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Resolvin E1 Regulates Inflammation at the Cellular and Tissue Level and Restores Tissue Homeostasis In Vivo

Hatice Hasturk,* Alpdogan Kantarci,* Emilie Goguet-Surmenian,* Amanda Blackwood,* Chris Andry,† Charles N. Serhan,‡ and Thomas E. Van Dyke2*

Resolvin E1 (RvE1) is a potent proresolving mediator of inflammation derived from omega-3 eicosapentaenoic acid that acts locally to stop leukocyte recruitment and promote resolution. RvE1 displays potent counter-regulatory and tissue-protective actions in vitro and in vivo. Periodontal disease is a local inflammatory disease initiated by bacteria characterized by neutrophil-mediated tissue injury followed by development of a chronic immune lesion. In this study, we report the treatment of established periodontitis using RvE1 as a monotherapy in rabbits compared with structurally related lipids PGE₂ and leukotriene B₄. PGE₂ and leukotriene B₄ each enhanced development of periodontitis and worsened the severity of disease. Promotion of resolution of inflammation as a therapeutic target with RvE1 resulted in complete restoration of the local lesion, and reduction in the systemic inflammatory markers C-reactive protein and II-1β. This report is the first to show that resolution of inflammation by a naturally occurring endogenous lipid mediator results in complete regeneration of pathologically lost tissues, including bone. The Journal of Immunology, 2007, 179: 7021-7029.

Inflammation is the normal host tissue response to infection and injury. However, uncontrolled and unresolved inflammation contributes to a range of acute and chronic human diseases such as arthritis and cardiovascular diseases. Chronic inflammation is characterized by the production of inflammatory cytokines, arachidonic acid-derived eicosanoids (prostaglandins, thromboxanes, leukotrienes, and other oxidative derivates), reactive oxygen species, and adhesion molecules (1–3). Periodontitis is a similar progressive inflammatory disease in which microbial etiologic factors induce an inflammatory cascade that leads to destruction of the organ supporting the teeth (periodontium), including soft tissues and bone (4–7). The very nature of periodontitis, being in the oral cavity and therefore easily observable, led to the use of periodontal diseases as a model system for other inflammatory diseases (8–10). As with all inflammatory diseases, the arachidonate-derived eicosanoids play a key role in the initiation and pathogenesis of the inflammatory lesion in periodontitis (11–13). In the case of periodontitis, the etiologic Gram-negative bacteria, such as Porphyromonas gingivalis, initiate an influx of neutrophils and neutrophil cyclooxygenase-2 activation leading to increased PGE₂ in situ (14). Indeed, many of the early pathophysiology events in periodontal diseases and its chronicity can be attributed to lipid mediators (15). Leukotriene B₄ (LTB₄),3 produced mainly by activated leukocytes, initiates accumulation and superoxide generation by neutrophils within inflamed sites, stimulating the release of granule-associated enzymes and bone resorption (16, 17). PGE₂ is a potent activator of osteoclast-mediated bone resorption, the hallmark of periodontal disease (13, 18) and, with other eicosanoids, mediates inflammation and periodontal tissue destruction (19, 20).

Resolution of inflammation is an actively regulated program rather than the passive termination of inflammation (21–24). The crucial identification of the cellular events and molecular signals that determine the end of inflammation and beginning of resolution has lead to a new appreciation of pathogenesis in inflammatory diseases (25–28). Neutrophils are present mainly in inflamed or injured tissues and their effective elimination is a prerequisite for complete resolution of an inflammatory response (29). Most current therapeutic approaches attempt to block activation of inflammation using anti-inflammatory drugs (nonsteroidal anti-inflammatory drugs, TNF inhibitors), or to promote healing with agents such as TGF-β1, bridging molecules, and phagocyte receptors (30). Prostaglandins and leukotrienes play essential roles in orchestrating inflammation and are well appreciated autacoids or local-acting mediators (17). Cyclooxygenase inhibitors are widely used examples of anti-inflammatory drugs that act by blocking prostaglandin biosynthesis (31) but can be toxic to resolution programs in vivo because their development anteceded the recognition of resolution as an active process (21, 22, 32).

