

RESEARCH ARTICLE

A High-resolution Immunohistochemical Method for studying Receptor Expression on the Periodontal Ligament of Whole-mount Human Tooth Roots

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ABSTRACT

Aims: Our laboratory has found that lysophosphatidic acid (LPA) and its cognate receptors [LPARs, (LPA1–6)] expressed by human gingival fibroblasts (GF) and periodontal ligament fibroblasts (PDLF) play key roles in oral fibroblast homeostasis and are implicated in the inflammation seen in periodontal disease. We have reported that PDLF express LPA1 and LPA3; however, information on the gross topographic distribution of LPARs in the periodontal ligament (PDL) was lacking, and therefore, we developed a simple method for *in situ* labeling of LPARs in the PDL of extracted teeth.

Materials and methods: Sectioning or grinding thin sections of demineralized or native teeth and periodontium have long been the standard methodologies used to assess biomarker distribution in the PDL; however, we modified traditional immunohistochemical labeling and used whole teeth with fixed, solvent permeabilized PDLs.

Results: LPA1 and LPA3 were specifically labeled in the PDL and could be visualized at both the macro- and micro-levels.

Conclusion: This technique effectively labeled LPARs, and it can serve as a basis for the *in situ* visualization of other biomolecules expressed in the PDL.

Clinical significance: The ability to observe PDL LPAR distribution at the macro-level complements the microscopic data, and it is useful for detecting and documenting molecular changes in the PDL/PDLF that were brought about by age, experimental treatments, or pathologies like periodontal disease.

Keywords: G-protein coupled receptor, Human, Immunohistochemistry, Lysophosphatidic acid receptor, Periodontal ligament.

How to cite this article: Cerutis DR, Headen KV, Ogunleye AO, Williams DE. A High-resolution Immunohistochemical Method for studying Receptor Expression on the Periodontal Ligament of Whole-mount Human Tooth Roots. *Int J Experiment Dent Sci* 2016;5(2):99-103.

Source of support: NIH/NIDCR 1 R15 DE016855 (D.R.C.)

Conflict of interest: None

INTRODUCTION

Lysophosphatidic acid (LPA) is a pleiotropic lipid growth factor with hormone-like properties. Activated platelets liberate LPA with platelet-derived growth factor (PDGF) and other platelet factors. All mammalian serum contains quantities of LPA (μM), which accounts for much of the serum's mitogenic and biological activity. Lysophosphatidic acid is a potent, complete mitogen for most cells. Other cell types, including fibroblasts, are known to make LPA.^{1,2}

Lysophosphatidic acid exists *in vivo* as multiple molecular species that have a fatty acid of varying chain length and degree of unsaturation covalently attached via an acyl, alkyl, or alkenyl linkage. It also controls multiple critical cellular responses by signaling through its high-affinity G-protein-coupled receptors (lysophosphatidic acid receptors, LPARs). These are designated as LPA1–6; LPA1, LPA2, and LPA3 are also known as EDG2, EDG4, and EDG7; the more recently identified LPARs include LPA4 (GPR23), LPA5 (GPR92), and LPA6 (P2Y5).^{3,4}

Lysophosphatidic acid (mainly the 18:1, 18:0, and 16:0 acyl species) are found in normal human saliva at physiological concentrations and were proposed to be involved in homeostasis and wound healing in the mouth and upper digestive tract.⁵ We have shown in our seminal studies that human oral fibroblasts express LPA1–3, as verified by immunohistochemistry (IHC) and flow cytometry,⁶ and that LPA controls their migration, proliferation, responses to PDGF, and intracellular calcium responses.^{7,8} Our data also show that the main salivary LPA species are 10-fold elevated in human saliva from moderate–severe periodontal disease.⁹

Lysophosphatidic acid regulates the expression of multiple genes associated with wound healing. It also acts as an inflammatory mediator in airway diseases, rheumatoid arthritis, atherosclerosis, and cancer, reportedly by acting through LPA1 and LPA3.² Our microarray analysis found that LPA controls the expression of multiple genes associated with wound healing and inflammation in human gingival fibroblasts (GF),¹⁰ cells key to the pathogenesis of periodontal disease. Thus, our cumulative findings suggest a regulatory role for LPA in periodontal disease.

