistically different from the 72-hour experimental group ($P > .01$).

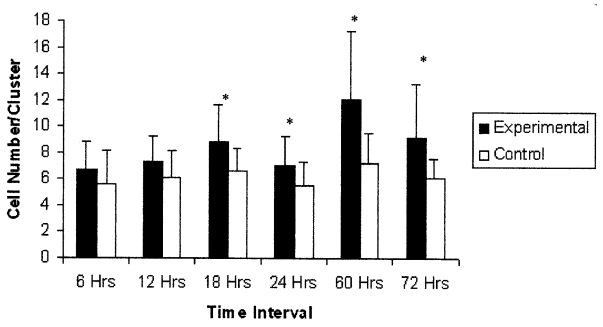

Fig 1. Mean ERM cell numbers from experimental and control specimens at different time intervals ($P < .01$).

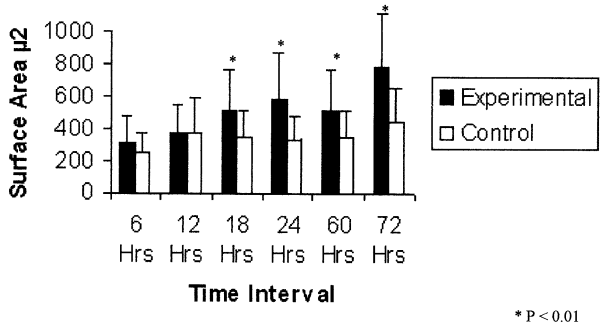

Fig 2. Mean surface areas of ERM clusters in experimental and control groups at different time intervals ($P < .01$).

On the experimental side, there was evidence of PDL narrowing at the marginal one third after 6 hours of force application. This narrowing is characteristic of a compression area due to tipping of the first and second molars. Hyalinization in the compression region was evident. Extravasations of red blood cells, on both the compression and the tension sides, were observed, but they were more extensive on the tension side. The ERM on the tension side were located near the root surface except in a few instances, where such cells were observed at a distance in the middle of the PDL. Thus the location of the ERM in the periodontium of teeth subjected to orthodontic forces was variable. In the 6-hour hyalinized regions, ERM were not observed (Fig 3), but, at later stages of remodeling during the 18-hour period, they were present in areas of compression (Fig 4).

In the 60-hour group, evidence of proliferation of the ERM was seen under light microscopy; they appeared as large clusters. The clusters consisted of

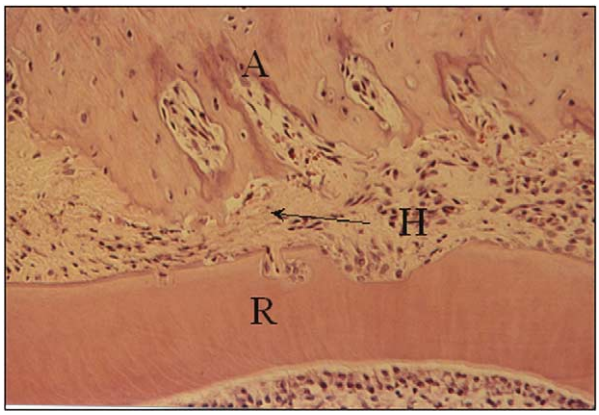

Fig 3. Photomicrograph showing narrowing of PDL in compression region. Lacunae of root (*R*) resorption can be seen in compression region with areas of hyalinization (*H*) in 6-hour experimental group (original magnification 160×).

numerous epithelial cells that appeared to be twice or triple the number in the control group. The epithelial cell size was also increased in the experimental side compared with the control side (Figs 5 and 6).

The ERM were labeled with anti-BrdU monoclonal antibody in the 60-hour experimental group; this indicated the incorporation of BrdU in the newly synthesized DNA during the S phase. There was no immunolabeling of the ERM on the control sides. The gingival epithelium in the same sections, which served as a positive control, was also immunolabeled (Fig 7). Negative controls for the immunolabeling procedure showed no labeling.

