Biochemical and Biophysical Research Communications 422 (2012) 339–343

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Enhancement of endothelial cell migration by constitutively active LPA_1-expressing tumor cells

Misaho Kitayoshi [a], Kohei Kato [a], Eriko Tanabe [a], Kyohei Yoshikawa [a], Rie Fukui [a], Nobuyuki Fukushima [b], 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] 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 1 May 2012
Available online 9 May 2012

Keywords:
LPA receptor
Cell migration
VEGF
Endothelial cell
B103

ABSTRACT

Lysophosphatidic acid (LPA) receptors belong to G protein-coupled transmembrane receptors (LPA receptors; LPA_1 to LPA_6). They indicate a variety of cellular response by the interaction with LPA, including cell proliferation, migration and differentiation. Recently, we have reported that constitutive active mutated LPA_1 induced the strong biological effects of rat neuroblastoma B103 cells. In the present study, we examined the effects of mutated LPA_1 on the interaction between B103 cells and endothelial F-2 cells. Each LPA receptor expressing B103 cells were maintained in serum-free DMEM and cell motility assay was performed with a Cell Culture Insert. When F-2 cells were cultured with conditioned medium from *Lpar1* and *Lpar3*-expressing cells, the cell motility of F-2 cells was significantly higher than control cells. Interestingly, the motile activity of F-2 cells was strongly induced by mutated LPA_1 than other cells, correlating with the expression levels of vascular endothelial growth factor (*Vegf*)-*A* and *Vegf*-*C*. Pretreatment of LPA signaling inhibitors inhibited F-2 cell motility stimulated by mutated LPA_1. These results suggest that activation of LPA signaling via mutated LPA_1 may play an important role in the promotion of angiogenesis in rat neuroblastoma cells.

1. Introduction

Lysophosphatidic acid (LPA) receptors belong to G protein-coupled transmembrane receptors [1–3]. As far, at least six types of LPA receptors (LPA_1 to LPA_6) have been identified [4,5]. LPA receptors provide a variety of cellular responses by the interaction with LPA, including cell proliferation, differentiation, migration and protection from apoptosis [1–5]. The distribution and pattern of LPA receptor expression are dependent on types of cells [6]. Each LPA receptor is not functionally equivalent and it acts as a positive or negative regulator of the biological effects [1].

In previous reports, the aberrant expressions of LPA receptors were found, such as ovarian, colon and breast cancer cells [7–9]. We have also reported that the reduced expressions of LPA receptor genes due to DNA hypermethylation occurred in human colon cancer and rodent cancer cells [10–12]. On the other hand, LPA *per se* can enhance malignant property of cancer cells, such as cell proliferation, migration and invasion [1,4]. In fact, a high level of LPA production was detected in plasma and ascites from patients with ovarian cancers [13,14]. Furthermore, frequent mutations of *Lpar1* gene occurred in rat lung and liver tumors, but not *Lpar2* to *Lpar5* genes [15–17]. Therefore, it is suggested that LPA signaling via LPA receptors may be involved in the pathogenesis of cancer cells. However, the biological function of LPA receptor is not fully understood in cancer cells.

To address this issue, we generated each LPA receptor expressing cells from rat neuroblastoma B103 cells which showed no detectable LPA response, and indicated that constitutive active mutated *Lpar1*-expressing B103 cells markedly enhanced malignant property of B103 cells [18,19]. In the present study, we examined the effects of mutated LPA_1 on the interaction between B103 cells and endothelial F-2 cells.

Angiogenesis is the important step of producing new blood vessels from the existing vasculature and promotes the activity of invasion and metastasis of cancer cells [20]. In the present study, to evaluate the role of mutated LPA_1 on the interaction between B103 cells and endothelial cells, we measured cell proliferation rate and motile activity of endothelial cells cultured with conditioned medium from mutated *Lpar1*-expressing B103 cells, compared with other LPA receptor expressing B103 cells.

Abbreviations: LPA, lysophosphatidic acid; LPA_1, LPA receptor-1; VEGF, vascular endothelial growth factor; RT, reverse transcription; PCR, polymerase chain reaction.

