No Involvement of Lysophosphatidic Acid Receptor-3 in Cell Migration of Mouse Lung Tumor Cells Stimulated by 12-O-Tetradecanoylphorbol-13-acetate

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Abstract: The tumor promoting agent 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulates cell migration of several tumor cells. Recently, we reported that loss of lysophosphatidic acid (LPA) receptor-3 (LPA_3) enhanced cell migration of murine lung tumor LL/2 cells. In the present study, we investigated whether LPA_3 is involved in cell migration of mouse lung tumor cells stimulated by TPA. Exogenous LPA_3 gene (*Lpar3*)-expressing (LL/2-a3) cells and LL/2-AB cells as a vector control generated from LL/2 cells were used. In a cell migration assay, TPA treatment significantly stimulated cell migration of LL/2-AB and LL/2-a3 cells, while the cell migration abilities of LL/2-a3 were markedly lower than those of LL/2-AB cells. Using quantitative real-time reverse transcription (RT)-polymerase chain reaction (PCR) analysis, no effect of TPA treatment on the expression levels of LPA_1, LPA_2 and LPA_3 genes was detected in either type of cells. These results suggest that the LPA_3 may not be involved in the enhanced migration ability by TPA in mouse lung tumor cells. (DOI: 10.1293/tox.24.183; J Toxicol Pathol 2011; **24**: 183–186)

Key words: LPA_3, 12-O-tetradecanoylphorbol-13-acetate, cell migration, lung, mouse

Lysophosphatidic acid (LPA) is a bioactive mediator and interacts with at least six G protein-coupled transmembrane receptors, LPA receptor-1 (LPA_1), LPA_2, LPA_3, LPA_4, LPA_5 and LPA_6[1–4]. After binding to LPA receptors (LPARs), LPA induces several biological effects, such as cell proliferation, differentiation, morphogenesis and protection from apoptosis[1–4]. In cancer cells, LPA can also enhance cell growth, migration, invasion and tumorigenicity[1,5,6].

Previously, aberrant expressions of LPAR genes have been detected in human malignancies, including ovary, colon and thyroid tumors[7–11]. Recently, we have also reported that the distinct expression patterns of LPAR genes due to DNA methylation were found in human and mouse tumor cells[12,13]. Therefore, this suggests that the biological functions of LPARs may be dependent on the type of cancer cells. In fact, LPA_3 enhanced cell migration and invasion of human ovarian cancer cells[14]. By contrast, we have reported that LPA_3 inhibited cell migration abilities of murine lung tumor cells[15].

It is well known that 12-O-tetradecanoylphorbol-13-acetate (TPA) is a tumor promoting agent that inhibits gap junctional intercellular communication in various cells types[16,17]. TPA also enhances cell migration through activation of protein kinase C (PKC) in some tumor cells[18,19]. In the present study, to clarify an involvement of LPA signaling pathway in TPA-stimulated cell migration of tumor cells, we investigated cell migration of mouse lung tumor cells treated with TPA and measured the expression levels of LPAR genes in those cells. We used the two cell lines, exogenous LPA_3 gene (*Lpar3*)-expressing (LL/2-a3) cells and LL/2-AB (vector) cells. LL/2-a3 cells showed significantly lower cell migration ability than control LL/2-AB cells[15].

LL/2-a3 cells were generated from mouse LL/2 lung tumor cells using retroviruses coexpressing green fluorescent protein (GFP) from an internal ribosomal entry site as described previously[15]. LL/2-AB cells were also used as a control clone. All cells were cultured in DMEM containing 10% fetal bovine serum in a 5% CO_2 atmosphere at 37 °C.

