Biochemical Characterization of Three BLT Receptors in Zebrafish

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Abstract

The leukotriene B4 (LTB4) receptor 1 (BLT1) is a high affinity receptor for LTB4, a chemotactic and inflammatory eicosanoid. The LTB4 receptor 2 (BLT2) was originally identified as a low affinity receptor for LTB4, and, more recently, as a high affinity receptor for 12-hydroxy-heptadecatrienoic acid (12-HHT). The zebrafish BLT receptors have not been previously identified and the in vivo functions of these receptors have been unknown. In this paper, we describe one zebrafish BLT1-like receptor, Blt1, and two zebrafish BLT2-like receptors, Blt2a and Blt2b. Cells expressing Blt1 exhibited LTB4-induced intracellular [Ca2+] increases, inhibition of cAMP production, ligand-dependent [35S]GTPγS binding, and transforming growth factor-α (TGFα) shedding activity in a dose-dependent manner, similar to human BLT1. Cells expressing Blt2a and Blt2b exhibited 12-HHT- and LTB4-induced intracellular [Ca2+] increases, inhibition of cAMP production, [35S]GTPγS binding, and TGFα shedding activity, with a dose-dependency similar to human BLT2. Reverse transcription (RT)-PCR analysis and whole-mount in situ hybridization revealed that blt1, blt2a, blt2b, zebrafish LTA4 hydrolase (lta4h), and zebrafish 5-lipoxiganase (5lo) are expressed in zebrafish embryos. Knockdown of blt1 by morpholino antisense oligonucleotides resulted in delayed epiboly at gastrulation. Consistently, knockdown of lta4h, an enzyme mediating LTB4 production, induced a phenotype similar to knockdown of blt1. These results suggest that the LTB4-BLT1 axis is involved in epiboly in zebrafish development.

Introduction

Leukotriene B4 (LTB4), an eicosanoid derivative of arachidonic acid metabolism produced by the sequential actions of 5-lipoxygenase (5-LO) and leukotriene A4 hydrolase (LTA4H), is a potent leukocyte chemoattractant [1]. Two G-protein-coupled receptors (GPCRs) for LTB4, BLT1 and BLT2, have been identified [2]. BLT1 is a high affinity LTB4 receptor [3], while BLT2 was originally identified as a low affinity LTB4 receptor [4]. Recently, we demonstrated that 12(S)-hydroxy-5-cis,8,10-trans-heptadecatrienoic acid (12-HHT), which had been considered as merely a by-product of thromboxane synthesis from prostaglandin endoperoxide, is an
endogenous high affinity ligand for BLT2 [5,6]. BLT1 and BLT2 form a gene cluster on both human and mouse chromosomes, suggesting that these receptors were generated by a gene duplication machinery [4]. BLT1 is expressed on various immune cells including neutrophils, eosinophils [7], monocytes, dendritic cells [8,9], activated T-cells [10], and osteoclasts [11], and induces the activation and migration of these cells [12]. The in vivo role of BLT2 has not been established, but our recent work revealed that BLT2 has an anti-inflammatory function in a mouse model of inflammatory colitis [13], as well as a protective role in allergic airway inflammation [14]. BLT2 also promotes wound healing by accelerating keratinocyte migration [15].

The zebrafish has emerged as a useful model system for genetic and pharmacological analyses of embryogenesis because fertilization and embryo development occur outside the maternal body and the embryos are transparent [16,17]. In addition, the zebrafish has been used as a model for studying inflammation and immunity because the immune system is largely conserved between zebrafish and mammals. Bischel et al. have shown that LTB4 induces neutrophil migration into the fins of live zebrafish [18], and recent forward genetic screening in zebrafish larvae has revealed that the lta4h locus modulates susceptibility to mycobacterial infections [19,20]. However, BLT receptors have not been definitively identified in zebrafish. In this study, we identified genes for zebrafish BLT receptors by bioinformatic and biochemical analyses, and revealed an unexpected function of zebrafish BLT1 in embryogenesis.

