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TITLE: Stimulation of Breast Tumor Cell Proliferative Recovery by the Peptide Hormone Erythropoietin

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<b>14. ABSTRACT</b> <b>Purpose, Scope and Major Findings:</b> Recent clinical trials have demonstrated that breast cancer patients receiving chemotherapy who were also treated with erythropoietin (EPO) had a shortened lifespan compared to patients receiving chemotherapy alone. We believe that these outcomes may be a result of EPO interfering with the effectiveness of chemotherapy either by inducing cytoprotective signaling pathways through the EPO receptor, which had been identified on breast tumor cells and/or stimulating growth of dormant breast tumor cells. Our studies in cell culture, however, failed to detect stimulation of breast tumor cell growth after treatment of breast tumor cells with erythropoietin, interference with the effectiveness of chemotherapeutic drugs including adriamycin, taxol and tamoxifen or attenuation of drug-induced senescence arrest. The EPO receptor was clearly active in breast tumor cells, based on signaling through JNK, ERK and p38 kinase pathways. However, EPO failed to activate Akt or STAT 5 signaling and in fact, STAT 5 expression was not detected. We conclude that the lack of protection by EPO may be related to failure to active Akt or STAT 5 signaling. Companion studies in leukemia cells indicate that chemotherapeutic agents have the ability to suppress EPO signaling. Consequently, the influence of EPO in the clinical trials described above is likely to have another basis, such as stimulation of the invasion and/or metastatic capacity of breast tumor cells or interference with drug delivery to the tumors, possibly by increasing interstitial fluid pressure.					
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## INTRODUCTION

In addition to surgery, radiation and hormonal therapy, treatment for breast cancer generally involves chemotherapy with cytotoxic drugs including doxorubicin, paclitaxel, cyclophosphamide, methotrexate and 5-fluorouracil. Unfortunately, cancer chemotherapy is almost always accompanied by severe and debilitating anemia, due to both inhibition of erythroid cell maturation in the bone marrow and interference with the kidney's ability to produce erythropoietin (EPO), the hormone that regulates red blood cell development. Chemotherapy-associated anemia is treated either with EPO in its native form (Epoetin-alpha, Procrit®) or with a genetically modified form of the drug (Darbepoetin).

Because of its potential to stimulate proliferation of hematopoietic and/or leukemic cells (which may express receptors for EPO and respond to EPO, even if not erythroid in nature), the use of EPO for chemotherapy-associated anemia is contraindicated for diseases of blood forming tissue. However, recent publications have reported the unexpected finding that EPO receptors are also expressed in tumors derived from the breast and in breast tumor cell lines<sup>1,2,3</sup>.

Injection of a typical pharmaceutical preparation of 4,000 units of EPO results in EPO concentrations much higher than the maximum physiological levels of ~ 1 unit/ml reached in anemic patients. The fact that EPO receptors are expressed on breast tumor cells raises concerns that EPO could stimulate the growth of indolent breast tumors and/or interfere with the action of anti-tumor therapies, either by enhancing tumor proliferation rates or interfering with apoptotic cell death.

A recent clinical trial supports the possibility that erythropoietin therapy for chemotherapy-induced anemia may have the undesired effect of interfering with the effectiveness of anticancer treatments and possibly accelerating tumor growth. A phase III trial demonstrated decreased survival and decreased local control in metastatic breast cancer patients undergoing chemotherapy who were treated with EPO<sup>4</sup>.

Our studies were designed to investigate the influence of EPO on breast tumor cell proliferative capacity, sensitivity to chemotherapeutic drugs, and promotion of apoptotic cell death – and to link these findings with signaling through the EPO receptor.

## BODY

We examined the influence of continuous exposure to a relatively high concentration (10 units/ml) of the form of recombinant human EPO used in the clinic (epoetin alpha; PROCRIT®) on both the rate of proliferation and survival of the p53 wild type estrogen receptor positive MCF-7 breast tumor cell line. Figure 1 indicates that: a) stimulation of proliferation of MCF-7 cells by EPO was minimal and not statically significant; b) sensitivity to the anti-estrogen, tamoxifen or the microtubule poison, taxol, was essentially unaltered by EPO; and c) that EPO failed to interfere with induction of apoptosis in MCF-7 cells by either taxol or tamoxifen. (Conditions for the MTT assay are: Control, DMSO and 0.5nM, 1nM, 5nM, and 10 nM taxol and 0.5uM, 1uM, 5uM and 10 um tamoxifen. Conditions for the apoptosis assay are Control, 0.5, 1, 5 and 10nM taxol, 0.5, 1, 5 and 10uM tamoxifen). Essentially identical results were generated when the drug utilized was doxorubicin and when similar studies were performed in the p53 mutant, estrogen receptor negative MDA-MB231 breast tumor cell line (not shown).

In view of the fact that the EPO receptor has been identified in both of these breast tumor cell lines, we proceeded to investigate whether the apparent lack of EPO on the response to chemotherapeutic drugs might be a consequence of deficiencies in signaling pathways downstream of the receptor<sup>5-8</sup>. Figures 2 through 6 indicate that EPO activated ERK, p38 kinase and JNK, but not Akt or STAT5 signaling in MCF-7 cells. These observations suggest that the absence of either EPO-activation of either STAT5 or Akt signaling might explain why EPO failed to protect MCF-7 cells from the apoptosis or inhibition of proliferation by chemotherapeutic drugs.

We further observed that STAT 5a was not expressed in MCF-7 cells. This observation indicates that the MCF-7 cells have mutated to no longer express or activate STAT5. Since STAT5 was first identified as “Mammary Gland Factor” and is the transcription factor required for both lactation and mammary development, the loss of STAT5 may be associated with the loss of differentiation common to virtually all cancers.

Although EPO is not required for proliferation or survival of F-MEL leukemia cells, we found that EPO strongly activates ERK in these cells (figure 7). To determine whether ERK activation by EPO might protect cells against apoptosis induced by chemotherapy, F-MEL leukemic cells treated with or without EPO were exposed to either cytarabine (Ara-C) or daunorubicin. Figure 7 indicates that the drugs dramatically suppressed ERK activation by EPO. EPO also failed to protect F-Mel cells from apoptosis induced by Ara-C or Daunorubicin (not shown). This study indicates that stimulation of at least one cytoprotective pathway by EPO is attenuated by chemotherapy, which may explain why EPO is unable to interfere with the drug action and promote survival.

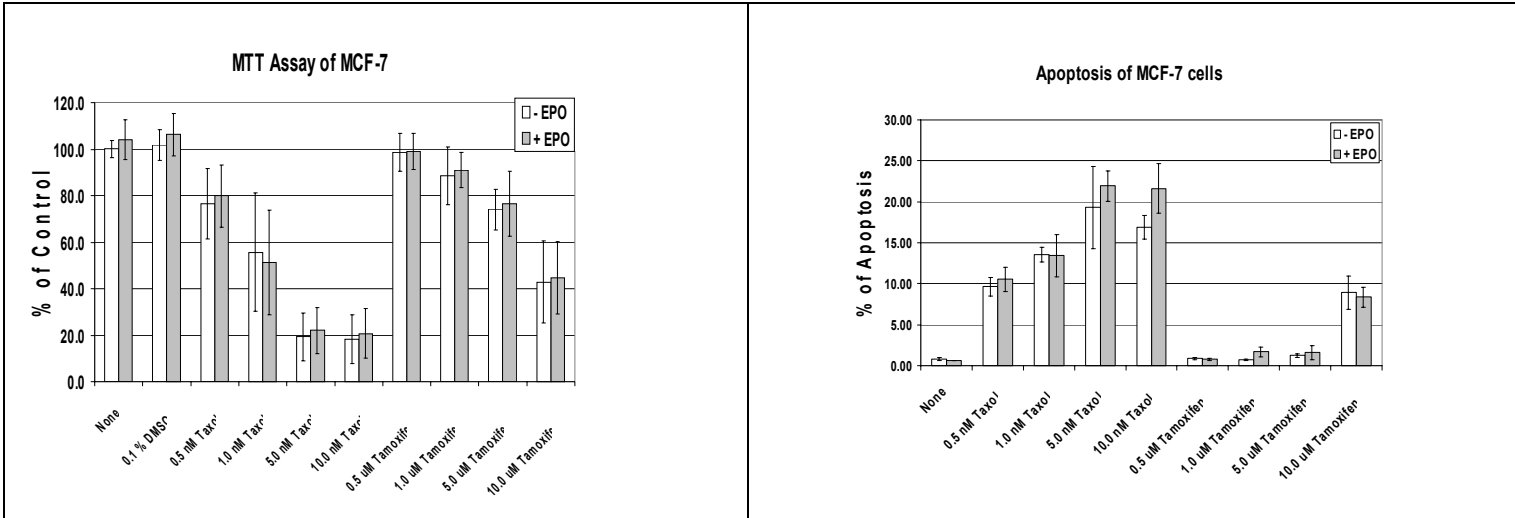


Figure 1. EPO does not stimulate proliferation of MCF-7 breast cancer cells or protect from apoptosis induced by anti-cancer drugs. Proliferation was estimated by a standard dye reduction of MTT assay (left panel) after 72 of exposure to 10 units EPO/ml and the indicated concentration of drug. In parallel experiments, the extent of apoptosis after 72 hours of exposure to drugs in the presence or absence of 10 units EPO/ml was estimated (right panel) by determination of the percent of cells that contained less than 2N DNA content as determined by propidium iodine staining and flow cytometry.

