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*J Immunol* 2003; 171:6856-6865; doi: 10.4049/jimmunol.171.12.6856

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Reduced Inflammation and Tissue Damage in Transgenic Rabbits Overexpressing 15-Lipoxygenase and Endogenous Anti-inflammatory Lipid Mediators

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PGs and leukotrienes (LTs) mediate cardinal signs of inflammation; hence, their enzymes are targets of current anti-inflammatory therapies. Products of arachidonate 15-lipoxygenases (LO) types I and II display both beneficial roles, such as lipoxins (LXs) that stereoselectively signal counterregulation, as well as potential deleterious actions (i.e., nonspecific phospholipid degradation). In this study, we examined transgenic (TG) rabbits overexpressing 15-LO type I and their response to inflammatory challenge. Skin challenges with either LTB4 or IL-8 showed that 15-LO TG rabbits give markedly reduced neutrophil (PMN) recruitment and plasma leakage at dermal sites with LTB4. PMN from TG rabbits also exhibited a dramatic reduction in LTB4-stimulated granular mobilization that was not evident with peptide chemoattractants. Leukocytes from 15-LO TG rabbits gave enhanced LX production, underscoring differences in lipid mediator profiles compared with non-TG rabbits. Microbe-associated inflammation and leukocyte-mediated bone destruction were assessed by initiating acute periodontitis. 15-LO TG rabbits exhibited markedly reduced bone loss and local inflammation. Because enhanced LX production was associated with an increased anti-inflammatory status of 15-LO TG rabbits, a stable analog of 5S,6R,15S-trihydroxyeicos-7E,9E,11Z,13E-tetraenoic acid (LXA4) was applied to the gingival crevice subject to periodontitis. Topical application with the 15-epi-16-phenoxyl-LXA4 stable analog (ATLa) dramatically reduced leukocyte infiltration, ensuing bone loss as well as inflammation. These results indicate that over-expression of 15-LO type I and LX4 is associated with dampened PMN-mediated tissue degradation and bone loss, suggesting that enhanced anti-inflammation status is an active process. Moreover, they suggest that LXs can be targets for novel approaches to diseases, e.g., periodontitis and arthritis, where inflammation and bone destruction are features.

The Journal of Immunology, 2003, 171: 6856–6865.
properties (10). These results, taken together, indicate that not all of the arachidonic acid-derived eicosanoids are proinflammatory, and that structurally distinct classes of lipid mediators can evoke sharply different actions during an inflammatory challenge.

The rabbit reticulocyte 15-LO was among the earliest studied at the structural level (11). Although this enzyme shares several features akin to 5-LO, it inserts molecular oxygen in arachidonate at the carbon 15 rather than at the carbon 5 position (12, 13). Two sequential hydrogen abstractions initiated by the 5-LO give LTA₄ biosynthesis (14). At the carbon 15 position, insertion of a hydroperoxide can also undergo epoxidation and/or transcellular biosynthesis to produce LXs (5, 15). Because substrate specificity of the 15-LO appears in vitro to be more flexible and/or versatile than that of 5-LO in that, depending on the local conditions (i.e., pH, substrate concentration, divalent cations, temperature, etc.), 15-LO may also oxygenate phospholipids as well as other polyunsaturated fatty acids (13, 15, 16). Hence, the notion that the 15-LO is uncontrolled relies heavily on in vitro observations with isolated enzyme preparations rather than physiologic studies, which has led to vastly differing interpretations of potential biologic role(s) and importance of the 15-LO in vivo.

Along these lines, inhibitors of 15-LO in a hypercholesterolemia-induced atherosclerosis model in rabbits reduced monocyte-macrophages in atherosclerotic lesions (see Ref. 17 and references within). Also, the hypothesis has been set forth that 15-LO could be involved in organelle degeneration/apoptosis (18), which likely arises from early notions of this enzyme’s activity with isolated mitochondria, and the elimination of mitochondria on RBC maturation (19). It is now clear that two forms of cyclooxygenase (COX) (1 and 2) play different physiologic roles (20), and, if we can consider the COX systems as examples, ample evidence from studies of LXs in the immune effector system (2, 5) and many others (4, 6, 7, 21) has recently emerged for at least two distinct structural and functional forms of the 15-LO. This heightened awareness raises the possibility that 15-LO types I and II can serve opposite functions and the likelihood that these first two forms of the mammalian enzymes are functionally distinct signaling systems in physiologic and pathophysiologic processes. It is now clear from results of numerous studies that 15-LO expression is increased in many human diseases. For example, 15-LO increases in human bronchotracheal epithelial cells (22) and aspirin-sensitive asthma (23), and 15-LO type I expression is enhanced at the cellular level by both IL-4 and IL-13 (24–26). Recent results show that IL-4 determines the eicosanoid profile of dendritic cells with counterregulatory expression of 5-LO and concomitant up-regulation of 15-LO type I gene expression (27), results that are consistent with the temporal relationship uncovered between the prostanoid-LT axis in contained exudates as antecedents and determinants in both assembly and production of LXs during inflammation and its self-limited progression to resolution (8). In this regard, PGE₂ and/or PGD₂ can direct the mobilization of 15-LO type I mRNA in human PMN.

