Lysophosphatidic Acid–Induced p21Waf1 Expression Mediates the Cytostatic Response of Breast and Ovarian Cancer Cells to TGFβ

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Abstract

Lysophosphatidic acid (LPA) is a multifunctional intercellular phospholipid mediator present in blood and other biological fluids. In cancer cells, LPA stimulates expression or activity of inflammatory cytokines, angiogenic factors, matrix metalloproteinases, and other oncogenic proteins. In this study, we showed that LPA upregulated expression of the cyclin-dependent kinase inhibitor p21Waf1 in TGFβ-sensitive breast and ovarian cancer cells, but not in TGFβ-resistant ones. We examined the possibility that LPA-induced p21 might contribute to the cytostatic response to TGFβ. In serum-free conditions, TGFβ alone induced p21 expression weakly in TGFβ-sensitive cells. Serum or serum-borne LPA cooperated with TGFβ to elicit the maximal p21 induction. LPA stimulated p21 via LPA1 and LPA2 receptors and Erk-dependent activation of the CCAAT/enhancer binding protein beta transcription factor independent of p53. Loss or gain of p21 expression led to a shift between TGFβ-sensitive and -resistant phenotypes in breast and ovarian cancer cells, indicating that p21 is a key determinant of the growth inhibitory activity of TGFβ. Our results reveal a novel cross-talk between LPA and TGFβ that underlies TGFβ-sensitive and -resistant phenotypes of breast and ovarian cancer cells. Mol Cancer Res; 9(11); 1562–70. © 2011 AACR.

Introduction

Lysophosphatidic acid (LPA; 1-acyl-sn-glycerol-3-phosphate) is a naturally occurring intercellular mediator of diverse biological processes including neurogenesis, angiogenesis, wound healing, immunity, and carcinogenesis (1). LPA is produced by activated platelets during coagulation, fibrinolysis, wound healing, immunity, and carcinogenesis (1). LPA is present in blood and other biological fluids. In cancer cells, LPA stimulates expression or activity of inflammatory cytokines, angiogenic factors, matrix metalloproteinases, and other oncogenic proteins. In this study, we showed that LPA upregulated expression of the cyclin-dependent kinase inhibitor p21Waf1 in TGFβ-sensitive breast and ovarian cancer cells, but not in TGFβ-resistant ones. We examined the possibility that LPA-induced p21 might contribute to the cytostatic response to TGFβ.

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interconnecting with the TGFB pathway (20). Mutations in the TβRII receptor gene (19) as well as missense mutation or deletion of Smad2 and Smad4 (21, 22) have been identified in different types of cancer. There is also evidence for overexpression of oncoproteins in inactivation of the cytosolic effect of TGFB in cancer, such as Myc–Miz-1 complex (23), Evi-1 (24), FoxG1 (25), CDK (26), and Ski and/or SnoN (27). However, these aberrations seen in only fractions of human tumors do not explain the generally altered responses to TGFB in a wide spectrum of cancers.

In this study, we examined the potential cross-talk between LPA signaling and TGFB in growth regulation of breast and ovarian cancer cells. We report that LPA upregulates expression of the CDK inhibitor p21 in breast and ovarian cancer cells sensitive to TGFB-induced growth arrest but not in TGFB-resistant cancer cells. In TGFB-sensitive cells, LPA cooperates with TGFB to elicit the maximal induction of p21 to mediate the cytostatic response to TGFB. Loss or gain of p21 expression led to a shift between TGFB-sensitive and -resistant phenotypes in these cells. Our results reveal a novel mechanism underlying the cytostatic program of TGFB in breast and ovarian cancer cells.

