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Temporary loss of plasma membrane integrity in orthodontic tooth movement

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Abstract

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In these studies, a rat model of orthodontic tooth movement was used to support the premise that periodontal ligament (PDL) cells experience plasma membrane disruption and resealing events upon application of mechanical stress. Immunoelectron microscopy, showed albumin in the cytoplasm of PDL and bone lining cells in the tension side of moved molars. The intracellular localization of this large molecule (60 KDa) suggests that these cells have undergone plasma membrane disruption and resealing. To further assess these and previous findings, fluorescent dyes (FITC-dextran and rhodamine-dextran) were delivered into the vascular system followed by application of 50 g of static load. These large dextran molecules (10 KDa) were preferentially taken up by PDL cells of the buccal (tension side) of moved molars. These cells were determined to be viable since dead cells do not retain these diffusible tracers. These studies provide evidence of a novel cellular mechanism for uptake and release of molecules and suggest a potential role for plasma membrane disruption in the mechanotransduction of orthodontic tooth movement.

Key words: orthodontic tooth movement; periodontal ligament; plasma membrane disruption

Introduction

Bone is continuously being modeled and remodeled to maintain volume, mechanical strength and calcium homeostasis. These macro- and microscopic changes in bone morphology depend greatly on

physical circumstances such as mechanical stress (1). During orthodontic tooth movement, the application of force will generate structural and bioche mical changes in the periodontal ligament (PDL) leading to remodeling of the surrounding alveolar bone (2).

Mechanotransduction, the process by which mechanical energy is converted into biochemical or electrical signals, could be mediated by several mechanisms. Cell-extracellular matrix interactions, cytokines, second messenger transmission trough gap junctions and intercellular adhesive junctions have been proposed to form a network that coordinates cellular responses in tissue remodeling (3). Several molecules such as nitric oxide and glutamate have also been implicated as mediators of mechanical signaling in bone (4, 5).

The precise mechanism by which bone cells convert mechanical stress into biochemical responses that ultimately lead to bone remodeling still remains unclear.

Temporary, survivable plasma membrane disruptions induced by mechanical stress have been established in many mammalian tissues. These sub-lethal 'cell woundings' are a commonplace occurrence in mechanically challenged tissues. The epidermis of the skin, aortic endothelium, gut epithelium, as well as cardiac and skeletal muscle undergo cell injury under physiological and pathological conditions (6). A rapid resealing response has been proposed as an important cellular adaptation to cope with these events and prevent disruption-induced death (7, 8). It is, therefore, logical to hypothesize that the PDL, a tissue subjected to mechanical stress, would experience plasma membrane disruption.

Cells wounds created by plasma membrane disruption are large enough for macromolecules such as serum albumin (66 KDa) to enter cells and become trapped by resealing. Previous research has demonstrated plasma membrane disruption events in the PDL using rat serum albumin as an endogenous 'wound marker' and light microscopic immunohistochemistry (9). In the present study, we provide further evidence through confocal and immunoelectron microscopy of 'cell wounding' in the PDL after orthodontic tooth movement as demonstrated by the uptake of fluorescent dextrans and endogenous albumin.

Material and 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

All procedures involving animals were reviewed and approved by the Committee for Care and Use of Laboratory Animals at the Medical College of Georgia. Fourteen Harlan Sprague Dawley retired breeder female rats were used in this study (average body weight of 300 g). Animals were provided with food and water *ad libitum*. The orthodontic device placed in our rodent model was built as described in our previous study (9). Briefly, a round stainless steel orthodontic wire (0.018 in, Sybron Dental Specialties, Glendora, CA, USA) was bent to construct rectangular springs with one helical loop (Fig. 1A,B).

