Leukotriene Receptor Antagonism and the Prevention of Extracellular Matrix Degradation During Atherosclerosis and In-Stent Stenosis

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Objective—The lipid-derived inflammatory mediators leukotrienes (LTs) are produced during vascular injury. The aim of the present study was to determine the role of LT receptor signaling in the pathophysiology of in-stent stenosis.

Methods and Results—New Zealand White rabbits were fed 0.3% cholesterol and subjected to angioplasty with balloon dilatation and stent implantation in the right carotid artery. Rabbits treated for 2 weeks with the BLT receptor antagonist BIIL284 (3 mg/kg once daily by oral gavage) displayed a significantly reduced in-stent intimal hyperplasia in carotid arteries compared with vehicle-treated rabbits. In addition, BIIL284 treatment significantly reduced the extracellular matrix metalloproteinase (MMP)-2 and MMP-9 activities in stented arteries. The inhibited MMP-9 activity was correlated with decreased macrophage content in the lesions. The LTB₄-induced migration of vascular smooth muscle cells was significantly inhibited by transfection with siRNA against MMP-2. Finally, human arteries subjected to ex vivo angioplasty and stent implantation displayed an increased in-stent intimal hyperplasia and higher MMP-2 and -9 activities in the presence of LTB₄.

Conclusions—These results suggest a key role of LT signaling in the extracellular matrix degradation associated with hyperlipidemia and in-stent stenosis. In conclusion, targeting LT receptors may represent a therapeutic strategy in atherosclerosis and interventional cardiology. (Arterioscler Thromb Vasc Biol. 2009;29:518-524.)

Key Words: lipocalyxine ■ macrophages ■ matrix metalloproteinases ■ restenosis ■ smooth muscle cells

The use of stents in percutaneous coronary interventions (PCI) has reduced the incidence of restenosis, particularly in combination with local administration of cell cycle inhibiting rapamycin derivatives by means of drug eluting stents (DES). However, the use of DES in interventional cardiology has also been associated with an increased risk of late stent thrombosis.⁴ Such side-effects suggest the importance of identifying novel targets for both local and systemic treatment, which have an inhibitory effect on in-stent restenosis.

Interestingly, PCI is a stimulus for intracoronary formation of leukotrienes (LTs).⁵ Derived from arachidonic acid through the 5-lipoxygenase (5-LO) pathway, LTs are potent inflammatory mediators implicated in several pathophysiological processes associated with atherosclerosis.⁶ Furthermore, the polymorphisms within the gene encoding the 5-LO activating protein (FLAP), which have been associated with an increased risk of myocardial infarction and stroke,⁷ were recently reproduced in restenosis patients.⁸ In the latter study, 2 of the FLAP gene polymorphisms studied were independent prognostic factors in predicting in-stent restenosis 6 months after PCI.⁹ Although no previous study has evaluated the effects of antileukotrienes on in-stent restenosis, the available data have provided indications for potential effects of LTs in the response to vascular injury. For example, LTB₄ induces migration and proliferation of human coronary artery smooth muscle cells (VSMCs).¹⁰ Furthermore, inhibition of LT biosynthesis decreases neutrophil deposition at sites of arterial injury in pigs. In addition to VSMC migration/proliferation and leukocyte recruitment, degradation of extracellular matrix may also be involved in the disease progress of restenosis, and recent studies have reported significant correlations between the LT formation and matrix metalloproteinase (MMP) activity in the context of atherosclerosis, aortic aneurysms, and smoking.¹¹-¹²

LTs induce their actions through two distinct receptor subtypes expressed on inflammatory cells as well as on structural cells in both airways and vessels. The CysLT receptors, denoted either CysLT₁ or CysLT₂ depending on their antagonist affinity, are activated by the cysteinyl-LTs (LTC₄, LTD₄, and LTE₄) and represent the target for currently used antileukotriene treatments in asthma.¹³ On the
other hand, LTB₄ activates BLT receptors, defined as BLT₁ and BLT₂, for the high- and low-affinity receptor subtype, respectively. The relative importance of these two pathways in restenosis is unknown. For example, although BLT receptor antagonism reduces intimal hyperplasia after different vascular injuries in rats, the results obtained with CysLT receptor antagonists in the latter model are conflicting, with both beneficial and neutral effects having been reported.

The present investigation was undertaken to evaluate BLT receptor antagonism in a model of in-stent restenosis using hyperlipidemic rabbits. The aim was to assess the therapeutic potential of anti-LTs in interventional cardiology and to determine the effects of BLT receptor signaling in MMP activity in hyperlipidemic rabbits both with and without vascular injury. To establish the pertinence of the rabbit model, an exploration of LT receptor activation on in-stent stenosis, and MMP activity was in addition performed in human arterial segments.