**Material and Methods**

**Materials**

LTB4 and 12-HHT were purchased from Cayman Chemical (Ann Arbor, MI). Probenecid was purchased from Sigma-Aldrich (St. Louis, MO). Pluronic F-127, Alexa-488-conjugated anti-Rat IgG (Molecular Probes), and fetal calf serum (FCS; GIBCO) were obtained from Invitrogen (Carlsbad, CA). A penicillin-streptomycin solution and geneticin (G418) were purchased from Nacalai Tesque (Kyoto, Japan). [35S]-guanosine 5'-O-(gamma-thio) triphosphate (GTPγS) was obtained from Perkin-Elmer Life Science (Boston, MA). Anti-hemagglutinin (anti-HA; clone 3F10) was purchased from Roche (Penzberg, Germany).

**cDNA cloning and plasmid construction of zebrafish Blts**

The cDNA of zebrafish blt1, blt2a, blt2b and lta4h were isolated by PCR from cDNA templates prepared from zebrafish embryos at 24–72 hours post fertilization (hpf). The following primers were used for cDNA cloning: blt1 (5'-atggcaacctttaactcgtgct-3' and 5'-tcaatgcaggggggtcagagtc-3'), blt2a (5'-atggcgttgaaccttctgtcccctc-3' and 5'-tcactttccattattctggggtgcg-3'), blt2b (5'-atggcattggaaaatggcagcttctc-3' and 5'-ttatagcctgatgacatccctagtc-3'). LTA4H and 5Lo were amplified by PCR and cloned into the pCS2+ vector. The following primers were used: lta4h (5'-tgcctggatcagcattggaaatggcagtcttc-3' and 5'-gcaatctgtttggtacctgttc-3'), and 5lo (5'-ctaggccggtcttc-3' and 5'-gggtttggtacctgttc-3'). All of the clones were verified by DNA sequencing.
Cell culture, transfection, and cell sorting

Chinese hamster ovary (CHO) cells were maintained in Ham’s F-12 medium (Wako) containing 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO₂ at 37°C. CHO cells were transfected with expression vectors using Lipofectamine LTX and the PLUS Reagent (Invitrogen) according to the manufacturer’s protocol. At 48 h post-transfection, the medium was changed to selection medium containing 1 mg/ml G418. After 2–3 weeks of selection, G418-resistant cells were stained with anti-HA (2 μg/ml) and Alexa-Fluor 488-conjugated anti-Rat IgG (10 μg/ml). Cells expressing the BLT receptors were collected as polyclonal populations by cell sorting using FACSaria II (Becton, Dickinson and Company, Franklin Lakes, NJ) and maintained in 0.3 mg/ml G418. A FACSCalibur instrument (Becton Dickinson) was used for flow cytometry.

Calcium mobilization assay

CHO cells (3 × 10⁴) stably expressing one of the receptors were seeded onto 96-well plates. After 16 h, cells were loaded with 4 μM Fluo-8 AM (ABD Bioquest) in 100 μl of HP buffer (HBSS containing 2.5 mM probenecid and 20 mM HEPES, pH 7.4) supplemented with 0.04% Pluronic F-127 and 1% FCS at 37°C for 30 min, followed by a further incubation at room temperature for 30 min. The cells were washed twice with HP buffer and agonist-induced intracellular calcium mobilization was determined by monitoring the fluorescence intensity (excitation at 485 nm, emission at 525 nm) using a FlexStation 3 plate reader (Molecular Devices, Sunnyvale, CA).

Membrane preparation and GTPγS binding assay

Cells were harvested and sonicated in ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 10 mM MgCl₂, and 2 mM EDTA) containing a protease inhibitor mixture (Nacalai Tesque, Kyoto Japan). The homogenate was centrifuged at 800 × g for 5 min at 4°C; the supernatant was collected and centrifuged at 100,000 × g for 1 h at 4°C. The resulting pellet was resuspended in the homogenization buffer. The membrane preparation (10 μg of protein) was incubated in 100 μl of GTPγS binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 5 μM GDP and 0.1% BSA) containing 1 nM [35S]GTPγS with LTB₄ or 12-HHT for 30 min at 30°C. To determine nonspecific binding, unlabeled GTPγS was added to the binding mixture to a final concentration of 10 μM. The bound [35S]GTPγS was separated from free [35S]GTPγS by rapid filtration through GF/C filters and washed with ~2 ml of ice-cold TMN buffer (10 mM Tris-HCl, pH 7.5, 25 mM MgCl₂, and 100 mM NaCl). The radioactivity of the filters was determined using a Top Count scintillation counter (Packard Instrument Co.).

