Role of the Low-Affinity Leukotriene B₄ Receptor BLT2 in VEGF-Induced Angiogenesis

Geun-Young Kim, Jin-Wook Lee, Sung-Hoon Cho, Ji-Min Seo, Jae-Hong Kim

Objective—The leukotriene B₄ (LTB₄) receptor BLT2 is expressed in endothelium, but no clear physiological function for it has yet been identified, especially in vascular angiogenesis. The purpose of this study is to characterize the potential function of BLT2 in vascular endothelial growth factor (VEGF)-induced angiogenesis.

Methods and Results—VEGF significantly upregulates BLT2 expression in human umbilical vein endothelial cells (HUVECs), and BLT2 knockdown by siRNA or inhibition of BLT2 by a specific BLT2 antagonist LY255283 attenuates VEGF-induced angiogenesis, which was determined by its effect on the formation of tube-like structures and on transmigration. The role of BLT2 in VEGF-induced angiogenesis was more evident in vivo, where BLT2 inhibition by LY255283 almost completely blocked VEGF-induced vessel formation in Matrigel-plug assays. In addition, we found that VEGF upregulates synthesis of the BLT2 ligand, 12(S)-hydroxyeicosatetraenoic acid (HETE). siRNA knockdown of 12-lipoxygenase (12-LO) expression attenuates VEGF-induced angiogenesis in HUVECs, and the addition of 12(S)-HETE to the 12-LO knockdown-HUVECs restores VEGF-induced angiogenesis. The activation of BLT2 itself by either 12(S)-HETE or LTB₄ evoked significant angiogenic phenotypes, both in vitro and in vivo.

Conclusion—Our findings indicate that BLT2 plays an essential role in mediating VEGF-induced angiogenesis. (Arterioscler Thromb Vasc Biol. 2009;29:915-920.)

Key Words: angiogenesis ■ VEGF ■ BLT2 ■ 12(S)-HETE ■ 12-lipoxygenase
induced angiogenesis. Our findings indicate that BLT2 expression is highly induced by VEGF and that the induced BLT2 plays an essential role in mediating VEGF-induced angiogenesis, which suggests a potential interplay among VEGF, BLT2, and BLT2 ligands in vascular angiogenesis.

Methods
All of the experiments were performed using human umbilical vein endothelial cells (HUVECs) and FVB mice. All experimental animals used in this study were treated according to guidelines approved by the Institutional Animal Care and Use Committee of Korea University. The detailed descriptions of the methods that were used in this study are provided in the supplemental materials (available online at http://atvb.ahajournals.org).

Results
A BLT2-Linked Pathway Is Necessary for VEGF-Induced Angiogenesis
To investigate the possible role of the BLTs in VEGF-induced angiogenesis, we initially used real-time PCR, semi-quantitative RT-PCR, and FACS analysis to examine the effect of VEGF on BLT expression in HUVECs. Incubation of the cells with VEGF (20 ng/mL) led to an upregulation of both BLT1 and BLT2 mRNA (Figure 1A and 1B) and a corresponding increase in the expression of the BLT proteins (Figure 1C).

VEGF is known to induce angiogenesis both in vivo and in vitro. Therefore, we next used Matrigel-plug assays to determine the extent to which the observed upregulation of BLT expression is necessary for VEGF-induced angiogenesis in vivo. When VEGF and growth factor–reduced Matrigel were subcutaneously injected into mice, there was a significant induction of new blood vessel formation over the course of 7 days. This was confirmed by positive staining for vascular endothelial cadherin (VE-Cadherin), which is a specific endothelial marker (Figure 2A). Notably, simultaneous injection of LY255283, which is a specific BLT2 antagonist, dramatically reduced blood vessel formation, whereas simultaneous injection of U75302, which is a BLT1 antagonist, had little effect (Figure 2A). We also examined the effects of these BLT inhibitors on the formation of VEGF-induced tube-like structures in vitro. When HUVECs were plated on Matrigel in the presence of VEGF, networks of tube-like structures were formed. Consistent with the in vivo findings, this effect was diminished by pretreatment with LY255283 but not pretreatment with U75302 (supplemental Figure IA). We used Transwell chambers to test the effect of the BLT inhibitors on VEGF-induced transmigration and found that pretreatment with LY255283, but not U75302, significantly reduced VEGF-induced transmigration (supplemental Figure IB). These observations were also confirmed by siRNA knockdown of BLT2. When BLT2 siRNA (50 nmol/L) was introduced into HUVECs, the level of BLT2 mRNA was specifically reduced (Figure 2B). This knockdown of BLT2 expression reduced both the formation of VEGF (20 ng/mL)-induced tube-like structures and the transmigration (Figure 2C and 2D). Based on these results, we conclude that VEGF-induced angiogenesis is considerably mediated via a BLT2-linked pathway.

