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ABSTRACT

Sublethal plasma membrane disruption (PMD) is an established mechanism for signaling in several cell types, including endothelial cells and skeletal muscle. We used a rat model of orthodontic tooth movement to test the hypothesis that periodontal ligament (PDL) cells communicate stretch to changes in bone cell activity in part *via* PMD. To produce PMD, we used a 50-g load from a spring activated in the buccal direction against the maxillary first molars for 5 min. Uptake of endogenous serum albumin was used as a PMD marker. Immunohistochemistry demonstrates albumin in PDL cells surrounding moved first molar tips. Image analysis shows significantly more albumin in cells of the buccal side (tension) of the moved teeth compared with those of the lingual, distal, and mesial sides, and those of the unmoved control. Albumin localization within cells of the PDL, after only 5 min of mechanical loading, suggests that PMD could promote uptake or release of signaling molecules.

KEY WORDS: mechanotransduction, orthodontic tooth movement, plasma membrane disruption, periodontal ligament.

Plasma Membrane Disruption in Orthodontic Tooth Movement in Rats

INTRODUCTION

Bone is a dynamic tissue that constantly undergoes remodeling. It is likely that the major reason for this process is to enable the bones to respond and adapt to the mechanical stress and strain that occur as a result of physiological function and during mechanical loading, as occurs during orthodontic tooth movement. Mechanical forces exerted on tooth roots and transmitted to the periodontal tissues initiate the remodeling activity that facilitates the movement of teeth through bone.

It has been shown that when mechanical stress is imposed on various tissues *in vivo*, transient, survivable disruptions of the plasma membrane are created. This form of cell injury has been termed “cell wounding”. Cells of the gut, skin, aortic endothelium, and skeletal muscle frequently suffer survivable plasma membrane disruption *in vivo* under physiological and pathological conditions of mechanical stress (McNeil and Ito, 1989, 1990; McNeil and Khakee, 1992; Yu, 1992).

Two important molecules in bone remodeling—IL-1 β (Auron *et al.*, 1984) and bFGF (Abraham *et al.*, 1986)—lack the signal peptide sequence, a prerequisite for protein secretion *via* the classic exocytotic pathway. Because of this, it has been proposed that these two polypeptides are released from dead, damaged, or injured cells (Klagsbrun and Vlodavsky, 1988; Young *et al.*, 1988). To date, there have been no studies linking the placement of orthodontic forces with plasma membrane disruption in cells of the PDL.

In the present study, we applied the plasma membrane disruption hypothesis to the cells of the periodontal ligament, a tissue frequently exposed to mechanical stress. We developed an *in vivo* rat model using an orthodontic device and endogenous albumin as a “wound marker”. These studies provide insight into this novel cellular mechanism, previously undescribed in the PDL.

MATERIALS & METHODS

All reagents used in this study were obtained from Sigma (St. Louis, MO, USA), except where indicated, and were of the highest quality available.

Tooth Movement

For this study, we used 15 Harlan retired breeder female rats with an average body weight of 300 ± 2 g (mean \pm SD). Rats were provided with feed and water *ad libitum*. Our animal protocol was reviewed and approved by the Committee for Care and Use of Laboratory Animals at the Medical College of Georgia. The animals were divided into two groups, one group with moved teeth and one control group with unmoved teeth, each composed of 7 animals. One additional control rat was used for Western blot analysis.

A round stainless steel orthodontic wire (0.018 inch, Sybron Dental Specialties, Glendora, CA, USA) was bent into a rectangular form with one helical loop to construct 7 customized uniform springs. The schematic occlusal view of the orthodontic appliance is shown in Fig. 1A, and a photograph of the spring placement is shown in Fig. 1B. We adjusted the initial expansion force for each rat

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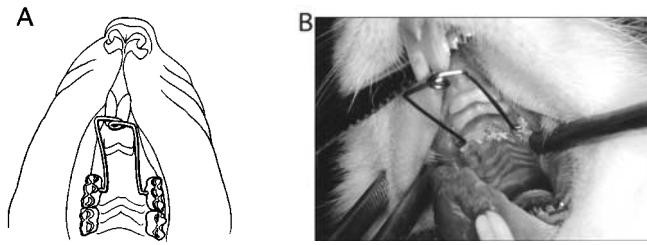


Figure 1. Orthodontic mechanics used in this study. **(A)** Schematic representation of orthodontic appliance for lateral movement of first upper molars. **(B)** Occlusal view of the activated appliance set on the rat maxilla. The initial expansional force was adjusted to 50 g.

