Myeloid Cell 5-Lipoxygenase Activating Protein Modulates the Response to Vascular Injury

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Rationale: Human genetics have implicated the 5-lipoxygenase enzyme in the pathogenesis of cardiovascular disease, and an inhibitor of the 5-lipoxygenase activating protein (FLAP) is in clinical development for asthma.

Objective: Here we determined whether FLAP deletion modifies the response to vascular injury.

Methods and Results: Vascular remodeling was characterized 4 weeks after femoral arterial injury in FLAP knockout mice and wild-type controls. Both neointimal hyperplasia and the intima/media ratio of the injured artery were significantly reduced in the FLAP knockouts, whereas endothelial integrity was preserved. Lesional myeloid cells were depleted and vascular smooth muscle cell (VSMC) proliferation, as reflected by bromodeoxyuridine incorporation, was markedly attenuated by FLAP deletion. Inflammatory cytokine release from FLAP knockout macrophages was depressed, and their restricted ability to induce VSMC migration ex vivo was rescued with leukotriene B4 (LTB4) FLAP deletion restrained injury and attenuated upregulation of the extracellular matrix protein, tenascin C, which affords a scaffold for VSMC migration. Correspondingly, the phenotypic modulation of VSMC to a more synthetic phenotype, reflected by morphological change, loss of α-smooth muscle cell actin, and upregulation of vascular cell adhesion molecule-1 was also suppressed in FLAP knockout mice. Transplantation of FLAP-replete myeloid cells rescued the proliferative response to vascular injury.

Conclusions: Expression of lesional FLAP in myeloid cells promotes leukotriene B4-dependent VSMC phenotypic modulation, intimal migration, and proliferation. (Circ Res. 2013;112:432-440.)

Key Words: angioplasty and stenting ■ animal model of human disease remodeling ■ inflammation ■ leukotrienes ■ restenosis ■ smooth muscle cell ■ vascular injury

Leukotrienes (LTs) are derived from arachidonic acid and are involved in a variety of inflammatory diseases, including asthma, arthritis, and psoriasis. 5-lipoxygenase (5-LO) activating protein (FLAP) binds to 5-LO and facilitates its translocation to the nuclear membrane, where 5-LO binds to arachidonic acid, triggering formation of LTA4 which is further metabolized to LTB4 and cysteinyl LTs (ie, LTC4, LTD4, and LTE4). Genetic disruption of FLAP suppresses LT synthesis.

Several key proteins within the LT cascade are expressed in human atherosclerotic plaques, including 5-LO, FLAP, 2 downstream enzymes (LTA4 hydrolase and LTC4 synthase) and 4 LT receptors, suggesting an active, proinflammatory circuit in the diseased vasculature. Preliminary evidence indicates that antagonism of the LTB4 receptor 1 (BLT1) or pharmacological inhibition of either FLAP or 5-LO restraints cholesterol absorption and augments to the hypolipidemic effects of a statin in mice and hamsters. LTB4 induces migration and proliferation of human coronary artery vascular smooth muscle cells (VSMCs) in vitro and inhibition of LT biosynthesis decreases neutrophil deposition at sites of arterial injury in pigs. Polymorphisms in the FLAP gene cosegregate with risk of myocardial infarction, stroke, and restenosis. Endothelial cell disruption by vascular injury results in increased permeability and recruitment of inflammatory cells and platelets. This is accompanied by the modulation of VSMCs to a more synthetic phenotype. VSMCs, which normally reside in the media, proliferate and migrate to the intima. They extend lamellipodia toward attractant mediators via actin polymerization, detach their trailing edges by degrading focal contacts, and generate force via myosin II to...
propel themselves forward.\textsuperscript{35,16} Inflammatory lipid mediators contribute to the initiation and progression of VSMC migration and proliferation.\textsuperscript{17}

Here, we show that FLAP expression is evident in lesional myeloid cells during the proliferative response to vascular injury. FLAP deletion attenuates the augmented expression of tenasin C (TNC) and vascular cell adhesion molecule (VCAM)-1, impairing the migratory response of VSMCs by suppressing macrophage release of inflammatory cytokines, LTB\textsubscript{4} and cysLTs. Reconstitution of knockout mice with FLAP-replete myeloid cells rescues this phenotype, implicating directly myeloid cell FLAP in the vascular response to injury.

