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Involvement of aberrant DNA methylation on reduced expression of lysophosphatidic acid receptor-1 gene in rat tumor cell lines ☆

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Abstract

Lysophosphatidic acid (LPA) is a bioactive phospholipid that stimulates cell proliferation, migration, and protects cells from apoptosis. It interacts with specific G protein-coupled transmembrane receptors. Recently, it has been reported that alterations of LPA receptor expression might be important in the malignant transformation of tumor cells. Therefore, to assess an involvement of DNA methylation in reduced expression of the LPA receptor-1 (lpa1) gene, we investigated the expression of the lpa1 gene and its DNA methylation patterns in rat tumor cell lines. Both rat brain-derived neuroblastoma B103 and liver-derived hepatoma RH7777 cells used in this study indicated no expression of lpa1. For the analysis of methylation status, bisulfite sequencing was performed with B103 and RH7777 cells, comparing with other lpa1 expressed cells and normal tissues of brain and liver. The lpa1 expressed cells and tissues were all unmethylated in this region of lpa1. In contrast, both B103 and RH7777 cells were highly methylated, correlating with reduced expression of the lpa1. Treatment with 5-aza 2′-deoxycytidine induced expression of lpa1 gene in B103 and RH7777 cells after 24 h. In RH7777 cells treated with 5-aza 2′-deoxycytidine, stress fiber formation was also observed in response to LPA in RH7777 cells, but not in untreated RH7777 cells. These results suggest that aberrant DNA methylation of the lpa1 gene may be involved in its reduced expression in rat tumor cells.

Keywords: Lysophosphatidic acid receptor-1; DNA methylation; Gene silencing; Rat

Lysophosphatidic acid (LPA) is a membrane-derived bioactive mediator and has several cellular effects, including regulation of cell proliferation, differentiation, transcellular migration, morphogenesis, and protection from apoptosis [1–6]. LPA can induce cell proliferation, migration, invasion, and production of angiogenic factors in human ovarian cancer cell lines, suggesting that LPA may play an important role in the development of tumor cells [2,3,6–9]. LPA interacts with at least five G protein-coupled transmembrane receptors, lysophosphatidic acid receptor-1 (LPA1), LPA2, LPA3, LPA4, and LPA5 [8–11]. LPA1 is ubiquitously expressed in normal tissues, but the expressions of other LPA receptor subtypes are relatively restricted, suggesting these receptors may have different biological functions regarding LPA [1,8,9]. Recently, aberrant expressions of LPA receptors (lpa1–lpa3) have been reported in human cancers, including ovarian, colorectal, and thyroid cancer, demonstrating that alteration of LPA receptor expression might be important

☆ *Abbreviations:* LPA, lysophosphatidic acid; RT, reverse transcription; PCR, polymerase chain reaction.

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in the malignant transformation of tumor cells as well as LPA *per se* [2,3,6,7,12,13]. However, the essential roles of LPA receptors in tumor biology have not been satisfactorily clarified.

Methylation of cytosine residues at CpG dinucleotides is a strong effector for the suppression of gene expression in mammalian genomes [14–18]. Furthermore, cytosine methylation can reduce the binding affinity of transcription factors and influence chromatin structure [15,16,19,20]. It has been proposed that aberrant DNA methylation of gene promoter regions is one of the major mechanisms of gene silencing in several tumors [15,21–24].

In the present study, to clarify the relationship between aberrant expression of the lpa1 and DNA methylation we measured DNA methylation status of the lpa1 gene in rat brain-derived neuroblastoma B103 and rat liver-derived hepatoma RH7777 cells, which did not express lpa1, and demonstrated that lpa1 gene silencing may be due to its aberrant DNA methylation.

Materials and methods

Cell culture and animals. The cell lines used in this study were the rat fibroblast cell WLfbt from normal lung tissue (passage 6), RLCNR from *N*-nitrosobis(2-hydroxypropyl)amine-induced rat lung adenocarcinoma [25], COS1NR from 4-hydroxy (amino) quinoline 1-oxide-induced rat osteosarcoma [26], and MFH1NR from 4-hydroxy (amino) quinoline 1-oxide-induced rat malignant fibrous histiocytoma (MFH) [26], all of which were previously established in our laboratory. B103 rat neuroblastoma and RH7777 rat hepatoma cells were gifts from Drs. D. Schubert and H. Leffert, respectively [27]. All cell lines were cultured in DMEM containing 10% fetal bovine serum in 5% CO_2 atmosphere at 37 °C. In order to obtain normal brain and liver tissues, F344 male rats (Japan SLC Inc., Shizuoka, Japan), 6 weeks old, were used.

Reverse transcription (RT)-polymerase chain reaction (PCR) amplification for the lpa1 gene expressions. Total RNA was extracted from frozen tissue using ISOGEN (Nippon Gene, Inc. Toyama, Japan) and first-strand cDNA was synthesized from 0.2 μg samples with Ready-To-Go YourPrime First-Strand Beads (Pharmacia Co. Ltd., Tokyo, Japan). To eliminate possible false positives caused by residual genomic DNA, all samples were treated with DNase.

