ORIGINAL ARTICLE

Role of 12-lipoxygenase in regulation of ovarian cancer cell proliferation and survival

Austin M. Guo · Xiuli Liu · Zaid Al-Wahab ·
Krishna Rao Maddippati · Rouba Ali-Fehmi ·
A. Guillermo Scicli · Adnan R. Munkarah

Abstract

Purpose Eicosanoid-related enzymes have been implicated in the pathogenesis of various cancers. Little is known about the relevance of lipoxygenase pathway to ovarian cancer growth. In this study, we examined the role of 12-lipoxygenase (12-LOX), the main human 12-HETE generating enzyme, in the regulation of proliferation and survival in epithelial ovarian cancer.

Methods Immunohistological analysis of 12-LOX expression in high-grade serous ovarian carcinoma and normal ovarian epithelium tissues was performed. The presence of 12-LOX-12-HETE system was confirmed in two epithelial ovarian cancer (EOC) cell lines, OVCAR-3 and SK-OV-3, using RT–PCR, Western blot and LC/MS analysis. The effects of N-benzyl-N-hydroxy-5-phenyl-pentanamide (BMD-122), a specific 12-LOX inhibitor, on cell growth, survival, apoptosis, and cell signaling were determined.

Results We found that a significantly higher level of 12-LOX expression in high-grade serous ovarian carcinoma compared to normal ovarian epithelium. OVCAR-3 and SK-OV-3 were found to express high level of 12-LOX mRNA and protein. Both EOC increased their 12-HETE production when incubated with arachidonic acid. BMD-122 inhibited the EOC growth in a dose-dependent fashion. Purified 12-HETE significantly reversed such inhibitory effects of BMD-122. In addition, BMD-122 blocked the MAPK signaling pathway by inhibiting the phosphorylation of ERK and induced a ~ 20–30% increase in the EOC apoptosis. Down-regulation of the 12-LOX expression using 12-LOX siRNA also resulted in markedly reduction in cell growth.

Conclusions These data suggest that 12-LOX is involved in the regulation of ovarian cancer cell growth and survival and is a potential new therapeutic target.

Keywords 12-lipoxygenase/12-HETE · BMD-122 · Proliferation · Ovarian cancer · Apoptosis · Caspases

A. M. Guo · X. Liu · A. R. Munkarah
Women's Health Services, Gynecological Oncology Research,
Henry Ford Health System, One Ford Place, Room 4D99,
Detroit, MI 48202, USA

Z. Al-Wahab
Department of Obstetrics and Gynecology,
Wayne State University/Karmanos Cancer Institute,
Detroit, MI 48201, USA

K. R. Maddippati · R. Ali-Fehmi
Department of Pathology, Wayne State University,
Detroit, MI 48201, USA

A. G. Scicli
Eye Care Services, Henry Ford Health System,
Detroit, MI 48201, USA

A. M. Guo
Department of Pharmacology, College of Medicine,
Wuhan University, Wuhan, People's Republic of China

A. M. Guo (✉)
Department of Pharmacology, New York Medical College,
15 Dana Road, BSB 546, Valhalla, NY 10595, USA
e-mail: mguo1@hfhs.org

Introduction

Epithelial ovarian cancer, the second most common gynecologic malignancy, accounts for 3% of all new cancer diagnoses in women in the United States. However, it is the most lethal gynecologic malignancy and the fifth leading cause of cancer death among women. In the year 2010,

21,880 new cases and 13,850 deaths were estimated [1]. Despite significant advances made in the field of cancer therapy, mortality rate associated with this disease continues to be high [1]. Therefore, there is a continued need to better understand the biology of the disease and identify novel effective therapies.

Eicosanoids have been shown to play an important role in the control of many cellular responses and cancer pathogenesis [2–4]. Eicosanoids are derived from the metabolism of arachidonic acid (AA) by three major families of enzymes known as cyclooxygenases (COX), lipoxygenases (LOX), and cytochromes P450 (CYP450) [5]. Among all three AA-metabolizing enzymes, one of the COX isoenzymes, COX-2, has been extensively studied and shown to play a crucial role in ovarian cancer progression. Studies on the possible role of the other pathways of AA metabolism are very scarce. Non-steroidal anti-inflammatory drugs (NASIDs) have been reported to have beneficial effects on reducing the risk of developing some solid tumors [6, 7] and have antitumor effects on many types of cancers. These studies were the basis to investigate the use of selective COX inhibitors in cancer treatment. However, the side effects associated with COX inhibitors have limited their therapeutic application.

Mammalian LOX mainly consists of three subfamilies of enzymes: 5-, 12- and 15-LOX [8, 9]. In general, 5- and 12-LOX have potential pro-carcinogenic roles, whereas 15-LOX-2 is believed to have an anti-cancer effect, and the role of 15-LOX-1 remains controversial [10, 11]. The two major isoforms of 12-LOX are platelet and leukocyte LOX. The platelet type was cloned from human platelets (*p*12-LOX) and the leukocyte type (*l*12-LOX) from porcine leukocytes. In humans, the main 12-LOX is *p*LOX. 12-LOX catalyzes the stereospecific oxygenation of AA to form 12(S)-hydroperoxyeicosatetraenoic acid (HPETE), which is reduced to the corresponding hydroxy compound, 12(S)-hydroxyeicosatetraenoic acid (12-HETE) [12]. Increased 12-LOX activity has been implicated in a variety of pathologies [13–15]. Accumulating evidence suggests that LOX pathways are involved in the regulation of the development of various types of cancer. 12-LOX has been shown to be expressed at low and basal levels in normal tissues [16, 17]. Whereas many tumor tissues, including prostate [18, 19], lung [20], gastric [21], colorectal [22], and breast cancers [23] over-express 12-LOX and produce 12-HETE. 12-HETE enhances tumor motility and secretion of proteinases and angiogenic factors. Increased 12-LOX and its metabolite, 12-HETE, have been reported to associate with increases in cancer angiogenesis and metastasis in several cancer types [18, 19, 22, 24].

Although 12-LOX expression and actions have been studied in a variety of tumors, the role of 12-LOX in ovarian cancer regulation has not been well defined. Freedman et al. reported a higher endogenous levels of 12-HETE in EOC tumor tissues than in benign peritonium tissues [25]. However, there are no data indicating whether 12-LOX expression is higher in human ovarian cancer, nor what possible functional role the 12-LOX-12-HETE system may have regulating ovarian cancer growth and survival. The objective of this study was to investigate the role of 12-LOX in EOC by (1) assessing levels of 12-LOX expression in ovarian cancer specimens as well as cell lines, (2) determining the effect of 12-LOX inhibition using pharmacologic agents as well as siRNA on cellular proliferation and apoptosis.

Materials and methods

Cell cultures

Normal human ovarian surface epithelial cells (HOSEpiC) were purchased and maintained in ovarian surface epithelial cell medium from ScienCell Research Laboratories as recommended by the manufacturer (Calsbad, CA). HOSEpiC at very early stages (passage 2–3) were used in all the experiments. Human epithelial ovarian cancer cells (OVCAR-3 and SK-OV-3) and culture reagents were purchased from American Type Culture Collection (Manassas, VA) unless otherwise indicated. OVCAR-3 cells were propagated in RPMI1640 (Invitrogen) with 20% non-heat activated FBS and 0.01 µg/ml bovine insulin (Sigma, St Louis, MO) and SK-OV-3 cells were grew in McCoy's 5A modified medium with 10% FBS. All cultures were maintained at 37°C in a humidified incubator containing 5% CO_2.

