Expression of 5-lipoxygenase and leukotriene A₄ hydrolase in human atherosclerotic lesions correlates with symptoms of plaque instability

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Leukotrienes (LT) are a group of proinflammatory lipid mediators that are implicated in the pathogenesis and progression of atherosclerosis. Here we report that mRNA levels for the three key proteins in LTB₄ biosynthesis, namely 5-lipoxygenase (5-LO), 5-LO-activating protein (FLAP), and LTA₄ hydrolase (LTA4H), are significantly increased in human atherosclerotic plaque ($n = 72$) as compared with healthy controls ($n = 6$). Neither LTC₄ synthase nor any of the LT receptors exhibits significantly increased mRNA levels. Immunohistochemical staining revealed abundant expression of 5-LO, FLAP, and LTA4H protein, colocalizing in macrophages of intimal lesions. Human lesion tissue converts arachidonic acid into significant amounts of LTB₄, and a selective, tight-binding LTA4H inhibitor can block this activity. Furthermore, expression of 5-LO and LTA4H, but not FLAP, is increased in patients with recent or ongoing symptoms of plaque instability, and medication with warfarin correlates with increased levels of FLAP mRNA. In contrast to human plaques, levels of 5-LO mRNA are not significantly increased in plaque tissues from two atherosclerosis-prone mouse strains, and mouse plaques exhibit segregated cellular expression of LTA4H and 5-LO as well as strong increases of CysLT₁ and CysLT₂ mRNA. These discrepancies indicate that phenotypic changes in the synthesis and action of LT in specific mouse models of atherosclerosis should be cautiously translated into human pathology. The abundant expression of LTA4H and correlation with plaque instability identify LTA4H as a potential target for pharmacological intervention in treatment of human atherosclerosis.

atherosclerosis | enzyme inhibitor | leukotriene B₄ | thrombosis

Leukotrienes (LTs) are potent lipid mediators implicated in a number of inflammatory diseases (1). In cellular biosynthesis of LTs, arachidonic acid is oxygenated by 5-lipoxygenase (5-LO), assisted by 5-LO-activating protein (FLAP), into the unstable epoxide LTA₄. This intermediate may be hydrolyzed by LTA₄ hydrolase (LTA4H) into LTB₄ or conjugated with GSH by LTC₄ synthase (LTC4S) to form LTC₄, the parent compound of the cysteinyl LTs (CysLTs) (2, 3). LTB₄ and the CysLTs exert biological effects through two pairs of receptors denoted BLT₁/BLT₂ and CysLT₁/CysLT₂, respectively (4–7). Diverse functions of LTs and their receptors in immune and inflammatory responses have been identified, and roles for these mediators have been demonstrated in several acute and chronic inflammatory diseases. A growing body of morphological, biochemical, pharmacological, and functional evidence collected from human and animal studies has implicated LTs in the development and progression of atherosclerosis (8–10), in particular LTB₄ (for review see ref. 11). Furthermore, genetic studies have identified variants of the 5-LO gene promoter and the FLAP gene as risk factors in human atherosclerosis and myocardial infarction (12, 13).

Atherosclerosis is an inflammatory disorder involving complex interactions between vascular wall cells and invading inflammatory cells (14). Genetically modified mouse models of

atherosclerosis, based on two pivotal genes of lipid metabolism, i.e., apolipoprotein E (ApoE) and the low-density lipoprotein receptor (LDLR), have been crossed with mice deficient in a specific gene of the LT cascade, e.g., 5-LO and BLT₁, to investigate the impact of the gene product on the atherosclerotic process (9, 15–17).

In the present study we examined the expression of 5-LO, FLAP, LTA4H, LTC4S, and the receptors BLT₁, BLT₂, CysLT₁, and CysLT₂ in human carotid plaques and aortic lesions from both ApoE (–/–) and compound ApoE (–/–) × LDLR (–/–) mice. In contrast to the mouse models, we find that both 5-LO and LTA4H are expressed at increased levels and colocalize within human atherosclerotic lesions, with expression levels correlating with recent or ongoing symptoms of plaque instability. We also report that a selective inhibitor of LTA4H can block LTB₄ biosynthesis in plaque tissue, thus identifying LTA4H as a potential target for development of drugs for prevention and treatment of atherosclerosis and acute atherothrombotic events.

Results

Expression of 5LO, FLAP, LTA4H, and LTC4S mRNA in Human Carotid Plaque and Mouse Atherosclerotic Aorta. In the human carotid atherosclerotic lesions, the transcript levels of 5-LO, FLAP, and LTA4H were high, corresponding to a 7.5-fold (7.5 ± 4.1 , $P < 0.001$), 2.7-fold (2.7 ± 1.3 , $P = 0.003$), and nearly 2-fold (1.9 ± 1.0 , $P = 0.03$) increase relative to normal iliac arteries, respectively (Fig. 1A). In contrast, the levels of LTC4S mRNA were not significantly different from controls.

