Erythropoietin inhibits apoptosis induced by photodynamic therapy in ovarian cancer cells

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Abstract

Recombinant human erythropoietin is widely used to treat anemia associated with cancer and with the myelosuppressive effects of chemotherapy, particularly platinum-based regimens. Erythropoietin is the principal regulator of erythroid cell proliferation, differentiation, and apoptosis. Recently, the antiapoptotic and proliferative effects of erythropoietin on nonhematopoietic cells were also established. We now show the effect of erythropoietin treatment on the response of A2780 and SKOV3 ovarian carcinoma cells to photodynamic therapy (PDT) using hypericin. SKOV3 exhibited an increased resistance to hypericin when cells were treated with erythropoietin. This resistance was reversed by treatment of SKOV3 cells with the specific Janus kinase 2 kinase inhibitor AG490 or the tyrosine kinase inhibitor genistein. These results support a role for the specific erythropoietin-induced Janus kinase 2/STAT signal transduction pathway in PDT resistance. Evidence of erythropoietin signaling was obtained by the demonstration of Akt phosphorylation in both A2780 and SKOV3 cells. Erythropoietin-treated SKOV3 cells exhibited decreased apoptosis induced by hypericin, an effect that was blocked by the phosphoinositide 3-kinase/Akt inhibitor wortmannin. These results may have important implications for ovarian cancer patients undergoing PDT and receiving erythropoietin. [Mol Cancer Ther 2008;7(8):2263–71]

Introduction

Erythropoietin is well established for its ability to alleviate anemia associated with cancer and with the myelosuppressive effects of various chemotherapeutic drugs, particularly platinum-based therapies (1, 2). Studies of patients with head and neck cancers (3), lung cancer (4), and pelvic malignancies (5), whose anemia has been corrected, or prevented, through the administration of erythropoietin have been reported to experience improved local/regional tumor control, quality of life, and survival in comparison with patients undergoing radiotherapy in the presence of anemia or whose anemia was partially corrected through the use of blood transfusions. Correction of anemia, especially with erythropoietin, also has been reported to be important in the outcome of radiotherapy and photodynamic therapy (PDT) treatments of cancer (3, 6). In a phase II trial of patients whose metastatic renal cell carcinoma was progressing on interleukin-2, results suggested that erythropoietin administration decreased interleukin-2 treatment toxicity, prevented drops in hemoglobin levels, and appeared to counteract the interleukin-2–driven increase in vascular endothelial growth factor levels, thereby inhibiting angiogenesis (7). In contrast, other clinical studies have raised concern about the use of erythropoietin (epoetin and darbepoetin) in cancer patients (see below).

Erythropoietin is a 30.4-kDa glycoprotein that regulates the growth, maturation, and survival of erythroid progenitor cells. Recently, erythropoietin has received newfound attention for its role(s) outside of hematopoiesis (reviewed in ref. 8). For instance, erythropoietin receptor (EpoR) expression in vascular endothelial cells, kidney, myoblasts, and intestine is associated with the ability of erythropoietin to induce cellular proliferation in these nonhematopoietic cell types (9–14). In the central nervous system, erythropoietin exerts a neuroprotective effect during the brain’s response to injury due to ischemia, trauma, and neurotoxins (15–17). Additionally, erythropoietin was also reported to enhance protein tyrosine phosphorylation in breast cancer cells and stimulate the in vitro proliferation of human breast cancer (18) and renal carcinoma cell lines (19). Recent evidence also supports a role for erythropoietin in the growth of human prostate cancer cells (20).

Erythropoietin has been cited as “safe and effective” in the treatment of anemia associated with multiple myeloma. However, myeloma cells and cell lines also have been shown to express EpoR (21), and erythropoietin was...
reported to directly stimulate the EpoR on myeloma cells and to result in phosphorylation of mitogen-activated protein kinase and other downstream signaling intermediates, some of which are essential for mitogenesis. An erythropoietin in breast cancer trial was terminated early and did not support the use of erythropoietin as an adjunct to first-line chemotherapy for patients with metastatic breast cancer who have normal hemoglobin concentrations (22). Although erythropoietin (epoetin β) efficiently corrects anemia among head and neck cancer patients undergoing radiotherapy, it was associated with increased locoregional progression and reduced survival (23).

