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Research paper

Lysophosphatidic acid induces expression of genes in human oral keratinocytes involved in wound healing

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ABSTRACT

Objective: Epithelial cells participate in wound healing by covering wounds, but also as important mediators of wound healing processes. Topical application of the phospholipid growth factor lysophosphatidic acid (LPA) accelerates dermal wound healing and we hypothesized that LPA can play a role in human oral wound healing through its effects on human oral keratinocytes (HOK).

Design: HOK were isolated from gingival biopsies and exposed to LPA. The LPA receptor profile, signal transduction pathways, gene expression and secretion of selected cytokines were analyzed.

Results: HOK expressed the receptors LPA_1, LPA_5 and LPA_6 and LPA activated the ERK1/2, JNK and p38 intracellular pathways, substantiated by secretion of IL-6 and IL-8. The early (2 h) and intermediate (6 h) gene expression profiles of HOK after LPA treatment showed a wide array of regulated genes. The majority of the strongest upregulated genes were related to chemotaxis and inflammation, and became downregulated after 6 h. At 6 h, genes coding for factors involved in extracellular matrix remodeling and re-epithelialization became highly expressed. IL-36γ, not earlier known to be regulated by LPA, was strongly transcribed and translated but not secreted.

Conclusions: After stimulation with LPA, HOK responded by regulating factors and genes that are essential in wound healing processes. As LPA is found in saliva and is released by activated cells after wounding, our results indicate that LPA has a favorable physiological role in oral wound healing. This may further point towards a beneficial role for application of LPA on oral surgical or chronic wounds.

1. Introduction

Lysophosphatidic acid (LPA) is a growth factor-like lipid, and its biological effects on cells are mediated via G protein-coupled receptors, termed LPA_{1-6} (Yanagida, Kurikawa, Shimizu, & Ishii, 2013). LPA is a natural constituent of mammalian tissue fluids such as plasma, broncho-alveolar lavage fluid (Zhao & Natarajan, 2009), saliva (Sugiura et al., 2002) and gingival crevicular fluid (GCF) (Bathena et al., 2011). LPA is produced by a number of cell types, including erythrocytes, leukocytes, activated platelets and human gingival fibroblasts (Blaho & Hla, 2011; Cerutis et al., 2015; Eichholtz, Jalink, Fahrenfort, & Moolenaar, 1993).

Several observations indicate that LPA plays a role in wound healing in skin and intestine. Wound healing is a dynamic process that involves four major processes that overlap in time (Eming, Krieg, & Davidson, 2007; Martin, 1999): hemostasis, inflammation, tissue formation, and remodeling and maturation. *In vitro*, LPA increases migration of human skin keratinocytes and rat intestinal epithelial cells (Sauer et al., 2004; Sturm, Sudermann, Schulte, Goebell, & Dignass, 1999). *In vivo*, it accelerates repair of injured epithelia in rats with colitis (Sturm et al., 1999), wound closing of rat skin (Balazs, Okolicany, Ferrebee, & Tigyi, 2000) and re-epithelialization during the early stages of wound healing in excisional ear wounds in mice (Demoyer, Skalak, & Durieux, 2000). Other studies have also shown that LPA increases rodent epidermal cell proliferation *in vitro* and also *in vivo* after topical application of LPA on intact skin (Piazza, Ritte, & Baracka, 1995). Wound healing in the oral cavity is mostly uncomplicated and without much scar formation (Wong et al., 2009), but it can be compromised in certain patients, e.g. those receiving radio- or chemotherapy (Raber-Durlacher et al., 2013) or medication against osteoporosis (Marx, 2003).

Little is known about the functions of LPA in the oral cavity. LPA, used at physiological concentrations in saliva, has been shown to

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increase proliferation of squamous carcinoma cells from the mouth, pharynx and esophagus (Sugiura et al., 2002). In patients with chronic periodontitis, the concentration of LPA has been shown to be 5–10 times higher in saliva and gingival crevicular fluid as compared with healthy subjects (Bathena et al., 2011). The gene expression profile of LPA-stimulated oral fibroblasts is characterized by up-regulation of inflammatory and wound healing genes as compared with unstimulated cells (Cerutis et al., 2015).

Transcriptional profiling is a valuable tool to indicate unknown activities and profiling of LPA-stimulated murine embryonal fibroblasts unveiled activities of LPA in tissue remodeling, angiogenesis, inflammation and tumor progression (Stortelers, Kerkhoven, & Moolenaar, 2008). The transcriptional response to LPA in oral epithelial cells is unexplored.

As LPA modulates wound healing in the skin and the intestine, we presently aim to find support for the hypothesis that LPA can contribute to wound healing in the oral cavity through its effects on oral epithelial cells. To this end, we wanted to determine the LPA receptor expression in human oral keratinocytes (HOK), test functional activity of its receptors, and examine the gene expression profile of HOK exposed to LPA. If indication can be found that LPA can improve oral wound healing, this physiological mediator might be considered for trials aimed at improving healing in acute or chronic oral wounds.