12(S)-HETE Is Involved in Mediating VEGF-Induced Angiogenesis
We then wanted to determine whether VEGF could induce the synthesis of the 2 known BLT2 ligands, 12(S)-HETE and LTB4. Platelet-type 12-LO had been previously reported to be expressed in HUVECs. In the present study, we observed that VEGF (20 ng/mL) upregulated both the expression of platelet-type 12-LO (data not shown) and the synthesis of 12(S)-HETE (Figure 3A). In contrast, VEGF had no effect on LTB4 synthesis in HUVECs. In addition, VEGF did not induce the expression of 5-LO or 5-LO activating protein (FLAP), which suggests that LTB4 is not involved in mediating VEGF signaling, at least in vitro (supplemental Figure II).
To further analyze the role of 12(S)-HETE in VEGF signaling, we investigated the effect of 12-LO inhibition on VEGF-induced angiogenesis by siRNA (100 nmol/L) knockdown of 12-LO in HUVECs (Figure 3B). The knockdown of 12-LO expression markedly suppressed both the formation of VEGF (20 ng/mL)-induced tube-like structures and the wound healing migration (Figure 3C and 3D). The addition of 12(S)-HETE (20 nmol/L) to the 12-LO siRNA transfected HUVECs significantly restored both effects. In contrast, pretreatment with LY255283 (10 μmol/L) diminished these recovery effects (Figure 3C and 3D). The 12-LO inhibitor baicalein25 had similar inhibitory effects, and the addition of 12(S)-HETE again clearly restored VEGF-induced angiogenesis (supplemental Figure III). Thus, 12-LO, its product 12(S)-HETE, and its involvement in the BLT2 pathway appear to play critical roles in mediating VEGF-induced angiogenesis.

Activation of the BLT2 Pathway Is Sufficient to Induce Angiogenesis

We then wanted to determine whether stimulation of BLT2 alone, or by treatment with LTB4 or 12(S)-HETE, could induce angiogenesis. Because HUVECs express only low levels of BLT2 under basal conditions,19 we induced BLT2 expression by pretreating HUVECs with VEGF for 9 hours (Figure 1C) and then assessed transmigration and the formation of tube-like structures under those conditions. When cells were stimulated with LTB4 (300 nmol/L) or 12(S)-HETE (100 nmol/L), transmigration and the formation of tube-like structures were greatly increased in the VEGF-pretreated group, although they were also increased somewhat in control cells pretreated with vehicle (PBS). Once again, LY255283, but not U75302, significantly reduced these effects (supplemental Figure IV), which suggested a specific mediatory role for BLT2. Similarly, BLT2 knockdown by siRNA caused significantly reduced angiogenesis in the VEGF-pretreated group (Figure 4A and 4B). We also enhanced BLT2 expression by transfecting HUVECs with a BLT2 expression plasmid using Lipofectamine reagent (Figure 4C) and carried out the same assays using the transfectants. We found that the activation of BLT2 by LTB4 (300 nmol/L) or 12(S)-HETE (100 nmol/L) greatly enhanced angiogenic phenotypes in BLT2 transfectants when compared with HUVECs transfected with the empty vector (Figure 4D and 4E). This indicates that BLT2 stimulation is sufficient to cause angiogenesis and that BLT2 is the receptor responsible for angiogenesis induced by 12(S)-HETE or LTB4.

Enhanced Blood Vessel Formation in BLT2-Overexpressing Transgenic Mice

To further assess the role of BLT2 in angiogenesis, BLT2 transgenic (TG) mice were prepared using the pCAGGS-B2 vector. After confirming proper BLT2 gene insertion, semiquantitative RT-PCR was carried out to verify the presence of elevated levels of BLT2 mRNA in the TG mice (supplemental Figure V). We then subcutaneously injected LTB4 and 12(S)-HETE together with growth factor–reduced Matrigel into wild-type (WT) and BLT2 TG mice. After 7 days, new blood vessel formation was dramatically increased in the
BLT2 TG mice compared with the WT mice (Figure 5). Notably, simultaneous injection of the BLT2 antagonist, LY255283, almost completely blocked new blood vessel formation in the BLT2 TG mice as well as in WT mice (Figure 5). This further demonstrated the critical role of BLT2 in angiogenesis.

Discussion

In the present study, we demonstrated that the induction of BLT2 expression and the stimulation of a BLT2-linked pathway are critical for VEGF-induced angiogenesis. VEGF stimulated the expression of BLT2 and BLT1 (Figure 1), and the inhibition of BLT2, but not BLT1, interfered with VEGF-induced angiogenesis in HUVECs (Figure 2). The role of BLT2 in VEGF-induced angiogenesis is more evident in vivo, where LY255283 almost completely suppressed VEGF-induced vessel formation in a Matrigel-plug assay (Figure 2). Notably, simultaneous injection of the BLT2 antagonist, LY255283, 12(S)-HETE, and LTB4 significantly inhibited angiogenesis (Figure 5). This further demonstrated the critical role of BLT2 in angiogenesis.