The springs were designed so that an activation of approximately 4 mm would deliver 50 *g* of lateral force to the first maxillary molars. The initial expansion force was adjusted by measuring the maxillary intermolar distance of each animal and customizing each spring accordingly. Calibration of the springs to ensure appropriate force delivery was conducted with a Vitrodyne V100 Universal Tester (Chantillon & Sons, Greensboro, NC, USA). With the animals under anesthesia, a small circular depression in the enamel of the lingual side of the crown of the right and left upper first molar was made utilizing a small round diamond burr in a dental hand-piece at slow speed. The appliances were engaged in the depressions so that they would not be dislodged during 5 min of spring activation and so that the force would be delivered predominantly in the lingual to buccal direction. The ipsilateral third molar was used as a control to evaluate the cellular response to mechanical stress.

Immunoelectron microscopy

Four animals were evaluated with immunoelectron microscopy. After spring removal, the animals were perfused transcardially with 120 ml of 0.02 M saline buffer for removal of extracellular albumin. This was

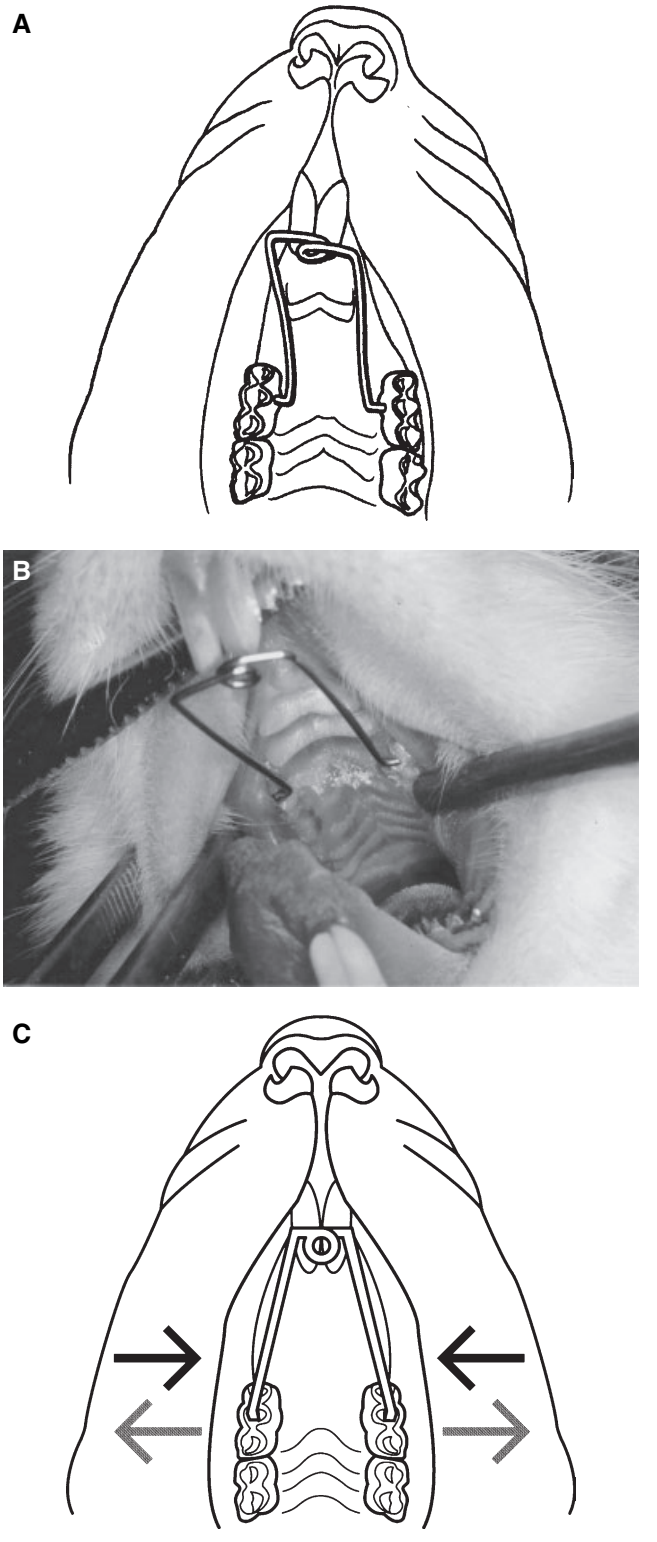

Fig. 1. (A) Schematic representation of the orthodontic appliance used for lateral movement of first upper molars. (B) Occlusal view of the activated appliance set on the rat maxilla. The initial expansion force was adjusted to 50 g. (C) Schematic representation of the orthodontic appliance used for the double label experiments. The lighter arrow represents the lingual-buccal movement of the first spring. The darker arrow shows the buccal-lingual direction of the second spring.

