Recombinant Human Erythropoietin in Combination with Chemotherapy Increases Breast Cancer Metastasis in Preclinical Mouse Models

Benjamin D. Hedley1, Jenny E. Chu2,3, D. George Ormond2, Michel S. Beausoleil2,3, Alexandra Boasie2, Alison L. Allan2,3,4, and Anargyros Xenocostas1,2,4,5

Abstract

Purpose: Erythropoiesis-stimulating agents (ESA) are used clinically for treating cancer-related anemia. Recent clinical trials have reported increased adverse events and reduced survival in ESA-treated breast cancer patients receiving chemotherapy, potentially related to erythropoietin (EPO)-induced cancer progression. However, minimal preclinical data are available about the impact of EPO on metastatic cell behavior and/or the metastatic process, and this was the goal of our study.

Experimental Design: Breast cancer cell lines were treated with recombinant human EPO (rHuEPO) and screened for expression of EPO receptors (EPOR). MDA-MB-231 and MDA-MB-435 cell lines were used for functional assays in vitro (two-dimensional/three-dimensional growth and survival) and in vivo (tumorigenicity and metastasis), in the presence or absence of EPO and/or cytotoxic agents.

Results: A large variation in EPOR expression across cell lines was observed. In vitro, rHuEPO had a protective effect on radiation-treated MDA-MB-435 cells (P < 0.05); however, rHuEPO treatment alone or combined with chemotherapy or hypoxia did not influence cell survival. In vivo, rHuEPO increased lung metastases in immunocompromised mice injected with MDA-MB-231 or MDA-MB-435 cells and treated with chemotherapy relative to mice treated with chemotherapy alone (P < 0.05).

Conclusions: The lack of an in vitro effect of rHuEPO highlights the importance of in vivo studies to delineate the effects of EPO on the metastatic process. These studies may begin to uncover the underlying functional explanation for the observed EPO-related adverse events and decreased survival in ESA-treated metastatic breast cancer patients undergoing chemotherapy.

Introduction

Over the past few decades, understanding of the physiologic function of erythropoietin (EPO) has evolved significantly. After being produced in the fetal liver or adult kidney, EPO binds to EPO receptors (EPOR), initiating signaling that stimulates growth, inhibits apoptosis, and induces the differentiation of erythroid progenitors to increase red blood cell mass (1). Originally known to be a critical component in the regulation of erythropoiesis, EPO has additionally been shown to exert tissue-protective effects on multiple tissues (2), suggesting a pleiotropic mechanism of action.

Following initial cloning and publishing of the human EPO gene sequence in 1985 (3), efforts were made to make recombinant human EPO (rHuEPO) and other erythropoiesis-stimulating agents (ESA) an accessible treatment for anemia. Chronic anemia and/or chemotherapy-induced anemia (CIA) is a frequent side effect in cancer patients, is associated with reduced quality of life, and may also enhance emergence of hypoxia-induced treatment resistance (4). In early studies, rHuEPO was shown to be a safe and effective treatment for CIA, reducing numbers of required transfusions and increasing patient quality of life (5). Recent meta-analyses have shown that ESA treatment results in an increased risk for venous thromboembolism (VTE) in cancer patients. In addition, these meta-analyses have provided conflicting data indicating that ESAs may or may not negatively impact overall patient survival, raising concerns about the potential for disease progression leading to increased mortality rates in cancer patients (6–9). There have been multiple trials evaluating the effect of ESAs on patients with breast cancer (5, 10–15). Of these, 2 studies (BRAVE and BEST) included only patients with...
Translational Relevance

Erythropoiesis-stimulating agents (ESA) are used clinically for treating cancer-related anemia (CIA). Recent clinical trials have reported increased adverse events/reduced survival in ESA-treated cancer patients receiving chemotherapy, potentially related to erythropoietin-induced cancer progression. However, minimal preclinical data are available about the underlying causes of these observations.

This article describes the effects of recombinant human erythropoietin (rHuEPO) on metastatic potential and chemotherapy response of human breast cancer cells using in vitro assays and preclinical animal models of metastasis. To the best of our knowledge, this is the first study to show potentiating effects on metastasis and reduction of chemotherapeutic efficacy in secondary sites by rHuEPO.

Despite concerns for disease progression related to ESA use for treatment of CIA, there are few, if any, studies addressing the question of metastasis. Thus, the novel findings presented here show for the first time positive effects on metastasis by rHuEPO in a clinically relevant animal model.

metastatic disease (10, 11). The BRAVE study evaluated a once weekly dose of rHuEPO and detected no difference in overall survival (10). The BEST study was ended early because of a higher mortality rate at 12 months in the rHuEPO-treated versus placebo arm (11). Additional trials in other cancer types, such as the ENHANCE (head and neck cancer), EPO-CAN 20 (non–small cell lung cancer), and GOG 191 (cervical cancer) trials, reinforced these concerns by reporting shorter progression-free survival and/or overall survival in patients treated with rHuEPO (16). Taken together, these clinical studies raise the question of whether there is an underlying EPO–tumor cell interaction that may lead to disease progression.

