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Role of leukotriene B_4 receptor signaling in human preadipocyte differentiation

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ABSTRACT

We investigated the role of leukotriene B_4 (LTB_4)–leukotriene receptor (BLT) signaling in preadipocyte differentiation into mature adipocytes. Blockade of BLT signaling by treatment with lipoxygenase inhibitors, a BLT antagonist, and small interfering RNAs for BLTs in human and mouse preadipocytes isolated from adipose tissues showed acceleration of differentiation into mature adipocytes. DNA microarray analysis revealed regulation of transforming growth factor, beta-induced 68 kDa (TGFBI) expression through the BLT signaling pathway during adipocyte differentiation. Knockdown of TGFBI also showed acceleration of preadipocyte differentiation. The LTB_4–BLT signaling pathway may negatively regulate preadipocyte differentiation via induction of TGFBI expression as a rate-limiting system to control adipocyte differentiation.

1. Introduction

The involvement of various inflammatory mediators such as tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6) in obesity that is closely related to insulin resistance has been reported [1–3]. One of the most important organs on obesity and insulin resistance is thought to be adipose tissue, because adipocytes in adipose tissue generate various adipocytokines that play important roles in the onset of metabolic syndrome [1–3].

Leukotrienes (LTs) such as leukotriene B_4, C_4, and D_4 (LTB_4, LTC_4, and LTD_4, respectively) that are generated through lipoxygenase (LOX) pathways are well-known to induce various inflammatory and allergic reactions such as leukocyte activation, capillary permeability, and bronchial contraction [4,5]. LTB_4 binds to specific receptors, BLT1 and BLT2, to activate the signaling pathways [6,7]. LTs have been reported to be involved in the proliferation of various cell types such as epithelial, endothelial and mesangial cells [8,9]. In addition, we have reported that LTB_4 controls immature neural stem cell proliferation and differentiation via the BLT signaling pathway [10]. Therefore, LTB_4 and its signaling pathway could be involved in various cell proliferations and differentiations. However, there are few reports about the role of LTs in adipocyte differentiation. Furthermore, most of reported papers about the adipocyte differentiation have used mouse fibroblastic 3T3-L1 cells, but not human preadipocytes. Therefore, the exact role of the LTB_4 signaling pathway in adipocyte differentiation of human preadipocytes is still unclear.

In this study, we investigated the role of LTB_4 and its receptor signaling pathway in human adipocyte differentiation and the potential mechanisms.

2. Material and methods

2.1. Reagents and antibodies

Insulin (INS), dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX) and rosiglitazone (ROSI) were purchased from Sigma Japan (Tokyo, Japan). LT synthetase, lipoxygenase (LOX) inhibitor, nordehydroguaiaretic acid (NDGA), and 5-LOX specific inhibitor, AA-861, were also purchased from Sigma Japan (Tokyo, Japan). ONO-4057, a specific BLT antagonist, was a kind gift from ONO Pharmaceutical Co. Ltd. (Osaka, Japan). Anti-BLT1 and -BLT2 polyclonal antibodies were purchased from Cayman Chemicals (Ann Arbor, MI, USA). An anti-TGF beta induced, 68 kDa (TGFBI,

Abbreviations: LT, leukotriene; LOX, lipoxygenase; TGFBI, transforming growth factor beta-induced 68 kDa; INS, insulin; DEX, dexamethasone; IBMX, 3-isobutyl-methylxanthine; ROSI, rosiglitazone; NDGA, nordehydroguaiaretic acid; TNFα, tumor necrosis factor alpha; IL-6, interleukin 6.

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synonym; Beta ig-h3) polyclonal antibody was purchased from Proteintech (Proteintech Group, Inc., Chicago, IL).

2.2. Cell culture and induction of adipocyte differentiation

Two types of human preadipocytes isolated from subcutaneous adipose tissue or visceral adipose tissue (Poietics Human Preadipocytes) were purchased from LONZA (LONZA Walkersville, Inc., Walkersville, MD). Study protocols for using human cells were approved by the Ethics Committee of Yokohama City University School of Medicine and Osaka University Graduate School of Dentistry.

Mouse preadipocytes isolated from epididymal adipose tissue were purchased from Primary Cell Ltd. (Sapporo, Japan) [11,12].