Throughout these experiments, the contribution of BLT2 to VEGF-induced angiogenesis has been more pronounced in vivo than in vitro (Figure 2 and supplemental Figure I), which suggests the former is much more dependent on the BLT2 pathway. We do not know the reasons for the difference between the in vitro and in vivo findings, but we suspect that the BLT2-linked pathway might be more effectively stimulated in vivo, perhaps because of the augmented production of 12(S)-HETE or LTB4 in the angiogenic area. In fact, the levels of 12(S)-HETE and LTB4 are reportedly increased at sites of tumor angiogenesis, and many tumor cells overexpress 12-LO, 5-LO or LTA4 hydrolase. In addition, we found that various types of tumor tissues express higher levels of BLT2 than their corresponding normal tissues (supplemental Figure VI). Therefore, those tumors are likely to produce elevated levels of the BLT2 ligands. The synthesis of LTB4 and 12(S)-HETE is somehow induced in angiogenesis via the BLT2-linked cascade in endothelium. We speculate that the contributory role of macrophages is likely to be minimal (data not shown). Given that BLT2 activation is suggested to occur downstream of VEGF, VEGF may also recruit leukocytes around the VEGF-secreting angiogenic area. As expected, there was an extensive accumulation of neutrophils within the plugs that were treated with VEGF, and there were no neutrophils within the plugs that were treated with PBS (supplemental Figure VIII). These observations are consistent with the finding that VEGF recruits myeloid progenitor cells, monocytes, and neutrophils to regions of ongoing angiogenesis. These cells likely produce elevated levels of the BLT2 ligands, which suggests the former is much more dependent on the BLT2 pathway. We do not know the reasons for the difference between the in vitro and in vivo findings, but we suspect that the BLT2-linked pathway might be more effectively stimulated in vivo, perhaps because of the augmented production of 12(S)-HETE or LTB4 in the angiogenic area.
Angiogenesis

Figure 6. Scheme for the involvement of BLT2 and its ligands in VEGF-induced angiogenesis.

culture system. We detected an approximately 1.4-fold increase in the synthesis of 12(S)-HETE in HUVECs stimulated with VEGF (Figure 3A), but we did not detect any accumulation of LTB4 (supplemental Figure IIA). HUVECs do not express FLAP, and VEGF did not induce its expression (supplemental Figure IIB and IIC). Therefore the inhibition of VEGF-induced angiogenesis by BLT2 siRNA or LY255283 in vitro is likely attributable to a block in the interaction of 12(S)-HETE with BLT2. In the same way, 12-LO siRNA or baicalein caused an inhibition of VEGF-induced angiogenesis, and the addition of supplemental 12(S)-HETE abolished their inhibitory effect on angiogenic responses (Figure 3C and 3D and supplemental Figure III).

The synthesis of 12(S)-HETE by 12-LO and its autocrine/paracrine action via BLT2 may therefore be implicated in VEGF-induced angiogenesis in vitro. VEGF has been reported to stimulate platelet-type 12-LO activity and induce the synthesis of 12(S)-HETE in an RV-ECT endothelial cell line, which was derived from rat brain microvessels, in the presence of exogenously added AA.24 In addition, the overexpression of 12-LO has been shown to increase the accumulation of VEGF in prostate cancer cells.25 There appears to be cross-talk between VEGF and 12-LO, such that each triggers the induction of the other. We could not, however, detect the induction of VEGF in response to treatment with 12(S)-HETE in HUVECs (data not shown).

The mechanism by which BLT2 stimulates angiogenesis remains to be further characterized, but our findings suggest that inhibition of the ERKs specifically interferes with BLT2-induced angiogenesis, such as by inhibiting HUVECs proliferation (supplemental Figure IX). This implies that ERKs occur downstream of BLT2 during angiogenesis. We have created a model that depicts how BLT2 and its ligands form an integrated pathway that affects VEGF signaling (Figure 6).

In this model, VEGF-activated endothelial cells upregulate BLT2 expression and synthesize its ligand, 12(S)-HETE. This process would enhance angiogenesis through an autocrine/paracrine mechanism. In addition, infiltrating leukocytes may produce more 12(S)-HETE or LTB4, which would further stimulate the BLT2 cascade and potentiate angiogenesis. Based on these data, we speculate that there is a self-reinforcing signaling network in the angiogenic microenvironment that involves VEGF-secreting cells (e.g., tumor cells), BLT2-overexpressing endothelium, and leukocyte-derived expression of LTB4/12(S)-HETE, and together they lead to fully activated angiogenesis. In summary, our findings point to BLT2 as a key mediator of VEGF signaling, and contribute to a better understanding of the mechanism responsible for vascular angiogenesis.

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Disclosures

None.

References


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Role of the low-affinity leukotriene B₄ receptor BLT2
in VEGF-induced angiogenesis

Geun-Young Kim, Jin-Wook Lee, Sung-Hoon Cho, Ji-Min Seo, and Jae-Hong Kim†

College of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea

†Corresponding author:

College of Life Sciences and Biotechnology, Korea University

5-1 Anam-dong, Sungbuk-gu, Seoul, 136-701, Korea

Tel: 82-2-3290-3452 Fax: 82-2-923-0851 E-mail: jhongkim@korea.ac.kr
Methods

Cell culture and chemicals

Human umbilical vein endothelial cells (HUVECs) were purchased from the BioBud Company (Seoul, Korea) and used at passages 5 to 6. The cells were grown in M199 medium (GIBCO, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS, Hyclone, Logan, UT), 100 units/mL penicillin (GIBCO), 100 μg/mL streptomycin (GIBCO), 25 mmol/L HEPES (Sigma, St Louis, MO), 10 units/mL heparin (Sigma), 25 mmol/L sodium bicarbonate (Sigma) and 20 ng/mL basic fibroblast growth factor (bFGF, BioBud Company). The BLT2 antagonist LY255283,1,3 LTB4 and 12(S)-HETE were all purchased from the Cayman Chemical Company (Ann Arbor, MI). The BLT1 antagonist U753021,2,4 was purchased from Biomol (Plymouth Meeting, PA). VEGF was purchased from BD Biosciences Pharmingen (San Diego, CA). PD98059 was purchased from Calbiochem (San Diego, CA).