Materials and Methods

Materials

- Anti-phospho CCAAT/enhancer binding protein beta (C/EBPβ), phospho-Erk1/2, tubulin α/β antibodies, and PD98059 were obtained from Cell Signaling. Anti-C/EBPβ, p21, and Erk antibodies were from Santa Cruz Biotech. LPA (oleoyleyl, 18:1) and sphingosine 1 phosphate (S1P) were obtained from Avanti Polar Lipids, Inc. Prior to use, LPA and S1P were dissolved in PBS containing 0.5% fatty acid–free bovine serum albumin (BSA) obtained from Roche. TGFB was obtained from PeproTech Inc. 12-O-tetradecanoylphorbol-13-acetate (TPA) was from Sigma. FBS was obtained from Atlanta Biological. Oligonucleotides were synthesized by Operon Biotechnologies, Inc. TRIZol and cell culture reagents were obtained from Invitrogen Inc. The transfection reagent Dharmafect 1 was obtained from Dharmacon. They were transfected into cells by using Dharmafect 1 following the manufacturer’s protocol. In brief, cells were plated in 6-well plates to reach 50% confluence before transfection for 12 to 16 hours with specific siRNA (100 pmol) and Dharmafect 1 (4 μL). The transfected cells were cultured in complete medium for approximately 48 hours before experiments.

Quantitative PCR

Total cellular RNA was isolated by using Trizol (Invitrogen). Complementary DNA (cDNA) was synthesized from RNA (1 μg, random primers), using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems. The relative levels of individual LPA receptors were determined by gene-specific probes, the TaqMan Universal PCR Master Mix and the 7900HT Fast Real-Time PCR System (Applied Biosystems).

Statistics

All numerical data were presented as mean ± SD. The statistical significance of differences was analyzed by the Student t test, where P < 0.05 was considered statistically significant.

Results

Induction of p21 by LPA in TGFB-sensitive breast and ovarian cancer cells

It has been well documented that LPA regulates expression of cytokines, angiogenic factors, and many other proteins involved in tumorigenesis and cancer metastasis (28–31). In contrast to these oncogenic mediators, we also found that in a subset of breast and ovarian cancer cell lines, LPA upregulates expression of p21, an inducible inhibitor of CDKs. As shown in Figure 1A, in the MDA-MB-231 breast carcinoma cells and the Caov-3 ovarian carcinoma cells, LPA stimulated p21 expression in a time-dependent manner. Following the addition of 10 μmol/L LPA to serum-starved cells, p21 protein was induced at 1 hour. The p21 protein levels reached the maximum by 4 hours. Although modest levels of p21 may promote assembly of active cyclin–CDK complex (32), excessive expression of
p21 generally causes cell cycle arrest. However, the strong and sustained induction of p21 by LPA was not associated with growth inhibition. Instead, LPA treatment led to increased proliferation in MDA-MB-231 and Caov-3 cells (data not shown; Fig. 5) as well as in other breast and ovarian cancer cell lines that LPA did not trigger p21 expression (Fig. 1B).

In an effort to understand the biological significance of LPA-mediated p21 expression, surprisingly we noticed that LPA-stimulated p21 expression only in cell lines sensitive to the TGFβ-induced growth arrest but not in cells refractory to TGFβ. As shown in Figure 1B, treatment of MDA-MB-231 and Caov-3 cells with TGFβ (2.5 ng/mL) for 48 hours resulted in a significant decrease in cell numbers compared with control cells cultured in the absence of TGFβ (Fig. 1B). In contrast, TGFβ did not inhibit the growth of cell lines such as BT-549, SK-BR-3, OVCA-432, and SKOV-3 in which LPA did not induce p21 (Fig. 1B).

**Correlation of LPA and TGFβ induction of p21**

We next explored the possibility that LPA-driven p21 expression might modulate the sensitivity of breast and ovarian cancer cells to TGFβ. Coincidently, the effect of TGFβ on p21 expression was identical to that of LPA in these breast and ovarian cancer cells. As shown in Figure 2, TGFβ-induced p21 expression at significant levels only in MDA-MB-231 and Caov-3 cells but not in TGFβ-resistant lines in which LPA failed to induce p21 (Fig. 2).

The loss of p21 inducibility by TGFβ could be due to abnormalities in TGFβ receptors or the TGFβ intracellular signaling through Smads. It is well known that TGFβ superfamily ligands bind to a TβRII, which recruits and phosphorylates a TβRI. TβRI then phosphorylates receptor-regulated Smads (RSmad) such as Smad2 and Smad3, which then bind to the common mediator Smad (coSmad). RSmad forms heterodimeric complexes with coSmads and accumulates in the nucleus where the complexes participate in regulation of TGFβ target genes involved in growth control (19). As shown in Figure 2, TGFβ induced phosphorylation of Smad3 in all breast and ovarian cancer cell lines examined, irrespective of their status of TGFβ sensitivity. Furthermore, we examined the effect of TGFβ on another TGFβ-target gene plasminogen activator inhibitor-1 (PAI-1). Upon treatment with TGFβ, all cell lines showed variable increases in expression of PAI-1 mRNA (Supplementary Fig. S1). This suggests that both TGFβ-sensitive and -resistant cells maintain functional TGFβ receptors and the Smad3 signal transducer.
Input of LPA signaling in TGFβ-induced p21 expression