A rapidly emerging body of evidence demonstrates that endogenous mediators actively participate in dampening host responses to orchestrate resolution of inflammation (33, 34). Lipoxins, the product of lipooxygenase: lipoxygenase interactions, actively drive resolution of inflammation. In addition, the role of previously unappreciated aspirin-triggered transformation circuits has led to a

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3 Abbreviations used in this paper: LTB₄, leukotriene B₄; RvE1, Resolvin E1; EPA, eicosapentaenoic acid; CRP, C-reactive protein; PUFA, polyunsaturated fatty acids; TRAP, tartrate-resistant acid phosphatase.
better understanding of proresolving signaling networks, including a series of complex cellular and chemical reactions, and tissue trafficking events (35). For example, lipoxins not only reduce influx of neutrophils, but also stimulate the nonphlogistic uptake of apoptotic neutrophils by tissue macrophages (21, 36).

Omega-3 polyunsaturated fatty acids (ω-3 PUFAs) are well known to decrease the production of inflammatory eicosanoids, cytokines and reactive oxygen species, and the expression of adhesion molecules (37). However, the molecular basis remains unknown. An ω-3 PUFA, eicosapentaenoic acid (EPA), is metabolized by aspirin-modified cyclooxygenase-2 to form a novel small molecule that promotes resolution of inflammation termed resolvins (39). Taken together, the findings suggest that a rational approach to the treatment of inflammatory diseases may involve the use of agonists of proresolving in addition to, or instead of, traditional anti-inflammatory approaches that block initiation pathways of inflammation and display unwanted side effects.

Materials and Methods

Animal model

The study was approved by Boston University Medical Center (BUMC) Institutional Animal Care and Use Committee before study initiation (IACUC protocol no. AN-13948). In addition, BUMC Institutional Biosafety Committee (IBC) approved the use of _P. gingivalis_ in this animal model to induce periodontal disease (IBC protocol no. 06-016).

New Zealand White rabbits (39 males, 3.5–4.0 kg) were purchased from Pine Acres Rabbit Farms (PARF), kept in individual cages, received water ad libitum, and fed standard rabbit chow at the Laboratory Animal Science Center at BUMC.

Bacteria growth and gel preparation

_P. gingivalis_ (strain A7436) was grown as previously described (9). In brief, bacteria were cultured on agar plates containing trypticase soy agar supplemented with 0.5% (w/v) yeast extract (Invitrogen Life Technologies), 5% defibrinated sheep RBC, 5 μg of hemin, and 1 μg/ml vitamin K (Sigma-Aldrich). Plates were incubated for 3 days at 37°C in jars anaerobically maintained through palladium-catalyzed hydrogen/carbon dioxide envelopes (Gas Pak Plus; BD Microbiology Systems). Colonies were randomly selected and anaerobically cultured overnight at 37°C in Schaeffler’s broth supplemented with vitamin K and hemin. Bacterial numbers were spectrophotometrically determined at 600 nm, adjusted to 10⁶ CFU (0.8 OD) and mixed with carboxymethylcellulose to form a thick slurry, which was applied topically to the ligated teeth every other day for the first 6 wk of the experiment.

_P. gingivalis*-induced periodontitis

Periodontitis in rabbits was used to monitor inflammatory events associated with bone disorders and the actions of the proresolving agonist, RvE1. Periodontitis was induced and established in all animals for a 6-wk period using a previously established protocol (9, 10). A 3.0 silk suture (ligature) was placed around the second premolar of both mandibular quadrants under general anesthesia (40 mg/kg ketamine, Ketaset (Fort Dodge Animal Health) and 5 mg/kg xylazine, Anased (Ben Venue Laboratories) injections). The slurry containing _P. gingivalis_ was applied to the ligatures on Monday, Wednesday, and Friday over a 6-wk period to induce periodontal inflammation and associated soft tissue and bone destruction at the disease baseline.

The RvE1 preparation was delivered in ethanol (8 μl) and the same volume of ethanol served as placebo. LTβ was purchased as a solution in ethanol (Cayman Chemicals) and delivered to the site via a Hamilton syringe in the same volume as RvE1. Concurrently, PGE₂ was purchased in salt form (Cayman Chemicals) and diluted in ethanol to obtain the same dose as RvE1. At the end of the 6-wk period, animals were euthanized using an overdose of pentobarbital (120 mg/kg Euthanasia-5 solution; Veterinary Laboratories) according to the approved protocol of IACUC.

