Biochemical and Biophysical Research Communications 405 (2011) 450–454

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Loss of lysophosphatidic acid receptor-3 enhances cell migration in rat lung tumor cells

Mai Hayashi [a], Kyoko Okabe [a], Yasuna Yamawaki [a], Miki Teranishi [a], Kanya Honoki [b], Toshio Mori [c], Nobuyuki Fukushima [d], Toshifumi Tsujiuchi [a,*]

[a] Division of Cancer Biology and Bioinformatics, Department of Life Science, Faculty of Science and Engineering, Kinki University, 3-4-1, Kowakae, Higashiosaka, Osaka 577-8502, Japan
[b] Department of Orthopedic Surgery, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521, Japan
[c] RI Center, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521, Japan
[d] Division of Molecular Neurobiology, Department of Life Science, Faculty of Science and Engineering, Kinki University, 3-4-1, Kowakae, Higashiosaka, Osaka 577-8502, Japan

ARTICLE INFO

Article history:
Received 13 January 2011
Available online 19 January 2011

Keywords:
Lpar3
Cell migration
Lung tumor
Rat

ABSTRACT

Lysophosphatidic acid (LPA) indicates several biological effects, such as cell proliferation, differentiation and migration. LPA interacts with G protein-coupled transmembrane LPA receptors. In our previous report, we detected that loss of the LPA receptor-1 (Lpar1) expression is due to its aberrant DNA methylation in rat tumor cell lines. In this study, to assess an involvement of the other LPA receptor, Lpar3, in the pathogenesis of rat lung tumor cells, we measured the expression levels of the Lpar3 gene and its DNA methylation status by reverse transcription (RT)-polymerase chain reaction (PCR) and bisulfite sequencing analyses, respectively. RLCNR lung adenocarcinoma cells showed reduced expression of the Lpar3, compared with normal lung tissues. In the 5′ upstream region of the Lpar3, normal lung tissues were unmethylated. By contrast, RLCNR cells were highly methylated, correlating with reduced expressions of the Lpar3. Based on these results, we generated the Lpar3-expressing RLCNR-a3 cells and measured the cell migration ability. Interestingly, the cell migration of RLCNR-a3 cells was significantly lower than that of RLCNR cells. This study suggests that loss of the Lpar3 due to aberrant DNA methylation may be involved in the progression of rat lung tumor cells.

1. Introduction

Lysophosphatidic acid (LPA) interacts with at least six G protein-coupled transmembrane receptors, LPA receptor-1 (Lpar1), Lpar2, Lpar3, Lpar4, Lpar5 and Lpar6 [1,2]. LPA exhibits several biological effects through the binding of LPA receptors, such as cell proliferation, differentiation, migration and morphogenesis [1–3]. LPA can also stimulate cell growth, migration and invasion of ovarian cancer cells, suggesting that LPA may play important roles in the development and progression of tumor cells [4–7]. Moreover, aberrant expression levels of LPA receptors have been reported in human ovarian, colorectal and thyroid tumors, indicating that alterations of LPA receptors might be also involved in the malignant transformation of tumor cells as well as LPA per se [4–9]. Recently, we have also demonstrated that aberrant expression levels of LPA receptor genes due to aberrant DNA methylation were found in rat and human tumor cells [10,11].

In our previous report, mutations of the Lpar1 gene occurred in not only lung adenocarcinomas, but also preneoplastic lesions induced by N-nitrosobis(2-hydroxypropyl)amine (BHP) in rats [12]. By contrast, no mutation of Lpar2, Lpar3, Lpar4 and Lpar5 genes was found in the same lung lesions [13]. Therefore, it suggests that alterations of the Lpar1 may be more important in rat lung carcinogenesis rather than other LPA receptor gene mutation. However, the biological roles of those LPA receptors in rat lung tumor cells remain to be clarified.