Semi-quantitative RT-PCR analysis was performed as described previously [28]. PCR amplification was carried out in a reaction volume of 20 μl containing 1 μl of each gene primer, 200 μl of each dNTP, 1× PCR buffer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA), 0.5 U of AmpliTaq Gold (Perkin-Elmer), and 0.5 μl of synthesized cDNA mixture. Primer pairs were as follows: for rat lpa1 (GenBank Accession No. AF090347), 5′-CGGGATTGGTCTTGCTACTG-3′ (sense) and 5′-CATCTCTTTGTCGCGGTAGG-3′ (antisense) (annealing temperature: 64 °C), and for rat glyceraldehyde-3-phosphate dehydrogenase (Gapdh), 5′-TTGTGAAGGTCGGTGTGAAC-3′ (sense) and 5′-AGGGGTCGTTGATGGCAACA-3′ (antisense) (annealing temperature: 55 °C). The rat Gapdh gene was used as an internal control gene. For each gene, multiple cycles of PCR amplification were tested. The cycle at which a sample having the highest expression reached an amplification plateau was determined, and a cycle number smaller than this was adopted for the analysis. The amplified products were then separated on 2% agarose gels containing 0.05 μg/ml ethidium bromide.

Bisulfite sequencing. Bisulfite treatment of genomic DNA was performed as previously described [28]. Briefly, genomic DNA was extracted with a DNeasy tissue kit (Qiagen, Hilden, Germany) from frozen tissues, and 500 ng of each sample was digested with the *Bam*HI (New England Biolabs Inc., MA, USA) restriction enzyme. The digested DNA was denatured in 0.3 N NaOH, then 2.9 M sodium bisulfite (Sigma, St. Louis, MO, USA) and 0.5 mM hydroquinone (Sigma) were added and the mixture underwent 15 cycles of 30 s denaturation at 95 °C and 15 min incubation at 50 °C. The sample was then desalted with the Wizard DNA cleanup system (Promega, Madison, WI, USA) and desulfonated by treatment with 0.3 N NaOH at room temperature for 5 min. After ethanol precipitation with ammonium acetate, DNA was dissolved in distilled water.

For bisulfite sequencing, PCR was performed with the following primer sets; lpa1-BS-F: 5′-GTGATAGAGGTGGGTGTGTTTGAT-3′, lpa1-BS-R: 5′-CACTATACTAAAAAACAAAAATCACA-3′ (annealing temperature: 62 °C) (GenBank Accession No. NW_047713). PCR products were subcloned with a TOPO TA cloning kit (Invitrogen Corporation, CA, USA) and sequenced with a BigDye terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems Japan Ltd.) and an ABI PRISM 310 genetic analyzer (Applied Biosystems Japan Ltd.). For each sample, eight clones were sequenced.

5-Aza 2′-deoxycytidine treatment for lpa1 induction. 5-Aza 2′-deoxycytidine (Sigma) was dissolved in phosphate saline buffer. Aliquots were prepared and frozen at −80 °C. Exponentially grown cells were treated with 5-aza 2′-deoxycytidine at a concentration of 10 μM for different times (24 and 48 h), and cells then harvested at each time point to analyze changes of Lpa1 expression [28].

Stress fiber formation assay. RH7777 cells were cultured on poly-L-lysine-coated coverslips (3000 cells per coverslip) in the absence or presence of 5-aza 2′-deoxycytidine at a concentration of 10 μM for 24 h. Cells were further cultured with or without 5-aza 2′-deoxycytidine under serum-free conditions for 15 h, exposed to 1 μM LPA for 15 min, and stained for actin filaments (f-actin) with tetramethyl rhodamine isocyanate (TRITC)-phalloidin (0.1 μg/ml) as previously described [27].

Results

The expression levels of Lpa1 in rat tumor cell lines were measured by semi-quantitative RT-PCR analysis. Representative results are shown in Fig. 1. While RLCNR, COS1NR, and MFH1NR cell lines expressed lpa1 at levels similar to the rat fibroblast cell WLfbt from normal lung tissue, its expressions were markedly reduced in B103 and RH7777.

To assess an involvement of aberrant DNA methylation on reduction of lpa1 expression, we performed a bisulfite sequence analysis to measure methylation status of the 5′ upstream region of lpa1 (between nt −742 and −340), which contains 20 CpG sites, in rat tumor cell lines. Normal fibroblast cell WLfbt and tumor cell lines RLCNR, COS1NR, and MFH1NR showed a demethylated status in this region. In contrast, B103 and RH777 were highly

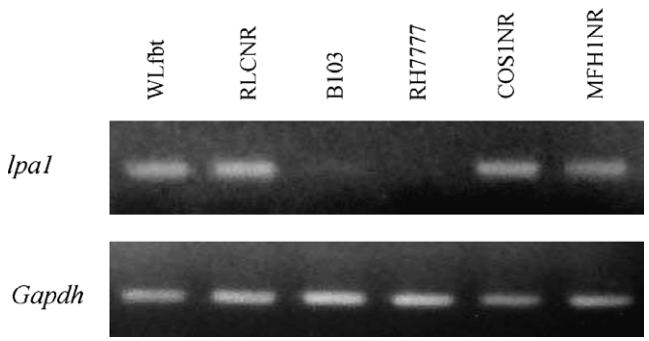

Fig. 1. Expression of lpa1 mRNA by semi-quantitative RT-PCR analysis.