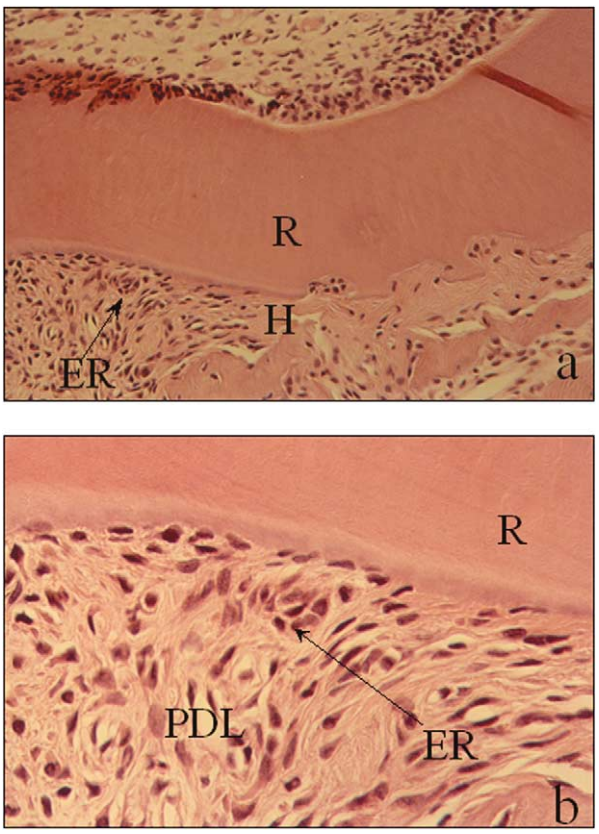

Fig 4. A, Low-power photomicrograph showing cluster of epithelial rests (*ER*) (*arrow*) in compression side of PDL very near root (*R*) surface in 18-hour experimental group. Note multinucleated cells in root resorption lacunae; *H*, hyalinization (original magnification 40×). **B**, Higher magnification of epithelial rests (*ER*) in compression side (original magnification 160×).

DISCUSSION

Mechanical stimulation of the ERM elicits responses that have been demonstrated in vitro.[9] In cell culture, ERM react to stimulation by proliferating, leading to an increase in number, a change in morphology, and an increased cytoplasmic volume.[10] The pathway of activation has been confirmed by the expression of cell surface epidermal growth factor receptors by the epithelial cells and the expression of keratinocyte growth factor in granulomas caused by the proliferation of the ERM. Keratinocyte growth factor is synthesized by fibroblasts.[11,12] The observed responses in this study agree with those reported in previous studies in which the ERM were stimulated by different ways.[13] The ERM in the experimental groups increased in number and size when compared with the controls. The proliferation of the epithelial cells was confirmed with immunofluorecence microscopy where the injected BrdU was incorporated in the newly synthesized DNA. In this study, the incorporation of BrdU in the proliferating ERM was observed in the 60-hour group, but other studies showed the incorporation of tritiated thymidine after 30 minutes of stretching in vitro.[8] This difference could be attributed to the sensitivity of detection of the BrdU in vivo. The ERM cell numbers in the 60-hour experimental group were statistically different from the other experimental groups as determined by ANOVA and Bonferroni tests. These increased numbers agree with the observed anti-BrdU detection in the same experimental group. There was a statistical difference in cell numbers between the experimental and the control groups at time intervals before the 60-hour group, but no BrdU was detected in these groups. However, that could be attributed to the sensitivity of BrdU detection at levels that are statistically different but biologically insignificant. The ERM cell numbers showed no statistical differences in the