Cell culture and induction of differentiation of preadipocytes were performed essentially according to the method described previously (Fig. 1A) [11,12]. Briefly, two human preadipocytes isolated from subcutaneous or visceral adipose tissue and mouse preadipocytes were cultured in preadipocyte basal medium-2 (PBM-2, LONZA Walkersville) supplemented with 10% fetal bovine serum,

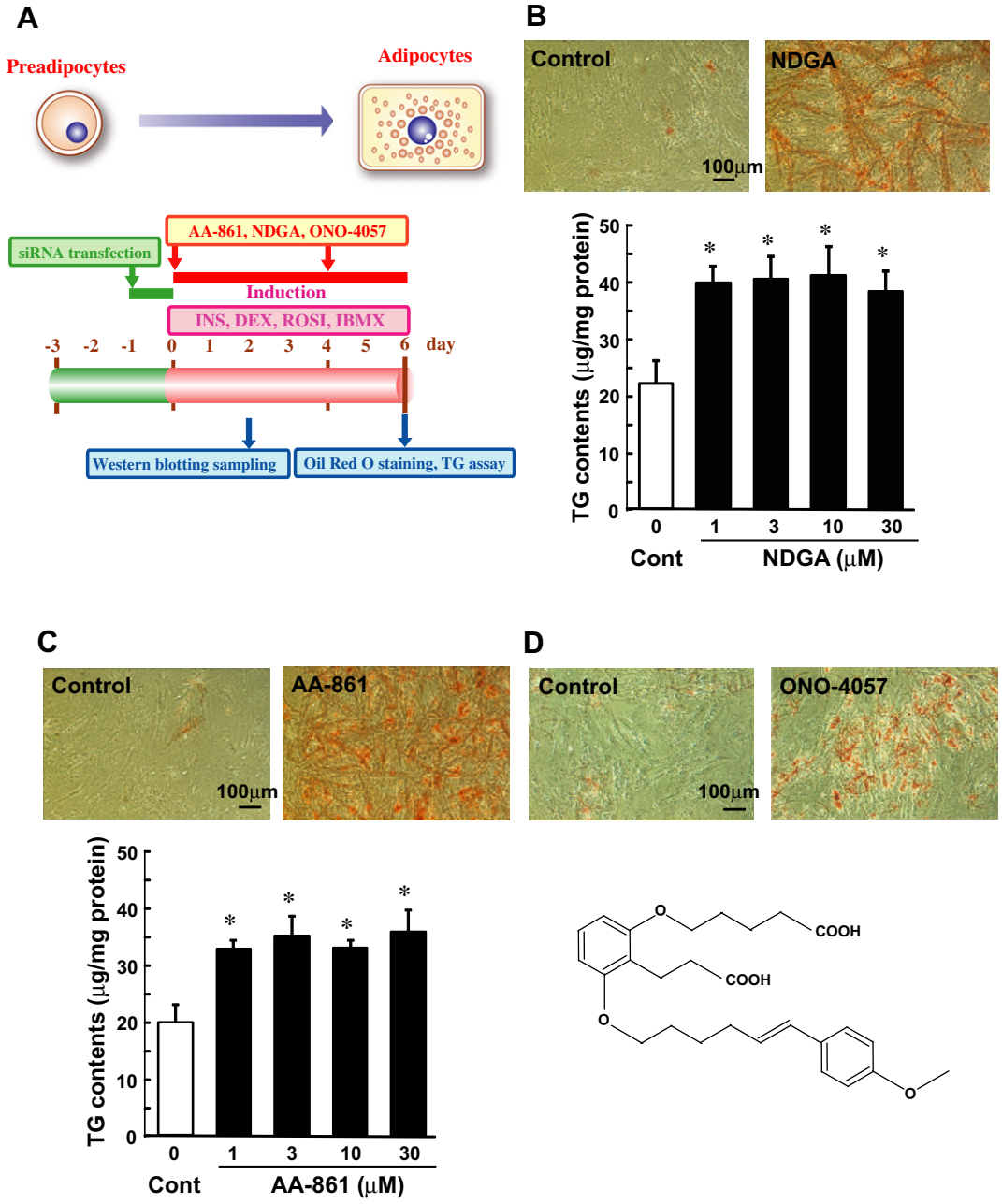

Fig. 1. Effects of LOX inhibitors and the BLT antagonist on human preadipocyte differentiation. (A) Schematic protocol of the preadipocyte differentiation. (B and C) Effects of NDGA (LOX inhibitor, B), or AA-861 (5-LOX inhibitor, C), on lipid accumulation in human preadipocytes isolated from subcutaneous adipose tissues. Human preadipocytes were treated with NDGA or AA-861 for 6 days. Then, accumulation of triacylglycerol (TG), a marker of lipid accumulation (right panel), in matured adipocytes was measured and expressed as TG contents (μg/mg protein). Each column represents the mean ± SEM from 3–5 independent experiments. $^{*}P < 0.05$ vs. vehicle control. Left panel represents typical photographs of differentiated adipocytes derived from human preadipocytes treated with NDGA, AA-861 or vehicle for 6 days. Cells were stained with Oil Red O method to visualize lipid accumulation. (D) Effect of ONO-4057, a specific BLT antagonist, on lipid accumulation in human preadipocytes. Left panel represents structure of ONO-4057, and right panel represents typical photographs of differentiated adipocytes from human preadipocytes treated with ONO-4057 (30 μM) or vehicle-only.

1% MEM non-essential amino acids, and 1% penicillin–streptomycin. At 3 days after reaching confluency, the medium was replaced with differentiation medium containing insulin (INS, 150 nM), dexamethasone (DEX, 1 μM), 3-isobutyl-1-methylxanthine (IBMX, 100 μM) and rosiglitazone (ROSI, 1 μM). The differentiation medium was changed every 4 days until analysis.

2.3. Evaluation of adipocyte differentiation

