Resolvin D1 protects periodontal ligament

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1Department of Clinical Dentistry–Center for Clinical Dental Research, University of Bergen, Bergen, Norway; 2Department of Periodontology, Forsyth Institute, Cambridge, Massachusetts; 3Department of Clinical Dentistry–Periodontics, University of Bergen, Bergen, Norway; 4Institute of Medicine, Section of Pharmacology, University of Bergen, Bergen, Norway; and 5Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative, and Pain Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts

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Mustafa M, Zarrough A, Bolstad AI, Lygre H, Mustafa K, Hasturk H, Serhan C, Kantarci A, Van Dyke TE. Resolvin D1 protects periodontal ligament. Am J Physiol Cell Physiol 305: C673–C679, 2013. First published July 17, 2013; doi:10.1152/ajpcell.00242.2012.—Resolution agonists are endogenous mediators that drive inflammation to homeostasis. We earlier demonstrated in vivo activity of resolvins and lipoxins on regenerative periodontal wound healing. The goal of this study was to determine the impact of resolvin D1 (RvD1) on the function of human periodontal ligament (PDL) fibroblasts, which are critical for wound healing during regeneration of the soft and hard tissues around teeth. Primary cells were cultured from biopsies obtained from three individuals free of periodontal diseases. Peripheral blood mononuclear cells were isolated by density gradient centrifugation from whole blood of healthy volunteers. PGE2, leukotriene B4 (LTB4), and lipoxin A4 (LXA4) in culture supernatants were measured by ELISA. The direct impact of RvD1 on PDL fibroblast proliferation was measured and wound closure was analyzed in vitro using a fibroblast culture “scratch assay.” PDL fibroblast function in response to RvD1 was further characterized by basic FGF production by ELISA. IL-1β and TNF-α enhanced the production of PGE2. Treatment of PDL cells and monocytes with 0.1–10 ng/ml RvD1 (0.27–27 M) reduced cytokine induced production of PGE2 and upregulated LXA4 production by both PDL cells and monocytes. RvD1 significantly enhanced PDL fibroblast proliferation and wound closure as well as basic FGF release. The results demonstrate that anti-inflammatory and proresolution actions of RvD1 with upregulation of arachidonic acid-derived endogenous resolution pathways (LXA4) and suggest resolution pathway synergy establishing a novel mechanism for the proresolution activity of the ω-3 docosahexaenoic acid-derived resolution agonist RvD1.

regeneration; inflammation; resolution

INFLAMMATION IS THE NORMAL host tissue response to bacteria in the periodontium that is protective in the acute phase enhancing the clearance of invading pathogens. Acute inflammation should normally be self-limiting as the ideal outcome to maintain tissue homeostasis (49). The proinflammatory pathways that initiate acute inflammation are mediated by cytokines, chemokines, and lipid mediators that signal between cells in a coordinated, well-orchestrated cascade. Excessive or unresolved inflammation becomes chronic leading to tissue injury and destruction or incomplete healing with fibrosis and scarring (47). Resolution of self-limited inflammation is a highly coordinated, active process that involves endogenously synthesized lipid mediators of resolution derived from the same eicosanoid pathways as proinflammatory mediators (47).

Eicosanoids derived from arachidonic acid, biosynthesized via the cyclooxygenase (COX) and lipoxigenase (LO) pathways, including prostaglandins and leukotrienes, exert a wide range of proinflammatory actions (10). By contrast, lipoxin A4 (LXA4) is derived from arachidonic acid later in inflammation through cell-to-cell interactions where different lipoxigenases from multiple cells act on a single arachidonate molecule (28). A distinct, longer acting form of LXA4 is produced in the presence of aspirin modified COX-2, such as aspirin-triggered 15-epi-LXA4; both have potent anti-inflammatory and proresolution actions (reviewed in Ref. 45). Proinflammatory eicosanoids such as PGE2 and PGD2 promote a shift to LXA4 production as the duration of the acute inflammatory lesion increases, thereby reprogramming host cells (e.g., neutrophils and mononuclear phagocytes) in the exudate to initiate resolution (28, 38). Lipoxins limit neutrophil trafficking to inflamed areas, induce neutrophil apoptosis, and attract monocytes to the lesion regulating their phenotype and enhancing phagocytosis of apoptotic neutrophils and bacteria by the resulting “resolution” macrophages (53). Collectively, lipoxins function to resolve the acute inflammatory response with minimal damage to the surrounding tissue (5, 47). More recently, the use of lipoxins for therapeutic indications on humans has revealed clinical benefit for dermatologic and ophthalmic indications (21, 60, 61).