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

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.05.012

2. Materials and methods

2.1. Cell culture

Each LPA receptor (*Lpar1*, *Lpar2* and *Lpar3*)-expressing (lpa1-1, lpa2-2 and lpa3-3-2, respectively) and mutated *Lpar1*-expressing (lpa1Δ-1) cells from B103 cells were used [18]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque Inc., Kyoto, Japan) containing 10% fetal bovine serum (FBS) in 5% CO_2 atmosphere at 37 °C. LPA receptor-unexpressing AB2-1bf (vector) cells were used as control [18].

2.2. Quantitative real time RT-PCR analysis for Vegf gene expressions

For quantitative real time RT-PCR analysis, a Smart Cycler II System (TaKaRa Bio, Inc., Shiga, Japan) and a SYBR Premix Ex Taq (TaKaRa) was used according to the manufacturer's protocol. The first strand cDNA was synthesized from 1.0 μg RNA sample of each cell with Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Co., Ltd., Mannheim, Germany). Primer pairs used in this assay were as follows: for *Vegf-A* (NCBI accession number NM_031836), F 5′-GCAATGATGAAGCCCTGGAG-3′, R 5′-GCTGGCTTTGGTGAGGTTTG-3′; for *Vegf-C* (NCBI accession number AY032729), F 5′-CCTGGAAAATGTGCCTGTGAA-3′, R 5′-GCTGCATGTTTGATGGTGGA-3′. The data for each gene was normalized to rat *Gapdh* [21].

2.3. Effects on cell proliferation of F-2 cells by LPA receptor expressing cells

To assess the interaction between LPA receptor expressing cells and endothelial cells, mouse endothelial F-2 cells were used [22]. F-2 cells were plated at 2000 cells/well in a 96-well plate and cultured with 100 μl of serum-free DMEM containing 30% supernatant from each LPA receptor expressing cells treated with LPA (10 μM) (Avanti Polar Lipid, Alabaster, AL, USA) for 48 h. For cell proliferation assay, a solution from a Cell Counting Kit-8 (CCK-8) (Dojin Chemistry, Kumamoto, Japan) was added to the plate 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 [18,19].

2.4. Effects on cell migration of F-2 cells by LPA receptor expressing cells

For cell motility assay, we used a Cell Culture Insert (BD Falcon, NJ, USA) with 8 μm pore size. F-2 cells were seeded in the filter at 1×10^5 cells in 200 μl serum-free DMEM (upper chamber) and placed in 24-well plates (lower chamber) containing 800 μl of supernatants from each LPA receptor expressing cells. After 24 h, cells remaining in the upper side of the filter were removed with cotton swabs. The number of cells migrated to the lower side of the filter was counted after Giemsa staining [19].

2.5. Effects on cell migration of F-2 cells by mutant Lpar1-expressing cells treated with LPA signaling inhibitors

To assess the effects of LPA signaling inhibitors on cooperation between mutant *Lpar1*-expressing cells and F-2 cells, pertussis toxin (PTX) (Pure Chemical Industries, Ltd.), U-73122 (Cayman Chemical Co., Ann Arbor, MI, USA) and Y-27632 (Mitsubishi Pharma Co., Osaka, Japan) were used. In the condition of serum-free DMEM, lpa1Δ-1 cells were pretreated with 100 ng/ml PTX for 24 h, 10 μM U-73122 for 15 min, or 10 μM Y-27632 for 15 min before the treatment of LPA (10 μM) [18,19]. After 48 h, supernatants from lpa1Δ-1 cells treated with those inhibitors were collected and used for the measurement of cell motile activity of F-2 cells.