Because phosphorylation of Smad3 by TGFβ was observed in both TGFβ-sensitive and -resistant cells, p21 induction by TGFβ seems to involve other signaling routes beyond the canonical Smad pathway in TGFβ-sensitive cells. In addition, both MDA-MB-231 and Caov-3 carry mutant p53 (33, 34). TGFβ-induced p21 expression in these cells is apparently mediated by a p53-independent process. We therefore examined the possibility that LPA contributes to TGFβ-induced p21 expression in the TGFβ-sensitive MDA-MB-231 and Caov-3 cells. When these cells were cultured in serum-free medium, TGFβ stimulated only weak to modest levels of p21 (Fig. 3A). The maximal p21 induction by TGFβ was seen when the cells were incubated in complete medium containing FBS (Fig. 3A), a condition in which the effects of TGFβ on cell proliferation and p21 expression were assessed in earlier experiments (Figs. 1B and 2). Serum itself induced p21 expression in MDA-MB-231 and Caov-3 cells. This suggests that induction of p21 by TGFβ that we had observed resulted from a combined action of TGFβ and a cofactor present in serum.

LPA is a prominent serum-borne factor responsible for many biological activities of serum (35). To determine whether LPA reproduces the action of serum in concert with TGFβ to maximize p21 induction, we examined the effect of LPA and TGFβ on p21 expression in MDA-MB-231 and Caov-3 cells. Indeed, p21 induction was maximized when both LPA and TGFβ were present (Fig. 3B). We also assessed other serum factors such as S1P and insulin for their ability to regulate p21 expression (36, 37). In contrast to LPA, S1P and insulin did not increase p21 expression. Nor did S1P and insulin potentiate the effect of TGFβ on p21 (data not shown). Taken together, these results suggest that a significant input of TGFβ-induced p21 is attributable to the action of LPA, which likely underlies the sensitivity of breast and ovarian cancer cells to TGFβ.

Role of p21 in mediating the cytostatic response to TGFβ

To confirm an essential role for p21 in mediating the TGFβ response, we used siRNA to knockdown p21 expression in the TGFβ-sensitive MDA-MB-231 and Caov-3 cells. As shown in Figure 4A, suppression of p21 induction by siRNA converted these cells into a resistant phenotype. The p21 knockdown cells became insensitive to the inhibitory effect of TGFβ, confirming that p21 induction is indeed a key component of TGFβ-induced cytostasis in breast and ovarian cancer cells.

If the p21 inducibility distinguishes TGFβ-sensitive cells from the resistant ones, we assume that the resistant cells could be rendered sensitive to TGFβ when p21 is induced somehow by other p21 stimuli. To test this possibility, we took advantage of the fact that phorbol ester (TPA) induces expression of p21 in cancer cells (38). We treated the TGFβ-resistant cell lines with TGFβ alone or TGFβ and TPA (0.1 μmol/L). The presence of TPA led to the induction of high and sustained expression of p21 whereas the cells treated with TGFβ alone did not show p21 expression (Fig. 4B). Treatment with TPA was not associated with inhibition of cell proliferation as shown in Figure 4B, suggesting that p21 induced by TPA was not sufficient to affect cell growth without TGFβ. However, the presence of TPA-induced p21 expression enables TGFβ to suppress growth of these otherwise TGFβ-resistant cells, consistent with the importance of p21 in mediating TGFβ sensitivity (Fig. 4B). The restoration of the growth-inhibitory effect of TGFβ was not due to induction of apoptotic cell death. TGFβ did not trigger apoptosis in TPA-treated BT-549 or OVCA-432 cells (Supplementary Fig. S2).