Methods

Animals
All animal studies were performed according to protocols approved by the Institute for Animal Care and Use Committee at the University of Pennsylvania. FLAP knockout (FLAP KO) mice have been fully backcrossed onto C57BL-6J background and were a kind gift from Merck Research Laboratories.

Experimental Model
Male FLAP KO and C57BL-6J wild-type (WT) mice, aged 12 weeks, were subject to injury of the right femoral artery by passage of a 0.38 mm diameter angioplasty guide wire, as previously described.\textsuperscript{18} Briefly, mice were anaesthetized with ketamine and acepromazine after which the right femoral artery was isolated through an incision in the right hind limb. Transluminal arterial injury was induced by insertion of a wire into the right femoral artery for 1 minute to induce endothelial damage. Sham surgeries were performed on the left femoral artery of the same mice. Mice were euthanized 2, 1, or 2 weeks after the surgery for immunohistochemistry.

Bromodeoxyuridine (BrdU) (0.7 mg/d for 28 days, Sigma, St. Louis, MO) was administrated to the mice via mini osmotic pumps (ALZET, Cupertino, CA) subcutaneously placed through a midback incision for each mouse. Mice were euthanized 4 weeks after the surgery for BrdU quantification, histology, and morphometric analysis.

Histology and Morphometric Analysis
Femoral arteries were perfusion-fixed with 4% paraformaldehyde and processed for paraffin embedding. Serial clusters of 6-μm-thick sections were cut 80 μm apart from each other along the length of the femoral artery. One section from each cluster was stained with hematoxylin-eosin (Sigma): sections with the most severe lesions from each femoral artery were selected for analysis using computerized morphometry (Image Pro Plus software, Media Cybernetics, Bethesda, MD). Measurements included luminal area, medial area, and intimal area both at baseline and 4 weeks after wire injury. The intima to media ratio was calculated as previously described.\textsuperscript{19} The percentage of stenosis was calculated as the ratio of the intimal area to the area inside the external elastic lamina.

Immunohistochemistry and BrdU Quantification
Representative sections were immunohistochemically stained for FLAP (1:300, a kind gift from Jilly Evans), α-smooth muscle actin (SMA, 0.6 μg/mL, Abcam, Cambridge, MA), BrdU (5 μg/mL, Hybridoma Bank, Iowa City, IA), TNC (10 μg/mL, Chemicon International Inc, Temecula, CA), VCAM-1 (10 μg/mL, Southern Biotech, Birmingham, AL), CD45 (2.5 μg/mL, BD Pharmingen, San Jose, CA), and Von Willebrand factor (0.5 μg/mL, Sigma). All sections were deparaffinized and pretreated with specific antigens to retrieve buffers. For FLAP immunostaining, sections were incubated with primary antibody and then incubated with a secondary antibody conjugated to horseradish peroxidase. FLAP positive cells were quantified using Image Pro Plus software. For immunofluorescence staining, Alex Fluor 488 was used as a secondary antibody for FLAP and Alexa Fluor 568 for CD45.

For TNC staining, sections were treated with Pronase (Boehringer Mannheim/Roche, Indianapolis, IN) for 10 minutes. For BrdU staining, sections were treated with 0.05% Trypsin (Sigma) in 0.1 mol/L Tris for 10 minutes. For VCAM-1 staining, antigen retrieval was accomplished by microwaving tissue for 15 minutes in unmasking solution (H-3300, Vector Laboratory Inc, Burlingame, CA). Endogenous peroxidase was then blocked with 3% hydrogen peroxide for 10 minutes. All sections were then incubated with primary antibody and species-specific secondary antibody and developed with a Vectastain Elite ABC kit (Vector Laboratory Inc). For Von Willebrand factor staining, sections were incubated with primary antibody overnight at 4°C and then incubated with Alexa Fluor 568 secondary antibody for 1 hour at 25°C. Vector M.O.M. kit was used for α-SMA staining following manufacturer instructions. α-SMA and VCAM-1 staining were quantified as the ratio of stain positive area to intimal area using Image Pro Plus software. Von Willebrand factor staining was quantified as ratio of positive length to lumen circumference using Image Pro Plus software. BrdU-positive cells and total cells in the intimal area were manually counted in a double blind manner and proliferation index was calculated as a percentage of the ratio between BrdU stained nuclei over the total number of nuclei in the intimal area.