EPOR has been shown to be expressed in normal and malignant cells (17). Many of these studies have relied on anti-EPOR antibodies that have since been shown to be nonspecific (18), detecting either proteins of incorrect molecular weight or detecting non-EPOR proteins of a similar weight to EPOR (19). In addition, there is no universally accepted molecular weight of EPOR because of cell type–specific posttranslational modifications or alternative splicing which may alter the apparent size(s) of EPOR on Western blotting (19–21). In vitro studies using tumor cell lines have reported conflicting results, with some studies reporting a functional effect of rHuEPO on the proliferative behavior of cell lines (22, 23), whereas others show a null effect (24, 25). Variable methodologic approaches were used in these conflicting studies with some limited to histopathologic, biochemical, or in vitro evaluation, thus highlighting the need for a more complete functional assessment of the influence of rHuEPO on tumor progression (17). Clinical disease progression in solid tumors requires evolution from localized disease to metastatic disease, often leading to patient death. Previous studies have investigated the effect of rHuEPO on cell lines in vitro or on the primary tumor but have not yet examined effects on metastasis in vivo.

The goal of this study was therefore to test the hypothesis that rHuEPO can influence metastatic cell behavior and/or the metastatic process in preclinical models of breast cancer. We first examined tumor cell lines for EPOR and EPO expression using multiple approaches. We next assessed the effects of rHuEPO on malignant potential in vitro. Finally, we used clinically relevant in vivo models of breast cancer tumorigenicity and spontaneous metastasis to determine the role of rHuEPO in disease progression. Our novel findings show that that rHuEPO can reduce the efficacy of chemotherapy in the metastatic setting in vivo, and in some cases, enhance the inherent metastatic growth potential of human breast cancer cells.

Materials and Methods

Cell lines and reagents

Cell lines and culture conditions used are listed in Supplementary Table S1. All media were purchased from Invitrogen. FBS was purchased from Sigma. Murine bone marrow (mBM) was obtained by aspiration from the tibia/femurs of nude mice into HBSS (Sigma).

PCR analysis

Cells were exposed to serum-free media in the presence or absence of rHuEPO (10 U/mL; EPREX, Janssen-Ortho Inc.) for 5 minutes before harvesting. RNA was isolated using TRIzol (Invitrogen) as per the manufacturer’s instructions. Isolated RNA was converted to cDNA by SuperScript III Reverse Transcriptase [0.5 μg oligo(dT)12–18, 0.5 mmol/L deoxynucleotide triphosphate, 1× strand buffer, 50 mmol/L diithiothreitol, 40 U RNase Out, and Superscript III Reverse Transcriptase (200 U/μg) RNA (Invitrogen)], with incubations at 50°C for 45 minutes followed by 70°C for 15 minutes. Reverse transcriptase PCR (RT-PCR) primers and cycling conditions are described in Supplementary Table S2.

Western blot analysis

Cells were exposed to serum-free media in the presence or absence of rHuEPO (10 U/mL) for 5 minutes before harvesting. Total protein samples consisting of 30 μg (cell lines), 15 μg (mBM), or 0.5 μg (rHuEPO; R&D Systems) were used for SDS-PAGE (10%). Following electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes (Immobilon, Millipore). Blocking and antibody dilution was done using 5% skimmed milk in TBS with 0.1% Tween-20. Because of previous observations noting the nonspecificity of commercially available EPOR antibodies (18), a panel of previously published and nonpublished primary antibodies was evaluated for EPOR binding (Supplementary Table S3). To confirm antibody specificity, primary antibodies were also preincubated with a 2-fold excess of rHuEPO overnight prior to use in...
immunoblotting. The most consistent and specific primary antibody was determined to be mouse anti-human EPOR MAB307 (R&D Systems; 1:2,000). Loading consistency was verified using mouse anti-actin antibody (Sigma; 1:2,000). The secondary antibody was goat anti-mouse conjugated to horseradish peroxidase (Calbiochem; 1:2,000). Proteins were visualized using Amersham ECL Plus chemiluminescent system (GE Healthcare).

Flow cytometric analysis
Cells were harvested into single-cell suspensions in ice-cold flow buffer (PBS + 2% FBS). The anti-EPOR antibody used was MAB307 (R&D Systems). The secondary antibody used was a phycoerythrin-conjugated anti-mouse antibody (BD Biosciences). Nonspecific binding was accounted for using an IgG1 isotype control antibody (Cedarlane Laboratories). Samples were incubated sequentially with primary and secondary antibodies (1 μg/10⁶ cells) and resuspended in cold flow buffer prior to analysis on an EPICS XL-MCL flow cytometer (Beckman Coulter).