Importantly, the generation of atherosclerotic lesions throughout the aortic tree was unexpectedly sharply reduced in 15-LO-overexpressing TG rabbits (28). The mechanism for this reduction in both lesion size and number, a histopathology marker of the disease and its severity (29), remains of interest. The finding that overexpression of 15-LO in rabbits led to protection from development of atherosclerosis is striking and again raises the possibility that, in vivo, the expression of 15-LO could play a key role in regulating cellular events relevant in the inflammatory response that are critical in establishing the magnitude and extent of atherosclerosis in vivo. In view of earlier results with LX generation and the role of 15-LO in LX biosynthesis (recently reviewed in Ref. 5), the results with 15-LO TG rabbits raised the possibility that overexpression of 15-LO could dampen the acute inflammatory response as a key determinant in coordinate expression of LXs in vivo. Along these lines, recent results indicate that 15-LO expression and LX production in acute inflammation is associated with resolution (8).

Because it is now increasingly appreciated that local inflammation plays an important role in cardiovascular disease and in particular in development of atherosclerosis (30), the present experiments were undertaken to address whether there is a reduced inflammatory response in 15-LO TG rabbits. We assessed experimental periodontitis as a well-appreciated marker of leukocyte-mediated bone loss and inflammation (31) that has pathogenic features similar to those observed in other inflammatory diseases such as arthritis (32–34). Results of the present study indicate that 15-LO-overexpressing TG rabbits show reduced leukocyte recruitment as well as ability to evoke tissue damage via release of granule-associated enzymes. These TG rabbits also generate enhanced levels of LXs, and in acute periodontitis, the 15-LO TG rabbits were protected from both bone loss and inflammation-induced tissue damage. Moreover, topical application of a metabolically more stable analog of 5S,6R,15S-trihydroxyeicosanoic acid (LXA₄₊) protected rabbits against excessive leukocyte-mediated tissue and bone damage.

Materials and Methods

15-LO-overexpressing TG rabbits were developed and maintained as described (28). For each series of experiments reported in this study, the expression of human 15-LO type I was monitored with isolated rabbit PMN by PCR and Northern blot using specific primers. The human 15-LO type I was overexpressed in the TG rabbits as described (28) and was functional (vide infra). The lysozyme promoter gives expression in monocytes/macrophages and PMN (35). Dimethylformamide (DMF), Evans blue, hexadecyltrimethylammonium bromide (HTAB), hydrogen peroxide, 3,3′,5,5′-tetramethylbenzidine, and DMLP were purchased from Sigma–Aldrich (St. Louis, MO), modiﬁed HBSS, PBS, and HEPEs buffer were from Life Technologies (Grand Island, NY), IL-8 was purchased from PeproTech (Rocky Hill, NJ), and LTβ⁺ was purchased from Cayman Chemicals (Ann Arbor, MI). All solutions for parenteral administration were diluted with pyrogen-free, sterile 0.9% NaCl (Baxter Travenol Laboratories, Malton, Ontario, Canada). Additional materials used in liquid chromatography (LC)-mass spectrometry (MS)-MS analyses were from vendors previously reported (36).

Rabbit skin myeloperoxidase (MPO) activity and Evans blue extravasation

MPO activity in skin biopsies was assessed as described (37), with minor modifications. Briefly, skin sites were homogenized in 100 mM acetic buffer containing 1% HTAB and 20 mM EDTA (pH 6.0). The homogenates were held at −80°C until assayed for MPO activity. Serial dilutions of the homogenates were incubated with 3,3′,5,5′-tetramethylbenzidine (3.2 mM) and hydrogen peroxide (1.0 mM) for 5 min at 37°C. The reactions were stopped with addition of 100 μl of 0.2 M sodium acetate (pH 3.0). MPO standard curves were prepared using isolated PMN obtained from fresh rabbit blood that was diluted in PBS/1% HTAB. The equivalent number of PMN per tissue site was calculated from the standard curve. Evans blue was extracted from rabbit skin biopsies in DMF; each biopsy was minced and left in 0.5 ml of DMF at room temperature for 24 h. The absorbance of the supernatant and of Evans blue standards was determined at 625 nm (with 450 nm as a reference wavelength) (38).