by measuring the interproximal maxillary molar distance and adjusting each spring to exert a 50-g load upon activation. This supra-clinical force was used to accentuate differences seen between groups during the short duration of this study. Calibration of the individual springs was conducted by means of a Vitrodyne V100 Universal Tester (Chantillon, Greensboro, NC, USA). With the animals under anesthesia, a small circular depression was made in the enamel of the lingual side of the crown of the right and left upper first molars, by means of a dental handpiece operating at slow speed with a coarse round diamond bur. The springs were engaged in the depressions so that the appliance would not be dislodged during the five-minute experimental period and so that the force was delivered primarily in the lingual-to-buccal direction. Rats were not killed until 2 hrs after removal of the spring, allowing for either cell recovery or cell dissolution. The control group followed the same protocol, but no spring was placed across the maxilla.

Preparation of Sections

Under deep anesthesia, the animals were perfused transcardially with 120 mL of 0.02 M heparinized saline buffer for removal of as much extracellular albumin as possible. This was followed by further perfusion with 40 mL of 10% buffered formalin solution and thus death. Using a low-speed saw (Isomet Buehler Ltd., Lake Bluff, IL, USA) under water irrigation, we isolated the maxilla and removed soft tissue from the bone. Each half of the maxilla was placed in 10% formalin solution overnight. The specimens were demineralized in EDTA decalcification fluid (41.3 gm disodium EDTA, 4.4 gm NaOH in 1000 mL distilled water) for 6 wks at 4°C. Specimens were then dehydrated in ascending grades of ethanol, cleared in xylene, and embedded in paraffin. Serial 5- to 10- μ m-thick sections were cut following the occlusal plane through the molar roots from the mesial of the first molar to the distal of the third molar. These sections were cut from the lowest one-fifth of the root tip end, to represent PDL presumably exposed to a large tipping force with lingual compression and buccal tension. Sections were then mounted on microscope slides for immunohistochemical localization of albumin.

Immunohistochemistry

The tissue sections for localization of intracellular albumin were processed by means of a modified avidin-biotin-peroxidase technique (Hsu *et al.*, 1981; Borke *et al.*, 1987). Briefly, paraffin sections on poly-L-lysine-coated slides were deparaffinized in limonene and rehydrated in descending concentrations of ethanol. Endogenous peroxidase activity was blocked by five-minute incubation in 0.3% H₂O₂. Non-specific binding of antibodies to tissue sections was blocked by incubation for 1 hr in 10 mg/mL

casein. Sheep anti-rat albumin (Accurate Antibodies, Westbury, NY, USA) was applied to the sections at a dilution of 1:2000 for 1 hr. Control sections were processed with normal sheep serum substituted for the anti-albumin antibody. After sections were washed in PBS, a 1:200 dilution of the secondary biotin-conjugated rabbit anti-sheep Ig antibody (Vector Laboratories, Burlingame, CA, USA) was applied for 30 min. After being further washed in PBS, the tissue sections were incubated for 30 min in the avidin-peroxidase complex reagent (ABC reagent, Vector). Following additional PBS washes, the peroxidase molecule in the immobilized avidin-peroxidase complex was used to reduce H₂O₂ in the presence of diaminobenzidine tetrahydrochloride (DAB), to produce a brown reaction product over the sites of antibody binding to albumin. After being stained, tissue sections were dehydrated in ascending concentrations of ethanol to xylene, and coverslipped with Permount. Some sections were also counterstained with Mayer's hematoxylin for histological identification.

Western Blot Analysis

We used Western blot analysis to determine if there was cross-reactivity of the anti-albumin antibody with other proteins of the rat maxilla. Samples from the soft tissues of the rat maxilla were homogenized in a solution containing 10 mM Tris-HCl (pH 7.4), 0.5 mM dithiothreitol, 1 mM EDTA, and 5 mM benzamidine. Rat plasma was also processed for Western blot analysis as a control. Each sample was assayed for total protein by means of the BCA protein assay (Pierce Inc., Rockford, IL, USA). Samples were standardized based on protein concentration and combined 1:1 with a 2X sample buffer containing 100 mM Tris-HCl (pH 7.4), 4% sodium dodecylsulfate (SDS), 10% β -mercaptoethanol, 20% glycerol, and 0.2% bromophenol blue. Samples containing 20 μ g of protein were applied to each lane of a 7.5% SDS-polyacrylamide gel (Laemmli, 1970). Marker proteins of known molecular weight were loaded onto the same gel for comparison with sample proteins. After approximately 45 min of electrophoresis, proteins were transferred to nitrocellulose membrane *via* a Trans-Blot apparatus (Bio-Rad, Richmond, CA, USA). Transferred proteins were processed for immunodetection of albumin by an avidin-biotin-peroxidase technique similar to that used for immunohistochemistry. Briefly, the nitrocellulose membrane was rinsed in PBS (pH 7.4) at room temperature before incubation for 5 min in 3% H₂O₂. The blot was then rinsed in PBS and incubated with 10 mg/mL casein in PBS for 1 hr with gentle shaking. This solution was removed, and a 1:5000 dilution of sheep anti-rat albumin antibody in PBS was applied to the blot for 1 hr. The blot was washed thoroughly with PBS-0.05% Tween-20 (PBS-Tween) before incubation in a 1:200 dilution of biotin-conjugated rabbit anti-sheep antibody for 30 min. The blot was washed in PBS-Tween and next incubated with the avidin-peroxidase complex solution for 30 min. After being washed in PBS-Tween, the blot was incubated with H₂O₂ in the presence of the DAB substrate solution for 2 min, and the reaction was terminated with de-ionized water.