Differentiation of preadipocytes to mature adipocytes was visually monitored by microscopic observation after Oil red O staining [11,12]. In addition, the amount of triglyceride, an index of lipid accumulation, was quantitatively measured using a Triglyceride *E*-test wako kit (Wako Pure Chemicals, Tokyo, Japan). The amount of triglyceride was normalized by protein amount and expressed as TG contents (μg/mg protein).

2.4. siRNA for knockdown of BLT1 and BLT2

We designed small interfering RNA (siRNA) for knockdown of BLT1 and BLT2 using an siRNA system (Qiagen, Tokyo, Japan). The sequences of the sense and antisense strand for BLT1 used were 5′-CAACCUACACUUCCUAUUA-3′, and 5′-UAAUAGGAAGUGUAGGUUG-3′, respectively. The sequences of the sense and antisense strand for BLT2 used were 5′-GGGACUUAACAUACUCUUA-3′, and 5′-UAAGAGUAUGUUAAGUCCG-3′, respectively.

For transfection, siRNAs or negative control siRNA (Stealth RNAi Negative Control Duplexes, Invitrogen, Tokyo, Japan) were combined with Lipofectamine RNAiMAX (Invitrogen) and incubated for 20 min at room temperature to produce the transfection mixture. Then, the transfection mixture was added to preadipocytes at a final concentration of 25, 50, and 100 nM siRNA (Fig. 1A). At 24 h after the start of transfection, the medium was replaced with differentiation medium to induce differentiation. Samples were collected at days 2 and 6.

2.5. siRNA for knockdown of TGFBI

Based on DNA microarray results, we designed two siRNAs for knockdown of transforming growth factor beta-induced, 68 kDa (TGFBI) using a stealth siRNA system (Invitrogen). The sequences of the sense and antisense strand for TGFBI used were; TGFBI-siRNA-sequence-1, 5′-AAUUCUGGUACAUAGAGGUGAGGGU-3′, 5′-ACCCUCACCUCUAUGUACCAGAUUU-3′, and TGFBI-siRNA-sequence-2, 5′-UAACCAGGAUUUCAUCACCAAUGUG-3′, 5′-CACAUUGGUGAUGAAAUCCUGGUUA-3′, respectively.

For transfection, experimental procedures were performed as those described in Section 2.4.

2.6. Statistical analysis

Results were expressed as the mean ± SEM. Statistical comparisons were performed using the Student's *t*-test or Scheffe's method after analysis of variance (ANOVA). The results were considered significantly different at $P < 0.05$.