cAMP assay

CHO cells (4 × 10⁴ cells/well) were plated on 96-well plates. On the following day, cells were washed twice with Krebs-Ringer Bicarbonate Buffer containing glucose (KRBG buffer: 0.49 mM MgCl₂, 4.56 mM KCl, 120 mM NaCl, 0.7 mM Na₂HPO₄, 1.5 mM NaHPO₄, 15 mM NaHCO₃ and 10 mM glucose (pH 7.4)). The cells were incubated with stimulation buffer (KRBG buffer containing 0.75 mM 3-isobutyl-1-methylxanthine (IBMX)) for 10 min at room temperature and then with 20 μM forskolin and LTB₄, or 12-HHT, for 15 min at 37°C. The incubation was terminated by the addition of lysis buffer (pH 7.3) supplied in the CatchPoint cAMP Fluorescent Assay Kit (Molecular Devices Corporation), and the concentration of cAMP in the lysate was determined according to the manufacturer’s protocol.
**TGFα shedding assay**

A TGFα shedding assay was performed as previously reported [22]. HEK cells (2 × 10^5 cells/well) were seeded into 12-well plates and incubated for 24 h. The cells were then transfected with the GPCR expression vector, pCAGGS-Gαq/i1 and pSS-AP-TGFα. At 24 h post-transfection, the cells were detached and resuspended in Hank’s balanced salt solution (HBSS) containing 5 mM HEPES (pH 7.4) and then seeded in a 96-well plate. After a 30 min incubation for 37°C, cells were stimulated with ligand for 1 h. The conditioned medium was transferred into another 96-well plate and p-nitrophenyl phosphate (p-NPP) solution (10 mM p-NPP, 40 mM Tris-HCl (pH 9.5), 40 mM NaCl and 10 mM MgCl₂) was added to both a conditioned medium plate and a cell plate. Absorbance at 405 nm (OD405) of both plates was read before and after a 1 h incubation at 37°C using a microplate reader (Bio-rad). To determine TGFα activity was calculated from the increase in OD405 in the conditioned medium (ΔODmedium) relative to the cell plate (ΔODcell) as follows. AP activity in the conditioned medium (APmedium) (%) was defined as the ratio of ΔODmedium to total ΔOD values (ΔODmedium plus ΔODcell).

**Zebrafish maintenance, morpholino injection and RT-PCR**

Zebrafish strain AB was maintained under standard conditions. All experimental animal care was performed in accordance with institutional and national guidelines and regulations. The study protocol was approved by the institutional review board of Kyushu University. All the experiments were performed at Kyushu University. Morpholino antisense oligonucleotides (MO) were designed and obtained from Gene Tools (Philomath, OR, USA). The sequences of translation-blocking MO and splice-blocking MO against blt1 were 5’—GGCCATTGGACTCAACCTTTATGGT—3’ (blt1 MO) and 5’—CTATTAGACATACCGGATAAATGTC—3’ (blt1 spl MO), respectively. The translation-blocking MO against lta4h (lta4h MO) has been described previously [19]. To test the specificities of the Blt1 MO and lta4h MO, the MO target sequences of Blt1 and lta4h were amplified by PCR and cloned into the pCS2P-EGFP vector (kindly provided by A. Kawahara, University of Yamanashi). MOs (5 ng) were injected into zebrafish embryos at the one- or two-cell stage. Total RNA from control MO- or blt1 spl MO-injected embryos was obtained at 24 hpf using the Trizol reagent (Invitrogen-Gibco, Carlsbad, CA) and was used as the template for generating cDNA (Superscript II reverse transcripase; Invitrogen-Gibco). A 338 base pair (bp) blt1 fragment was amplified using the following primers: blt1_fw, 5’—aggctgaggacagagaggtggagca—3’, and blt1_rv, 5’—ggcaacagaaagaggggaagca—3’. The 392 bp nonsplicing transcript of Blt1 (lta4h) fragment was amplified using the following primers: blt1_fw and blt1_rv 2 5’—aatgtcggccgtgccttattgat—3’. A 264 bp blt2a fragment was amplified using the following primers: blt2a_fw, 5’—tgaggactttgcaccgcttttgac—3’, and blt2a_rv, 5’—gtcagaaggtgacagaaatcaccag—3’. A 299 bp blt2b fragment was amplified using the following primers: blt2b_fw, 5’—gcgtctgtcgacagactgaccgt—3’, and blt2b_rv, 5’—ggctgaaaggtggcttgctgctgctg—3’. A 251 bp lta4h fragment was amplified using the following primers: lta4h_fw, 5’—gcctctctctgctgccaaatgtg—3’, and lta4h_rv, 5’—agaggggaggttctcaagaagggg—3’. A 330 bp 5lo fragment was amplified using the following primers: 5lo_fw, 5’—tgaaaaatgcctgccacgctacaggtgac—3’, and 5lo_rv, 5’—ctttgctcaacaccaacacggaag—3’.