3. Results and discussion

Since naive B103 cells express very low levels of endogenous each LPA receptor and indicate no response to LPA treatment, they have been widely used to exogenously introduce LPA receptor genes and investigate the cellular function of LPA signaling [18,19]. Using B103 cells, we generated constitutive active mutated *Lpar1*-expressing cells, which lacked the carboxyl terminal region of LPA_1 [18]. This clone indicated significantly high cell proliferation, motility, invasion and tumorigenicity, whereas wild-type LPA_1 inhibited those activities [19]. Moreover, mutated LPA_1 also enhanced matrix metalloproteinase-2 (Mmp-2) expression and activation in B103 cells [21]. In the present study, we examined the effects of mutated LPA_1 on the interaction between B103 cells and endothelial F-2 cells, using this clone.

Angiogenesis is regulated by various growth factors, including VEGF family. VEGFs are produced by tumor cells and promote angiogenesis, and its suppression leads to the inhibition of tumor growth [23,24]. Therefore, we first measured the expression levels of *Vegf-A* and *Vegf-C* genes in LPA receptor expressing cells by quantitative real time RT-PCR analysis. The expressions of *Vegf-A* gene in lpa1-1, lpa3-3-2 and lpa1Δ-1 cells were significantly higher than those of control cells. lpa1Δ-1 cells also indicated high expression levels of *Vegf-C* gene, but not other cells (Fig. 1A).

Next, to assess the effects on cell growth of F-2 cells by LPA receptor expressing cells, LPA expressing cells were maintained in serum-free DMEM with LPA treatment (10 μM) for 48 h. Then, F-2 cells were cultured with serum-free DMEM containing 30% supernatants from each LPA expressing cell. lpa1-1 and lpa3-3-2 cells showed slightly high growth rate, compared with other cell (Fig. 1B). For cell motility assay, supernatants from each LPA expressing cell were also used. When F-2 cells were cultured with supernatants from lpa1-1 and lpa3-3-2 cells, cell motility of F-2 cells was significantly higher than AB2-1bf cells, but not lpa2-2 cells. Interestingly, lpa1Δ-1 cells induced markedly high cell motile activity than other cells, correlating with the expression levels of *Vegf-A* and *Vegf-C* genes (Fig. 2).

Since lpa1Δ-1 cells induced the highest cell motile activity of F-2 cells with *Vegf* expressions, we examined whether LPA signaling via G proteins may participate in the stimulation of *Vegf* expressions and F-2 cell motility by mutated LPA_1. It is well known that LPA_1 couples to Gi, Gq and G12/13 [18,19]. Therefore, lpa1Δ-1 cells were pretreated with PTX, U-73122 or Y-27632 before the treatment of LPA. After 48 h, cells were harvested and RNA was extracted from each cell for expression assay. Supernatants were also collected for the motility assay. PTX is an inhibitor of Gi protein. U-73122 inhibits PLC which is a downstream effector of Gq and Y-27632 inactivates ROCK which is a downstream effector of G12/13 [18,19]. The expression of *Vegf-A* gene in lpa1Δ-1 cells was significantly inhibited by PTX, U-73122 and Y-27632. The treatment of U-73122 significantly suppressed *Vegf-C* expression, but not PTX and Y-27632 (Fig. 3A). Cell motile activity of F-2 cells was significantly suppressed by conditioned medium from lpa1Δ-1 cells pretreated with PTX, U-73122 and Y-27632 (Fig. 3B).

To confirm the activation of F-2 cell migration by mutated LPA_1, we also cultured mouse fibroblast 3T3 cells and mutated *Lpar1*-expressing fibroblast (3T3-a1Δ) cells [18,19]. The expression levels of *Vegf-A* in 3T3-a1 (*Lpar1*-expressing) and 3T3-a1Δ cells were significantly higher than those of 3T3-AB (control) cells. 3T3-a1Δ cells also indicated the high expression of *Vegf-C* (Fig. 4A). Using supernatants from each cell, the effects on cell proliferation and motile activities of F-2 cells were mea-