Most ovarian cancer patients are treated with chemotherapy. In selected cases in which the cancer has responded well to prior treatment but in which there remains a high risk of tumor recurrence, radiation therapy may be needed as well. One alternative treatment is PDT. PDT usually involves systemic administration of a tumor-localizing photosensitizer and its subsequent activation by light of an appropriate wavelength to create a photochemical reaction causing photodamage to the tumor (24).

Studies report a high incidence of anemia in ovarian cancer patients at admission and further increases following the initiation of chemotherapy. Erythropoietin appears to be effective and well tolerated in preventing hemoglobin decline in patients undergoing aggressive cyclic platinum-based chemotherapy for advanced ovarian carcinoma (25). However, recent studies showed that several ovarian cancer cell lines and breast cancer cell lines expressed EpoR (26). Furthermore, we have shown that these EpoR are functional and that long-term erythropoietin treatment of ovarian cancer cells in vitro leads to paclitaxel resistance (27, 28). Interestingly, destruction of erythropoietin- and EpoR-expressing malignant tumors, as well as their capillaries, could be induced by deprivation of erythropoietin signaling via the injection of anti-erythropoietin monoclonal antibody or soluble EpoR into xenografts of malignant uterine and ovarian tumors (29). These data suggest that erythropoietin, in addition to its direct mitogenic effects on malignant cells, may promote tumor growth via an increase in vascular supply (29).

We have now investigated the in vitro effect of erythropoietin exposure on the response of less aggressive ovarian adenocarcinoma cell line A2780 and highly aggressive ovarian adenocarcinoma cell line SKOV3 to PDT with hypericin. The results show that erythropoietin treatment can induce a resistance to PDT and that this effect is due to specific intracellular signaling pathways triggered by erythropoietin in these cells.

Materials and Methods

Cell Lines

Human ovarian carcinoma cell lines A2780, A2780cis (cisplatin-resistant derivative of A2780), and SKOV3 were obtained from the European Collection of Animal Culture. Ovarian adenocarcinoma cell line A2780cis90 (cisplatin-resistant derivative of A2780) was kindly provided by Dr. Kozubik (Institute of Biophysics, Czech Academy of Science). BaF3 cells, a murine pre-B-cell line, was used as an EpoR-negative control. A2780, A2780cis, A2780cis90, and BaF3 cells were grown as monolayers in RPMI 1640 with l-glutamine (Life Technologies) and SKOV3 cells were grown in McCoy’s medium (Life Technologies). Medium was supplemented with 10% FCS (Life Technologies) and antibiotic/antimycotic solution (100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B; Life Technologies) and, in the case of BaF3 cells, was supplemented with 5% WEHI-3B cell conditioned medium. The cells were maintained under standard tissue culture conditions of 37°C, 95% air/5% CO2. The number of cells was determined using a Coulter counter (model ZF, Coulter Electronics), and total cell viability was analyzed by staining with 0.15% eosin followed by light microscopy.

Immunohistochemical Staining

Study protocols involving human material were approved by the P.J. Šafárik University Institutional Review Board. Immunohistochemical detection of EpoR was done on formalin-fixed, paraaffin-embedded tissue blocks. Tissue sections (7 μm thick) were deparaffinized with xylene and rehydrated in graded alcohols. Slides were then steamed in citrate buffer solution in a microwave oven for 15 min. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in methanol followed by blocking of nonspecific binding using milk buffer for 30 min at room temperature. Rabbit polyclonal antibody against EpoR (Santa Cruz Biotechnology) was applied overnight in humidified chamber at 4°C. After washing in 2% milk buffer (3 × 5 min), the sections were subsequently incubated with secondary antibody (mouse antirabbit antibody conjugated with horseradish peroxidase; Pierce Biotechnology) for 30 min at room temperature. Slides were then washed in 2% milk buffer (2 × 5 min) and Tris buffer (1 × 5 min). The slides stained for EpoR were visualized with 3,3-diaminobenzidine tetrachloride at a concentration of 0.5 mg/mL in Tris (pH 7.6) and 0.015% hydrogen peroxide. The slides were rinsed with tap water, counterstained with hematoxylin for 2 min, washed in tap water, dried, mounted, and coverslipped. Slides processed by omission of primary antibody served as a negative control of immunohistochemical procedure. We quantified EpoR protein expression into four categories: 3+, high level (90-100% positive cells); 2+, medium level (10-90% positive cells); 1+, low level (up to 10% positive cells); and −, negative cells (0% positive cells). For statistical analysis, only samples with high level (3+) and medium level (2+) protein expression were considered as positive. Samples scored as 1+ or − were considered negative.

