In Vivo and in Vitro Ovarian Carcinoma Growth Inhibition by a Phosphatidylinositol 3-Kinase Inhibitor (LY294002)

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ABSTRACT

Phosphatidylinositol 3-kinase (PI3-K) induces mitogenesis, cell growth, and cell transformation. Amplification of the gene encoding the PI110α subunit likely is an important event in ovarian cancer progression, and PI3-K inhibitors are possible therapeutic agents for this disease. We evaluated effects of LY294002, a potent inhibitor of PI3-K, on growth of ovarian carcinoma in vivo and in vitro, and on ascites formation in vivo. Athymic mice were inoculated i.p. with the ovarian cancer cell line OVCAR-3. Seven days after inoculation, mice were treated with or without LY294002 (100 mg/kg of body weight) for 3 weeks. Body weight and abdominal circumference were measured twice weekly. At the end of the experiment, mice were sacrificed, ascites volume was measured, and tumors were excised. Mean tumor burden in the LY294002-treated group was reduced by ~65% vs controls. Virtually no ascites developed in the treatment group; mean volume of ascites in controls was 3.3 ± 0.38 ml. OVCAR-3 cells also were cultured in vitro and with LY294002 (1, 5, and 10 μM) for 24 h. The number of cells in 1, 5, and 10 μM LY294002-treated wells was reduced by 27, 56, and 75%, respectively, vs controls. In vivo and in vitro morphological studies demonstrated that LY294002 induced marked nuclear pyknosis and diminished cytoplasmic volume in the tumor cells, confirmed as apoptosis. Thus, LY294002 significantly inhibits growth and ascites formation in ovarian carcinoma in vivo and markedly inhibits ovarian cancer cell proliferation in vitro, suggesting an important role of PI3-K inhibitors as a potentially useful treatment for women with ovarian carcinoma.

INTRODUCTION

Ovarian cancer is characterized by i.p. carcinomatosis and the development of large volumes of ascites fluid (1). It is the major cause of death from gynecological malignancy and the fifth most common cause of death from cancer in American women. Except for some improvement in survival length with the introduction of cisplatin and paclitaxel therapy, the likelihood of success in the treatment of women with advanced, recurrent, or persistent ovarian cancer has remained largely unchanged for four decades (2). Recent observations indicate that the gene encoding the PI110α catalytic subunit of PI3-K³ is increased in copy number in approximately 80% of primary ovarian cancer cells and several ovarian epithelial carcinoma cell lines (including the OVCAR-3 line used in the present study), and an inhibitor of this pathway has been shown to inhibit growth of an ovarian cancer cell line in vitro (3).

Class I PI3-K plays a central role in cellular proliferation, motility, angiogenesis, viability, and senescence. It phosphorylates and dephosphorylates the D3 portion of membrane phosphoinositols (4, 5). This cytosolic enzyme consists of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit (6). Although the role of PI3-K and its lipid products in signal transduction processes is still unclear, the activity of this enzyme on tyrosine kinases induces mitogenesis, cellular growth, and cellular transformation (5, 7, 8). Moreover, PI3-K is involved in differentiation of certain cell types, e.g., those of Leydig cell and prostate cancers (9, 10). Thus, the PI3-K signaling cascade may play a critical role in ovarian epithelial tumorigenesis, invasion, and metastasis. Recently, several studies have focused on the role of inhibitors of PI3-K as potential tumor suppressor agents (11).

We have developed a model of i.p. ovarian carcinoma in athymic immunodeficient mice (12). This model closely resembles stage 3 ovarian epithelial carcinoma, with both extensive dissemination of ovarian carcinoma cells to peritoneal surfaces and the development of massive ascites. Therefore, in the present study, we assessed the effects of an inhibitor of PI3-K on tumor growth and ascites formation in this mouse model of i.p. ovarian carcinoma.