The results of the present study indicate key effects of BLT receptor signaling in the VSMCs and inflammatory response associated with vascular injury and hyperlipidemia, with implications not only for in-stent restenosis but also atherosclerosis.

**Methods**

**Animal Experiments**

All animal experiments were approved by the Bichat University Institutional Animal Care and Use Committee. Nineteen male New Zealand White rabbits (3.5 to 4.0 kg; CPA, Olivet, France) received headed once daily by oral gavage of either 2 mL tylose (1%) or 2 mL water. Three more rabbits, receiving ordinary chow without cholesterol (1 mg/kg; Aspegic) for 14 days, delivered through the drinking water. This protocol significantly inhibited MMP-2. A second group of rabbits carotid arteries were harvested, flushed with saline, and cleaned of adipose tissue followed by fixation in 4% paraformaldehyde. Stented arteries were included in methyl methacrylate, sectioned with a motorized microtome (HM355, matoxyline, Mircom) followed by staining with hematoxylin, Masson trichrome, and orceine. The intimal and medial areas were quantified using a light microscope couplèd to HistoLab image analysis software. At least 3 sections of each staining were used for quantification, representing different levels of the stented arterial segment. Nonstented rabbit carotid arteries were embedded in paraffin after paraformaldehyde fixation and used for immunohistochemical stainings with antibodies against αSMA-actin (Dako), Ki-67 (a nuclear proliferation marker, Immunotech), or RAM-11 (a marker of rabbit macrophage cytoplasm, Dako), as previously described.

**Gelatin Zymographic Assays**

Gelatinolytic activities of pro-MMP-2, MMP-2, pro-MMP-9, and MMP-9 were measured as previously described. Briefly, 20-µL samples were mixed with loading buffer (50 mmol/L TRIS–HCl, pH 6.8, 0.1% glycerol, 2% SDS, 0.5 mg/mL bromophenol blue). The electrophoresis was performed in 10% polyacrylamide gels containing 2.5 mg/mL of gelatin (type IV) in TRIS-base-glycine buffer (0.124 mol/L TRIS, 0.95 mol/L glycine, 0.01% SDS) at 200 V during 1 hour. Gels were subsequently soaked for 30 minutes in 2.5% Triton X-100 at room temperature followed by brief rinse in distilled water. The incubation with 50 mmol/L TRIS-HCl, pH 7.8, 10 mmol/L CaCl₂ buffer was performed for 19 hours at 37°C. Gels were stained with Coomassie Brilliant Blue buffer (0.5% Coomassie Brilliant Blue R-250, 10% propanol, 5% acetic acid) on a shaker at room temperature, destained in 10% acetic acid, 30% ethanol (v/v) during 10 minutes and stored in 10% acetic acid solution (v/v). The MMP activities were estimated from the 70, 60, 98, and 96 kDa gelatinolytic bands corresponding to pro-MMP-2, MMP-2, pro-MMP-9, and MMP-9, as previously described. Densitometric analyses of scanned gelatinolytic bands were performed with NIH (Scion Image Software and National Institutes of Health, Release Beta 3b software). The cumulative sum of the activities in supernatants collected at 24, 48, and 72 hours was used in the analysis. Total MMP activity was defined as the sum of the proform and the active form of MMP-2 and -9, respectively.

**Cell Culture and Transfection**

The rabbit aortic VSMC line Rh-16 was cultured in Dulbecco modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum and antibiotics (penicillin 100 U/mL, streptomycin 100 µg/mL and amphotericin 0.25 µg/mL; Gibco-BRL). One day before transfection, VSMCs were plated in 24-well plates at a density of 5x10⁴ cells per well and cultured at 37°C in 95% air/5% CO₂. At 50% to 70% confluence, cells were washed with PBS followed by addition of 50 mmol/L Tama-tagged siRNA against rabbit MMP-2 and 1 µL of cationic lipid Si-ENDO (Eurogentec) in 600 µL of culture media for 24 hours at 37°C, as previously described. This protocol significantly inhibited MMP-2. A second group of VSMCs were transfected with 50 mmol/L Tama-tagged scramble siRNA using the same protocol. Results obtained with untransfected VSMCs (no siRNA and no jet Si-ENDO) used as controls, were not significantly different compared with those obtained in cells transfected with scramble siRNA (data not shown).
Migration Assay
The in vitro migratory activity of VSMCs was measured using a wound migration assay, as previously described. Briefly, a line was created with a single scratch at the center of a VSMC monolayer (50% to 70% confluence) using a sterile 1.15 mm diameter pipette tip. VSMCs were photographed with phase contrast microscopy (OLYMPUS CK40, X10 objective) immediately after the wound and at 24 and 48 hours after transfection (15 images at each time point). Distance between cells at both sides of the wound was measured for 5 pairs of cells per image. The VSMC migration induced by LTB4 (1 nmol/L to 1 μmol/L) was expressed cellular displacement during 24-hour migration after subtraction of baseline (ie, without LTB4).