Morphometric analysis

After sacrificing the animals, the mandible was dissected free of muscle and soft tissue, keeping the attached gingiva intact. The mandible was split into two halves from the midline between the central incisors. Half was taken for morphometric analysis of bone loss, and the other half was used for histological evaluation of periodontitis. For morphometric analysis, the sectioned mandible was dehydrated by immersion in 10% hydrogen peroxide followed by 3% hydrogen peroxide with 0.5% (w/v) yeast extract (Invitrogen Life Technologies) and 0.5% (w/v) yeast extract (Invitrogen Life Technologies) and 0.5% (w/v) yeast extract (Invitrogen Life Technologies). To quantify bone loss, the length of the tooth from cusp tip to apex of the root was measured, as was the length of the tooth structure outside the bone that was measured from the cusp tip to the coronal extent of the periodontal pocket. From this, the individual percentages of the teeth within the bone were calculated (42). Bone values are expressed as the percentage of bone loss.

In addition, the soft tissue (pocket) depth and infrabony defect depth were measured in all groups using a 0.5 mm calibrated periodontal probe. The tip of the tooth at the measured site was used as the reference point for these measurements. Furthermore, tooth mobility was also calculated using Muhleman’s mobility index (43) as follows: 0 = no mobility; 1 = >0.5 and <1 mm mobility buccolingually; 2 = >1 mm mobility buccolingually; 3 = >2 mm mobility buccolingually; 3+ = both vertical and buccolingual mobility.

Qualitative histological evaluations

Half of the mandible was immersed in 10 volumes of ImmunoPure (Decal Corporation) and the solution was replaced every 24 h for 2 wk. Decalcification was confirmed by serial radiographs, which were taken every other day. After the decalcification, the tissues were rinsed for 1–3 min in running water, placed in Cal-Arrest (Decal Corporation) to bring tissues to alkaline pH, and placed in 4% formalin/1% CaCl₂ (46). The specimens were dehydrated and embedded in methylmethacrylate. Eight to ten un-decalcified sections of the cellular composition of inflammatory infiltrates or with tartrate-resistant acid phosphatase (TRAP) to examine osteoclastic activity of the cellular composition of inflammatory infiltrates or with tartrate-resistant acid phosphatase (TRAP) to examine osteoclastic activity. For qualitative assessments, three areas were analyzed on each tooth corresponding to the coronal, middle, and apical third of the root. In addition, some of the sections per animal were stained with Masson’s trichrome. Sections were analyzed for qualitative histological findings.
Quantitative histomorphometry

To quantify the changes in bone, the mean value (±SD) of the linear distance and the area of bone loss were calculated for each group. Previously developed measurement technique (47) was used to calculate the bone changes at three different sections of the root using the Prolmage software. The linear measurements were made at three levels each corresponding to one third of the root and alveolar bone interface: crestal, mid, and apical. Linear distance is reported as the distance from the base of the epithelium to the alveolar crest border at the three chosen levels, the apical, middle, and coronal third of the root and is expressed as the difference between treated and untreated sites. Likewise, area measurements were presented as the difference between the treated and untreated total area.

In addition, osteoclastogenesis was examined in all TRAP-stained sections by calculating the osteoclasts in affected areas. The total number of osteoclasts at the surface of the bone was compared between the groups.

Microbial sampling

Microbial dental plaque was sampled at baseline at 6 and 12 wk using paper points. The area was isolated to prevent saliva contamination, air-dried and 30 s samples were collected using sterile paper points according to previously reported methods (48). Each sample was placed in an individual Eppendorf tube containing 0.15 ml TE (10 mM Tris-HCl, 1 mM EDTA [pH 7.6]) and 0.5 M NaOH was added for stabilization. Eighteen species representing periodontal organisms including P. gingivalis, Actinobacillus actinomycetemcomitans, Actinobacillus odontolyticus, Actinomyces viscosus, Actinomyces israelii, Peptostreptococcus micros, Prevotella intermedia, Prevotella nigrescens, Capnocytophaga curvata, Capnocytophaga recta, Streptococcus oralis, Streptococcus intermedius, Tannerella forsythensis, Treponema denticola, Eikenella corrodens, Fusobacterium nucleatum, Escherichia coli, and Esterococcus faecalis were investigated in each plaque sample using the checkerboard DNA-DNA hybridization technique (49). Evaluation of the chemiluminescent signals is performed by radiographic detection, comparing the obtained signals with the signals generated by pooled standard comparisons of 10^4, 10^5, and 10^6 of each of the 18 species. Chemiluminescence units are transformed into a scale of 0 to 5, 0: no signal; 1, signal density weaker than the low standard (i.e., undetectable <10^4 bacteria); 2, signal density equal to the next standard (=10^4 bacteria); 3, signal density higher than the 10^4 standard but lower than that of the 10^5 standard (>10^4 but <10^5 bacteria); 4, signal density equal to the high standard (=10^5 bacteria); and 5, signal density higher than the high standard (>10^5 bacteria).