Resolution pathways are not limited to arachidonic acid-derived molecules. The proresolving resolvins are bioactive products derived from essential ω-3 polyunsaturated fatty acids that participate in the resolution of inflammation and tissue repair. The members of this family are endogenously produced products biosynthesized from the ω-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid; termed the E-series resolvins (RvE) or D-series resolvins (RvD), respectively. Aspirin-triggered forms of resolvins are generated by the aspirin-modified-COX-2 pathway; both the aspirin-triggered and natural pathways are LO-initiated mechanisms (46, 48). Resolvins display potent proresolving actions (52). Systemic treatment with RvD1 or RvD2 improved disease activity in colitis possessing greater efficacy than conventional therapies (2). Resolvins also provide significant benefit in pain management and rheumatoid arthritis (8, 11, 21, 29, 51, 62). New data also demonstrated a beneficial impact of resolvins in diabetic wound healing (55). Administration of resolvins in nanomolar doses blocks osteoclast differentiation and bone resorption (15). This observation was supported in vivo in an experimental periodontitis model where RvE1 treatment resulted in al-

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most complete regeneration of the periodontium, leading to the formation of new attachment, new cementum, and new periodontal ligament (PDL; Ref. 14). These observations further support the negative role of chronic inflammation in tissue healing and regeneration and suggest that the control of inflammation with natural, endogenous mediators of inflammation resolution, not inhibitors of proinflammatory pathways, is a rational approach to modification of wound healing and tissue engineering. The precise mechanism of action of resolution agonists in wound healing and regeneration is not known and is of significant interest. The regeneration of an organ induced by a proresoluto mediator suggests that the actions of resolvins go beyond interactions with only inflammatory cells. In addition to inflammatory cells, cells of the connective tissues are hypothesized to respond to the resolvins during the healing process.

Fibroblasts are involved in collagen production, wound healing, and tissue repair. Fibroblasts are responsive to the surrounding inflammatory environment and cytokine activity (39). PDL cells are a highly specialized phenotype of fibroblasts with unique functions important to regeneration of the attachment apparatus between the hard tissues of teeth and alveolar bone, including the production of extracellular proteins such as basic fibroblast growth factor (bFGF), platelet-derived growth factor and epidermal growth factor (22, 25, 34, 40, 56). PDL cells also produce several types of collagen, including type V collagen, which is involved in tissue repair and regeneration (44). Gingival and PDL fibroblasts express a variety of different genes (13). PDL fibroblasts also express alkaline phosphatase, which plays a key role in the mineralization of bone and acellular cementum formation, indicating their osteogenic potential and capability for producing mineralized tissues (1). bFGF regulates this process inducing chemotactic migration of the PDL cells and promoting angiogenesis through differentially expressed specific receptors (34, 40). PDL cells actively participate in bone remodeling through the production of lipid mediators including prostaglandins (35). Recent studies have demonstrated that PDL fibroblasts in close contact with bacteria produce cytokines and chemokines and amplify inflammation suggesting the fibroblast as a target for pharmacological intervention in periodontitis (12, 17). This study focused on the response of PDL fibroblasts to RvD1. The production of pro- and anti-inflammatory eicosanoids as well as bFGF by PDL cells, their proliferation, and the capacity of PDL fibroblasts to migrate in a wound closure model were assessed. Together these results provide evidence that resolution agonists play an important role in periodontal wound healing.