LC-MS-MS analysis of eicosanoids

Incubations with rabbit whole blood and/or isolated leukocytes from both 15-LO TG and non-TG rabbits were stopped with 2 vol of cold methanol and kept at −20°C overnight. Protein precipitates were obtained by centrifugation and washed twice with methanol. The resulting supernatants from individual incubations were pooled, and the eicosanoids were extracted with Extract-Clean solid-phase cartridges (C₁₈; Alltech Associates, Deerfield, IL), using PGB₂ (M⁻ + H⁺ = m/z 333) as an internal standard for calculating extraction recovery (39). Isolated methyl formate fractions
from each incubation were taken to dryness with a gentle stream of nitrogen and suspended in mobile phase for LC-MS-MS analyses. LC-MS-MS was performed using a quadrupole ion trap mass spectrometer system equipped with an electrospray ionization probe (LCQ; Finnigan MAT, San Jose, CA). Samples were injected into the HPLC component, comprised of a Spheri-5 C18 column (4.6 × 250 mm; 5 μM; column) and a SpectraSYSTEM UV2000 (Thermo Separation Products) UV-vis absorbance detector. The column was initially eluted isocratically for 20 min with methanol-water-acetic acid (65:34:99.01, v/v/v) at a flow rate of 0.2 ml/min, followed by a linear gradient for 20 min with methanol-acetic acid (99.01:0.99, v/v), and then into the electrospray probe. The sprayer voltage was set to −5−6 kV, and the heated capillary to 250°C. Eicosanoids were quantitated via selected ion monitoring for analytical molecular anions (e.g., [M − H] = m/z 351.5 for LXA4 and m/z 335.5 for LTB4). 20-Hydroxy-LTB4 and LXA4 share molecular mass. Thus, additional criteria were used to distinguish 20-hydroxy-LTB4 and LXA4, namely MS-MS spectra, UV absorbance, and HPLC retention times. In the HPLC system used, 20-LTB4 elutes ∼6 min earlier than LXA4. The MS-MS spectrum of LXA4 is different from that of 20-LTB4, LXA4 possesses diagnostic ions 115, 144, 205, 217, 233, 251, 264, 271, 289, and 307, whereas 20-hydroxy-LTB4 possesses diagnostic ions m/z 161, 195, 277, and 243. Also, the LTB4 metabolite 20-OH-LTB4 carries a 270-nm λmax whereas LXB4 and LXA4 carries 300-nm λmax. All chromatophores were used to distinguish them. Other LTB4-derived products, 20-COOH-LTB4 and 19-OH-LTB4, eluted before LXA4 in this system. Product ion mass spectra (MS-MS or MS3) were also acquired for each to obtain definitive identification of the eicosanoids of interest that were present in these incubations.

PMN enzyme release

Rabbit PMN were isolated from whole blood (40, 41), and the indicated numbers of PMN obtained from 15-LO TG or the non-TG rabbits were exposed to either eMLP (10−6 M) or LTB4 (10−6 M) for 10 min (37°C; pH 7.4). PMN enzyme release was determined by a luciferase assay that was performed after the addition of 100 μl of a solution of 2.2′-azino-di-(3-ethyl)thiazoline sulfonic acid and 0.06% H2O2 in 100 mM citrate buffer (pH 4.2). The calibration curve and assay were linear in the range of 0.3–50.0 × 10−6 PMN/ml.

Mandible harvesting and periodontal lesion quantification

In parallel, 15-LO TG rabbits and non-TG rabbits were given oral exam-

Radioactive and histologic analyses of bone destruction

The percentage of the tooth within the bone was calculated radiographi-
cally using Bjoern technique (42, 43). The radiographs were taken with a
digital x-ray (Schick Technologies, Long Island City, NY). To quantify
bone loss, the length of the tooth from the cusp tip to the apex of the root was
measured, as was the length of the tooth structure outside the bone, measured from the cusp tip to the coronal extent of the proximal bone. From this, the percentage of the tooth within the bone was calculated. Bone values are expressed as the percentage of the tooth in the bone (length of tooth in bone × 100/total length of tooth).

For histologic analysis, the other half of the mandible was immersed in
a volume of Umakon (Decal, Tallman, NY) equal to at least 10 times the size of section; solution was replaced every 24 h for 72 h. After the
decalcification, the tissues were rinsed for 1–3 min in running water and
placed in Cal-Arrest (Decal) to neutralize the pH of the tissue, enhance
embedding and staining characteristics, and stop further decalcification so
that the tissue does not become over-decalcified. The tissue was kept in this
solution for 2–3 min, rinsed again in flowing deionized water for at least 3 min, and embedded in paraffin. Thin sections (0.7 μm) were cut and stained
with H&E to identify the cellular composition of the inflammatory infiltrates.

Results

Reduced dermal leukocyte accumulation, plasma extravasation, and degranulation

The 15-LO type I-overexpressing TG rabbits were evaluated for their acute inflammatory phenotype to investigate potential con-
troller regulatory actions that might be linked in vivo to overexpression of 15-LO type I enzyme, because earlier results showed that 1) overexpression of 15-LO type I in TG rabbits reduced and/or protected the magnitude of atherosclerosis (28), and 2) LX and aspirin-triggered 15-epi-LX (ATL) each regulate leukocyte recruitment in vivo (5). To this end, we first evaluated PMN responses to dermal administration of chemotaxants with both 15-LO TG and non-TG rabbits. Following intradermal administration of either LTB4 or the peptide ligand IL-8, we determined their ability to mount PMN infiltration in vivo as well as evoke PMN-mediated leakage permeability changes. PMN isolated from 15-LO TG rabbits expressed the human 15-LO type I as determined by PCR and Northern blot analyses (data not shown). Results in Fig. 1 indicate that the accumulation of PMN as well as plasma leakage were sharply diminished in the skin of 15-LO TG rabbits exposed to LTB4. Of interest, the 15-LO type I-overexpressing TG rabbits did not show statistically significant changes in PMN recruitment

standard hematological parameters, including clinical chemistry (complete
blood count and total cholesterol), were recorded for all animals.