Cytokine levels

Blood samples were obtained from the central ear artery using standard venupuncture techniques from each animal at baseline; 6 and 12 wk. Serum samples were collected and stored at 80°C for analysis of IL-1β and C-reactive protein (CRP). CRP was determined using a rabbit-specific ELISA kit following the manufacturer’s instructions (Immunology Consultants Laboratory). IL-1β was analyzed by ELISA specific for human IL-1β according to the manufacturer (Abcam).

Statistical analysis

Mean values for linear and area measurements were used to determine the changes in bone level. In addition, TRAP plus stained cell counts were calculated to detect the osteoclastogenesis. Multiple comparisons within groups were made using ANOVA with Bonferroni correction (α = 0.05). Mean values for IL-1β and CRP levels were used to detect the changes in inflammatory markers before and after treatment. Statistical analysis was performed using nonparametric tests. Comparisons between the six study groups were performed using the Kruskal-Wallis test. In case of significant differences, post hoc two-group comparisons were made with the χ² test. The statistical significance was set at p = 0.05.

Results

Treatment of established inflammatory disease with RvE1

To examine the potential of proresolving molecules such as RvE1 for the treatment of inflammatory diseases, we used a unique in vivo system to assess both soft and hard tissue destruction, namely rabbit periodontitis (9, 10). A common human periodontal pathogen, P. gingivalis, induced periodontitis. Thirty nine male New Zealand White rabbits were used as described in Materials and Methods. Disease was induced for 6 wk in all groups (Fig. 1). One group was sacrificed at 6 wk to determine baseline disease. The other groups then entered treatment for an additional 6 wk. The treatment arms of the study included RvE1 as a monotherapy; vehicle-alone (95% ethanol) as a placebo control; and two structurally related lipid mediators, LTB4 and PGE2, as alternative monotherapies. Results revealed that P. gingivalis induced significant periodontal disease (Fig. 2, A1/B1). The progression of disease was unaffected by placebo therapy, and progression was significantly more severe when either LTB4 or PGE2 was used (Fig. B1/C1). RvE1 treatment resulted in significant bone and soft tissue attachment gain, whereas other treatment groups showed progression characterized by soft tissue and bone destruction (*, p < 0.05) (BSL, baseline periodontitis; VHC, vehicle-alone).
RESOLUTION OF INFLAMMATION RESTORES TISSUE HOMEOSTASIS

 FIGURE 3. RvE1 induces restoration of lost bone. Radiographic bone loss was quantified (see Materials and Methods). Baseline periodontal disease (6 wk) resulted in ~30% bone loss (inset). Radiographic analyses revealed that RvE1 restored the lost bone (~95%), whereas the vehicle-alone, LTB4-, and PGE2-treated groups showed ~13, 9, and 18% more bone loss, respectively.

2, A2/B2, A4/B4, and A5/B5, respectively). Topical RvE1 treatment resulted in essentially complete resolution of periodontal inflammation and restoration of both soft and hard tissues clinically (Fig. 2, A3/B3). The irregular, edematous and hyperemic appearance of soft tissues was also resolved, and no clinical signs of inflammation were identifiable in RvE1-treated rabbits. Surprisingly, RvE1 treatment resulted in bone regrowth to pre-disease levels. Periodontal disease progressed from baseline disease (6 wk) in each of the other groups (Fig. 2, A2/B2, A4/B4, and A5/B5).