3. Results

3.1. Effects of LOX inhibitors and the BLT antagonist on human preadipocyte differentiation

Preadipocytes can differentiate into mature adipocytes in the induction medium containing INS, DEX, IBMX, and ROSI (PPARγ-ligand) [11,12]. We used this induction condition for differentiation of human preadipocytes derived from subcutaneous adipose tissues (Fig. 1A). NDGA, a pan-LOX inhibitor, enhanced the accumulation of lipids and the increase in TG contents, the index of human preadipocyte differentiation into mature adipocytes (Fig. 1B). No alteration of cell proliferation was observed under our experimental conditions (data not shown). AA-861, a 5-LOX inhibitor, also enhanced the differentiation of human preadipocytes into mature adipocytes (Fig. 1C). These accelerated effects occurred in a concentration-dependent manner. In addition, ONO-4057, a specific antagonist of LTB_4 receptor, accelerated the preadipocyte differentiation (Fig. 1D). These results suggest that the LTB_4–BLT signaling pathway may be involved in preadipocyte differentiation.

3.2. Effect of BLT1 and BLT2 knockdown by siRNA on human preadipocyte differentiation

We measured the release of LTB_4 from preadipocytes into the culture medium. The level of LTB_4 in the medium was 31.8 ± 8.4 nmol/L (mean ± SEM, $n = 3$). In addition, Western blot analysis revealed the expression of both BLT1 and BLT2 in human preadipocytes from the start to late phases of differentiation (Fig. 2A).

Therefore, we designed siRNAs specific for BLT1 or BLT2 to knockdown the receptors, respectively. SiRNAs against BLT1 and BLT2 successfully suppressed the expression of BLT1 and BLT2 (Fig. 2B). Indicators of preadipocyte differentiation such as the lipid accumulation and the increase of TG contents were observed by suppression of BLT1 using the specific siRNA (Fig. 2C–E). Similar results were observed by the suppression of BLT2 using the specific siRNA (Fig. 2F–H). We also checked the effects of the 5-LOX inhibitor and BLT1, BLT2-siRNAs on visceral fat-derived human preadipocyte differentiation. Differentiation of visceral fat-derived human preadipocytes was also enhanced by treatment with the 5-LOX inhibitor, BLT1- or BLT2-siRNAs (Fig. 2I–K). These results clearly indicate that the LTB_4–BLT signaling pathway suppresses human preadipocyte differentiation, and blockade or knockdown of BLTs leads to the stimulation of preadipocyte differentiation.

3.3. Investigation of the potential mechanisms of BLT signaling-mediated inhibition of human preadipocyte differentiation

To investigate the potential mechanisms of BLT signaling-mediated inhibition of human preadipocyte differentiation, we performed DNA microarray analysis to identify the molecules regulated by BLT signaling. Many molecules were significantly altered by treatment with the LOX inhibitor or BLT-1 siRNA (data not shown). Among them, we focused on transforming growth factor, beta-induced 68 kDa (TGFBI), because the alteration of TGFBI mRNA was significantly higher and the net amount of TGFBI mRNA was markedly larger. To confirm the DNA microarray data, we performed Western blot analysis. As shown in Fig. 3A, increased expression of TGFBI protein was observed at day 1 after the start of induction of differentiation. In addition, the expression of TGFBI protein was markedly suppressed by treatment with the LOX inhibitor, or BLT1- and BLT-2 siRNAs (Fig. 3B and C, respectively). These results indicate that the expression of TGFBI may be related to preadipocyte differentiation.

We, therefore, designed TGFBI-specific siRNA for knockdown the molecules. Two different siRNAs for TGFBI (sequence-1 and -2) successfully suppressed the expression of TGFBI protein (Fig. 3D). Stimulation of preadipocyte differentiation such as the lipid accumulation and the increase of TG contents were observed after knockdown of TGFBI using the specific siRNAs (Fig. 3E–H). These results strongly suggest that BLT-signaling-induced expression of TGFBI suppresses preadipocyte differentiation.

