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Rapamycin by itself and additively in combination with carboplatin inhibits the growth of ovarian cancer cells☆

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ABSTRACT

Objective. The current standard treatment for ovarian carcinoma, consisting of surgery followed by chemotherapy with carboplatin and paclitaxel, is fraught with a high rate of recurrences. We hypothesized that targeted inhibition of specific signaling pathways in combination with conventional drugs may increase chemotherapeutic efficacy.

Methods. We analyzed the expression and activation profiles of various signaling pathways in nine established ovarian cancer cell lines (CAOV-3, ES2, PA-1, SKOV-3, NIHOVCAR3, OV90, TOV112D, A1847, A2780) and 24 freshly procured human ovarian tumors. The PI3 kinase pathway component Akt was frequently overexpressed and/or activated in tumor cells. The effect of several PI3K pathway inhibitors (rapamycin, LY294002, SH-6) and rapamycin in combination with carboplatin on various tumor cell growth characteristics was tested in cell lines and fresh tumor-derived transient monolayer and organ cultures.

Results. Rapamycin by itself and additively with carboplatin inhibited the growth and invasion, and increased the sensitivity to anoikis of most of the ovarian cancer cell lines and fresh tumors. The additive inhibitory effect may be due to enhanced apoptosis as demonstrated by Poly-ADP-Ribose Polymerase (PARP) cleavage and Annexin V staining in cells treated with both rapamycin and carboplatin.

Conclusions. Rapamycin in combination with standard chemotherapeutic agents may improve the efficiency of ovarian cancer treatment.

Introduction

The mortality rate of ovarian cancer, the second most frequent malignancy of the female genital tract in the western world, is higher than that of all other gynecologic malignancies combined. Serous carcinoma accounts for almost 60% of all ovarian malignancies. Of these, more than 70% present at an advanced stage with widespread disease in the peritoneal cavity and/or distant metastases [1]. The standard treatment of ovarian carcinoma consists of cytoreductive surgery with subsequent chemotherapy including carboplatin and paclitaxel. Despite initially high response rates to this treatment, most patients develop recurrent disease within few years [1]. Second line treatment regimens are less standardized and include platinum-based agents, gemcitabine, topotecan, etoposide, 5-fluorouracil, doxorubicin and combinations thereof [1,2]. Median survival has improved with these regimens, but long-term survival and disease mortality have remained largely unchanged due to drug-resistant recurrent tumor. Therefore, more effective treatment options for ovarian carcinoma are needed.

The molecular mechanisms of ovarian oncogenesis are poorly understood. Among cell growth and survival controlling mechanisms, the phosphatidylinositol-3 kinase (PI3K) signaling pathway is often activated. Aberrations of its signaling molecules are frequently found in ovarian cancer cells. This includes overexpression of the upstream receptor protein kinases (RPTKs) [3–6], mutations/amplifications of the PI3K catalytic (PIK3CA, p110) and regulatory (p85) subunits [7–9], Akt activation [10], and Akt 2 amplification [11]. Loss of the negative regulator PTEN due to deletion, inactivating mutations or epigenetic silencing has been associated with ovarian carcinomas of endometrioid histology [12–14]. Aside from uncontrolled growth, the capacity of invasion and metastatic spread are pathogenic features of cancer cells. Numerous reports suggest a role of PI3K signaling in invasion and metastasis [15–21]. Therefore, the component molecules of the PI3K signaling pathway are logical targets for new anti-cancer drug development.

Rapamycin, a macrolide produced by *Streptomyces hygroscopicus*, leads to cell cycle arrest in the G1 phase by inhibition of the mammalian target of rapamycin complex 1 (mTORC1). mTORC1 controls protein translation and several cytokine-driven signaling pathways involved in cell cycle progression [22,23]. Rapamycin and its analogues have been in clinical use for about a decade as immunosuppressants. Recently they have been tested as potential anti-cancer

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drugs against breast, gastrointestinal, head and neck, renal and other solid tumors [22,24–27]. Temserolimus was FDA approved for the treatment of advanced renal cell carcinoma in 2007 and is currently being evaluated in several clinical trials for its use in the treatment of gynecologic malignancies, including ovarian carcinoma [28]. Cell culture studies [10,29–31] and mouse models [32–37] also suggested that PI3K pathway inhibitors are efficient in suppressing ovarian cancer cell growth.

We analyzed the effect of the PI3K, AKT and mTORC1 inhibitors LY294002, SH-6 and rapamycin, respectively, on ovarian cancer cell growth using established cell lines and fresh human tumor tissue. Since the biologic targets of PI3K pathway inhibitors and carboplatin are different, we hypothesized that combination treatment may result in an additive effect. Our results show that rapamycin alone and additively in combination with carboplatin effectively inhibits various oncogenic properties of ovarian cancer cells.

Materials and methods

Cell lines and cultures

The ovarian cancer cell lines CAOV-3, ES-2, PA-1, SKOV-3, OV-90, NIHOVCAR3, TOV-112D (obtained from ATCC), A1847 and A2780 (obtained from Dr. Stuart Aaronson, Mount Sinai School of Medicine) were cultured in DMEM with 10% fetal calf serum (FCS).