After euthanizing the rabbits with pentobarbital overdose (120 mg/kg;
Harvard Medical Area Standing Committee on Animals protocols 02570 and
3192), the mandible was dissected free of the muscles and the soft tissue
covering the attached gingiva and bone. Then the mandible was split into two
halves from the midline between the central incisors. Half was taken for morphometric analysis of the bone loss, and the other half was used for histological evaluation of periodontitis.

Morphometric analysis of bone destruction

Half of the sectioned mandible was defleshed by immersion in 10% hy-
drogen peroxide (10 min; room temperature). The soft tissue was removed
completely and then the mandible was stained with methylene blue for good
visual distinction between the tooth and the bone. Next, the bone level
around the second premolar was measured directly by a 0.5-mm calibrated
periapical probe. Measurements were made at three points each, at buccal and lingual sides, for crestal bone level. A mean crestal bone level around
the tooth was calculated. Similarly, for the proximal bone level, measure-
ments were made at mesial and distal aspects of the tooth. The measure-
ments were taken from both the buccal and lingual side on both proximal
aspects of the second premolar, and the mean proximal bone level was
calculated. The bone level was also quantified by Image Analysis (Image-
Pro Plus 4.0; Media Cybernetics, Silver Spring, MD). The sectioned mandible
was mounted and photographed using an inverted microscope at ×10. The
captured image was also analyzed as above, and the mean crestal bone level
around the tooth was calculated in millimeters.

Morphometric analysis of bone destruction

The percentage of the tooth within the bone was calculated radiographi-
cally using Bjoern technique (42, 43). The radiographs were taken with a
digital x-ray (Schick Technologies, Long Island City, NY). To quantify
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embedding and staining characteristics, and stop further decalcification so
that the tissue does not become over-decalcified. The tissue was kept in this
solution for 2–3 min, rinsed again in flowing deionized water for at least 3 min, and embedded in paraffin. Thin sections (0.7 μm) were cut and stained
with H&E to identify the cellular composition of the inflammatory infiltrates.
were identified using their coelution with authentic standard and signature ions diagnostic for each (44). Results in Table I demonstrate a 3- to 4-fold increase in LXS generated by 15-LO TG rabbit leukocytes compared with activated cells from non-TG rabbits. Representative MS-MS spectrum shown for LXA4 in Fig. 3A displayed signature ion fragmentations that were diagnostic and consistent with those reported for LXA4, namely m/z M-H 351, 333 [351-H2O], 315 [351-2H2O], 307 [351-CO2], 289 [351-H2O-CO2], 271 [351-2H2O-CO2], 251 [351-CHO(CH2)3CH3], 235 [351-CHO(CH2)3COOH], 233 [235-2H2], 207 [351-CO2-CHO(CH2)3CH3] 155 [351-CHO(CH2)3COOH-CHO(CH2)4CH3], and 115 [CHO(CH2)3COO-] (cf Ref. 44). These results indicate an increased production of LXS by PMN isolated from 15-LO TG rabbits.

The other classes of LO pathway products monitored are shown in Fig. 3B. These included the monohydroxy-eicosanoids 5S-hydroxy-eicosatetraenoic acid (HETE), 12S-HETE, 15S-HETE, and several dihydroxy-eicosanoids such as LTB4, as well as the double dioxygenation product 5,15-diHETE that were generated by both groups of rabbit leukocytes. As shown in Fig. 3B, we observed that both 15-HETE and 5,15-diHETE (C) were produced from endogenous substrate by non-TG rabbits. Their levels produced from endogenous sources of arachidonate were significantly increased from those from non-TG cells activated in parallel. 15-HETE was two to six times higher in the 15-LO TG cells. This was anticipated, because we expected the 15-LO TG rabbits to express higher levels as well as to accumulate 15-HETE. The amounts of 5,15-diHETE generated from endogenous arachidonic acid upon activation of PMN were approximately four times higher than with the non-TG PMN. Addition of exogenous arachidonic acid to these incubations increased 5,15-diHETE production by approximately four to five times in TG above the non-TG values. Unexpectedly, upon stimulation with ionophore and exposure to exogenous arachidonate, the 15-LO TG rabbits shift LO product profile, giving elevated levels of 12-HETE (8–10 times higher) as well as 5-HETE that were ~30–70 times higher with exogenous arachidonic acid. Also noted were increased quantities of 14,15-diHETE and LXB4 (not shown) in addition to LXA4. However, the appearance of LXB4 in these rabbits was not consistent. Taken together, these results suggest that 15-LO overexpression in rabbits gives rise to decreased PMN responses, reduced infiltration, and granule mobilization both in vitro and in vivo, and upon challenge ex vivo, leukocytes from 15-LO TG gave increased production of 15-HETE, 5,15-diHETE, and LX.