followed by further perfusion with 40 ml of 4% glutaraldehyde. Using a low-speed saw (Isomet Buehler Ltd, Lake Bluff, IL, USA), the maxilla was isolated and soft tissue removed from the bone. Each maxilla yielded two hemi-maxillae samples. Samples were fixed in 2% gluteraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 and decalcified for 18 days (EDTA, 2% gluteraldehyde in 0.1 M phosphate buffer pH 7.2). Specimens were then fixed in 1% osmium tetroxide in distilled water and embedded in EM ged 812 (Electronic Microscopy Sciences, Fort Washington, PA, USA). The most distal root of the first upper molar was isolated and cut in 1 μm thick sections with glass knives. Ultrathin sections (120 nm) were cut with an ultramicrotome (Reichert Scientific Instruments, Buffalo, NY, USA), stained with a mixture of equal parts acetone and water-saturated uranyl acetate, and mounted on nickel 200 mesh thick/thin grids coated with a formvar membrane. Non-specific binding sites were blocked by placing sections in 1% casein in phosphate buffered saline solution for 30 min at room temperature. After excess blocking solution was removed, sections were incubated overnight at 4°C in 1:1000 specific goat anti-rat serum albumin (Nordic Immunological Labs, San Clemente, CA, USA). After rinsing and blotting, grids were next immersed in 1:20 dilution of 10 nm colloidal gold-conjugated antibody (Chemicon International, Temecula, CA, USA) for 2 h at room temperature. Samples were then washed with distilled water and counter-stained with uranyl acetate before viewing with a Zeiss EM902 electron microscope.

Fluorescent microscopy

Fluorescein isothiocyanate (FITC)-dextran (FDx) and rhodamine-dextran (RDx) (MW 10 KDa) were used as 'wound markers'.

Four animals were used for the single label fluorescence microscopy study; 12.5 μg/ml FITC-dextran/saline solution was delivered by means of an infusion pump (Harvard Apparatus, South Natick, MA, USA) into the vascular system *via* the right carotid artery for 1 min followed by 5 min spring activation. Upon spring removal, the animals were perfused with isotonic saline solution followed by formaldehyde perfusion. Portions of the maxilla containing the roots were mounted on microscope slides. Cells of the PDL

that suffered membrane disruption, and thus became labeled with FDx, were identified and photographed on a confocal scanning laser microscope (Molecular Dynamics System, Sunnyvale, CA, USA) and a Nikon Diaphot 200 inverted microscope at the Medical College of Georgia Imaging Core Facility.

Four animals were used for the double label experiments. Two different springs were made to be placed in the occlusal surface of the molars (Fig. 1C). One spring moved the same molar from lingual to buccal and the other from buccal to lingual to create two opposite tension and compression areas. Saline solution containing FDx was delivered in the circulation for 1 min before activation of the first spring; after 5 min the spring was removed followed by infusion of 5 ml of saline solution to wash any excess dye from the vasculature. A second dye RDx/saline solution was infused for 1 min followed by 5 min of spring activation to move molars in the opposite direction. After removal of the spring, the vasculature was rinsed with saline solution followed by formaldehyde. Each dye was delivered for a total of 6 min. The fluorescence of the rhodamine dye was excited at 570 nm and the emitted light captured with a 595/30-nm bandpass filter. The fluorescence of the FITC-dextran was excited at 488 nm and captured with a 530/30-nm bandpass filter.

