Selective induction of cyclooxygenase-2 plays a role in lysophosphatidic acid regulated Fas ligand cell surface presentation

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Abstract Previous studies found that lysophosphatidic acid (LPA) upregulated Fas ligand (FasL) presentation on the ovarian cancer cell surface and lead to apoptosis of activated lymphocytes. In this report, we investigated the role of selective induction of cyclooxygenase-2 (Cox-2) in FasL cell surface presentation stimulated by LPA. Ovarian cancer cells pretreated with general aspirin derivative acetylsalicylic acid and specific Cox-2 inhibitor (NS-398) before stimulation with LPA, FasL cell surface presentation was significantly blocked, so was the apoptosis of activated lymphocytes mediated by increasing FasL on the ovarian cancer cell surface. Using the specific inhibitors PD98059, AG1478 or dominant-negative epidermal-growth-factor receptor (EGFR-DN) plasmid, we found that the activation of ERK1/2 played a role in Cox-2 induction, and the transactivation of EGFR worked as an upstream signaling pathway in ERK1/2 phosphorylation. This study first revealed the selective induction of Cox-2 by LPA led to FasL presentation on ovarian cancer cell surface and provide cancer cell immune privilege, and might provide important information of Cox-2 in cancer progression and Cox-2 inhibitors' application in cancer chemoprevention.

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1. Introduction

The lack of early effective diagnostic approaches with high mortality rate makes ovarian cancer a strong candidate for chemoprevention\cite{1}. The recent reports revealed that both cyclooxygenase-1 (Cox-1) and cyclooxygenase-2 (Cox-2) were expressed in ovarian epithelial cancers and might contribute to ovarian cancer development or metastasis \cite{2}. But Cox-1 expression was constitutive in epithelia ovarian cancer and found also in normal ovarian epithelium, while Cox-2 expression was inducible and frequently found at the advancing margin of tumor invasion or in new metastases \cite{2–4}. The increased Cox-2 expression is associated with chemotherapy resistance and outcome in these patients \cite{5,6}. Reports indicated that overexpression of Cox-2 may lead to cell phenotypic changes, which may alter the angiogenic and metastatic potential of cancer cells \cite{7,8}. Epidemiological observations suggest that inhibition of Cox-2 by non-steroidal anti-inflammatory drugs (NSAIDs) may decrease the development of ovarian cancer and serve as a strategy of chemoprevention for ovarian cancers \cite{9,10}. However, the role of Cox-2 in Fas ligand (FasL) expression and immune regulation has not been explored in ovarian cancer cells.

The cellular messenger molecule lysophosphatidic acid (LPA, 1-acyl-sn-glycerol 3-phosphate) is a naturally occurring glycerophospholipid that elicits growth factor-like cellular responses. LPA is highly elevated in ascites from ovarian cancer patients \cite{11,12}. LPA evokes a diverse range of physiological and pathological responses, including mitogenesis, inhibited apoptosis, actin cytoskeleton reorganization and increased ovarian cancer cell motility \cite{11,13–15}. Type II secretory PLA2 (sPLA2-IIA), involved in lipid metabolism, functions as a cell-associated enzyme and regulated by extracellular cytokines or growth factors, is critical to signal transduction of pro-inflammatory lipid mediators \cite{16,17}. Co-localization and functional coupling of Cox-2 and sPLA2-IIA have been reported \cite{18,19}. Our previous works revealed that LPA strongly upregulated FasL presentation on ovarian cancer cell surface and induced apoptosis of activated T lymphocytes \cite{17}. Though the results indicated that extracellular signal regulated kinase (ERK) kinase cascade was required for FasL upregulation, the inhibitor of sPLA2-IIA, however, only partially inhibited ERK phosphorylation and activation, suggesting that additional pathways may participate in ERK upstream activation in response to LPA. Epidermal-growth-factor receptor (EGFR) transactivation and tyrosine phosphorylation linked to the G protein-coupled receptors (GPCRs) activation have been clearly elucidated \cite{20,21}. While others argue that ERK activation may also through the platelet-derived growth factor receptor (PDGFR) transactivation \cite{22,23}. Besides, the correlations of signaling pathways and the abnormal expressions of Cox-2 and EGFR are still questionable \cite{5,24,25}.

We therefore, first investigated the selective Cox-2 induction by LPA and its role in FasL cell surface presentation stimulated by LPA in ovarian cancer cells. We found the specific
Cox-2 inhibitors significantly inhibited FasL cell surface presentation induced by LPA. These results may support the possible clinical application of Cox-2 inhibitors in ovarian cancer chemoprevention. Secondly, we explored the upstream signaling pathways in ERK activation by EGFR transactivation upon LPA stimulation.