In the ApoE (–/–) mouse aorta, 5-LO and LTA4H mRNA levels were essentially unaltered, whereas FLAP and LTC4S mRNA showed a tendency to increase relative tissue from wild-type C57BL/6J mice (Fig. 1B). In ApoE (–/–) × LDLR (–/–) mice, the expression pattern of the enzymes was essentially the same as in ApoE (–/–) mice, except that LTA4H mRNA levels were significantly increased (1.7 ± 0.3 , $P = 0.01$) (Fig. 1C).

Expression of 5-LO, FLAP, and LTA4H Protein in Human and Mouse Atherosclerotic Lesions. Staining of human carotid plaque demonstrated prominent expression of 5-LO, FLAP, and LTA4H in intimal lesion areas that also stained positively for human macrophage marker CD163 (Fig. 2). The distribution of 5-LO,

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Abbreviations: LT, leukotriene; CysLT, cysteinyl-LT receptor; 5-LO, 5-lipoxygenase; LTA4H, LTA₄ hydrolase; FLAP, 5-LO-activating protein; LTC4S, LTC₄ synthase; LDLR, low-density lipoprotein receptor; ApoE, apolipoprotein E; EIA, enzyme immunoassay.

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Fig. 3. Segregated expression of LTA4H and 5-LO protein in mouse atherosclerotic lesions. (A–C) Serial sections of mouse aortic lesions stained for LTA4H and macrophage marker CD68 show positive signals in intima lesion. Preadsorption of antiserum with LTA4H protein abolishes the LTA4H staining. (Magnification: $\times 10$. Scale bar: 200 μm .) (D and E) Serial sections stained for LTA4H and 5-LO show segregated distribution with 5-LO- and LTA4H-positive cells mainly in tunica adventitia and intima, respectively (black arrows). (Magnification: $\times 5$. Scale bar: 200 μm .) (F–I) Immunofluorescence staining for LTA4H (Cy3, red), CD68 (FITC, green), and DNA (DAPI, blue) and merged picture show partial colocalization of LTA4H and CD68 (indicated by white arrows), with other LTA4H-positive cells in close proximity to CD68-positive cells (indicated by *). Preadsorption of antiserum with LTA4H abolishes the Cy3 red LTA4H staining. (Scale bar: 50 μm .) (K–O) Immunofluorescence staining for 5-LO (Cy3, red), CD68 (FITC, green), and DNA (DAPI, blue) and merged picture show 5-LO and CD68 colocalized in cells in adventitia (indicated by white arrows); preadsorption of antiserum with 5-LO abolishes the Cy3 (red) 5-LO staining. (Scale bar: 50 μm .) L, lumen.

Discussion

During the past few years, the focus of the LT research field has expanded from allergic and inflammatory diseases of the respiratory tract to another disease characterized by chronic inflammation, namely, atherosclerosis (14). Early data in the literature associated LT production with ischemic heart disease (18), but for long 5-LO was generally believed to be absent from advanced human atherosclerotic lesions (19). Three almost simultaneous studies sparked new interest in this area; first, the demonstration that 5-LO is abundantly expressed in human atherosclerotic lesions and correlates with disease stage (8); second, the observation that a selective BLT₁ antagonist could protect against atherogenesis in two mouse models of atherosclerosis (10); and, third, the key report that deletion of the 5-LO gene affords protection in atherosclerosis-prone mice (9). These reports were followed by genetic studies identifying promoter variants of 5-LO and the FLAP gene as significant risk factors for development of atherosclerosis and myocardial infarction in man (12, 13), which in turn led to the exciting prospect of anti-LTs as potential therapeutics for cardiovascular disease (11). However,

studies conducted thus far have generated information that does not conform to a consistent picture. Studies have usually focused on a single enzyme or receptor using human tissues or a specific mouse model of atherosclerosis. Here we exploited the large Biobank of Karolinska Endarterectomies collection of human atherosclerotic vascular tissues obtained from patients undergoing carotid endarterectomy as well as plaque tissues from two mouse models of atherosclerosis to generate a comprehensive expression profile of key enzymes and receptors of the LT cascade in humans and mice.