**MATERIALS AND METHODS**

**Reagents.** RvD1 (resolvin D1, 75,S,R,17S trihydroxydocosa-4Z,9E, 11E,13Z,15E,19Z-hexanoic acid; 10012554), PGE2, and leukotriene B4 (LTB4) Luminescent kit were obtained from Cayman (Ann Arbor, MI). DMEM, HEPES buffer, FCS, Histopaque 1119, Histopaque 1077, penicillin, and streptomycin were obtained from Sigma (St. Louis, MO). Human recombinant IL-1β, human recombinant TNF-α (specific activity >2 × 10^6 U/mg), and bFGF ELISA kit were purchased from R&D Systems (Minneapolis, MN). LXA4 ELISA was supplied by Neogen (Lexington, KY).

**Cell cultures.** This study was approved by the Ethics Committee at Bergen University, the Institutional Review Board at Boston University Medical Center (BUMC-IRB) and the IRB of the Forsyth IRB, and signed informed consent was obtained from all subjects participating in the study. Primary PDL cells were cultured from periodontal biopsies obtained from three healthy individuals with no clinical signs of periodontal disease as previously described (42). Minced pieces of periodontal tissue were explanted to 25-cm^2 Falcon tissue culture flasks containing 5 ml DMEM supplemented with sodium-glutamine (4 mM), 10% FCS, penicillin (50 IU/ml), and streptomycin (50 mg/ml). PDL cells were isolated by trypsinization of the primary outgrowth of cells, incubated at 37°C in a humidified incubator aerated with 5% CO2 in air, and routinely passaged using 0.025% trypsin in PBS containing 0.02% EDTA. For characterization, PDL cells were stained with crystal violet solution; the cells exhibited a characteristic spindle-shape when visualized under light microscopy. In parallel with previous reports (26), PDL expression of collagen-I and bone sialoprotein was measured. Further characterization of the PDL cells was confirmed by positive staining for alkaline phosphatase; PDL cells also produce calcified nodules when seeded in the presence of osteogenic medium as previously reported (41). Purity of the cells was 90% or above. Receptor expression for ALX/FPR1 was demonstrated to be positive in primary cultures using FACS analysis. The cells used for the experiments proliferated in logarithmic phase between the 8th and 15th passages.

For coculture experiments, PDL cells (2 × 10^4 cells) were seeded in 24-well dishes in DMEM (2 × 10^4) containing 5% FCS and incubated for 24 h at 37°C. Peripheral blood was obtained from different healthy individuals according to the IRB-approved protocol. Peripheral blood mononuclear cells were isolated using a discontinuous gradient system (0.25 × 10^6/ml) as described by Kalmar et al. (18). PBS was added to the mononuclear cell suspension and centrifuged at 350 g for 10 min at room temperature. Contaminating nonmonocytic cells were removed. Monocytes were resuspended in DMEM (0.2 × 10^6/ml) with 10% FCS. DMEM containing primary monocytes (0.2 × 10^6/ml) were cultured either alone or with PDL cells. After 4 h, the medium was removed and the cells were rinsed twice with serum-free medium. DMEM containing 1% FCS with IL-1β (500 pg/ml) or TNF-α (10 ng/ml) in the presence or absence of 0.1–10 ng/ml RvD1 (0.27–27 nM) was added, and cells were incubated for 24 h. The culture supernatants were collected and stored at −70°C until analyzed. For the bFGF experiments, PDL fibroblasts (1.5 × 10^5 cells/cm^2) were seeded in Nunc multiwell dishes (24-well plate) in DMEM containing 5% FCS and grown for 48 h at 37°C. The cells layers were then rinsed three times with serum-free medium. Cultures were then incubated in DMEM medium containing 1% FCS with and without human recombinant human recombinant IL-1β and RvD1 for 24 h.