Cell Culture
Peritoneal macrophages were collected as previously described.\textsuperscript{20} Cells were treated with calcium ionophore, A23187 (Sigma) 10 μmol/L for 20 minutes, and cell culture media were collected for cell migration studies and detection of 5-Hydroxyeicosatetraenoic acid (5-HETE), LTB\textsubscript{4}, LTC\textsubscript{4}, LTD\textsubscript{4}, and LTE\textsubscript{4}. Cell culture media were collected for measurements of cytokines from macrophages stimulated with lipopolysaccharide (5 μg/mL, Sigma) for 12 hours. Primary VSMCs from the aorta of WT or FLAP KO mice were cultured as previously described.\textsuperscript{21} Briefly, mouse aortas were isolated and placed under cell culture coverslips (Nalge Nunc International, Rochester, NY) on tissue culture plates in Dulbecco Eagle Medium/Ham aortic media were placed and cultured under cell culture coverslips (Nalge Nunc International, Rochester) for 7 days. Cells were then routinely passaged and used from passages 2 to 4.

5-Ethynyl-2′-Deoxyuridine Cell Proliferation Assay
5-ethyl-2′-deoxyuridine (EdU) incorporation assay using Click-It EdU Imaging Kits (Invitrogen, C10339) was performed to evaluate new DNA synthesis in WT and FLAP KO mVSMC. 2.5×10\textsuperscript{4} cells in 12-well dish were seeded in DMEM/F12-10% fetal bovine serum. After 24 hours, cells were incubated in DMEM/F12-1 mg/mL BSA for 48 hours. Then, the cells were incubated in DMEM/F12-10% fetal bovine serum with 10 μmol/L solution of EdU for...
Mass Spectrometric Analysis of 5-HETE and LTB₄

Macrophage production of 5(S)-HETE and LTB₄ was measured by mass spectrometry. Briefly, 1 mL of cell culture media was spiked with stable isotope-labeled internal standards: d₅-LTB₄ and d₅-5(S)-HETE (Cayman Chemical). Samples were acidified with formic acid, extracted with ethyl acetate, dried, and stored in 80 µL acetonic tritile until analysis, when 120 µL water was added. A TSQ Quantum Ultra mass spectrometer interfaced with a heated electrospray probe and an Accela UHPLC solvent delivery system (Thermo Scientific) were used with Hypersil Gold 200x2.1 mm with 1.9 µ particle size columns (Thermo Scientific). The mobile phase was generated from (A) water and (B) acetonitrile: methanol (95:5), each containing 0.005% acetic acid and adjusted to pH 5.7 with ammonium hydroxide. The gradient consisted of a 2 minutes isocratic segment at 40% B followed by a linear increase to 50% B at 3 minutes, then a linear increase to 70% B at 20 minutes. After each sample, the column was washed for 2 minutes with 100% B and equilibrated at 40% B for 10 minutes. The flow rate was 350 µL/min. Transitions were monitored at m/z 319→301 and m/z 327→309, collision energy 12 v, for 5(S)-HETE and d₅-5-HETE, and m/z 335→195 and 339→197, collision energy 16 v, for LTB₄ and d₅-LTB₄. Retention times were ≈7.7 minutes for LTB₄ and 15.2 minutes for 5(S)-HETE. Quantitation was by peak area ratios and normalized by media protein concentration.

Boyden Chamber Assay

Aliquots of 10,000 primary VSMCs were added to the top wells of Costar Transwell modified Boyden chambers (6.5-mm-diameter tissue culture-treated polycarbonate membranes containing 8-µm pores, Corning, NY) and grow confluent. Cells were primed with tumor necrosis factor-α (20 ng/mL, Sigma) for 24 hours in RPMI-1620 medium (Sigma) with 2% serum. Culture media from stimulated peritoneal macrophages were then added in the bottom wells. In other studies, medium containing exogenous LTB₄ (100 nmol/L, Cayman Chemical, Ann Arbor, MI) and LTD₄ (80 nmol/L, Cayman Chemical) were added into the bottom wells. Six replicates were used for each condition. After 12 hours, cells remained in the top wells were scraped off using cotton swabs from 3 replicates of each condition, leaving the other 3 untouched serving as cell loading controls. All remaining cells were stained with 0.1% crystal violet (Sigma) for 20 minutes. Stained cells in all 6 replicates were washed off by 1% deoxycholic acid (Sigma) solution and absorbance was measured at 595 nm.

