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Short communication

Enhancement of Drug Resistance by Lysophosphatidic Acid Receptor-3 in Mouse Mammary Tumor FM3A Cells

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Abstract: Lysophosphatidic acid (LPA) acts as a simple phospholipid that interacts with G protein-coupled transmembrane LPA receptors. Recently, it has been reported that each LPA receptor plays different biological roles in acquisition of the malignant property of tumor cells. In this study, to assess the involvement of LPA receptor-3 (LPA_3) in cell survival after treatment with anticancer drugs, we generated *Lpar3*-expressing FM3A-a3A9 cells from mouse mammary tumor FM3A cells and examined the cell survival rate after treatment with anticancer drugs compared with *Lpar3*-unexpressing cells. Cells were treated with 0.005 to 10 μM of cisplatin (CDDP) or doxorubicin (DOX) for 3 days. For the CDDP and DOX treatments, the cell survival rate of FM3A-a3A9 cells was significantly higher than that of *Lpar3*-unexpressing cells. The expression level of the *Mdr1a* gene in FM3A-a3A9 cells was higher than that of *Lpar3*-unexpressing cells, whereas no significant difference in *multidrug resistance 1b (Mdr1b)* and *glutathione S-transferase mu1 (Gstm1)* expressions was found. These results suggest that LPA_3 may enhance the cell survival rate after treatment with anticancer drugs in mouse mammary tumor cells, correlating with increased expression of the *Mdr1* gene. (DOI: 10.1293/tox.25.225; J Toxicol Pathol 2012; 25: 225–228)

Key words: LPA, LPA receptor-3, drug resistance, FM3A, mammary, mouse

Lysophosphatidic acid (LPA) is a simple phospholipid that interacts with at least G protein-coupled LPA receptors, LPA_1 to LPA_6[1–3]. LPA has several cellular effects through the binding of LPA receptors, such as cell proliferation, migration, differentiation, morphogenesis and protection from apoptosis[1–3]. There are various expression patterns of LPA receptors in not only normal tissues but also tumor cells, suggesting that their biological functions may be essentially different[1–5]. In our recent studies using endogenous LPA_3 unexpressed cells, exogenous LPA_3 stimulated cell migration and tumorigenicity in rat liver tumor cells, whereas cell migration ability was inhibited by exogenous LPA_3 in lung tumor cells of the rat and mouse[6,7].

In cancer cells, multidrug resistance (MDR) refers to a phenomenon of simultaneous resistance to structurally and functionally unrelated anticancer drugs. It is considered that one of the most important mechanisms underlying the acquisition of multidrug resistance is the activation of efflux transporter proteins, such as P-glycoprotein, which is encoded by MDR genes[8,9]. Moreover, the induction of detoxification enzyme is also involved in multidrug resistance in cancer cells[10]. It is well known that glutathione S-transferases (GSTs) represent a major family of detoxification enzymes[10]. Recently, we reported that exogenous LPA_3 increased the cell survival rate after treatment with cisplatin (CDDP) and doxorubicin (DOX) in rat liver tumor cells[6].

Although aberrant expression levels of LPA_1 were detected in breast cancer cells, the biological functions of LPA_2 and LPA_3 in the pathogenesis of breast cancer are still unclear[11]. Multidrug resistance and poor clinical outcome are closely linked in several cancers, including breast cancer[8]. In the present study, to better understand the involvement of LPA_3 in the acquisition of multidrug resistance, we generated *Lpar3*-expressing cells from mouse mammary tumor FM3A cells, and investigated cell survival rate after CDDP and DOX treatment. Furthermore, the expression levels of *multidrug resistance 1a (Mdr1a)*, *Mdr1b* and *glutathione S-transferase mu1 (Gstm1)* genes were also measured. FM3A cells expressed *Lpar2*, but not *Lpar1* and *Lpar3* due to DNA methylation[12].