The flavonoid derivative, LY294002 (Eli Lilly, Indianapolis, IN), a potent inhibitor of PI3-K, is a competitive, reversible inhibitor of the ATP binding site of PI3-K (13). LY294002 induces specific G₁ arrest in cell growth, leading to almost complete inhibition of melanoma cell proliferation (14) and partial inhibition of MG-63 (osteosarcoma cell line) proliferation (15). It completely inhibits the retinoblastoma protein hyperphosphorylation that normally occurs during G₁ progression.

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³ The abbreviations used are: PI3-K, phosphatidylinositol 3-kinase; PVC, University of California, San Francisco; VEGF, vascular endothelial growth factor; VPF, vascular permeability factor.
and induces up-regulation of the cyclin-dependent kinase inhibitor p27 (14). The effect of LY294002 on cell cycle progression may provide insights into a possible link between the PI3-K activation pathway and cancer cell cycle regulation.

In the present studies, we investigated the effects of LY294002 on the growth of ovarian carcinoma. Here we demonstrate that this inhibitor significantly inhibits growth and ascites formation of ovarian carcinoma in vivo and markedly inhibits ovarian cancer cell proliferation in vitro.

MATERIALS AND METHODS

Materials. LY294002, a PI3-K inhibitor, was obtained from Sigma Chemical Co. (St. Louis, MO). The human OVCAR-3 cell line was kindly provided by Dr. T. Hamilton, Fox Chase Cancer Center (Philadelphia, PA). Athymic immunodeficient mice were obtained from Simonsen Laboratories (Gilroy, CA). Plastic coverslips used for in vitro studies were obtained from Nunc, Inc. (Naperville, TN). The ApopTag kit for the study of apoptosis was obtained from Intergen Co. (Purchase, NY). All cell culture reagents were obtained from the Cell Culture Facility, UCSF.

In Vivo Studies

Experimental Animals. Two groups of athymic immunodeficient mice (Simonsen Laboratories, Gilroy, CA) were delivered to the UCSF Animal Care Facility, housed in isolated conditions, fed autoclaved standard pellets and water, and allowed to adapt to their new environment. All protocols involving immunodeficient mice were approved by the Committee on Animal Care, UCSF.

Preparation of OVCAR-3 Cells. Cells were collected from the ascitic fluid of athymic mice inoculated with OVCAR-3. Ascites fluid was collected and placed in a 4°C refrigerator for 1–2 h. The supernatant was then discarded. The cells were diluted with RPMI 1640 supplemented with 2.0 g/liter glucose and 0.3 g/liter L-glutamine that was prewarmed in a 37°C incubator.

In Vivo Inoculation of OVCAR-3 Cells. Two groups of athymic nude mice (5–7 weeks) were inoculated i.p. with OVCAR-3 cells (n = 24; 2 × 10⁶ cells/mouse in 500 μl of RPMI 1640). Seven days after inoculation, one group (n = 12) of mice (two groups of n = 6 each) was treated with 2 mg of LY294002 dissolved in DMSO plus 0.25 ml of PBS i.p. daily for 3 weeks. This dose was based on a preliminary dose-ranging study from 0–100 mg/kg body weight of LY294002 (i.p.) in which 100 mg/kg was found to result in significant inhibition of ascites and tumor burden. The rest of the mice (n = 12; two groups of n = 6 each) received vehicle only. The concentration of DMSO in the vehicle was 8%. Body weight and abdominal circumference were measured twice weekly. At the end of the experiment, mice were euthanized with CO₂. The volume of ascites was measured. The tumor was excised, weighed, and fixed in 4% paraformaldehyde, pH 7.4, at 4°C for 24 h and embedded in paraffin. Paraffin sections (5 μm) were used for histochemical analysis.