**Whole-mount in situ hybridization**

Whole-mount in situ hybridization was performed according to a standard protocol. A digoxigenin-labeled antisense RNA probe was prepared using pCS2-blt1, pCS2-blt2a, pCS2-lta4h, and pCS2-lta4h. Capped mRNA was synthesized using an SP6 mMessage mMachine kit (Ambion, Austin, TX, USA) and purified using Micro Bio-Spin columns (Bio-Rad, Hercules, CA, USA).
Statistical Analysis

Statistical analysis was performed using Prism (Graphpad Software) for all comparisons.

Results

Cloning of blt1 and blt2 homologs in zebrafish

To obtain cDNAs for BLT receptors in zebrafish, we searched for putative zebrafish BLT receptors and identified three BLT-like sequences (XP_002662767, XP_009301152, XP_003197923) from the NCBI database. We amplified full-length cDNAs corresponding to these three sequences and cloned each into an expression vector. From the results of the pharmacological experiments described below, XP_002662767, XP_009301152, and XP_003197923 were named blt1, blt2a, and blt2b, respectively. We aligned the amino acid sequences of zebrafish, human, and mouse BLT1 (Fig. 1), and found that they shared moderate homology, with sequence identities of 38% between zebrafish and human or mouse. We also aligned the amino acid sequences of zebrafish, human and mouse BLT2 (Fig. 2), and found that they shared relatively low homology with sequence identities of 29–34% between zebrafish and human or mouse (Fig. 3A). A phylogenic analysis showed that zebrafish BLTs form a clade independent from mammalian BLT1 and BLT2 (Fig. 3B). In human and mouse, the blt1 and blt2 genes are located on chromosome 14. In zebrafish, the blt1 and blt2a genes are located on chromosome 7, and the blt2b gene is located on chromosome 2 (Fig. 3C).

Ligand identification and intracellular signaling of Blt1, Blt2a, and Blt2b

To examine the ligands and intracellular signaling of zebrafish Blt1, Blt2a, and Blt2b, we established CHO cells stably expressing N-terminally HA-tagged zebrafish Blt1, Blt2a, and Blt2b, as well as human BLT1 (hBLT1) and BLT2 (hBLT2). CHO cells expressing the receptors were sorted as polyclonal populations and analyzed using a flow cytometer. The hBLT1 and zebrafish Blt1 were expressed on the cell surface at similar levels (Fig. 4A), and hBLT2, zebrafish Blt2a, and Blt2b were expressed on the cell surface at similar levels (Fig. 4B). Previously, we showed that human BLT1 and BLT2 are coupled to the Gi and Gq families of G-proteins [23–25]. To investigate intracellular signaling through zebrafish BLTs, we performed calcium mobilization assays using the transfected cells. In hBLT1 (Fig. 5B) and zebrafish Blt1 (Fig. 5C) cells, intracellular free calcium concentration increased in a dose-dependent manner in response to LTB4 stimulation, whereas 12-HHT did not induce calcium mobilization in these cells, suggesting that zebrafish Blt1 is a zebrafish ortholog of human BLT1. In hBLT2 (Fig. 5D), zebrafish Blt2a (Fig. 5E), and zebrafish Blt2b (Fig. 5F) cells, the intracellular free calcium exhibited a dose-dependent increase in response to either 12-HHT or LTB4 stimulation, and 12-HHT activated zebrafish Blt2a and Blt2b at lower doses than LTB4, suggesting that zebrafish Blt2a and Blt2b are zebrafish orthologs of hBLT2.