2. Materials and methods

2.1. Cell culture and reagents
Acetylsalicylic acid (ASA) was from Sigma Chemical Co., St. Louis, MO; Cox-2 inhibitor (NS-398, NS), PD98059, AG1478, and AG1296 were from Calbiochem (California). All cell lines, culture conditions and other materials were previously described [15].

2.2. FasL and Cox-2 immunodetection
FasL staining and analytic procedures were previously described [17]. To increase the levels of membrane-bound FasL, cells were pretreated with 10 μM matalloproteinase (MMP) inhibitor GM6001 (Calbiochem, CA). For intracellular Cox-2 staining, cells were permeabilized and fixed first, then incubated with anti-huCox-2-FITC mAb (BD Pharmingen). Isotype murine IgG1-FITC serves as control. Stained cells were analyzed by flow cytometry (FACS).

2.3. Western blot analysis
As previously described [15]. Simply, cell lyate was separated on 12% SDS-PAGE gels and transferred to membranes, then stained with the primary anti-Cox-2, anti-Cox-1 or anti-FasL (Santa Cruz Biotechnology), anti-ERK1/2 or anti-phosphoryted-ERK1/2 (p-ERK1/2, Cell Signaling Technology). The membranes were washed and then stained with the secondary anti-rabbit immunoglobulin-horseradish peroxidase (Santa Cruz Biotechnology). Protein bands were visualized by ECL Western blotting detection system (Amershan).

2.4. RT-PCR
Total RNA was isolated using Sigma GenElute Mammalian Tatal RNA kit. Reverse transcription was performed using Promega Access-Quick RT-PCR System according to the manufacturer's instructions. Cox-2 mRNA were amplified by the primers for human Cox-2: 5'-CTTTGGAAGCTTCTCTAGGC-3' (forward), 5'-CGGGAGGAGGATACATCTC-3' (reverse), with product length 388 bp. β-Actin was amplified using specific primers 5'-AGCCGAAAAATCGTGGCTG-3' (forward) and 5'-CAGGTTATATGGTGGTGGC-3' (reverse) as control, yielding a 309 bp fragment. The PCR products were analyzed on a Tris–EDTA (TAE) 1.5% agarose gel with ethidium bromide.

2.5. Assay of Cox-2 activity
Cells were serum starved for 2 h and incubated with different concentrations of LPA for 12 h in the presence or absence of arachidonic acid (AA), the spent medium was harvested and prostaglandin E2 (PGE2) was measured utilizing negative ion chemical ionization–mass spectrometry [26].

2.6. Co-culture of ovarian cancer cells with CD4+ T cells and apoptosis detection
Ovarian cancer cells were pretreated with different reagents. CD4+ cells were purified and activated, then added to adhesive ovarian cancer cells mono-layer [17]. Following co-culture for 24 h, non-adhesive T cells were collected and stained with Annexin V-FITC and PI (BD Pharmingen) and then analyzed by FACS.

2.7. Plasmid construction and transfection
The cDNA of EGFR-CD533 without the cytoplasm C-terminal 533 amino acids was previously described [27,28]. PCR product of the full length of EGFR-CD533 was cloned into pcDNA3.1/NT-TOPO using GFP Fusion TOPO TA Expression Kits (Invitrogen, Carlsbad, CA) according to the instruction manual. Clones acquired were digested and shown to be the right size inserted, and the correct coding and direction was confirmed by sequencing. The constructed pcDNA3.1/NT-EGFR-CD533-GFP-TOPO was referred as EGFR-DN plasmid thereafter. Ovarian cancer cells growing in 10 cm dish were transfected with 5 μg EGFR-DN plasmid or 5 μg control vector pcDNA3.1/NT-GFP (Invitrogen, Carlsbad, CA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transfection efficiencies (>80%) were detected by fluorescence microscopy and FACS after 48 h.