FIGURE 1. 15-LO-overexpressing TG rabbits give reduced leukocyte recruitment to skin and reduced plasma extravasation with LTB4. Plasma extravasation was quantitated using Evans blue from skin biopsies (see Materials and Methods), and MPO activity was monitored in rabbit skin as an index of PMN infiltration. Non-TG rabbits (•; solid line) gave significantly higher (p < 0.05) levels of leukocyte recruitment (A) and leukocyte-dependent leakage permeability (B) in response to LTB4 compared with 15-LO TG rabbits (○; dashed line). Statistical significance was not observed with human IL-8 addition to the skin (C and D). Results represent the mean ± SEM of 12–16 sites obtained from four 15-LO TG and non-TG rabbits.

FIGURE 2. PMN from 15-LO TG rabbits show reduced granule-associated enzyme release with LTB4. Statistically significant differences in degranulation monitored as MPO release were obtained when isolated PMN were exposed to LTB4 (10 nM; 10 min; 37°C) (A) but not to an equimolar concentration of fMLP (10 nM) (B). Isolated PMN were incubated in parallel: non-TG (●) vs 15-LO TG rabbits (○). Results represent n = 3 at each experimental condition; p < 0.01 by ANOVA. Values obtained with 15-LO TG vs non-TG with fMLP were not statistically significant.
Periodontitis is a focal inflammation characterized and driven by aberrant PMN-mediated tissue damage (31). Because earlier results in a mouse model showed protection from tissue damage with addition of LX analogs (45), we conducted the next experiments with rabbits to evaluate the potential impact of 15-LO overexpression and local LXA₄/ATLa application to this form of acute inflammation. In the

**FIGURE 3.** LC-MS-MS analysis of eicosanoid classes. Rabbit leukocytes were isolated from both non-TG and 15-LO TG rabbits and incubated with ionophore A23187 (15 μM; 20 min; 37°C). Products were extracted and identified as in Materials and Methods and Table I. A, MS-MS spectrum of LXA₄ diagnostic ions is indicated (see inset and text). B, LC profiles from 15-LO TG rabbits. C, MS-MS spectrum of 5,15-diHETE diagnostic ions is indicated (see inset and text). Results are representative of those from four separate TG vs non-TG experiments.
first experimental setting (experiment A), ligatures were tied around the second mandibular premolar of the rabbits for 6 wk to create an environment where a periodontopathogen \textit{P. gingivalis} could be retained. As negative controls, a group of animals only received ligatures without microbial challenge, another group did not receive any application, and systemic metronidazole was used as a positive control to prevent \textit{P. gingivalis}-induced infection. As expected, the no-treatment group did not demonstrate any pathologic change over the course of the study and application of ligature alone resulted in a mild loss of bone (Table II). The application of \textit{P. gingivalis} together with ligatures led to the development of periodontitis as observed by changes in the gross features as well as radiologic and histologic parameters (Fig. 4, A, 1–4; B, 1–4; C, 1 and 2; D, I and 2; Table II). In Fig. 4A, soft tissue destruction was observed in non-TG animals that received \textit{P. gingivalis} (2 and 4) as compared with those to which only ligature was applied (1 and 3) (arrows). Corresponding defleshed specimens show the impact of bone loss in Fig. 4B. Non-TG animals that received \textit{P. gingivalis} (Fig. 4B, 2 and 4) demonstrated significant bone destruction, whereas there was no resorption in the absence of \textit{P. gingivalis} (1 and 3) (arrows). Further radiographic examination (Fig. 4C) confirmed loss of bone in response to \textit{P. gingivalis} in non-TG animals (2) compared with control animals (1) (arrows). Histological analysis demonstrated prominent leukocyte infiltrates as well as massive bone resorption in specimens from non-TG rabbits that received \textit{P. gingivalis} (Fig. 4D2) (arrows), whereas no loss of bone was observed in control non-TG animals where only ligature was applied (D1). Soft and hard tissue destruction due to \textit{P. gingivalis}-induced periodontitis in non-TG rabbits as assessed by clinical, radiographic, and histological evaluations was not observed in 15-LO-overexpressing TG rabbits subjected in parallel to the same procedures (Fig. 4, A5, B5, C3, and D3). Quantitation of bone levels revealed crestal and proximal bone levels, and percentage of tooth in bone was essentially without damage in the TG 15-LO-overexpressing TG rabbits (percentage of bone loss in the 15-LO TG = 0, whereas the non-TG with disease gave \textasciitilde 43% bone loss; \( n = 5 \); Table II). Metronidazole treatment prevented bone loss induced by \textit{P. gingivalis} challenge (Table II).