Reagents

BMD-122 (also known as BHPP, N-benzyl-N-hydroxy-5-phenylpentanamide) was synthesized by one of our co-authors (KRM) according to published procedure [21]. Annexin-V and propidium iodide was purchased from Sigma (St. Louis, MO). Baicalein was purchased from Cayman chemical company (Ann Arbor, MI). Cell culture regents were obtained from Invitrogen. Anti-12-LOX antibody was purchased from Abcam (Cambridge, MA). Anti-12(R)-LOX antibody was purchased from Novus Biologicals (Littleton, CO). Anti-phospho-ERK1/2 and anti-phospho-AKT antiserum was purchased from Upstate (Waltham, MA). Anti-procaspase-8 antibody was purchased from Santa Cruz. Horseradish peroxidase-coupled secondary antibodies were all from Pierce, Rockford, IL.

Immunohistological analysis of 12-LOX expression in ovarian cancer tissues sections

Twenty benign ovarian tissues specimens, obtained from cases that underwent salpingo-oophorectomy for benign uterine pathology, and 20 high-grade serous ovarian carcinomas were retrieved from archival materials from the Detroit Medical Center/Karmanos Cancer Center pathology department. Case distribution by FIGO stage was as follows: Fifteen were stage III and 5 were stage IV at diagnosis. H- and E-stained slides from each case were reviewed for validation/confirmation of diagnosis and histology by one of the authors (RAF). Immunohistochemistry for 12-LOX expression was performed on 5-μm tissue sections from the selected cases. After de-paraffinizing and hydrating with phosphate-buffered saline (PBS, pH 7.4), the sections were pretreated with hydrogen peroxide (3%) for 10 min to remove endogenous peroxidases and incubated in goat serum for 10 min. A primary antibody for 12-LOX (Santa Cruz, SC-32939) dilution (1:200) was then applied to each section, followed by washing and incubation with the biotinylated secondary antibody for 20 min at room temperature. Detection was performed with AEC, and counterstaining was done with Mayer's hematoxylin followed by mounting.

The expression of 12-LOX was assessed based on the presence of cytoplasmic staining. The scoring was assigned based on the percentage of positive epithelial cells: a zero score was used if no cytoplasmic staining was noted in any cell; score 1 with <5% of cell staining positive; score 2 with 6–30%; and score 3 with >30% of cells staining positive. For statistical analysis, cases with score 0 or 1 were considered as being negative and cases with score 2 or 3 as positive.

Cell proliferation assay

Cell growth studies were performed with cultures plated at a density that ensured exponential growth for at least 5 days. All test agents were dissolved in ethanol (EtOH) unless indicated otherwise, and an equal volume of EtOH was added to the cultures as a vehicle control. The concentration of EtOH in the medium never exceeded 0.1%. In most of the experiments, MTT assay was performed by plating OVCAR-3 at 5×10^3 and SK-OV-3 at 2×10^3 cells in 96-well plates. Cells were allowed to grow overnight before changed to serum-free media prior to the addition of test reagents. Eight hours later, the serum-free media containing test reagents were discarded and cells were washed twice with $1\times$ PBS. Then, normal growth media containing the test agents were added. MTT assay were performed 40 h later as recommended by the manufacturer. For 12-HETE rescue experiment, cell counting

were used to assess proliferation after treating OVCAR-3 and SK-OV-3 with V (vehicle control), 10 μM BMD122, 1 μM 12(S)-HETE, BMD + 12(S)-HETE, 1 μM 12(R)-HETE, and BMD122 + 12(R)-HETE for 48 h.

12-LOX RT–PCR

Total RNA was extracted from normal human ovarian epithelial cells, OVCAR-3 and SK-OV-3 cells, using TRIZOL reagent and treated with DNAse and quantitated. Five micrograms of RNA was used as template for the RT reactions using SuperScript First-Strand Synthesis System for RT–PCR (Invitrogen). Primers used for PCR were 12-LOXF (5′-CTTCCCGTGCTACCGCTG-3′) and 12-LOXR (5′-TGGGGTTGGCACCATTGAG-3′). Amplification was performed at 95°C for 3 min, followed by 35 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 45 s. The PCR reaction was extended at 72°C for 10 min. The products were checked by electrophoresis on 1% agarose gels.

Western blotting

Normal human ovarian epithelial cells, OVCAR-3 and SK-OV-3, were plated and allowed to grow to 80% confluency before protein lysates were harvested. Western blot using anti-human platelet-type 12-LOX antibody, anti-12(R)-LOX, anti-phospho-ERK1/2, anti-phospho-AKT, and anti-procaspase-8 was performed as previously described [26]. In brief, 40–80 μg of protein was separated on a 14% Tris–glycine gel (Invitrogen) and electroblotted on a PVDF membrane (Biotrace, Bothell, WA). Membranes were blocked for 1 h at room temperature with blocking buffer [0.2% I-Block reagent (Tropix, Bedford, MA), 0.1% Tween-20 in $1\times$ PBS] before incubation with primary antibodies in blocking buffer (overnight at 4°C). All primary antibodies were used at a dilution of 1:500–1:1,000. After washing $3\times$ in washing buffer ($1\times$ TBS and 0.1% Tween-20), membranes were incubated for 1 h at room temperature with a peroxidase-conjugated secondary antibody (Thermo Scientific, Rockford, IL) (diluted 1:3,000 in blocking buffer). Membranes were then washed 3 times in washing buffer, and chemiluminescence detection was performed using an enhanced chemiluminescence kit (Thermo Scientific) according to the manufacturer's protocol. Stripped membranes were re-probed with actin (Santa Cruz) that served as a loading control. Similar protocol was repeated in EOC in which 12-LOX were down-regulated using siRNA.

LC/MS analysis of 12-HETE formation

Cultured OVCAR-3 and SK-OV-3 cells were incubated with 10 μM AA in serum-free medium for 8 h. Cell pellets and cell media were separated by centrifugation. Cell

pellets were resuspended in 3 ml of cold 1× PBS and homogenized by sonication. Then, harvested culture media and cell homogenization samples were spiked with 10 ng of 15(S)-HETE-d8 (internal standard), applied to pre-conditioned SEP-Pak C18 cartridges (100 mg adsorbent, Waters), washed with water followed by hexane. Eicosanoids were eluted with 500 µl of ethyl acetate-hexane (3:1). The eluate was dried under nitrogen and reconstituted in methanol: 25 mM aqueous ammonium acetate (7:3). The extracted and reconstituted sample was subjected to HPLC on a Max-RP C18 column (2×150 mm, 3μ, Phenomenex) eluted isocratically with methanol:13 mM aqueous ammonium acetate (8:2) at a flow rate of 0.4 ml/min. The eluent was monitored for HETEs by mass spectrometer (QuattroLC, Micromass) in the negative ion mode using Multiple Reaction Monitoring for transitions of m/z 319 to m/z 179 for 12-HETE (Source block: 120°C, Desolvation: 350°C, Cone voltage: –24 V, Collision energy: 14 eV, and Collision gas pressure: 3.2×10^{-4} Bar). 15(S)-HETE-d8 (MRM transition of m/z 327–226 under identical conditions) was used as internal standard for recovery and quantitation. Under these conditions, retention times for 15(S)-HETE-d8 and 12-HETE were 2.6 and 2.9, respectively. Minimum detection limit is 50 pg for each compound on the column under these conditions. Quantitations of 12-HETE production were normalized against total cellular protein concentrations (determined against BSA standard).

siRNA silencing of 12-LOX in EOC

12-LOX in both OVCAR-3 and SK-OV-3 was down-regulated using the Stealth RNAi siRNA Card for human Alox12 (Catalog # 1299003; three 12-LOX-specific 20–25 nt siRNA construct) from Invitrogen. Cells were transfected using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, 12-LOX-specific (180 pmol for SK-OV-3; 240 pmol for OVCAR-3) and scrambled control siRNA were mixed with Lipofectamine 2000 reagent and added to the two EOC cultures at 37°C in the opti-MEM medium. The transfection was carried out for 48 h. Protein extracts were harvested from these cultures, and Western blot analysis was performed using antibodies against 12-LOX. In a different experiment, transfected OVCAR-3 and SK-OV-3 cultures were trypsinized and plated for assessing the effects of siRNA on cell growth and survival using MTT assay. Greater than 80% of transfection was confirmed using green fluorescent protein (GFP)-linked control siRNA primers.