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Many studies have investigated the makeup and function of the human periodontal ligament (PDL). It contains the microscopic viscoelastic periodontal ligaments that anchor teeth to the alveolar bone, vasculature, innervation, and extracellular matrix (ECM). The main cell type in the PDL is the periodontal ligament fibroblast (PDLF), whose major functions are making, maintaining, and remodeling/repairing the periodontal ligaments^{11,12} in response to mechanical and inflammatory stimuli. The health of the PDLF determines the rate of remodeling of the PDL and the turnover and replacement of major ECM molecules (like collagen) in response to physiologic stresses and mechanical loading.^{13,14} Lysophosphatidic acid controls collagen I biosynthesis and regulates fibrocartilage formation,¹⁵ and therefore, any disease process that affects PDLF and its LPARs will affect the ability of the periodontal ligament to homeostatically adjust to mechanical and inflammatory stressors. Because of the multiple regulatory roles LPA plays in PDLF biology, we need a way to study these receptors on the whole PDL.

Because teeth are composed of hard mineralized tissues, the majority of published reports studying aspects of PDL biology in humans or animal models have utilized demineralized, sectioned teeth with surrounding periodontal tissue, while a few studies have used protocols with cut and ground fixed, non-demineralized teeth. However, as we need to visualize LPAR expression *in situ* on the PDL, we developed the immunohistochemical technique, which is reported here.

MATERIALS AND METHODS

Our Creighton University Dental Clinic routinely performs tooth extractions. Teeth ($n=24$) were obtained from healthy (age range 35–60) nonsmoking patients with or without periodontal disease. The study conformed to the Declaration of Helsinki guidelines and was approved by the Creighton University Institutional Review Board. Informed consent was obtained from all donors.

Chemicals and Reagents

All were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. The kit for immunohistochemical staining [VECTASTAIN Elite ABC Kit (Standard) Catalog Number PK-6100] was purchased from Vector Laboratories, Burlingame, CA, USA. The rabbit polyclonal antibody (Ab) for LPA1 (Lysophosphatidic Acid Receptor Edg2/LPA1 Polyclonal Antibody, N-terminal; reactivity, anti-human) was obtained from MBL International Corporation (Woburn, MA, USA) and used at a 1:800 dilution. The anti-LPA3 Ab (LPA3 rabbit polyclonal Ab, # 10004840) was from Cayman Chemical (Ann Arbor, MI, USA) and

was used at a dilution of 1:1,000. All washes were done at 25°C with gentle shaking unless indicated otherwise.

In situ Immunohistochemical LPAR Detection on the PDL

Freshly extracted teeth were immediately collected into ice-cold Dulbecco's modified Eagle's cell culture medium (Invitrogen, Grand Island, NY, USA). The teeth were rinsed six times (in ice-cold Hank's balanced salt solution, Invitrogen, Grand Island, NY, USA) in order to diminish microbial contaminants and remove extraction debris.

After rinsing, teeth were fixed with freshly prepared ice-cold 1:1 acetone:methanol for 10 minutes, and then allowed to air dry and stored at 4°C if they were to be immunostained at a later time. The teeth were rehydrated through graded alcohols (100, 90, 70, and 50%) and then washed twice in excess fresh Tris-buffered saline (TBS), pH 7.4 for 5 minutes. To verify that PDL remained attached to the tooth roots, staining was done with 4',6-diamidino-2-phenylindole (DAPI, 1:5,000 in TBS) for 15 minutes in the dark to stain nucleic acid and enable detection of cell nuclei in the PDL by fluorescence microscopy. The teeth were then washed three times (5 minutes/wash) with TBS to remove excess DAPI, and then, they were transferred to aqueous mounting media (1:1 TBS/glycerol with 0.001% thymol). The PDL was visualized and imaged where transmitted light could pass (i.e., around the edges of the roots) using a Leica DMIL inverted microscope (Leica Microsystems, Buffalo Grove, IL, USA) with an ultraviolet filter set [excitation is ~359 nm and emission is ~461 nm for deoxyribonucleic acid (DNA)-bound DAPI].