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical Industries, Ltd.,

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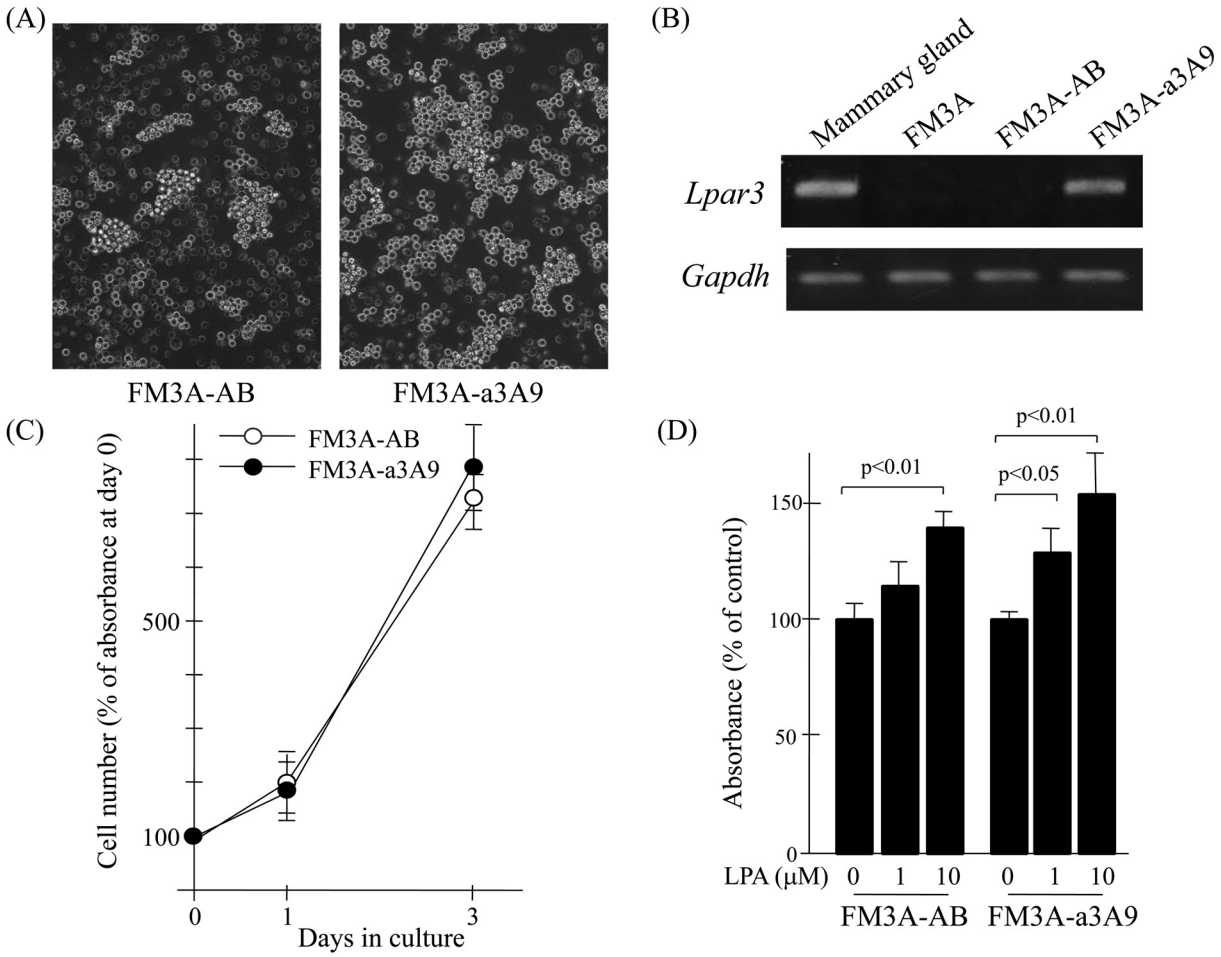

Fig. 1. (A) Morphology of FM3A-AB (control vector), and the *Lpar3*-expressing FM3A-a3A9 cells in serum-containing medium. (B) Expression patterns of the *Lpar3* gene in mouse mammary tumor cells by semiquantitative RT-PCR analysis. (C) Cell proliferation rate of mouse mammary tumor cells. Cells were cultured in serum-containing medium. Data are expressed as percentage of cell numbers on day 0. (D) The effects of LPA on cell growth in FM3A-AB and FM3A-a3A9 cells. Cells were cultured with or without LPA (1 or 10 μM), and cell proliferation was measured using a CCK-8. Columns indicate the means of three studies; bars indicate SD.

Osaka, Japan) containing 10% fetal bovine serum (FBS) in 5% CO_2 atmosphere at 37°C. To generate *Lpar3*-expressing (FM3A-a3A9) cells from FM3A cells, we used retroviruses co-expressing green fluorescent protein (GFP) from an internal ribosomal entry site as described previously[13,14]. As control cells, FM3A-AB (vector) cells were also used.