Assessment of apoptosis

The effects of BMD-122 to induced apoptosis in OVCAR-3 and SK-OV-3 cells were examined by fluorescence-activated cell-sorting (FACS) analysis after labeling the cells with an annexin V-FITC antibody (Sigma, St. Louis, MO). After incubation of EOC cells with either vehicle or various concentrations of BMD-122 for 20 h, the cells were trypsinized, washed, and pelleted. Non-attached floating cells were also collected by centrifugation. The cells were resuspended in a binding buffer, treated with 5 µl of annexin V-FITC conjugate and 10 µl of propidium iodide and incubated in the dark at room temperature for 20 min before performing a FACS analysis using a Becton–Dickinson FACScan (Franklin Lakes, NJ) cell sorter. A minimum of 10^4 events per sample was collected. Data were analyzed using Cell Quest Pro software (San Jose, CA). Alternatively, fluorescent caspase-9 and caspase-3 apoptosis assay (Santa Cruz Biotechnology, Santa Cruz, CA) were performed in BMD-122 treated OVCAR-3 and SK-OV-3 cells to determine whether BMD-122 activate caspase-9 and caspase-3 activities in these cultures. Equal numbers of cells in 96-well plate were exposed to various concentrations of BMD-122 for 20 h again. Caspase-9 and caspase-3 activities were measured following the manufacturer's recommendations.

Statistical analysis

Data were analyzed using ANOVA followed by Tukey's test or a Student's t test when only 2 groups were studied. A $P < 0.05$ was considered to be significant.

Results

Ovarian tumor tissue express higher level of 12-lipoxygenase than normal ovarian tissue

To determine whether there is a differential expression of 12-LOX in normal human ovarian tissues and ovarian cancer tissues, immunohistological analysis of 12-LOX expression was performed in 20 high-grade serous ovarian carcinomas samples and compared with normal human ovarian epithelium sections. 12-LOX expression with positive cytoplasmic staining was seen in 75% of ovarian cancer tissue sections examined by immunohistochemistry (Fig. 1). We did not detect significant 12-LOX expression (only basal) in the normal ovarian epithelial tissue we tested. These data suggest that 12-LOX expression is markedly higher in ovarian cancer tissues than in normal ovarian epithelium.

12-LOX in OVCAR-3 and SK-OV-3 human ovarian epithelial cancer cells

To determine whether 12-LOX-12-HETE system was present and functional in two commonly used human

Fig. 1 Immunohiostochemical analysis of 12-LOX expression in normal and human ovarian cancer tissues. Normal ovarian epithelium showed no staining. Strong cytoplasmic staining for 12-LOX is shown in a case of high-grade serous carcinoma. Representative histological images were shown from 20 normal tissues and 20 high-grade serous tissues based on the scoring description in the Sect. "Materials and methods"
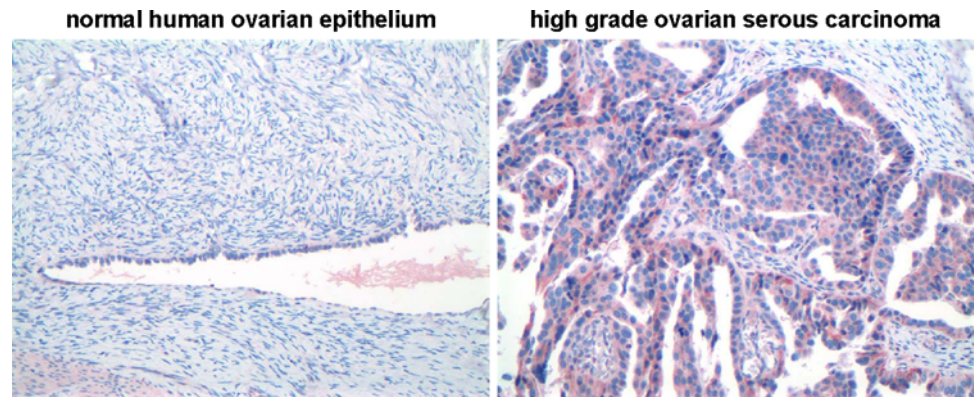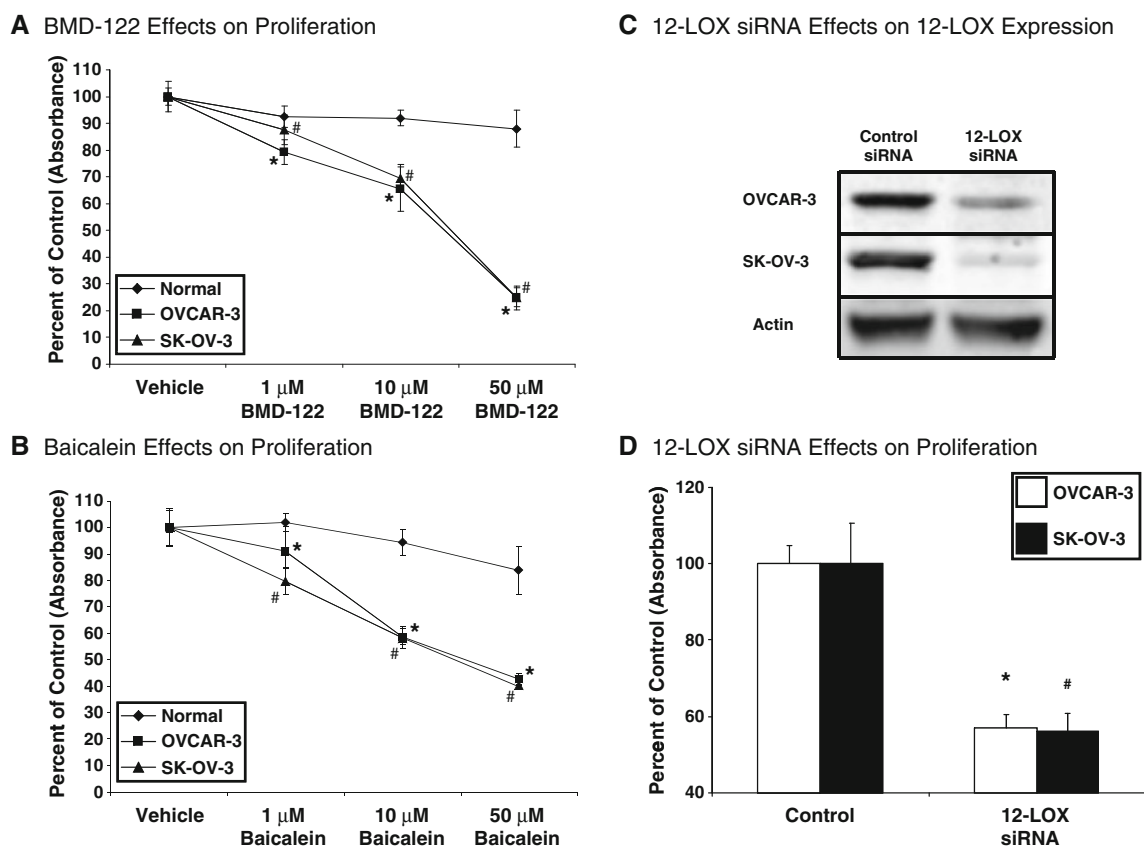