Reverse Transcription-PCR

Total RNA was isolated using Trizol (Life Technologies) and reverse transcription-PCR (RT-PCR) was done according to the protocol provided by the Qiagen One-Step RT-PCR kit. Briefly, for each sample, 150 ng total RNA was
mixed with RT-PCR buffer, deoxynucleotide triphosphate mix (10 mmol/L of each deoxynucleotide triphosphate), primers (10 μmol/L each), and enzyme mix. For EpoR detection, forward primer 5'-ACCGTGTACCATCACCATCA-AT-3' and reverse primer 5'-GCTTTCAAACTCGCT-CTCTG-3' were used. 18S rRNA was determined by the primers designed by SuperArray (SuperArray Bioscience).

Thermal cycling variables were 30 min at 50°C for reverse transcription and 15 min at 95°C for initial PCR activation. PCR cycles were used as follows: 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. The amplified DNA was fractioned by electrophoresis and stained with ethidium bromide.

**Cell Treatments and 3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyltetrazolium Bromide Assay**

A2780 and SKOV3 cells were seeded into 96-well cell culture plates at a density of 1 × 10^4 and 6.5 × 10^3 per well, respectively. After overnight incubation and after discarding the medium with FCS, the cells were treated in serum-free medium with 5, 30, or 150 units/mL erythropoietin (epoetinum α; Janssen-Cilag International) for 2 h followed by 16 h cotreatment with hypericin in the dark (Appli-Chem). Hypericin was activated by light with a total dose of 4.4 J/cm² (fluence rate 4.4 mW/cm²/s) using a set of 11 white L18W/30 lamps (Osram) with maximum emission range 530 to 620 nm. For Janus kinase 2 inhibition studies, the specific Janus kinase 2 kinase inhibitor AG490 (30 μmol/L; Calbiochem-Novabiochem International) or genistein (50 μmol/L; AppliChem) was used 2 h before erythropoietin treatment. For inhibition studies of phosphoinositide 3-kinase (PI3K)/Akt, wortmannin (200 nmol/L; Calbiochem-Novabiochem International) or anti-phosphoinositide 3-kinase (PI3K)/Akt, wortmannin (200 nmol/L; Sigma-Aldrich) was applied 30 min before erythropoietin treatment. The effect of erythropoietin on the response of ovarian cancer cells to hypericin was determined using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) assay. Briefly, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide was added into 96-well culture plates (0.2 mg/mL) 24 h after the photoactivation of hypericin. Cell culture plates with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide were placed back into the incubator for an additional 4 h, and after discarding the medium, the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide-formazan product was solubilized using 10% SDS (Sigma-Aldrich). The absorbance measurements were carried out using a universal microplate reader (Fluostar Optima; BMG Labtech) at 584 nm.

**Photometric Cell Death Detection**

A photometric enzyme immunoassay for the quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) was used (Roche Applied Science). Briefly, cell lysate (20 μL) prepared 6 h after the photoactivation of hypericin was placed into a streptavidin-coated microplate. A mixture of anti-histone-biotin and anti-DNA-POD (80 μL) was added and incubated for 2 h at room temperature. During the incubation period, the anti-histone antibody bound to the histone component of the nucleosomes and simultaneously bound the immunocomplex to the streptavidin-coated microplate via its biotin group. Additionally, the anti-DNA-POD antibody reacted with the DNA component of the nucleosomes. ABTS solution (100 μL) was added as a substrate for POD and the resultant chromophore was measured at 405 nm against ABTS solution as a blank (reference wavelength, 490 nm) using a universal microplate reader. The specific enrichment of mononucleosomes and oligonucleosomes released into the cytoplasm was calculated using the following formula: enrichment factor = mU [absorbance (10⁻³)] of the sample / mU of the corresponding negative control.

**Phosphatidylinerse Externalization to Detect Apoptosis**

For phosphatidylinerse externalization analysis, 1.5 × 10⁶ cells were seeded in serum-free medium and allowed to settle for 24 h. Then, 150 units/mL erythropoietin and 60 mmol/L hypericin were added to the medium of some experimental groups for 16 or 24 h, respectively, and then irradiated with a total light dose of 4.4 J/cm². Two hours later, phosphatidylinerse externalization was detected together with cell viability by Annexin V/propidium iodide double-staining kit (Bender MedSystems). Cells were treated according to the manufacturer’s instruction. Briefly, adherent and floating cells (1.5 × 10⁶) were harvested together and stained with Annexin V/FITC in binding buffer for 10 min, washed, stained with propidium iodide for 10 min, and then analyzed using a FACSCalibur flow cytometer (Becton Dickinson). Results were analyzed with CellQuest Pro software. The results are presented as mean ± SD of three independent experiments together with one representative set of data. Graphical output was generated using WinMDI software.

