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Original Contribution

PLA2R1 kills cancer cells by inducing mitochondrial stress

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ABSTRACT

Little is known about the biological functions of the phospholipase A2 receptor (PLA2R1) except that it has the ability to bind a few secreted phospholipases A2 (sPLA2′s). We have previously shown that PLA2R1 regulates senescence in normal human cells. In this study, we investigated the ability of PLA2R1 to control cancer cell growth. Analysis of expression in cancer cells indicates a marked PLA2R1 decrease in breast cancer cell lines compared to normal or nontransformed human mammary epithelial cells. Accordingly, PLA2R1 ectopic expression in PLA2R1-negative breast cancer cell lines led to apoptosis, whereas a prosenescence response was predominantly triggered in normal cells. PLA2R1 structure–function studies and the use of chemical inhibitors of sPLA2-related signaling pathways suggest that the effect of PLA2R1 is sPLA2-independent. Functional experiments demonstrate that PLA2R1 regulation of cell death is driven by a reactive oxygen species (ROS)-dependent mechanism. While screening for ROS-producing complexes involved in PLA2R1 biological responses, we identified a critical role for the mitochondrial electron transport chain in PLA2R1-induced ROS production and cell death. Taken together, this set of data provides evidence for an important role of PLA2R1 in controlling cancer cell death by influencing mitochondrial biology.

Introduction

The phospholipase A2 receptor (PLA2R1) is a 180-kDa type I transmembrane protein. Its extracellular domain is composed of a cysteine-rich domain (Cys-R), a fibronectin-like type II domain (FNII), and eight C-type lectin-like domains (CTLDs). It also contains a transmembrane domain and a short cytoplasmic tail with unknown functions, if any [1]. Via its distinct domains, PLA2R1 can bind several secreted phospholipases A2 (sPLA2) and various types of collagens and carbohydrates [1–4].

We have previously demonstrated that PLA2R1 knockdown in normal human fibroblasts delays replicative senescence, whereas its ectopic expression induces premature senescence [5]. This senescence-regulating effect in normal human fibroblasts involves reactive oxygen species (ROS) accumulation and a subsequent activation of the DNA damage and p53 pathway [5].

To extend these observations, we have now screened for PLA2R1 expression in cancer cell lines and found that its expression decreases in breast cancer cells. We next investigated the effect of its ectopic expression in PLA2R1-negative breast cancer cell lines and found that it strongly induces cancer cell death. Structure–function studies done by deleting the various extracellular domains of PLA2R1 and by using chemical inhibitors of sPLA2 signaling pathways suggest an sPLA2-independent mechanism. Finally, we found that PLA2R1 induces cell death in a mitochondrial ROS-dependent manner.

Materials and methods

Cell culture

Human cancer cell lines (ATCC) were cultured in the following media: Dulbecco′s modified Eagle′s medium (DMEM; Invitrogen) for MDAMB-453 and WI38 and minimal essential medium (Invitrogen)

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for Cama-1 and BT-20 (Invitrogen). Virus-producing GP293 cells (Clontech) were cultured in DMEM. All media were supplemented with 10% fetal bovine serum (FBS; Lonza), 1% penicillin/streptomycin (Invitrogen), 0.36% gentamycin (Invitrogen). For Cama-1 and BT-20 cell lines, 1% nonessential amino acids (Invitrogen) was added. HMECs (Clonetics) were cultured in mammary epithelial cell growth medium (Promocell) in the presence of 1% penicillin/streptomycin and 0.36% gentamycin.

Vectors

Human wild-type membrane-bound PLA2R1 (GenBank NM 007366) was generated by PCR from the PLA2R1-encoding pSupF vector and ligated into the pGEMTeasy vector (Promega), fully sequenced, and subsequently subcloned in the pLPCX retroviral vector (Clontech) using XhoI/NotI restriction sites.

Transfection and infection

GP293 cells were transfected by means of PEI reagents according to the manufacturer's recommendations (Euromedex). Two days after transfection, the viral supernatant mixed with fresh medium (1/2) and Polybrene (final concentration 8 μg/ml) was used to infect target cells. Cells were infected for a period of 12–24 h depending on the cell type. Importantly, the infection protocols were designed so that practically all cells were infected, as judged by the results of infection with a green fluorescent protein control. One day postinfection, cells were selected with puromycin at the final concentration of 0.75–1.5 μg/ml depending on the cell type.