In Vitro Studies

Cell Cultures. OVCAR-3 cells were seeded (2 × 10⁶) on plastic 6-well culture plates. Some of the plates were prepared with plastic coverslips in the wells (Nunc, Inc.). The cells were cultured in RPMI 1640 containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained in a 37°C incubator in a humidified atmosphere of 95% O₂/5% CO₂. Twenty-four hours after the cells were seeded, the medium was removed and replaced with culture medium in the presence of either LY294002 dissolved in DMSO or DMSO only for an additional 24 h. The concentration of DMSO in both control and test groups was maintained at 0.5%. At the initial 24 h time point, a baseline cell number was established by trypsinizing and counting one plate of cells using a hemocytometer (Improved Neubauer, VWR Scientific Counting Chamber) while the original morphological appearance of the cells was captured from one well of cells growing on plastic coverslips by fixing with 2% paraformaldehyde. This was used as the control for assessing morphological alterations caused by the treatment. At the end of the second 24 h time point, cells from each of the LY294002 and DMSO treatment groups were trypsinized, and cell number was counted. To determine whether cells would recover after withdrawal of LY294002, some of the 10 μM LY294002-treated wells were withdrawn from LY294002 and cultured for an additional 24 h. The cells on the plates with plastic coverslips were prepared for morphological studies.

Determination of OVCAR-3 Cell Apoptosis. OVCAR-3 cells were cultured on plastic 6-well culture plates. The cells were cultured in RPMI 1640 containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin while being maintained in a 37°C incubator in a humidified atmosphere of 95% O₂/5% CO₂. Twenty-four hours after the cells were seeded, the medium was removed and replaced with culture medium in the presence or absence of LY294002 (10 μM dissolved in DMSO) for an additional 24 h. Cells were collected by trypsinizing and fixed at a density of 5 × 10⁶ cells/ml in freshly diluted 1% paraformaldehyde. The cell suspension (100 μl) was dropped on sialized gelatin-coated microscope slides. DNA labeling with digoxigenin-dUTP and terminal transferase, followed by immunocytochemical staining with peroxidase-coupled antidigoxigenin antibody and diamino benzidine, was carried out with the reagents supplied in the Apoptag kit (Intergen, Purchase, NY) according to the manufacturer’s instructions, except that Tris was substituted for phosphate in the wash buffer. After light counterstaining with hematoxylin, nuclei that stained brown were scored as positive for apoptosis, and those that stained blue were scored as negative. At least 100 cells in at least three × 200 microscopic fields were scored, and the apoptotic index was calculated as the percentage of cells that were scored positive. The same Apoptag kit was used for determination of cell apoptosis in ovarian cancer tissue from OVCAR-3-inoculated mice treated with LY294002.

Light Microscopy and Analysis. The H&E-stained OVCAR-3 cells on the plastic coverslips were examined with a Leica DMRB or Leica Ortholux II photomicroscope at low and high magnifications. Images were collected with a Photometrics DEI-470 CCD camera and a RasterOps 24XLTv frame grabber, imported directly into Adobe Photoshop 4.0, and stored on an external 100 MB Zip drive (Iomega). Photomicrographic plates were composed from the original data in Photoshop, without alteration or manipulation, and annotated with rub-on letters and symbols.
Statistics. Data were analyzed using the unpaired Student’s t test for comparison between groups. Differences between groups were considered statistically significant at \( P < 0.05 \). Experiments in vivo were performed in duplicate, whereas experiments in vitro were performed in triplicate.

RESULTS

Effects of LY294002 on OVCAR-3 Tumor Growth and Ascites Formation. To examine the effects of LY294002 on OVCAR-3 tumor growth, we used a model of i.p. ovarian carcinoma in athymic immunodeficient mice that was developed previously in our laboratory (12). In all animals treated with LY294002, treatment was initiated 8 days after OVCAR-3 cell inoculation and continued for 21 days. Two animals in the treatment group \( (n = 12) \) died 2 weeks after LY294002 treatment. One of the mice was suspected of having small bowel obstruction. The reason for the death of the other was unknown. The remaining animals were sacrificed 28 days after OVCAR-3 inoculation. At postmortem examination, tumors were found on the surface of the peritoneum, intestines, and uterus in both treatment and control groups. However, in the control group, tumors were also found on the diaphragm and in the hilus of the liver.