**Western Blotting**

The cells were washed twice with ice-cold PBS and scraped into radiolimunoprecipitation buffer (1 × PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Protease inhibitor cocktail “Complete” (Roche Diagnostics) was added to the lysates. The lysates were transferred into microcentrifuge tubes and passed through a 21-gauge needle to shear the DNA. After incubation on ice for 45 min and after centrifugation at 10,000 × g for 10 min at 4°C, the supernatants were transferred into new microcentrifuge tubes. Protein samples were separated on 10% SDS-polyacrylamide gel, electrophlotted onto Immobilon-P transfer membrane (Millipore), and detected using anti-EpoR (1:200; Santa Cruz Biotechnology), anti-Bcl-2 (1:200; Santa Cruz Biotechnology), anti-Akt (1:1,000; Cell Signaling Technology), anti-phospho-Akt (Ser473; 1:1,000; Cell Signaling Technology), and anti-β-actin (1:10,000; Sigma-Aldrich) primary antibodies. Then, the membranes were incubated with secondary horseradish peroxidase-conjugated antibodies (goat anti-rabbit IgG F(ab)₂, 1:10,000, and goat anti-mouse IgG F(ab)₂, 1:10,000; Pierce) for 1 h. The antibody reactivity was visualized with enhanced chemiluminescence Western blotting substrate (Pierce) using Kodak Biomax film (Sigma-Aldrich). Protein bands were quantified using ELLIPSE software version 2.0.7.1 (ViDiTo, SR).
Statistical Analysis
Data were processed by scientific graphing and analysis software Origin (OriginLab) and statistically analyzed using one-way ANOVA followed by Tukey's multiple comparison tests.

Results
Expression of EpoR in Ovarian Cancer Cells
Different types of primary human ovarian carcinomas were immunohistochemically analyzed for EpoR expression. Twenty-six carcinomas (6 serous cystadenocarcinomas, 7 mucinous cystadenocarcinomas, 7 endometrioid carcinomas, and 6 clear cell carcinomas) at different stages were positive for EpoR expression. Six healthy control tissue samples showed low or no expression of EpoR (considered as negative). Figure 1 shows representative examples of control (Fig. 1A) and positive carcinoma samples (Fig. 1B).

RT-PCR (Fig. 2A) as well as Western blot (Fig. 2B) analysis revealed the presence of EpoR in the less aggressive ovarian carcinoma cell line A2780, in A2780cis- and A2780cis90-resistant derivative of A2780 cells, and in highly aggressive ovarian carcinoma cell line SKOV3. SKOV3 cells exhibited lower expression of EpoR than did the A2780 cells. Verification of EpoR specificity was done using a blocking peptide specific for EpoR as recommended by the manufacturer (data not shown). The upper band of ~120 kDa may represent a dimerized form of EpoR or an irrelevant cross-reacting species.

Erythropoietin Induces SKOV3 Cell Resistance to Hypericin
We determined the sensitivity of SKOV3 and A2780 cells to PDT. SKOV3 cells were more resistant to PDT than were A2780 cells (Fig. 3A). Although we have not studied detailed mechanism of SKOV3 cell resistance in detail, initial experiments showed a five times lower intracellular concentration (accumulation) of hypericin (measuring the fluorescence of hypericin at 485 and 590 nm) in SKOV3 than in A2780 cells after 16 h incubation of cells with hypericin (data not shown). To investigate the effect of erythropoietin on the response of the cells to PDT, A2780 and SKOV3 cells were treated with either 5, 30, or 150 units/mL erythropoietin for 18 h in serum-free medium. Treatment of SKOV3 cells with 150 units/mL erythropoietin resulted in a significant increase in resistance to PDT (Fig. 3A). Importantly, this erythropoietin-induced increase in PDT resistance was reversed by treatment of SKOV3 cells with the specific Janus kinase 2 kinase inhibitor AG490 (Fig. 3B) or with the tyrosine kinase inhibitor genistein (data not shown). Under the experimental conditions
employed, erythropoietin treatment did not change the PDT sensitivity of A2780 cells (Fig. 3A). We did not observe any significant changes in the cell response to PDT when A2780 and SKOV3 cells were treated with 5 or 30 units/mL erythropoietin (data not shown).