epithelial ovarian cancer cells, OVCAR-3 and SK-OV-3, RT–PCR for 12-LOX mRNA was performed in both cell lines as well as in normal human ovarian surface epithelial cells (HOSEpiC). Both OVCAR-3 and SK-OV-3 express significant amount of 12-LOX mRNA whereas HOSEpiC express significantly lower levels (Fig. 2a). Similar results were also observed in the 12-LOX protein expression level in these cells (Fig. 2b). We did not detect any expression of the 12(R)-LOX protein in the two EOC cultures using Western blot analysis (*data not shown*), which suggesting

12(R)-LOX is not be expressed in EOC. More importantly, both OVCAR-3 and SK-OV-3 cells have basal level of 12-HETE production. When incubated with free AA, these cultures responded with significant increases in 12-HETE production (Fig. 2c). HOSEpiC cells produced significantly less 12-HETE than the two EOC both in the presence and in the absence of AA, again consistent with the finding that increased 12-LOX-12-HETE system in ovarian cancer cells. Taken together, these data suggest that 12-LOX is present and functional in epithelial ovarian cancer cells.
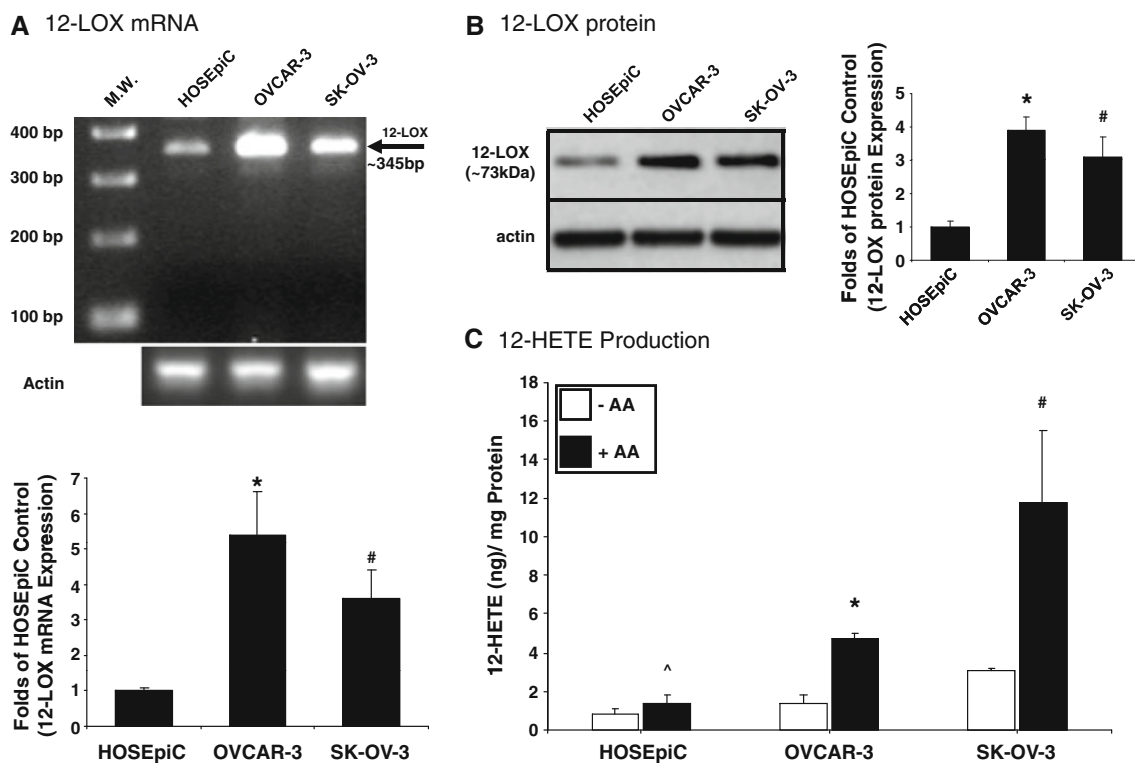
Fig. 2 12-Lipoxygenase in normal human ovarian epithelial cells and two epithelial ovarian cancer cells, OVCAR-3 and SK-OV-3. RT–PCR was first performed for the analysis of 12-lipoxygenase expression (**a**). Western blot analysis was also carried out for 12-LOX protein expression (**b**). Actin was used as loading control. Representative blot of three experiments is shown. Densitometrical analyses for relative 12-LOX mRNA and protein expression levels in

these cells were shown. $*P < 0.05$ for OVCAR-3 cells versus HOSEpiC controls, and $^\#P < 0.05$ in SK-OV-3 cells versus HOSEpiC controls. **c** LC/MS quantitation of 12-HETE was performed in normal human ovarian epithelial cells and EOC cultures treated with or without exogenous AA as described in the "Materials and methods". $^\wedge P < 0.05$ for HOSEpiC, $*P < 0.05$ for OVCAR-3 cells, and $^\#P < 0.05$ in SK-OV-3 cells

A BMD-122 Effects on Proliferation

C 12-LOX siRNA Effects on 12-LOX Expression

B Baicalein Effects on Proliferation

D 12-LOX siRNA Effects on Proliferation

Fig. 3 Effects of 12-LOX inhibition by BMD-122, Baicalein, and 12-LOX siRNA on the proliferation and survival of OVCAR-3 and SK-OV-3. Normal human ovarian epithelial cells and EOC cultures were treated with various concentrations of BMD-122 (**a**) for 48 h. Cell proliferation and survival were assessed using MTT assay. **b** A different 12-LOX inhibitor Baicalein was also used to treat normal human ovarian epithelial cells and EOC for 48 h. MTT assay was again performed to assess its effects on cell growth. **c** siRNA against 12-LOX was delivered as described in "Materials and methods" using Lipofectamin 2000. Scrambled siRNA was used as control siRNA. Protein extracts were harvested at the end of transfection and Western blot analysis were performed using anti-12-LOX antibody. Actin was used as loading control. Representative blot from three separate experiments is shown. **d** In parallel, MTT assay was also performed to assess the effects of 12-LOX siRNA on the proliferation and survival of these cultures. Data represent mean ± SD of at least three different experiments. *$P < 0.05$ in OVCAR-3 cells and #$P < 0.05$ in SK-OV-3 cells versus corresponding controls

Inhibition of 12-LOX decreases OVCAR-3 and SK-OV-3 growth and survival in a dose-dependent fashion

To examine the effects of blocking 12-LOX on cell proliferation, we treated the OVCAR-3 and SK-OV-3 cells with two structurally different 12-LOX inhibitors, BMD-122 and Baicalein, followed by measurement of cell viability using MTT assay. Treatment with BMD-122 (1, 10, and 50 μM) produced little or no effects on HOSEpiC cells, whereas it resulted in a dose-dependent inhibition of the growth of OVCAR-3 and SK-OV-3 cultures. There was 35% and 75% inhibition 2 days after treatment at 10 and 50 μM concentrations, respectively (Fig. 3a). Similar dose-dependent growth inhibition was observed in OVCAR-3 and SK-OV-3 cells treated with Baicalein (1, 10, and 50 μM) with minimal effects on HOSEpiC cells (Fig. 3b).