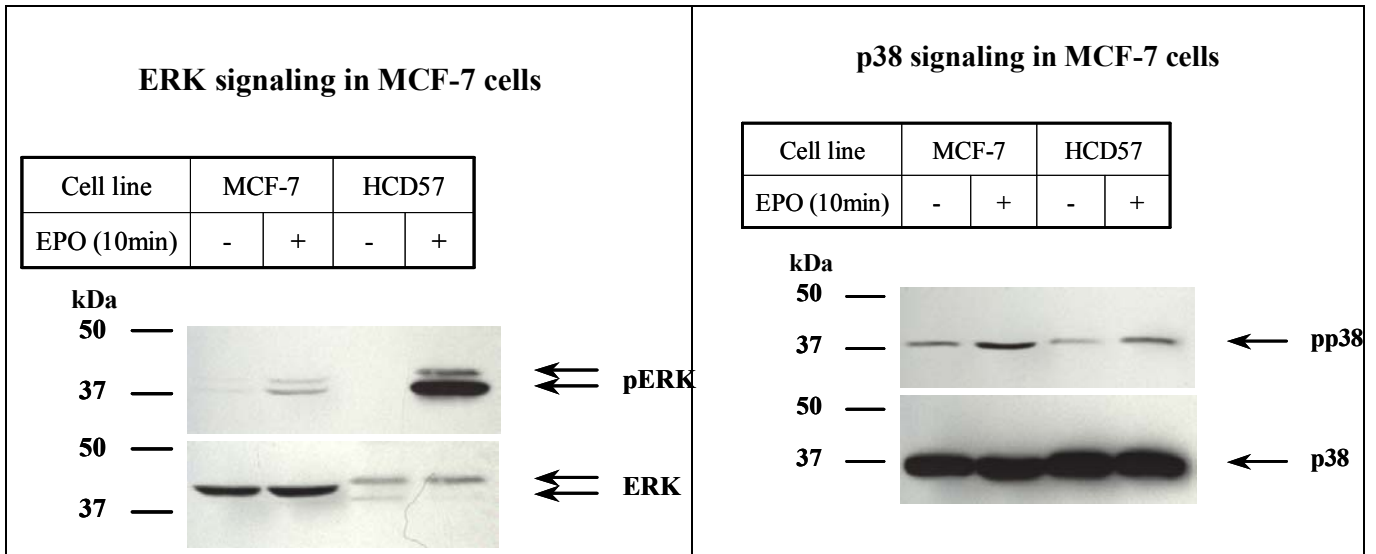


Figure 2. EPO activated ERK-1/2 MAP kinase in MCF7 cells. MCF7 breast cancer cells or HCD57 erythroid cells (positive control) were treated with 10 units of EPO/ml for 10 minutes. 10<sup>6</sup> cells were dissolved in SDS-PAGE sample buffer and western blotted with anti-phospho ERK antibody. The blot was stripped of bound antibody and reprobed with anti-ERK.

Figure 3. EPO activates p38 MAP kinase in MCF7 cells. MCF7 breast cancer cells or HCD57 erythroid cells (positive control) were treated with 10 units of EPO/ml for 10 minutes. 10<sup>6</sup> cells were dissolved in SDS-PAGE sample buffer and western blotted with anti-phospho p38 antibody. The blot was stripped of bound antibody and reprobed with anti-p38.

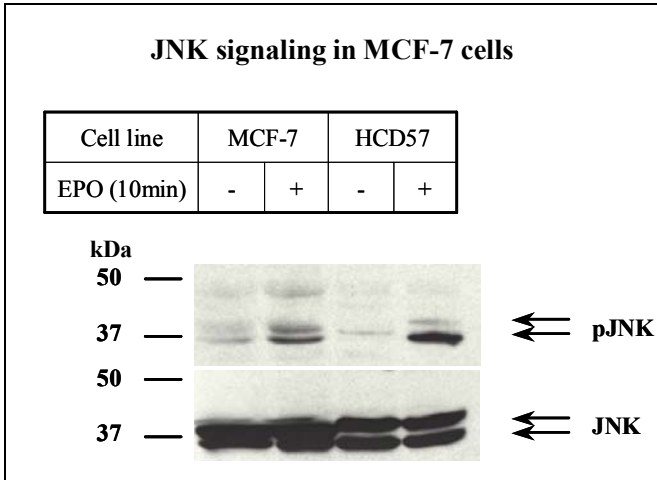


Figure 4, EPO activated JNK-1/2 MAP kinase in MCF7 cells. MCF7 breast cancer cells or HCD57 erythroid cells (positive control) were treated with 10 units of EPO/ml for 10 minutes.  $10^6$  cells were dissolved in SDS-PAGE sample buffer and western blotted with anti-phospho JNK antibody. The blot was stripped of bound antibody and reprobed with anti-JNK.

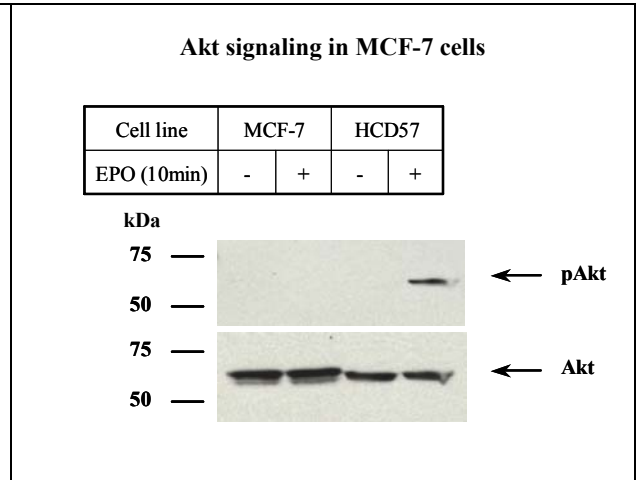


Figure 5. EPO does not activate Akt (Protein Kinase B) in MCF7 cells. MCF7 breast cancer cells or HCD57 erythroid cells (positive control) were treated with 10 units of EPO/ml for 10 minutes.  $10^6$  cells were dissolved in SDS-PAGE sample buffer and western blotted with anti-phospho Akt antibody. The blot was stripped of bound antibody and reprobed with anti-Akt.

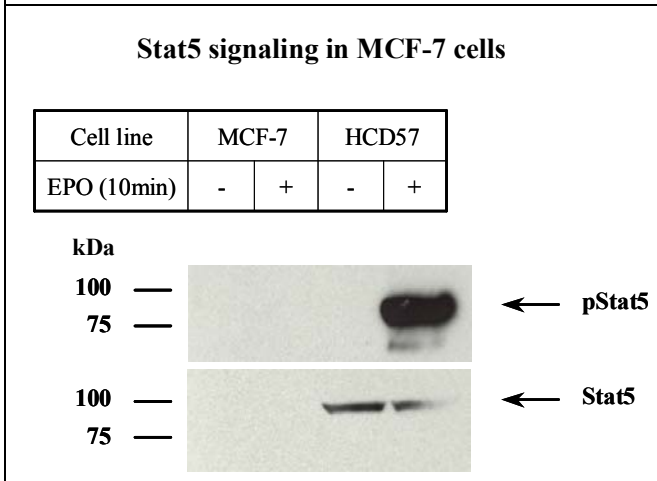


Figure 6 (above) STAT5a is neither expressed nor is STAT5a/b activated by EPO in MCF 7 cells. MCF7 breast cancer cells or HCD57 erythroid cells (positive control) were treated with 10 units of EPO/ml for 10 minutes.  $10^6$  cells were dissolved in SDS-PAGE sample buffer and western blotted with anti-phospho STAT5 antibody (recognized both phospho-STAT5a and 5b). The blot was stripped of bound antibody and reprobed with anti-STAT5a.