Fig. 1 illustrates two representative mice, treated with LY294002 (mouse B) or with vehicle (PBS + DMSO) only (mouse A). Mouse A (32 g) was larger than mouse B (18 g) and had significant abdominal swelling. Mouse B developed dry skin, which may be a side effect of LY294002 (Fig. 1A). At autopsy (Fig. 1B), in mouse A, tumors were found on the surface of the peritoneum, intestines, uterus, diaphragm, and hilus of the liver. In mouse B, tumors were only found on the peritoneum, intestines, and uterus. Mouse A had a tumor burden of 3.8 g with 7 ml of bloody ascites, whereas mouse B had a 0.9-g tumor burden without ascites.

The histological appearance of tumor tissue from the OVCAR-3-inoculated mice with and without PI3-K inhibitor (LY294002) treatment is illustrated in Fig. 2. LY294002 induced pyknosis and nuclear condensation, as well as reduced cytoplasmic volume in the tumor cells. Some nuclei separated from the cytoplasm.

As i.p. tumor growth and development of ascites could not be measured directly, body weight and abdominal circumference, which reflect both increasing ascites accumulation and tumor burden, were measured twice weekly. Abdominal circumference \( (7.2 \pm 2 \ cm) \) significantly increased in the control group compared to the LY294002-treated group \( (6.35 \pm 0.42 \ cm; \text{Fig. 3A}) \). Body weight increased in both groups for the first week after inoculation (Fig. 3B). In the control group, body weight continued to increase, whereas there was no significant change in body weight after treatment with LY294002.

Because i.p. tumor growth could not be monitored directly, and because of diffuse spread within the abdomen, tumor burden could not be accurately assessed quantitatively. Therefore, i.p. tumor burden was assessed at postmortem examination. All visible i.p. tumors were excised and weighed. The mean tumor burden in the LY294002-treated group \( (0.62 \pm 0.1 \ g) \) was reduced by \( \sim 65\% \) compared to the control group \( (2.1 \pm 0.28 \ g) \).

In the control group, five of the 12 animals exhibited abdominal swelling with ascites \( (3–7.5 \ ml) \), and 3 of these had bloody ascites. Four animals had ascites volumes of less than 3 ml, and the remaining three had no detectable ascites. In the LY294002-treated group \( (n = 12) \), 2 animals developed ascites of less than 3 ml; the remaining 10 had no detectable ascites. LY294002 significantly inhibited ascites formation in the mice inoculated with OVCAR-3. Virtually no ascites developed in the treatment group, whereas the mean volume of ascites in the control group was \( 3.3 \pm 0.38 \ ml \).

Effects of LY294002 on OVCAR-3 Cell Proliferation. To determine the effects of LY294002 on cell proliferation as well as possible morphological alterations, OVCAR-3 cells were treated with different concentrations of LY294002 in static culture. After 24 h of treatment, the number of cells in 1, 5, and 10 \( \mu M \) LY294002-treated wells was reduced by 27, 56, and 75%, respectively, compared to the control group. The morphological appearance of proliferating OVCAR-3 cells is illustrated in Fig. 4. LY294002 \( (1–10 \ \mu M) \) markedly inhibited cell proliferation (Fig. 4, A–E). When the cells were treated with 10 \( \mu M \)
LY294002 for 24 h, the effects appeared toxic (Fig. 4E). Cellularity was decreased, and the cell clusters appeared shrunken with poor cellular cohesion. Cells had hyperchromatic, pyknotic nuclei, and the amount of cytoplasm was decreased. However, after withdrawal of 10 μM LY294002 for 24 h, the OVCAR-3 cells at least partially recovered (Fig. 4F). LY294002 induced nuclear pyknosis and diminished cytoplasmic volume, which was clearly demonstrated in the 5 μM-treated wells (Fig. 5).

Fig. 6A shows apoptosis of OVCAR-3 cells after 24 h of treatment with 10 μM LY294002 in vitro. The brown nuclei indicate cells that have undergone apoptosis. Approximately 15% of OVCAR-3 cells were apoptotic. Fig. 6B shows apoptosis of OVCAR 3 cells in ovarian cancer tissue from OVCAR-3-inoculated mice with LY294002. The brown nuclei and debris reflect apoptosis.