For the cell migration assay, an uncoated Cell Culture Insert (BD Falcon, Franklin Lakes, NJ, USA) with an 8-μm pore size was used. Cells were pretreated with TPA (5 nM)
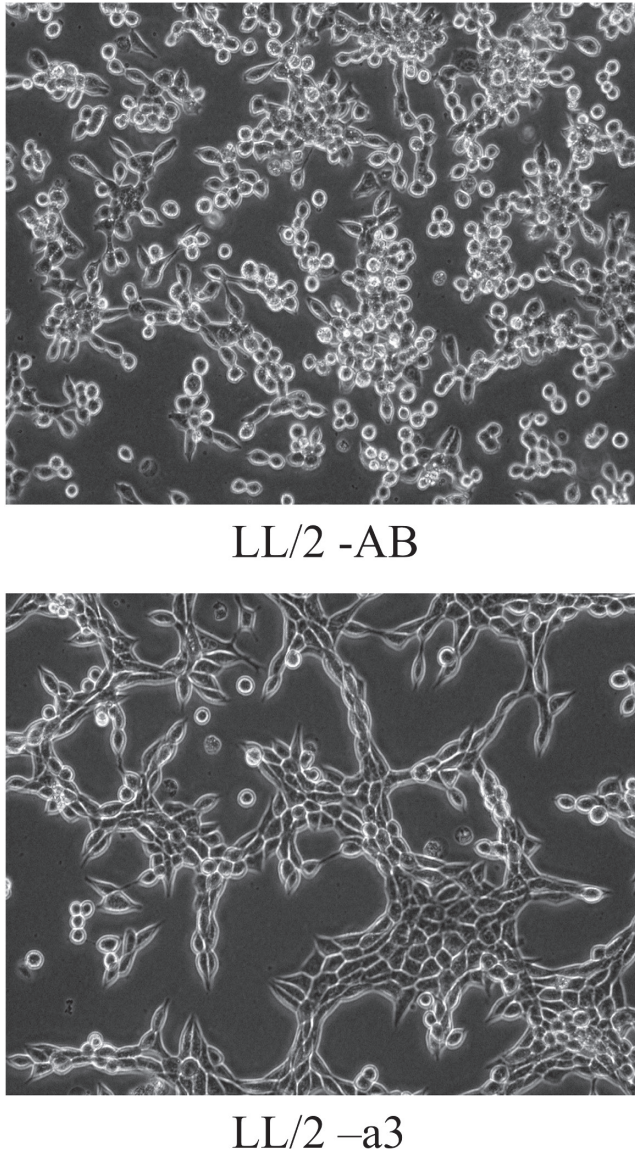
LL/2 -AB

LL/2 –a3

Fig. 1. Morphology of LL/2-AB (vector) and LL/2-a3 (*Lpar3*-expressing) cells in serum-containing medium.

(Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 48 h and seeded into the filter at 1×10^5 cells in 200 µl of serum-free DMEM (upper chamber) and placed in 24-well plates (lower chamber) containing 800 µl of 5% charcoal stripped FBS (Sigma Biochemicals, St. Louis, MO, USA) –DMEM with or without LPA (10 µM) (Avanti Polar Lipids, Inc., Alabaster, AL, USA). After incubation for 24 h, cells remaining in the upper side of the filter were removed with cotton swabs. The percentage of cells migrated to the lower side of the filter was counted after Giemsa staining. Each experiment was repeated three times[15].

Total RNA was extracted from each cell using ISO-GEN (Nippon Gene Co., Ltd., Toyama, Japan), and first-strand cDNA was synthesized with Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH,

Mannheim, Germany). To assess the expression patterns the *Lpar1, Lpar2 and Lpar3 genes* in TPA-treated cells, reverse transcription (RT)-polymerase chain reaction (PCR) analysis was performed[15]. The amplified PCR products were separated on 2% agarose gels containing 0.05 µg/ml ethidium bromide. The expression levels of those genes were also measured by quantitative real-time RT-PCR analysis using a SYBR Premix Ex Taq (TaKaRa Bio, Inc., Shiga, Japan) and a SmartCycler II System (TaKaRa)[13]. The rat *Gapdh* gene was used as an internal control gene. The data of the target genes were normalized to *Gapdh*. Each assay was repeated at least twice for confirmation.

The morphological appearances of the LL/2-AB and LL/2-a3 cells used in this study are shown in Fig. 1. The cell growth rate between the two types of cell indicated the same levels in DMEM containing 10% FBS (data not shown).