2. Material and methods

2.1. Biopsies and cell cultures

Biopsies were obtained from healthy volunteers during third molar extractions. The study was approved by Regional Ethical Committee of Health (REK South-East), and was carried out according to the Declaration of Helsinki's principal for biomedical research. Written, informed consent was obtained from all donors. Primary human oral keratinocytes (HOK) were prepared from the biopsies. After transport in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza BioWhittaker, Portsmouth, NH, USA) with 2% penicillin-streptomycin-fungizone (PSF) (Lonza BioWhittaker), the biopsies were transferred to DMEM with 1.25 mg/ml dispase (GIBCO, Paisley, UK) and incubated over night at 4 °C. The epithelial sheets were peeled off, cut into small pieces and incubated in 10 X trypsin EDTA (Sigma-Aldrich, St Louis, MO, USA) for 7 min at 37 °C. A Pasteur pipette was used to loosen the cells. Trypsinization was stopped by adding fetal calf serum (FCS). The cells were then cultured in keratinocyte serum-free medium (KSFM; GIBCO), supplemented with 25 μg/ml bovine pituitary extract (BPE; GIBCO), 1 μg/ml epidermal growth factor (EGF; GIBCO) and 1% PSF in a humidified atmosphere of 5% CO_2 in air at 37 °C. In the experiments, 2nd – 6th passaged cells were used. For all experiments, the cells were seeded at a desired density, incubated overnight and then grown in starvation medium (KSFM without addition of BPE and EGF), 24 h before stimulation. All experiments were carried out on cells cultured from individual donors and examined separately.

2.2. Lysophosphatidic acid (LPA)

LPA 18:1 was purchased from Avanti Polar Lipids (Alabaster, Alabama, USA) and dissolved in fatty acid-free bovine serum albumin (Sigma-Aldrich) in PBS to 2 mM and stored at – 20 °C until use. Fatty acid-free BSA was used as vehicle control in all experiments.

2.3. RNA isolation, reverse transcription and real-time qPCR

RNA was extracted from cell cultures obtained from three different individual donors by the use of RNeasy mini kit (74106; Qiagen, Hilden, Germany). The experiments were run separately. The quantity and purity of the RNA was assessed with a NanoDrop spectrophotometer (ThermoFisher Scientific, Frederick, MD, USA). Total RNA (400 ng) was transcribed into cDNA by using a mixture of reverse transcriptase enzyme and random nonamers (Eurogentec, Seraing, Belgium). Each cDNA synthesis was performed in a total volume of 20 μl for 10 min at 25 °C, then 30 min at 48 °C and terminated by heating for 5 min at 95 °C. Detection of mRNA was performed by using a Fast EvaGreen qPCR Master Mix (Biotium, Hayward, CA, USA). The cDNA was diluted 2.33 times with RNase-free water. Each real time PCR reaction consisted of 2.5 μl RNase-free water, 12.5 μl Master Mix, 1 μl each of forward and reverse primers(LPA1 forward CTTCTACA ACGAGTCCATT and reverse AACAGTGATTCCAAGTCC; LPA2 forward GTTTGTAACAGACATTATTC and reverse AATCAGACTCAGGTAGAT; LPA3 forward CATAGAGGATAGTATTAGC and reverse TTAGAGACA GGTAATCAT; LPA4 forward TGACTTCCAATTCCAAGATTC and reverse AGACAGCACCATTGAGAT:, LPA5 forward AACAACATCCAAGTCCAA and reverse GAAGAGAGAAACGAGAGAG; LPA6 forward TTGGAGTAT TCATCTTGACTAC and reverse ACTGACCAGCAACCTT) and 8 μl of template. Reactions were carried out on an Stratagene MX3005p (Agilent Technologies, CA, USA) for 40 cycles (95 °C for 15 s, 56 °C for 1 min) after an initial 10 min incubation at 95 °C. All reactions were performed in duplicate. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene.

2.4. Illumina® bead array

Genome-wide transcriptional analysis was performed using Illumina® bead array technique Norwegian Microarray Consortium in Oslo, Norway (project no.: NMC-OSLO-0222). Utilizing the Illumina® TotalPrep™-96 RNA Amplification Kit, biotin-labeled cRNA was synthesized from 500 ng total RNA by first- and second strand reverse transcription followed by *in vitro* transcription of cRNA. RNA quantity was determined using a NanoDrop Spectrophotometer while RNA size and integrity were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and RNA integrity number (RIN) values. Values above 7 were considered acceptable. All samples had RIN values above 8. 750 ng of biotin-labeled cRNA were analyzed using a HumanHT-12 v4 Expression BeadChip. Data from the microarray were filtered and processed using the software program TIBCO Spotfire® (TIBCO Spotfire, Boston, MA, USA). Datasets from the arrays were grouped in Spotfire® and filtered for empty records and genes with signal intensities below the cut-off of 400 set equal to background intensity. Cell cultures were obtained from four different individual donors and examined separately. Triplicates of datasets from stimulated and unstimulated cells were averaged and fold changes of gene regulation were calculated relative to unstimulated cells.

2.5. Western blotting

HOK were seeded at the desired density (dependent on type of experiments) in 6-well plates and incubated overnight. The cells were then cultured for 24 h in keratinocyte SFM medium (without BPE) before stimulation with LPA (10 μM) for another 24 h. The cells were washed twice in cold sterile PBS and then scraped directly in CelLytic M Cell Lysis Reagent (Sigma-Aldrich) with Halt™ Phosphatase Inhibitor Cocktail, Halt™ Protease Inhibitor Cocktail and 5 μM EDTA (Pierce, Rockford, IL, USA) on ice. The samples were centrifuged at 4 °C for 3 min at 1957 g and the supernatants were collected. Total protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) with gamma globulin as a standard. In brief, 15 μg protein samples were mixed with Laemmli sample buffer containing dithiothreitol (DTT), heated to 100 °C for 4 min, and loaded on 15% Tris/glycine SDS-polyacrylamide gels. The proteins were transferred to 0.45 μm nitrocellulose membranes using a semi-dry transfer system (Bio-Rad). The membranes were blocked with 4% bovine serum albumin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in Tris buffered saline containing Tween-20 (TBST, 50 mM Tris, 154 mM NaCl, 0.1% Tween-20, pH 7.6) for 1 h at room temperature.