For the FDx/Hoechst experiment, FDx was delivered and a spring was activated as described above from the lingual to the buccal direction. After spring removal, 10 μg/ml of Hoechst 33342 dye was infused for 3 min. Animals were perfused with saline solution followed by formalin before viewing with a Zeiss LSM 510 confocal laser scanning microscope equipped with a Coherent Mira 900 tunable Ti: sapphire laser for multi-photon excitation.

Results

Immunoelectron microscopy

Serum albumin has been previously used as an endogenous marker for plasma membrane disruption (10, 11) because of its large size (66 kDa) it is not found within cells unless wounding and resealing events have taken place. In our preceding work, immunohistochemistry revealed significant amounts of serum albumin in the tension side of the PDL of moved maxillary first molars (9). In this study, we used electron microscopic immunogold histochemistry to localize intracellular albumin in PDL cells after exposure to mechanical stress.

Gold particles were observed in fibroblasts in the tension side of the root of moved molars. This labeling was seen in the cytoplasm, nuclei and nuclear membrane. The presence of gold particles in the nuclei and nuclear membrane is likely a consequence of albumin, already in the cytoplasm due to plasma membrane disruption, going through the 70 nm nuclear pores (Fig. 2A,B).

Cells in the experimental tissue appear neither necrotic nor apoptotic when compared with the third molar ipsilateral control tooth, suggesting that nonlethal plasma membrane disruptions had occurred in the cells of the PDL. No gold particles were detected in any of the cytoplasmic organelles.

Fluorescence microscopy

Plasma membrane disruption can be detected by measuring the influx of a normally impermeant fluorescent marker. Exogenous 'wound reporters' FDx (green) and RDx (red) (MW 10 KDa) were used to confirm our observations made with the endogenous albumin (9).

When 50 g of force was applied to the first upper molars in the presence of FDx, fibroblasts from the tension side exhibited intense fluorescence for FDx. These must be viable cells, since dead cells do not retain this freely diffusible tracer (Fig. 3A). The density of FDx-positive cells was qualitatively and quantitative larger in the tension side of moved molars when compared with the other sides and to the unmoved control (Fig. 3B,C).

To further correlate the uptake of fluorescent tracers with PDL cell wounding and resealing events, mechanical stress was applied to the molars in the presence of both RDx and FDx. After injecting FDx, molars were subjected to a lateral tipping force from the lingual to buccal direction for 5 min. After flushing with saline solution, RDx was administered followed by activation of the second spring to move the molars in the oposite direction. The spatial distribution of cells containing these fluorescent dyes was consistent with the direction of the applied force. In the combined FDx and RDx images, the areas with labeled cells correspond clearly with the tension area when