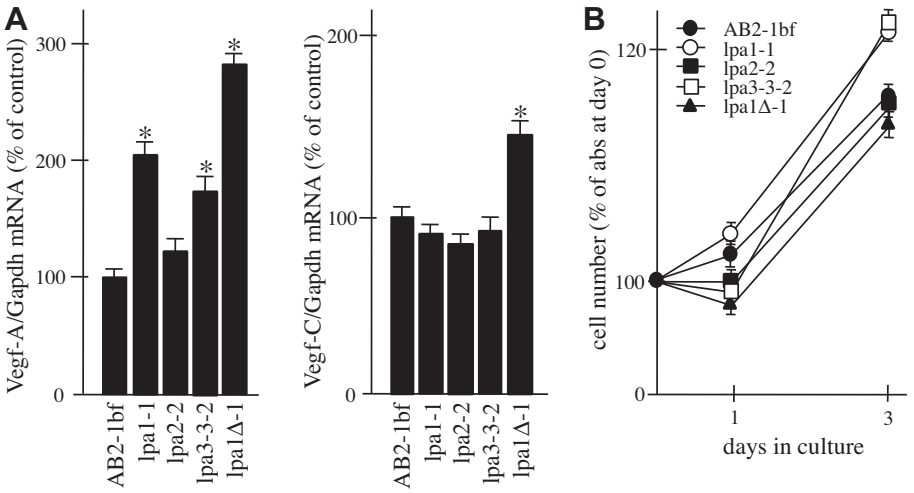

Fig. 1. (A) Expression levels of *Vegf-A* and *Vegf-C* genes mRNAs relative to *Gapdh* mRNA in LPA receptor expressing B103 cells by quantitative real time RT-PCR analysis. Cells were maintained in serum-containing medium. Columns indicate the mean of three studies; bars indicate SD. $^{*}p < 0.01$ vs. AB2-1bf (control) cells. (B) Cell proliferation rate of endothelial F-2 cells cultured with conditioned medium from LPA receptor expressing B103 cells treated with LPA for 48 h. F-2 cells were cultured in serum-free DMEM containing 30% supernatant from each cell. Data are expressed as the percentage of cell number on day 0.