Given that LX levels were elevated with cells from TG rabbits and that TG rabbits gave diminished responses to both dermal and oral challenges (Figs. 1 and 4), we administered an analog of LXA4/ATLa that is stable to rapid metabolic inactivation (46). This add-back/treatment was conducted with rabbits that were given the periodontal ligature challenge accompanied by \textit{P. gingivalis} to determine whether a product of 15-LO overexpression in vivo could alter the pathogenesis of this disease. Results in Fig. 5 and Table II show that administration of the LX analog (denoted ATLa) at \( \text{5--6 g/kg/} \text{wk} \) given three times per week for the duration of the experiment (6 wk) prevented gross as well as microscopic changes in the periodontium. These clinical parameters in rabbits treated with ATLa (Fig. 5, A, I and 3; B, I and 3; C1; and D1) were noted as sharply diminished leukocyte accumulation, and obvious protection from both bone loss and focal inflammation, compared with those rabbits with ligatures that were not treated with LX analog (A, 2 and 4; B, 2 and 4; C2; and D2). Quantitative analyses showed that topical application of ATLa prevented periodontitis-induced bone loss (Table II).

### Discussion

Results of the present studies demonstrate that overexpression of the arachidonate 15-LO type I in TG rabbits gives, upon challenge, a phenotype that leads to enhanced endogenous anti-inflammation. Other phenotypic changes in these rabbits were not apparent, and they appeared as essentially healthy rabbits of normal life expectancy. Of interest, the isolated PMN from these TG rabbits showed essentially normal responses to the chemotactic peptides fMLP and IL-8, yet showed a marked reduction in their ability to release granule-associated enzymes upon exposure to secretagogue levels of LTB4 compared with non-TG rabbits. In addition, a reduction in

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### Table I. \textit{LX} production is elevated in activated leukocytes from 15-LO TG rabbitsa

| Group          | LXA4  |
|----------------|-------|
| Non-TG         | 6.3   |
| 15-LO TG       | 20.6  |

a The amounts of LXs were determined by LC-MS-MS analysis following extraction (see Materials and Methods). Rabbit leukocytes were isolated from both groups (15-LO TG and non-TG), enumerated, and incubated in PBS (pH 7.45) with ionophore A23187 (15 \( \mu \)M; 20 min; 37°C). PGB2 was used as an internal standard, and PGB2 extraction gave only a 1.6% recovery difference between values for non-TG and TG rabbits. Results are expressed as nanograms per 5 \( \times \) 10\(^6\) cells and are representative of four separate experiments where individual incubations were performed in duplicate.

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### Table II. Protection from disease-associated bone loss by ATLa

| Treatment Group                                                                 | Percentage of Bone Loss | \( n \) |
|---------------------------------------------------------------------------------|-------------------------|-------|
| **Experiment A**                                                                |                         |       |
| No treatments                                                                   | 0.0 ± 0.0               | 3     |
| Ligature alone                                                                  | 10.0 ± 1.5              | 6     |
| Ligature + \textit{P. gingivalis}                                                | 43.0 ± 4.6†             | 6     |
| 15-LO TG + ligature + \textit{P. gingivalis}                                     | 0.0 ± 0.5†              | 5     |
| Ligature + \textit{P. gingivalis} + metronidazole                               | 16.0 ± 2.2              | 3     |
| **Experiment B**                                                                |                         |       |
| No treatments                                                                   | 0.0 ± 0.0               | 3     |
| Ligature alone                                                                  | 10.0 ± 1.2              | 3     |
| Ligature + \textit{P. gingivalis}                                                | 46.1 ± 3.6†             | 6     |
| Ligature + \textit{P. gingivalis} + topical 15-epi-LXA4 stable analog           | 3.0 ± 0.8†              | 6     |

a Results from separate experimental designs (experiments A and B) are expressed as percentage of bone loss as determined by the Bjorn technique (see Materials and Methods). Values are expressed as the mean ± SD. In experiment A, periodontitis was induced with silk ligature and \textit{P. gingivalis} as described in Materials and Methods. Rabbits were examined at 6 wk. In the absence of ligature, bone loss was essentially not present. In these healthy rabbits, bone loss is expressed as mean ± SD. Application of \textit{P. gingivalis} was necessary to induce robust periodontal disease (bone loss) that was sensitive to antimicrobial therapy with metronidazole. In experiment B, the effect of an LX/ATLa on the progression of the periodontal bone loss was evaluated. ATLa was applied as described in Materials and Methods, with vehicle alone as the parallel control. Application of ATLa significantly prevented bone loss induced by \textit{P. gingivalis}, as well as bone loss induced by the ligature alone.

†, \( p < 0.05 \) compared with ligature alone.
PMN infiltration to the skin and reduced vascular permeability were also evident in 15-LO TG rabbits in response to challenges with LTB\(_4\), but not IL-8 administration. The cellular basis for reduced responsiveness to LTB\(_4\) in the 15-LO type I TG rabbits is not clear at present, but it is of interest to point out that LXA\(_4\) and its analogs can down-regulate LTB\(_4\) signaling via activation of postreceptor binding events (47). Also, LXA\(_4\) does not compete for LTB\(_4\) binding at its recombinant receptor BLT\(_1\), or with human PMN (48). These findings from skin challenges in vivo and with isolated PMN in vitro suggest that the overexpression of 15-LO type I leads to dampened LTB\(_4\)-dependent PMN activation as well as recruitment.

