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Research Paper

Blocking LPA-dependent signaling increases ovarian cancer cell death in response to chemotherapy

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ABSTRACT

The paradoxical role of reactive oxygen species in cell death versus cell survival establishes a delicate balance between chemotherapy efficacy and management of detrimental side effects. Normal proliferative signaling requires that cells remain inside a redox range that allows reversible protein oxidation to occur. Shifting the redox environment toward highly reducing or oxidizing states leads to cellular stress and cell death. Reactive oxygen species produced in response to Taxol and cisplatin treatment are necessary for effective cancer cell killing but the same ROS leads to damaging side effects in normal tissues. Combining antioxidants with chemotherapeutics to alleviate the unwanted side effects produces variable and often undesirable effects on cancer treatment. Here, we describe a more targeted method to improve ovarian cancer cell killing without the need for antioxidants. In ovarian cancer cells, lysophosphatidic acid (LPA) is a prominent growth factor that contributes to tumor survival and proliferation. We find that blocking LPA-dependent signaling with a specific receptor antagonist consistently increases cell death in response to both Taxol and cisplatin. We propose that inhibiting the upregulated growth factor-dependent signaling in cancer cells will target chemo-insensitivity, potentially lowering the necessary dose of the drugs and preventing harmful side effects.

1. Introduction

The damage to normal tissues by reactive oxygen species (ROS) produced in response to chemotherapeutics is a major complication in cancer treatment. Taxol and cisplatin are two common chemotherapeutic agents often used in combination as a first line of defense to treat many cancers, including ovarian carcinomas [1–3]. Both drugs non-specifically target rapidly proliferating cells, but in mechanistically different ways. Taxol directly interacts with tubulin and reduces depolymerization of the microtubules [1,2,4,5]. This blocks cells in the G2/M phase of the cell cycle and prevents proliferation [2,4,6]. Cisplatin crosslinks purine bases in genomic DNA which interferes with DNA repair and causes a DNA damage response resulting in apoptosis in cancer cells [7–10]. Both drugs increase ROS production, not only in tumor cells where the increased oxidative stress leads to a favorable outcome, but also in surrounding tissues which leads to painful neuropathy, kidney damage, hearing loss, and gastrointestinal side effects [9–11]. The ROS-induced damage to normal tissues increases dose responsively, often causing the course of treatment to remain below a maximally effective level.

Both dietary and pharmaceutical antioxidant supplements have been used in clinical trials with modest success in preventing side effects [12–17]. Clinically, broad range or systemic antioxidant approaches have been applied such as *n*-acetylcysteine (NAC), a potent ROS scavenger, or all trans retinoic acid (ATRA), the animal form of Vitamin A [5,12,16]. Additionally, patients often self-medicate with naturally occurring antioxidants such as green tea, Vitamin E, muscadine extract, resveratrol, and fish oil [5,12,13,18–22]. Clinical studies are currently underway with NAC, in conjunction with chemotherapy (NIH Clinical Trial #NCT01878695) to test the effects of decreasing ROS production on tumor metabolism, as well as fatigue and post-treatment recovery in patients with breast cancer. Global inhibition of ROS has previously been shown to inhibit peripheral neuropathy in patients treated with Taxol [1], and a separate study observed that kidney damage was reduced when using antioxidants in combination with cisplatin therapy [8,23]. However, the predictability of a cancer cell's response to combining these types of treatments with a chemotherapy or radiation regimen is complicated, with some clinical trials reporting lowered rates of survival for patients treated in combination with antioxidant therapies as opposed to those treated with chemotherapeutics alone [5,16,24]. The overarching conclusion is that decreasing the ROS produced in response to chemotherapy has

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variable, and sometimes undesirable, effects on the efficacy of the treatment.

Reactive oxygen species also play essential roles in normal cell proliferation and metabolism. They have been established as important signaling molecules in response to growth factors and cytokines allowing cells to respond to environmental changes [25–29]. Reversible protein oxidation plays a significant role in cell survival and proliferative pathways that protect mitochondrial membrane potential, inhibit apoptosis, and increase proliferative signaling. These pathways require the redox state of the cell to remain within a specific range, referred to as the redox window, where reversible redox-dependent proliferative signaling can occur [30]. When cellular redox homeostasis shifts outside the window to either excess oxidation or reduction, normal cellular signaling is disrupted, and apoptosis or cell death occurs [29,31].

In cancer cells, constitutive growth factor-dependent signaling promotes sustained proliferation and resistance to cell death [32–42]. Growth factor-stimulated ROS production and alterations in metabolism contribute to higher intracellular oxidative states in cancer than in normal tissues [9,32,43,44]. In ovarian cancer, a predominant growth factor responsible for stimulating proliferation and survival is lysophosphatidic acid (LPA). Levels of LPA found in the ascites fluid of ovarian carcinomas reach concentrations between 2–20 μM, indicating its importance in tumorigenesis and making it a potential biomarker for disease [45–49]. LPA interacts with endothelial differentiation gene (Edg) family G-protein coupled receptors (GPCR) that signal through various mechanisms to increase the expression of survival signaling molecules and growth factors that promote cancer proliferation and survival [50–52]. We previously reported that LPA also stimulates NADPH oxidase-dependent generation of ROS in endosomes containing LPA receptors [53,54]. This is essential to NF-κB, ERK, and Akt signaling and leads to increased proliferation in SKOV3 ovarian cancer cells [53]. Upregulation of these pathways in cancer also allows tumor cells to resist oxidative stress and apoptosis through the increased production of Bcl-2, Bcl-xL and other proteins that protect mitochondrial membrane potential, as well as increased resistance to ER stress-induced mitochondrial ROS production [55–57]. Treatment of these cells with a specific LPA receptor antagonist eliminates the LPA-dependent ROS production, protein oxidation, and leads to apoptosis [53,54].