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 rate for 3 days, solution from a Cell Counting Kit-8 (CCK-8) (Dojin Chemistry, Kumamoto, Japan) was added to each well at 0, 1 or 3 days and further incubated for 1 h. The absorbance of the culture medium at 450 nm was then determined. To assess the effects of LPA on cell growth, cells were also cultured in serum-free DMEM with or without 1 or 10 μM LPA (Avanti Polar Lipids, Inc., Alabaster, AL, USA) for 3 days, which were added every 24 h[6,7,13–15]. To evaluate the effects of CDDP and DOX in the *Lpar3*-expressing cells, cells were treated with 0.005 to 10 μM of CDDP (Sigma Biochemicals) or DOX (Sigma Biochemicals) in DMEM containing 10% FBS for 3 days, which were added every 24 h[6]. Cell viability was also measured with CCK-8 (Dojin Chemistry)[6].

To evaluate the expression levels of *Mdr1a*, *Mdr1b* and *Gstm1* genes, quantitative real-time RT-PCR (RT, reverse transcription; PCR, polymerase chain reaction) analysis using SYBR Premix Ex Taq (TaKaRa Bio, Inc., Shiga, Japan) and a Smart Cycler II System (TaKaRa) was performed[6,14,15]. The primer pairs used in this study were as follows: for mouse *Mdr1a*, F 5'-AAATCCAGCGGCAGAACAGC-3', R 5'- TGCCAAATGTGAAGCCCTGA-3'; for mouse *Mdr1b*, F 5'-TCATGAAACTGCCCCACCAA-3', R 5'-GGGCAATGGCGATTCTCTGT-3'; and for mouse *Gstm1*, F 5'-AAGTTCAAGCTGGGCCTGGA-3', R 5'-GATGGCATTGCTCTGGGTGA-3'. The data of the target genes were normalized to *Gapdh*[6,14,15].

Since the *Lpar3* gene expression in FM3A cells was undetectable[12], we generated *Lpar3*-expressing FM3A-a3A9 cells (Fig. 1A). The *Lpar3* expression levels in each

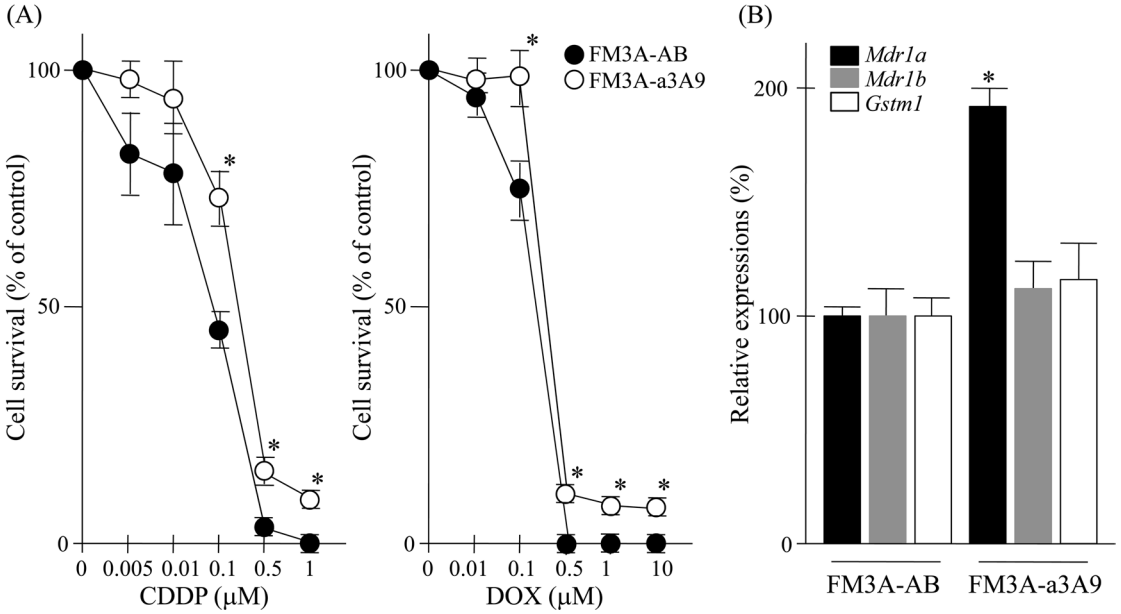

Fig. 2. (A) Cell survival rate of FM3A-AB and FM3A-a3A9 cells treated with CDDP or DOX. *P<0.01 vs. FM3A-AB cells. (B) Expression levels of *Mdr1a*, *Mdr1b* and *Gstm1* gene mRNAs relative to Gapdh mRNA by quantitative real-time RT-PCR analysis.