p21-dependent inhibition of LPA-driven cell proliferation by TGFβ

LPA stimulated p21 expression in MDA-MB-231 and Caov-3 cells (Fig. 3B). However, in spite of the robust and sustained induction of p21, LPA is mitogenic toward these cells. To determine whether TGFβ was able to block the mitogenic effect of LPA, we compared the growth of MDA-MB-231 and Caov-3 cells incubated with LPA in the absence or presence of TGFβ. Figure 5A showed that TGFβ effectively inhibited cell number increases stimulated by LPA. Moreover, siRNA knockdown of p21 expression resulted in resistance of these cells to TGFβ (Fig. 5B), confirming an essential role for p21 in TGFβ repression of LPA-induced cell proliferation. In TGFβ-resistant breast and ovarian cancer cell lines, LPA also acted as a mitogen. The mitogenic activity of LPA, however, was not affected by
TGFβ (data not shown), consistent with the lack of induction of p21 by LPA, TGFβ, or LPA and TGFβ in these cells.

Mechanisms for LPA induction of p21

Ovarian and breast cancer cells express multiple LPA receptors including LPA1, LPA2, LPA3, and LPA5 as described previously (39–41). Expression of the LPA4 and LPA6 receptors was undetectable in the breast and ovarian cancer lines (data not shown). We thus used siRNA to knockdown expression of LPA1, LPA2, LPA3, or LPA5. The cells treated with LPA were then examined for p21 protein expression. LPA-induced p21 was drastically reduced by downregulation of LPA1 or LPA2 (Fig. 6A). Knockdown of LPA3 or LPA5 did not attenuate the effect of LPA on p21 expression. Therefore, we conclude that LPA-stimulated p21 expression in MDA-MB-231 and Caov-3 cells occurs through the LPA1 and LPA2 receptors.

LPA induced strong and sustained activation of extracellular signal-regulated kinases (Erk) in MDA-MB-231 and Caov-3 cells (29, 42). When Erk1 and Erk2 were silenced by siRNAs, LPA induction of p21 was blocked (Fig. 6B), indicating that the Erk pathway is linked to activation of p21 expression in response to LPA.

In contrast to Erk, phosphatidylinositol 3-kinase was dispensable for LPA-induced p21 induction because its inhibitor LY-294002 did not attenuate the effect of LPA on p21 expression (data not shown).

Erk couples directly or indirectly to diverse downstream effectors and transcription factors that could culminate in p21 induction. We used siRNA to screen for transcription factors required for LPA-induced p21 expression including AP-1, SRE, NF-kB, and C/EBPβ (30, 43). In this group of transcription factors, C/EBPβ was found to be critical to the p21 induction. Knockdown of C/EBPβ expression

Figure 4. Essential role of p21 in the cytostatic response to TGFβ. A, the TGFβ-sensitive cells lost sensitivity to TGFβ following siRNA knockdown of p21 expression. MDA-MB-231 and Caov-3 cells in 6-well plates were transfected with control siRNA (ctrl si) or p21 siRNA. The cells were treated for 48 hours with or without TGFβ (2.5 ng/mL) before quantification of cell numbers with a Coulter counter. Efficiency of p21 siRNA knockdown was confirmed by immunoblotting. B, the TGFβ-resistant cell lines gained sensitivity to TGFβ following TPA induction of p21. BT-549 and OVCA-432 in 6-well plates were treated for 48 hours with or without TGFβ in the presence of TPA (0.1 μmol/L) or vehicle before quantification of cell numbers. Expression of p21 in these cells treated with TGFβ, TPA, or TGFβ þ TPA was analyzed by immunoblotting. *, P < 0.05; **, P < 0.01. DMSO, dimethyl sulfoxide.
prevented LPA-induced p21 expression (Fig. 6C). Finally, inhibition of Erk activity with the MEK inhibitor PD98059 prevented C/EBP\(_b\) phosphorylation and the subsequent p21 induction in LPA-treated MDA-MB-231 and Caov-3 cells (Fig. 6D). These findings show that LPA stimulates p21 expression through the LPA₁/₂-Erk-C/EBP\(_b\) signaling network.