Next, we demonstrated that 12-LOX inhibition by silencing 12-LOX gene using siRNA resulted in growth inhibition of OVCAR-3 and SK-OV-3 cells. 12-LOX protein expression was markedly lower in both EOC cell lines transfected with 12-LOX-specific siRNA versus scrambled control siRNA (Fig. 3c). Furthermore, MTT assay indicates that the proliferation of 12-LOX down-regulated cultures was decreased by ∼50% compared to cultures treated with scrambled siRNA (Fig. 3d). Thus, these data support the results obtained from pharmacological inhibition of 12-LOX by BMD-122 and Balcalein, reaffirming the involvement of 12-LOX in regulation of ovarian cancer cell growth.

Effects of 12(S)-HETE and 12(R)-HETE on basal and BMD122 inhibited EOC growth

The AA metabolite of human platelet-type 12-LOX is 12-HETE [27]. Of the three known isoforms of 12-LOX, the epidermal-type 12-LOX is the only enzyme known to generate 12(R)-HETE from AA [43]. All other lipoxygenases
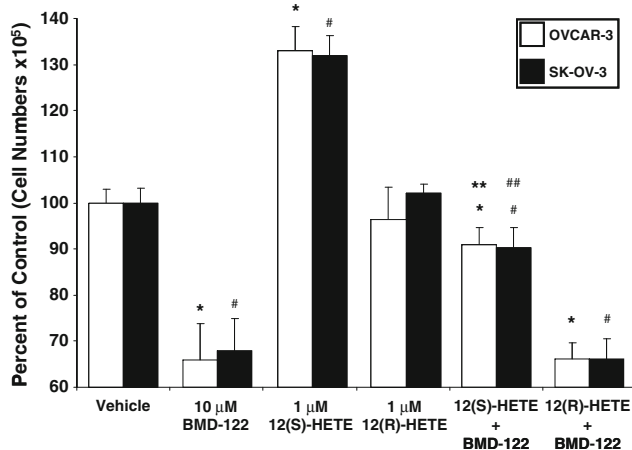
Fig. 4 12(S)-HETE but not 12(R)-HETE reverses the inhibitory effects of BMD122 on the growth of OVCAR-3 and Sk-OV-3. OVCAR-3 and SK-OV-3 cultures were treated with either 1 µM 12(S)-HETE, 1 µM 12(R)-HETE or 10 µM BMD-122 alone, or 12-HETE and BMD-122 together as before. Cell numbers were counted 48 h later, and EtOH-treated cultures were used as control. Data are expressed in percent of control in cell numbers Data are mean ± SD of two separate experiments, each by triplicate. $*P < 0.05$ in OVCAR-3 and $\#P < 0.05$ in SK-OV-3 cells versus their normal controls. $**P < 0.05$ in OVCAR-3 and $\#\#P < 0.05$ in SK-OV-3 cells treated with BMD-122 + 12-HETE versus cultures treated with BMD-122 only

generate the S-stereoisomer. To determine the stereoselectivity of the 12-LOX product responsible for the observed effects on ovarian cancer cells, we have tested both 12(S)-HETE and 12(R)-HETE on the proliferation of OVCAR-3 and SK-OV-3 cells. As the data shown in Fig. 4 demonstrate, 12(S)-HETE but not 12(R)-HETE stimulates cell growth by ~35%. Additionally, enzymatic inhibition of 12-LOX by BMD-122 that led to the growth inhibition of ovarian cancer cells was reversed only by 12(S)-HETE and not by 12(R)-HETE (Fig. 4). These findings represent evidence that suggests a significant growth stimulatory effect of 12(S)-LOX and its metabolite 12(S)-HETE on EOC cells. 12(R)-LOX and 12(R)-HETE do not appear to be involved in the regulation of EOC proliferation.

12-LOX inhibition by BMD-122 induces apoptosis in OVCAR-3 and SK-OV-3 cells

Inhibition of 12-LOX has been shown to induce apoptosis in several cancer cell types [20, 21, 28, 29]. To further study the effects of BMD-122 on apoptosis in EOC, OVCAR-3 and SK-OV-3 cell cultures were treated with various concentrations of BMD-122 for 20 h followed by annexin-V and propidium iodide labeling. The numbers of apoptotic cells were analyzed using flow cytometry based on previously described protocols [26]. All doses (1, 10, and 50 µM) of BMD-122 induced significant increases in
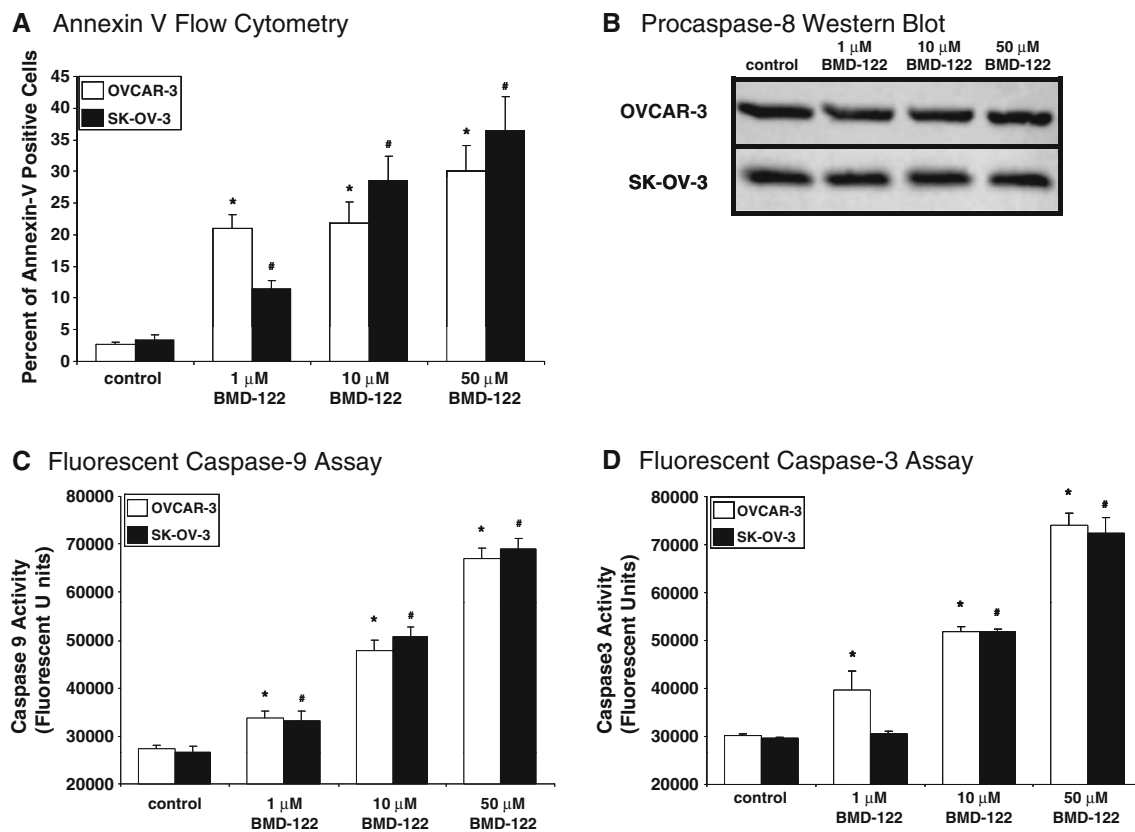
the percentage of cells labeled by annexin V, an indicator of cells in the early stages of apoptosis (Fig. 5a). This apoptosis seemed to be caspase 8-independent since BMD-122 failed to cleave and thus activate pro-caspase 8 as shown in Western blot (Fig. 5b). On the other hand, the levels of caspase-9 and caspase-3 in BMD-122-treated OVCAR-3 and SK-OV-3 cultures were markedly increased, suggesting the activation of caspase-9 and caspase-3 pathway (Fig. 5c, d). Thus, 12-LOX inhibition by BMD-122 decreases OVCAR-3 and SK-OV-3 cell survival by inducing caspase-9- and caspase-3-mediated apoptosis.