In this study, to assess an involvement of other LPA receptors in the pathogenesis of tumor cells, we measured the Lpar3 gene expression and its DNA methylation status in rat lung tumor cells. Moreover, we generated the Lpar3-expressing cells and investigated the cell migration ability in these cells, using a cell migration assay. RLCNR lung tumor cells used in this study were derived from lung adenocarcinoma induced by BHP in rats [14].

2. Materials and methods

2.1. Cell culture and animals

The rat tumor cells used in this study were RLCNR, COS osteosarcoma, B103 neuroblastoma and C6 glioma cells. All cell lines

Abbreviations: LPA, lysophosphatidic acid; Lpar3, LPA receptor-3; RT, reverse transcription; PCR, polymerase chain reaction; BHP, N-nitrosobis(2-hydroxypropyl)-amine.

* Corresponding author. Fax: +81 6 6721 2721.
E-mail address: ttujiuch@life.kindai.ac.jp (T. Tsujiuchi).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.01.051

were cultured in DMEM containing 10% FBS in a 5% CO_2 atmosphere at 37 °C. Mouse LL/2 lung tumor cells were also cultured under the same condition. In order to obtain normal lung and brain tissues, F344 male rats (Japan SLC Inc., Shizuoka, Japan), 6 weeks old, were used.

2.2. Reverse transcription (RT) – polymerase chain reaction (PCR) analysis for the Lpar3 gene expression

Total RNA was extracted from each cell and tissue using ISOGEN (Nippon Gene, Inc., Toyama, Japan) and first-strand cDNA was synthesized from 0.5 μg samples with Transcriptor First-Strand cDNA Synthesis Kit (Roche Diagnostics Co. Ltd., Mannheim, Germany). Semi-quantitative RT-PCR analysis was performed as described previously [10,11]. Primer pairs were as follows: for rat Lpar3 (NCBI Accession No. NC_005101), 5′-CTCGTACAAGGACGAGGACAT-3′ and 5′-TGAGACAGGCAAGGACTCTTA-3′, and for rat glyceraldehyde-3-phosphate dehydrogenase (Gapdh), 5′-TTGTGAAGGTC GGTGTGAAC-3′ and 5′-AGGGGTCGTTGATGGCAACA-3′. The amplified products were then separated on 2% agarose gels containing 0.05 μg/ml ethidium bromide.

2.3. Bisulfite sequencing analysis for DNA methylation status of the Lpar3 gene

Genomic DNA was extracted with a DNeasy tissue kit (QIAGEN, Hilden, Germany) from each cell and tissue, and bisulfite treatment of genomic DNA was performed with an EpiTect Bisulfire Kit (QIAGEN). For bisulfite sequencing, PCR was performed with the following primer sets; Lpar3-BS-F: 5′-GGTTTGGATGTTATTAGTAGG AAA-3′, Lpar3-BS-R: 5′-CATCCACTTATCATAATAACACTC-3′ (NCBI Accession No. NW_047633.2). 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) and an ABI PRISM 310 genetic analyzer (Applied Biosystems Japan). For each sample, eight clones were sequenced [10,11].

2.4. Establishment of Lpar3-expressing cells

To generate Lpar3-expressing cells, we used retroviruses co-expressing green fluorescent protein (GFP) from an internal ribosomal entry site. Retroviruses expressing FLAG-tagged Lpar3 were prepared by transfection of Phoenix cells with S001-AB plasmids containing Lpar3, as described previously [15]. Control retroviruses were generated by using S001-AB plasmids. RLCNR and LL/2 cells were infected with retroviruses expressing Lpar3 gene in the presence of 5 μg/ml polybrene (Sigma Biochemicals, St. Louis, MO). Infected cells were harvested, and about 100 cells were sparsely replated in a 15-cm-diameter plate. Colonies in which all cells expressed GFP were picked up and propagated further [15].

