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Lysophosphatidic acid triggers cathepsin B-mediated invasiveness of human endometriotic cells

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ABSTRACT

Extracellular lysophosphatidic acid (LPA) and the G-protein-coupled LPA receptors (LPAR) are involved in cell migration and invasion and found in the human endometrium. However, underlying mechanisms resulting in cellular invasion have been rarely investigated. We used stromal endometrial T-HESC, epithelial endometriotic 12Z, 49Z and Ishikawa cells. Interestingly, proliferation of T-HESC cells was strongly increased after LPA treatment, whereas the epithelial cell lines only showed a moderate increase. LPA increased invasion of 12Z and 49Z strongly and significantly. The LPAR inhibitor Ki16425 (LPAR1/3) attenuated significantly LPA-induced invasiveness of 12Z, which was confirmed by LPAR1 and LPAR3 siRNAs, showing that both LPA receptors contribute to invasiveness of 12Z cells. Investigation of cell invasion with an antibody-based protease array revealed mainly differences in cathepsins and especially cathepsin B between 12Z compared to the less invasive Ishikawa. Stimulation with LPA showed a time- and dose-dependent increased secretion of cathepsin B which was inhibited by the Gq inhibitor YM-254890 and Gi/o inhibitor pertussis toxin in the 12Z cells, again highlighting the importance of LPAR1/3. The activity of intracellular and secreted cathepsin B was significantly upregulated in LPA-treated samples. Inhibition of cathepsin B with the specific inhibitor CA074 significantly reduced LPA-increased invasion of 12Z. Our results reveal a novel role of LPA-mediated secretion of cathepsin B which stimulated invasion of endometriotic epithelial cells mainly via LPAR1 and LPAR3. These findings may deepen our understanding how endometriotic cells invade into ectopic sites, and provide new insights into the role of LPA and cathepsin B in cellular invasion.

1. Introduction

Lysophosphatidic acid is a small phospholipid and is synthesized mainly by two pathways with the key enzymes autotaxin (ENpp2) and phospholipase A_2 [1]. It can be found extracellularly in plasma, serum, saliva, tears, follicular fluid and cerebrospinal fluid [2]. Especially high levels were found in serum of patients with multiple myeloma and in ascites fluid of ovarian cancer [3].

On the cellular level, LPA influences proliferation, survival, migration, adhesion, invasion, hypertension, smooth muscle cell contraction, neurite retraction, platelet aggregation, and secretion of cytokines and chemokines [1,4]. This broad spectrum of LPA actions is mediated through its interactions with six known LPA receptors (LPAR1–6), all of them being class A rhodopsin-like G protein coupled receptors (GPCRs) [5]. For example, LPAR1/3 predominantly couple to $G_{q/11}$ and $G_{i/o}$ [5] leading to diverse cellular processes such as cell proliferation, migration and invasion [3]. While LPAR1 is found in neurons, bone, renal and lung fibrosis, as well as in ovarian, pancreas, and breast cancer LPAR2 is found in testis, leucocytes, prostate, spleen, intestine, and pancreas [4]. Furthermore, LPAR3 is present in brain, heart, pancreas, lung, prostate, endometrium and ovary. All three LPARs are abundant on nearly all immune cells [5] and are often overexpressed in certain cancers [3]. In contrast, expression profiles of LPAR4–6 are less well characterized.

Because of its stimulatory effects on cell proliferation, migration, and invasion, LPA has been in the focus of cancer research for several years [3]. Its interactions with LPAR2 induce invasiveness of endometrial cancer cells through LPAR2 [6], with LPAR1 invasiveness of ovarian cancer [7], and its binding to LPAR3 triggers migration of oral squamous carcinoma cells [8]. While at this point, there is no question

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about the involvement of LPA and LPAR in pathological conditions, the apparent redundancy caused by different receptors using similar ligands and downstream signaling elements, makes a general clinical treatment approach challenging [9].

Endometriosis is a life quality impairing, pathological condition that affects up to 10% of women most often associated with severe pain and infertility [10]. It is usually classified as deep infiltrating (DIE), peritoneal or ovarian endometriosis, which are characterized by the presence of endometrial tissue outside the uterine cavity [11]. While several explanations for the pathogenesis of endometriosis exist [12], the mechanisms involved in the invasive actions of the endometrial cells and their translocation and invasion of extrauterine tissues are, however, poorly understood [13].

Stromal cells of the human endometrium express high quantities of LPAR1 and LPAR6 [14]. LPAR3, which is cyclically regulated and highly abundant in the secretory phase in epithelial and stromal endometrial cells [15], was also found to be involved in the decidualization of the uterine epithelium in mice [16]. Of note, LPAR1 was described to induce IL-8 expression via a nuclear factor-κB-dependent signal pathway in endometrial stromal cells [17] as well as trophoblast cells [18]. Thus, the authors suggested that LPAR1 might play a role in angiogenesis of endometrium and placenta during pregnancy [17,18]. However, a possible involvement of the LAP receptors in the manifestation of endometriosis has not been assessed to date. It was therefore the intention of the present investigation to address LPA/LPAR effects on molecular mechanisms that might be associated with proliferation and invasive behavior of endometrial and endometriotic cells. As a model served human endometrial T-HESC, endometriotic 12Z and 49Z cells and epithelial Ishikawa cells.