Erythropoietin Inhibits Apoptosis Induced by PDT in SKOV3 Cells

SKOV3 cells were incubated with either 14 nmol/L (H1) or 42 nmol/L (H2) of hypericin for 16 h in the dark and then activated by light. The results of the quantitative sandwich enzyme immunoassay (Fig. 4A and B) showed significant induction of apoptosis detected at 6 h as well as 24 h after the photoactivation of hypericin. Hypericin decreased also the number of adherent cells (data not shown) and increased the number of floating cells (Fig. 4C) with apoptotic morphology (data not shown). To investigate whether erythropoietin affects apoptosis induced by PDT, SKOV3 cells were incubated in serum-free medium with 5, 30, or 150 units/mL erythropoietin for 2 h followed by hypericin treatment. Figure 4 shows the antiapoptotic effect of 150 units/mL erythropoietin with the two different concentrations of hypericin. The antiapoptotic effect of erythropoietin was evidence by a reduced concentration of mononucleosomes and oligonucleosomes (Fig. 4A and B) as well as by a reduced number of floating cells (Fig. 4C) in erythropoietin + hypericin–treated cells versus hypericin-treated cells. The two lower concentrations of erythropoietin did not show any effect on hypericin/PDT-induced apoptosis (data not shown). We used the PI3K/Akt inhibitor wortmannin to show the role of Akt in this erythropoietin-induced antiapoptotic effect. Preincubation of SKOV3 cells with wortmannin for 30 min blocked the antiapoptotic activity of erythropoietin (Fig. 4) and confirmed the role of Akt in erythropoietin-induced cell protection/antiapoptosis in response to PDT.

Erythropoietin Reduces PDT-Induced Phosphatidylserine Externalization

An analysis of phosphatidylserine externalization confirmed that preincubation of SKOV3 cells with erythropoietin protects tumor cells from hypericin/PDT-mediated apoptotic process. In this regard, erythropoietin suppressed the onset of apoptosis significantly even at the higher concentration of hypericin (60 nmol/L; Fig. 4D). The insignificant alterations in cell cycle distribution together with decreased cell number are consistent with marked cell death without any effect on cell cycle regulation (data not shown). Furthermore, up-regulation of the antiapoptotic protein Bcl-xL and down-regulation of proapoptotic protein Bax in erythropoietin + hypericin–treated cells versus single hypericin treatment (Fig. 5) and the presence of Bad/
Bcl-xL heterodimer in hypericin-treated cells but not in erythropoietin + hypericin–treated cells (data not shown) confirmed the antiapoptotic effect of erythropoietin. No significant changes in Bcl-xL or Bax were observed in erythropoietin + hypericin–treated A2780 cells (Fig. 5).

**Erythropoietin Triggers Akt Phosphorylation in Ovarian Cancer Cells**

To confirm EpoR signal transduction in these two ovarian cancer cell lines, we investigated whether erythropoietin treatment resulted in phosphorylation of Akt, a critical mediator of the antiapoptotic action of erythropoietin. As seen in Fig. 6A, erythropoietin treatment of A2780 cells resulted in a time-dependent increase in phospho-Akt, specifically at Ser473 with the maximum at 2 h. No phosphorylation of Ser473 was seen in A2780 cells when cells were incubated for 24 h with erythropoietin (Fig. 6B). In contrast, SKOV3 cells exhibited increased phosphorylation of Akt after 6 h (Fig. 6A) and 24 h (Fig. 6B) incubation of cells with erythropoietin. Importantly, the phospho-Akt band was not seen in cells treated with erythropoietin and wortmannin, an inhibitor of PI3K, which is upstream of Akt. Despite using monoclonal anti-phospho-Akt (Ser473) antibody, a second immunoreactive band appeared in SKOV3 cell lysate. However, we were able to distinguish the specific phospho-Akt product (top band) using the PI3K inhibitor wortmannin. In regard to the phosphorylation of Akt at Ser473, we found two differences between A2780 and SKOV3 cells. Firstly, a basal level of Akt phosphorylation was observed in control SKOV3 cells without erythropoietin treatment (Fig. 6A and B). Secondly, after 24 h of erythropoietin treatment, Akt phosphorylation remained