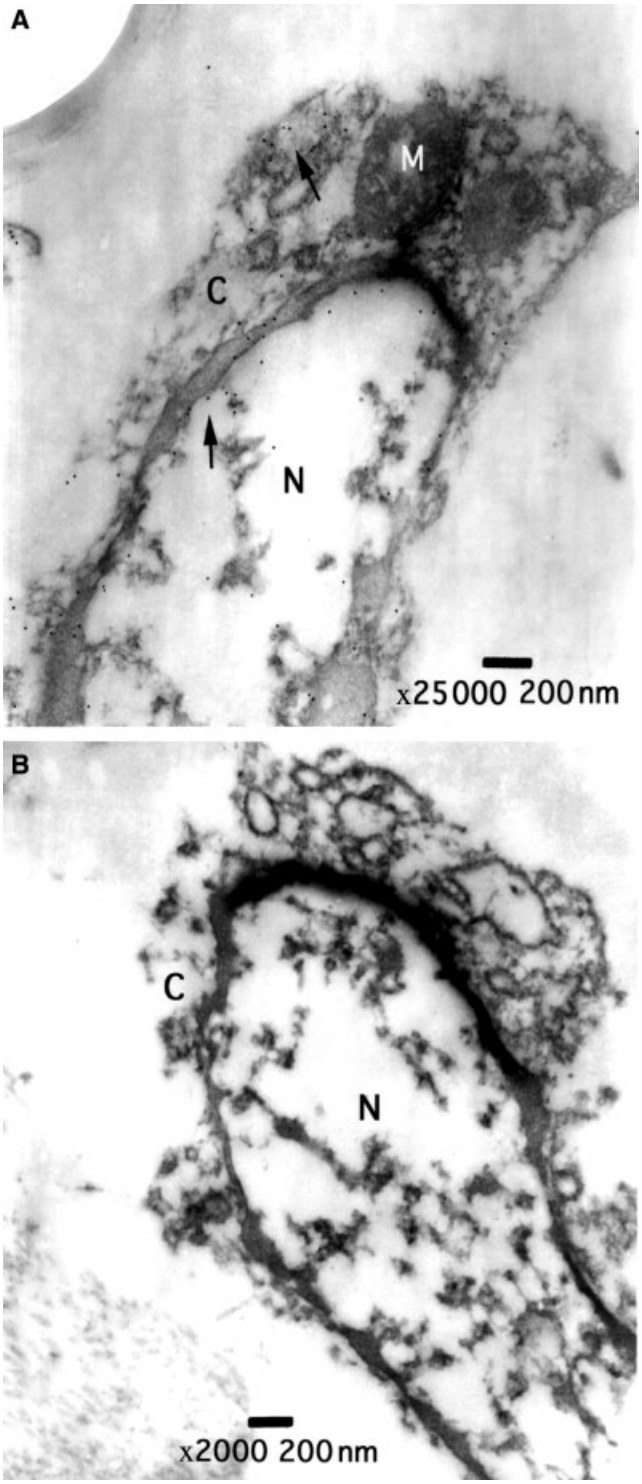

Fig. 2. (A) Transmission electron micrograph of rat molar fibroblast, after 5 min of a 50 g spring activation. Tissue sections were incubated without anti-rat serum albumin antibody followed by 10 nm colloidal gold-conjugated antibody. Gold particles (arrows) are associated with the cytoplasm and nuclei but not mitochondria. C, cytoplasm; M, mitochondria; N, nuclei. Magnification ×25000. (B) Transmission electron micrograph of rat molar fibroblast, after 5 min of a 50 g spring activation. Tissue sections were incubated without anti-rat serum albumin antibody. C, cytoplasm; M, mitochondria; N, nuclei. Magnification ×20000.

the red or green dye was present, cells with yellow cells are those that incurred and survived the two plasma membrane disruptions (when molars were moved in the two directions) and hence retained both the red and green dyes (Fig. 3D). Possible disruption caused by injecting the dyes through the sulcus or the gingiva was avoided by delivering them through the carotid artery. This technique also prevents the uneven distribution of the dyes throughout the maxillary tissues. Third molar roots were used as a control within the same animal and did not reveal any labeled cells in the side corresponding to the tension in the experimental group.

The nuclei acid marker Hoechst 33342 was delivered to the circulation in the presence of FDx to confirm our prediction that these fluorescent tracers entered the cell cytoplasm after cell stretching. The association of the FDx dye with the nuclei is seen in Fig. 4A. The intracellular localization of the FDx was confirmed by switching to bright field without moving the specimen during confocal observation (data not shown). Micrographs of the lingual (tension) side of moved molars taken with a two photon confocal laser scanning microscope showed the specific localization of FDx around the blue (Hoechst 33342) stained nuclei (Fig. 4A). Lingual side of third molars on the same sections (these teeth had not been subjected to orthodontic force) was used as a control. A micrograph from the lingual side of a third molar root (control) revealed the cell nuclei (blue stained) but not FITC-dextran (Fig. 4B).

Discussion

Temporary loss of the plasma membrane integrity is a common event and represents a normal physiologic mechanism for cells residing in mechanically challenged environments (6). Rapid resealing of the disruptions permits survival and, therefore, constitute an important cellular adaptation (7). The mechanism used to reseal could differ in relation to the length of the injury (12).

Mechanotransduction from plasma membrane disruption is explained through the 'wound hormone' hypothesis: growth factors and other molecules can flux through the membrane wound eliciting changes in gene expression (13, 14).

