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

Hanna Hlawaty, Marie-Paule Jacob, Liliane Louede, Didier Letourneau, Charles Brink, Jean-Baptiste Michel, Laurent Feldman, Magnus Bäck

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: lipoygenase ■ 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 derivates 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. 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). Derived from arachidonic acid through the 5-lipoxygenase (5-LO) pathway, LTs are potent inflammatory mediators implicated in several pathophysiological processes associated with atherosclerosis. 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, were recently reproduced in restenosis patients. In the latter study, 2 of the FLAP gene polymorphisms studied were independent prognostic factors in predicting in-stent restenosis 6 months after PCI.

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 vascular smooth muscle cells (VSMCs). 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.

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. 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 anesthesia under surveillance in a temperature-controlled room. Intravenous anesthesia with 0.1% pentobarbital sodium, local anesthesia with xylocaine was also administered s.c. at the site of the femoral incision. Heparin (500 IU i.v.) was administered once the arterial access was obtained. A 5-French introducer was installed into the femoral artery through which a 3.5-mm diameter/30-mm length angioplasty balloon was placed in the right common carotid artery under fluoroscopic guidance using a Philips BV Endura fluoroscope. The balloon was inflated three times (30 s, 10 ATM) in the carotid artery covering its departure from the brachiocephalic trunk to the base of the skull. After balloon dilatation, two consecutive stents were implanted with approximately 1 cm space into the right common carotid artery by 30 s inflation at 12 ATM. After repair of the femoral skin wound, rabbits were left to recover from anesthesia under surveillance in a temperature-controlled room. Treatment protocols were commenced 24 hours before angioplasty and continued for 15 days until sacrifice. Treatment was administered once daily by oral gavage of either 2 mL tylolose (1%) or 2 mL tylolose containing the BLT receptor antagonist BIIL284 (3 mg/kg; a gift from Boehringer-Ingelheim). All rabbits undergoing the angioplasty procedure received antibiotics for 3 days and low dose aspirin (1 mg/kg; Aspicéig) for 14 days, delivered through the drinking water. Three more rabbits, receiving ordinary chow without cholesterol and not being subjected to either angioplasty or treatment protocols were also included in the study. The rabbits were euthanized by an overdose of pentobarbital sodium 14 days after angioplasty and not being subjected to either angioplasty or treatment protocols were also included in the study. The rabbits were euthanized by an overdose of pentobarbital sodium 14 days after angioplasty.

Morphological Studies

Rabbit 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, Microm) followed by staining with hematoxylin, Masson trichrome, and orceine. The intimal and medial areas were quantified using a light microscope coupled to a computer 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 α SMA-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 200V during 1 hour. Gels were subsequently soaked for 30 minutes in 2.5% TritoX-100 at room temperature followed by brief rinse in distilled water. The incubation with 50 mmol/L TRIS–HCl, pH 7.8, 10 mmol/mL 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-116 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 nmol/L Tamra-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 nmol/L Tamra-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, 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 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 substraction of baseline (ie, without LTB4).
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 (Figure 3). 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).

The intimal hyperplasia observed in-stent in the present model is associated with VSMC activation, and LTB4 has previously been shown to induce VSMC migration and proliferation. 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, 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 VSMCs 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.8 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.6 In addition, the increased macrophage production of LTβ4 in the absence of dampening effects of TGFβ on T-cells has suggested LTβ4 signaling as a potential link between innate and adaptive immunity in the context of atherosclerosis.20

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.21 Previous studies have in addition suggested a relationship of MMP and 5-LO activities in carotid atherosclerotic lesions with clinical signs of cerebral ischemia.9 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.10,11 The suggestion of an LTβ4-induced MMP activity in the present and previous10,11 animal models were extended to human tissues through the LTβ4-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 LTβ4-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 LTB₄-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, LTB₄-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 LTB₄-induced migration of VSMCs. Third, human stented arteries exhibited significantly increased MMP activities in the presence of LTB₄. 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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References

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Supplement Material

**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.

![Plasma concentrations of BIIL315](image)

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

<table>
<thead>
<tr>
<th></th>
<th>Control (N=5)</th>
<th>BIIL284 (N=4)</th>
</tr>
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<tbody>
<tr>
<td>White Blood Cells (10⁶/μL)</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>
<td>3.2±0.56</td>
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<tr>
<td>Eosinophils (%)</td>
<td>0.0±0.02</td>
<td>0.1±0.13</td>
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<tr>
<td>Basophils (%)</td>
<td>3.1±0.64</td>
<td>2.8±0.51</td>
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</tbody>
</table>

**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 LTB₄ (100 nM, 24h).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LTB₄</th>
<th>N</th>
<th>P</th>
</tr>
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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>
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</table>