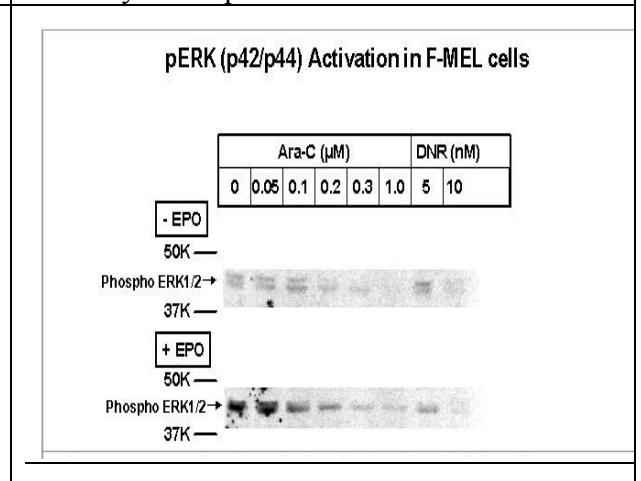


Figure 7 (above) ERK phosphorylation (and activation) in either EPO treated or untreated F-MEL erythroleukemia cells is suppressed by treatment with cytarabine (Ara C) or daunorubicin. Cells were incubated in the absence or presence of 10units/ml of EPO and the indicated concentrations of drug for 72 hours.  $10^6$  cells were analyzed by western blotting with anti phosphor ERK antibody.

## **KEY RESEARCH ACCOMPLISHMENTS**

Demonstration that EPO fails to stimulate growth of breast tumor cells.

Evidence that EPO fails to suppress growth inhibition or induction of apoptosis by chemotherapeutic drugs in breast tumor cells.

Establishment of EPO signaling in breast cancer.

Evidence that select cytoprotective pathways are not induced by EPO in breast cancer cells.

Preliminary indications that chemotherapeutic drugs may suppress EPO mediated signaling pathways.

## **REPORTABLE OUTCOMES**

Presentation of findings at Era of Hope Meeting in June 2005

Presentation of findings at Annual Meeting of American Association for Cancer Research 2005.

Recent Submission of grant application to extend this work to the Department of Defense Breast Cancer Research Program as a “Synergistic Grant”. In this proposal, we hypothesized that Epo may promote breast tumor cell metastasis, protect breast tumor cells from anoikis ( thereby facilitating invasion and/or migration) and possibly stimulate the growth of breast tumor stem cells.

Manuscript published in Clinical Cancer Research (appended).

## **CONCLUSIONS AND SIGNIFICANCE**

Taken together, our studies show that while the EPO receptor is expressed and functional in breast tumor cells, this does not automatically translate into either the stimulation of additional proliferation or protection from apoptosis by EPO. The absence of EPO’s protective effect might be limited to only a select few cancers (such as breast cancer) where the critical elements of the cytoprotective signaling (Akt or STAT-5) are not activated by EPO. Alternatively, a widespread ability of chemotherapy drugs to attenuate EPO signaling may explain why EPO cannot protect breast cancer cells from apoptosis induced by these drugs.

It is however possible that EPO may prove to influence such elements of breast tumor cell function as invasion and metastasis<sup>9-11</sup>. Additional possibilities we propose to investigate should our proposal be funded are protection from anoikis ( a form of apoptosis that occurs when cells lose adherence to the cellular substratum) or stimulation of proliferation of breast tumor stem cells.

**REFERENCES**

1. Acs G, Chen M, Xu X, Acs P, Verma A and Koch CJ. Autocrine erythropoietin signaling inhibits hypoxia-induced apoptosis in human breast carcinoma cells. *Cancer Lett* 214: 243-251, 2004.
2. Acs G, Acs P, Beckwith SM, Pitts RL, Clements E, Wong K and Verma A. Erythropoietin and erythropoietin receptor expression in human cancer. *Cancer Research* 61: 3561-3565, 2001.
3. Arcasoy MO, Amin K, Karayal AF, Chou SC, Raleigh JA, Varia MA and Haroon ZA. Functional significance of erythropoietin receptor expression in breast cancer. *Lab Invest* 82: 911-918, 2002.
4. Leyland-Jones B. *Lancet Oncol* Breast cancer trial with erythropoietin terminated unexpectedly. 4: 459-460, 2003.
5. Bao H, Jacobs-Helber S M, Lawson AE, Penta K, Wickrema A, and Sawyer ST, Protein Kinase B (C-Akt), Phosphatidylinositol 3-Kinase And STAT5 Are Activated By Erythropoietin In HCD57 Erythroid Cells But Are Constitutively Active In An EPO-Independent, Apoptosis Resistant Subclone (HCD57-SREI Cells) *Blood* 93: 3757-3773, 1999.
6. Bittorf T, Seiler J, Ludtke B, Buchse T, Jaster R and Brock J. Activation of STAT 5 during EPO directed suppression of apoptosis. *Cell Signaling* 12:23-30, 2000.
7. Bouscary D, Pene F, Claessens Y-E, Muller O, Chretien S, Fontenay-Roupie M, Gisselbrecht S, Mayeux P and Lacombe C. Critical role for PI 3-kinase in the control of erythropoietin induced erythroid progenitor proliferation. *Blood* 101: 3436-3437, 2003.
8. Jacobs-Helber, S.M, Ryan, J. and Sawyer, S.T. JNK and p38 are activated by EPO but are not induced in apoptosis following EPPO-withdrawal in EPO-dependent HCD57-cells, *Blood*. 96: 933-940, 2000.
9. Lai SY, Childs EE, Xi S, Coppeli FM, Gooding WE, Wells A, Ferris RL and Grandis JR. Erythropoietin-mediated activation of JAK-STAT signaling contributes to cellular invasion in head and neck squamous cell carcinoma. *Oncogene* 24: 4442-4449, 2005.
- 10 Nakamatsu K, Nishimura Y, Suzuki M, Kanamori S, Maenishi O and Yasuda Y. Erythropoietin/erythropoietin-receptor system as an angiogenic factor in chemically induced murine hepatic tumors. *Int J Clin Oncol* 9: 184-188, 2004.
11. Ribati D. Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulated neovascularization. *Blood* 93: 2627-2636, 1999.

## **Erythropoietin Fails to Interfere with the Antiproliferative and Cytotoxic Effects of Antitumor Drugs**

David A. Gewirtz, Xu Di, Teneille D. Walker, and Stephen T. Sawyer

**Abstract Purpose:** Erythropoietin (EPO) therapy is widely used for the prevention and treatment of anemia resulting from cancer chemotherapy. Native EPO regulates erythropoiesis, at least in part, by protecting erythroid progenitor cells from apoptotic cell death. The recent discovery of the EPO receptor (EPOR) on cancer cells raises the concern that EPO therapy might stimulate tumor growth and/or protect cancer cells from drug-induced apoptosis. Therefore, the capacity of EPO to interfere with the effects of conventional chemotherapeutic drugs on proliferation, apoptosis, and the induction of senescence was investigated in MCF-7 and MDA-MB231 breast tumor cells, which express the EPOR as well as in F-MEL erythroleukemia cells.

**Experimental Design:** Breast cancer cells and F-MEL leukemic cells were cultured in the presence or absence of EPO and then exposed to antitumor drugs. Cell proliferation was assessed by a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction assay 72 hours after drug exposure. Cytotoxicity was monitored by clonogenic survival. Apoptosis was evaluated either by the terminal deoxynucleotidyl transferase-mediated nick-end labeling assay or fluorescence-activated cell sorting analysis, and senescence was monitored by  $\beta$ -galactosidase staining. EPO signaling was assessed by monitoring the phosphorylation/activation of specific signaling proteins.

**Results:** EPO failed to stimulate the proliferation of MCF-7 or MDA-MB231 breast tumor cells or F-MEL leukemic cells. EPO treatment also failed to interfere with the antiproliferative and/or cytotoxic effects of Adriamycin, Taxol, and tamoxifen in breast tumor cells (or of cytarabine and daunorubicin in F-MEL cells). EPO failed to prevent apoptosis induced by Taxol or senescence induced by Adriamycin in MCF-7 cells. EPO stimulated the activation of extracellular signal-regulated kinase, p38, and c-Jun-NH<sub>2</sub>-kinase in MCF-7 cells but did not activate Akt or signal transducers and activators of transcription 5 (STAT5). EPO failed to activate any of these signaling pathways in MDA-MB231 cells. Cytarabine and daunorubicin interfered with EPO signaling in F-MEL cells.

**Conclusions:** These findings suggest that EPO is unlikely to directly counteract the effectiveness of cancer chemotherapeutic drugs. This may be a consequence of either ineffective signaling through the EPOR or drug-mediated suppression of EPO signaling.

Cancer chemotherapy is often accompanied by severe and debilitating anemia due to both inhibition of erythroid cell maturation in the bone marrow and interference with the ability of the kidney to produce erythropoietin (EPO), the

hormone that regulates red blood cell development. Chemotherapy-associated anemia is treated either with EPO in its native form (epoetin- $\alpha$  or epoetin- $\beta$ , Epogen or Procrit) or a genetically modified form of the drug (Darbepoetin, Aranesp) or transfusion.