**Discussion**

TGF\(\beta\)-mediated cytostasis is induced, at least in part by Smad-dependent activation of TGF\(\beta\) target genes involved in cell cycle control, primarily CDK inhibitors p15, p21, and p27. In addition, TGF\(\beta\) activation of Smad represses expression of proteins that promotes cell cycle progression including c-Myc, Id1, Id2, E2F, and Sp-1 (44, 45). These TGF\(\beta\)-induced cytostatic transcriptional programs, however, are subverted in a majority of cancers, leading to cytostatic resistance to TGF\(\beta\) (46). In addition to genetic and epigenetic aberrations in TGF\(\beta\) receptors or Smad proteins, emerging data suggest that in most malignancies, abrogation of TGF\(\beta\)-induced growth arrest is mediated by abnormal expression or function of intracellular proteins implicated in Smad regulation of its target genes (18). In theory, environmental cues that influence expression or activity of Smad, Smad regulatory circuits or Smad responsive genes could also alter cellular responses to TGF\(\beta\). However, there have been few studies to analyze potential cross-talk between extracellular factors such as LPA and TGF\(\beta\)-Smad to regulate the responsiveness of cancer cells to TGF\(\beta\).

Using breast and ovarian cancer cells as model systems, we showed that LPA upregulates expression of the prototype Smad target gene p21, contributing to the TGF\(\beta\)-mediated growth inhibition. In these cells, the ability of LPA to stimulate p21 expression through the LPA₁/₂-Erk-C/EBP\(_b\) signaling network confirmed an essential role of p21 in mediating the cytostatic response to TGF\(\beta\). Previous studies in breast and ovarian cancer cells also supported the involvement of p21 as a key mediator of TGF\(\beta\)-induced growth inhibition (44). Another observation in ovarian cancer indicates that abrogation of TGF\(\beta\)-induced growth arrest is associated with overexpression of FoxG1, a negative regulator of p21 expression (47). Therefore, p21 seems to be a general mediator of TGF\(\beta\)-induced growth arrest in multiple types of cancer cells. The findings of this work highlight the possibility that the sensitivity to TGF\(\beta\)
in breast and ovarian cancer cells could be reconstituted through upregulation of p21 expression. It will be of interest to develop and test agents that can specifically activate p21 expression or stabilize p21 protein in cancer cells.

An interesting finding in this study is that p21 induction in TGFβ-sensitive cells is accomplished through cooperative effects of TGFβ and the serum-borne factor LPA. A significant input of p21 expression is evoked from LPA activation of its receptors, namely LPA₁ and LPA₂. Using molecular and pharmacologic approaches, we further showed that LPA upregulates p21 expression in TGFβ responsive cells through the Erk-C/EBPβ signaling pathway. We have previously shown that C/EBPβ is a transcription factor activated by LPA which accounts for LPA-induced expression of Cox-2 and sphingosine kinase 1 in various cancer cells (30, 48). The results in this work links C/EBPβ to the induction of p21 by LPA in TGFβ growth arrest program in breast and ovarian cancer cells, suggesting...
a general role for this transcription factor in regulation of LPA target genes. Consistent with their resistance to TGFβ, the stimulatory effect of LPA on p21 was not seen in most breast and ovarian cancer cell lines. The differential effects of LPA on p21 in different cell lines are not fully understood but could be due to distinct expression patterns of LPA receptors in these cells. The receptor knockdown experiments in the TGFβ-sensitive MDA-MB-231 and Caov-3 cells indicated that both LPA1 and LPA2 receptors are required for induction of p21 by LPA. Among the TGFβ-resistant cell lines, SKOV-3 and BT-549 express low levels of LPA2 (28, 49) and OVCA-432 exhibits elevated LPA3 (28). It is conceivable that coexpression of two or more receptors at appropriate levels is important for optimal induction of p21 by LPA. Alternatively, it is also possible that certain LPA receptors including the conventional LPA3, novel LPA receptor subtypes, and other unknown LPA receptors could be present in the resistant cells and serve as negative regulators of certain biological functions of LPA.

References


Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Dr. Michael W. Maceyka for reading the manuscript and his constructive suggestions.

Grant Support

This work was supported by the NIH/NCI grant 2R01CA102196 (X. Fang), the Department of Defense ovarian cancer research program grant W81XWH-11-1-0541 (X. Fang), the Jeffress Memorial Fund (X. Fang), and the NIH grant P30 CA16059 to Massey Cancer Center of Virginia Commonwealth University School of Medicine.

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Received July 14, 2011; revised August 24, 2011; accepted August 25, 2011; published onlineFirst September 2, 2011.


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Molecular Cancer Research

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