When profiled for lipid mediators, approximately two to four times greater LX\(_A_4\), four to five times greater 5,15-diHETE, and two to six times greater 15-HETE generation were characteristics of isolated PMN from 15-LO TG rabbits. In addition to the LXs, both 14\(R,15\(S\)-diHETE and increased 12\(S\)-HETE were noted with these TG rabbits when isolated PMN were exposed to exogenous arachidonic acid (vide infra). Along these lines, results from earlier studies have shown that, with human leukocytes in vitro, 14\(R,15\(S\)-diHETE down-regulates LT-stimulated superoxide anion generation (49) and NK cell cytotoxicity (50). Hence, both LXs and 14\(R,15\(S\)-diHETE, which were observed and elevated in profiles from activated PMN obtained from 15-LO TG rabbits when incubated with arachidonic acid, could serve as local protective autacoids in vivo (recently reviewed in Ref. 5). That the accumulation of 12-HETE upon activation of PMN from these rabbits with ionophore A23187 and arachidonate was 8–10 times higher than in incubations without added arachidonate is of interest and suggests additional regulatory mechanisms for this LO activity in the TG rabbits in vivo. Enhanced 12-HETE was also found by Shen et al. (28). This increased arachidonate 12-LO activity per se can also contribute to production of bioactive counterregulatory eicosanoids such as LXA\(_4\) and 14\(R,15\(S\)-diHETE. These can be formed via several regulatory levels in situ including enhanced conversion of LTA\(_4\) by 12-LO activity that are 15-LO independent. One mechanism involves up-regulation by 12-hydroperoxyeicosatetraenoic acid, which in turn increases
FIGURE 5. Prevention of periodontal tissue destruction by topical application of LX/ATLa. Periodontal disease was induced in non-TG rabbits with ligature and \textit{P. gingivalis} as described in Materials and Methods and Fig. 4. Half of the animals received topical application of LX/ATLa, and the other half received vehicle (ethanol) alone, as described in Materials and Methods. Six micrograms of vehicle alone or of LX/ATLa was applied to the ligature tied around the second premolars three times per week for 6 wk. A, 1 and 3, Buccal and lingual periodontal tissues of an LX/ATLa-treated animal. 2 and 4, Vehicle control. Note the tissue destruction in the control animal (arrows), which is absent in the LX/ATLa-treated animal. A, 1 and 3, Buccal and lingual periodontal tissues of an LX/ATLa-treated animal. 2 and 4, Vehicle control. Note the tissue destruction in the control animal (arrows), which is absent in the LX/ATLa-treated animal. B, The protection against periodontal destruction is seen in 1 and 3 compared with 2 and 4 after the jaws have been defleshed (arrows). C, Radiographically, inhibition of bone loss is apparent in the LX/ATLa-treated animal in 1 compared with vehicle in 2 (arrows). D, Histologic evaluation reveals a well-organized, noninflamed periodontium in the LX/ATLa-treated animal in 1 compared with vehicle in 2 where there is loss of connective tissue, a severe inflammatory infiltrate, and loss of alveolar bone (arrows; magnification, ×400).

the generation of LXs catalyzed by 12-LO from multiple precursors including LTA4 by increasing the enzymatic activity (5, 51). Another explanation for increased 12-HETE in 15-LO type I TG may lie in the substrate presentation with overexpression of 15-LO TG. The substrate ratio to enzyme in vivo may favor 15-LO type I production of 12-HETE. Changes from position 15 to 12 are known to occur when the substrate-binding pocket is enlarged (52).

Excessive recruitment of PMN to the periodontium contributes to the progression of periodontal disease and to the destruction of periodontal tissue (31, 53), which appears to be similar to PMN-dependent tissue damage in certain arthritic diseases that can contribute to chronic inflammation and panus formations (32, 33, 54). In the present experiments, we found that inflammation associated with excessive PMN recruitment to the rabbit periodontium was sharply diminished in TG rabbits overexpressing 15-LO. Hence, it appears that the overexpression of 15-LO can dramatically alter the outcome and pathogenesis for a focal inflammatory event in vivo. Because it has been shown earlier that PMN degranulation and superoxide anion generation, when released to the extracellular milieu, contributes to the degradation of joint tissues (32, 33) as well as to damaging periodontal ligaments and bone surrounding teeth (53), which is exacerbated in active disease in periodontitis patients (31), we examined the magnitude of bone loss, using both radiologic and morphometric analyses. Here, too, the enhanced expression of 15-LO gave sharp decreases in these rabbits in PMN-mediated bone degradation (cf Figs. 4 and 5). This is of particular interest, because there are, to date, few if any mechanisms where endogenous local-acting counterregulatory substances can prevent bone degradation that results from PMN-mediated local inflammation.