Effects of BMD-122 on the MAPK and PI-3 pathways in OVCAR-3 and SK-OV-3 cells

The phosphorylation and activation of mitogen-activated protein kinases (MAPK) is known to play a key role in the regulation of cell growth and proliferation. Treatment of OVCAR-3 and SK-OV-3 with 10 and 50 µM of BMD-122 for 20 h significantly reduced the phosphorylation of p42/p44 MAPK (Fig. 6a, b). However, there were no changes in the phosphorylation of Akt in BMD-122 treated-cultures even at the highest concentration (Fig. 6a, b). No changes were seen in the levels of total ERK1/2 or AKT in either cell lines (*data not shown*). These data indicate that 12-LOX in EOC may regulate MAPK but it does not regulate PI-3 kinase. BMD-122 induced apoptosis in OVCAR-3 and SK-OV-3 may be PI-3 kinase-independent.

Discussion

The precise genetic and molecular defects underlying epithelial ovarian cancer (EOC) remain largely unknown, and treatment options for patients with advanced disease are limited. Eicosanoids, the bioactive metabolites of AA, are known to play an important role in the growth and development of many cancers. Inducible cyclooxygenase (COX-2) is constitutively expressed in many cancer tissues, and COX-2 selective inhibitors were developed as potential anticancer agents [6, 7]. The other major pathway of AA metabolism, the lipoxygenase pathway, was shown to also play an important role on cancers. 12(S)-HETE, the metabolite of 12-LOX, was shown to induce tumor angiogenesis, migration and proliferation of cancer cells as well as inhibit apoptosis in many types of cancers including prostate, breast, lung, and skin [18–20, 30]. However, little is known about the role of 12-LOX in ovarian cancer. We have found that ~75% of high-grade serous ovarian carcinoma tissues showed marked over-expression of 12-LOX, a result suggesting that the 12-LOX-12-HETE system might play a role in regulating ovarian cancer growth and/or survival in some ovarian cancers. These data

A Annexin V Flow Cytometry

B Procaspase-8 Western Blot

C Fluorescent Caspase-9 Assay

D Fluorescent Caspase-3 Assay

Fig. 5 Inhibition of 12-LOX by BMD-122 induces apoptosis in OVCAR-3 and SK-OV-3. **a** Cultures were treated with various doses of BMD-122 (0, 1, 10, and 50 μM) for 20 h. Cell apoptosis was assessed using Annexin-V apoptosis kit according to the manufacturer's protocols. Labeled cells were then quantitated with flow cytometric analysis. Data represent mean $\pm$ SD of at least four individual experiments. *$P < 0.05$ in OVCAR-3 and #$P < 0.05$ in

SK-OV-3 cells versus untreated controls. **b** Same experiments were performed with fluorescent caspase-3 apoptosis assay using the manufacturer's recommended protocol. Data were obtained using a Fluorometer with excitation set at 400 nm and emission set at 535 nm. Data represent mean $\pm$ SD of at least three different experiments. *$P < 0.05$ in OVCAR-3 cells and #$P < 0.05$ in SK-OV-3 cells versus corresponding controls

are consistent with the elevated levels of 12(S)-HETE, reported by Freedman et al. in samples of EOC tumor, tumor-free malignant peritoneum (MP), and benign peritoneum (BP) [25]. This prompted us to undertake a detailed examination of the role of 12-LOX in ovarian cancer.

To better define the role of 12-LOX in ovarian cancer, we studied two widely used ovarian cancer cell lines, OVCAR-3 and SK-OV-3. Western blotting and RT–PCR clearly indicated that 12-LOX was much higher in these cell lines compared with a normal human ovarian surface epithelial cells. These data are consistent with the immunohistochemical observations with samples from human subjects affected by ovarian cancer. The protein detected in the cells was enzymatically active since 12-HETE production increased markedly when the cells were incubated with the 12-HETE precursor, AA. We did not detect the expression of 12(R)-LOX in both EOC cells, suggesting that 12(R)-LOX may not be involved in regulation of ovarian cancer. It should be noted that the primary products of lipoxygenases are the hydroperoxides (e.g. 12(S)-

HpETE from 12-LOX metabolism of AA). However, these hydroperoxides are reduced to the corresponding hydroxyl compounds by the intracellular peroxidases (e.g. glutathione peroxidase) and only the reduced products are released from the cells but not the hydroperoxides themselves.

In order to assess the potential role of the 12-LOX in the ovarian cancer cell lines, we examined the effects of suppressing its activity on EOC cell growth. We exposed the cells to two different and highly selective inhibitors of 12-LOX, BMD-122 [22], and Baicalein [29]. Both led to a marked and dose-dependent inhibition of growth even in the presence of serum, a potent source of growth factors. Thus, the growth-promoting effects of serum were absent if the ability of the cells to synthesize 12-HETE was inhibited. This suggests that 12-HETE is a very important element in the control of ovarian cancer cell growth, at least in the cell lines studied. The effects of the inhibitors was due in large part to reduction in 12(S)-HETE, since the addition of purified 12(S)-HETE but not 12(R)-HETE significantly reversed the inhibitory effects of BMD-122 on both

A OVCAR-3
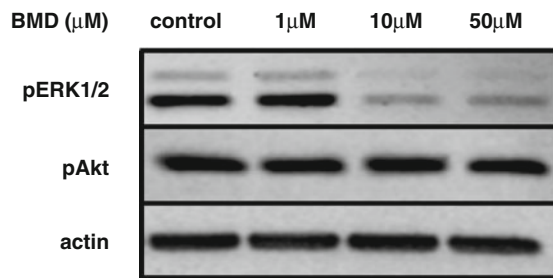
B SK-OV-3
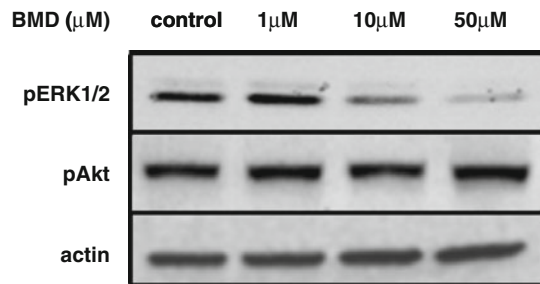
Fig. 6 Effects of 12-LOX inhibition by BMD-122 on MAPK and PI-3 kinase signaling pathway. OVCAR-3 (**a**) and SK-OV-3 (**b**) were treated with various concentrations of BMD-122 for 20 h as indicated. Western blot analysis was performed using anti-phospho-ERK/1/2 and anti-phospho-Akt antibody. Actin was used as loading control. Representative blots from three separate experiments were shown

OVCAR-3 and SK-OV-3 cells. To confirm the data obtained with the pharmacological inhibitors, we studied the effects on growth resulting from reducing levels of 12-LOX by silencing its gene using siRNA technology. Substantial, although incomplete, reduction in 12-LOX protein was concomitantly accompanied by marked reduction in cell growth and survival.