**Quantification of PGE2, LTB4, LXA4, and bFGF.** PGE2 and LTB4 in supernatants were analyzed using a commercially available kit for multiplex immunoassay (Cayman, Ann Arbor, MI). Human recombinant IL-1β and bFGF ELISA kit (R&D Systems) following the instructions of the manufacturer and analyzed on a Luminex 100 (Austin, TX) platform. LXA4 was analyzed using ELISA kit (Neogen, Lansing, MI). The assay was performed following the instructions of the manufacturer and analysis was performed on a Luminex 100 (Austin, TX) platform. LXA4 was analyzed using ELISA kit (Neogen, Lansing, MI). The assay was performed following the instructions of the manufacturer and measured by a microplate reader at 650 nm. bFGF was measured using ELISA kit (R&D Systems) following the instructions of the manufacturer.

**Cell proliferation.** Cell proliferation and cell viability were analyzed using a colorimetric assay that quantifies cleavage of tetrazolium salts to formazan by mitochondrial dehydrogenase in viable cells. The color change is directly proportional to the amount of mitochondrial dehydrogenase in a given culture; the assay measures tissue metabolic activity of cells thereby reflecting the cell number. PDL fibroblasts (1.5 × 10^6 cells/cm^2) were seeded in 24-well plates in a medium containing 5% FCS at 37°C for 48 h. The cells were then treated in a serum-free medium with RvD1 at different doses for 24 h. Ten microliters of cell proliferation reagent WST-1 (Roche Molecular Biochemicals, Mannheim, Germany) were added to each well and
seeded in 12-well plates at a density of 3 cells were passaged using Trypsin/EDTA (Mediatech), counted, and incubated at 37°C in a humidified incubator for 4 h. The samples were shaken for 1 min, and absorbance at 450 nm was measured on a microplate spectrophotometer (BMG LABTECH). Fresh medium was used as a negative control. Scratch wound healing model. PDL fibroblasts were grown in 10% FBS, DMEM medium (ATCC) with 1% MEM NEAA (GIBCO), and were incubated at 37°C in a humidified atmosphere of 95% air-5% CO2. Medium was changed every 3 days. When confluent, they were incubated at 37°C in a humidified atmosphere of 95% air-5% CO2. Medium was changed every 3 days. When confluent, cells were passaged using Trypsin/EDTA (Mediatech), counted, and seeded in 12-well plates at a density of 3 × 10^4 cells using the same growth medium until they reached confluence for experiments. Then, a linear scratch wound (3-mm wide) was created using a rubber policeman as previously described (37). PDL cell monolayers were incubated with RvD1 (100 ng/mL; 270 nM) or vehicle in 1% FBS DMEM medium until the scratch completely closed. Standardized computer images were taken daily until the wounds were closed, and distance was measured using the computer software.

Statistical analysis. Student’s t-test (two-tailed) was used to compare the difference between the groups with or without RvD1 treatment. For multiple comparisons, one-way ANOVA with Bonferroni corrections was performed.

RESULTS

PDL cell response to IL-1β and TNF-α. PDL cells generated significant levels of PGE2 in response to IL-1β (500 pg/mL) or TNF-α (10 ng/mL) demonstrating their capacity to be actively involved in the inflammatory process as responders to proinflammatory cytokines (Fig. 1; P < 0.05). Addition of RvD1 to PDL cultures resulted in a statistically significant reduction of IL-1β and TNF-α-induced PGE2 release by PDL cells (P < 0.05). The magnitude of reduction was 47% (range 33–57% for TNF-α and 45–48% for IL-1β) demonstrating that the proinflammatory changes in PDL cell function are significantly countered by the action of RvD1. LTB4 generation by PDL cells was not detectable (data not shown). In parallel, IL-1β stimulated substantial release of LXA4 by PDL cells suggesting that arachidonic acid metabolism along with proresolution pathways might be involved in the PDL cell response to a proinflammatory cytokine (Fig. 2; P < 0.05). This response, although increased, was not significant for TNF-α suggesting the difference between signaling pathways responding to various cytokines. The data suggest that the proinflammatory activation of PDL cells is coupled with the capacity to resolve inflammation through the release of specific agonists in response to proinflammatory challenge.