Next, we quantified cAMP levels in CHO cells expressing the receptors. In hBLT1 (Fig. 6B) and zebrafish Blt1 (Fig. 6C) cells, 10 nM LTB4 inhibited forskolin-induced cAMP formation, but 12-HHT did not, suggesting that zebrafish Blt1 is a high affinity LTB4 receptor similar to hBLT1. In hBLT2 (Fig. 6D), zebrafish Blt2a (Fig. 6E), and zebrafish Blt2b (Fig. 6F) cells, 10 nM 12-HHT inhibited cAMP formation, but LTB4 had only a minimal effect on cAMP, suggesting that zebrafish Blt2a and Blt2b are high affinity 12-HHT receptors similar to hBLT2. To confirm that zebrafish BLT receptors directly activated G-proteins, we performed GTPγS binding assays using a membrane preparation of CHO cells expressing the receptors. Incubation with 1 μM LTB4 induced robust GTPγS binding, but 12-HHT did not, in hBLT1 (Fig. 7B) and zebrafish Blt1 (Fig. 7C) cells. Incubation with 1 μM 12-HHT induced robust GTPγS binding, and
μM LTB4 induced moderate but significant GTPγS binding, in hBLT2 (Fig. 7D), zebrafish Blt2a (Fig. 7E), and zebrafish Blt2b (Fig. 7F) cells. These results suggest that zebrafish Blt1 is a BLT1-type receptor, and zebrafish Blt2a and Blt2b are BLT2-type receptors.

TGFα shedding activities of zebrafish Blt1, Blt2a, and Blt2b

To confirm the ligands and intracellular signaling of zebrafish BLT receptors, we performed a TGFα shedding assay. In the TGFα shedding assay, GPCR activation is measured by the ectodomain shedding of a membrane-bound pre-form of alkaline phosphatase-tagged TGFα (AP-TGFα) into the medium [22]. HEK293 cells were transfected with a GPCR expression vector, a Gq/11 chimeric plasmid, and an expression plasmid encoding AP-TGFα, and then
stimulated with a ligand, which resulted in the accumulation of released AP-TGF\(\alpha\) into the medium (conditioned medium). LTB4 stimulation resulted in a dose-dependent increase in TGF\(\alpha\) shedding that was similar between hBLT1 (Fig. 8A) and zebrafish Blt1 (Fig. 8B) cells. In hBLT2 (Fig. 8C), zebrafish Blt2a (Fig. 8D), and Blt2b (Fig. 8E) cells, TGF\(\alpha\) shedding activity was
Fig 3. Sequence analysis of the zebrafish Blt receptors. (A) The amino acid identities among BLT1s (left) and BLT2s (right) are illustrated. (B) A phylogenic tree of BLT homologs in chordates was generated by GENETEX-MAC using the Unweighted Pair Group Method using the arithmetic Average (UPGMA). (C) Chromosomal locations of the human, mouse, and zebrafish blot1 and blot2 genes are shown. Chr., chromosome; cideb, cell death-inducing dff4-like effector b; nop9, nucleolar protein; adcy4, adenylate cyclase 4; gimap7-like, GTPase IMAP family member 7-like; ftr66, fin TRIM family, member 66; nfatc4, nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4; sdr39u1, short chain dehydrogenase/reductase family 39U.

doi:10.1371/journal.pone.0117888.g003
dose-dependently increased by either 12-HHT or LTB₄ stimulation. The lower AP activity of zebrafish Blt2a (Fig. 8D) may be affected by the lower expression of Blt2a on the cell surface in this assay (data not shown). In zebrafish Blt2b cells (Fig. 8E), the TGFα shedding activity induced by LTB₄ was much lower than that induced by 12-HHT. Thus, Blt2b may exhibit greater specificity for 12-HHT than that shown by hBLT2.