Quantitative Analysis of Intracellular Albumin

Densitometric evaluation of immunostained PDL was performed by means of an Olympus BX40 microscope (Olympus Optical Co, Ltd., Tokyo, Japan). Microscope illumination intensity and magnification remained constant during the acquisition of images *via* a mounted video camera. The first root of each sample was captured, and the optical densities of immunoreactions at the tension, compression, mesial, and distal sides of the most mesial root of the maxillary first

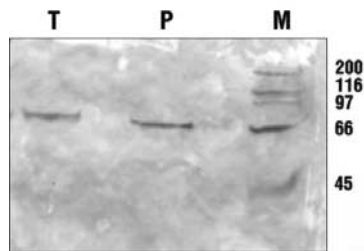


Figure 2. Western blot analysis demonstrating the specificity of the sheep anti-rat albumin antibody. Immunostaining of rat maxillary soft tissue homogenate (lane T) and rat plasma (lane P) shows single bands corresponding to the molecular weight of albumin. Lane M contains biotinylated marker proteins.

molar were measured by means of the Image Tool software (University of Texas Health Science Center, San Antonio, TX, USA). We obtained 12 measurements from each root (3 for tension, 3 for compression, 3 for mesial, and 3 for distal). Areas of identical size (64-pixel squares) were outlined in each image and digitized by the program to yield light intensity levels ranging from 0 (black) to 255 (white). The light intensity *per* outlined area was recorded and logged directly into the Excel program for further analysis.

Statistical Analysis

A nested repeated-measures factorial analysis of variance model was used to determine if differences in the mean density readings existed between areas of PDL surrounding the tooth roots and between control and treatment (moved teeth) groups. The following two hypotheses were tested. First, within each group (control and experimental), do differences exist between sides (lingual, distal, mesial, and buccal)? Second, within each side, do differences exist between groups? We used a Tukey multiple-comparison procedure to determine where differences within groups between sides or within each side between groups existed. The significance level was set at $p < 0.05$.

RESULTS

Western Blot Analysis

The specificity of the sheep anti-rat albumin antibody was confirmed by Western blot analysis (Fig. 2). Our blots showed a single band in both maxilla tissue homogenates and rat plasma corresponding to the known molecular weight of albumin (~ 66 kDa). No cross-reactivity was seen with proteins of either higher or lower molecular weight.

Immunohistochemistry

Serum albumin was used as an endogenous marker because its large size (~ 66 kDa) prohibits its uptake by cells during the short 5 min of exposure of the cells to the load. The presence of intracellular albumin in the cells of the PDL shows that plasma