Expression of 5-LO, FLAP, and LTA4H Is Increased and Colocalized in Human Atherosclerotic Lesions. Analysis of atherosclerotic tissues from 72 patients revealed a striking increase in the expression of 5-LO mRNA, confirming previous data on human atherosclerotic specimens (8). In addition, we found significant increases in the mRNA levels of FLAP and LTA4H, thus completing the triplet of key proteins required for LTB₄ biosynthesis (Fig. 1) and corroborating the notion that this specific LT is critically involved in human atherosclerosis (10, 13, 16, 17, 20). In contrast, neither LTC₄S nor any of the LT receptors displayed significantly elevated levels of mRNA. Furthermore, 5-LO, FLAP, and LTA4H proteins colocalize in intimal lesions (Fig. 2), particularly in macrophages, presumably facilitating efficient enzyme coupling and LTB₄ synthesis. Moreover, the lack of symmetrical increases of all LT cascade proteins suggests the existence of regulatory mechanisms, yet to be identified, which influence their expression levels within the atherosclerotic lesions, as very recently demonstrated for 5-LO expression in unstable plaque (21).

Expression of 5-LO and LTA4H mRNA Correlates with Recent or Ongoing Atherothrombotic Events. We found a significant correlation between the levels of 5-LO and LTA4H mRNA and recent or ongoing symptoms of plaque instability (Fig. 5). Such events are caused by atherothrombosis in unstable plaques, which eventually go through a healing process (22). Presumably, the time interval between the last recorded symptom and the surgical procedure represents unstable plaques at different stages of healing/instability. Therefore, the data point to an association between expression of 5-LO and LTA4H and plaque

Fig. 4. Synthesis of LT_{B4} in human carotid plaque and inhibition by an LTA4H inhibitor. Homogenates of human plaque tissue (*n* = 8) were preincubated for 30 min in the absence (*n* = 8) or presence (*n* = 4) of a hydroxamic acid inhibitor of LTA4H (HA) followed by incubation with arachidonic acid. LT_{B4} was measured by HPLC/EIA, and data are presented as mean ± SD. **, *P* < 0.01.

Fig. 5. Correlation of 5-LO, FLAP, and LTA4H mRNA levels with clinical symptoms and medication with warfarin. (A) Levels of 5-LO, FLAP, and LTA4H mRNA in human carotid plaques were correlated with the occurrence of symptoms of plaque instability. The mRNA levels of 5-LO and LTA4H were significantly higher in patients with symptoms within 1 month ($n = 18$) than in patients with the last symptoms >3 months ago ($n = 10$). Data are presented as mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (as compared with normal control tissues). (B) Comparison of the mRNA levels of 5-LO, FLAP, and LTA4H in plaques from patients with ($n = 11$) and without ($n = 55$) warfarin treatment. Data are presented as mean $\pm$ SD. **, $P < 0.01$.

instability. Together with genetic data identifying 5-LO as a risk factor for atherosclerosis (12), it is tempting to speculate that LTB₄ may be involved in driving local acute inflammatory processes that precede and precipitate an acute thrombotic event. Hence, these findings are potentially of clinical significance and suggest that anti-LTs may be useful, not only in reducing atherogenesis, but also in the prevention and treatment of acute atherothrombotic complications.

Treatment with Warfarin Correlates with Increased Expression of FLAP mRNA in Atherosclerotic Plaques. We were surprised to find that levels of FLAP mRNA were significantly higher in patients taking warfarin (Fig. 5B). This drug inhibits blood clotting by means of reduced liver production of several vitamin K-dependent coagulation factors (II, VII, IX, and X), and a mechanism for its effect on FLAP mRNA expression is not obvious. Warfarin medication is usually withdrawn 2–3 days before endarterectomy and substituted with other drugs, which may also influence FLAP gene expression. In addition, we noted a tendency of decreased FLAP mRNA levels in patients taking aspirin, a drug that is seldom, if ever, combined with warfarin treatment.

Differences in mRNA and Protein Expression Between Human and Murine Lesions. Although several lines of evidence indicate a role for LTs, in particular LTB₄, in atherosclerosis, the data presented thus far are not fully consistent. For instance, deletion of the 5-LO gene in mice with an LDLR-deficient genetic back-

Fig. 6. Expression of LT receptor mRNA in human carotid plaques and in aortic plaques from two atherosclerosis-prone mouse models. Shown is expression of CysLT₁, CysLT₂, BLT₁, and BLT₂ transcripts in human carotid plaques (A) and plaques from ApoE^{-/-} mice (B) and ApoE^{-/-} × LDLR^{-/-} mice (C) as determined by real-time quantitative PCR. Data for human plaques ($n = 72$) were compared with normal controls ($n = 6$), whereas ApoE^{-/-} mice ($n = 8$) and ApoE^{-/-} × LDLR^{-/-} mice ($n = 5$) were compared with the wild-type C57BL/6J strain ($n = 8$). Data are presented as mean $\pm$ SD. **, $P < 0.01$.