To assess cell migration of LL/2-AB and LL/2-a3 cells stimulated by TPA, the cells were treated with TPA for 48 h. While LL/2-a3 cells showed lower cell migration than LL/2-AB cells, the cell migration abilities of LL/2-AB and LL/2-a3 cells were significantly stimulated by TPA treatment (Fig. 2A). Furthermore, to evaluate the effect of LPA on cell migration stimulated by TPA, LPA was added into the lower chamber of the Cell Culture Insert and TPA-treated cells were incubated for 24 h. LPA treatment significantly increased the cell migration of both types of cells treated with TPA (Fig. 2B).

The LPAR gene expressions in LL/2-AB and LL/2-a3 cells treated with TPA were measured by semiquantitative RT-PCR and quantitative real-time RT-PCR analyses. No effect of TPA treatment on the expression levels of *Lpar1*, *Lpar2* and *Lpar3* was detected in either type of cells (Fig. 3A, B).

LPA$_3$ acts as a positive or negative regulator of cell migration, depending on the cell type. In human ovarian cancer cells, exogenous *LPAR1*, *LPAR2* or *LPAR3*-expressing cells acquired high malignant potency, and transfection of small interfering RNAs suppressed cell migration and invasion of those cells[14]. By contrast, the exogenous *Lpar3*-expressing LL/2-a3 cells showed lower cell migration than control LL/2-AB cells[15].

TPA has several biological effects, including the inhibition of gap junctional intercellular communication and stimulation of cell migration through activation of PKC in various cell types[16–19]. Thus, to clarify the involvement of LPA$_3$ in cell migration of mouse lung tumor cells treated with TPA, we investigated cell migration of LL/2-a3 and LL/2-AB cells treated with TPA, and measured the expression levels of LPA receptor genes in those cells. The results indicated that TPA stimulated the cell migration abilities of both LL/2-a3 and LL/2-AB cells, but did not affect the expression levels of their LPAR genes, suggesting that LPA$_3$ may not be involved in cell migration of mouse lung tumor cells stimulated by TPA.