Quantitative morphologic assessments demonstrated that administration of topical RvE1 (4 μg/tooth) resulted in statistically significant bone regrowth (>95%) compared with baseline periodontitis (Fig. 2, A6). Both the infrabony (vertical) defects and horizontal bone loss were completely restored. Teeth in all groups were mobile at 6 wk due to destruction of periodontal attachment and local inflammation. Mobility became quite severe by 12 wk in all groups except the RvE1 treatment group where the teeth exhibited essentially no mobility at 12 wk.

Radiographic bone levels were calculated using the modified Bjorn technique (42) (Fig. 3). Baseline periodontal disease (6 wk) displayed ~30% bone loss (Fig. 3, inset). Analyses of radiographic images demonstrated that RvE1 treatment restored the lost bone, whereas those treated with vehicle alone, LTB4, or PGE2 progressed with ~13, 9, and 18% more bone loss, respectively, compared with baseline disease.

In addition, clinical periodontal disease parameters including pocket depth (distance between crest of the gingiva and soft tissue attachment on the tooth surface), infrabony defect depth (vertical dimension of bone loss) and tooth mobility (43) were evaluated (Table I). Pocket depths and infrabony defect depths reflected established periodontitis at 6 wk (baseline periodontitis). Evaluation after a 6-wk treatment phase (at 12 wk) revealed that topical RvE1 treatment resulted in statistically significant decreases in pocket depth and infrabony defect depth when compared with baseline periodontitis and all other treatment groups (p < 0.05). At 12 wk, the other treatment groups (i.e., vehicle-alone, LTB4, and PGE2) exhibited significantly greater pocket depth, infrabony defect depth and tooth mobility than at 6 wk, indicating progression of the periodontal disease.

Composition of the rabbit oral microflora in health and disease

Although the pathogenesis of periodontitis appears to be inflammatory, the etiology is the bacterial biofilm that forms on teeth commonly known as dental plaque. These biofilms appear to release chemorepellent, which stimulates recruitment of neutrophils that, without control, can destroy surrounding tissues. To this end, P. gingivalis was introduced to the rabbit oral microflora as an exogenous pathogen to initiate local inflammation and periodontal disease during the first 6 wk of the study. At 6 wk, addition of the pathogen ceased (Fig. 1). Dental plaque samples were collected at baseline and 6 and 12 wk to determine the persistence of the pathogen and the response of the resident microflora. The rabbit oral microflora comprised anaerobic and aerobic bacteria; predominantly A. viscosus, P. micros, and C. curvus and C. rectus in health (50) (Table II). When P. gingivalis was introduced, there was a shift to a more anaerobic flora in the biofilm and an overall increase in bacterial load. Previously undetected species including S. intermedius and F. nucleatum were observed. P. gingivalis was detectable throughout the experiment except in the RvE1-treated group (Table II). There is a precedent for the observation of Gram-negative pathogens altering the dynamics of the endogenous biofilm (51) suggesting biofilm behavior in rabbits is similar to that observed in humans.

Histological evaluation

H&E-stained sections showed significant inflammatory cell infiltration in connective tissue and bone resorption with irregular bone surfaces and resorptive lacunae in all groups except the RvE1-treated group, where inflammatory changes and bone loss were essentially completely reversed (Fig. 4a). Histomorphometric analysis of H&E-stained sections complemented clinical assessments. Linear and area measurements revealed significant changes in bone levels between treatment groups (Fig. 4b). The vehicle-alone, PGE2-, and LTB4-treated groups progressed significantly by 12 wk to ≥50% bone loss. In sharp contrast, RvE1 treatment resulted in 30% bone gain restoring bone to pretreatment levels.

To differentiate between bone growth and regeneration of the periodontal organ (periodontal ligament, cementum, and bone),

Table I. Clinical impact of treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pocket Depth (mm)</th>
<th>Infrabony Defect Depth (mm)</th>
<th>Tooth Mobilityb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buccal</td>
<td>Lingual</td>
<td>Buccal</td>
</tr>
<tr>
<td>Baseline periodontitis</td>
<td>3.9 ± 1.1</td>
<td>3.2 ± 1.0</td>
<td>4.2 ± 0.9</td>
</tr>
<tr>
<td>Vehicle-alone</td>
<td>7.0 ± 0.8d</td>
<td>6.1 ± 0.7d</td>
<td>6.9 ± 0.7d</td>
</tr>
<tr>
<td>RvE1-treated</td>
<td>0.7 ± 0.8g</td>
<td>0.4 ± 0.7g</td>
<td>0.6 ± 0.3g</td>
</tr>
<tr>
<td>LTB4-treated</td>
<td>5.1 ± 0.4d</td>
<td>4.5 ± 0.4d</td>
<td>5.2 ± 0.8d</td>
</tr>
<tr>
<td>PGE2-treated</td>
<td>6.6 ± 0.4d</td>
<td>5.9 ± 0.7d</td>
<td>7.2 ± 0.4d</td>
</tr>
</tbody>
</table>