For immunostaining, the teeth were blocked in TBS/5% goat serum for 1 hour at room temperature [RT (25°C)], and subsequently, they were incubated with each of the primary rabbit Abs for 1.5 hours at RT with gentle shaking. They were then washed 3×1 hour in excess TBS (~15 mL/tooth) before being incubated overnight at 4°C with goat antirabbit biotinylated secondary Ab. This immunohistochemistry protocol required longer wash times to remove all non-specifically bound Abs from the PDL; its thickness (~0.2mm) varies among individuals and age groups.¹⁶ After washing three times, 1 hour for each wash in excess TBS, any endogenous peroxidase activity was blocked by incubating with 0.1% fresh H₂O₂ in TBS/5% goat serum for 0.5 hours. The teeth were then washed three times, 15 minutes for each wash with TBS, to remove all H₂O₂ before incubating with the avidin–biotin complex reagent for 1 hour. Three more 1 hour TBS washes (RT) were done, then Ab localization was visualized using 3,3'-diaminobenzidine (DAB), with nickel enhancement [Vector Laboratories; DAB Peroxidase (horseradish peroxidase) Substrate Kit (with Nickel), 3,3'-diamino-

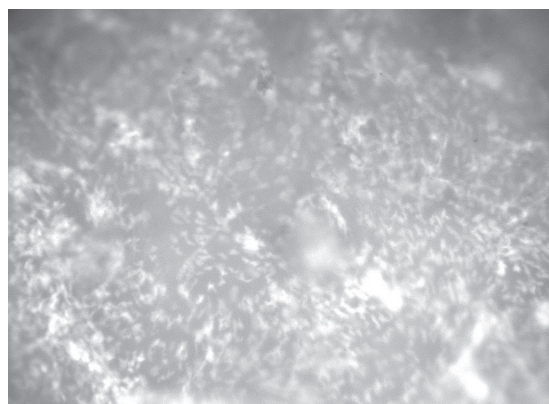


Fig. 1: DAPI nuclear stain of the whole periodontal ligament. Freshly extracted teeth were fixed in 1:1 acetone:methanol for 10 minutes, rehydrated, and stained with DAPI. A typical view is shown. This representative image of n=24 was taken with an inverted fluorescent cell culture microscope. NB: Because of the curved nature of the tooth root and the thickness of the PDL, only a portion of the picture can be in focus at any one time. Magnification, 100×

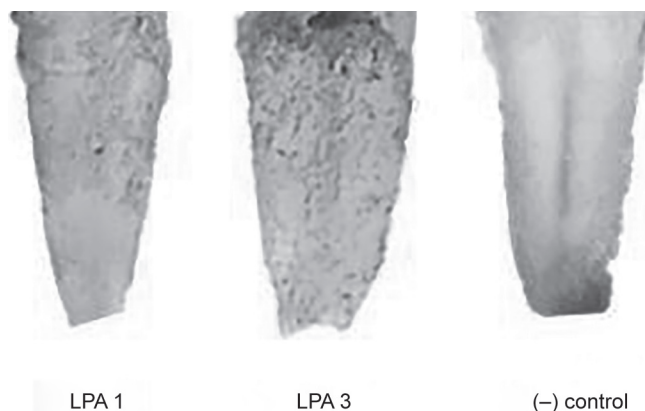


Fig. 2: Macro images of LPAR-immunostained tooth roots. Typical images (6.3×) of tooth roots immunostained for LPA1 and LPA3 and a control tooth – primary antibody omitted – (gray areas are photography shadows) are shown. The crowns and dissecting microscope stage backgrounds were removed for clarity