Fluorescence-activated cell sorting (FACS) analysis

HUVECs were incubated for 4 hours in M199 medium containing 5% FBS and then stimulated with VEGF. After 9 hours, the cells were detached by treatment with trypsin, washed with PBS, fixed and permeabilized with Cytofix/Cutoperm™ Fixation/Permeabilization Kit (BD Biosciences) for 20 minutes at 4°C. The cells were then incubated with primary antibodies against BLT1 or BLT2 (Cayman Chemical Company) for 1 hour. Cells were washed three times with PBS and incubated with a FITC-conjugated secondary anti-rabbit IgG (1:200). The cells were analyzed with a FACSCalibur flow cytometer using Cell Quest software (BD Biosciences). Background fluorescence was obtained by measuring the fluorescence of cells that were incubated with an isotype-matched control antibody (rabbit IgG) and FITC-conjugated secondary anti-rabbit IgG. The specific mean fluorescence intensity (MFI) was calculated by
subtracting the MFI of the cells that were incubated with the isotype-matched control antibody from that of the cells that were incubated with antibodies against BLT1 or BLT2.

**Measurement of 12(S)-HETE**

HUVECs were seeded onto gelatin-coated 6-well plates and grown to 90% confluence. The cells were incubated for 4 hours in M199 medium containing 1% FBS and then treated with VEGF for 30 minutes. The plates were washed twice with 1 mL of cold PBS, mixed with 1 mL of 100% ethanol and left for 30 minutes at 4°C. The resulting precipitate was removed by centrifugation at 10,000 rpm for 30 minutes at 4°C. The ethanol supernatant that contained 12(S)-HETE was collected, evaporated under vacuum and reconstituted in the assay buffer that was supplied with the 12(S)-HETE ELISA kit (Assay Designs, Ann Arbor, MI). 12(S)-HETE was then measured according to the manufacturer’s instructions. According to the 12(S)-HETE ELISA kit manual, the 12(S)-HETE antibody showed a 100% cross-reactivity with 12(S)-HETE, a 2.5% cross-reactivity with 12(R)-HETE and low cross-reactivity (less than 0.3%) with 5(S)-HETE, 15-HETE and other eicosanoids.

**Preparation and identification of BLT2 transgenic (TG) mice**

The pCAGGS-B2 vector carries a strong and constitutive expression cassette for gene expression under the control of the CAG promoter (CMV immediate early enhancer/chicken-β-actin promoter). The use of the CAG promoter leads to a ubiquitous expression profile in eukaryotes. For example, the pCAGGS vector was successfully used to express GFP in all tissues of transgenic mice, with the exception of hair and red blood cells. In addition, the pCAGGS vector was recently used to drive transgene expression during the differentiation of murine embryonic stem cells into vascular progenitor cells such as Flk-1+ mesodermal cells, endothelial cells and smooth muscle cells. The complete rat BLT2 gene was subcloned from
pcDNA3-HA-rat BLT2 and inserted into the pCAGGS-B2 vector, followed by restriction enzyme digestion and purification. The construct was then injected into fertilized eggs, which were subsequently implanted in foster females. Potential TG founder mice were screened by PCR with two different primer sets using genomic DNA extracted from the tails of 3-week-old mice to detect transgene integration. Briefly, the mouse tail biopsy was placed into 200 μL of TES buffer (50 mmol/L Tris-Cl, pH 8.0, 50 mmol/L EDTA, 0.5% SDS) and digested with proteinase K at a final concentration of 200 μg/mL overnight at 55°C. Mouse genomic DNA was obtained after phenol extraction and ethanol precipitation, and PCR was performed with the two primer sets. The primers used to screen for BLT2 TG mice were 5'-GCGCAGGGACTTCTTTGTC-3' (forward), 5'-GCTCTAGAGCCTCTGCTAACC-3' (forward) and 5'-CCGATGGGTGGCACAATTGAC-3' (reverse). The PCR protocol entailed 35 cycles of denaturation at 96°C for 60 seconds, annealing at 52°C for 30 seconds, and elongation at 72°C for 120 seconds, followed by a final extension at 72°C for 10 minutes. The amplified PCR products (924 bp and 677 bp, respectively) were subjected to electrophoresis on a 1.0% agarose gel, after which the bands were visualized by ethidium bromide staining. The positive founder that was obtained was bred with female mice of the same strain (FVB). Potential positive litters (F1) were screened by PCR as described above. To verify the overexpression of BLT2, total RNA was isolated from 6-week-old BLT2 TG mice and age-matched controls using Easy Blue™ (Intron Company, Sungnam, Korea). 1 μg of total RNA was reverse transcribed for 60 minutes at 42°C and amplified by PCR using primers for mouse BLT2 (forward, 5'-CAGCATGTACGCCAGCGTGC-3'; reverse, 5'-CGATGGCGCTCACCAGACC-3'). The PCR protocol entailed 28 cycles of denaturation at 95°C for 30 seconds, annealing at 69°C for 30 seconds, and elongation at 72°C for 45 seconds, followed by a final extension at 72°C for 10 minutes. The PCR products were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. The expected product size was 380 bp.
**Real-time PCR**