Despite the reported specificity of the lipoxygenase inhibitors, selectivity of the inhibition toward one lipoxygenase is generally concentration dependent. Hence, pharmacological inhibition of AA-metabolizing enzymes often have cross-inhibitory effects on more than one metabolizing systems, a potentially confounding effect. To examine whether the effects of BMD-122 could be due to inhibition of AA-metabolizing enzymes other than 12-LOX, we tested the effects of 4 other AA-metabolizing enzymes inhibitors on the proliferation and survival of the two EOC cells: MK886 (a 5-LOX activating protein inhibitor), MS-PPOH (an epoxygenase metabolizing enzyme inhibitor), 15-Lipoxygenase inhibitor 1 (a 15-LOX inhibitor), and HET0016 (a cytochrome P450 4 A and 4F inhibitor). No significant inhibitory effects were observed within the range of concentrations used (>50 μM) (*data not shown*). This suggests that the effects observed with the 12-LOX inhibitors are due to inhibition of 12-HETE synthesis and not to

interference with some other pathway. Thus, 12-LOX appears to play an important role regulating the growth and proliferation of ovarian cancer cells, suggesting that it may be a therapeutic target.

In some other cancer cell lines such as prostate, lung, gastric and pancreas, it has been demonstrated that the 12-LOX pathway appears to be required for the survival of these cells [20, 21, 28, 29]. In gastric cancer, Baicalein leads to apoptosis involving the activation of caspase-7 but not caspase-3 and decreased bcl-2/Bax ratio [21]. In prostate cancer, blocking 12-LOX resulted in the activation of both caspase-3 and caspase-7 [31]. These differences in the mechanism of apoptosis induced by 12-LOX inhibition seem to be cell specific. Our data indicated that inhibition of 12-LOX in ovarian cancer induced apoptosis through the caspase-9 and caspase-3 pathway.

To better understand the signaling pathways affected by inhibition of 12-LOX activity, we investigated changes in phosphorylation of two key signaling pathways. Extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) are members of the mitogen-activated protein kinase super family that can mediate cell proliferation and apoptosis [32, 33]. Extracellular stimuli such as growth factors, cytokines, mitogens, hormones, and oxidative or heat stress trigger a signal by interacting with a multimolecular complex of receptors such as receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs) or epidermal growth factor receptor (EGFR) ultimately leading to the phosphorylation of MAPK. Once activated, MAPKs regulate cellular activities ranging from gene expression, mitosis, cell differentiation, movement, and programmed death. ERK1/2 signaling pathway promotes cell survival by a dual mechanism comprising the posttranslational modification and inactivation of a component of the cell death machinery and the increased transcription of pro-survival genes [33–35]. We observed that ovarian cancer cells exposed to BMD-122 have markedly reduction in the phosphorylation of p42/p44 MAPK (ERK1/2). It is possible that such reduction in MAPK activation contributes to the observed growth inhibition and cell death.

The PI-3 kinase-Akt system is a recognized pro-survival pathway and plays a very important role in the survival ability of many types of cancers [36, 37]. Inhibition of PI-3 kinase reduces phosphorylation of Ser^{473} of AKt, a downstream mediator of the PI-3 kinase survival pathway and is associated with cell death [38, 39]. If BMD-122 acted by inhibiting PI-kinase, we would have expected reduction in AKt Ser^{473} phosphorylation. However, we did not observe such a reduction even on a shorter time course in BMD-122-treated cultures (*data not shown*). This suggests that the reduced growth induced by BMD-122 is unlikely due to changes in the PI-3 kinase/Akt pathway. A more detailed study on the precise pathways associated

with the inhibition of growth and survival induced by inhibition of 12-LOX in ovarian cells is beyond the scope of the present work, but it will be necessary to better understand the role of 12-LOX in the growth of ovarian cancer cells.

12-LOX, the enzymes responsible for 12-HETE generation, are generally absent in normal epithelia. They can be induced by pro-inflammatory stimuli and are often constitutively expressed in various epithelial cancers including colon, esophageal, lung prostate and breast cancer [16, 18, 40–42]. The present data suggest that epithelial ovarian cancer also constitutively express 12-LOX. Its product, 12-HETE, appears to exert pro-tumorigenic effects and it has been proved to have a strong association with progression of various cancers [4, 18, 19, 22, 23]. The present data suggest that it may also play an important role in ovarian cancer growth. In a recent study, Gao et al. found that the level of 12-LOX mRNA expression in prostate cancer is correlated with tumor stage [18]. In their work, the data suggested that elevation of 12-LOX mRNA expression occurs more frequently in advanced-stage, high-grade prostate cancer. The data presented here suggest that it may be worthwhile to determine if the same applies to ovarian cancer.

In summary, we presented data suggesting that the AA-metabolizing enzyme 12-LOX and its metabolite 12(S)-HETE are important regulators of ovarian cancer growth. We have shown that 12-LOX is highly expressed in ovarian tumors and in the ovarian cancer cell lines OV-CAR-3 and SK-OV-3. 12-LOX inhibition resulted in a significant inhibitory effect on the growth of the ovarian cancer cell lines and in marked cellular apoptosis, suggesting that the 12-LOX pathway is essential for the growth and survival of ovarian cancer cells. Thus, 12-LOX inhibition may have potential therapeutic role in the treatment of epithelial ovarian cancer.

Conflict of interest None.