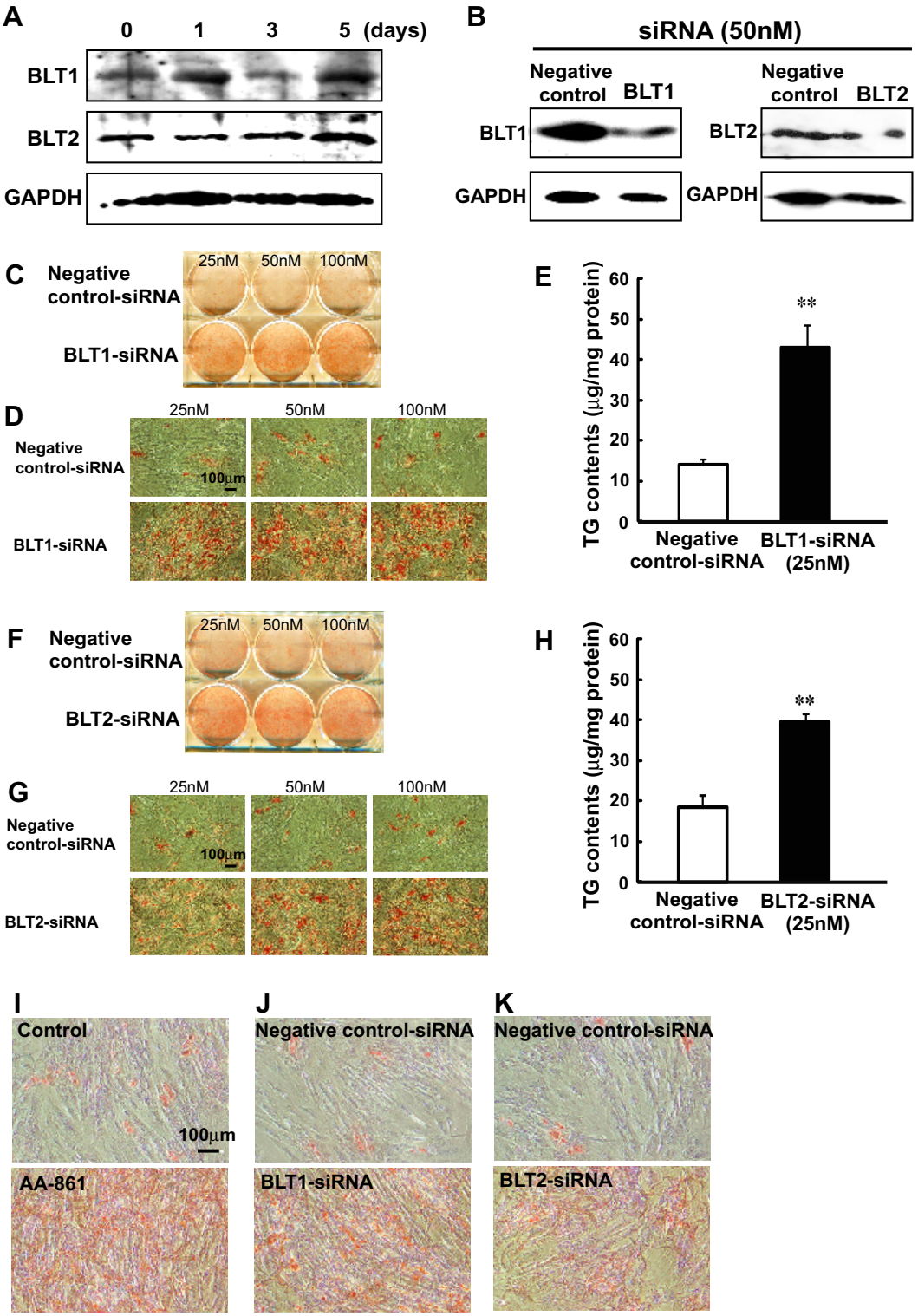

Fig. 2. Effect of BLT1 and BLT2 knockdown by siRNA on human preadipocyte differentiation. (A) Expression of BLT1 and BLT2 during preadipocyte differentiation. Detection of BLT1 and BLT2 was performed by Western blot analysis using anti-BLT1 or -BLT2 antibodies. GAPDH was used as an internal standard for confirmation of equally applied. (B) Confirmation of BLT1 and BLT2 knockdown by specific siRNAs. Negative controls represent the treatment with the negative control siRNA (Stealth RNAi Negative Control Duplexes). (C–E) Effect of BLT1 knockdown by siRNA. Typical macroscopic (C) and microscopic (D) pictures of differentiated adipocytes derived from human preadipocytes by Oil Red O staining. (E) Accumulation of TG in mature adipocytes was measured and expressed as TG contents μg/mg protein). Each column represents the mean ± SEM from 3–5 independent experiments. $^{**}P < 0.01$ vs. negative control-siRNA treatment. (F–H) Effect of BLT2 knockdown by siRNA. Typical macroscopic (F) and microscopic (G) pictures of differentiated adipocytes derived from human preadipocytes by Oil Red O staining. (H) Accumulation of TG in matured adipocytes was measured and expressed as TG contents (μg/mg protein). (I–K) Effects of the LOX inhibitor and siRNAs for BLT1 and BLT2 on visceral fat-derived human preadipocyte differentiation. Typical microscopic pictures of mature adipocytes by the Oil Red O staining.