PGE2 production by PDL cells in monocyte cocultures. Next, we tested the impact of RvD1 on PDL cell PGE2 production when cultured with peripheral blood monocytes to identify whether cell-to-cell contact with an inflammatory cell population has functional impact on PDL cells or if PDL cell stimulation is driven by the proinflammatory cytokines alone. As shown in Fig. 1, proinflammatory cytokines lead to significant production of PGE2 by PDL fibroblasts. Since monocytes and macrophages are the major cell types that produce these proinflammatory cytokines, we have cocultured the PDL fibroblasts with peripheral blood monocytes obtained from healthy donors. The first experiment demonstrated that in the absence of an inflammatory stimulant, monocytes lead to a statistically significant increase in PGE2 production by PDL cells (Fig. 3A; P < 0.05). RvD1 (10 ng/mL; 27 nM) did not prevent this increase. When PDL cells were stimulated with IL-1β (500 pg/mL), significant levels of PGE2 were produced and the response was reduced by ~50% in the presence of RvD1 (Fig. 3B). When IL-1β was used in cocultures of PDL cells and monocytes, the PGE2 release increased significantly compared with unstimulated cells confirming the observations above (Fig. 1A; P < 0.05). RvD1 significantly reduced IL-1β-induced PGE2 production by PDL cells and PDL-monocyte cocultures (P < 0.05), while there was no significant impact on monocytes alone. LTB4 generation by PDL cells in response to monocytes was not detectable (data not shown). These findings suggest that PDL cells are stimulated by both cellular and extracellular inflammatory signals, while only cytokine-induced PDL cell function is responsive to the RvD1.

LXA4 production by PDL cells in response to monocytes. LXA4 production by PDL cells in coculture with primary
monocytes was assessed. PDL cells generate significantly higher levels of LXA\(_4\) in the presence of monocytes compared with baseline levels and monocyte production alone (Fig. 4A; \(P < 0.05\)). When the cells were treated with IL-1\(\beta\) (500 pg/ml), there was a parallel response to the unstimulated cells and cocultures (Fig. 4B). IL-1\(\beta\) stimulated LXA\(_4\) generation in PDL-alone or PDL-monocyte cocultures suggesting that the LXA\(_4\) production by PDL cells increased in response to stimulation with proinflammatory cytokines. This finding further supported the notion that resolution of the inflammatory response is associated with PDL fibroblast function involved during the regeneration of tissues.

**Proliferation of PDL fibroblasts and migration in a wound closure.** RvD1 dose dependently increased the proliferation of the PDL fibroblasts over 24 h of culture (Fig. 5A). Peak response was observed at 10 ng/ml of RvD1 treatment. The “scratch assay” is a widely used procedure for monitoring cell migration in vitro as an indicator of wound closure. Primary human PDL cells were grown to confluence and treated with RvD1 (100 ng/ml) as described above. Figure 5B demonstrates that the PDL migration and the resulting wound closure is significantly increased in cultures treated with RvD1 compared with the vehicle control \((P < 0.05)\) suggesting that the RvD1 enhances wound healing activity of PDL cells both through enhanced cell motility and proliferation in parallel with the resolution of inflammation. In parallel to these proliferative events, bFGF release by the PDL fibroblasts was enhanced in the presence of RvD1 at both concentrations tested compared with the vehicle control when cells were stimulated by IL-1\(\beta\) \((P < 0.05;\) Fig. 5C).

**DISCUSSION**

PDL cells are highly specialized fibroblasts with unique involvement in the regeneration of soft and hard tissues of the periodontium lost to disease. The goal of regenerative therapies for periodontitis is enhancement of wound healing and restoration of tissue integrity. As such, PDL cells play a critical role for regeneration of the periodontal tissues as the primary target in tissue engineering (32). Several approaches have been de-
scribed to enhance PDL cell repopulation of the dental root surface in an attempt to create a new attachment between the tooth and the alveolar bone, while preventing the apical migration of epithelial cells; all of these approaches have failed to fully restore tissue integrity (4). We demonstrated earlier that resolution of inflammation results in full regeneration of the lost periodontal tissues in a rabbit model of periodontitis (14). All tissues and cells of the periodontium were actively involved in this process. Therefore, we hypothesized that non-inflammatory cells of the periodontal tissues also respond to the resolvins during wound healing and regeneration. The present results demonstrate that PDL fibroblasts respond to RvD1 with reduced PGE2. Coculture experiments with monocytes demonstrate that this activity is dependent on the cytokines present rather than cell-cell interactions and that an inflammatory milieu potentiates the action of RvD1. In parallel, RvD1 enhances the proliferation, rate of PDL migration and wound closure, and bFGF production collectively providing a potential link in the active coordination of resolution of inflammation, wound healing, and tissue regeneration.