ground effectively protected against atherosclerosis (9), whereas a more extensive study failed to detect any similar effects but rather a protection against aortic aneurysm (15). Because 5-LO deletion leads to a complete loss of all LTs, the latter study is difficult to reconcile with data demonstrating that pharmacological antagonism or genetic deletion of BLT₁ in mice also affords protection against atherosclerosis (10, 16, 17). We found that expression of 5-LO mRNA was not significantly up-regulated in ApoE^{-/-} or ApoE^{-/-} × LDLR^{-/-} mice, and the distribution of 5-LO and LTA4H proteins was segregated (Fig. 3). Furthermore, CysLT₁ and CysLT₂ expression was strongly up-regulated in the mouse models (Figs. 6 and 7). These findings are consistent with the report by Zhao *et al.* (15), in which the atherosclerosis-prone mouse strains displayed expression of 5-LO in the adventitia (rather than intimal lesions)

Mm00446703_g1, Mm00521839_m1, and Mm00498491_s1, respectively.

Immunohistochemistry. Cryostat sections (10 μ m) of human carotid plaque or mouse aorta were fixed in acetone for 10 min, air-dried, and stained by using biotin–avidin–immunoperoxidase technology (20). Rabbit polyclonal antibodies against human LTA4H (prepared in-house), human 5-LO (a gift from Olof Rådmark; Karolinska Institutet, Stockholm), human FLAP (a gift from Jilly Evans; Merck Frosst Labs, Pointe Claire, PQ, Canada), human CysLT₁ (Cayman Chemical, Ann Arbor, MI), and CD68 (Serotec) were detected with biotinylated goat anti-rabbit IgG (Vector Laboratories). Mouse monoclonal antibodies against human CD163 (DakoCytomation) were detected with biotinylated horse anti-mouse IgG (Vector Laboratories), and rat polyclonal anti-mouse CD68 was detected with biotinylated rabbit anti-rat IgG (Vector Laboratories). All antibodies were used at optimal dilutions determined by titration on mouse spleen and human tonsils, respectively. For immunofluorescence double staining, after preincubation with 10% normal goat serum, primary antibodies from rabbit against LTA4H, 5-LO, or CysLT₁ receptor were combined with rat anti-mouse CD68 (1:10,000), applied on sections overnight at 4°C, rinsed, and incubated with Cy3-conjugated goat anti-rabbit-IgG (1:400; Jackson ImmunoResearch) and FITC-conjugated goat anti-rat-IgG (1:200; Jackson ImmunoResearch) at room temperature for 1 h. After staining DNA with DAPI, slides were mounted with an antifading mounting medium (Vector Laboratories) and observed under epifluorescence. The specificity of primary antibodies was assessed by neutralizing the primary antibody with a 20-fold excess of blocking protein or peptide at room temperature for 1 h before use.

Reverse-Phase HPLC Coupled to EIA. Human carotid plaques were collected immediately after endarterectomy, dissected, weighed,

homogenized in 0.1 M PBS containing proteinase inhibitor mixture (Roche), and sonicated three times for 10 s on ice. Homogenates were centrifuged at 10,000 $\times$ g for 10 min at 4°C, and the resulting tissue extracts were preincubated in the presence or absence of 10 μ M aminohydroxamic acid, synthesized as described (25), for 30 min at room temperature. Samples were incubated with 2 mM ATP, 2 mM Ca²⁺, and 80 μ M arachidonic acid for 10 min and quenched with an equal volume of methanol. After acidification to pH 3–4, samples were purified by solid-phase extraction (Supelclean LC18, Supelco) and analyzed by reverse-phase HPLC. The column (Nova-Pak C18, Waters) was eluted with acetonitrile/methanol/water/acetic acid (30:35:35:0.01 by volume) at 1.0 ml/min, and absorbance was monitored at 270 nm. Fractions corresponding to the retention time of an LTB₄ standard (Cayman Chemical) were collected, dried under nitrogen, and resuspended with EIA buffer. Levels of LTB₄ were determined in triplicate assays with an LTB₄ EIA kit (Cayman Chemical) by using dilutions within the linear portion of the standard curve.

Statistical Analysis. Data are presented as mean $\pm$ SD. Differences between the means were evaluated by one-way ANOVA, Student's *t* test, or the Mann–Whitney test when appropriate. A value of *P* < 0.05 was considered statistically significant.

Note. During the preparation of this article, it was reported that the LTA4H gene, alone or in combination with the FLAP gene, constitutes a significant risk factor for myocardial infarction in several human populations (26), thus further corroborating the results and conclusions of the present study.

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