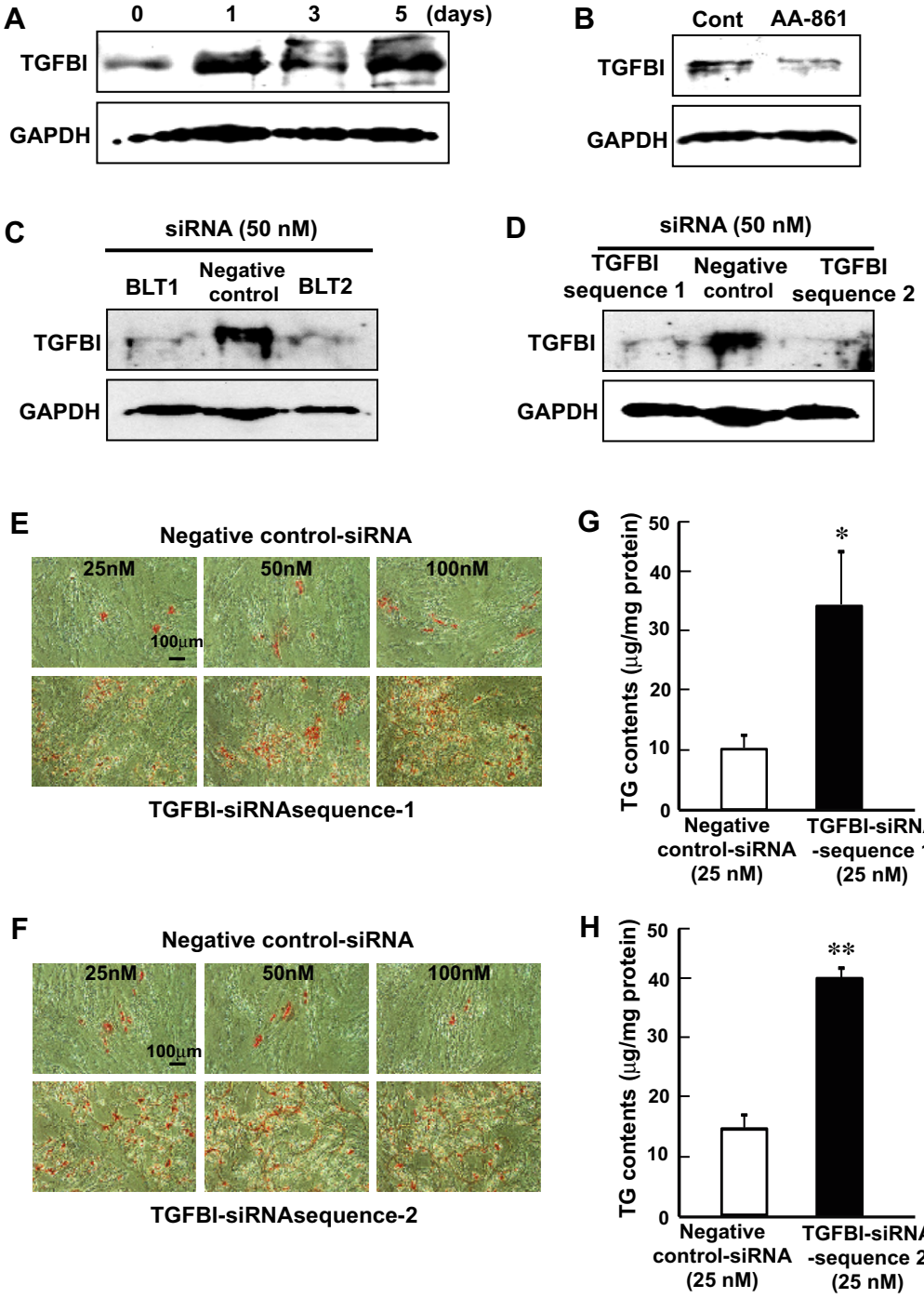

Fig. 3. Effect of transforming growth factor, beta-induced 68 kDa (TGFBI) knockdown by siRNA on human preadipocyte differentiation. (A) Time-dependent expression of TGFBI during preadipocyte differentiation. Detection of TGFBI was performed by Western blot analysis using an anti-TGFBI antibody. GAPDH was used as internal standard for confirmation of equally applied. (B) Effect of the LOX inhibitor on the expression of TGFBI in human preadipocytes. Detection of TGFBI was performed by Western blot analysis using anti-TGFBI antibody. (C) Effect of the knockdown of BLT1 or BLT2 by siRNA on the expression of TGFBI in human preadipocytes. Detection of TGFBI was performed by Western blot analysis using an anti-TGFBI antibody. (D) Confirmation of TGFBI knockdown by specific siRNAs for two different sequences (sequence-1 and -2). Negative controls represent the treatment with the negative control siRNA (Stealth RNAi Negative Control Duplexes). (E–G) Effect of TGFBI knockdown by siRNA. (E and F) Typical microscopic pictures of differentiated adipocytes from human preadipocytes by Oil Red O