ELISA for Determination of Cytokines

Supernatants collected from lipopolysaccharide (5 µg/mL) stimulated macrophage cells were measured for cytokines: tumor necrosis factor-α, interferleukin (IL)-6, IL-1 β, and IL-10 by Mouse ELISA Kits (Thermo Scientific, Madison WI) following manufacturer instructions.

Bone Marrow Transplantation

Bone marrow transplantation experiments were performed with methods similar to those previously described. Bone marrow cells were flushed out from the femurs and tibias of WT (CD45.1⁺) and FLAP KO (CD45.2⁺) mice with cell culture medium (RPMI 1640). Bone marrow cells were meshed and cleared of erythrocytes by treating with ammonium-chloride-potassium (ACK, Gibco) lysis buffer. Recipient mice, WT, and FLAP KO mice were lethally irradiated with two 525-rad doses spaced 3 hours apart (totally 1050 rads). Bone marrow cells were injected into recipient mouse through the tail vein (1x10⁶ cells per mouse). Repopulation of the immune system was monitored by flow cytometric analysis of the blood cells using fluorescent isothiocyanate antimouse 45.2 and PE mouse antimouse 45.1. In the chimeric mice, >95% of the myeloid cells were derived from donor bone marrow. Chimeric mice were subjected to femoral artery injury, and intimal hyperplasia was quantified as described above.

Statistical Analysis

Data were subject to ANOVA with post hoc pairwise comparisons as appropriate. Nonparametric approaches were employed when the variable distributions departed from normality or if the sample sizes were low (<6 per group). One-sided or 2-sided tests were performed, as appropriate. Tukey multiple comparison corrections were used for post hoc comparisons if the multiple-testing corrected ANOVA P values were significant at the 0.05 level.

Results

FLAP Expression Was Markedly Increased in Response to Vascular Injury

Lesional FLAP expression appeared increased at 2 hours, 1, 2, and 4 weeks after vascular injury (Figure 1A), and FLAP positive cells were colocalized with those staining for the leukocyte marker, CD45⁺ (Figure 1B). This increase in FLAP expression was most marked in the acute inflammatory phase 2 hours after injury and declined comparatively by 1 week as the lesions had become relatively depleted of inflammatory cells. During the early phase of the response (2 hours), the denuded luminal surface was covered with a monolayer of leukocytes positive for both CD45⁺ and FLAP (Figure 1B). Augmented FLAP expression was detectable in the neointimal lesions persisted at 1, 2, and 4 weeks after vascular injury (Figure 1A). However, insufficient antibody was available to quantitate expression precisely 2 and 4 weeks after injury.

FLAP Deletion Protects Against the Intimal Hyperplastic Response to Injury

FLAP KO mice and controls were subjected to femoral artery wire injury. Lesion formation in response to injury 4 weeks later was markedly reduced in FLAP KO mice (Figure 2A). The mean intimal and medial area, intima/media ratio, and
the percentage vascular stenosis did not differ between WT and FLAP KO at baseline (Figure 2B). The mean intimal area, intima/media ratio, and percentage vascular stenosis of FLAP KO mice were reduced on average by 57% (40436±6842 versus 17244±4066 μm²; *P<0.05), 66% (3.34±0.73 versus 1.15±0.2; *P<0.05) and 42% (57.3±10.1 versus 33.1±5.9%; *P<0.05), respectively, compared with WT mice at 4 weeks after wire injury (Figure 2B). The medial area, by contrast, did not differ between genotypes (15937±1953 versus 16334±873 μm²; *P>0.05) (Figure 2B).

FLAP Deficiency Results in Decreased VSMC Proliferation

The response to vascular injury is believed to involve proliferation of VSMCs and their migration into the neointima. FLAP KO mice displayed a significant decrease in VSMC proliferation as reflected by BrdU staining (Figure 3A). The BrdU index, calculated as a percentage of the ratio of BrdU positive nuclei over total number of cells in the neointima, was significantly depressed in FLAP KO mice compared with WT mice (87±3.3 versus 65.3±3.8%; *P<0.01) (Figure 3B). The absolute number of BrdU positive cells was also significantly reduced in the FLAP KOs (254±48 versus 73±14%; *P<0.01) (Figure 3B).