a Distance between tip of the gingiva and soft tissue attachment on the tooth surface.

b Vertical dimension of bone loss (distance between the tip of the tooth and the base of the bone defect).

c Movement of the tooth as a result of bone and soft tissue attachment loss.

p < 0.05 compared with baseline periodontitis, vehicle-alone, LTB4-, and PGE2-treated groups.

p < 0.01 compared with baseline periodontitis, vehicle-alone, LTB4-, and PGE2-treated groups.
un-decalcified sections were stained with Van Gieson’s, and evaluated by light and polarized microscopy (46). The examination of the un-decalcified sections revealed regeneration of tissues including the periodontal ligament, cementum, and bone at the sites treated with RvE1 (Fig. 5). The regenerated tissues were indistinguishable from the native structures found in the apical areas of the same teeth. Adjacent to the cementum on the root surface, newly formed and continuous periodontal ligament with oblique connective tissue fibers was observed (Fig. 5a). Sharpey’s fibers inserting in newly formed cementum and bone were observed. The lamellar nature of new bone was apparent when viewed under polarized light (Fig. 5b), and the regenerated bone was generally rather mature with only remnants of woven bone.

Masson’s Trichrome stained sections were also evaluated for evidence of new bone and connective tissue formation (Fig. 6).

<table>
<thead>
<tr>
<th>Microbial Species</th>
<th>Healthy</th>
<th>Baseline periodontitis</th>
<th>RvE1 treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. actinomycetemcomitans</td>
<td>0</td>
<td>1.5 ± 0.7</td>
<td>0</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>0</td>
<td>4.7 ± 1.3</td>
<td>0</td>
</tr>
<tr>
<td>A. odontolyticus</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A. viscosus</td>
<td>2.4 ± 0.7</td>
<td>3.9 ± 1.6</td>
<td>2.2 ± 1.1</td>
</tr>
<tr>
<td>A. israelii</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P. micros</td>
<td>2.6 ± 0.9</td>
<td>4.1 ± 1.4</td>
<td>2.4 ± 0.9</td>
</tr>
<tr>
<td>P. intermedia</td>
<td>0.1 ± 0.4</td>
<td>1.7 ± 0.9</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td>P. nigrescens</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. curva</td>
<td>0.3 ± 0.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. rectus</td>
<td>0.7 ± 0.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S. oralis</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S. intermedius</td>
<td>0.5 ± 0.4</td>
<td>2.1 ± 0.8</td>
<td>1.1 ± 0.7</td>
</tr>
<tr>
<td>T. forsythensis</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T. denticola</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E. corrodens</td>
<td>1.5 ± 0.7</td>
<td>3.8 ± 1.2</td>
<td>1.4 ± 0.8</td>
</tr>
<tr>
<td>F. nucleatum</td>
<td>0</td>
<td>2.4 ± 1.1</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td>E. coli</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Chemiluminescence units (scale of 0 to 5 with 0 indicating no signal): 1, undetectable <10⁴ bacteria; 2, 10⁵ bacteria; 3, >10⁵ but <10⁶ bacteria; 4, 10⁶ bacteria; and 5, >10⁶ bacteria.

* p < 0.05 compared with healthy.
RvE1 prevents osteoclast-mediated bone resorption

To determine whether the proresolving actions of RvE1 are mediated through the suppression of osteoclastogenesis, TRAP staining was used to identify clastic cells. Resorbing bone lacunae contained large numbers of TRAP-positive cells in vehicle-alone, LTB4-, and PGE2-treated animals, whereas RvE1-treated specimens contained few or nondetectable TRAP-positive cells (Fig. 7, a and b).