Proliferation and enhanced migration of fibroblasts may lead to fibrotic changes in various organs including the gingival tissues (19, 58). This process is regulated by CCN2/CTGF and TGF-β1 and through prostaglandin receptors with high specificity (3, 19, 54). However, it should be noted that gingival fibroblasts and PDL fibroblasts, while phenotypically sharing the same characteristics, have substantial functional differences (6, 24, 31). Our finding that PDL fibroblasts respond to RvD1 and generate significant levels of LXA4 supports a potential link in the active coordination of resolution of inflammation, wound healing, and tissue regeneration.

Our findings are consistent with those of earlier reports that demonstrated that the inflammatory mediators IL-1β and TNF-α increase the production of PGE2 by human PDL cells (36). Herein, we report for the first time that the production of PGE2 is enhanced in PDL cells and monocyte cocultures similar to the interactions between gingival fibroblasts and lymphocytes (63). Prostaglandins are important biological mediators involved in the inflammatory response, as well as regulation of bone formation and bone resorption (43). PGE2 is implicated in the pathogenesis of periodontal diseases and suggested to be a molecular marker for disease as the level is enhanced in gingival crevicular fluid, as well as in inflamed periodontal tissues during active disease (7). The exogenous application of PGE2 enhanced neutrophil recruitment and mediated tissue injury in rabbit periodontitis (14). Since PGE2 is strongly associated with the progression of periodontal disease, it is reasonable to assume that compounds that act as modulators of the inflammatory response, shifting the response to the resolution phase, may be of importance in the treatment of periodontal diseases. In this context, the finding that RvD1 reduces the production of PGE2 by PDL cells may be of relevance for tissue protection (52). Biosynthesis of PGE2 involves the translocation of NF-κB (9) and expression of COX and PGE synthase (16). Historically, blocking agents have been used to counteract PGE2 production (57, 59). Most of these agents were found to abolish PGE2 production through complete blockage of COX (33). However, inhibition of COX activity may also prevent the generation of the resolution phase by inhibition of lipoxin production (20). Lipoxins in turn promote resolution actively by retarding the entry of new neutrophils to sites of inflammation and promoting neutrophil apoptosis (50).

Active resolution of inflammation is a process driven by novel compounds such as lipoxins and resolvins that provide a potential set of pharmacologic tools to dampen the damaging aspects of the inflammatory response without inhibition of enzyme pathways or receptor antagonism (47). RvD1 induced a decrease in PGE2 enhancing the wound healing capacity of PDL cells. PDL cells may also act as immune accessory cells...
in addition to their central role maintaining the structural integrity of the connective tissues in injury and healing processes. In this context, the capability of PDL cells to produce LXA₄ emphasizes their role contributing to healing and tissue regeneration. RvD1 exerts its resolving actions through specific receptors (23). While expression of receptors for RvD1 on PDL cells remains to be investigated, the results from the present study provide evidence for a novel mechanism for the proresolving actions of RvD1 on PDL cells and open new terrain for exploiting the healing and regenerative actions of resolvins demonstrated in vivo.

REFERENCES

AUTHOR CONTRIBUTIONS

C. Serhan and T. Van Dyke are inventors on patents [resolvins] assigned to BWH and BU and licensed to Resolvyx Pharmaceuticals. C. Serhan and T. Van Dyke are scientific founders of Resolvyx Pharmaceuticals and own equity in the company. The interests of C. Serhan and T. Van Dyke interests were reviewed and are managed by BWH and Partners HealthCare and The Forsyth Institute, respectively, in accordance with their conflict of interest policies.

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