FLAP Deficiency Suppresses VSMC Phenotype Transition and Attenuates TNC Deposition While Preserving Endothelial Integrity

Although VSMCs continue to predominate in the intima, their loss of α-SMA after injury was attenuated in FLAP KO mice (Online Figure IA). Moreover, the transformation of VSMC from elongated spindle-shaped cells, aligned perpendicular to the blood vessel lumen to a more disordered orientation and morphology was prominent in WT mice after injury, but was suppressed in FLAP KO mice. The injury induced upregulation of medial and neointimal VCAM-1, and TNC was also markedly attenuated in VSMCs from FLAP KO mice (Online Figure IB and 1D). Despite its effects on VSMC proliferation, FLAP deficiency did not affect endothelial integrity, as reflected by staining with an antibody directed against Von Willebrand factor (Online Figure IC), or endothelial function, as assessed by measurement of isometric tension in aortic rings (Online Figure IIA and IIB). Endothelium-dependent relaxation in response to either acetylcholine or sodium nitroprusside was not different between WT and FLAP KO mice. Moreover, there was no significant difference in systolic and diastolic blood pressure between WT and FLAP KO mice (Online Figure IIIA and IIB).

Figure 2. 5-lipoxygenase activating protein (FLAP) deficiency is associated with a decreased intimal hyperplastic response to injury. A, Hematoxylin eosin staining of representative sections of mice femoral arteries 28 days after wire injury, from wild-type (WT) (n=6) or FLAP knockout (FLAP KO) mice (n=8). Solid arrowheads: internal elastic lamina; Open arrowheads, external elastic lamina, defining the borders of intima and the media. B, Quantification of intimal and medial areas. The ratio of intima to media is shown. Measurements were taken at baseline and 4 weeks after wire injury in both WT and FLAP KO mice. *P<0.05 *P<0.01, WT versus FLAP KO. Scale bar, 50 μm.
FLAP Deficiency Decreased Macrophage LT and Proinflammatory Cytokine Production

FLAP deficiency disrupted LT synthesis as measured by 5-HETE, LTB4, LTC4, LTD4, and LTE4 production in peritoneal macrophages (Figure 4A). Lipopolysaccharide induced macrophage generation of tumor necrosis factor-α, and IL-6 was also attenuated by FLAP deletion (Figure 4B). IL-1β and IL-10 levels were not altered by FLAP deficiency (Online Figure IV).

Effect of FLAP Deficiency on VSMC Migration and Proliferation

Culture medium derived from FLAP KO macrophages was considerably less effective than that from WT VSMCs in inducing VSMC migration in a Boyden Chamber assay (75.9% versus 40.1%, respectively, \( P < 0.05 \)). Replacing the upper chamber with exogenous LTB4, but not LTD4, restores VSMC migration (Figure 4C).

Exogenous LTB4 and LTD4, but not LTC4 or LTE4, stimulate WT VSMC proliferation in a dose-dependent manner (Online Figure VA). We also observed a small decrease in the proliferative ability, as measured by EdU incorporation, of VSMCs from FLAP KOs compared with those from WT mice (49.7% versus 40.5% after 48 hours, \( P < 0.01 \) and 67.9% versus 45.7% at 72 hours \( P < 0.01 \), respectively) (Online Figure VB). Both LTB4 (10 nmol/L) and LTD4 (10 nmol/L) fully or partially restored this proliferation defect in FLAP KO VSMCs (Online Figure VA; \( P < 0.001 \)).

Bone Marrow Cell-Derived FLAP Regulates Neointima Formation

Bone marrow was transplanted from WT or FLAP KO donors into WT or FLAP KO recipient mice. After repopulation, 95% of the bone marrow cells were shown to derive from donor mice (Online Figure VI). The intimal hyperplastic response was significantly reduced in FLAP KO→FLAP KO (FLAP KO donor into FLAP KO recipient) mice comparing with that in WT→WT (WT donor into WT recipient) mice, consistent with what we observed previously, when comparing global FLAP KO mice with WT controls (Figure 5). This intimal proliferative phenotype was rescued by restoring FLAP in bone marrow cells as observed in WT→FLAP KO (WT donor into FLAP KO recipient) mice. The intimal hyperplastic response was again attenuated if WT bone marrow cells were replaced by FLAP deficient myeloid cells, as observed in FLAP KO→WT (FLAP KO donor into WT recipient) mice (Figure 5). No difference in intimal formation was detected between WT→WT and WT→FLAP KO as well as between FLAP KO→FLAP KO and FLAP KO→WT mice. FLAP KO→WT mice displayed a significantly lower vascular proliferative response compared with WT→FLAP KO mice (Figure 5).