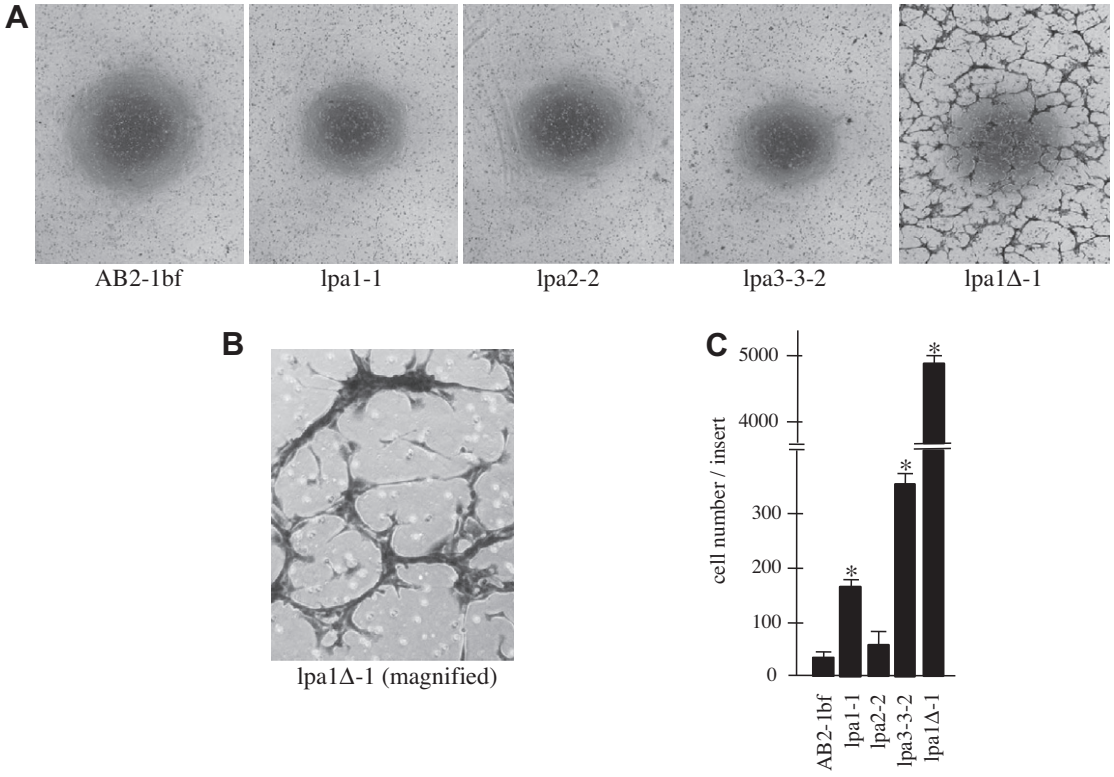

Fig. 2. The effects of mutated *Lpar1*-expressing B103 cells on cell motile ability of endothelial F-2 cells with a Cell Culture Insert. F-2 cells were incubated in serum-free DMEM (upper chamber) and supernatants (lower chamber) from each LPA receptor expressing cells treated with LPA (10 μM). (A) Representative phase contrast micrographs of F-2 cells migrated to lower side of the filter. Cells were stained by Giemsa solution. (B) Phase contrast micrographs of F-2 cells incubated with supernatants from lpa1Δ-1 cells (magnified). (C) The number of F-2 cells migrated to the lower side. Columns indicate the mean of three studies; bars indicate SD. $^{*}p < 0.01$ vs. F-2 cells incubated with supernatants from AB2-1bf cells.

sured. Although no significant difference of cell proliferation rate was found (Fig. 4B), F-2 cells cultured with supernatants from 3T3-a1Δ cells showed markedly high cell motile activity as well as lpa1Δ-1 cells (Fig. 4C and D).

Previously, the relevance between LPA signaling and angiogenesis in cancer cells has been reported. In ovarian cancer cells, LPA induced VEGF expression and production via LPA_2, but not LPA_1 [25]. The production of VEGF was inhibited by knockdown of *LPAR2* or *LPAR3*, suggesting that LPA signaling via LPA_2 and LPA_3 may play a critical role in the progression of ovarian cancer cells [26]. By contrast, LPA induced VEGF secretion in colon cancer cells which expressed LPA_1 or LPA_2 exclusively [27]. Moreover, LPA increased

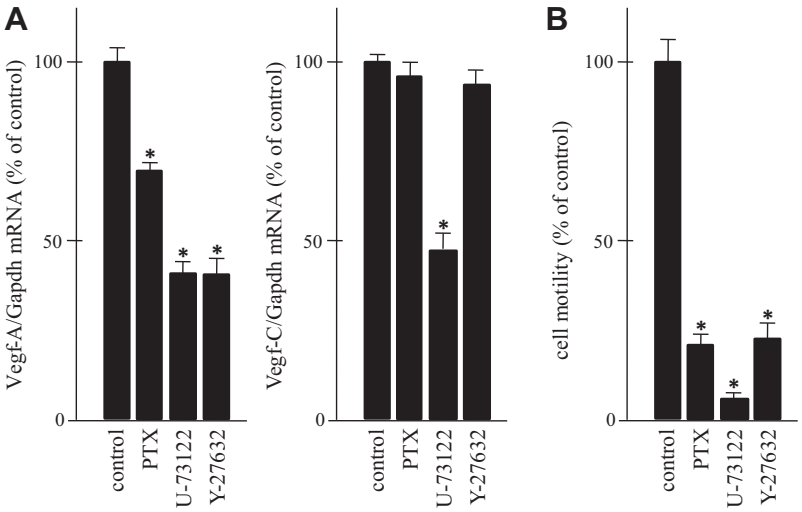

Fig. 3. (A) Effects of LPA signaling inhibitors on *Vegf-A* and *Vegf-C* expressions in lpa1Δ-1 cells. lpa1Δ-1 cells were pretreated with 100 ng/ml PTX for 24 h, 10 μM U-73122 for 15 min, or 10 μM Y-27632 for 15 min before the treatment of LPA (10 μM). After 48 h, cells were harvested and RNA was extracted from each cell. (B) Effects of LPA signaling inhibitors on F-2 cell motility by lpa1Δ-1 cells. Data are indicated as a percentage of control without inhibitor treatment. Columns indicate the mean of three studies; bars indicate SD. $^{*}p < 0.01$ vs. untreated control lpa1Δ-1 cells.