**DISCUSSION**

Several lines of evidence suggest that PI3-K is implicated in a wide array of biological and pathophysiological responses. Among these are the observations that it is necessary for a full mitogenic response (16) and can inhibit apoptosis (17). *PIK3CA*, the gene encoding the P110α catalytic subunit of PI3-K, is present in increased copy numbers in primary ovarian cancer cells and several ovarian epithelial cell lines (3). Therefore, inhibition of PI3-K may provide a mechanism for inhibiting ovarian carcinoma growth. Our studies support this tenet and indicate that LY294002 markedly inhibited ovarian cancer growth in athymic mice inoculated with OVCAR-3 cells in vivo and reduced OVCAR-3 cell proliferation in vitro. These observations confirm and extend a previous study demonstrating that LY294002 significantly decreased OVCAR-3 cell proliferation in vitro in a dose-dependent manner (3). A noteworthy finding in our study is the inhibition of growth of ovarian carcinoma by LY294002 in vivo, which has not been reported previously.

Previous studies indicated that LY294002 completely abolished PI3-K activity in several cell types, including neutrophils,
endothelial cells, and breast cancer cells (18–20). Two mechanisms may explain the inhibitory effects of LY294002 on ovarian cancer cell proliferation. One is that LY294002 inhibits cell cycle progression, inducing specific G1 arrest, leading to an inhibition of cell proliferation. As demonstrated previously in human melanoma cells, LY294002 inhibits cell proliferation by inhibiting G1 cyclin-dependent kinase activity and the subsequent phosphorylation of retinoblastoma protein (14). These inhibitory effects are, at least in part, a result of induced up-regulation of cyclin-dependent kinase inhibitory p27 (14).

The second possibility is that LY294002 increases apoptosis of ovarian carcinoma, as recently suggested (3). Ras was found to protect cells from apoptosis through activation of protein kinase B/Akt via PI3-K (21). LY294002, by inhibiting PI3-K activity, blocks the signal transduction pathway, which in turn may inhibit Ras-mediated protection from apoptosis in ovarian cancer. Our morphological studies demonstrate that LY294002 induced pyknotic and condensed nuclei, as well as reducing cytoplasmic volume in the tumor cells both in vivo and in vitro, indicating apoptosis. Using digoxigenin-UTP and terminal transferase labeling with immunocytochemistry, we have also confirmed that LY294002 induces OVCAR-3 cell apoptosis both in vivo and in vitro. However, necrosis cannot be ruled out completely, as apoptosis may subsequently lead to necrosis (22).

Our studies also demonstrate that LY294002 markedly inhibits the ascites formation associated with ovarian carcinoma. Recent observations indicate that VEGF induces the processes leading to endothelial cell survival through the PI3-K/Akt signal transduction pathway (23). VEGF, also known as VPF, plays a major role in the development of malignant ascites (24). We recently demonstrated that immunoneutralization of VEGF/VPF inhibited ascites formation associated with ovarian carcinoma in this same i.p. athymic mouse model (12). In the present studies, LY294002, by inhibiting PI3-K activity, may have blocked the signal transduction pathway of VEGF/VPF, which in turn may

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**Fig. 4** Appearance of cultured OVCAR-3 cells with and without PI3-K inhibitor (LY294002).

A, OVCAR-3 cells were cultured for 24 h in RPMI with 10% fetal bovine serum. The cells form small and medium-sized clusters with some anastomoses of clusters.

B, appearance after an additional 24 h without treatment. Many more cells are present, and the cell clusters are larger and are fused.

C, appearance after 24 h of treatment with 1 μM LY294002. Cellularity is decreased, and there is little anastomosis between cell groups.

D, appearance after 24 h of treatment with 5 μM LY294002. Cellularity is decreased, as is the size of cell groups. No confluent growth is present.

E, appearance after 24 h of treatment with 10 μM LY294002. Cellularity is decreased, and the cell clusters appear shrunken with poor cell cohesion. The cells have hyperchromatic, pyknotic nuclei, and the amount of cytoplasm is decreased.