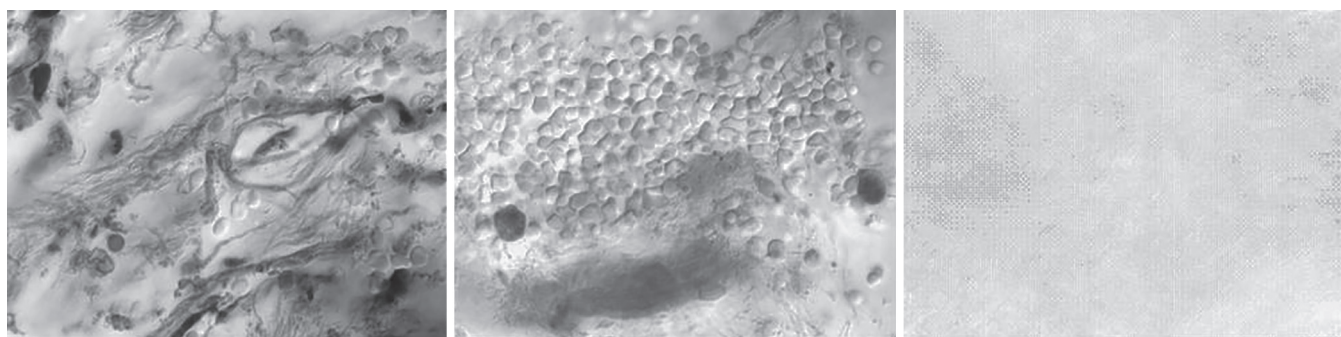


Fig. 3: Microscopic immunolocalization of LPARs in the periodontal ligament. Portions of the immunostained periodontal ligaments see Fig. 2 were removed from the roots for examination at high power. The typical distribution and labeling of LPA1 and LPA3 are shown (600×)

benzidine Catalog Number: SK-4100] as specified by the manufacturer. The teeth were then washed for 3 minutes under cold tap water at a gentle flow, and then, they were transferred into aqueous mounting media for storage prior to further evaluation.

At the macro-level, LPAR immunostaining density and distribution on the tooth roots were determined using a dissecting microscope (6.3×) to visualize and photograph the complete tooth roots.

To determine the information value and quality of the microscopic detail obtainable after the *in situ* IHC labeling, small sections of the PDLs were carefully removed without deformation by gently cutting/peeling them from the roots. This was accomplished using new, sharp scalpels and forceps. The PDL fragments were mounted in aqueous media on glass slides and then imaged at 600×.

RESULTS

For proof of concept that extracted healthy or moderately periodontally involved teeth retain PDL covering, they were fixed, permeabilized, and stained with the DNA-

intercalating dye DAPI to reveal cell nuclei on the tooth roots. Figure 1 shows a representative tooth root covered with fluorescent nuclei. Periodontal ligament covering was present on all teeth examined (n=24), including on teeth extracted from moderate cases of periodontal disease.

Figure 2 shows the macro (6.3×) views of representative tooth roots immunostained with anti-LPA1 or anti-LPA3 Abs. For both receptors, staining is clearly visible on all the PDL (except in areas where mechanically stripped by the forceps during the extraction process). A typical control tooth (primary Ab omitted) is absent any labeling, confirming the specificity of the anti-LPAR immunostaining.

In Figure 3, the representative segments of PDL separated from the roots show extensive labeling of cell nuclei and periodontal fibers with the anti-LPA1 or anti-LPA3 Abs. While most nuclei are labeled, some are not labeled.

DISCUSSION

All the examined teeth retained PDL covering following the extraction (Fig. 1, DAPI stain), and thus, they

were appropriate to use for developing this *in situ* IHC G-protein coupled receptor (GPCR) detection technique.