For real-time PCR, total RNA was purified from HUVECs using Easy Blue™ (Intron Company) and reverse-transcribed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). For the PCR reactions, LightCycler® 480 SYBR Green I Master (Roche, Germany) was used, and glyceraldehyde-3-phosphate (GAPDH) served as a reference gene for normalization. The primer sequences used were as follows: for BLT1,$^{12}$ 5'- CCTGAAAAGGATGCAGAAGC-3' (forward) and 5'- AAAAAGGGAGCAGTGAGCAA-3' (reverse); for BLT2,$^{12}$ 5'- CTTCTCATCGGGCATCACAG-3' (forward) and 5'- ATCCTTCTGGCCTACAGGT-3' (reverse); and for GAPDH,$^{13}$ 5'-ACGGATTTGGGTATTGATGG-3' (forward) and 5'- TGAATTTGGAGGATCTCGC-3' (reverse). The thermal cycling protocol entailed 45 cycles of denaturation at 95°C for 10 seconds, annealing at 57°C (for BLT1), 60°C (for BLT2) or 58°C (for GAPDH) for 20 seconds, and elongation at 72°C for 10 seconds (for BLT1 and BLT2) or 15 seconds (for GAPDH). Melt curves were analyzed to ensure amplification specificity of the PCR products.

**Transfection, RNA interference and semiquantitative RT-PCR**

The human *BLT2* expression plasmid and its control vector (pcDNA3) were introduced into HUVECs using Lipofectamine (Invitrogen), according to the manufacturer’s instructions. Human platelet-type 12-LO siRNA (Santa Cruz Biotechnology, Santa Cruz, CA), BLT2 siRNA (Bioneer, Daejeon, Korea) and scrambled siRNA (Santa Cruz Biotechnology) were introduced into HUVECs using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. The BLT2 siRNA sequence is as follows: sense 5'-CCACGCGAGUCAA CCUUCUGtt-3' and antisense 5'-CAGAAGGUGACUGCGUGGta-3'.$^{14}$ Forty-eight hours later, cells were harvested, and the levels of *12-LO* and *BLT2* were determined by
semiquantitative RT-PCR. Total RNA was extracted using Easy Blue™ (Intron Company), after which 2 μg of the extracted RNA was reverse-transcribed using M-MLV reverse transcriptase. For the semiquantitative analysis of transcripts, we first determined the optimal PCR conditions for linear amplification of GAPDH. The primers for BLT1 were 5'-TATGTCTGCGGAGTCAGCATGTACGC-3' (forward) and 5'-CCTGTAGCCGACGCCCTATGTCCG-3' (reverse). The PCR protocol for BLT1 entailed 30 cycles of denaturation at 94°C for 30 seconds, annealing at 67°C for 30 seconds, and elongation at 72°C for 30 seconds, followed by a final extension at 72°C for 10 minutes. The primers for BLT2 were 5'-TCTCATCGGGCATCACAGGT-3' (forward) and 5'-CCAAGCTCCACACCACGAAG-3' (reverse). The PCR protocol entailed 35 cycles of denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds, and elongation at 72°C for 30 seconds, followed by a final extension at 72°C for 10 minutes. To detect platelet-type 12-LO, nested RT-PCR was performed. The primers for platelet-type 12-LO were 5'-CCTTCCCGTGCTACCGCTGG-3' (first-run forward), 5'-TGGGGTTGGCACCATTGAGG-3' (first-run reverse), 5'-CCAGAAGCATCGAGAGAAGG-3' (nested PCR forward), and 5'-CAGGAGCTCAGGAGGGTGTA-3' (nested PCR reverse). The first-run PCR protocol entailed 25 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 60 seconds, followed by a final extension at 72°C for 10 minutes. The nested PCR protocol entailed 20 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, and elongation at 72°C for 30 seconds, followed by a final extension at 72°C for 10 minutes. The primers for FLAP were 5'-CTGCGTTTGCTGGACTGATG-3' (forward) and 5'-GGAGATGGTGGGTGGAGATCG-3' (reverse). The PCR protocol entailed 28 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, and elongation at 72°C for 30 seconds, followed by a final extension at 72°C for 10 minutes. The protocol for GAPDH entailed 20 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and elongation at 72°C for 30 seconds,
followed by a final extension at 72°C for 10 minutes. Amplified PCR products were subjected to electrophoresis on 1.5% agarose gels. The bands were then visualized by ethidium bromide staining and photographed with a Gel Doc 2000 Documentation system (Bio-Rad, Hercules, CA). The expected product sizes for $BLT1$, $BLT2$, platelet-type 12-LO, FLAP and GAPDH were 346 bp, 220 bp, 219 bp, 219 bp and 230 bp, respectively. The specificity of all of the primers was confirmed by sequencing the PCR products.