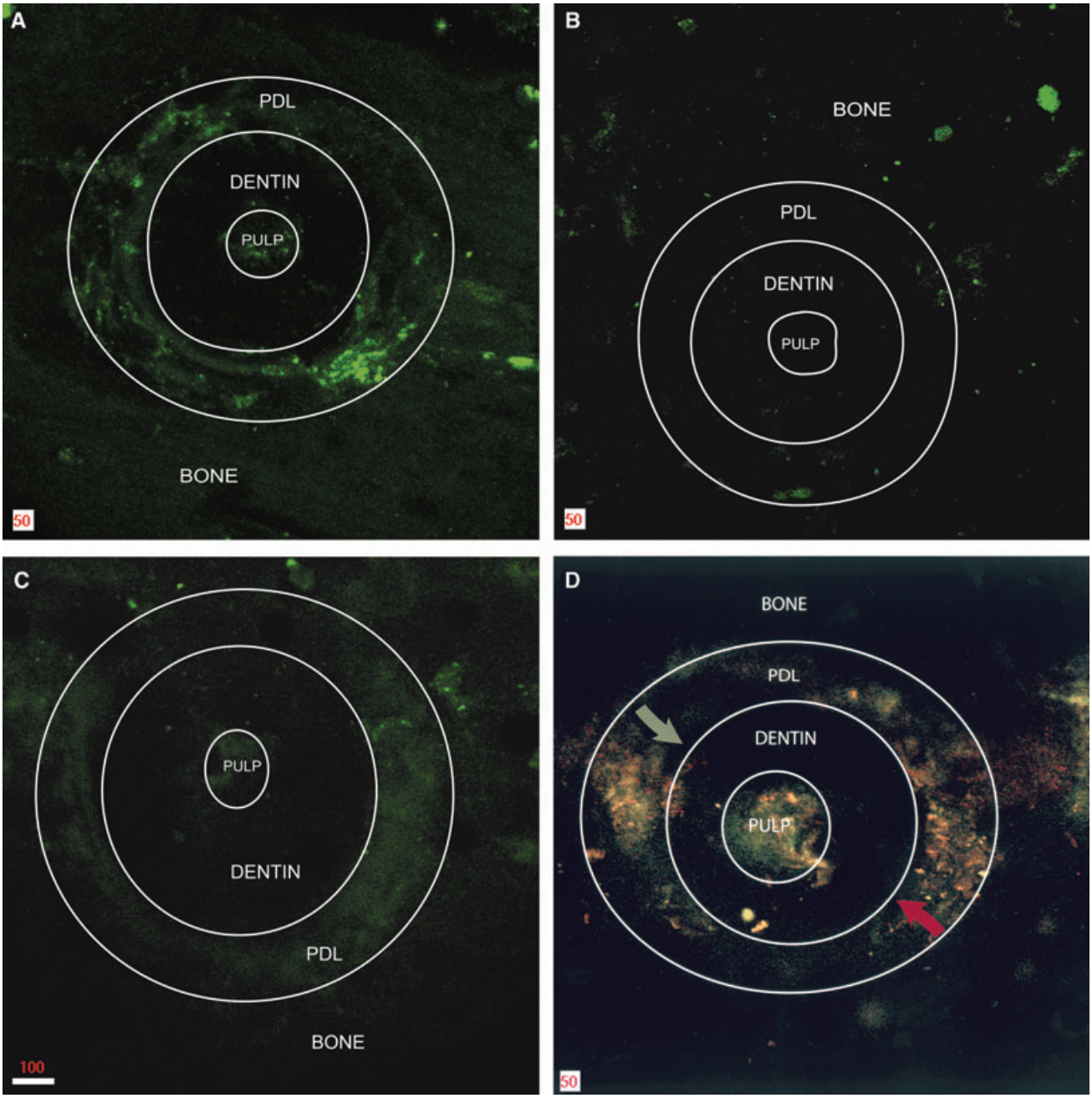

Fig. 3. (A) Projection micrograph of first molar root showing uptake of FITC-dextran after 5 min of spring activation (50 g force). Inner circle identifies pulpal soft tissue. Outer ring designated PDL identifies the location and width of the periodontal ligament. (B)Micrograph of third molar root of the same section used as a control. (C) Micrograph of first molar root of a control animal. Fluorescent dyes were delivered to the circulation. Force was not applied to this animal. (D) Micrograph of a first molar after 5 min of spring activation (50 g force). Red arrow indicates the direction of the force (therefore, the tension side) in the presence of rhodamine–dextran. Green arrow indicates the direction of force in the presence of FITC-dextran. PDL, periodontal ligament.