In addition, osteoblast activity was evaluated by immunohistochemistry in sections stained with osteocalcin as a marker of new bone formation (52, 53). In sections from RvE1-treated specimens, cells consistent with osteoblasts were demonstrable on newly formed alveolar bone (Fig. 7c). The bone surface was intact and demonstrated the normal characteristics of alveolar bone suggesting that RvE1 treatment regenerated and completely restored the healthy architecture of osseous tissue surrounding the teeth including well organized connective tissue, periodontal ligament, and cementum deposition.

Local inflammation: a modifier of systemic inflammation

There is substantial epidemiologic evidence and some experimental evidence in humans suggesting that periodontal inflammation can influence the course of systemic disease, in particular, cardiovascular disease, diabetes and low-birth weight premature birth (54–57). CRP is considered a component of normal serum, occurring in concentrations ranging from 0.07 to 2.9 μg/ml with a median value of 0.6 to 1.9 μg/ml; however, elevated CRP reflects an elevation in systemic inflammation that is associated with increased risk for cardiovascular disease (58). Elevated serum CRP levels in periodontitis subjects were initially reported by Ebersole et al. (59). IL-1β is produced at the initial stages of inflammation primarily by monocytes and macrophages. IL-1β induces capillary endothelial cells to secrete chemokines and to increase the expression of cell adhesion molecules (60). IL-1β also induces the expression of metalloproteinases (61). To evaluate the influence of a local inflammation on the systemic inflammatory response, we evaluated serum IL-1β and CRP levels. Serum samples were collected from peripheral blood obtained at baseline, 6 and 12 wk (end of the treatment period). Local inflammation (periodontitis) induced elevations of IL-1β and CRP in all animals. Treatment with vehicle-alone resulted in no additional changes, whereas PGE2 treatment induced a marked increase in IL-1β compared with baseline disease. Also, the systemic marker CRP (58) was markedly increased with LTB4 treatment. Topical RvE1 therapy gave a statistically significant reduction in both systemic IL-1β and CRP levels compared with the other groups (Fig. 8) (p <
The levels of IL-1β and CRP with RvE1 treatment were comparable with levels associated with health. No adverse events were apparent throughout the study and no animals were prematurely lost during the study. The rabbits tolerated these procedures well and showed no indication of adverse events related to the pathogen, e.g., lethargy or fever. To examine the potential for local and systemic side effects of RvE1, intraoral soft and hard tissue examinations were performed during the course of the study. In addition, biopsies were obtained from specific internal organs including, esophagus, lung, liver, spleen, and kidney at the end of the study. Clinical assessments of oral soft and hard tissues did not reveal any untoward events including irritation, redness, suppuration or any lesions of oral tissues. Histological evaluations of the tissue biopsies were normal, with the exception of one PGE2-treated animal that exhibited focal areas of inflammation in the liver.

Discussion

In this study, we report for the first time the regeneration of hard and soft tissues lost to inflammatory disease by the activation of inflammation resolving pathways with an endogenous mediator, RvE1, recently identified from ω-3 fatty acids, used as a topical treatment. Homeostasis is a fundamental characteristic of living things. It is the maintenance of the internal environment within tolerable limits and is often described as a process of balance. The physiologic resolution of a well orchestrated inflammatory response is essential to maintain homeostasis at the cellular and tissue level generating specific mediators that can dampen the magnitude of the leukocyte infiltrate during inflammation and promote resolution (24, 62). Recently, a new family of local-acting mediators was discovered that are products of PUFA aspirin-triggered transformation circuits (63). These new chemical mediators are endogenously generated from EPA in inflammatory exudates collected during the resolution phase and were termed resolvins because specific members of the family control the magnitude and duration of inflammation in animal models (10, 24, 39, 40). There is a body of evidence that proresolutes molecules from the new families derived from ω-3 PUFAs, resolvins, and protectins, counterregulate neutrophil infiltration and promote resolution (40). RvE1 specifically interacts with the LTB4 receptor BLT1 on neutrophils and ChemR23 on monocytes to regulate leukocytes during inflammation (41). RvE1 also stimulates the uptake and clearance of local cytokines (29).