Reagents

LY294002 is a specific inhibitor (IC50 = 1.40 μM) of PI3 kinase. Rapamycin (sirolimus) binds to *FK*506 *b*inding *p*rotein *12* (FKBP12) and selectively inhibits the catalytic activity of the *m*ammalian *t*arget *o*f *r*apamycin (mTOR) when complexed to the *r*egulatory *a*ssociated *p*rotein of m*TOR* (raptor) in the mTOR-complex 1 (mTORC1). SH-6 (Akt inhibitor III) is a phosphatidylinositol analog that inhibits the activation of Akt without decreasing phosphorylation of other kinases downstream of Ras. Carboplatin (*cis*-Diamine[1,1-cyclobutanedicarboxylato]platinum[II]), an analog of cisplatin with reduced nephrotoxicity, is an alkylating agent that cross-links DNA strands and induces apoptosis in cancer cells, possibly via caspase-3 activation, and is used in the standard chemotherapy for ovarian carcinoma. LY294002 and rapamycin were purchased from L.C. Laboratories, Woburn, MA. SH-6 and carboplatin were from Calbiochem, San Diego, CA. The dosage of these reagents was based on our previous experience [38]. For SH-6, we empirically determined to use 20 ng/ml to effectively inhibit more than 80% of the phospho-AKT. For drug combination experiments, we targeted 50% inhibition by either drug.

Fresh tumor cells and organ culture

This study was approved by the Institutional Review Board. A waiver of informed consent was obtained for studies of human tissue. Between April 2003 and June 2005, de-identified fresh tumor and, when possible, matched normal tissue samples were harvested from 24 different ovarian carcinoma patients. In order to prevent confounding results due to the great variety of histologic subtypes of ovarian malignancies, we focused on primary ovarian papillary serous carcinomas (OPSC), which constitute the vast majority of our study cohort (Supplementary Table 1). The histopathologic diagnoses, tumor grade and stage were verified according to the World Health Organization (WHO) classification and the American Joint Committee on Cancer (AJCC) staging system [39,40]. Due to limited tissue availability, not all tissue samples could be subjected to all tests.

Monolayer and organ cultures were prepared as follows: fresh tissue was harvested within 15 min of surgical removal, placed in DMEM containing 10% FCS and kept at 4 °C. The specimen was divided into 3 pieces, which were used for protein extraction, preparation of monolayer cell culture and organ culture, respectively.

For monolayer culture, the normal or tumor tissue was minced and treated with trypsin (0.5%) and collagenase (1 mg/ml) in phosphate buffered saline containing 0.2% EDTA at room temperature for 30 min with constant stirring. After sedimentation for 5 min, the supernatant was harvested, while the sediment was subjected to a second cycle of trypsin and collagenase treatment. The combined supernatant cell suspension was centrifuged, and the collected cells were resuspended in DMEM and seeded in culture dishes. In some cases, the cells were cultured in DMEM containing 1.3% methylcellulose for 48 h to enrich for anoikis resistant tumor cells before seeding on the culture dishes [41]. With this protocol, about 50% of the initial cells survived. Significant growth of monolayer tumor cells could be observed during the initial 3 to 4 passages. The monolayer tumor cell cultures with or without preselection in methylcellulose medium could be maintained for 3 to 6 weeks (Supplementary Fig. 1A). The epithelial origin was confirmed by immunostaining for cytokeratins (Supplementary Fig. 2).

For transient organ culture, 1–2 mm^3 tumor pieces were placed on a nylon mesh platform floating on DMEM and cultured in the air-medium interphase. Changing the medium every three days, the tumor tissue remained viable for 3 to 4 weeks. Cell growth could be observed around the periphery of the tissue fragments (Supplementary Fig. 1B) and was assessed after one week by ^{3}H-thymidine incorporation assay.

Protein analysis

Growth factor receptors and signaling molecules were analyzed by Western blots as described [16]. Total lysates from either cell cultures or freshly frozen tumor tissues were prepared using RIPA buffer [16,18,41]. The frozen tissues were minced and dounce homogenized prior to RIPA extraction. Protein lysates were resolved by SDS-PAGE, probed with primary antibodies against ErbB2, EGFR (Calbiochem, San Diego, CA), IGF1R [42], IR, AKT-1, AKT-2, phospho-AKT(S473) (Santa Cruz Biotechnology, Santa Cruz, CA), MAP kinase, phospho-MAP kinase, phospho-AKT(T308) (Cell Signaling, Danvers, MA), Stat3 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Stat3 (Cell Signaling, Danvers, MA), PARP (Beckton Dickinson, Franklin Lakes, NJ), GAPDH (Chemicon International, Temecula, CA) and β-tubulin (Sigma, St Louis, MO), and visualized by chemiluminescence (Perkin-Elmer, Boston, MA, and Roche, Nutley, NJ).

Monolayer growth assay

10^5 cells were seeded in 6 cm Petri dishes and maintained in the growth medium with or without rapamycin (5 or 10 ng/ml), carboplatin (1 or 2 μg/ml, as indicated in the figure legends) or both. 72 to 96 h later the cells were trypsinized and counted.

Colony assay

10^5 cells were seeded in a 6 cm Petri dish and cultured in agar containing DMEM without or with LY294002 (10 μM), SH-6 (20 ng/ml), rapamycin (5 ng/ml), carboplatin (2 μg/ml), or rapamycin (5 ng/ml) and carboplatin (2 μg/ml) combined. After 14 days, colonies were quantified as described previously [18].

Invasion assay

10^5 cells were plated in a Boyden chamber, and the number of invaded cells was quantified as described [18], except that the insert was precoated with 100 μl of 0.5 μg/ml growth factor reduced Matrigel (Beckton Dickinson, Franklin Lakes, NJ) in DMEM containing 0.1% bovine serum albumin (BSA) and incubated at 37 °C for 30 min before use. The observation time was 6 to 24 h.