Discussion

Here, we report a striking impact of FLAP deletion on the proliferative response to vascular injury. Pharmacological inhibitors of either 5-LO or FLAP, both of which disrupt formation of LTB4, are being developed for use in asthma, a condition in which antagonists of receptors for the sulfido-peptide LTs, LTC4, LTD4, and LTE4, already have established clinical utility.23,24

The functional importance of LTs in cardiovascular disease is more controversial. Variations in genes relevant to LT biosynthesis, including FLAP, have cosegregated with cardiovascular events in humans,9–12 and expression of genes relevant to synthesis of LTB4 has been detected in human atherosclerotic plaques.3

Antagonism of BLT1 attenuates the intimal hyperplastic response to vascular injury in both rats and rabbits.7,25 However, trials of drugs that perturb the LT synthesis/response pathway are yet to be reported to influence cardiovascular outcomes in patients. Furthermore, attempts to study the impact of 5-LO deletion on atherogenesis in rodents have yielded conflicting results.26–28
Figure 4. 5-lipoxygenase activating protein (FLAP) deficiency attenuates vascular smooth muscle cell migration by disruption of leukotriene (LT) B₄ and proinflammatory cytokine synthesis. A, Peritoneal macrophages from wild-type (WT) or FLAP knockout (FLAP KO) mice were cultured and stimulated with A23187 (10 µmol/L) for 20 minutes or lipopolysaccharide (LPS) (5 µg/mL) overnight. 5-Hydroxyeicosatetraenoic acid (5HETE), LTB₄, LTC₄, LTD₄, and LTE₄ were measured using cell culture supernatant. **P<0.01; n=5. B, Macrophage cell culture supernatants were collected at 0 hour and 12 hours after LPS stimulation. Tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 levels were measured by ELISA. *P<0.05, n=5–10. C, Vascular smooth muscle cells (VSMCs) isolated from the aortas of WT were placed in modified Boyden chambers and chemotaxis assessed to stimulated peritoneal macrophage medium harvested from either WT or FLAP KO mice as indicated. As noted, LTB₄ or LTD₄ are added into the lower wells. *P<0.05, **P<0.01, n=3.
Although FLAP inhibition does not influence atherogenesis induced in hyperlipidemic mice with a high fat diet, it does have an impact when atherogenesis is accelerated in such models by additional manipulation like cyclooxygenase-2 deletion or T cell activation. Thus, although it may be unimportant as a phenotypic modulator when atherosclerosis is accelerated purely by dyslipidemia, additional proinflammatory manipulations appear to render mice susceptible to a contribution from FLAP-dependent pathways. It is always difficult to extrapolate the relative importance of a particular pathway from experiments in mice to the human condition. The clear impact of FLAP disruption here does not preclude a similar phenotypic response to disruption of an apparently unconnected biological pathway. However, such data as ours do frame hypotheses which can then be addressed in clinical trials where the issue of potential functional redundancy will become apparent.

After vascular injury, platelets and leukocytes are activated and adhere to the damaged vessel wall. Activated leukocytes release LTs, which may induce leukocyte chemotaxis, vasoconstriction, and an increase in vascular permeability. Infiltrated leukocytes persist in the vessel wall long after experimental balloon induced vascular injury. Release of LTB4 by activated leukocytes would be presumed to promote further recruitment of inflammatory cells, production of proinflammatory cytokines, and activation of VSMCs. Mature, fully differentiated contractile VSMCs express 4 actin isoforms, the most abundant of which is α-SMA. VSMCs modulate their phenotypes in response to injury coincident with downregulation of contractile proteins, such as α-SMA, and an increase of VCAM-1 expression. They gain the ability to proliferate and to upregulate extracellular matrix proteins, including TNC, a large oligomeric extracellular matrix glycoprotein, that plays an important role during the early cellular events of restenosis and provides a milieu that facilitates cell migration and proliferation of VSMC.