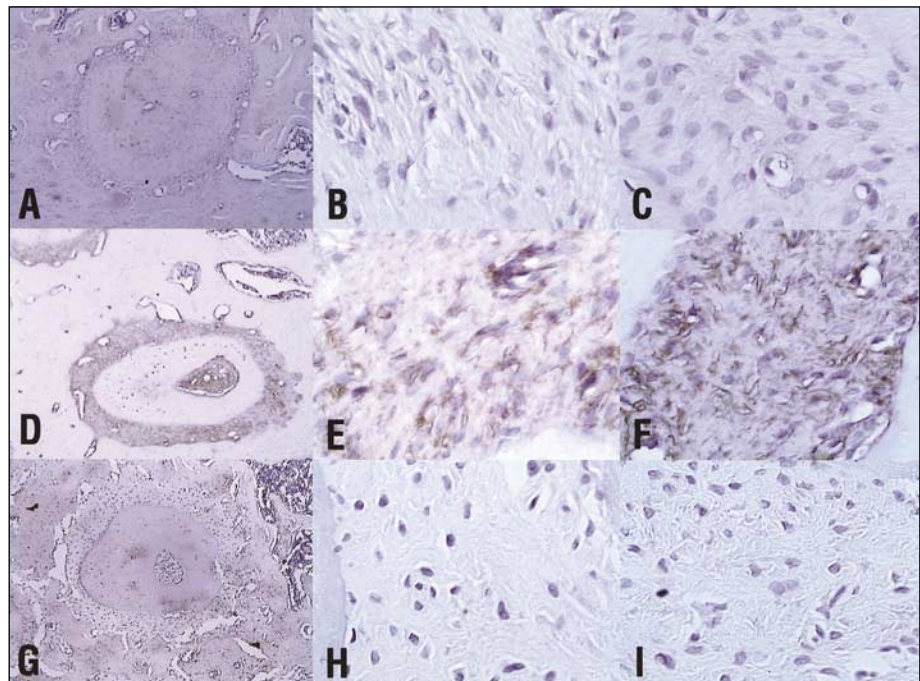


Figure 3. Immunohistochemistry of rat maxillary first molar root tips demonstrating cellular uptake of albumin. (A) Negative control section showing PDL stained by immunohistochemistry without anti-albumin antibody (x25). (B & C) Higher-magnification (x100) micrographs of buccal and lingual areas (respectively) from the same PDL seen in (A), showing no background staining. (D) Tissue section showing immunolocalization of albumin in the PDL from a tooth after 5 min of loading and 2 hrs of recovery (x25). (E & F) Higher-magnification (x100) micrographs of buccal and lingual areas (respectively) from the same PDL seen in (D), showing intracellular localization of albumin. (G) Tissue section from a control rat where teeth were not loaded, showing no apparent staining of the PDL (x25). This section was stained by immunohistochemistry with anti-albumin antibody. (H & I) Higher-magnification (x100) micrographs of buccal and lingual areas (respectively) from the same PDL seen in (G), showing no intracellular or background staining.

membrane disruption has occurred, or that uptake of the albumin preceded the application of force. No background staining was seen in tissue sections processed in the absence of the primary sheep anti-rat antibody (Figs. 3A-3C). This indicates that the other reagents used in the immunohistochemical localization studies did not cross-react with tissue molecules.

Fig. 3D shows a representative section of rat maxilla processed after exposure of the first molar for 5 min to a heavy spring exerting an initial force of 50 g. Immunohistochemical localization with anti-rat albumin antibody shows intracellular albumin in cells of the PDL (Figs. 3D-3F). Areas of the PDL under tension (buccal, Fig. 3E) and compression (lingual, Fig. 3F) show a broad distribution of cells demonstrating immunoreactivity for albumin. No albumin is seen in the extracellular connective tissue. Figs. 3G-3I show a representative cross-section of rat first molar root from a control tooth that did not undergo orthodontic tooth movement. These Figs. represent sections that were processed identically to those of the moved teeth represented by Figs. 3D-3F. No localization of intracellular albumin is seen in these control sections, suggesting that plasma membrane disruption occurred as a result of the applied force and did not occur prior to loading.

A statistically significant difference was found between groups ($p < 0.0001$) and between sides within the experimental group ($p = 0.0023$). No statistically significant differences were found between sides of the control group ($p = 0.4207$). For the experimental group, although the immunohistochemistry

Table. Semi-quantitative Analysis^a of Intracellular Albumin in Cells of the Rat PDL

Group	Mean	Std. Error
Control	0.00	0.83
Experimental	4.08 ^{a,b}	1.39
Side		
Lingual (compression)	0.90	1.77
Distal	1.26	1.83
Mesial	0.88	1.84
Buccal (tension)	5.11*	1.63
Group x Side		
Control		
Lingual	0.00	1.49
Distal	0.00	1.83
Mesial	0.00	1.84
Buccal	0.00	1.53
Experimental		
Lingual (compression)	1.81	3.23
Distal	2.52	3.23
Mesial	1.76	1.72
Buccal (tension)	10.23*	2.45

^a Analysis of variance results (total integrated density minus background) of intracellular albumin concentration. The values are means \pm Std. Error, $n = 7$. Statistical significance between groups and sides was determined by a nested repeated-measures factorial analysis of a variance model.

^b Asterisk (*) indicates values significantly higher than other values in each group as discussed in the text.

demonstrates the uptake of albumin by cells on all sides of the moved teeth, the buccal (tension) side shows a significantly higher mean density reading than the lingual (compression, $p = 0.0002$), the distal ($p = 0.0011$), and the mesial ($p = 0.0002$) sides. Comparison of buccal (tension) sides from control and experimental groups shows that the control group has a significantly lower mean density reading than the experimental group ($p = 0.0001$) (Table).