**In vitro wound healing migration assay**

Confluent HUVECs were incubated for 4 hours in M199 medium containing 5% FBS and then scratched with a universal blue tip (Axygen T-1000-B). The cells were then rinsed twice with PBS and incubated in fresh M199 medium containing 5% FBS. In some cases, inhibitors or antagonists were added 30 minutes before agonist stimulation. After 8 hours of stimulation, the cells were stained with hematoxylin and images were generated using a CKX41 microscope equipped with a DP71 digital camera (Olympus, Tokyo, Japan, 50×). The number of migrating cells was counted in three areas chosen at random.

**Transmigration assay**

Transmigration assays were performed using Transwell chambers (Corning Costar, Cambridge, MA) with 6.5-mm diameter polycarbonate filters (8-μm pore size), as previously described. Briefly, confluent HUVECs were incubated for 4 hours in M199 medium containing 5% FBS. The lower surfaces of the filters were coated with 10 μL of 1% gelatin for 1 hour at 37°C. Cells were trypsinized and suspended in M199 medium containing 1% FBS before being loaded into the upper chambers to a final concentration of $1 \times 10^5$ cells/well. The cells were then allowed to migrate to the lower side of the chambers, which contained LTB₄, 12(S)-HETE or VEGF. If called for, inhibitors or antagonists were applied to the cells in suspension for 30 minutes before
seeding. After incubation for 3 hours at 37°C in 5% CO₂, the filters were disassembled, and the upper surface of each filter was scraped free of cells by wiping with a cotton swab. Cells that had migrated to the underside of the filter were fixed for 1 minute in methanol, stained for 1 minute in hematoxylin and finally stained for 30 seconds in eosin. Cell migration was quantified by counting the cells on the lower side of the filter after they were photographed using a CKX41 microscope equipped with a DP71 digital camera (Olympus). Five fields were counted in each assay. Each sample was assayed in triplicate, and the assays were repeated three times.

**Tube-like structure formation assay**

Tube-like structure formation was assayed as previously described, with slight modifications. Briefly, 300 μL of growth factor-reduced Matrigel (BD Biosciences) was added to each 24-well plate and polymerized for 12 hours at 37°C. HUVECs that had been incubated in M199 medium containing 5% FBS for 4 hours were trypsinized and suspended in M199 medium containing 1% FBS. If called for, the cells were pre-treated with inhibitors or antagonists for 30 minutes before being seeded onto the Matrigel layer to a density of 5 × 10⁴ cells/well. Cells were then stimulated with LTB₄, 12(S)-HETE or VEGF. After 12 hours, five randomly selected areas were photographed using a CKX41 microscope equipped with a DP71 digital camera (Olympus), and tube lengths were measured and quantified using Image J software, which is the image processing program developed at the U.S. National Institutes of Health (NIH).

**In vivo Matrigel-plug assay**

The in vivo matrigel plug assay was performed as previously described, with slight modifications. Briefly, FVB wild-type mice or BLT2 TG mice (female, 8-10 week old) were subcutaneously injected with 400 μL of growth factor-reduced Matrigel from BD Biosciences that also contained heparin (20 units) and the agents to be tested. After 7 days, the mice were
killed, and the solidified Matrigel was excised, fixed in 10% formalin, embedded in paraffin, cut into 5-μm sections and stained with anti-VE-Cadherin antibody (BD Biosciences) using an IHC Select™ Complete Immunoperoxidase Secondary Detection System (Chemicon, Temecula, CA). Each stained Matrigel section was photographed using a BX51 microscope equipped with a DP71 digital camera (Olympus), and the area of VE-Cadherin-positive blood vessels was calculated using Image J software.

[^3H]Thymidine incorporation assay

HUVECs were seeded onto gelatin-coated 96-well plates to a density of 2 × 10^3 cells/well. After incubation for 30 hours in M199 medium supplemented with 20% FBS and 20 ng/mL bFGF, the cells were washed twice with PBS and incubated for 4 hours in M199 medium containing 5% FBS. In some cases, inhibitors or antagonists were added 30 minutes before stimulation with LTB₄ or 12(S)-HETE. [³H]Thymidine (2 μCi/mL) was added 14 hours after agonist stimulation and, after an additional 24 hours, the cells were harvested on a filtermat. The filtermat was dried and packaged in a sample bag. Finally, Betaplate scint (4 mL) was added to the filtermat, and the radioactivity was counted in a liquid scintillation counter (MicroBeta, Wallac/Perkin-Elmer, Boston, MA). [³H]Thymidine, filtermats, sample bags and Betaplate scint were all purchased from PerkinElmer Life Sciences (Waltham, MA).