Immunoblot

Cell lysates were prepared in ice-cold Giordano buffer (50 mM Tris–HCl, pH 7.4, 250 mM NaCl, 0.2% Triton X-100, 5 mM EDTA) supplemented with protease and phosphatase inhibitors (Roche). Lysates were clarified by centrifugation at 14,000 rpm for 30 min at 4 °C. Protein concentrations were measured by means of the Bradford protein assay (Bio-Rad 500-0006). Cell extracts were resolved by SDS–PAGE under nonreducing conditions for PLA2R1 detection or reducing conditions for detection of other proteins and transferred onto nitrocellulose membranes. For detection, the following primary antibodies were used: anti-PLA2R1 (HPA012657, Atlas), anti-active caspase-3 (ab32042, Abcam or 9661, Cell Signaling Technology), anti-actin (A5316, Sigma, St. Louis, MO, USA), and anti-H3-pSer10 (ab14955; Abcam). After overnight incubation at 4 °C with primary antibodies, blots were washed with phosphate-buffered saline (PBS) containing 0.05% Tween, incubated with a peroxidase-coupled secondary antibody, and washed again in PBS containing 0.05% Tween. Antigen–antibody complexes were detected by ECL (Amersham).

RNA extraction, retrotranscription, and PCR

Total RNA extraction was prepared using a phenol–chloroform method using Tri reagent (Sigma–Aldrich). PhaseLockGel tubes (Eppendorf, Hamburg, Germany) were used for phase separation. The synthesis of cDNA was performed from 3 μg of total RNA using the First-Strand cDNA Synthesis Kit (GE Healthcare, Chalfont St. Giles, UK). The reverse transcription reaction was diluted 1/60 and used as cDNA template for qPCR analysis. TaqMan quantitative PCR analysis was carried out on a LightCycler 2.0 system (Roche Applied Science). PCR mixtures contained LightCycler TaqMan mix, 200 nM primers, and 1.67 μl of cDNA template in a 6.67-μl reaction volume. Housekeeping genes ACTB and PGK1 were used in breast cancer cell lines. Real-time PCR intron-spanning assays were designed using the ProbeFinder software (Roche Applied Science).

Colony formation assays

Colony formation assays were carried out in 12-well plates. Five to 10 days after seeding, the cells were washed with PBS, fixed with 4% paraformaldehyde, and stained with 0.05% crystal violet (Sigma–Aldrich).

TUNEL assays

Cells cultured under various conditions were harvested by trypsinization and then pooled with their culture supernatants (so as to include nonadherent cells in the test samples). After centrifugation the cells were resuspended in FBS (100%) and 1×10^5 cells were cytospun on microscopic slides. After fixation with 3.7% paraformaldehyde, the cells were washed three times in 1 × PBS and permeabilized 15 min in PBS/0.2% Triton X-100. Cells were serially washed in 1 × PBS and then with terminal deoxynucleotidyl transferase (TdT) buffer (Tris 30 mM, sodium cacodylate 150 mM, pH 7.5) to which was added extemporaneously $CoCl_2$ (1 mM). dUTP addition at the 3′-OH DNA termini was performed by incubating cells 1 h at 37 °C in a solution of TdT/$CoCl_2$ buffer in the presence of biotin–dUTP (Roche 11093070910; 6 μM final) and the TdT enzyme (Roche 03333566001; 2.4 U/μl final). Reaction was stopped by washing with TB buffer (NaCl 300 mM, sodium citrate 34.1 mM) for 15 min at room temperature. Cells were then washed in 1 × PBS, blocked 10 min in a solution of 1 × PBS containing 2% bovine serum albumin, and incubated 1 h with Cy3-coupled streptavidin in PBS. Nuclei were stained with Hoechst for 10 min, washed three times in 1 × PBS, and mounted using Fluoromount-G (SouthernBiotech). At least 500 events were recorded.

Senescence-associated-β-galactosidase (SA-β-Gal) analysis

SA-β-Gal analyses were performed as in [6]. At least five different fields were counted for each condition representing at least 500 events.

ROS assays

Cellular ROS content was assayed by incubating cells with 3 μM H_2DCF-DA probe for 60 min. The cells were washed in 1 × PBS buffer, trypsinized, and resuspended in 300 μl 1 × PBS buffer. They were counterstained with propidium iodide (PI) to exclude dead cells from the analysis. ROS levels were analyzed by FACS with a FACSCalibur flow cytometer, with 10,000 events recorded. The final data were analyzed using the Flow Jo 7.5.5 software.

ROS production was also determined using Amplex red assay (Molecular Probes) as described in [7]. Briefly, Amplex red mix (20 μM Amplex red and 0.2 U/ml horseradish peroxidase (HRP)) was added to the fresh culture medium before measurement. Amplex red was excited at 530 nm and emission was measured at 590 nm using Tecan M1000 PRO and fluorescence was recorded every 2 min for 18 min. Fluorescence was normalized to the number of cells and represented as relative fluorescence intensity.

Mitochondrial lipid oxidation assessment

Cardiolipin oxidation was assessed using 10-*N*-nonyl acridine orange (NAO) as in [8].

Annexin V/PI analysis

Cells collected by centrifugation were counted and then incubated with annexin V–FITC in 1 × binding buffer (10 mM Hepes/NaOH, pH