2. Methods

2.1. Cell lines

The endometrial T-HESC and endometriotic 12Z and 49Z cell lines were chosen because they represent both, the stroma and the glandular epithelial component of the endometrium which might play a role in the pathogenesis of endometriosis. The stromal T-HESC cells (ATCC, CRL-4003) resemble fibroblasts from normal endometrium and show typical endometrial characteristics [19]. We test the T-HESC cells regularly with immunofluorescence for positivity for CD10, which is a very specific marker for stromal endometrial and endometriotic cells [20] and negativity of keratins to avoid epithelial contamination. The epithelial cells 12Z and 49Z (provided by Prof. Starzinski-Powitz, Frankfurt, Germany) from active peritoneal endometriotic lesions [21] show characteristics of the active phase of endometriosis and thus are suitable for studying cellular behavior of endometriosis [19] and especially for invasion [22]. The 12Z cells are positive for keratin 19, ER-α, ER-β, PR [21] and negative for CD10. The 49Z cells are positive for keratin 19, but weakly for ER-α, PR, and negative for ER-β and CD10. The Ishikawa cells originate from an endometrial adenocarcinoma and were purchased from ATCC just prior to this study. While this cell line certainly does not represent healthy or endometriotic tissue, it retains characteristics of endometrial glandular epithelium and thus is used in several studies on endometriosis as a representative cell line for the human endometrium [23,24]. In our study, we used the epithelial Ishikawa cells as a low-invasive control [25] for comparison with the epithelial 12Z cells which are highly invasive [21].

2.2. Cell culture

Each cell line was cultured in 175 cm^2 flasks in a humidified incubator at 37 °C and 5% CO_2. Medium was renewed every 3–4 days. Cells were detached with 0.25% accutase and passaged at 70–80% confluency. Growth medium composition has been as follows: T-HESC; DMEM/F12 with 2 mM glutamine supplemented with 10% FCS, 1% penicillin/streptomycin (pen-strep), 1% insulin, transferrin and selenium solution; Ishikawa: MEM supplemented with 5% FCS, 1% non-essential amino acids, 2 mM glutamine and 1% penicillin/streptomycin; 49Z and 12Z: DMEM, 4.5 g/L glucose supplemented with 10% FCS, 2 mM glutamine and 1% pen-strep. All cell culture reagents were from Invitrogen/Thermo Scientific (Karlsruhe, Germany).

Table 1
List of primer sequences used for RT-PCR.

Oligo name	Sequence (5′ → 3′)	Position/ Refseq	Amplicon size
LPAR1 fwd	GCGCCAGGTACACAGCTT	239-256, NM_001401.3	497 bp
LPAR1 rev	GTCAATGAGGCCCTGACGAA	735-716	
LPAR2 fwd	ACTGGAGGCCCAGATGGTCA	127-146, NM_004720.5	633 bp
LPAR2 rev	ACAGCCACCATGAGCAGGAA	759-740	
LPAR3 fwd	GGGTCCATAGCAACCTGACC	620-639, NM_012152.2	340 bp
LPAR3 rev	AACGCCCCTAAGACAGTCATC	959-939	
LPAR4 fwd	TGTTTGCTAAAGGCATGCGG	24-43, NM_001278000.1	312 bp
LPAR4 rev	TCTGTCTTCCCCAAGAAAGAGTG	335-313	
LPAR5 fwd	AATGCTTCCTCTTCACTCTCTCC	24-46, NM_020400.5	950 bp
LPAR5 rev	TCTCGAAGCATAGGCGCAC	973-955	
LPAR6 fwd	ATGGCAAACATTTGCTGCAT	246-265, NM_005767.5	700 bp
LPAR6 rev	CGCTTTCTCACAATTCTTGGT	945-925	
GAPDH fwd	TCAGAACACCTATGGGGAAGGT	23-44, M17851.1	258 bp
GAPDH rev	AGGGATCTCGCTCCTGGAAG	280-261	

2.3. LPAR1–6 expression profile by RT-PCR

A total of $5 * 10^5$ cells of each cell line were grown as described above. Total RNA was extracted with the RNAeasy Kit (Qiagen, Hilden, Germany) in accordance to the user manual. Reverse transcription was performed using a cDNA Synthesis Kit H-Minus (PeqLab/VWR, Erlangen, Germany) according to the supplier's instructions. Primers were designed with the NCBI Primer-Blast algorithm and were intron-spanning except for LPAR5 (Invitrogen/Thermo Scientific, Table 1). The other PCR reagents were purchased from Bio&Sell (Nürnberg, Germany). Semi-quantitative PCR was performed with 1 µg cDNA. GAPDH was used as a positive control. After an initial heating to 95 °C for 4 min, each cycle consisted of denaturing at 95 °C for 30 s, annealing at 58 °C for 20 s and elongation at 72 °C for 40 s except for the final extension which lasted 5 min. The program consisted of 35 cycles.

2.4. LPA ELISA

Each cell line ($1.5 * 10^5$ cells) was cultured in 5 cm dishes in serum-free medium for 48 h and supernatants analyzed with the LPA ELISA Kit (KA1041, Abcam, range 1.563–50 ng/mL, detection limit 0.85 ng/mL) according to the manufacturer's instructions.

2.5. Basement membrane invasion assays

Invasion assays were performed with fluorometric CytoSelect 24-well Cell Invasion assays (Biocat, Heidelberg, Germany) coated with a basement membrane protein matrix. The assays were carried out according to the manufacturer's instructions. Briefly, $1.5 * 10^5$ cells were cultured on 24-well inserts for 48 h in 300 µL serum-free medium with all cell line specific supplements. The lower compartment contained the respective cell culture medium with 10% FCS as a chemoattractant. LPA (18:1 LPA sodium salt, Santa Cruz Biotechnology Heidelberg, Germany), the LPAR1/3 specific inhibitor Ki16425 (ABSource Diagnostics Munich, Germany) or the cathepsin B specific inhibitor