Statistics

The results are presented as means ± SD. Analyses were performed with Student’s t test using SigmaPlot 8.0. Values of p<0.05 were considered significant. p<0.05 and p<0.01 are represented in the figures as * and **, respectively.
Supplemental Figure I. BLT2 antagonist inhibits VEGF-induced angiogenesis in vitro. (A) HUVECs were incubated in M199 medium containing 5% FBS for 4 hours; LY255283 (10 μmol/L) and U75302 (1 μmol/L) were added to cells suspended in M199 medium containing 1% FBS for 30 minutes. The cells were then used for tube-like structure formation assay by the stimulation of VEGF (20 ng/mL). After 12 hours, five randomly selected areas were photographed, and tube lengths were measured using Image J software. Data are expressed as the mean fold increase over that of the control ± SD (n = 3). Bars: 100 μm. (B) HUVECs were incubated in M199 medium containing 5% FBS for 4 hours, after which LY255283 (10 μmol/L) or U75302 (1 μmol/L) was added to cells suspended in M199 medium containing 1% FBS for 30 minutes. The cells were then used for the transmigration assay by the stimulation of VEGF (20 ng/mL). After 3 hours, cells that had transmigrated to the lower well were stained and counted. Data are expressed as the mean fold increase over that of the control ± SD (n = 3).

Supplemental Figure II. HUVECs do not synthesize LTB₄ in response to VEGF. (A) Once released by cPLA₂, arachidonic acid is converted to LTA₄ by 5-LO and 5-LO activating protein (FLAP), after which LTA₄ is converted to LTB₄ by LTA₄ hydrolase or to LTC₄ by LTC₄ synthase. Endothelial cells synthesize LTC₄ in response to LTA₄, which is largely released from polymorphonuclear leukocytes. To mimic this intercellular transfer, we added LTA₄ to HUVECs before treating them with VEGF. For this experiment, HUVECs were seeded onto gelatin-coated 6-well plates and grown to 90% confluence. For the LTB₄ assays, the cells were incubated for 4 hours in M199 medium containing 1% FBS and then treated with LTA₄ (10 nmol/L, Biomol) for 5 minutes. VEGF (20 ng/mL) was added 15 minutes prior to the addition of LTA₄. LTB₄ was measured using an LTB₄ ELISA kit (Amersham Pharmacia Biotech, Ltd,
UK), as previously described.\(^5\) (B) Cells were incubated for 4 hours in M199 medium containing 1% FBS and stimulated with VEGF (20 ng/mL) for the indicated times. Total RNA was then extracted, and semiquantitative RT-PCR was performed. (C) Cells were incubated for 4 hours in M199 medium containing 1% FBS and stimulated with VEGF (20 ng/mL) for the indicated times. The cells were harvested and western blotting was performed. Differentiated HL60 cells\(^{15}\) were used as positive controls for the level of FLAP.

**Supplemental Figure III. The addition of 12(S)-HETE restores VEGF-induced angiogenic responses in baicalein-treated HUVECs.** The 12-LO inhibitor baicalein was purchased from Biomol. Pretreatment with baicalein (25 \(\mu\)mol/L) for 30 minutes reduced the formation of VEGF-induced tube-like structures (A, bars: 100 \(\mu\)m) and wound healing migration (B, 50\(\times\)). When 12(S)-HETE (20 nmol/L) was added in the presence of baicalein and VEGF (20 ng/mL), the inhibitory effect of baicalein on VEGF-induced angiogenic responses was diminished.

**Supplemental Figure IV. BLT2 activation enhances angiogenic responses in HUVECs.** (A-B) HUVECs were incubated for 4 hours in M199 medium containing 5% FBS, after which either VEGF (20 ng/mL, right panel) or vehicle (PBS, left panel) was added for an additional 9 hours. The cells were washed with PBS, trypsinized, and assayed for transmigration (A) and the formation of tube-like structures (B) as described in Supplemental Figure I. All data are expressed as the mean fold increase over that of the control \(\pm\) SD \((n = 3)\).

**Supplemental Figure V. Generation of BLT2-overexpressing transgenic mice.** (A) Schematic of the DNA construct used to generate BLT2 TG mice (see Methods for details). The two sets of forward and reverse primers are indicated. (B) Identification of founders using PCR. Genomic DNA from a tail biopsy was amplified using the two indicated primer sets. The 677-bp
PCR product formed with primer set 1 and the 924-bp product formed with primer set 2 are shown. The DNA construct used to generate BLT2 TG mice was used as a template for the positive control. L: DNA ladder, W: WT mice, B: BLT2 TG mice, P: positive control. (C) Overexpression of BLT2 mRNA in various organs of BLT2 TG mice. Total RNA was isolated, reverse transcribed and amplified using specific primers for BLT2 and GAPDH. W: WT mice, B: BLT2 TG mice.

**Supplemental Figure VI. Overexpression of BLT2 in human tumors.** Sections of several human tumors and corresponding normal tissues were purchased from Pentagen Inc. (Seoul, Korea). They were deparaffinized with xylene, rehydrated with ethanol, treated with proteinase K (100 μg/mL) and incubated with blocking buffer for 30 minutes. Thereafter, the tissues were stained with anti-human BLT2 antibody (LifeSpan BioScience) and alkaline phosphatase substrate (Alkaline phosphatase substrate system, Chemicon). Each stained tissue section was photographed using a BX51 microscope equipped with a DP71 digital camera (Olympus). SCC: squamous cell carcinoma, DCC: ductal cell carcinoma. Bars: 20 μm.