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**Figure 4.** Effect of erythropoietin on the apoptotic response of ovarian adenocarcinoma SKOV3 cells to hypericin treatment. SKOV3 cells were serum starved and treated with 150 units/mL erythropoietin for 2 h followed by hypericin treatment for additional 16 h. **A,** identification of apoptotic cells was done 6 h after the photoactivation of hypericin measured by the release of mononucleosomes and oligonucleosomes. Treatment groups: C, control without erythropoietin; Epo, 150 units/mL erythropoietin; H1, 14 nmol/L hypericin; H2, 42 nmol/L hypericin; Epo + H1, 150 units/mL erythropoietin + 14 nmol/L hypericin; Epo + H2, 150 units/mL erythropoietin + 42 nmol/L hypericin; W, 200 nmol/L wortmannin; W + Epo + H1, 200 nmol/L wortmannin + 150 units/mL erythropoietin + 14 nmol/L hypericin; W + Epo + H2, 200 nmol/L wortmannin + 150 units/mL erythropoietin + 42 nmol/L hypericin. **B,** identification of apoptotic cells was measured by the release of mononucleosomes and oligonucleosomes 24 h after the photoactivation of hypericin. **C,** confirmation of apoptosis was done counting detached cells 24 h after the photoactivation of hypericin. Mean ± SD of at least three independent experiments. +, P < 0.05; ++, P < 0.01; ++++, P < 0.001, H versus C. *, P < 0.05; **, P < 0.01, erythropoietin + H versus H. oo, P > 0.05; oo, P > 0.01, W + erythropoietin + H versus erythropoietin + H. **D,** phosphatidylserine externalization was detected by Annexin V/PI/propidium iodide double staining 2 h after the photoactivation of hypericin (60 nmol/L). Representative result of three experiments. Note reduction in apoptotic cells when treated with erythropoietin.
only in SKOV3 cells and not in A2780 cells (Fig. 6B). Furthermore, 24 h incubation of SKOV3 cells with erythropoietin revealed a concentration-dependent activation of Akt (Fig. 6B).

Discussion
Several studies have shown the presence of EpoR on tumor cells (whereas, in some cases, not in the adjacent normal tissue). Among these are ovary and uterus (29, 30), melanoma (31), renal carcinoma (32), and clinical specimens of breast cancer as well as monolayer cultures of tumor cell lines derived from breast cancer (33, 34), prostate cancer (20), and ovarian cancer (27, 28). Using RT-PCR, Arcasoy et al. (35) isolated and characterized several cDNAs for EpoR splice variants expressed in human cancer cells, which may modulate the reported cellular effects of recombinant human erythropoietin and erythropoietin–EpoR antagonists. These findings provide evidence that erythropoietin might directly stimulate the survival, growth, and proliferation of malignant cells. Recent studies from our laboratory show the presence of EpoR on ovarian cancer cells (27, 28). We have confirmed and extended the results of McBroom et al. (26) and in the present study show EpoR on primary human ovarian tumor cells and on ovarian cancer cell lines A2780 and SKOV3, also showing higher expression of EpoR on A2780 cells compared with SKOV3 cells (Figs. 1 and 2).

Erythropoietin is a hormone/cytokine that interacts with an EpoR homodimer on RBC progenitors and promotes differentiation and cell growth/survival/antiapoptosis. The antiapoptotic and cytoprotective effect of erythropoietin on neurons (36) and cardiomyocyte cells (37) is also mediated via the EpoR. In this regard, erythropoietin binds to the extracellular domain of the EpoR, which is a single transmembrane protein that lacks protein tyrosine kinase or other enzymatic activity. The formation of EpoR dimers (38) initiates recruitment and activation of Janus kinase 2, which subsequently activates signaling cascades by recruitment and phosphorylation of STAT5, PI3K, and...
mitogen-activated protein kinase proteins. There is evidence that, in some nonhematopoietic cell types, erythropoietin binds to a heteroreceptor comprising the EpoR and common β receptor subunit, also known as CD131 (39).

Motility assays have revealed that erythropoietin exposure increased the cell motility of both SKOV3 and OVCA1 ovarian cancer cells (26). Another very recent in vitro study indicates that exogenous erythropoietin results in a significant in vitro resistance to ionizing radiation and to cisplatin in the human malignant glioma U87 and the primary cervical cancer HT100 cell lines that express EpoR (40). The effect of erythropoietin exposure was abolished in both U87 and HT100 cell lines using the specific Janus kinase 2 kinase inhibitor tyrphostin (AG490). In our experimental model, SKOV3 cells did not exhibit any increased resistance to paclitaxel or irradiation (data not shown); they did exhibit increased resistance to PDT (Fig. 3A). This resistance was blocked by treatment of SKOV3 cells with AG490 (Fig. 3B).