Fig 4. Establishment of CHO cells expressing the zebrafish Blt receptors. CHO cells stably expressing hBLT1, zebrafish Blt1, hBLT2, zebrafish Blt2a, or Blt2b were sorted after staining the cell surface Blts using an anti-HA antibody, and the surface expression was analyzed by flow cytometry.

doi:10.1371/journal.pone.0117888.g004
Fig 5. Calcium mobilization in CHO cells expressing the zebrafish Blt receptors. Intracellular calcium mobilization in CHO cells stably expressing hBLT1, zebrafish Blt1, hBLT2, zebrafish Blt2a, or Blt2b was analyzed using a FlexStation plate reader to measure fluorescence intensity. Data represent the mean ± s.e.m. (n = 4). These data are representative of at least two independent experiments with similar results.

doi:10.1371/journal.pone.0117888.g005
Fig 6. Levels of cAMP in CHO cells expressing the zebrafish Blt receptors. CHO cells stably expressing the human or zebrafish receptors were stimulated with 20 μM forskolin and 10 nM LTB4 or 12-HHT, and the levels of cAMP in cell lysates were determined. Data represent the mean ± s.e.m. (n = 3). **, P < 0.005; *, P < 0.05, one-way ANOVA with Bonferroni post-hoc test. These data are representative of at least two independent experiments with similar results.

doi:10.1371/journal.pone.0117888.g006
Fig 7. G-protein activity in CHO cells expressing the zebrafish Blt receptors. Membrane preparations of CHO cells expressing human or zebrafish receptors were incubated with 1 nM [35S] GTPγS and 1 μM LTB4 or 12-HHT, and specific binding was measured. Data represent the mean ± s.e.m. (n = 3). **, P < 0.005, one-way ANOVA with Bonferroni post-hoc test. These data are representative of at least two independent experiments with similar results.

doi:10.1371/journal.pone.0117888.g007
Expression of \textit{blts}, \textit{lta4h}, and \textit{5lo} in zebrafish embryos

To confirm the gene expression of \textit{blt1}, \textit{blt2a}, \textit{blt2b}, \textit{lta4h}, and \textit{5lo} in zebrafish embryos, reverse transcriptase (RT)-PCR was performed using mRNA of zebrafish embryos at 24 hpf. The mRNAs of \textit{blt1}, \textit{blt2a}, \textit{blt2b}, \textit{lta4h}, and \textit{5lo} were detected in zebrafish embryos (Fig. 9A) and

Fig 8. Ligand-dependent TGF\(\alpha\) release via zebrafish Blt receptors. HEK293 cells expressing Blt receptors, G\(_{\alpha q/i1}\), and AP-TGF\(\alpha\) were stimulated with LTB\(_4\) or 12-HHT. AP-TGF\(\alpha\) release (\%) was quantified using a colorimetric AP assay using p-NPP as a substrate. Data are representative of at least two independent experiments with similar results.

doi:10.1371/journal.pone.0117888.g008
whole-mount in situ hybridization revealed that blt1, lta4h, and 5lo were widely expressed at 3 hpf (Fig. 9B).

The LTB4-BLT1 axis is required for epiboly

To investigate the roles of Blt1 in zebrafish embryogenesis, we used two blt1 morpholino antisense oligonucleotides: blt1 MO blocks the translation of mature mRNAs and blt1 spl MO blocks the normal splicing of blt1 (Fig. 10A). We confirmed the efficiency and specificity of the blt1 MO using a modified EGFP construct harboring the morpholino target sequence upstream of the respective start codon (S1 Fig. A and B). To confirm the knockdown of blt1 by the blt1 spl MO, we performed RT-PCR analysis using primer sets that amplify normal transcripts (blt1_fw and blt1_rv2, Fig. 10A) and abnormal transcripts with un-spliced introns (blt1_fw and blt1_rv, Fig. 10A). Injection of the blt1 spl MO dramatically reduced the correctly spliced blt1 transcripts (Fig. 10A, middle) and increased levels of the abnormal transcripts (Fig. 10A, low). These results suggested that the blt1 spl MO efficiently knocked down blt1 in zebrafish. We confirmed the efficiency and specificity of an lta4h MO using a modified EGFP construct harboring the morpholino target sequence upstream of the respective start codon (S1 Fig. C and D).