The paradoxical role of ROS in cell death versus cell survival establishes a delicate balance between chemotherapy efficacy and management of detrimental side effects. This is further complicated by the shift in the redox window necessary for optimal survival of normal tissues versus that of cancer. Presently it is not practical to measure the changing redox state of a tumor. Drug treatment, angiogenesis, hypoxia, and increased proliferation and survival signaling all play a role. Thus, using broad range ROS scavengers and systemic antioxidants to prevent unwanted side effects of chemotherapy is not widely successful. Here, we examine the role of LPA-dependent survival signaling as a source of chemo-resistance to Taxol and cisplatin in SKOV3 ovarian cancer cells. We propose that inhibiting growth factor-dependent signaling to abrogate survival and proliferative signaling in cancer cells is a more specific target to combat chemo-insensitivity and prevent harmful side effects. A better understanding of the source of ROS in response to each chemotherapeutic agent and the time and location of ROS production are needed to maximize efficacy with minimal toxicity.

2. Materials and methods

2.1. Reagents and antibodies

Cisplatin, Taxol, and NAC were purchased from Sigma Aldrich. Primary antibodies for Western blots to detect cleaved caspase-3, cleaved caspase-7, and anti-rabbit secondary antibody were from Cell Signaling Technology. Dichlorofluorescein diacetate, RPMI 1640 medium, and Opti-MEM media were from Invitrogen. MitoSOX reagent was purchased from Molecular Probes. FuGENE 6 Transfection Reagent was purchased from Promega. The plasmid for expression of HyPer was from Evrogen. Fetal bovine serum was from Lonza. Nitrocellulose membranes were from Bio-Rad and Super Signal chemiluminescence reagent was from Pierce. VPC32183 and alkyl-linked 18:1 lysophosphatidic acid (LPA) [1-(9Z-octadecenyl)-2-hydroxy-*sn*-glycero-3-phosphate (ammonium salt)] was from Avanti Polar Lipids, Inc.

2.2. Cell culture and treatments

SKOV3 cells (from ATCC stocks) were grown, maintained, and treated at 37 °C with 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin. LPA, supplied in chloroform, was dried under a stream of nitrogen, resuspended to a concentration of 1 mM in phosphate buffered saline (PBS) containing 1% fatty acid-free bovine serum albumin (BSA), and then diluted into culture medium to the indicated concentrations.

2.3. Proliferation assay

SKOV3 cells were plated in 96 well plates at 1.5×10^3 cells per well and incubated overnight at 37 °C, 5% CO_2. Cells were treated with Taxol, cisplatin, or vehicle control concurrently with NAC where indicated. Cellular reactions were stopped by removing the culture media and fixing the cells with 10% (w/v) trichloroacetic acid, followed by staining with sulforhodamine B (0.4% w/v in 1% acetic acid) for 10 min. The excess dye was removed by washing repeatedly with 1% (vol/vol) acetic acid. The protein-bound dye was finally dissolved in 10 mM Tris base solution (pH unadjusted) for OD determination at 564 nm using a Molecular Devices VersaMax tunable microplate reader.

2.4. Western blotting

For Western blotting, cells were plated at 5×10^5 cells per dish in 100 mm dishes, treated or not treated with pharmacological agents, washed with cold, calcium-free PBS, scraped into lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 50 mM NaF, and 1 mM sodium vanadate), and centrifuged to remove cell debris after one freeze/thaw cycle. Protein concentration was measured (Pierce BCA protein assay) and samples (typically 40 μg protein/lane) were resolved on SDS polyacrylamide gels, then transferred to nitrocellulose membranes, probed with protein-specific antibodies and visualized using Super Signal chemiluminescence reagent.

2.5. Microscopy

Confocal Microscopy. For mitochondrial ROS production measurements using MitoSOX, 4×10^4 cells were plated in 0.5 mL of media in 4-well Lab-Tek II Chambered #1.5 Coverglass and incubated for 24 h. To monitor cytoplasmic ROS production, cells were transiently transfected with pHyPer-cyto, a reporter for H_2O_2 levels in the cytoplasm, using FuGene6 Transfection Reagent according to manufacturer's protocol, and incubated for 24 h [58]. Where indicated, cells were pretreated with LPA or receptor antagonist before labeling with 1 μM MitoSOX for 10 min [59]. MitoSOX reagent was removed and cells were washed three times with media. The field of view was located and focused before the addition of the Taxol or cisplatin. Images were collected over time using Zeiss LSM510 laser scanning confocal microscope.

Fluorescent Microscopy. For measurement of long term ROS production, SKOV3 cells (5×10^4) were plated in 2 mL RPMI supplemented with 10% FBS in 35 mm dishes. Cells were treated 24 h after plating with indicated concentrations of cisplatin for 24 h then incubated with dichlorofluorescein diacetate for 10 min, washed and visualized using an Olympus inverted epi-fluorescent microscope with