Periodontal tissues are constantly exposed to mechanical stress during mastication influencing tissue remodeling and repair. In orthodontic tooth movement, where external loads are applied to teeth, the PDL plays an important role on regulating alveolar bone response (15). It is our hypothesis that plasma membrane disruption occurs in cells of the PDL and constitutes an additional mechanism for mechanotransduction in orthodontic tooth movement.

In our previous study, we established plasma membrane disruption and resealing events in a rodent model during orthodontic tooth movement by means of an endogenous 'wound marker'. Serum albumin was significantly higher in the buccal (tension) side of

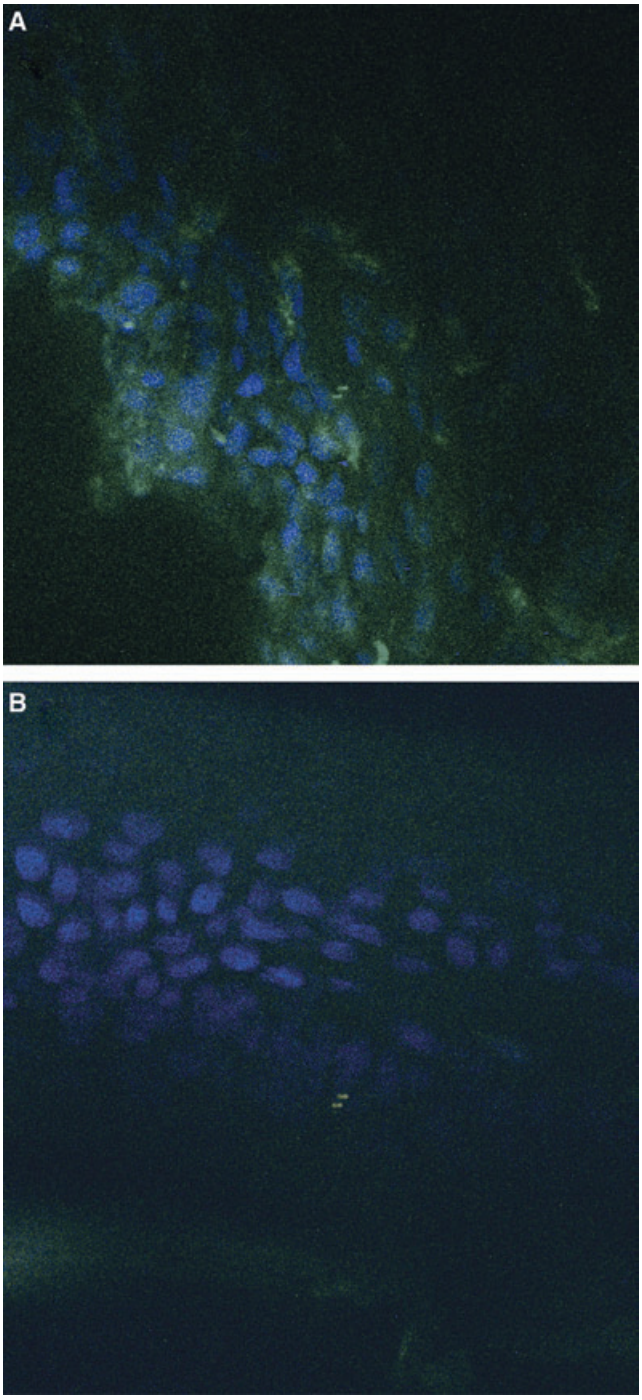

Fig. 4. (A) Micrograph of the lingual (tension) side of a first molar after 5 min of spring activation (50 g force). The specific localization of FITC-dextran around the blue (Hoeschst 33342) stained nuclei, confirms the intracellular localization of the green dye. (B) Lingual side of a third molar on the same section (this tooth had not been subjected to orthodontic force). The only fluorescent label is the Hoeschst dye seen in the nuclei.