2.8. Statistics
Statistical analysis was performed on GraphPad Instat. P < 0.05 was accepted for significance.

3. Results

3.1. Selective induction of Cox-2 by LPA
To date, Cox-2 protein induction by LPA and/or sPLA2-IIA has been controversial [8,19,29]. We tested if LPA may affect Cox-2 protein and mRNA expression in ovarian cancer cells.
Western blot showed that Cox-2 protein expression was induced by LPA dose-dependently, meanwhile the constitutive expression of Cox-1 was not affected by LPA (Fig. 1A). Intracellular FACS analysis further confirmed the upregulation of Cox-2 protein by LPA (Fig. 1B). RT-PCR analysis suggested the increase of Cox-2 mRNA transcription by LPA, in both dose-dependent and time-course increasing manner (Fig. 1C). In the presence or absence of AA, cells were stimulated with different doses of LPA and the levels of PGE2 were detected. Results confirmed the increasing Cox-2 activity with increasing doses of LPA treatment (Fig. 1D).

3.2. ASA and NS-398 inhibit FasL upregulation on the ovarian cancer cell surface induced by LPA

Previous studies found LPA regulated FasL cell surface expression and secretion [17]. To investigate if Cox-2 activity may participate in FasL cell surface expression induced by LPA, we pretreated OVCAR3 cells with ASA and Cox-2 specific inhibitor NS-398 before LPA treatment. We found that both ASA and NS-398 dose-dependently blocked FasL cell surface presentation induced by LPA (Fig. 2). The same experiments were repeated with Dov13 cells for 3 times and similar results were observed (data not shown). Cells treated as above were lysed, however, Western blot did not show significant FasL protein level changes, suggesting the regulations of FasL by ASA/LPA or NS-398/LPA were most at post-translational level (Fig. 2D).

3.3. ASA and NS-398 inhibit FasL-mediated apoptosis of activated lymphocytes by ovarian cancer cells

When activated purified human peripheral CD4+ lymphocytes were co-cultured with ovarian cancer cells, we found that ovarian cancer cells induced the apoptosis of CD4+ lymphocytes [17]. Treatment Dov13 cells with LPA enhanced CD4+ lymphocytes undergoing apoptosis (Fig. 3A). However, pretreatment Dov13 cells with ASA or NS-398 before LPA treatment significantly blocked the enhancement (Fig. 3B). The experiments were performed using OVCAR3 cells and the similar results were observed (Fig. 3B). When anti-FasL neutralizing antibody was added, the apoptosis of CD4+ lymphocytes decreased, confirming the apoptosis indeed was induced through FasL and Fas interactions (Fig. 3B).

3.4. LPA regulates Cox-2 induction and FasL cell surface expression through EGFR activation and ERK1/2 phosphorylation

By binding to pertussis-toxin-sensitive GPCRs, LPA rapidly induced Cox-2 protein expression [29]. Though the activation of ERK1/2 has often been reported to be the common downstream signaling pathway [30–32], multiple ligand-independent...
protein tyrosine kinase receptors transactivations by LPA, including the EGFR and PDGFR, might work in several cellular systems [20,22,23]. To investigate the complicated regulating pathways involved in Cox-2 induction and FasL cell surface presentation, we pretreated ovarian cancer cells with specific kinase or receptor kinase inhibitors PD98059 (for ERK1/2), AG1478 (for EGFR), AG1296 (for PDGFR) before LPA stimulation. FACS revealed that both PD98059 and AG1478 time consequentially inhibited intracellular Cox-2 expression and FasL cell surface presentation induced by LPA (Fig. 4A, B). AG1296 had no effect on either Cox-2 or FasL expression (Fig. 4C). Western blot reaffirmed that the Cox-2 protein expression was regulated by PD98059 and AG1478 during LPA stimulation (Fig. 4D).

To further clarify FasL and Cox-2 expression signaling pathways regulated by LPA, EGFR-DN plasmid was constructed and transient transfection was performed in OVCAR3 cells (Fig. 5A). In normal culture or control vector cells, LPA stimulation significantly induced ERK1/2 phosphorylation and Cox-2 expression (Fig. 5B, lane 2 and lane 4). In cells pretreated with Ag1478 or EGFR-DN cells, ERK1/2 phosphorylation status or Cox-2 expression were close to basal levels, confirming that the ERK1/2 kinase activation was induced through EGFR transactivation (Fig. 5B). After LPA treatment, a slight but not significant increase of FasL protein expression was observed, confirming the regulation of FasL by LPA through induction of Cox-2 was most at post-translational. The basal level FasL protein level was relatively lower in EGFR-DN cells (lane 5 and lane 6), but there was no significant increase of FasL protein after LPA treatment either. However, FasL cell surface translocation induced in control cells was significantly blocked in EGFR-DN cells (Fig. 5C).