Because of the potential of EPO to stimulate proliferation of hematopoietic and/or leukemic cells (which may express receptors for EPO and respond to EPO, even if not erythroid in nature), the use of EPO for chemotherapy-associated anemia is contraindicated for diseases of blood-forming tissue. Recent publications, however, have reported the unexpected finding that EPO receptors (EPOR) are also expressed in various malignant tissues, including tumors derived from the breast and in breast tumor cell lines (1–6). The fact that EPORs are expressed on tumor cells raises concerns that EPO could stimulate the growth of indolent tumors and/or interfere with the action of antitumor therapies, either by enhancing tumor proliferation rates or interfering with apoptotic cell death.

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A number of studies have examined whether expression of the EPOR allows EPO to affect tumor growth or responsiveness to chemotherapy. Stimulation of the proliferation of prostate tumor cell lines by EPO has been reported (7), whereas HeLa cells transfected with the EPOR showed a 50% increase in clonogenic survival with EPO treatment (5). Autocrine EPO may also stimulate the proliferation and survival of uterine and ovarian cancers as addition of either anti-EPO or soluble EPOR-attenuated proliferation (1, 8).

With regard to breast cancer, EPO was reported to stimulate the proliferation of various human breast tumor cell lines that express the EPOR, including MCF-7, BT-549, T-47D, MD-134, and MDA-231 cell lines (2). MCF-7 and T47-D cell lines were shown to respond to hypoxia by enhanced secretion of EPO, which protected against hypoxia-induced apoptosis (9). Finally, a neutralizing EPO antibody interfered with growth of rat syngeneic mammary tumors in a tumor chamber model (3), suggesting that autocrine EPO facilitated breast tumor growth.

There is a relatively limited body of literature relating to the effect of EPO on tumor cell response to chemotherapy. EPO has been shown to promote resistance to imatinib in human leukemia cells through the stimulation of phosphatidylinositol 3-kinase and mitogen-activated protein kinase (10). EPO also induced resistance to cisplatin in human malignant glioma cells and in a primary cervical cancer cell line (11). In contrast to these observations, one study showed that EPO failed to interfere with the antitumor effects of cisplatin against xenografts of lung cancer (12), whereas another report suggests that EPO chemosensitized human renal carcinoma and myelomonocytic leukemia cells to danorubicin and vinblastine (13). With regard to radiation, studies have shown contradictory results, with EPO conferring resistance in glioma and cervical cancer cells (11), acting as a radiosensitizer through reduction of hypoxia in glioblastoma cells (14, 15) or failing to alter radiation sensitivity in HeLa cells (5).

Two recent clinical trials support the possibility that EPO therapy for chemotherapy-induced anemia may have the undesired effect of interfering with the effectiveness of anticancer treatments and possibly accelerating tumor growth. A phase III trial showed decreased survival and decreased local control in metastatic breast cancer patients undergoing chemotherapy who were treated with EPO (16). Similar findings were reported in trials for head and neck cancer (17).

To address the potential effect of EPO on the response of tumor cells to chemotherapy, we evaluated whether EPO affected the sensitivity of MCF-7 and MDA-MB231 breast tumor cells to drugs used in the treatment of breast cancer [the anthracycline antibiotic, Adriamycin (doxorubicin); the antiestrogen, tamoxifen; and the microtubule poison, Taxol]. Similar studies were performed in F-MEL leukemia cells. We further determined the influence of EPO on the induction of apoptosis by Taxol and tamoxifen and on the promotion of senescence by Adriamycin.

## Materials and Methods

**Cells and treatment.** The p53 wild type, estrogen receptor-positive MCF-7 human breast tumor cell line was obtained from the National Cancer Institute Facility (Frederick, MD). The p53 mutant, estrogen receptor-negative MDA-MB231 breast tumor cells were obtained from the American Type Culture Collection (Manassas, VA). F-MEL murine

leukemic cells were obtained from Dr. Tim Bender (University of Virginia, Charlottesville, VA) and UT7-EPO human erythroleukemia cells were obtained from Dr. Dwayne Barber (University of Toronto, Toronto, Ontario, Canada). Clinical grade EPO (epoetin  $\alpha$ , Procrit) was purchased from the Medical College of Virginia Hospital pharmacy.

MCF-7 and MDA-MB231 cells were cultured in basal RPMI 1640 supplemented with 10% FCS. F-MEL cells and UT7-EPO cells were cultured in DMEM with 10% FCS. Cells were treated with or without 10 units EPO/mL; 24 hours later, cells were either maintained in EPO alone or with additional exposure to one of the following antitumor drugs: Adriamycin, Taxol, or tamoxifen.

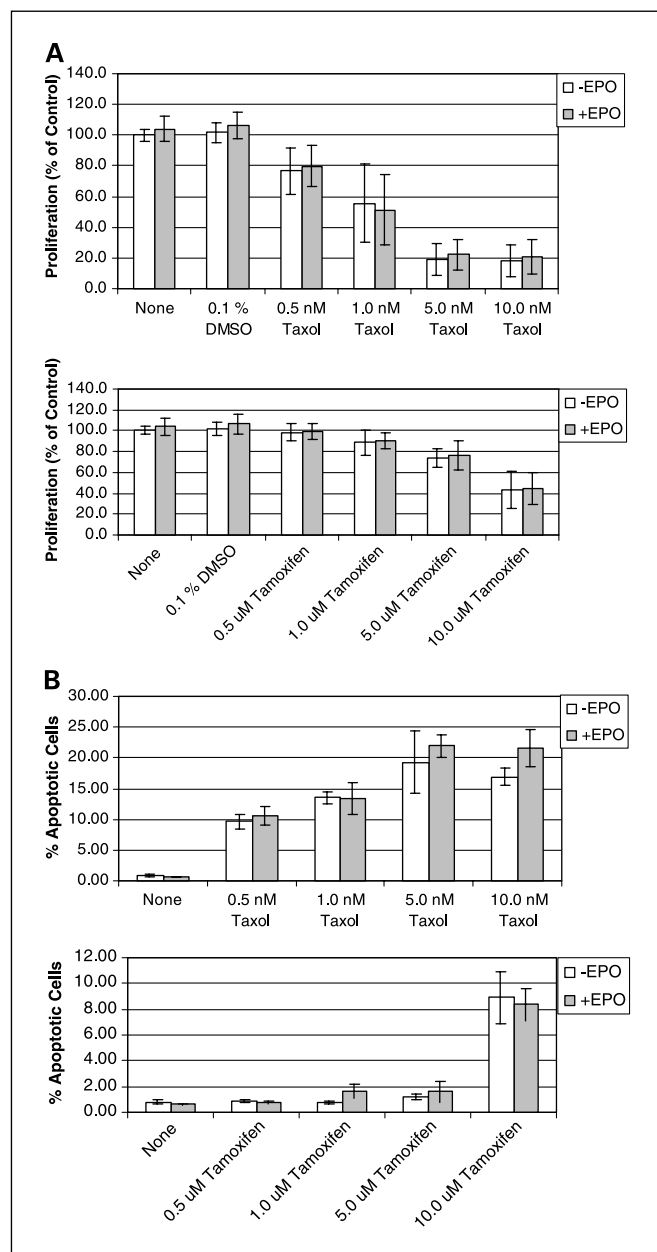
**Proliferation, apoptosis, and senescence assays.** After 3 days, cells were washed free of drug and cell proliferation/survival was estimated based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye assay, as described previously (18). Clonogenic survival of MCF-7 cells was determined as described previously (19) after cells were treated by continuous (72 hours) exposure to Adriamycin or Taxol, either alone or with prior exposure to EPO for 24 hours and in the continuous presence of EPO. Senescence induction was determined based on alterations in cell morphology (enlargement and flattening) and expression of a pH 6 dependent  $\beta$ -galactosidase (20). Apoptosis was detected by the TUNEL assay (21) using the *In situ* Cell Death Detection kit (Roche, Applied Science, Indianapolis, IN). Apoptosis studies were also conducted by flow cytometry analysis of propidium iodide-stained cells using the FACScan flow cytometer (Becton Dickinson, Rutherford, NJ), as previously described (22, 23). Cells containing sub-G<sub>0</sub>-G<sub>1</sub> DNA indicative of apoptosis were gated and shown as a percentage of the total cell numbers.