Periodontitis is characterized by destruction of connective tissue and bone by the host response (64). Lipid mediators of inflammation play an important role throughout the pathogenesis of periodontitis. In particular, PGE2 and LTB4 are strongly associated with progressive disease (65, 66) and are, in large part, drivers of the chronic lesion. Conversely, recent new discoveries demonstrate that resolution of inflammation is an active process and that homeostasis cannot be achieved until the lesion is free of neutrophils (67). These principles are supported by these studies where we demonstrate that exogenous PGE2 and LTB4 enhance the local inflammatory response leading to neutrophil recruitment and enhanced neutrophil-mediated tissue damage. Monocytes recruited to the chronic lesion enhance the inflammatory response through secretion of more PGE2, IL-1β, TNF-α, and other proinflammatory molecules. The resolving molecules stop neutrophil infiltration and drive neutrophils to apoptosis (23, 29, 68), while at the same time attracting monocytes to the lesion (23). However, the phenotype of the resolvin-recruited monocyte is nonphlogistic (69), and they phagocytose apoptotic neutrophils without contributing to further inflammation or tissue damage.

Much has been written in recent years about the relationship between periodontitis and systemic disease (for review, see Ref. 70). The reported work in this area suggests that the local inflammatory burden presented by periodontal infection causes a systemic inflammatory burden. CRP and IL-1β are most often reported markers of systemic inflammatory burden. As indicated earlier, RvE1 seems to work through specific receptors on cells, but the systemic effect is almost certainly indirect. RvE1 therapy lowers the inflammatory burden locally, which results in a lower systemic inflammatory burden.

Periodontitis is unique among the inflammatory diseases because the etiology is well known and well characterized: the biofilm, dental plaque. Our work complements the earlier observations of Marsh et al. (51), in chemostat biofilm systems that indicated that Gram-negative pathogens have an impact on the stability and dynamics of the biofilm. They observed, as we have here, that the Gram-negative pathogen causes an overgrowth of the resident flora and a shift to a more pathogenic flora that incorporates other Gram-negative pathogens. Of particular interest in this study is the observation that control of inflammation through pro-resolution pathways resulted in elimination of the Gram-negative pathogen from the flora and a return to pre-disease homeostasis of both the resident flora and the host. RvE1 has no inherent antibacterial activity (10). The elimination of the pathogen is likely related to the physiology of the organism.

P. gingivalis is a Gram-negative, obligate anaerobic, asaccharolytic organism. Unable to use sugars for energy, it metabolizes essential amino acids. The source of amino acids is collagen breakdown products provided by the host through inflammation. In fact, P. gingivalis possesses an array of proteolytic enzymes, the gingipains (71), to accomplish this. We hypothesize that resolution of inflammation effectively eliminates P. gingivalis from the lesion by removing the food source. We suggest that while the etiology of periodontitis is bacteria, the pathogenesis is inflammatory. The chronic inflammation supports the growth of the pathogen through production of tissue breakdown products. Resolution of the inflammatory lesion removes the ecological niche of the pathogen. This is supported by our earlier observation that prevention of inflammation with resolving molecules precludes establishment of the pathogen and tissue breakdown (10).

Regeneration of tissues lost to disease is problematic in many human diseases, such as arthritis and periodontitis. Therapies that are aimed at regeneration of lost tissues attempt to recapitulate development with the assumption that the ability to recapitulate development is lost as the organism ages. Hence, the therapeutic strategy is to add back growth factors, substrates, and other molecules to mimic development. The present results emphasize the role of local inflammation in tissue regeneration.

In summary, our results are the first to demonstrate RvE1 as a therapeutic agent in an in vivo leukocyte- and osteoclast-mediated inflammatory disease. RvE1 acts as a modulator of the inflammatory response shifting the response to more rapid resolution and effectively preventing the chronic phase. Elimination of inflammation in the healing lesion promotes tissue regeneration. These principles may be applicable to other inflammatory diseases including arthritis and cardiovascular disease due to the similarities between these diseases, such as the neutrophil induced panus formation in arthritis (72, 73) and the inflammatory tissue damage to blood vessels stimulating atherogenesis (74). These observations taken together provide novel evidence that Resolvin E1 not only plays a key role in controlling inflammation but also might be useful for a wide range of complex inflammatory conditions including bone disorders, such as periodontitis and arthritis, by restoration of stem
cells (75) thereby promoting regeneration of lost tissues, including connective tissue and bone.

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Disclosures

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