Interestingly, injection of either the blt1 MO, blt1 spl MO, or lta4h MO, but not injection of a control MO, resulted in a severe delay in epiboly during gastrulation (Fig. 10B and 10C). These results suggest that the LTB4-BLT1 axis is required for normal epiboly in zebrafish development.

Discussion

In this study, we identified and characterized one zebrafish BLT1-like receptor and two zebrafish BLT2-like receptors. Knockdown of blt1 delayed epiboly at gastrulation of zebrafish
Fig 10. Morpholino-mediated knockdown of \textit{blt1} and \textit{lta4h} affects epiboly. (A) Diagram of a partial map of \textit{blt1} genomic DNA. Exons and introns are shown as boxes and lines, respectively. Two nonoverlapping \textit{blt1} MOs, \textit{blt1} MO and \textit{blt1} spl MO, were designed to target translation and splicing of Blt1, respectively. The \textit{blt1} MO should eliminate the transcription of exon 1, which contains the translation start site, resulting in aberrant protein synthesis. The efficacy of \textit{blt1} spl MO was validated by RT-PCR using \textit{blt1} \_fw and \textit{blt1} \_rv or \textit{blt1} \_rv2. Total RNA was isolated from \textit{blt1} spl MO or control MO-injected embryos at 24 hpf. Reduced expression of normal spliced \textit{blt1} transcripts and increased mis-spliced \textit{blt1} transcripts are shown. (B) Representative images of delayed epiboly of \textit{blt1} and \textit{lta4h} morphants from 4 to 10 hpf. Embryos were
embryos. Database screening by sequence homology to human BLT1 and BLT2 identified three putative zebrafish Blts (Fig. 1–3) that share relatively low homologies to human and mouse BLT1 (~40%, Fig. 3A and 3B). Crystal structures of BLT1 and BLT2 have not been reported, but a structural model of the ligand binding site of BLT1 has been [26]. Alanine substitution of residues predicted to be potential ligand contact points in human BLT1, H94, Y102 (helix III), R156 (helix IV), E185 (helix V), and N241 (helix VI) resulted in reduced binding affinity, and all of these residues are conserved among zebrafish Blt1 and human and mouse BLT1 (Fig. 1). The R156A mutant of human BLT1 failed to show any [3H]LTB4 binding; R156 is the predicted binding site for the carboxyl group of LTB4 [26]. Among zebrafish Blt2a and Blt2b and human and mouse BLT2, all of these amino acids with the exception of H94 are conserved (Fig. 2), suggesting that H94 in helix III may be important in distinguishing between the chemical structures of LTB4 and 12-HHT. An evolutionary tree (Fig. 3B) suggests that the gene duplication of BLT1 and BLT2 occurred after the fish branched off from the other vertebrates. The presence of two BLT2-type receptors in zebrafish suggests that BLT2 may have specific roles in zebrafish that are not required in mammals.

To identify the ligands of putative zebrafish BLTs, we constructed expression vectors for the zebrafish Blts and conducted experiments to monitor GPCR-dependent signaling (Fig. 5–8). Previously, we found that the level of BLT1 on the plasma membrane is always higher than BLT2 in various cultured cells when overexpressed under the same promoter. Both human BLT1 and zebrafish Blt1 were expressed on the cell surface at a higher level than human BLT2, or zebrafish Blt2a or Blt2b (Fig. 4). The GPCR assays conducted here all suggest that Blt1 is a zebrafish ortholog of BLT1, and that Blt2a and Blt2b are zebrafish orthologs of BLT2 (Fig. 5–8). RT-PCR and whole-mount in situ hybridization of zebrafish embryos showed that mRNAs of blt1, as well as lta4h and 5lo, enzymes mediating LTB4 production, were detected in zebrafish embryos (Fig. 9). The blt1 and lta4h knockdown experiments indicated that the LTB4–Blt1 axis is involved in epiboly at gastrulation (Fig. 10).