2.5. Cell proliferation assay

Cells were plated at 2000 cells/well in a 96-well plate and cultured with 100 μl of DMEM containing 10% FBS. To measure cell growth for 3 days, solution from a Cell Counting Kit-8 (CCK-8) (Dojin Chemistry, Kumamoto, Japan) was added to the plate at 0, 1, 2 or 3 days and cells were further incubated for 1 h. The absorbance of the culture medium at 450 nm was determined. The assay was always done in triplicate [15].

2.6. Scrape assay

Cells were plated onto a 6-well plate and cultured until confluence in DMEM containing 10% FBS. A 1000 μl tip was used to make a scrape line. The wells were washed twice with PBS and then incubated in serum-free DMEM with or without LPA (10 μM). The photographs were taken at 0 and 7 h after scrape.

2.7. Cell motility assay

For motility assay, an uncoated Cell Culture Insert (BD Falcon, NJ, USA) with 8 μm pore size was used. Briefly, cells were seeded in the filter at 1×10^5 cells in 200 μl of serum-free DMEM (upper chamber) and placed in 24-well plate (lower chamber) containing 600 μl of serum-free DMEM with or without LPA (10 μM). Cells were incubated 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.

3. Results

The expression levels of the Lpar3 in rat tumor cells were measured by the semi-quantitative RT-PCR analysis. To compare the Lpar3 expression patterns, we used not only RLCNR cells but also other three tumor cells. Representative results are shown in Fig. 1(A). While the Lpar3 was expressed in COS and C6 cells, RLCNR and B103 cells markedly reduced the Lpar3 expressions. By contrast, normal lung and brain tissues expressed the Lpar3 gene (Fig. 1(B)). We next performed the bisulfite sequence analysis to measure methylation status of the 5′ upstream region of Lpar3 (between nt. −418 and 12), which contains 15 CpG sites, in rat tumor cells. COS and C6 cells were weakly or moderately methylated, but RLCNR and B103 cells were highly methylated, correlating with reduced expressions of the Lpar3. Normal lung and brain tissues showed weakly methylated status of the Lpar3 (Fig. 1(C)).

To address biological roles of the Lpar3 in the pathogenesis of rat lung tumor cells, we generated the Lpar3-expressing RLCNR-a3 cells (Fig. 2), and assessed the cell migration ability, compared with the Lpar3-unexpressing control RLCNR-AB cells. In scrape assay, both cells were treated with or without LPA for 7 h after scrape. RLCNR-AB cells showed high cell migration and LPA treatment enhanced it. By contrast, RLCNR-a3 indicated lower cell migration than RLCNR-AB cells (Fig. 3(A)). We next performed cell motility assay with Cell Culture Insert. The cell motility of RLCNR-a3 cells showed significantly lower than that of RLCNR-AB cells. LPA treatment significantly increased the cell motility of both cells (Fig. 3(B)). In our recent report, the reduced Lpa3 gene expression due to aberrant DNA methylation was found in mouse lung tumor LL/2 cells [16]. Therefore, we also generated Lpar3-expressing LL/2-a3 cells and measured cell motility, compared with the Lpar3-unexpressing control LL/2-AB cells. LL/-a3 cells indicated significantly lower cell motility than LL/2-AB cells as well as rat lung tumor cells (Fig. 3(C)).

4. Discussion

In this study, we indicated that the reduced Lpar3 expression due to aberrant DNA methylation was detected in rat lung tumor cells, while the normal lung tissue expressed the Lpar3. Previously, it has been reported that various levels of the Lpar3 gene expression were found in human cancer cells. The Lpar3 gene expression elevated in ovarian cancer cells, compared with normal ovarian epithelial cells [5]. By contrast, Lpar3 expression levels were relatively low in human colorectal cancers [8]. In our recent studies, we have also reported that a variety of the Lpar3 expression levels were found in human colon cancer cells and mouse tumor cells, correlating with DNA methylation status of the Lpar3 gene [11,16]. Therefore, suggests that the Lpar3 expression levels may be dependent on the type of cancer cells.