Experimental Cell Research 352 (2017) 139–145

Contents lists available at ScienceDirect

Experimental Cell Research

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

Lysophosphatidic acid signaling via LPA_1 and LPA_3 regulates cellular functions during tumor progression in pancreatic cancer cells

Kaori Fukushima[a], Kaede Takahashi[a], Eri Yamasaki[a], Yuka Onishi[a], Nobuyuki Fukushima[b], Kanya Honoki[c], Toshifumi Tsujiuchi[a,*]

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

ARTICLE INFO

Keywords:
LPA
LPA receptor
Cisplatin
Tumor progression
Pancreatic cancer cells

ABSTRACT

Lysophosphatidic acid (LPA) signaling via G protein-coupled LPA receptors exhibits a variety of biological effects, such as cell proliferation, motility and differentiation. The aim of this study was to evaluate the roles of LPA_1 and LPA_3 in cellular functions during tumor progression in pancreatic cancer cells. LPA_1 and LPA_3 knockdown cells were generated from PANC-1 cells. The cell motile and invasive activities of PANC-1 cells were inhibited by LPA_1 and LPA_3 knockdown. In gelatin zymography, LPA_1 and LPA_3 knockdown cells indicated the low activation of matrix metalloproteinase-2 (MMP-2) in the presence of LPA. Next, to assess whether LPA_1 and LPA_3 regulate cellular functions induced by anticancer drug, PANC-1 cells were treated with cisplatin (CDDP) for approximately 6 months. The cell motile and invasive activities of long-term CDDP treated cells were markedly higher than those of PANC-1 cells, correlating with the expression levels of *LPAR1* and *LPAR3* genes. In soft agar assay, the long-term CDDP treated cells formed markedly large sized colonies. In addition, the cell motile and invasive activities enhanced by CDDP were significantly suppressed by LPA_1 and LPA_3 knockdown as well as colony formation. These results suggest that LPA signaling via LPA_1 and LPA_3 play an important role in the regulation of cellular functions during tumor progression in PANC-1 cells.

1. Introduction

Lysophosphatidic acid (LPA) is an extracellular physiological lipid, consisting of a glycerol, a phosphate and a fatty acid [1–4]. LPA exhibits a variety of biological responses with G protein-coupled transmembrane LPA receptors. At least six subtypes of LPA receptors have been identified as LPA receptor-1 (LPA_1) to LPA_6 so far. LPA receptors can be divided structurally into two subtypes. LPA_1, LPA_2 and LPA_3 are members of the endothelial cell differentiation gene (Edg) family. LPA_4, LPA_5 and LPA_6 have been classified as non-Edg LPA receptors which belong to the purinergic receptor family [1–4].

It has been reported that alterations of LPA receptors are involved in the pathogenesis of cancer cells as well as LPA per se [5,6]. Indeed, the production of LPA was highly detected in blood and ascites from advanced ovarian cancer patients [7]. In our previous studies, frequent mutations of *Lpar1* gene occurred in lung and liver tumors of rats [8,9]. The reduced expressions of LPA receptor genes due to DNA hypermethylation were found in human colon cancer cells [10]. On the other hand, LPA signaling via LPA receptors regulates malignant property in cancer cells, such as cell proliferation, migration, angiogenesis and tumorigenicity. However, the biological effects of each LPA receptor are not uniform [5].

Recently, we have indicated that LPA_4 and LPA_5 negatively and LPA_6 positively regulated the cell motility, invasion and tumorigenicity of human pancreatic cancer PANC-1 cells [11]. In the present study, to evaluate the effects of other LPA receptors on cellular functions during tumor progression in pancreatic cancer cells, LPA_1 and LPA_3 knockdown cells were generated from PANC-1 cells. Since chemotherapy accelerated regrowth and elevated MMP-9 expression and metastatic potency of cancer cells [12–14], we also examined an involvement of LPA_1 and LPA_3 in the regulation of cellular functions induced by cisplatin (CDDP).

2. Materials and methods

2.1. Cell culture and CDDP treatment

PANC-1 cells were cultured in Dulbecco's modified Eagle's medium

* Corresponding author.
E-mail address: ttujiuch@life.kindai.ac.jp (T. Tsujiuchi).

http://dx.doi.org/10.1016/j.yexcr.2017.02.007
Received 24 November 2016; Received in revised form 6 February 2017; Accepted 7 February 2017
Available online 08 February 2017

(DMEM) (Wako Pure Chemical Industries Ltd., Osaka, Japan) containing 10% fetal bovine serum (FBS) in a 5% CO_2 atmosphere at 37 °C. To generate the long-term CDDP treated (PANC-CDDP) cells, cells were maintained by the stepwise treatment of CDDP (Sigma, St. Louis, MO, USA) at a range of 0.01–3.0 μM for approximately 6 months [15,16].

2.2. Cell motility and invasion assays

Cells were seeded at 5×10^4 cells on a Cell Culture Insert (8 μm pore size) (BD Falcon, Franklin Lakes, NJ) in 200 μl serum-free DMEM (upper chamber) and placed in a 24-well plate (lower chamber) containing 800 μl of 5% charcoal stripped FBS (Sigma) – DMEM with or without LPA (10 μM). After incubation for 16 h, the numbers of cells that had moved to the lower side of the filters were counted after Giemsa staining. Before cell motility, some cells were pretreated with 100 ng/ml pertussis toxin (PTX) (Wako) for 24 h, 5 μM U-73122 (Cayman Chemical Co., Ann Arbor, MI, USA) for 30 min, 10 μM Y-27632 (Mitsubishi Pharma Co., Osaka, Japan) for 30 min or 10 μM dioctanoylglycerol pyrophosphate (DGPP) (Avanti Polar Lipids) for 30 min. For invasion assay, the filter was coated with Matrigel (12.5 μg/filter) (BD Falcon) and dried. Cells were seeded at 1×10^5 cells on the filter and incubated for 20 h [11,16].