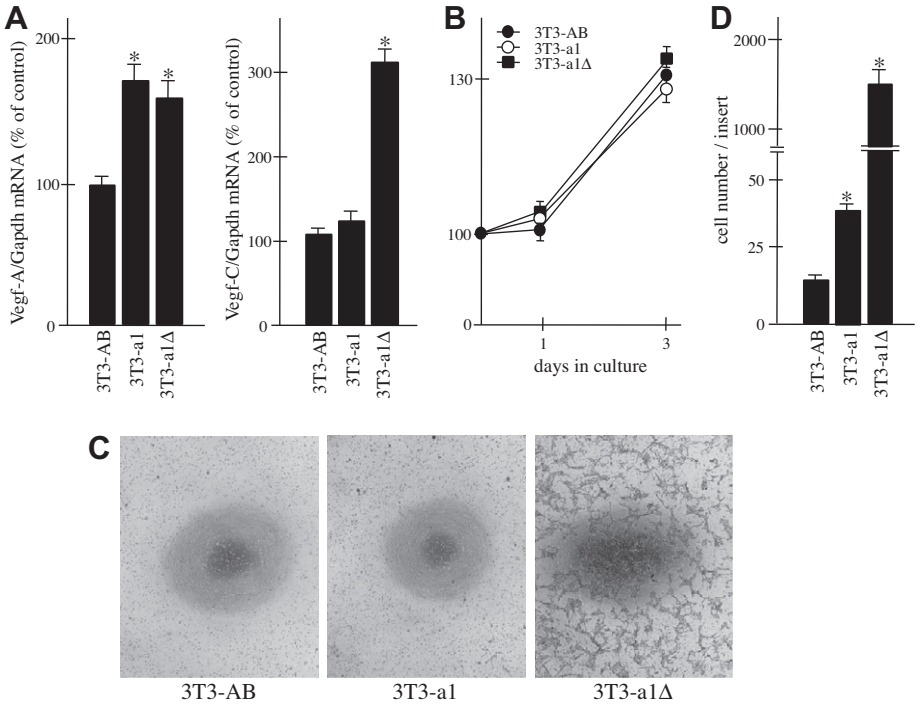

Fig. 4. (A) Expression levels of *Vegf-A* and *Vegf-C* genes mRNAs relative to *Gapdh* mRNA in 3T3-AB, 3T3-a1 and 3T3Δ-1 cells by quantitative real time RT-PCR analysis. Columns indicate the mean of three studies; bars indicate SD. $^{*}p < 0.01$ vs. 3T3-AB (control) cells. (B) Cell proliferation rate of endothelial F-2 cells cultured with conditioned medium from 3T3-AB, 3T3-a1 and 3T3Δ-1 cells treated with LPA for 48 h. F-2 cells were cultured in serum-free DMEM containing 30% supernatant fr0m each cell. Data are expressed as the percentage of cell number on day 0. (C) Representative phase contrast micrographs of F-2 cells migrated to lower side of the filter. Cells were stained by Giemsa solution. (D) The number of F-2 cells migrated to the lower side. Columns indicate the mean of three studies; bars indicate SD. $^{*}p < 0.01$ vs. F-2 cells incubated with supernatants from 3T3-AB cells.

VEGF-C expression and tube formation in endothelial cells through LPA_1 and LPA_3 [28].

In summary, the present study demonstrated that mutated LPA_1 markedly enhanced cell motile activity of endothelial cells, correlating with *Vegf* expressions. Since frequent *Lpar1* mutations were detected in rat lung and liver tumors [15,16], it is suggested that LPA signaling via mutated LPA_1 may play important roles in the acquisition of malignant potency of cancer cells.

Acknowledgments

This study was supported in part by a Grant-in-Aid (20591765) for Scientific Research from Ministry of Education, Culture, Sports, Science and Technology of Japan, and by Grants (21321201) from the Ministry of Health, Labor and Welfare of Japan, and by Grants from the Faculty of Science and Engineering, Kinki University.