DISCUSSION

Many mechanisms have been proposed to explain the locally induced adaptive alveolar bone remodeling seen in orthodontic tooth movement (Burger and Klein-Nulend, 1999; Ducy *et al.*, 2000; Teitelbaum, 2000). It is clear that growth factors and cytokines are potential molecular mediators in this process (Hill, 1998). Two polypeptides known to be involved in bone remodeling, basic fibroblast growth factor (bFGF) (Abraham *et al.*, 1986) and interleukin-1 β (IL-1 β) (Auron *et al.*, 1984), lack the signal peptide sequence normally considered a prerequisite for secretion *via* the classic pathway (Walter and Lingappa, 1986). Disruption of plasma membrane integrity, if it occurs in cells *in vivo*, would constitute an additional molecular route to and from the cell cytoplasm for these and other important molecules. It has been observed that FGF is released from the cytoplasm of mechanically wounded cells by diffusion through disruptions in the plasma membrane (McNeil *et al.*, 1989; Clark *et al.*, 1993, 1995). Observations made in our laboratory using a similar model in the cranial suture support this hypothesis.

Coronal sutures from a week-old rat pup were subject to 0.59 N of tensile force by means of engaging a 40-g-load microspring for 5 min. The amount of bFGF present in the protein-free medium was significantly higher, approximately 150%, than that in the control (Yu *et al.*, 2001). These observations have led to the concept of bFGF as a "wound hormone". Growth factor release following mechanical disruption ensures that the repair process initiated by the growth factors is accurately localized to sites damaged as a result of cell stress. This "wound hormone" hypothesis, if applied to cell damage associated with orthodontic tooth movement, would provide a new explanation as to how bone remodeling is initiated in response to mechanical loads.

To investigate this hypothesis, we developed a rat model of orthodontic tooth movement. We used a spring design that was simple to construct and able to deliver a consistent force that was adjustable for calibration and to compensate for anatomical differences between rats in terms of the maxillary intermolar distance (Fig. 1). In our model, we considered the possibility of the applied force being diminished by transmission to the midline suture. However, this force was dispersed over a large area and is therefore negligible.

The forces used to produce orthodontic tooth movement in rats vary among studies and authors, even when the same method or device is applied. Heavy forces that produce tooth movement have been shown to range from 40 g to 60 g (Bridges *et al.*, 1988; Gibson *et al.*, 1992; Kyomen and Tanne, 1997; Shirazi *et al.*, 1999). In our model, we used 50 g of force.

In our studies, we followed the uptake by rat PDL cells of the ~ 66-kDa rat serum albumin molecule. We used the animal's native albumin as a probe by postulating that this molecule would be unable to permeate the phospholipid bilayer barrier in only 5 min unless a disruption of the plasma membrane had occurred. Intracellular albumin as a marker for cell injury has been used in different tissues, such as rat tricep, endothelial cells of the rat aorta, and cardiac myocyte of normal and overloaded rat heart (McNeil and Khakee, 1992; Yu and McNeil, 1992; Clark *et al.*, 1995; Fischer *et al.*, 1997). Although the intracellular albumin was observed on all sides of experimentally moved teeth, cytoplasmic staining with antibodies to endogenous albumin was significantly higher in cells from the tension side of the moved teeth when compared with the other sides within the same group and when compared with cells from the PDL of unmoved control teeth. This suggests that when high mechanical forces are imposed on the PDL, wounding of the plasma membrane occurs in numerous cells. Whether wounding also occurs with lesser force, permitting the passage of smaller molecules, seems likely but was not investigated in the present study.

The inclusion of 2 hrs of recovery time following the release of force ensured that if the cells had not resealed or had died, the intracellular albumin would have had sufficient time to diffuse from the cytoplasm. Further, the morphology of the PDL cells appeared essentially normal, also suggesting that the cells had survived plasma membrane disruption (Fig. 3).

The lack of intracellular albumin in our control group suggests that for membrane disruption to allow passage of a 66-kDa molecule may require a large mechanical force. The possibility remains open that normal occlusal forces may generate lesser disruptions.

In summary, the work described here provides the first *in vivo* evidence of a role for plasma membrane disruption in the mechanotransduction of orthodontic tooth movement. Studies in other tissues and species show that this cellular phenomenon occurs frequently *in vivo*. Our work suggests that this overlooked but important mechanism also occurs in cells of the PDL.

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