staining. (G) The accumulation of TG in matured adipocytes was measured and expressed as TG contents (μg/mg protein). Each column represents the mean ± SEM from 3–5 independent experiments. $^{*}P < 0.05$, and $^{**}P < 0.01$ vs. negative control-siRNA treatment.

3.4. Effects of the LOX inhibitor and knockdown of BLTs by siRNA on mouse preadipocyte differentiation

To confirm the effects of BLT-signaling on preadipocyte differentiation, we used mouse preadipocytes isolated from mouse epididymal adipose tissue. As shown in Fig. 4, treatment with the LOX inhibitor, or BLT1- and BLT2-siRNA showed the acceleration of lipid accumulation in mouse preadipocytes. The results observed in mouse preadipocytes were quite similar to those in human preadipocytes.

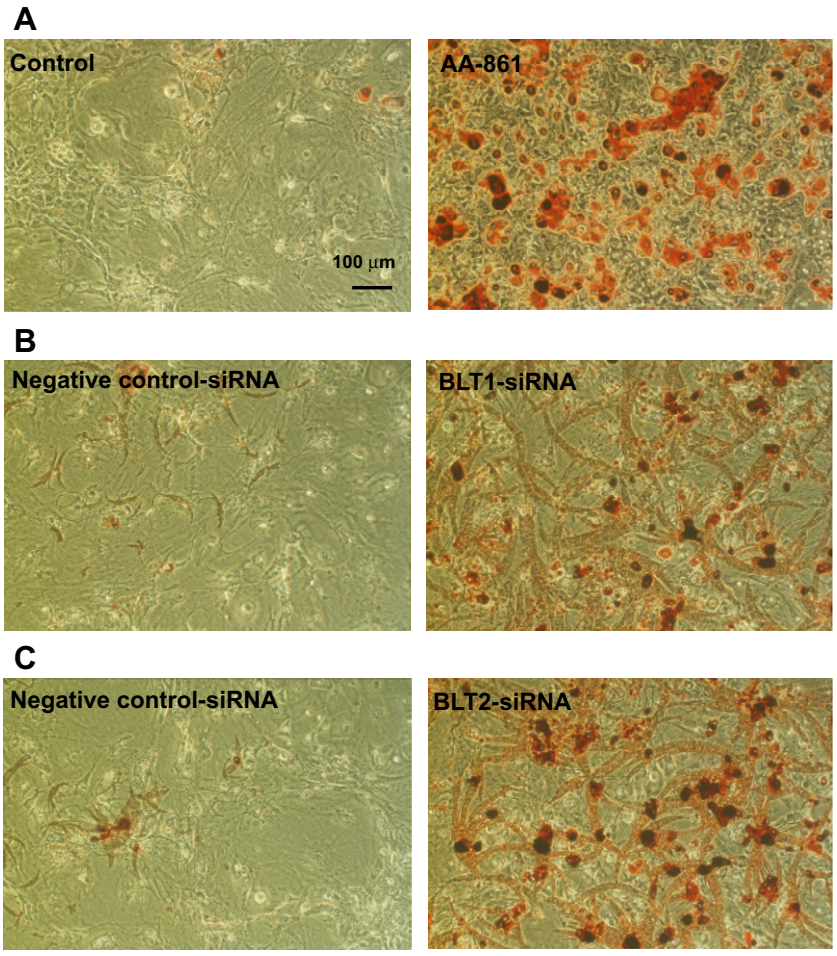

Fig. 4. Effects of the LOX inhibitor and siRNAs for BLT on mouse preadipocyte differentiation. Typical microscopic pictures of mature adipocytes differentiated from mouse preadipocytes isolated from epididymal adipose tissues by the Oil Red O staining. Mouse preadipocytes were treated with AA-861 (A), BLT1-siRNA (B), or BLT2-siRNA (C), respectively.