F, treatment effects appeared toxic, but after 24 h of withdrawal of treatment, there is some recovery. Cellularity is decreased, but the cells have regained cohesion, and their nuclei and cytoplasm more closely resemble the appearance of untreated cells. Scale bar, 150 μm; magnification, × 50.
have inhibited the ascites formation associated with ovarian carcinoma.

The increasing body weight was proportionate to the increasing abdominal circumference, suggesting that the body weight reflected the combination of ascites volume and tumor burden. At autopsy, both body weight and abdominal circumference were also proportionate to ascites volume and tumor burden. Assessment of body weight and abdominal circumference may be a practical, albeit somewhat imprecise, way to assess the increasing volume of ascites and tumor burden in vivo. Currently, we are assessing the utility of dynamic magnetic resonance imaging in athymic immunodeficient rats (25) as a means of quantifying vascular permeability, tumor mass, and microvascular density.

Two animals in the treatment group died 2 weeks after LY294002 treatment. One was suspected of having small bowel obstruction. The reason for the death of the other was unknown. Eighty % of the treated mice developed dry and scaly skin. However, 3 days after cessation of LY294002, the skin returned to a normal appearance. Because LY294002 is a competitive reversible inhibitor of PI3-K, skin changes were reversible. Dry and scaly skin is one of the indications of hyperkeratosis, and a previous report indicates that transgenic mice transfected with collagenase developed dry and scaly skin, which demonstrated hyperkeratosis upon histological analysis (26). Because keratinization has been regarded as a special form of apoptosis (27), dry and scaly skin in the treated mice could represent hyperkeratosis in the epidermis as a result of increasing LYP294002-induced apoptosis.

Similar to the in vivo studies, when OVCAR-3 cells were

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Fig. 5 Appearance of cultured OVCAR-3 cells with and without PI3-K inhibitor (LY294002) in high magnification light microscopy. Cells were cultured in RPMI with 10% fetal bovine serum. A, cells cultured for 24 h without inhibitor have vesicular nuclei, one or more prominent nucleoli, and a moderate amount of cytoplasm. B, cells cultured for 24 h with 5 μM LY294002 show degenerative changes, including decreased nuclear size, hyperchromasia, and smudging of the nuclear chromatin. Some cells (left) are swollen, with an increased nuclear to cytoplasmic ratio, whereas in others the cytoplasm is dense and decreased in amount (right). Scale bar, 100 μm; magnification, × 300.

Fig. 6 OVCAR-3 cell apoptosis in vivo and in vitro. A, apoptosis of OVCAR-3 cells after 24 h of treatment with 10 μM LY294002 in vitro. Brown nuclei indicate apoptotic cells. B, apoptosis of OVCAR-3 cells in tissue from mice inoculated with OVCAR-3 cells following LY294002 treatment. Brown nuclei and debris indicate apoptosis. Scale bar, 100 μm; magnification, × 150.
treated in vitro with the higher (10 μM) dose of LY294002 for 24 h, the effects appeared toxic. Cellularity was decreased, and the cell clusters appeared shrunken, with poor cellular cohesion. The cells had hyperchromatic, pyknotic nuclei, and the amount of cytoplasm was decreased. However, 24 h after withdrawal of 10 μM LY294002, there was partial recovery. The cellularity was decreased, but the cells regained cohesion, and their nuclei and cytoplasm more closely resembled the appearance of the untreated cells. These observations both in vivo and in vitro imply that the side effects appearing in the LY294002-treated mice are largely dependent on the dose and the time course of the LY294002 treatment, both of which might be reduced to avoid these side effects. Combining LY294002 at lowered doses with other antitumor chemotherapeutic agents might be an effective way to control the growth of ovarian carcinoma more completely with fewer side effects. We are currently exploring this possibility.

In summary, our data demonstrate that LY294002 significantly inhibits growth and ascites formation in ovarian carcinoma in vivo and markedly inhibits cancer cell proliferation in vitro, suggesting an important role of PI3-K in the growth of ovarian carcinoma. Intraperitoneal inhibition of PI3-K is a potentially useful treatment modality for women with ovarian carcinoma.

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