References

[1] M.-E. Lin, D.R. Herr, J. Chun, Lysophosphatidic acid (LPA) receptors: signaling properties and disease relevance, Prostaglandins Other Lipid Mediat. 91 (2010) 130–138.

[2] I. Ishii, N. Fukushima, X. Ye, J. Chun, Lysophospholipid receptors: signaling and biology, Annu. Rev. Biochem. 73 (2004) 321–354.

[3] N. Fukushima, I. Ishii, J.A. Contos, J.A. Weiner, J. Chun, Lysophospholipid receptors, Annu. Rev. Pharmacol. Toxicol. 41 (2001) 507–534.

[4] K. Noguchi, D. Herr, T. Mutoh, J. Chun, Lysophosphatidic acid (LPA) and its receptors, Curr. Opin. Pharmacol. 9 (2009) 15–23.

[5] S. Ishii, K. Noguchi, K. Yanagida, Non-Edg family lysophosphatidic acid (LPA) receptors, Prostaglandins Other Lipid Mediat. 89 (2009) 57–65.

[6] J.J.A. Contos, I. Ishii, J. Chun J. Lysophosphatidic acid receptors, Mol. Pharmacol. 58 (2000) 1188–1196.

[7] E.J. Goetzl, H. Dolezalova, Y. Kong, Y.L. Hu, R.B. Jaffe, K.R. Kalli, C.A. Conover, Distinctive expression and functions of the type 4 endothelial differentiation gene-encoded G protein-coupled receptor for lysophosphatidic acid in ovarian cancer, Cancer Res. 59 (1999) 5370–5375.

[8] D. Shida, T. Watanabe, J. Aoki, K. Hama, J. Kitayama, H. Sonoda, Y. Kishi, H. Yamaguchi, S. Sasaki, A. Sako, T. Konishi, H. Arai, H. Nagawa, Aberrant expression of lysophosphatidic acid (LPA) receptors in human colorectal cancer, Lab. Investigation 84 (2004) 1352–1362.

[9] N. Panupinthu, H.Y. Lee, G.B. Mills, Lysophosphatidic acid production and action: critical new plays in breast cancer initiation and progression, Br. J. Cancer 102 (2010) 941–946.

[10] M. Tsujino, M. Fujii, K. Okabe, T. Mori, N. Fukushima, T. Tsujiuchi, Differential expressions and DNA methylation patterns of lysophosphatidic acid receptor genes in human colon cancer cells, Virchows Arch. 457 (2010) 669–676.

[11] T. Tsujiuchi, K. Shimizu, M. Onishi, E. Sugata, H. Fujii, T. Mori, K. Honoki, N. Fukushima, Involvement of aberrant DNA methylation on reduced expression of lysophosphatidic acid receptor-1 gene in rat tumor cell lines, Biochem. Biophys. Res. Commun. 349 (2006) 1151–1155.

[12] K. Okabe, M. Hayashi, N. Wakabayashi, Y. Yamawaki, M. Teranishi, N. Fukushima, T. Tsujiuchi, Different expressions and DNA methylation patterns of lysophosphatidic acid receptor genes in mouse tumor cells, Pathobiology 77 (2010) 278–282.

[13] X. Fang, D. Gaudette, T. Furui, Lysophospholipid growth factors in the initiation, progression, metastases, and management of ovarian cancer, Ann. NY Acad. Sci. 905 (2000) 188–208.

[14] X. Fang, M. Schummer, M. Mao, S. Yu, F.H. Tabassam, R. Swaby, Y. Hasegawa, J.L. Tanyi, R. LaPushin, A. Eder, R. Jaffe, J. Erickson, G.B. Mills, Lysophosphatidic acid is a bioactive mediator in ovarian cancer, Biochim. Biophys. Acta 1582 (2002) 257–264.