**Western blotting.** MCF-7 or MDA-MB231 cells were cultured to confluence in 35 mm culture dishes. After the addition of fresh culture medium for 4 hours, either no additions were made or 10 units EPO/mL added for 10 minutes. The culture medium was aspirated and replaced with ice-cold Iscove's modified Dulbecco's medium. The cells were scraped on ice, collected by low-speed centrifugation, and suspended in a Triton X-100 lysis buffer. Soluble proteins were recovered in the supernatant following high-speed centrifugation for 30 minutes. The amount of protein was measured in each cell extracts and equal concentrations of protein from control and EPO-treated MCF-7 cells were mixed with hot SDS-PAGE sample buffer and analyzed by SDS-PAGE and Western blotting. For a positive control, HCD57 erythroid cells were treated with EPO or left untreated in a parallel experiment. Control and EPO-treated HCD57 cell extracts were also analyzed in the same SDS-PAGE gels as the breast tumor cell extracts. The signaling proteins of interest were detected first by antiphosphospecific antibodies that recognized only the activated form of the signaling molecules. The blot was stripped of bound antibody and reprobed with an antibody that recognized both phosphorylated and nonphosphorylated forms of the signaling molecules as described previously (22).

## Results

We examined the influence of continuous exposure to a relatively high but pharmacologically relevant concentration of recombinant human EPO (10 units/mL) on both the rate of proliferation and survival of the p53 wild type, estrogen receptor-positive MCF-7 breast tumor cell line. As a positive control, the identical batch of EPO used for all the studies in breast tumor cells stimulated proliferation of HCD57 erythroid cells by ~20-fold (data not shown), consistent with the known dependence of these cells on EPO for both survival and proliferation. A single preliminary experiment suggested a moderate stimulatory effect of EPO ( $125.7 \pm 7.9\%$  of the control;  $P < 0.05$ ) on the proliferation of MCF-7 breast tumor cells, but this effect was not reproduced in further studies. As

shown in Fig. 1A, proliferation and/or survival of MCF-7 cells were essentially identical in the absence and presence of EPO. No effects were observed even at higher doses of EPO (not shown). Figure 1A further indicates that EPO failed to interfere with the antiproliferative and/or antisurvival effects of either the microtubule poison, Taxol or the antiestrogen, tamoxifen, over a broad range of drug concentrations. Similarly, EPO did not interfere with the inhibition of proliferation by the



**Fig. 1.** A, influence of EPO on the antiproliferative effects of Taxol and tamoxifen in MCF-7 breast tumor cells. Cells were exposed to tamoxifen or Taxol either alone or in the presence of EPO (10 units/mL) for a period of 3 days before assessment of viable cell number by a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction assay. Columns, means for three replicate experiments; bars, SE. B, influence of EPO on apoptosis induced by Taxol and tamoxifen in MCF-7 cells. Cells were exposed to Taxol or tamoxifen (as in A) over the indicated range of drug concentrations either alone or with a prior 24-hour exposure to and continuous exposure to EPO (10 units/mL). Apoptosis was monitored by fluorescence-activated cell sorting analysis based on the percent of sub-G<sub>0</sub>-G<sub>1</sub> events after 72 hours of culture. Columns, means for three replicate experiments; bars, SE.

topoisomerase II poison, Adriamycin over a concentration range from 50 to 250 nmol/L (data not shown). We also failed to detect protection from drug cytotoxicity when using elevated concentrations of EPO (up to 100 units/mL) or low serum (1%; data not shown). Clonogenic survival studies at an Adriamycin concentration of 50 nmol/L and a Taxol concentration of 100 nmol/L confirmed that EPO failed to interfere with the cytotoxic effects of these antitumor drugs (data not shown).

In our hands, treatment of MCF-7 cells with Adriamycin results primarily in an accelerated senescence response (24) whereas Taxol or tamoxifen promote apoptosis. EPO is known to promote survival of erythroid cells, in large part through protection from apoptosis (25, 26). However, Fig. 1B shows that EPO failed to suppress apoptosis in MCF-7 cells over a broad range of concentrations of either Taxol or tamoxifen. Figure 2 indicates that EPO also failed to alter the widespread and extensive senescence induced by Adriamycin.

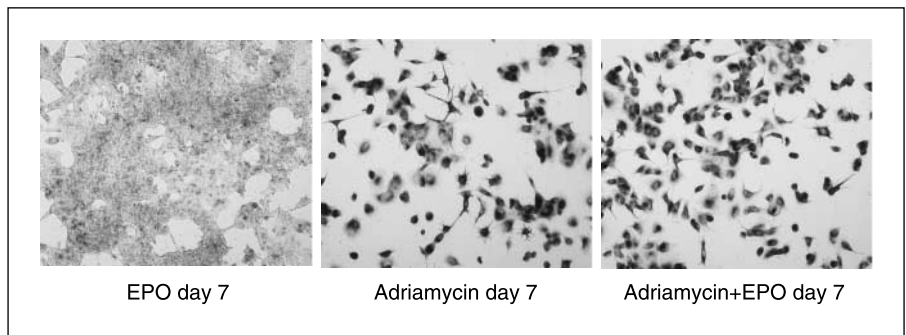
In view of the evidence for expression of the EPOR in breast tumor cell lines (which we have confirmed in MCF-7 cells by PCR, not shown), the lack of an EPO effect on proliferation, apoptosis, or senescence due to chemotherapy was unexpected. To address the basis for the failure of EPO to interfere with the chemotherapeutic agents, we evaluated the ability of EPO to signal in MCF-7 cells through well-established pathways that are thought to promote proliferation or cytoprotection, specifically the extracellular signal-regulated kinase (ERK), p38 and c-Jun-NH<sub>2</sub>-kinase (JNK) kinases, Akt (also known as protein kinase B), and signal transducers and activators of transcription (STAT) 5 (22, 23, 25, 27, 28). Figure 3 indicates that EPO was effective in promoting the phosphorylation (i.e., activation) of p38, JNK, and ERK in MCF-7 cells. Activation of these pathways in HCD57 cells is shown as a positive control. EPO, however, failed to activate either Akt or STAT5a or STAT5b. We further confirmed the published finding that STAT5 is not expressed in MCF-7 cells (29).

To test if the lack of effects of EPO on the responsiveness of breast tumor cells to chemotherapy was limited to a single cell line, similar proliferation and survival studies were also performed in MDA-MB231 cells, which are p53 mutant and estrogen receptor independent. In agreement with the results in MCF-7 cells, EPO failed to stimulate MDA-MB231 cell proliferation or to significantly attenuate the antiproliferative actions or apoptosis induced in MDA-MB231 cells by either Taxol or Adriamycin (data not shown).

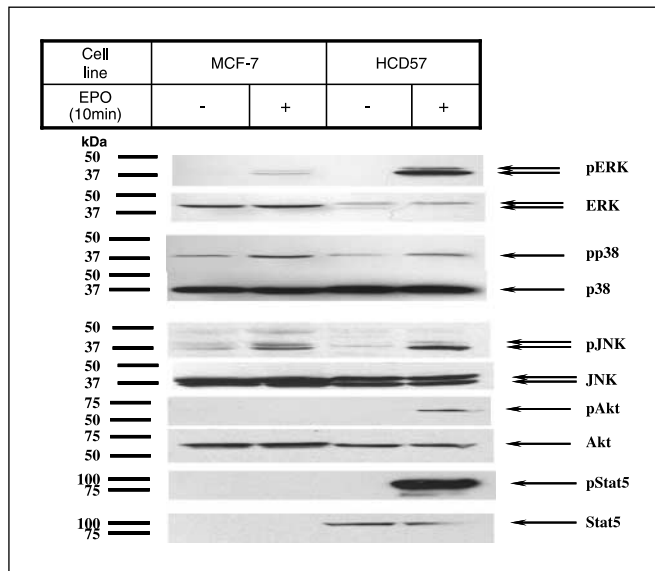
As in the studies with MCF-7 cells, we further examined the ability of EPO to signal through ERK, JNK, Akt, and STAT5 in MDA-MB231 cells. Figure 4 indicates that EPO did not activate any of these cytoprotective signaling pathways in MDA-MB231 cells. In a separate study, we also failed to observe any affect of EPO on p38 phosphorylation/activity in the MDA-MB231 cells (not shown).

Although EPO is not required for proliferation or survival of F-MEL leukemia cells, the wild-type EPOR was first cloned from these cells and the EPOR expressed on the surface of F-MEL cells is functional (30). Exposure of the F-MEL cells to EPO led to initial activation of ERK of 10- to 50-fold; the EPO-stimulated ERK1/2 then declined after 1 hour to a steady-state level that was 3- to 5-fold higher than in controls and levels remained elevated as long as 72 hours after EPO treatment (Fig. 5). To determine whether the long-term activation of ERK by EPO might protect these cells against apoptosis induced by

**Fig. 2.** Influence of EPO on senescence in MCF-7 cells. Cells were exposed to 1  $\mu$ mol/L Adriamycin for 2 hours either alone or with a prior 24-hour exposure to EPO (10 units/mL) followed by continuous exposure to EPO. Senescence was monitored based on  $\beta$ -galactosidase staining following 3 or 7 days in the absence or presence of 10 units EPO/mL. (Original magnification,  $\times 100$ ).



chemotherapy, F-MEL leukemic cells cultured with or without 10 units EPO/mL were exposed to increasing concentrations of either cytarabine or daunorubicin. These drugs suppressed the proliferation of F-MEL cells by 90% or more as indicated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction assay (Fig. 6A) and also induced apoptosis in 70% to 90% of the cells (Fig. 6B). The addition of EPO, however, had no effect on either the inhibition of proliferation or induction of apoptosis by either drug. EPO at a concentration of 5 units/mL also failed to significantly alter the growth of the F-Mel cells (not shown). These observations are likely to be related to the data presented in Fig. 5, which indicates that treatment of the F-MEL cells with either cytarabine or daunorubicin dramatically suppressed ERK1/2 activation by EPO. Consequently, it seems that EPO failed to protect F-MEL cells from drug-induced apoptosis and inhibition of proliferation due to attenuation of EPO-dependent signaling by the antitumor drugs.