Fig. 4. Signaling pathways involved in LPA induced Cox-2 induction and FasL cell surface presentation. (A, B and C) OVCAR3 cells were pretreated with PD98059, AG1478 or AG1296 for 1 h, then incubated with 80 lM LPA for 16 h in the presence of the different inhibitors. FACS analysis of cell surface FasL and intracellular Cox-2. Artificial gates were set according to the isotype control and relative Cox-2 expressing cells were calculated. *P < 0.01 LPA treatment compared with control; †P < 0.01 PD98059 or AG1478 pretreatment before LPA compared with LPA treatment alone; §P > 0.05 AG1496 pretreatment before LPA compared with LPA treatment alone. The same experiments were performed on Dov13 cells and the identical results were observed (data not shown). (D) Cells treated as above were lysed and Western blots were performed for Cox-2 protein expression. Figure shown is a typical experiment of three with identical results.
4. Discussion

Though the induction of COX-2 has been reported to contribute to cancer growth and metastasis [8,33,34], our research first found that it might also provide ovarian cancer cell immune privilege by upregulation of FasL. Numerous studies have shown that FasL expression in vivo plays a role in cancer cell counter attacking immune cells and tumor development [35–37], since in tumor microenvironment, deaths of activated immune T cells are FasL-dependent and might be directly regulated by FasL expression level [38,39]. Furthermore, the very recent finding in animal model confirmed that reduced FasL expression by tumor cells led to increased lymphocyte infiltration [37]. When COX-2 inhibitors significantly blocked FasL cell surface presentation and ovarian cancer cell induced apoptosis of activated lymphocytes, immune balance and anti-tumor response in tumor microenvironment might be restored. Our study therefore provides support for COX-2 inhibitors’ clinical applications in ovarian cancer chemoprevention [9,10].

In our experimental system, CD4+ helper T cells instead of FasL expression CD8+ cytolytic cells were used as target cell during coculture to secure the FasL surface expressing ovarian cancer cells as the one-way apoptosis inducing factor. In tumor microenvironment, Fas expressing cancer cells may also face FasL expressing CD8+ cytolytic T cells or NK cells, the fate of individual cell may depend on the significance of pro- or anti-apoptosis signals. LPA quickly downregulates Fas on the ovarian cancer cell surface first [40], so the later FasL upregulation would have little disadvantage on its own fate. To the surrounding activated lymphocytes dramatic apoptosis may happen since these cells usually upregulated Fas upon activation [41]. A biomathematical prediction model shall apply into the tumor microenvironment and may guide clinical combinational therapies. The combination of COX-2 inhibitors and MMP inhibitors have been providing additive therapeutic benefits in cancers [42–45]. Since LPA provides ovarian cancer cell immune privilege while promote its invasiveness, we would like to suggest to target the signaling initiator, LPA, which might be of primary importance in ovarian cancers prevention and treatment [14,17].

Our study demonstrated that COX-2 induction coupled to ERK signaling activation played a role in LPA regulated FasL cell surface presentation. This extends previous understanding regarding LPA promote FasL cell surface translocation and function through sPLA2-IIA via ERK activation [17,46]. LPA elicits multiple and complex biochemical signaling cascades. The ligand-independent transactivation of protein tyrosine kinase receptors has been shown in several cellular systems and seems to be a general possibility of signaling networks in cancer cells [20,22,23,28]. Moreover, our study revealed the ERK phosphorylation via EGFR transactivation played a significant role in LPA-mediated COX-2 induction and FasL cell surface presentation. The inhibition of fos gene expression and DNA synthesis may explain the decrease of COX-2 protein and mRNA by inhibition of EGFR function through either the specific inhibitor AG1478 or EGFR-DN [20,28]. Since LPA also increased FasL microvesicle exocytosis, we cannot exclude that LPA might regulate FasL protein expression, somehow slightly, at translational level [17,46,47]. Together with our previous investigations, detailed upstream signaling pathways involved in FasL ovarian cancer cell surface presentation regulated by LPA were explored and summarized (Fig. 6).

5. Conclusion

We found the selective induction of COX-2 plays a role in FasL cell surface presentation stimulated by LPA. COX-2 inhibitors significantly blocked FasL cell surface presentation and ovarian cancer cell mediated apoptosis of activated lymphocytes. This research provides support for COX-2 inhibitors’ clinical application in ovarian cancer chemoprevention. By binding to GPCR on the ovarian cancer cell surface, LPA transactivates EGFR followed by ERK1/2 activation to induce COX-2 mRNA and protein expression. These consequent
events substantially affect FasL protein cell surface presentation, providing ovarian cancer cells proinflammatory phenotype and immune privilege.

References