2.3. Establishment of LPA_1 and LPA_3 knockdown cells

Using X-tremeGENE HP Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Germany), a HuSH short hairpin RNA plasmid (29-mer) against target LPA_1 or LPA_3 (Origene, Rockville, MD) was transfected into PANC-1 cells. After 3 days, cells were treated with puromycin (WAKO) for at least 3 weeks and a stable clone was obtained. Control cells were also generated using a vector plasmid without the target sequence [11,16].

2.4. Cell proliferation assay

Cells were seeded at 4000 cells/well in 96-well plates and maintained in DMEM containing 10% FBS. To measure the cell growth rate for 3 days, a solution from the Cell Counting Kit-8 (CCK-8) (Dojin Chemistry, Kumamoto, Japan) was added to each well at 0, 1 or 3 days. After 1 h, the absorbance of the conditioned medium at 450 nm was measured. In addition, to investigate the effects of LPA on cell proliferation activity, some cells were cultured with DMEM containing 5% charcoal stripped FBS (Sigma) and treated with LPA (Avanti Polar Lipids, Inc., AL, USA) at concentrations of 1 or 10 μM every 24 h for 3 days. These assays were performed in triplicate [11,16].

2.5. Gelatin zymography

Cells were cultured in serum-free DMEM with or without LPA (10 μM) for 2 days. The supernatants obtained from the individual cells were loaded on a 10% SDS-PAGE containing 0.1% gelatin. The gels were washed twice with washing buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl and 2.5% Triton X-100) for 30 min and incubated in reaction buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$ and 0.02% NaN_3) at 37 °C for 16 h. The gels were then stained with 0.25% Coomassie Brilliant Blue R250 (Wako) and destained. The bands were quantitated with image analysis software (NIH Image, Bethesda, MD) [11].

2.6. Quantitative real-time reverse transcription (RT) – polymerase chain reaction (PCR) analysis

The first strand cDNAs were synthesized with a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Co. Ltd., Mannheim, Germany) for quantitative real-time RT-PCR analysis. The expression levels of LPA receptor genes were measured by SYBR *Premix Ex Taq* (Tli RNaseH Plus) (TaKaRa Bio Inc., Shiga, Japan) and a Smart Cycler II System (TaKaRa), and normalized to those of *GAPDH* gene [16].

2.7. Synthesis of extracellular LPA by autotaxin (ATX)

To assess whether extracellular LPA was synthesized by ATX in conditioned mediums, cells were maintained in serum-free DMEM for 2 days. Before cell motility assay, lysophosphatidylcholine (LPC) (10 μM) (Avanti Polar Lipids) was added in 800 μl of conditioned mediums obtained from the individual cells (lower chamber). After incubation for 30 min, mouse endothelial F-2 cells were seeded at 1×10^5 cells on the filters in 200 μl serum-free DMEM (upper chamber) and incubated for 16 h. In addition, S32826 (5 μM) (Cayman Chemical Co.) which is an ATX inhibitor was added in conditioned mediums before LPC treatment [16].

2.8. Soft agar assay

Cells were suspended in DMEM containing 10% FBS and 0.4% low-melting-point agarose, and seeded at 1×10^4 cells on a bottom layer containing 0.8% agarose in 3-cm diameter well. The cells were photographed on day 14 after plating and measured the size of at least 8 colonies [17].

2.9. Statistical analysis

Analysis of variance (ANOVA) was performed to evaluate statistical significance. The data were recognized to differ significantly for values of $p < 0.01$. The results are given as means ± SD.

3. Results

3.1. Effects of LPA signaling inhibitors on cell motile activity of PANC-1 cells

The expression patterns of the *LPAR1* and *LPAR3* genes in PANC-1 cells by semi-quantitative RT-PCR analysis are shown in Fig. 1A. Before cell motility assay, cells were pretreated with PTX, U-73122 and Y-27632. The cell motile activity of PANC-1 cells was significantly suppressed by PTX, U-73122 and Y-27632. In addition, cells were pretreated with DGPP. In the presence of LPA (10 μM), DGPP inhibited the cell motile activity of PANC-1 cells (Fig. 1B).

3.2. Effects of LPA receptor knockdown on cellular functions of PANC-1 cells

To assess the effects of LPA_1 and LPA_3 on cellular functions of PANC-1 cells, LPA_1 and LPA_3 knockdown cells were generated (PANC-L1 and PANC-L3 cells, respectively) (Fig. 2A). LPA_1 or LPA_3 knockdown did not affect the expression levels of other LPA receptor genes in PANC-1 cells (data not shown). The cell growth rates of PANC-L1 and PANC-L3 cells were higher than that of control PANC-G cells (Fig. 2B). PANC-L1 and PANC-L3 cells showed the low cell motility, compared with PANC-G cells. The cell motile activities of PANC-L1 and PANC-L3 cells were reduced by LPA, but not PANC-G cells (Fig. 2C). In cell invasion assay, the invasive activities of PANC-L1 and PANC-L3 cells were markedly lower than that of PANC-G cells. LPA inhibited the cell invasive activities of PANC-L1 and PANC-L3 cells, while no change of cell invasive activity of PANC-G cells was observed (Fig. 2D). In gelatin zymography, PANC-L1 and PANC-L3 cells showed the low matrix metalloproteinase-2 (MMP-2) activities of PANC-L1 and PANC-L3 cells in the presence of LPA, compared with PANC-G cells. No activation of MMP-9 in all cells was detected (Fig. 3E and F).