moved molars when compared with lingual (compression), distal and mesial sides, and those of the unmoved control molars (6). Serum albumin, a 66 KDa molecule, would not be able to negotiate the plasma membrane over the short-time frame of our study unless a disruption had occurred (10, 11, 14).

To further support our findings, it was necessary to demonstrate that the albumin detected was intracellular. In the present study, transmission electron micrographs showed the localization of 10 nm-gold particles conjugated to anti-albumin antibodies exclusively in fibroblasts exposed to mechanical force. The labeling was in the cytoplasm and nuclei and insignificant outside the cell's boundaries. Nuclei labeling with anti-albumin antibodies were probably not due to disruption of the nuclear membrane but to cytoplasmic albumin entering the nuclei through the 70 nm nuclear pores. An interesting observation was that gold particles were not localized in organelles such as mitochondria or endoplasmic reticulum. The lack of albumin inside the mitochondria suggests that its double membrane was intact and therefore the cell had not gone through irreversible cell injury. Indeed, there was no ultrastructural evidence of mitochondrial swelling or other signs of cell damage.

To further validate the occurrence of plasma membrane disruption events in the PDL, a 50 g spring was activated in the presence of FDx and RDx for 5 min each in opposing directions. These fluorescent tracers were introduced into the vascular system and the uptake of these dyes by PDL cells was documented using confocal microscopy.

Periodontal ligament cells of orthodontically moved molars displayed intense fluorescence, particularly cells in the tension side. Fluorescent cells were less frequent in the compression, distal and mesial side of moved teeth and almost non-existent in the unmoved controls.

These observations are consistent with our previously reported (9) immunohistochemistry data as well as with our gold labeling findings. The short-time frame of the uptake of these large molecules in all of our experiments suggests that a plasma membrane disruption and resealing event has occurred. Periodontal ligament cells most likely survived these disruptions. First, the morphology of the cells detected was normal. Second, only living cells would be capable of retaining these highly diffusible tracers. The advantages of PDL cells surviving these injuries are self-explanatory.

To demonstrate the cytoplasmic localization of the fluorescent markers, the nuclear specific dye Hoechst 33342 was delivered to the circulation after the FDx.

The cell nucleus (bright blue) was observed surrounded by the green fluorescent dye in moved molars. The localization of the blue and green fluorescence points to an intracellular limitation of the dyes.

At the cellular level, tooth movement is accomplished by bone formation and bone resorption. The mitotic, locomotory and other cellular responses that accomplish such a stress induced remodeling are activated by several known mechanisms. Local mediators such as prostaglandins, interleukins and growth factors are key players in bone remodeling induced by orthodontic forces (16, 17).

Basic fibroblast growth factor (bFGF) and interleukin-1 beta (IL-1β), are known to be involved in bone remodeling but lack the signal peptide required for protein secretion *via* the classic pathway (18, 19). Temporary disruptions of the plasma membrane would likely provide an additional molecular route to and from the cell cytoplasm for these and other important molecules. Fibroblast growth factor has been experimentally shown to be released through disruptions of the plasma membrane of endothelial cells, cardiac myocyte, ventricular myocytes and skeletal muscle cells (14, 20–22). Injury of the plasma membrane appears to regulate bFGF and c-fos expression as well (13, 23).

We propose that PDL cells undergo temporary survivable disruptions of the plasma membrane. These open disruptions would allow normally impermeant molecules to be released and taken up by cells; communicating, in this manner, mechanical strain to changes in bone cell activity.

The present work provides additional evidence of this novel and important cellular phenomenon. It presents a potential role for plasma membrane disruption in mechanotransduction in orthodontic tooth movement.

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