Human Artery Organ Culture
From 15 patients undergoing coronary artery by-pass surgery, internal mammary arteries (IMAs) were obtained as surgical waste in accordance with French ethical laws (L.1211-3-9). Arterial segments measuring approximately 2 cm were subjected to balloon dilatation (3×30 s, 10 ATM) followed by stent implantation by 30 s inflation at 10 ATM, as previously described. The stented arterial samples were subsequently cultured in serum-containing medium for 14 days at 37°C in 95% air/5% CO2 in the absence or presence of LTB4 (100 nmol/L), with change of medium every 2 days. Morphological analysis of intimal hyperplasia was performed as described above. In some experiments, arterial samples with and without stent were cultured for 24 hours in serum-free medium for analysis of MMP activity. Protein concentrations of MMP-2, MMP-9, TIMP-1, and TIMP-2 were measured by ELISA kits from Amersham, according to the manufacturers’ instructions.

Data Analysis
All results are expressed as mean±SEM. Statistically significant differences were determined using either a Signed Rank Test (pairwise comparisons) or an ANOVA test followed by Bonferroni test (multiple comparisons). *P<0.05 was considered significant.

Results
Plasma and Blood Analyses
The plasma levels of cholesterol at the time of sacrifice are shown in Table 1. The 0.3% cholesterol regimen significantly increased plasma cholesterol, whereas the triglyceride concentrations were not significantly altered (data not shown). The different treatment protocols had no significant effects on the plasma lipid concentrations measured (Table 1). Determinations of the white blood cell counts in rabbits treated with either vehicle or BIIL284 revealed no significant differences (supplemental Table I, available online at http://atvb.ahajournals.org). Monitoring of the plasma concentrations of the active metabolite of BIIL284 (BIIL315) revealed a Cmax of 145±42 ng/mL obtained 6 hours after gavage, and that the concentration 24 hours after gavage was 4±1.8 ng/mL (supplemental Figure 1). The latter concentration has previously been reported be sufficient to reach 100% inhibition of LTB4-induced Mac-1 expression on ex vivo stimulated neutrophils.

In-Stent Stenosis
Rabbits receiving vehicle displayed intimal hyperplasia 14 days after angioplasty (Figure 1). Both the area of the in-stent intimal hyperplasia and the intima/media ratio were significantly lower in rabbits treated with the BLT receptor antagonist BIIL284 (Figure 1).

Matrix Metalloproteinase Activities
The sum of either MMP-2 or -9 activities in conditioned media of rabbit carotid arteries at 24-, 48-, and 72-hour ex vivo incubation are shown in Figure 2. Rabbits fed cholesterol exhibited significantly greater levels of total MMP-2 compared with those fed normal chow, which was further increased after vascular injury and stent implantation (Figure 2, top left panel). Furthermore, the gelatinolytic activity corresponding to the active form of MMP-2 was significantly increased by high cholesterol and stenting (Figure 2, top right panel). Treatment with BIIL284 significantly decreased total MMP-2 in both uninjured and stented arteries, and the active fraction of MMP-2 in stented arteries (Figure 2, top panels). Whereas MMP-9 activity was undetectable in carotid arteries derived from rabbits on normal chow, detectable levels were obtained by high-cholesterol diet (Figure 2, bottom left panel), with the active form of MMP-9 only being detectable after stenting (Figure 2, bottom right panel). Treatment with BIIL284 significantly reduced both total and active MMP-9 activity in stented arteries (Figure 2, bottom panels).

Macrophage Infiltration
Immunohistological stainings with RAM-11 revealed the presence of macrophages infiltrating the deeper layers of the...
intima and the media of carotid arteries derived from rabbits on high cholesterol regimen (Figure 3, top panels), whereas RAM-11 stainings were undetectable in arteries derived from control rabbits on normal chow. In balloon injured arteries, BIIL284 treatment significantly reduced the number of RAM-11–positive cells within the vascular wall (Figure 3, top panels). Furthermore, a significant correlation was found between macrophage content of the arterial segment and the MMP-9 activity detected in the supernatant (Figure 3, bottom panel).