Here, we show that FLAP deletion disrupts several elements of this process, restraining the intimal proliferation consequent to vascular injury. First, detection of FLAP is consistent with its involvement in the early margination of CD45+ leukocytes. Second, FLAP is fundamental to the capacity of lesional leukocytes to elaborate both LTB4 and the proinflammatory cytokines, tumor necrosis factor-α, and IL-6. Deletion of FLAP impairs these properties and LTB4 rescues the phenotype. Third, FLAP deletion restrains both the phenotypic transition of VSMCs from a contractile to a more synthetic phenotype and the upregulation of TN-C in response to injury. TN-C is upregulated during neointimal hyperplasia and is associated with the synthetic proliferative phenotype of VSMCs after vascular injury, in pulmonary hypertension, and after vascular engraftment. It provides a scaffold along which proliferating VSMCs can migrate to form the neointima. Thus, these effects are mechanistically congruent with the marked effect of FLAP deletion on both VSMC migration, evoked either by lipopolysaccharide-stimulated macrophage culture media or by authentic LTB4 and on the intimal proliferative response to femoral vascular injury in vivo. Finally, we provide evidence from transplantation experiments that implicate myeloid cell FLAP as the primary influence on VSMC migration and proliferation. Transplantation of FLAP deficient myeloid cells recapitulates the impact of global FLAP deletion on the response to vascular injury, whereas delivery of FLAP-replete myeloid cells rescues the impact of global FLAP deletion in this model.

Although myeloid FLAP predominates, these results do not exclude a minor contribution from FLAP deficiency in other cells to the phenotype observed in FLAP KO mice. For example, we noticed a small decrease in cycling of FLAP KO cells relative to WT (Online Figure VB), which could be attributable...
to trace amounts of FLAP in VSMCs as detected by reverse transcriptase-polymerase chain reaction (Online Figure VC), albeit that we failed to detect FLAP protein. Alternatively, it may be a compensatory effect. 5-LO has been detected in human pulmonary artery endothelial cells, human VSMCs, and cultured mouse VSMCs.

Although we find minimal expression of FLAP message in VSMCs and are unable to detect FLAP protein, deletion of FLAP reduces VSMC proliferation (Figure 3A and 3B). This could contribute to the reduction of the neointimal hyperplasia and the intima/media ratio observed in FLAP KO mice after vascular injury. In summary, we have shown that deletion of myeloid cell FLAP markedly attenuates the proliferative response to vascular injury, whereas preserving endothelial integrity. Disruption of the LT synthesis/response pathway consequent to FLAP deletion restrains several elements of the response to injury, including CD45 positive leukocyte accumulation, VSMC phenotype transition, TNC expression and macrophage polarization, and induction of VSMC migration. FLAP deletion protects against the intimal hyperplastic response to injury predominantly by disrupting LT synthesis in myeloid cells and, thereby, depressing local inflammation and VSMC migration and proliferation.

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Disclosures
None.

References

**Novelty and Significance**

**What Is Known?**

- 5-lipoxygenase activating protein (FLAP) is required for enzymatic metabolism of arachidonic acid to leukotriene (LT) B$_2$ by lipoxygenase.
- LT$_B$ promotes cellular migration and proliferation in vitro, whereas downstream products, sulfidopeptide LTs, modulate vascular tone.
- Elements of the 5-lipoxygenase pathway are expressed in human atherosclerotic plaque.

**What New Information Does This Article Contribute?**

- Deletion of FLAP attenuates vascular smooth muscle cell phenotypic modulation, intimal migration, and proliferation in response to vascular injury.
- This phenotype reflects depletion of FLAP-dependent LT$_B$ formation by myeloid cells.
- Local delivery of macrophage-targeted FLAP inhibitors may attenuate restenosis after angioplasty.

Although LTs have been clearly implicated in asthma, their importance as drug targets in cardiovascular disease is unclear.
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In the article by Yu et al. “Myeloid Cell 5-Lipoxygenase Activating Protein Modulates the Response to Vascular Injury,” that appeared in the February 1, 2013 issue of the journal (Circ Res. 2013;112:432–440. DOI: 10.1161/CIRCRESAHA.112.300755), a correction was needed.

An author’s name was spelled incorrectly. The correct name is Gavin Landesberg.

The authors apologize for the error.