The activation of Akt signal transduction mediates the antiapoptotic effects of erythropoietin in primary human erythroid progenitor cells (41), in vascular endothelial cells (42), and in neural tissue (43). A recent study showed the association between erythropoietin and dose-dependent inhibition of apoptosis in response to Taxol treatment and serum starvation of rodent mammary adenocarcinoma (R3230) cells in vitro (44).

We observed a difference between A2780 and SKOV3 cells in duration of erythropoietin-induced Akt phosphorylation. SKOV3 cells showed basal level of Akt phosphorylation and enhanced Akt phosphorylation observed after 24 h incubation of SKOV3 cells with erythropoietin (Fig. 6A and B). On the other hand, there was no Akt phosphorylation found in A2780 cells after 24 h incubation with erythropoietin despite the increased Akt phosphorylation observed after 2 h incubation (Fig. 6A and B). Tokunaga et al. (45) found that HER-2/neu overexpression was significantly (p < 0.0001) associated with Akt phosphorylation at Ser473. Therefore, it is possible that the presence of constitutive expression of Akt and the basal level of phosphorylated Ser473 might be related to the higher expression of HER-2/c-erbB-2 observed in SKOV3 cells. On the contrary, A2780 cells did not show any expression of HER-2/c-erbB-2 gene (data not shown).

Activation of erythropoietin-mediated signaling (Akt and ERK1/2) in R3230 cells was associated with a dose-dependent inhibition of apoptosis in response to Taxol treatment and serum starvation, an effect that was blocked by the addition of a PI3K inhibitor (44). In our study, erythropoietin induced increase of Akt phosphorylation did not protect SKOV3 cells against paclitaxel toxicity (data not shown) but did protect against the cytotoxic effect induced by PDT. Additional studies are needed to determine whether there is any relation among c-erbB-2 overexpression, Akt phosphorylation, and erythropoietin-induced resistance to PDT in SKOV3 cells.

In this regard, Hwang et al. (46) showed that hypericin inhibited the expression of c-erbB-2 and induced apoptosis of SKOV3 cells. In our experimental model, 16 h incubation of SKOV3 or A2780 cells with hypericin did not induce apoptosis (data not shown). The only apoptotic process was induced by the photoactivation of hypericin after 16 h incubation with hypericin (Fig. 4). Duran and Song (47) showed that photoactivation of hypericin in the presence of oxygen leads to the generation of superoxide anion radicals and singlet oxygen (1O2). It is generally accepted that 1O2 plays a major role in the biological activity of hypericin. Furthermore, the exposure of NIH 3T3 cells to 1O2 elicited a strong and sustained phosphorylation of Akt. Significantly, cell death induced by 1O2 was enhanced by inhibition of PI3K, suggesting that activation of Akt by 1O2 may contribute to fibroblast survival under this form of oxidative stress (48). Similarly, increased Akt phosphorylation in SKOV3 cells induced by 150 units/mL erythropoietin showed cell protecting and antiapoptotic effect in response to hypericin treatment. Indeed, erythropoietin exposure of SKOV3 cells decreased the percentage of apoptotic cells observed 6 and 24 h after hypericin photoactivation, an effect that was blocked by PI3K/Akt inhibitor wortmannin (Fig. 4).

Multiple studies have shown that erythropoietin has a positive effect in regards to tumor therapies. Indeed, systemic recombinant erythropoietin resulted in improved oxygenation of tumors, an effect associated with higher efficacy of radiotherapy and PDT (6, 49). Our in vitro results suggest that the presence of EpoR on cancer cells alone does not necessarily predict the cell protection against the cytotoxic effect induced by different therapies when cells are pretreated or cotreated with erythropoietin. Nevertheless, in some cases, erythropoietin-induced Akt phosphorylation might be related to cancer cell protection. Erythropoietin may have potential risk and protect those cancer cells that have the basal constitutive Akt phosphorylation. To confirm this and to investigate any other deleterious effect of erythropoietin on cancer cells, further studies using different tumor models in vitro and in vivo will be required.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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etin stimulates angiogenesis.


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