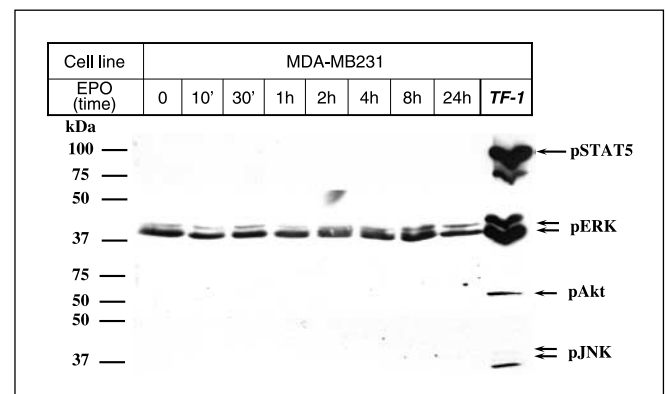


**Fig. 3.** Effect of EPO on signaling pathways in MCF-7 cells. Equal amount of protein from cell lysates was fractionated in 10% to 12% SDS-PAGE, and subjected to Western blot analyses; membranes were stripped and reprobed. For a positive control, HCD57 erythroid cells were treated with EPO or left untreated in a parallel experiment. *A*, the blot was probed with anti-phospho-ERK antibody and then reprobbed with anti-ERK. *B*, the blot was probed with anti-phospho-p38 antibody and then reprobbed with anti-p38. *C*, the blot was probed with anti-phospho-JNK antibody and then reprobbed with anti-JNK. *D*, the blot was probed with anti-phospho-Akt antibody and then reprobbed with anti-Akt. *E*, the blot was probed with anti-phospho-STAT5 antibody (recognized both STAT5a and STAT5b) and then reprobbed with anti-STAT5a. The migration of molecular mass markers on SDS-PAGE are indicated as kDa.

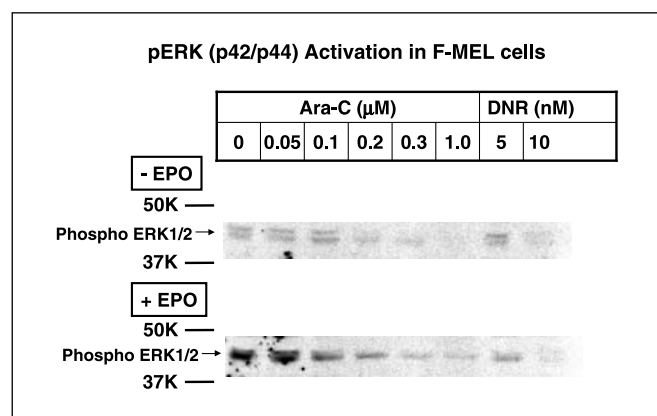
### Discussion

One recent clinical trial with breast cancer patients indicated a deleterious effect of EPO therapy on patient survival (16) but this finding has not yet been confirmed by additional studies. Although a similar report of a deleterious effect of EPO therapy in patients suffering head and neck squamous cell carcinoma (17) suggests that EPO therapy for the anemia of chemotherapy is potentially harmful to cancer patients, there are some concerns with the design of these clinical trials (31). Other studies that have analyzed clinical data relating to the effect of EPO on patient response to chemotherapy have supported continued use of EPO to ameliorate chemotherapy-induced anemia (32, 33). One report indicates that EPO enhances the response to chemotherapy in breast cancer (33), whereas another suggests that EPO may reduce the risk of tumor progression (34). Finally, questions have been raised regarding the approaches used for detection of the EPOR on tumor cells (35). Given this continuing controversy, our studies were designed to determine if EPO might directly stimulate proliferation and/or act as a cytoprotective agent to promote survival of breast cancer cells exposed to chemotherapeutic drugs.

Acs et al. (2) reported stimulation of proliferation in MCF-7 cells by EPO in the absence of serum; however, the



**Fig. 4.** Effect of EPO on signaling pathways in MDA-MB231 cells. Equal amount of protein from the lysate from each cell was fractionated in 10% to 12% SDS-PAGE and subjected to Western blot analyses; membranes were stripped and reprobed. For a positive control, the phosphorylation state of signal molecules in TF1 human erythroleukemia cells that were continuously cultured with EPO was measured. Individual blots were probed with anti-phospho-STAT5, anti-phospho-ERK antibody, anti-phospho-JNK antibody, and anti-phospho-Akt antibody. The blots were reprobed with antibodies that recognized both phosphorylated and nonphosphorylated signaling molecules to assure equal loading (not shown due to absence of detectable activation). The migration of molecular mass markers on SDS-PAGE is indicated as kDa.

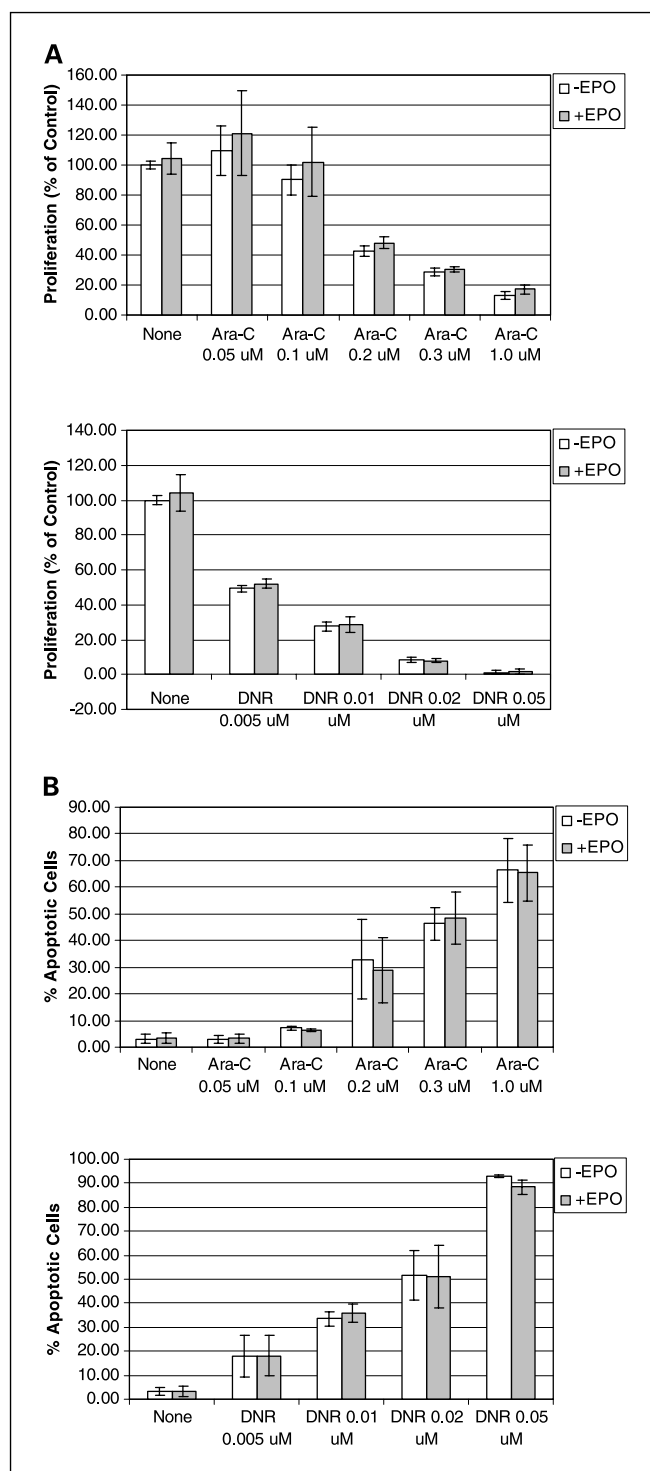


**Fig. 5.** Effect of EPO on signaling pathways in F-MEL cells. F-MEL cells were resuspended in fresh culture medium and cultured without EPO or with 10 units EPO/mL for 72 hours. Indicated concentrations of either cytarabine (*Ara-C*) or daunorubicin (*DNR*) were also added to some of the F-MEL cultures at the start of the experiment. After culture of 72 hours, the cells were recovered by centrifugation and extracts were prepared as described in Materials and Methods. The amount of protein was measured in each cell extracts and equal concentrations of protein from control and EPO-treated cells and cell treated with both EPO and drugs were mixed with hot SDS-PAGE sample buffer and analyzed by SDS-PAGE and Western blotting. The blot was then probed with antiphosphospecific ERK antibodies and reprobed with antibodies that recognize both phosphorylated and nonphosphorylated ERK to assure equal loading of ERK in each lane (not shown). The migration of molecular mass makers on SDS-PAGE is indicated as kDa.