A previous report showed that LPARs may form heterodimers with other receptors, resulting in novel signaling and different functional behaviors[20]. Therefore, the involve-
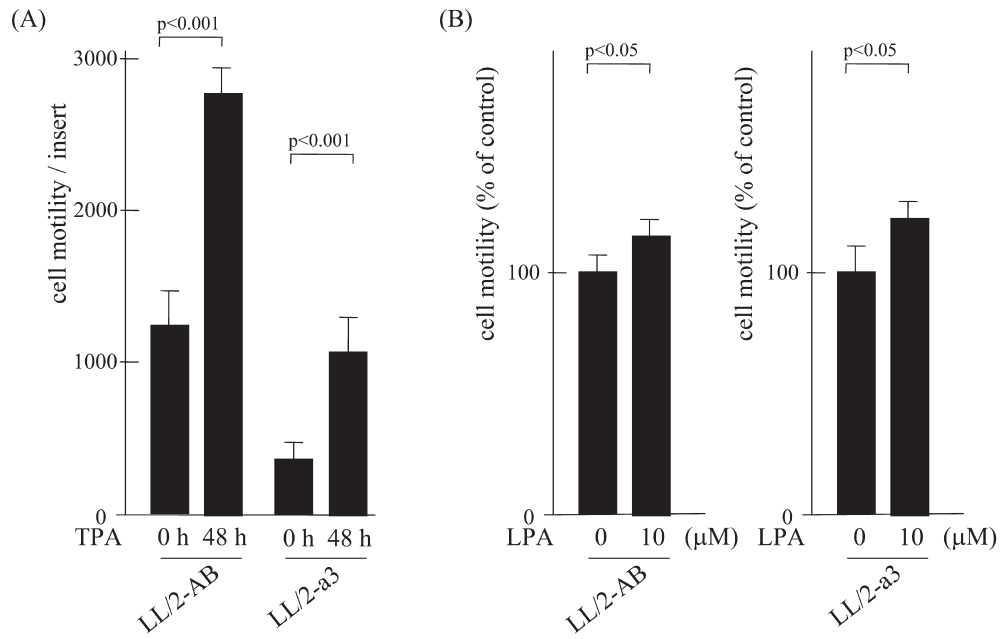
Fig. 2. The cell migration assay. Columns indicate the means of three studies; bars indicate SD. (A) Cell migration of LL/2-AB and LL/2-a3 cells stimulated by TPA. Cells were treated with or without TPA for 48 h. (B) Effects of LPA on TPA-stimulated cell migration of LL/2-AB and LL/2-a3 cells. TPA-treated cells were incubated for 24 h with or without LPA (10 μM). Cells incubated without LPA were used as the control.
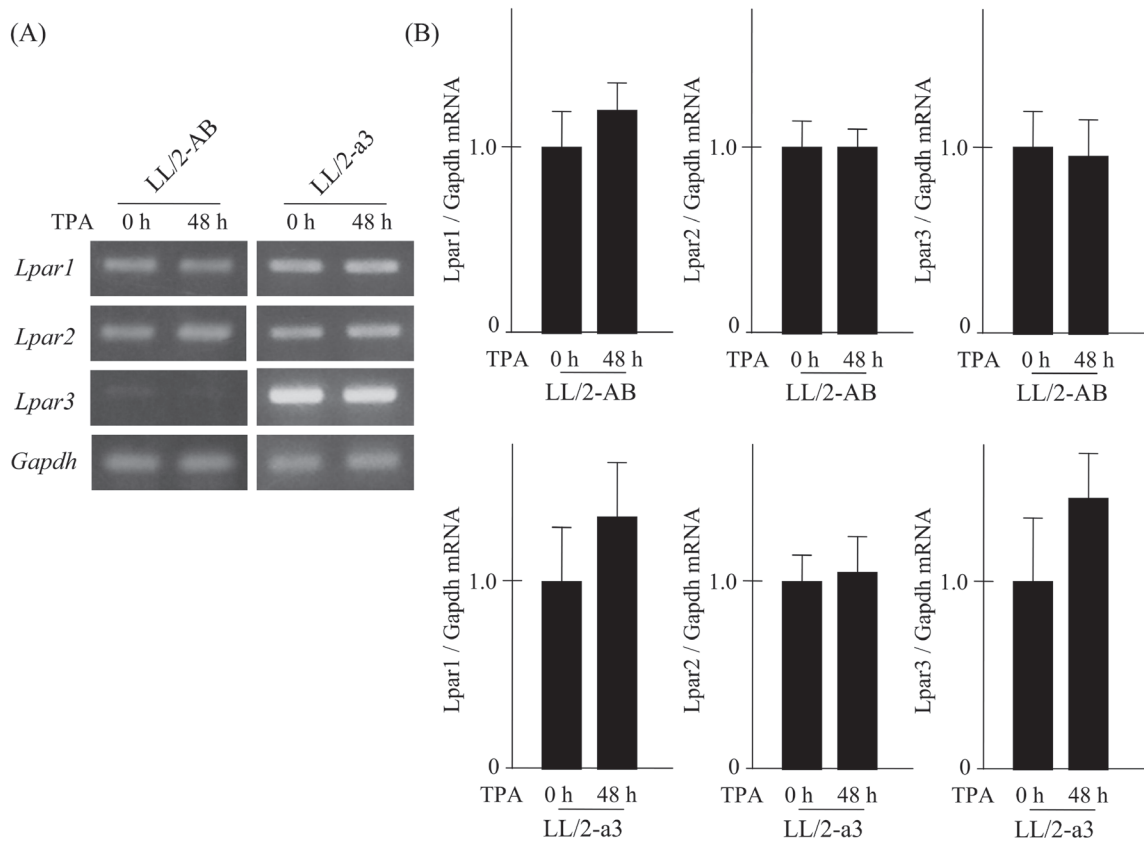

Fig. 3. (A) Expression patterns of LPAR gene mRNAs by semiquantitative RT-PCR analysis. (B) Relative expression levels of LPAR gene mRNAs relative to *Gapdh* mRNA by quantitative real-time RT-PCR analysis. LL/2-AB and LL/2-a3 cells were treated with TPA for 48 h.

ment of other LPARs in cell migration of tumor cells treated with TPA should be further studied.

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