\textit{P. gingivalis} stimulates the brisk recruitment of PMN into murine air pouches (45). In the infected mice, LX stable analogs regulated the degree and magnitude of PMN recruitment and reduced the release of PMN-derived agents that lead to tissue degradation, as well as protected from blood-borne microbes and their relocalization in murine heart (45). In the present studies with rabbits, the administered LX stable analog given topically to the periodontium reduced PMN infiltration and associated pathologies. Bone degradation, namely loss of attachment and tissue injury, was prevented by topical administration of the LX/ATL stable analog in amounts as low as 5–6 μg/2–3 μl/tooth applied three times a week for a duration of 6 wk. These results are quite striking and together provide clear evidence that LX generation can play a role in limiting inflammation in vivo in animals larger than mice in experimental disease models and that topical application of LX/ATL stable analogs can indeed reduce the magnitude of disease.

To date, there are apparently no small molecules or agents presently available that can significantly reduce periodontal disease by regulating PMN traffic and the onset of PMN-mediated tissue damage. Given the gross similarities in pathogenesis of inflammation-induced bone loss in periodontal disease (31) and joint inflammation in arthritic patients (32, 33), it is likely that the present results open new avenues to investigate the role of LX biosynthesis via 15-LO as potential targeted components of this pathway in new approaches for the treatment of acute and focal inflammatory diseases where endogenous injury from within leads to chronic bone loss and/or irreversible tissue damage. Given the now-appreciated
contributions of focal oral inflammation and oral hygiene to cardiovascular disease (55) and the heightened awareness that inflammation is a key component contributing to cardiovascular disease (30), it is possible that enhancing endogenous anti-inflammation and resolution (5, 8) via LX and/or related compounds in the treatment of periodontal disease may have a systemic salutary impact on the state of vascular inflammation.

Along these lines, earlier results are of particular interest, because they unexpectedly indicate a lower atherosclerotic potential in these 15-LO TG rabbits (28). In humans, atherosclerotic plaque rupture by balloon angioplasty releases appreciable levels of LXs within the lumen of the vessel (56). In view of our present results and that an important component of atherosclerosis is the dysregulated inflammatory response (30), our results underscore the dramatic reduction in acute inflammatory sequelae and the signs of inflammation such as leakage (see Fig. 1) evoked in 15-LO TG rabbits. The overexpression of 15-LO and enhanced production of LXs and related lipid mediators that can activate endogenous anti-inflammatory responses suggest that the overall set point or checkpoints (57) for governing the magnitude and duration of the response is altered by the overexpressing 15-LO type I (28). Taken together, these results in rabbits provide further evidence for multilevel regulation and endogenous counterregulation as an active process to self-limit acute inflammation. This counterregulatory set of mediators, when added back as a metabolic inactivation-resistant stable LX/ATL analog, diminished the extent of inflammation and provide additional lines of evidence pointing to the products of the 15-LO type I pathway as key effectors in the diminished inflammatory response phenotype observed for 15-LO TG rabbits. This change in anti-inflammation status uncovered in the present study may also be related to the protective impact of 15-LO overexpression in the hypercholesterolemia-fed rabbits noted earlier (28) and are consistent with 15-LO gene therapy and reduction of murine glomerular disease with enhanced LXA₄ production in vivo (58).

In view of the current perception that 15-LO is involved in a variety of in vivo responses including cancer genesis, organelle degradation (15), inflammation, and expedited resolution (5), the present results underscore the need to further elucidate the compartmentalization and factors regulating expression of 15-LO-related mechanisms (59). This is of particular interest in view of the recent findings that there is more than one form of 15-LO in mammalian systems, currently denoted as 15-LO-1 and -2. Perhaps akin to the COXs 1 and 2, (20), these different 15-LO enzymes will likely serve specialized functions in physiologic as well as pathophysiologic mechanisms. Hence, the seemingly unrelated functional roles of 15-LO may reflect system specificity (i.e., mamma- lian, plant, etc.), species, and organ selectivity (see Ref. 4) as well as event-specific activation for substrate use by specific forms of 15-LO to generate required lipid mediators/autacoids (16) that can carry highly specialized functional roles. This appears to be the case in that LX generation from arachidonate is tightly regulated in each of the immune effector cell systems as well as in mucosal immune function. LXs are stereospecific in the subnanomolar range in regulating leukocyte traffic and gene regulation relevant in acute inflammation and its timely resolution (5), whereas the oxygenation of phospholipids and organelle destruction at the enzyme level appears to be less specific (15). The present results emphasize that prolonged 15-LO overexpression gives apparently healthy rabbits, which is associated, upon challenge, with enhanced LX generation, where the outcome is reduced local inflammatory response in vivo.

Acknowledgments

We thank Mary H. Small for expert assistance in manuscript preparation, Margaret Morrissey for expert assistance in caring for the rabbits, and Katherine Gotliner and Dr. Song Hong for expert assistance with mass spectral analysis.

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