concentration of EPO used was significantly higher than therapeutic concentrations, which tend to fall within the range of 1.7 to 3.6 units/mL (36). Similarly, McBroom et al. (37) found that EPO concentrations of 100 and 200 units/mL were required to produce relatively modest stimulation of the growth of ovarian carcinoma cells. In our studies, exposure of MCF-7 cells cultured in 10% FCS to a concentration of EPO near (or exceeding) the highest possible physiologic concentration for 3 days failed to reproducibly increase the rate of proliferation. We also tested if lowering the concentration of FCS in the medium or increasing EPO concentration 10-fold to exceed physiologic levels enhanced the effect of EPO on proliferation. However, the absence or presence of serum or 10-fold more EPO had no effect on the proliferation of the MCF-7 cells. As the incubation medium for these studies contained phenol red, which has some estrogenic properties, this experimental condition could be considered somewhat analogous to the hormonal environment in postmenopausal women, where low levels of estrogen are derived from the adrenal glands. Our studies in serum-containing medium would also more closely reflect EPO present in the circulation whereas those reported by other investigators in the absence of serum might be more indicative of the interstitial fluid environment surrounding the cell.

Studies in the estrogen receptor-negative MDA-MB231 breast tumor cell line similarly found no effect of EPO on proliferation. These observations are consistent with two other recent reports that failed to detect growth stimulation by EPO in a number of tumor cell lines (36, 38).

EPO is apparently secreted as an autocrine factor from MCF-7 cells under hypoxia (9). The presence of autocrine EPO could limit the effects of exogenously added EPO. This interference, however, is unlikely in our experiments because EPO was only synthesized in these cells under extreme hypoxic conditions (9) whereas our studies only tested EPO affects in a normal



**Fig. 6.** *A*, lack of EPO-mediated interference with the antiproliferative activity of cytarabine and daunorubicin in F-MEL leukemic cells. F-MEL cells were cultured in 96-well plates in the presence or absence of 10 units EPO/mL and the indicated concentration of cytarabine or daunorubicin. After a period of 3 days, assessment of viable cell number by a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction assay was done. Columns, means for three replicate experiments; bars, SE. *B*, lack of EPO-mediated protection from apoptosis induced by cytarabine and daunorubicin in F-MEL leukemic cells. F-MEL cells were exposed to cytarabine and daunorubicin (as in *A*) over the indicated range of drug concentrations either alone or with a prior 24-hour exposure to and continuous exposure to EPO (10 units/mL). Apoptosis was monitored by fluorescence-activated cell sorting analysis based on the percent of sub-G<sub>0</sub>-G<sub>1</sub> events after 72 hours of culture. Columns, means for three replicate experiments; bars, SE.

incubator atmosphere of 5% CO<sub>2</sub> and 20% O<sub>2</sub>. We further confirmed that EPO secretion from MCF-7 cells was insignificant under these oxygen conditions by showing that conditioned medium from MCF-7 cells did not support the growth and proliferation of the EPO-dependent HCD57 erythroid cell line (data not shown). It is more likely that lack of effect of EPO on proliferation of MCF-7 and MDA-MB231 cells is due to autoactivating mutations or alteration in gene expression that result in constitutive activation of signaling pathways to drive proliferation at a near-maximal rate. Alternatively, autocrine growth factors other than EPO secreted by the breast tumor cells may maintain the near-maximal rate of proliferation.

Using both p53 wild-type MCF-7 cells and p53 mutant MDA-MB231 cells, we failed to detect attenuation of the antiproliferative, apoptotic, or senescence-promoting effects of the respective drugs with EPO. Although we detected activation of multiple mitogen-activated protein kinase cascades by EPO in MCF-7 cells, our past work in erythroid cell suggests that mitogen-activated protein kinases could be involved in proliferation but less so in survival (23, 27, 28, 39). The strong activation of JNK by EPO in MCF-7 cells is interesting because JNK activity is widely associated with the induction of apoptosis in many cell and tissues and only infrequently linked to the stimulation of proliferation. Our recent work showed JNK activation by EPO in erythroid cells stimulated proliferation (23); however, it is possible that EPO fails to enhance proliferation or survival of MCF-7 cells because of the strong activation of JNK that counteracts the protective or stimulatory effect of ERK. The lack of STAT5 and Akt activation by EPO in either MCF-7 or MDA-MB231 breast cancer cell line is also consistent with EPO failing to promote survival because STAT5 and Akt signaling pathways have previously been associated with resistance to apoptosis in erythroid cells (23, 27, 28, 39). The inability of EPO to stimulate proliferation or to activate any of the signaling pathways tested in the MDA-MB231 cells suggests that the EPOR may not be functional in these cells, despite evidence for its expression (2). Alternatively, the tyrosine protein kinase, Janus-activated kinase 2, which couples EPO binding to signal transmission, may not be expressed at sufficient levels to allow signaling.

Overall, the absence of EPO activation of either STAT5 or Akt signaling in MCF-7 cells or any of four potential cytoprotective signaling pathways in MDA-MB231 cells suggests that expression of the EPOR in the breast tumor cells may be dissociated from the EPO-activated signaling pathways that direct erythroid cells to survive, proliferate, and differentiate. In support of this conclusion, evidence has been presented that EPO may not activate signaling pathways or promote tumor proliferation despite the expression of the EPOR (6).

It is of particular interest that EPO-dependent ERK signaling in F-MEL cells was attenuated by the antitumor drugs daunorubicin and 1- $\beta$ -D-arabinofuranosylcytosine. This study

indicates that chemotherapeutic drugs may suppress the potential cytoprotective actions of EPO, which may explain why EPO is unable to interfere with the drug action and promote survival even in the leukemic erythroid cells. It should be noted that we could not test the long-term drug effects on ERK activity in the MCF-7 cells because the activation of ERK1/2 by EPO was only significantly increased over the basal levels for a few hours.

Taken together, our studies show that whereas the EPOR is expressed and apparently functional in at least some breast tumor cells, this does not automatically translate into either a high degree of EPO-dependent proliferation or protection from antiproliferative or cytotoxic drug actions. The absence of the protective effect of EPO might be limited to only a select few cancers (such as breast cancer) where the critical elements of the cytoprotective signaling (Akt or STAT5) are not activated by EPO. Alternatively, a widespread ability of chemotherapy drugs to attenuate EPO signaling may explain why EPO cannot protect cancer cells from drug-induced apoptosis.

Although detectable levels of the EPOR are expressed in most human breast carcinomas (2), this work nevertheless suggests that EPO is unlikely to interfere with the effectiveness of cancer chemotherapy through a direct effect on the breast cancer cells. Of particular significance, the failure of EPO to interfere with senescence arrest due to Adriamycin further suggests that EPO cannot stimulate cell cycle reentry of arrested or indolent cells.

This work provides only limited insights as to the basis for the deleterious effect of EPO in the recent clinical trials involving breast cancer patients. The findings here do not support a direct protective effect of EPO on the breast cancer cells during chemotherapy. Our finding that expression of receptors for EPO does not necessarily correlate with protection from chemotherapeutic drugs may somewhat lessen the concern of dangers of EPO therapy for breast cancer patients. Our studies tend to support the findings that EPO is likely to be relatively safe and effective for patients undergoing chemotherapy for the treatment of solid tumors (31–34).

In contrast to the encouraging findings in this study relating to the continued effectiveness of chemotherapy in the presence of EPO, EPO responsiveness has also been identified in capillary endothelial cells (1, 40–42), indicating that EPO may have a role in tumor angiogenesis. EPO has also been reported to contribute to the invasive capacity of head and neck squamous cell carcinoma (4). In the late stages of preparing this article, a report was published that suggests that EPO-activated ERK increases the migration of MCF-7 cells (43). Consequently, it is formally possible that EPO may be promoting angiogenesis, tumor invasion, or metastasis.