References

1. Jemal A, Siegel R, Xu J, Ward E (2010) Cancer statistics, 2010. CA Cancer J Clin 60:277–300
2. Wang D, Dubois RN (2010) Eicosanoids and cancer. Nat Rev Cancer 10:181–193
3. Funk CD (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294:1871–1875
4. Nie D, Honn KV (2002) Cyclooxygenase, lipoxygenase and tumor angiogenesis. Cell Mol Life Sci 59:799–807
5. Roman RJ (2002) P-450 metabolites of arachidonic acid in the control of cardiovascular function. Physiol Rev 82:131–185
6. Chen X, Wang S, Wu N, Sood S, Wang P, Jin Z, Beer DG, Giordano TJ, Lin Y, Shih WC, Lubet RA, Yang CS (2004) Overexpression of 5-lipoxygenase in rat and human esophageal adenocarcinoma and inhibitory effects of zileuton and celecoxib on carcinogenesis. Clin Cancer Res 10:6703–6709
7. de Groot DJ, de Vries EG, Groen HJ, de Jong S (2007) Non-steroidal anti-inflammatory drugs to potentiate chemotherapy effects: from lab to clinic. Crit Rev Oncol Hematol 61:52–69
8. Brash AR (1999) Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. J Biol Chem 274:23679–23682
9. Kuhn H, Thiele BJ (1999) The diversity of the lipoxygenase family. Many sequence data but little information on biological significance. FEBS Lett 449:7–11
10. Bhattacharya S, Mathew G, Jayne DG, Pelengaris S, Khan M (2009) 15-lipoxygenase-1 in colorectal cancer: a review. Tumour Biol 30:185–199
11. Shureiqi I, Lippman SM (2001) Lipoxygenase modulation to reverse carcinogenesis. Cancer Res 61:6307–6312
12. Pace-Asciak CR, Granstrom E, Samuelsson B (1983) Arachidonic acid epoxides. Isolation and structure of two hydroxy epoxide intermediates in the formation of 8,11,12- and 10,11,12-trihydroxyeicosatrienoic acids. J Biol Chem 258:6835–6840
13. Quintana LF, Guzman B, Collado S, Claria J, Poch E (2006) A coding polymorphism in the 12-lipoxygenase gene is associated to essential hypertension and urinary 12(S)-HETE. Kidney Int 69:526–530
14. Coffey MJ, Jarvis GE, Gibbins JM, Coles B, Barrett NE, Wylie OR, O'Donnell VB (2004) Platelet 12-lipoxygenase activation via glycoprotein VI: involvement of multiple signaling pathways in agonist control of H(P)ETE synthesis. Circ Res 94:1598–1605
15. Huber J, Furnkranz A, Bochkov VN, Patricia MK, Lee H, Hedrick CC, Berliner JA, Binder BR, Leitinger N (2006) Specific monocyte adhesion to endothelial cells induced by oxidized phospholipids involves activation of cPLA2 and lipoxygenase. J Lipid Res 47:1054–1062
16. Yoshimoto T, Takahashi Y (2002) Arachidonate 12-lipoxygenases. Prostaglandins Other Lipid Media 68–69:245–262
17. Furstenberger G, Krieg P, Muller-Decker K, Habenicht AJ (2006) What are cyclooxygenases and lipoxygenases doing in the driver's seat of carcinogenesis? Int J Cancer 119:2247–2254
18. Gao X, Grignon DJ, Chbihi T, Zacharek A, Chen YQ, Sakr W, Porter AT, Crissman JD, Pontes JE et al (1995) Elevated 12-lipoxygenase mRNA expression correlates with advanced stage and poor differentiation of human prostate cancer. Urology 46:227–237
19. Timar J, Raso E, Dome B, Li L, Grignon D, Nie D, Honn KV, Hagmann W (2000) Expression, subcellular localization and putative function of platelet-type 12-lipoxygenase in human prostate cancer cell lines of different metastatic potential. Int J Cancer 87:37–43
20. Leung HW, Yang WH, Lai MY, Lin CJ, Lee HZ (2007) Inhibition of 12-lipoxygenase during baicalein-induced human lung nonsmall carcinoma H460 cell apoptosis. Food Chem Toxicol 45:403–411
21. Wong BC, Wang WP, Cho CH, Fan XM, Lin MC, Kung HF, Lam SK (2001) 12-Lipoxygenase inhibition induced apoptosis in human gastric cancer cells. Carcinogenesis 22:1349–1354
22. Chen YQ, Duniec ZM, Liu B, Hagmann W, Gao X, Shimoji K, Marnett LJ, Johnson CR, Honn KV (1994) Endogenous 12(S)-HETE production by tumor cells and its role in metastasis. Cancer Res 54:1574–1579
23. Connolly JM, Rose DP (1998) Enhanced angiogenesis and growth of 12-lipoxygenase gene-transfected MCF-7 human breast cancer cells in athymic nude mice. Cancer Lett 132:107–112
24. Yoshimura R, Inoue K, Kawahito Y, Mitsuhashi M, Tsuchida K, Matsuyama M, Sano H, Nakatani T (2004) Expression of 12-lipoxygenase in human renal cell carcinoma and growth prevention by its inhibitor. Int J Mol Med 13:41–46

25. Freedman RS, Wang E, Voiculescu S, Patenia R, Bassett RL Jr, Deavers M, Marincola FM, Yang P, Newman RA (2007) Comparative analysis of peritoneum and tumor eicosanoids and pathways in advanced ovarian cancer. Clin Cancer Res 13:5736–5744

26. Guo M, Roman RJ, Fenstermacher JD, Brown SL, Falck JR, Arbab AS, Edwards PA, Scicli AG (2006) 9L gliosarcoma cell proliferation and tumor growth in rats are suppressed by N-hydroxy-N′-(4-butyl-2-methylphenol)Formamidine (HET0016), a selective inhibitor of CYP4A. J Pharmacol Exp Ther 317:97–108

27. Nie D, Tang K, Diglio C, Honn KV (2000) Eicosanoid regulation of angiogenesis: role of endothelial arachidonate 12-lipoxygenase. Blood 95:2304–2311

28. Matsuyama M, Yoshimura R, Tsuchida K, Takemoto Y, Segawa Y, Shinnka T, Kawahito Y, Sano H, Nakatani T (2004) Lipoxygenase inhibitors prevent urological cancer cell growth. Int J Mol Med 13:665–668

29. Tong WG, Ding XZ, Witt RC, Adrian TE (2002) Lipoxygenase inhibitors attenuate growth of human pancreatic cancer xenografts and induce apoptosis through the mitochondrial pathway. Mol Cancer Ther 1:929–935

30. Timar J, Raso E, Honn KV, Hagmann W (1999) Human melanoma cell lines12-lipoxygenase expression in. Adv Exp Med Biol 469:617–622

31. Pidgeon GP, Kandouz M, Meram A, Honn KV (2002) Mechanisms controlling prostate cell cycle arrest and induction of apoptosis after 12-lipoxygenase inhibition in cancer cells. Cancer Res 62:2721–2727

32. Mebratu Y, Tesfaigzi Y (2009) How ERK1/2 activation controls cell proliferation and cell death: is subcellular localization the answer? Cell Cycle 8:1168–1175

33. Yoon S, Seger R (2006) The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors 24:21–44

34. Balmanno K, Cook SJ (2009) Tumour cell survival signalling by the ERK1/2 pathway. Cell Death Differ 16:368–377

35. Sawe N, Steinberg G, Zhao H (2008) Dual roles of the MAPK/ERK1/2 cell signaling pathway after stroke. J Neurosci Res 86:1659–1669

36. Osaki M, Oshimura M, Ito H (2004) PI3K-Akt pathway: its functions and alterations in human cancer. Apoptosis 9:667–676

37. Berra E, Milanini J, Richard DE, Le Gall M, Vinals F, Gothie E, Pages G, Pouyssegur J (2000) Signaling angiogenesis via p42/p44 MAP kinase and hypoxia. Biochem Pharmacol 60:1171–1178

38. Downward J (2004) PI 3-kinase, Akt and cell survival. Semin Cell Dev Biol 15:177–182

39. Stiles BL (2009) PI-3-K and AKT: onto the mitochondria. Adv Drug Deliv Rev 61:1276–1282

40. Jiang WG, Douglas-Jones A, Mansel RE (2003) Levels of expression of lipoxygenases and cyclooxygenase-2 in human breast cancer. Prostaglandins Leukot Essent Fatty Acids 69:275–281

41. Ohd JF, Nielsen CK, Campbell J, Landberg G, Lofberg H, Sjolander A (2003) Expression of the leukotriene D4 receptor CysLT1, COX-2, and other cell survival factors in colorectal adenocarcinomas. Gastroenterology 124:57–70

42. Gupta S, Srivastava M, Ahmad N, Sakamoto K, Bostwick DG, Mukhtar H (2001) Lipoxygenase-5 is overexpressed in prostate adenocarcinoma. Cancer 91:737–743

43. Boeglin WE, Kim RB, Brash AR (1998) A 12R-lipoxygenase in human skin: mechanistic evidence, molecular cloning, and expression. Proc Natl Acad Sci USA 95:6744–6749