4. Discussion

In the present study, we clearly showed that the LTB_4–BLT signaling pathway provides a potent regulatory signal that inhibits the differentiation of preadipocytes isolated from adipose tissues.

It is not fully understood whether inflammation-related lipid mediators, such as LTs and PGs, promote or inhibit the onset of metabolic syndrome. Several previous reports indicated that PGD_2-derived 15-deoxy-$\Delta^{12,14}$-PGJ_2 promoted adipocyte differentiation via activation of the PPARγ pathway [13,14]. Therefore, cyclooxygenase-related prostanoids are considered to be involved in the enhancement of adipocyte differentiation. In contrast, there are few reports about the involvement of LOX-related metabolites in adipocyte differentiation. These studies still do not indicate whether all inflammation-related molecules promote adipocyte differentiation or not. In our present study, we showed the expression of both LTB_4 receptors, BLT1 and BLT2, in human preadipocytes. We also confirmed the release of LTB_4 itself from preadipocytes into the culture medium. These results indicate that paracrine or autocrine pathways of BLT-signaling operate in preadipocytes, which shows negative regulation of human and mouse preadipocyte differentiation of adipocyte progenitors isolated from adipose tissues, because inhibition of this pathway by treatment with LOX inhibitors, the BLT antagonist, and siRNA for BLTs showed the enhancement of preadipocyte differentiation.

We also found that TGFBI is an important molecule involved in the suppression of human preadipocyte differentiation mediated by the BLT-signaling pathway. TGFBI is a 68 kD extracellular matrix molecule that was initially cloned from human adenocarcinoma cells treated with TGFβ [15]. The precise function of TGFBI remains unclear, however several studies have demonstrated that TGFBI is involved in cell adhesion, migration, corneal dystrophy, tumorigenesis and nephropathy [16]. In addition, it has been reported that TGFBI is involved in keratinocyte differentiation [17]. Therefore, it is possible that TGFBI plays important roles in the differentiation of various cell types. In the present study, we clearly showed that TGFBI is a negative regulator of human preadipocyte differentiation using specific siRNAs. We also confirmed that the expression of TGFBI was closely regulated by the BLT-signaling pathway. These results suggest that the LTB_4–BLT signaling pathway negatively regulates preadipocyte differentiation via the induction of TGFBI expression as rate-limiting system to control excessive adipocyte differentiation. However, the role of TGFBI in preadipocyte differentiation may be limited, and we cannot rule out the involvement of other molecules in BLT-signaling-mediated preadipocyte differentiation. In addition, we could not clarify the downstream mechanisms of TGFBI in adipocyte differentiation. Integrins, Akt signaling, or other pathways might be involved in TGFBI-mediated adipocyte differentiation [17]. Further investigation is required to clarify the downstream effect of TGFBI in adipocyte differentiation.

Recently, BLT1 and BLT2 knockout mice were generated [18,19]. However, it was reported that no marked differences between wild-type and knockout mice were observed in terms of adipocyte

differentiation and lipid accumulation [18,19]. It is considered that compensative mechanisms might be existed in vivo. Otherwise, the local action of BLT-signaling in adipose tissues may be different from the systemic actions. Conditional knockout mice for BLT1 and BLT2 specific to adipose tissues are required to answer the questions. In contrast, it has been reported that an increase of adipose tissues is observed in 5-LOX-deficient mice compared with that in wild-type mice [20]. This observation indicates that 5-LOX metabolites are involved in the inhibition of the increase of adipose tissues, and also supports our results in which the LOX metabolites/BLT-signaling pathway negatively regulate preadipocyte differentiation that results in lipid accumulation.

In conclusion, the LTB_4–BLT signaling pathway negatively regulates preadipocyte differentiation via the induction of TGFBI expression as rate-limiting system to control excessive adipocyte differentiation.

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