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## References

1. Yasuda Y, Fujita Y, Matsuo T, et al. Erythropoietin regulates growth of human malignancies. *Carcinogenesis* 2003;24:1021–9.
2. Acs G, Acs P, Beckwith SM, et al. Erythropoietin and erythropoietin receptor expression in human cancer. *Cancer Res* 2001;61:3561–5.
3. Arcasoy MO, Amin K, Karayal AF, et al. Functional significance of erythropoietin receptor expression in breast cancer. *Lab Invest* 2002;82:911–8.
4. Lai SY, Childs EE, Xi S, et al. Erythropoietin-mediated activation of JAK-STAT signaling contributes to cellular invasion in head and neck squamous cell carcinoma. *Oncogene* 2005;24:4442–9.
5. Pajonk F, Weil A, Sommer A, Suwinski R, Henke M. The erythropoietin receptor pathway modulates survival of cancer cells. *Oncogene* 2004;23:8987–91.
6. Westphal G, Niederberger E, Blum C, et al. Erythropoietin and G-CSF receptors in human tumor cells: expression and aspects regarding functionality. *Tumors* 2002;88:150–9.
7. Lang SH, Miller RW, Duncan W, Habib FK. Production

- and response of human prostate cancer cell lines to granulocyte macrophage-colony stimulating factor. *Int J Cancer* 1994;59:235–41.
8. Yasuda Y, Musha T, Tanaka H, et al. Inhibition of erythropoietin signaling destroys xenografts of ovarian and uterine cancers. *Br J Cancer* 2001;84:836–43.
  9. Acs G, Chen M, Xu X, Acs P, Verma A, Koch CJ. Autocrine erythropoietin signaling inhibits hypoxia-induced apoptosis in human breast carcinoma cells. *Cancer Lett* 2004;214:243–51.
  10. Kirschner KM, Baltensperger K. Erythropoietin promotes resistance against the Abl tyrosine kinase inhibitor imatinib (STI571) in K562 leukemia cells. *Mol Cancer Res* 2003;1:970–80.
  11. Belenkov AI, Shenouda G, Rizheuskaya E, et al. Erythropoietin induces cancer cell resistance to ionizing radiation and to cisplatin. *Mol Cancer Ther* 2004;3:1525–32.
  12. Sigounas G, Sallah S, Sigounas VY, et al. Erythropoietin modulates the anticancer activity of chemotherapeutic drugs. *Cancer Lett* 2004;214:171–9.
  13. Carvalho G, Lefaucheur C, Cherbonnier C, et al. Chemosensitization by erythropoietin through inhibition of the NF- $\kappa$ B rescue pathway. *Oncogene* 2005;24:737–45.
  14. Pinel S, Barberi-Heyub M, Cohen-Jonathan E, et al. Erythropoietin-induced reduction of hypoxia before and during fractionated irradiation contributes to improvement of radioresponse in human glioma xenografts. *Int J Radiat Oncol Biol Phys* 2004;59:250–9.
  15. Stuben G, Thews O, Pottgen C, et al. Impact of anemia prevention by erythropoietin on the sensitivity of xenografted glioblastomas to fractionated radiation. *Strahlenther Onkol* 2003;179:620–5.
  16. Leyland-Jones B. Best Investigators and Study Group. Breast cancer trial with erythropoietin terminated unexpectedly. *Lancet Oncol* 2003;4:459–60.
  17. Henke M, Laszig R, Rube C, et al. Erythropoietin to treat head and neck cancer patients with anaemia undergoing radiotherapy: randomised, double-blind, placebo-controlled trial. *Lancet* 2003;362:1255–60.
  18. Fornari FA, Randolph JK, Yalowich JC, Ritke MK, Gewirtz DA. Interference with DNA unwinding by doxorubicin in MCF-7 breast tumor cells. *Mol Pharmacol* 1994;45:649–56.
  19. Chaudhry M, Sundaram S, Gennings C, Carter H, Gewirtz DA. The vitamin D3 analog, ILX-23–7553, enhances the response to Adriamycin and irradiation in MCF-7 breast tumor cells. *Cancer Chemother Pharmacol* 2001;47:429–36.
  20. Dimri GP, Lee X, Basile G, et al. A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc Natl Acad Sci U S A* 1995;92:9363–7.
  21. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992;19:493–501.
  22. Chen J, Jacobs-Helber SM, Barber DL, Sawyer ST. Erythropoietin-dependent autocrine secretion of tumor necrosis factor- $\alpha$  in hematopoietic cells modulates proliferation via MAP kinase-ERK-1/2 and does not require tyrosine docking sites in the EPO receptor. *Exp Cell Res* 2004;298:155–66.
  23. Jacobs-Helber SM, Sawyer ST. Jun N-terminal kinase promotes proliferation of immature erythroid cells and erythropoietin-dependent cell lines. *Blood* 2004;104:696–703.
  24. Elmore LW, Rehder CW, Di X, et al. Adriamycin induced replicative senescence in tumor cells requires functional p53 and telomere dysfunction. *J Biol Chem* 2002;277:35509–15.
  25. Bittorf T, Seiler J, Ludtke B, Buchse T, Jaster R, Brock J. Activation of STAT5 during EPO directed suppression of apoptosis. *Cell Signal* 2000;12:23–30.
  26. Koury MJ, Bondurant MC. Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. *Science* 1990;248:378–81.
  27. Bao H, Jacobs-Helber SM, Lawson AE, Penta K, Wickrema A, Sawyer ST. Protein kinase B (c-Akt), phosphatidylinositol 3-kinase, and STAT5 are activated by erythropoietin (EPO) in HCD57 erythroid cells but are constitutively active in an EPO-independent, apoptosis-resistant subclone (HCD57-SREI cells). *Blood* 1999;93:3757–73.
  28. Jacobs-Helber SM, Ryan JJ, Sawyer ST. JNK and p38 are activated by EPO but are not induced in apoptosis following EPO-withdrawal in EPO-dependent HCD57-cells. *Blood* 2000;96:933–40.
  29. Yamashita H, Nevalainen MT, Xu J, et al. Role of serine phosphorylation of STAT5a in prolactin-stimulated  $\beta$  casein gene expression. *Mol Cell Endocrinol* 2001;183:151–63.
  30. D'Andrea AD, Lodish HF, Wong GG. Expression cloning of the murine erythropoietin receptor. *Cell* 1989;57:277–85.
  31. Gaspy JA. Cancer patient survival and erythropoietin. *J Natl Compr Cancer Network* 2005;3:796–804.
  32. Hedenus M, Vansteenkiste J, Kotasek D, Austin M, Amado RG. Darbepoetin  $\alpha$  for the treatment of chemotherapy-induced anemia: disease progression and survival analysis from four randomized double-blind, placebo controlled trials. *J Clin Oncol* 2005;23:6941–8.
  33. Larsson A-M, Landberg G, Pahlman S, Albertsson M. Erythropoietin enhances response to treatment in patients with advanced breast cancer. *Acta Oncol* 2005;43:594–7.
  34. Pronzato P, Jassem J, Mayordomo J. Epoietin  $\beta$  therapy in patients with solid tumors. *Crit Rev Oncol Hematol* 2005. Epub ahead of print.
  35. Elliott S, Busse L, Bass MB, et al. Anti-Epo receptor antibodies do not predict Epo receptor expression. *Blood* 2005. Epub ahead of print.
  36. Hardee ME, Kirkpatrick JP, Shan S. Human recombinant erythropoietin (rEPO) has no effect in tumor growth or angiogenesis. *Br J Cancer* 2005;93:1350–5.
  37. McBroom JW, Acs G, Rose GS, Krivak TC, Mohyeldin A, Verma A. Erythropoietin receptor function and expression in epithelial ovarian carcinoma. *Gynecol Oncol* 2005;99:571–7.
  38. Hale SA, Wong C, Lounsbury KM. Erythropoietin disrupts hypoxia-inducible factor signaling in ovarian cancer cells. *Gynecol Oncol* 2006;100:14–9.
  39. Lawson AE, Bao H, Wickrema A, Jacobs-Helber SM, Sawyer ST. Phosphatase inhibition promotes anti-apoptotic but not proliferative signaling pathways in erythropoietin-dependent HCD57 cells. *Blood* 2000;96:2084–92.
  40. Ribatti D, Presta M, Vacca A, et al. Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulated neovascularization. *Blood* 1999;93:2627.
  41. Nakamatsu K, Nishimura Y, Suzuki M, Kanamori S, Maenishi O, Yasuda Y. Erythropoietin/erythropoietin-receptor system as an angiogenic factor in chemically induced murine hepatic tumors. *Int J Clin Oncol* 2004;9:184–8.
  42. Anagnostou A, Liu Z, Steiner M, et al. Erythropoietin receptor mRNA expression in human endothelial cells. *Proc Natl Acad Sci U S A* 1994;91:3974–8.
  43. Lester RD, Jo M, Campana WM, Gonias SL. Erythropoietin promotes MCF-7 breast cancer cell migration by a ERK/MAP kinase-dependent pathway and is primarily responsible for the increase in migration observed in hypoxia. *J Biol Chem* 2005;280:39723–77.