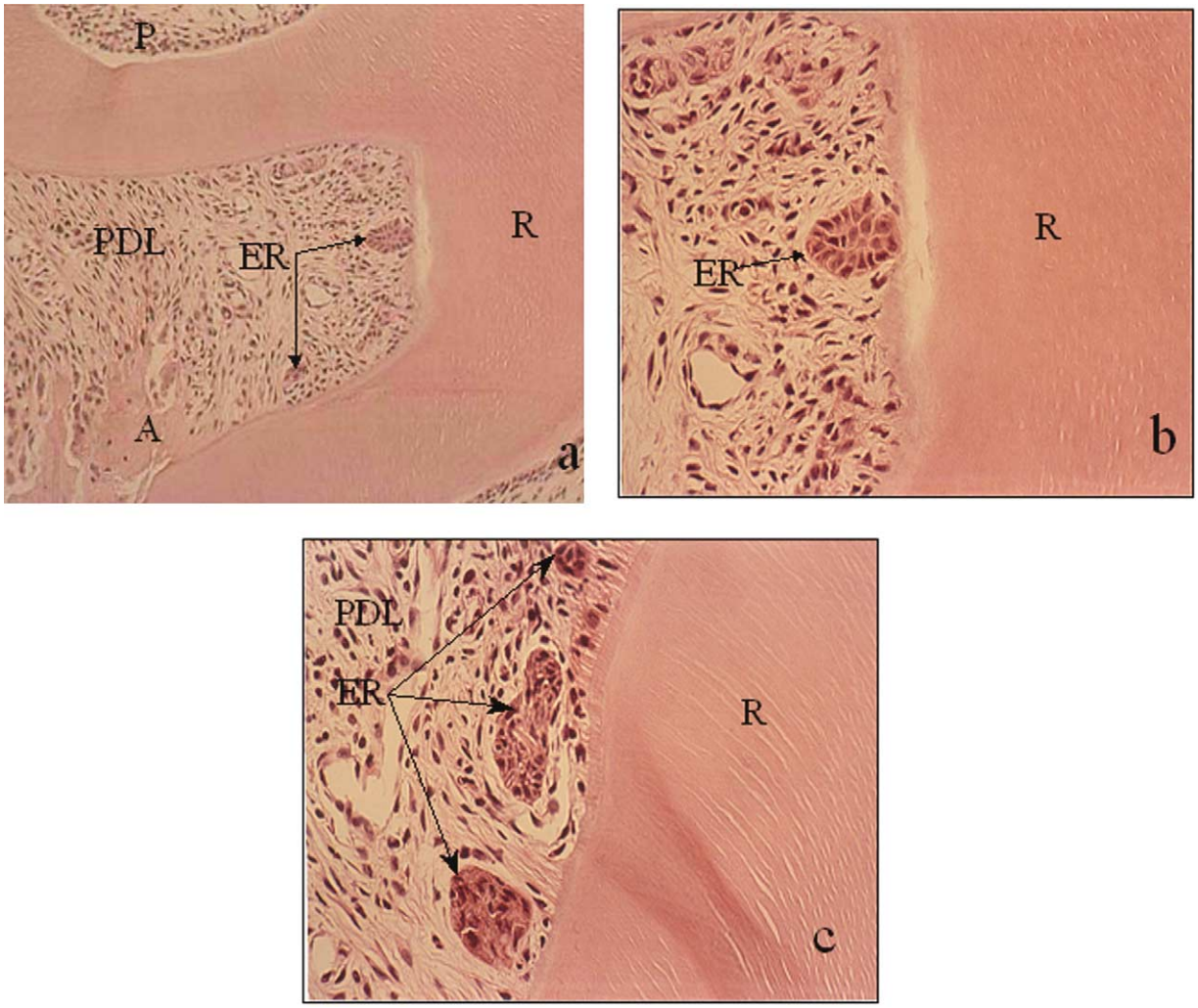

Fig 5. A, Photomicrograph showing increased number and cluster size of epithelial rests (*ER*) in PDL of 60-hour experimental group near root surface (*R*); *A*, alveolar bone; *P*, pulp (original magnification 60×). **B**, Higher magnification of epithelial rest cluster (*ER*) (original magnification 160×). **C**, Photomicrograph showing 3 enlarged epithelial rest clusters (*ER*) in tension side of 60-hour experimental group near root surface (*R*) (original magnification 160×).

6- and 12-hour groups; this might be explained by the fact that these time intervals are associated with inflammatory responses to forces. Furthermore, these early stages are associated with changes in vascularity and hyalinization of the compressed regions when no cells are present. It is to be expected that it takes time for these regions of the PDL to reorganize.

The increased numbers of ERM in the experimental groups where periodontal remodeling is accelerated suggest that these cells might be involved in remodeling activities. Because ERM synthesize collagenase,[4,5] they are natural candidates to initiate remodeling of PDL collagen fibers. In 1961, Reitan[14] reported that the ERM in humans and primates have the same fate as other periodontal cells in the hyalinized areas and that they did not reappear after healing of those areas. In the present study of rats, the ERM were seen in the compression area near the root surface in the 60-hour group. The ERM are arranged in strands or networks of cells. The reappearance of the ERM in the compressed areas is explained by the cells' ability to migrate from the surrounding undamaged areas of the PDL and their arrangement in strands and networks in the PDL. Also in this study, the ERM were seen in other spatially different locations relative to the root surface.