This correction has been made to the current online version of the article, which is available at http://circres.ahajournals.org/content/112/3/432.full
Methods

Isometric tension measurement

Concentration-response studies of vasodilation stimulated by acetylcholine (Ach) and sodium nitroprusside (SNP) were performed as described previously (1). Briefly, thoracic aortas were cut into rings and mounted in a myograph. Resting tension was set to 5 mN. After equilibration (at least 60 minutes at 37°C), rings were pre-contracted with 3x10^{-7} mol/L phenylephrine (PE), which corresponded to 50% maximum PE stimulation in both WT and FLAP KO mice (data not shown), and relaxed with 1x10^{-9} to 1x10^{-6} mol/L Ach or with 1x10^{-10} to 1x10^{-5} mol/L SNP at 37°C in bubbled (95% O2/5% CO2) Krebs-Henseleit (KH) buffer. At the beginning of the experiment, potassium chloride (100 mM) was used to induce maximal contraction. Each experiment was performed in parallel with 3 aortic rings derived from WT and FLAP KO mice.

Blood pressure measurement

Systolic and diastolic blood pressure were measured in conscious mice (age- and sex-matched) by using a computerized noninvasive tail-cuff system (Visitech Systems, Apex, NC) as described elsewhere (2).

References


Supplemental Figures Legend

Figure I FLAP deficiency suppresses VSMC phenotype transition and attenuates TNC deposition while preserving endothelium integrity. Representative staining and quantification of anti α-SMC actin (A), anti VCAM-1 (B) and anti VWF staining (C) of mice femoral arteries 4 weeks after injury, from WT (n=3) or FLAP KO mice (n=3). D, Representative anti TNC staining of femoral arteries from WT and FLAP KO mice obtained 4 weeks after injury.
Figure II Endothelium function assessment. Endothelium and vascular smooth muscle cell-mediated vascular relaxation were assessed by isometric tension measurement of aorta rings. Acetylcholine (A) and sodium nitroprusside (B)-induced relaxation of phenylephrine preconstricted thoracic aortas were not different among WT and FLAP KO mice (n=3).

Figure III Systolic and diastolic blood pressure. Systolic (A) and diastolic blood pressure (B) were recorded by tail cuff in WT and FLAP KO mice on LDLR background (n=9-10).

Figure IV FLAP deficiency did not alter macrophage generation of IL-1β and IL-10. Macrophage cell culture supernatants were collected at baseline and 12 hours after LPS stimulation. IL-1β and IL-10 levels were measured by ELISA, n=4-5.

Figure V Leukotrienes stimulate VSMC proliferation. A, Exogenous authentic LTB₄, LTC₄, LTD₄ or LTE₄ were added at the concentrations indicated and Edu incorporation was measured (* P<0.05, **P<0.001, n=3). B, EdU incorporation of cultured aorta VSMCs from either WT or FLAP KO mice was measured after 24, 48 hours and 72 hours incubation (**P<0.01, n=4). C, FLAP expression was measured in VSMCs by RT-PCR (n=2).

Figure VI Repopulation of bone marrow cells. Repopulation of the immune system was monitored by flow cytometric analysis of the blood cells using FITC anti-mouse 45.2 and PE mouse anti-mouse 45.1.
Supplemental Figure I

A

WT

FLAP KO

α-SMC actin

WT

FLAP KO

α-SMC actin in positive area %
C

WT

FLAP KO

D

injured non-injured

WT

FLAP KO
Supplemental Figure II

A

% Relaxation

Log [ACh] (M)

B

% Relaxation

Log [SNP] (M)
Supplemental Figure III

A

Systolic blood pressure (mmHg)

B

Diastolic blood pressure (mmHg)
Supplemental Figure IV

![Graph showing IL-1b and IL-10 levels with WT and FLAP KO conditions at 0h and 12h after LPS stimulation.](image-url)
Supplemental Figure V

A

![Graphs showing EdU positive nuclei percentage for different treatments.](image)

B

![Graph showing EdU positive nuclei percentage over time.](image)

C

![Graph showing FLAP and 10% iSS mRNA levels.](image)
Supplemental Figure VI

CD45.1

WT -> WT

3.14e-3

97.7

FLAP KO -> FLAP KO

98.4

0.01

CD45.2

WT -> FLAP KO

1.02

97.7

FLAP KO -> WT

97.5

1.29