cell were confirmed by semi-quantitative RT-PCR analysis (Fig. 1B). The cell growth rate of each clone was the same in DMEM containing 10% FBS (Fig. 1C). To examine the effects of LPA on cell proliferation of each clone, cells were cultured in serum-free DMEM with or without LPA treatment. LPA significantly increased cell proliferation ability at 1 and 10 μM in FM3A-a3A9 cells, while the proliferation rate of FM3A-AB cells was stimulated at 10 μM of LPA (Fig. 1D). The stimulation of growth rate of FM3A-AB cells may be due to other expressed LPA receptors.

Next, to assess whether *Lpar3*-expressing cells may show multidrug resistance, two chemotherapeutic drugs with different modes of action, CDDP and DOX, were used. Cells were treated with CDDP or DOX for 3 days. The cell viability of FM3A-a3A9 cells was significantly higher at a dose of 0.1, 0.5 and 1 μM of CDDP than that of FM3A-AB cells (LC_{50}=0.24 μM and 0.09 μM, respectively). For DOX treatment, FM3A-a3A9 cells showed significantly higher cell viability at a dose of 0.1, 0.5, 1 and 10 μM than FM3A-AB cells (LC_{50}=0.23 μM and 0.18 μM, respectively) (Fig. 2A). Using quantitative real-time RT-PCR analysis, the expression levels of *Mdr1a*, *Mdr1b* and *Gstm1* genes in FM3A-a3A9 and FM3A-AB cells were measured. No significant differences were found in the *Mdr1b* and *Gstm1* genes between FM3A-a3A9 and FM3A-AB cells. By contrast, the expression of the *Mdr1a* gene in FM3A-a3A9 cells was approximately 2.0-fold higher than that in FM3A-AB cells (P<0.01) (Fig. 2B). Mdr1a and Mdr1b proteins are multidrug transporters and reduce the intracellular drug content to sublethal levels in cells. Overexpression of these transporters has been detected in several cancer cells and correlated with poor responses to chemotherapeutic agents[8,9]. Therefore, this suggests that LPA_3 may enhance the cell survival rate after treatment with CDDP and DOX through induction of the *Mdr1a* gene in FM3A cells. However, the mechanism underlying the induction of *Mdr1a* expression by exogenous LPA_3 remains to be clarified.

In cancer cells, the distinct expression levels of LPA_3 have been reported. In our recent report, a variety of LPA_3 expression patterns were found in human colon cancer cells, correlating with the DNA methylation status of the *LPAR3* gene[16]. Therefore, it seems that the biological role of LPA_3 may not be equivalent, depending on the types of cells. So far, functional analyses for LPA_3 in cancer cells have been performed by the induction of exogenous LPA_3 or shRNAs. In ovarian cancer cells, LPA_3 increased these cell abilities by lentivirus constructs, and inhibited cell migration and invasion by shRNAs[17]. Moreover, *LPAR3*-expressing cells increased primary tumor size, ascites volume and metastatic potency to distance organs and reduced the survival rate of mice in a mouse xenograft model[17]. In rat liver cells, LPA_3 induced by 12-O-tetradecanoylphorbol-13-acetate, which is a tumor promoting agent, stimulated cell migration ability[18]. In our recent report, the cell survival of *Lpar3*-expressing liver tumor cells treated with anticancer drugs was elevated, correlating with the expression levels of *Mdr1a*, *Mdr1b* and *Gstp1*[6]. Therefore, this evidence suggests that LPA_3 may contribute to the acquisition of malignant potency in cancer cells. By contrast, exogenous LPA_3 inhibited cell motility of *Lpar3*-expressing lung cancer cells in the murine[7].

In conclusion, LPA_3 is involved in the enhancement of cell survival rate after treatment with CDDP and DOX in mouse mammary tumor cells, correlating with the increased *Mdr1a* expression. Taken together with our previous report[6],

LPA_3 may be a target molecule for a novel chemotherapeutic approach in cancer cells.

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