Gastrulation involves a series of coordinated cell movements that establish the germ layers and the major body axes of the embryos [27]. Epiboly, which involves the thinning and spreading of a multilayered cell sheet, is the first coordinated cell movement of zebrafish gastrulation and occurs after the ninth or tenth zygotic cell division. Epiboly is visualized as the thinning and spreading of the blastoderm over the yolk. Since epiboly is initiated prior to the other cell movements in zebrafish gastrulation, the initial events in gastrulation can be studied in isolation from later, more complex cell movements [28–30]. Numerous molecules are involved in epiboly including microtubules, microfilaments, cell adhesion proteins, kinases, and transcription factors [27]. The prostaglandin biosynthetic enzymes cyclooxygenase-1 (Cox-1) and prostaglandin E2 synthase (Ptges) are also involved in epiboly. Treatment with 50 μM of indomethacin caused gastrulation arrest at 50% epiboly stage and 25 μM of indomethacin resulted in milder defect. Indomethacin-dependent epiboly defects were rescued by co-incubation with 1 μM PGE2 [31,32].

Lin et al. reported that Gaα12/13 regulates epiboly formation through two distinct mechanisms: limiting E-cadherin activity and modulating the organization of the actin cytoskeleton [33,34]. In mammals, BLT1 activates small GTPases and induces the reorganization of actin cytoskeleton [35–37]; BLT1 activates leukocyte migration and firm adhesion to endothelial cells.
Thus, BLT1 may modulate the organization of the actin cytoskeleton and mediate cell migration in epiboly of zebrafish embryo.

We were not able to rescue the delay in epiboly by co-injection of MOs and blt1 mRNA, suggesting that the localization and levels of Blt1 expression may be critical for proper epiboly. Although further analyses are required to understand the in vivo roles of Blt1 in epiboly, the molecular identification of zebrafish Blts will be useful for studying the in vivo roles of these receptors in zebrafish.

Supporting Information

S1 Fig. Specificity of blt1 and lta4h morpholinos. (A) 5’ sequence of EGFP mRNA designed to evaluate the function of the blt1 MO. The underline indicates the blt1 MO target sequence, the start codon is highlighted in blue, and the beginning of the open reading frame (ORF) of EGFP is highlighted in green. (B) Representative images of the effects of the blt1 MO on the expression of control EGFP and blt1 MO-EGFP. Translation of EGFP mRNA containing the blt1 MO target sequence is blocked by the blt1 MO. Control EGFP is not blocked by the blt1 MO. Embryos were injected with a mix of mRNA (250 pg) and MO (2.5 ng) at the one cell stage. EGFP fluorescence and bright-field (BF) images were taken at 7 hpf. (C) 5’ sequence of EGFP mRNA designed to evaluate the function of the lta4h MO. The underline indicates the lta4h MO target sequence, the start codon is highlighted in blue, and the beginning of the ORF of EGFP is highlighted in green. (D) Representative images of the effects of the lta4h MO on the expression of the lta4h MO-EGFP. Translation of EGFP mRNA containing the lta4h MO target sequence is blocked by the lta4h MO but not by a control MO. Embryos were injected with a mix of mRNA (250 pg) and MO (2.5 ng) at the one cell stage. EGFP fluorescence and BF images were taken at 6.5 hpf.

(TIF)

Acknowledgments

We thank Dr. Hiroyuki Toh (CBRC, AIST) for valuable advice on searching for the zebrafish BLTs and Dr. Atsuo Kawahara (University of Yamanashi) for useful suggestions. We appreciate the technical support provided by the Research Support Center, Graduate School of Medical Sciences, Kyushu University.

Author Contributions

Conceived and designed the experiments: TO TI TY. Performed the experiments: TO TI. Analyzed the data: TO TI. Wrote the paper: TO TI TY.

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