The Ab labeling for each of the tested LPARs showed the expected plasma and nuclear membrane staining patterns; the EDG family of LPARs (LPA1–3) has been reported to be present on both of these membranes in other systems.^{17,18} We have seen the same distribution for LPA1 and LPA3 in both human GF and PDLF grown *in vitro* on plastic cell culture plates or glass slides.¹⁹

For our laboratory, the IHC assay of whole tooth was described here, and further, it has been a valuable tool to complement our pharmacologic studies that have characterized the LPARs expressed by human PDLF and the role they play in homeostasis and in periodontal disease. We propose that this technique may be useful to other researchers in the oral system, whether they are utilizing human or animal models, to best study the periodontal disease-related (or other) question they are posing for investigation. It can also be used to study any marker (for which an Ab is available) that correlates with an oral disease-related process and/or disease outcome. The following caveats and suggestions are offered:

Ab validation: Before use in this assay, the Ab staining must first be validated using cryosections and/or deparaffinized tissue sections. We first validated these Abs in our system before proceeding; when exploring this technique for the desired application (at least for the first few experiments), it is best to verify that this technique is working by doing a side by side known positive control(s), which can include PDLF cytopins or PDLF that have been grown on glass coverslips.

We used polyclonal Abs for this study, as they usually better tolerate small degrees of antigen denaturation and are more tolerant of minor polymorphic and glycosylation variations between individuals.²⁰ While it was not a problem in our system (since LPA1 is moderately expressed and LPA3 occurs in very high abundance in human PDLF), a low abundance protein will benefit from the amplification provided by the use of a polyclonal Ab, as the protein of interest will bind > 1 Ab on the multiple epitopes. However, this characteristic makes it imperative to rigorously check the desired antigen sequence for any possible undesired cross-reactivity.

Monoclonal Abs are very likely to perform well; however, they should be verified by each researcher wishing to use this technique for their particular application. They only recognize one epitope per antigen and if that is compromised by experimental procedure or other variables, IHC staining may be low or absent, leading to erroneous conclusion. Monoclonal Abs from other species (rabbits,²¹ chickens,²² or camelids²³) may offer advantages and refinements to this technique. The latter monoclonal

Abs penetrate better, especially to normally inaccessible sites like the clefts in folded proteins, as they are smaller in size. This may allow for gentler fixation and/or permeabilization techniques to be used if needed. They also are more robust and enduring due to their high tolerance to temperature and pH variations.^{24,25} The reader is referred to two excellent reviews of the advantages and issues of polyclonal and monoclonal Abs and for the technical aspects of IHC and rabbit monoclonal Abs.^{20,21} **Antibody sources and experimental variables:** For each system, Abs from different vendors may have to be trialed, as variability in performance between individual Ab lots and different suppliers is well known.²⁶ It is advisable to test several positive controls, a range of primary Ab dilutions, incubation times, and temperatures (e.g., 4°C overnight or 1 hour shaking at RT).

Antigen retrieval: In our system, it was not necessary to perform any antigen retrieval procedure, as the LPARs, which we have studied, remain recognizable by these particular Abs even after long-term storage in 10% buffered formalin or paraformaldehyde, followed by a 10-minute ice-cold (1:1 ethanol:methanol, methanol, or 1:1 acetone:methanol) permeabilization step. Each researcher should determine if antigen retrieval is required in their system for this technique to work.

In conclusion, we report here for the first time (to the best of our knowledge) the ability to detect GPCRs on and inside cells located in the PDL of extracted, fixed whole human tooth roots. The ability to obtain immunohistochemical information at both the macro- and micro-levels from whole teeth enhances the assessment of molecular changes in the PDL.

CLINICAL SIGNIFICANCE

The ability to observe PDL LPAR distribution at the macro-level complements the microscopic data, and it is useful for detecting and documenting molecular changes in the PDL/PDLF that were brought by age groups, experimental treatments, or pathologies like periodontal disease.

ACKNOWLEDGMENT

Authors thank Drs Laura Barritt and Sonia Sanchez, Department of Oral Biology, Creighton University Dental School, USA, for their helpful advice on imaging. This study was supported by NIH/NIDCR 1 R15 DE016855 (D.R.C.). As it utilized anonymized extracted teeth, it is not considered as a research involving human subjects.

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