VSMC Proliferation and Migration

The proliferative activity of VSMCs (determined by the colocalization of nuclear Ki-67 staining with αSM-actin) was low in the intima and media 14 days after vascular injury. There were no significant differences in Ki-67 staining between arteries derived from vehicle- and BIIL284-treated rabbits (114±40 and 274±99 Ki-67-stained cells/mm², respectively).

LTB₄ (1 nmol/L to 1 µmol/L) induced migration of rabbit aortic Rb-1 VSMCs with a bell-shaped concentration-response curve and a maximum migration at 10 nmol/L of LTB₄ (Figure 4). Transfection with siRNA against MMP-2 significantly inhibited the migratory response induced by LTB₄ (Figure 4). Furthermore, when performed in the presence of rabbit plasma, the LTB₄-induced migration was significantly inhibited in the presence of plasma derived from BIIL284-treated compared with untreated rabbits (Figure 4).

Ex Vivo Angioplasty of Human Arteries

Balloon dilatation and stent implantation in human arterial samples resulted in a small intimal layer covering the luminal side of the stent after 14 days of culture (Figure 5, left panel; dotted line: basal membrane; arrowheads: intima; small arrows: stent struts). Arterial segments cultured for 14 days in the presence of LTB₄ (100 nmol/L) displayed a significantly greater intima/media ratio compared with controls (Figure 5).
In addition, gelatinolytic activities corresponding to both MMP-2 and MMP-9 detected in the supernatants of IMAs were significantly increased by LTB₄ after 24-hour incubation (Figure 5). Furthermore, ELISA measurements of MMP and TIMP protein concentrations revealed a significant increase in MMP-2 protein levels by LTB₄, whereas the levels of TIMP-1 were significantly decreased (supplemental Table II).

Discussion

The results of the present study indicate that LT receptor signaling may be involved in both VSMCs and leukocyte activation in the context of in-stent stenosis. The BLT receptor antagonist BIIL284 reduced the in-stent intimal hyperplasia 14 days after angioplasty with stent implantation in the carotid artery of hyperlipidemic rabbits. Furthermore, LT-dependent matrix metalloproteinase activity in the context of hyperlipidemia and in-stent stenosis was revealed. Taken together, these data suggest anti-LTs as a potential treatment of vascular inflammatory conditions associated with increased extracellular matrix degradation, such as in-stent restenosis and atherosclerosis.

The intimal hyperplasia observed in-stent in the present model is associated with VSMC activation,⁸ and LTB₄ has previously been shown to induce VSMC migration and proliferation.⁶ In the present study, the LTB₄-induced effects on VSMC migration was confirmed, and extended to also increased MMP-2 activity. Extracellular MMP-2 activity was significantly increased in the rabbit carotid artery both by hypercholesterolemia and stenting in the present study. Furthermore, rabbits treated with the BLT receptor antagonist BIIL284 exhibited significantly reduced MMP-2 activity in stented arteries. Because MMP-2 expression is limited to VSMCs in the present model,⁸ these results extend the previously described LT-induced effects on VSMCs by associating BLT receptor signaling with also increased MMP-2 activity. This notion was further supported by the inhibition of the LTB₄-induced VSMC migration after silencing of MMP-2 message, indicating that part of the recently described BLT receptor signaling in VSMCs⁶,¹⁹ is mediated through MMP-2.

Also MMP-9 activity was detected in conditioned media of carotid arteries derived from cholesterol-fed rabbits but not in those derived from rabbits receiving normal chow. Further-
more, only stented arteries yielded gelatinase activity corresponding to the active form of MMP-9, suggesting that MMP-9 activity may be limited to atherosclerosis and vascular injury. The significant correlation of macrophage number with MMP-9 activity furthermore suggests these inflammatory cells as a major source of MMP-9, which corroborates the previously reported colocalization of MMP-9 with macrophages after vascular injury in rabbits. The decrease in MMP-9 activity in carotid arteries by BIIL284 was associated with a reduced number of macrophages infiltrating the vascular wall, whereas the circulating levels of leukocytes were not significantly altered by the treatment. These data suggest that the prevention of in-stent stenosis by BLT receptor antagonism was associated with local inhibitory effects on both VSMCs and inflammatory cells. BLT receptors are expressed on macrophages within human atherosclerotic lesions. In addition, the increased macrophage production of LTB₄ in the absence of dampening effects of TGFβ on T-cells has suggested LTB₄ signaling as a potential link between innate and adaptive immunity in the context of atherosclerosis.