**Supplemental Figure VII. Neutrophil accumulation in LTB₄ or 12(S)-HETE-treated Matrigel in vivo.** LTB₄ or 12(S)-HETE-containing Matrigel sections were stained for rat anti-mouse Gr-1 monoclonal antibody (BD Biosciences) using an IHC Select™ Complete Immunoperoxidase Secondary Detection System (Chemicon). Each image was recorded using a BX51 microscope equipped with a DP71 digital camera (Olympus). Positive immunoreactivity is shown in brown (arrowheads). The results shown are representative of three independent experiments. Bars: 50 μm.

**Supplemental Figure VIII. Neutrophil accumulation in VEGF-treated Matrigel in vivo.**
PBS or VEGF-containing Matrigel sections were stained for rat anti-mouse Gr-1 monoclonal antibody, as described in Supplemental Figure VII. Bars: 50 μm.

**Supplemental Figure IX. ERK activation is necessary for the LTB₄- and 12(S)-HETE-induced cell proliferation.** (A) HUVECs were incubated for 4 hours in M199 medium containing 1% FBS and then stimulated with LTB₄ (300 nmol/L) or 12(S)-HETE (100 nmol/L) for 10, 20 or 30 minutes. The cells were then harvested, and western blotting was performed in order to detect ERK phosphorylation. The results shown are representative of three independent experiments with similar results. (B) HUVECs were incubated with PD98059 (20 μmol/L), LY255283 (10 μmol/L) or U75302 (1 μmol/L) for 30 minutes prior to treatment with LTB₄ (300 nmol/L) or 12(S)-HETE (100 nmol/L), and proliferation assays were performed. Data are expressed as the mean fold increase over that of the control ± SD (n = 5).

**References**


21. Feinmark SJ, Cannon PJ. Endothelial cell leukotriene C4 synthesis results from
Kim et al. Supplemental Figure II

A

![Bar graph showing LTB4 levels.](image)

Control  | LTA4
---|---
PBS    | 0.5
VEGF   | 1.5

B

![Western blot showing FLAP and GAPDH expression.](image)

VEGF (hours): 0, 1, 3, 6
DHL50
FLAP
GAPDH

C

![Western blot showing FLAP and β-actin expression.](image)

VEGF (hours): 0, 5, 9, 12, 18, 24
DHL50
FLAP
β-actin
Kim et al. Supplemental Figure III

A

PBS  VEGF + DMSO + EtOH  VEGF + baicalein + EtOH  VEGF + baicalein + 12(S)-HETE

Relative tube length (Fold increase)

PBS  VEGF + DMSO + EtOH  VEGF + baicalein + EtOH  VEGF + baicalein + 12(S)-HETE

B

PBS  VEGF + DMSO + EtOH  VEGF + baicalein + EtOH  VEGF + baicalein + 12(S)-HETE

Wound healing migration (Fold increase)

PBS  VEGF + DMSO + EtOH  VEGF + baicalein + EtOH  VEGF + baicalein + 12(S)-HETE
Kim et al. Supplemental Figure IV

A

PBS-pretreated group

Transmigration (Fold Increase)

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VEGF-pretreated group

Transmigration (Fold Increase)

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B

Relative tube length (Fold Increase)

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VEGF-pretreated group

Relative tube length (Fold Increase)

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Kim et al. Supplemental Figure V

A

Chicken β-actin promoter

BLT2

Rabbit β-globin poly A

5'-GTCTAGAGCTCTGCTAAACC-3'

5'-CGATGGGTGGCACAATTGAC-3'

primer set 1

5'-GCTCTAGAGCCTCTGCTAACC-3'

5'-GGCAGGAGCTGCTTTTCG-3'

primer set 2

5'-GTCTAGAGCTCTGCTAAACC-3'

5'-GGCAGGAGCTGCTTTTCG-3'

primer set 1

B

L W B P W B P

1000 bp
850 bp
650 bp
500 bp

1000 bp
850 bp
650 bp
500 bp

primer set 1
(677 bp)

primer set 2
(924 bp)

C

lungs

spleen

colon

small intestine

brain

heart

kidney

liver

W B W B W B W B W B W B W B W B W B

BLT2

GAPDH
Kim et al. Supplemental Figure VI

Parotid gland, normal
Parotid gland, SCC

Esophagus, normal
Esophagus, SCC

Breast, normal
Breast, DCC

Thymus, normal
Thymus, thymoma
Kim et al. Supplemental Figure VII

EtOH  12(S)-HETE  LTB₄
Kim et al. Supplemental Figure VIII
A

**Graph A**

- **LTB₄**
  - 0, 10, 20, 30 minutes
  - p-ERK 1/2
  - ERK 1/2

- **12(S)-HETE**
  - 0, 10, 20, 30 minutes
  - p-ERK 1/2
  - ERK 1/2

B

**Graph B**

- [3H]-Thymidine incorporation (Fold Increase)
  - 0.5
  - 1
  - 1.5
  - 2

- **etOH**
- **LTB₄**
- **12(S)-HETE**
- **LY294002**
- **U73122**
- **PD98059**
- **DMSO**

**Supplemental Figure IX**

Kim et al.