A rat model widely used in previous studies was also used in this experiment. This model has many similarities to the supporting structures in human teeth,

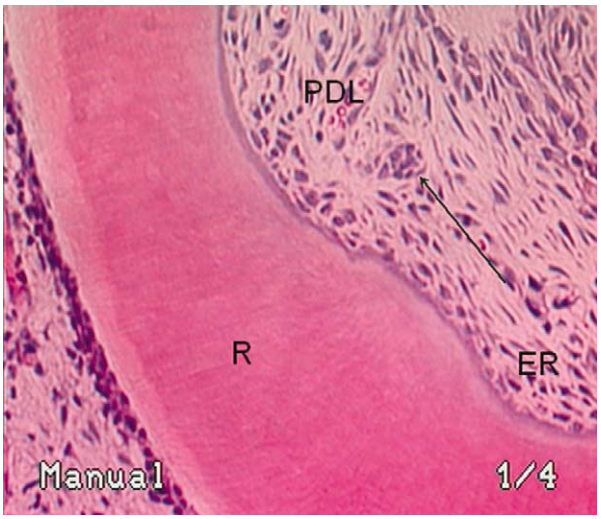

Fig 6. Photomicrograph showing epithelial rest cluster (*ER*) in PDL in 6-hour control group near root surface (*R*) (original magnification 160×).

but there are also some differences. For example, the alveolar bone in rats is denser and contains fewer marrow spaces. In addition, rats have fewer ERM than do humans and other animals.[15] These observations were confirmed by the present findings; the ERM clusters were abundant primarily in the root furcation and cervical regions. Future studies are needed to clarify the role of the ERM in collagen turnover, particularly during orthodontic tooth movement. The in vivo production of collagenase by these cells—essential for collagen degradation during active tooth movement and retention periods—needs additional investigation. These studies should try to demonstrate the production of collagenase by the ERM during experimental tooth movement. Future studies should also consider the fate of the proliferating ERM in the PDL after the elimination of the stimulus.

CONCLUSIONS

The ERM are groups of cells in the PDL near the root surface as remnants of Hertwig's root sheath that is present during early stages of root development. Their role in the PDL is still not fully understood. It has been shown in this study that these cells can be stimulated to proliferate by mechanical stresses in the PDL during orthodontic tooth movement.

The size of the ERM clusters also increased as observed under light microscopy. Furthermore, the increased proliferative activity was confirmed by the incorporation of BrdU into the newly synthesized DNA during the cell cycle. The reactions of the ERM to mechanical stresses are consistent with a significant role in the metabolism and remodeling of the PDL during orthodontic tooth movement.

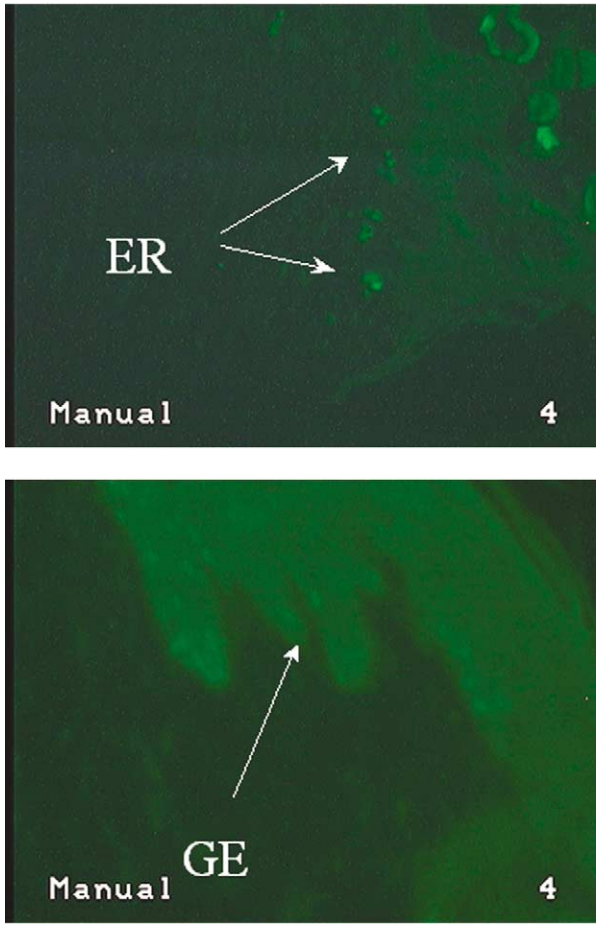

Fig 7. Photomicrograph of epithelial rests (*ER*) labeled with anti-BrdU, showing immunofluorescence in coronal region of PDL in 60-hour experimental group (original magnification 100×); **B**, photomicrograph showing immunolabeling of basal region of gingival epithelium (*GE*) using anti-BrdU antibody that served as positive control (original magnification 100×).

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