[15] T. Yamada, M. Furukawa, M. Hotta, A. Yamasaki, K. Honoki, N. Fukushima, T. Tsujiuchi, Mutations of lysophosphatidic acid receptor-1 gene during progression of lung tumors in rats, Biochem. Biophys. Res. Commun. 378 (2009) 424–427.

[16] Y. Obo, T. Yamada, M. Furukawa, M. Hotta, K. Honoki, N. Fukushima, T. Tsujiuchi, Frequent mutations of lysophosphatidic acid receptor-1 gene in rat liver tumors, Mutat. Res. 660 (2009) 47–50.

[17] N. Wakabayashi, M. Tsujino, M. Tajiri, M. Taki, A. Koshino, H. Ikeda, N. Fukushima, T. Tsujiuchi, No mutations of lysophosphatidic acid receptor genes in lung adenocarcinomas induced by N-nitrosobis-(2-hydroxypropyl)amine in rats, J. Toxicol. Pathol. 3 (2010) 63–66.

[18] S. Shano, K. Hatanaka, S. Ninose, R. Moriyama, T. Tsujiuchi, N. Fukushima, A lysophosphatidic acid receptor lacking the PDZ-binding domain is constitutively active and stimulates cell proliferation, Biochim. Biophys. Acta 1783 (2008) 748–759.

[19] M. Hayashi, K. Okabe, Kato, M. Okumura, R. Fukui, K. Honoki, N. Fukushima, T. Tsujiuchi, Differential function of lysophosphatidic acid receptors in cell proliferation and migration of neuroblastoma cells, Cancer Lett. 316 (2012) 91–96.

[20] J. Folkman, Tumor angiogenesis: therapeutic implications, N. Engl. J. Med. 285 (1971) 1182–1186.

[21] K. Kato, R. Fukui, K. Okabe, E. Tanabe, M. Kitayoshi, N. Fukushima, T. Tsujiuchi, Constitutively active lysophosphatidic acid receptor-1 enhances the induction of matrix metalloproteinase-2, Biochem. Biophys. Res. Commun. 417 (2012) 790–793.

[22] K. Tei, N. K-Kimura, O. Taguchi, K. Kumamoto, S. Higashiyama, N. Taniguchi, K. Toda, R. Kawata, Y. Hisa, R. Kannagi, Roles of cell adhesion molecules in tumor angiogenesis induced by cotransplantation of cancer and endothelial cells to nude rats, Cancer Res. 62 (2002) 6289–6296.

[23] N. Ferrara, H.P. Gerber, J. LeCouter, The biology of VEGF and its receptors, Nat. Med. 9 (2003) 669–676.

[24] K.J. Kim, B. Li, J. Winer, M. Armanini, N. Gillett, H.S. Phillips, N. Ferrara, Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*, Nature 362 (1993) 841–844.

[25] Y.-L. Hu, C. Albanese, R.G. Pestell, R.B. Jaffe, Dual mechanisms for lysophosphatidic acid stimulation on human ovarian carcinoma cells, J. Natl. Cancer Inst. 95 (2003) 733–740.

[26] S.Yu, M.M. Murph, Y. Lu, S. liu, H.S. Hall, J. Liu, C. Stephens, X. Fang, G.B. Mills, Lysophosphatidic acid receptors determine tumorigenicity and aggressiveness of ovarian cancer cells, J. Natl. Cancer Inst. 100 (2008) 1630–1642.

[27] D. Shida, J. Kitayama, H. Yanaguchi, Y. Okaji, N.H. Tsuno, T. Watanabe, Y. Takuwa, H. Nagawa, Lysophosphatidic acid (LPA) enhances the metastatic potential of human colon carcinoma DLD1 cells through LPA1, Cancer Res. 63 (2003) 1706–1711.

[28] C.-I. Lin, C.-N. Chen, M.-T. Huang, S.-J. Lee, C.-H. Lin, C.-C. Chang, H. Lee, Lysophosphatidic acid upregulates vascular endothelial growth factor-C and tube formation in human endothelial cells through $LPA_{1/3}$, COX-2, and NF-κB activation-and EGFR transactivation-dependent mechanisms, Cell Signal. 20 (2008) 1804–1814.