The decrease in MMP activity by antileukotriene treatment in the present study was observed also in uninjured vessels from hypercholesterolemic rabbits. This finding suggests that the leukotriene-induced MMP activity may not solely be associated with in-stent stenosis, but may potentially also represent a pathophysiological process of atherosclerosis in the absence of angioplasty. The degradation of vascular extracellular matrix by MMPs is a key component in atherosclerotic plaque rupture, causing for example myocardial infarction and stroke. Previous studies have in addition suggested a relationship of MMP and 5-LO activities in carotid atherosclerotic lesions with clinical signs of cerebral ischemia. Furthermore, the reduced aneurysm formation by genetic targeting of either 5-LO or the BLT₁ receptor in hyperlipidemic ApoE⁻/⁻ mice is associated with decreased MMP activity. The suggestion of an LTB₄-induced MMP activity in the present and previous animal models were extended to human tissues through the LTB₄-induced in-stent intimal hyperplasia as well as increased activity of both MMP-2 and MMP-9 after ex vivo angioplasty of human arteries.

MMP activities measured by zymography may reflect effects both on both MMPs and their endogenous inhibitors, TIMPs. Measures of those protein concentrations in the present study indicated that the LTB₄-induced MMP-2 activ-
ity was associated with increased levels of MMP-2 protein in conditioned media from stented human arteries. In contrast, the MMP-9 activity may in addition be associated with a LTβR-induced decrease in TIMP-1 protein concentrations.

In summary, the BLT receptor antagonist BIIL284 reduced in-stent stenosis 14 days after carotid angioplasty in hypercholesterolemic rabbits. This effect was associated with effects on both VSMCs and macrophage infiltration. Furthermore, LTβR-dependent MMP activity was demonstrated through different approaches. First, BLT receptor antagonism decreased MMP activity in stented lesions. Second, disruption of MMP-2 signaling through siRNA inhibited the LTβR-induced migration of VSMCs. Third, human stented arteries exhibited significantly increased MMP activities in the presence of LTβR. In conclusion, the inhibition of extracellular matrix degradation and macrophage infiltration by BLT receptor antagonists represents a potential therapeutic possibility in the treatment of in-stent restenosis and atherosclerosis.

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Disclosures
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Supplemental Fig I. Plasma concentrations of BIIL315, the active metabolite of BIIL284 (n=4). The $C_{\text{max}}$ of 145±57 ng/mL was obtained 6h post gavage, and the concentration 24h after gavage was 4±1.8 ng/mL.

Supplemental Table I. White blood cell count in hypercholesteremic rabbits treated with either vehicle (Control) or BIIL284 (3 mg/kg).

<table>
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<tr>
<th></th>
<th>Control ($N=5$)</th>
<th>BIIL284 ($N=4$)</th>
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<tbody>
<tr>
<td>White Blood Cells ($10^6/\mu L$)</td>
<td>12.8±1.8</td>
<td>13.8±2.1</td>
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<tr>
<td>Neutrophils (%)</td>
<td>33.3±4.1</td>
<td>27.7±2.7</td>
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<tr>
<td>Lymphocytes (%)</td>
<td>58.2±5.4</td>
<td>66.2±2.2</td>
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<tr>
<td>Monocytes (%)</td>
<td>5.4±1.2</td>
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<tr>
<td>Eosinophils (%)</td>
<td>0.0±0.02</td>
<td>0.1±0.13</td>
</tr>
<tr>
<td>Basophils (%)</td>
<td>3.1±0.64</td>
<td>2.8±0.51</td>
</tr>
</tbody>
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Supplemental Table II. MMP and TIMP protein concentrations (ng/mg tissue) in conditioned media derived from stented human internal mammary arteries in the absence or presence of LTB$_4$ (100 nM, 24h).

<table>
<thead>
<tr>
<th></th>
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<th>LTB$_4$</th>
<th>$N$</th>
<th>$P$</th>
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<tbody>
<tr>
<td>MMP-2</td>
<td>2.5±0.41</td>
<td>5.4±2.8</td>
<td>(6)</td>
<td>$P&lt;0.05$</td>
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<tr>
<td>TIMP-2</td>
<td>0.41±0.12</td>
<td>0.41±0.07</td>
<td>(11)</td>
<td>NS</td>
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<tr>
<td>MMP-9</td>
<td>0.32±0.08</td>
<td>0.49±0.18</td>
<td>(11)</td>
<td>NS</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>0.23±0.09</td>
<td>0.10±0.04</td>
<td>(6)</td>
<td>$P&lt;0.05$</td>
</tr>
</tbody>
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