ELISA
Secretion of rHuEPO by cancer cell lines was assessed by an EPO-specific ELISA (Stem Cell Technologies). Conditioned media was prepared as described previously (26). Volumes were normalized to an equivalent of 1 × 10⁵ cells, and absorbance at 450 nm was recorded by a Bio-Rad plate reader. Values were compared with a standard curve generated by rHuEPO to quantify the amount of rHuEPO secreted by cancer cell lines.

Anchorage-dependent growth assays
MDA-MB-231 or MDA-MB-435 cells (harvested at 70%–80% confluency) were plated on 60 mm dishes (5.0 × 10⁴ cells per dish; n = 3 per time point) in the presence or absence of rHuEPO (10 U/mL) in normal growth media containing serum. Triplicate plates were harvested and counted by hemocytometer every 48 hours for 14 days. Doubling time was calculated according to \( T_d = t \times \ln(2)/\ln(N_{t+1}/N_t) \), where \( t \) = time of the experiment (hours), \( N_{t+1} \) = number of cells after time \( t \), and \( N_t \) = initial number of cells plated.

Plating efficiency/colony formation assay
MDA-MB-231 and MDA-MB-435 cells were seeded onto 100 mm dishes (n = 3; 100 cells per dish) in the presence or absence of rHuEPO (10 U/mL) and incubated for 2 weeks to determine the effect on plating efficiency/colony formation. Plates were fixed with 1% gluteraldehyde in PBS, stained with hematoxylin, and quantified for number and size of colonies using ImageJ software (NIH).

Anchorage-independent growth assay
Anchorage-independent growth assays were conducted as previously described (26) in normal growth media in the presence or absence of rHuEPO (10 U/mL) for 3 to 4 weeks. Plates were fixed in 10% neutral-buffered formalin and scanned at 100× magnification. Colonies of size 20 μm or more were counted and scored using ImageJ software.

In vitro cytotoxic treatment
MDA-MB-231 and MDA-MB-435 cells were plated in triplicate in 6-well dishes (1 × 10⁵ cells per well) for chemotherapy and radiation experiments, or 60 mm dishes (5 × 10⁴ cells per dish) for hypoxia in the presence or absence of rHuEPO (10 U/mL). Cells were treated with chemotherapy (10 mg/mL paclitaxel, 24 hours), radiation (two 5 Gy fractions, 24 hours apart, from a Cobalt-60 irradiator), or hypoxia [24 (MDA-MB-231) or 48 (MDA-MB-435)] hours of treatment in 1% O₂, 5% CO₂, and 94% N₂ with normoxic controls incubated in 5% CO₂]. The media and any floating/dead cells in the media were then collected, combined with cells harvested by trypsinization, and subjected to centrifugation. Cells were stained with propidium iodide (50 μg/mL) for 30 minutes and analyzed for viability on an EPICS XL-MCL flow cytometer.

In vivo tumorigenicity and metastasis assays
Animal procedures were carried out in accordance with protocols approved by the University of Western Ontario Council on Animal Care. MDA-MB-435 and MDA-MB-231 cells were suspended in HBSS (Sigma) at a concentration of 2 × 10⁷ cells/mL. A solution of 100 μL was injected into the second thoracic mammary fat pad of 6- to 7-week-old female mice as described elsewhere (26). Athymic nude mice [Hsd:Athymic Nude-Foxn1nu (nu/nu); Harlan Sprague-Dawley] were injected with MDA-MB-435 cells, and nonobese diabetic (NOD)/severe combined immunodeficient (SCID) mice (NOD.CB17-Prkdcscid/NCrHsd; Harlan Sprague-Dawley) were injected with MDA-MB-435 cells. Tumor volume (mm³) was determined using weekly caliper measurements in 2 perpendicular dimensions and calculated using the formula: \( volume = 0.52 \times (width)^2 \times (length) \). Mice were sacrificed 12 weeks post-injection, and tissues were collected for analysis.

To determine an in vivo dose of rHuEPO that would minimize adverse effects of polycythemia and VTE, mice were injected with either MDA-MB-231 or MDA-MB-435 cells as described above. Three weeks postinjection, control cohorts received weekly saline subcutaneous injections (n = 4), whereas other groups received rHuEPO [300 U/kg, 600 U/kg, or 1,200 U/kg (n = 4 per group)]. Blood was collected via weekly saphenous vein puncture and hemoglobin (Hb) levels were determined by colorimetric assay (BioPacific Diagnostics) at each time point. Assay results were validated at endpoint using a calibrated clinical LH780 hematology analyzer (Beckman Coulter) by determining Hb levels in blood obtained through cardiac puncture. Correlation between methods is shown in Supplementary Table S4.

To investigate any potential chemoprotective effects of rHuEPO, mice were injected with either MDA-MB-231 or MDA-MB-435 cells as noted above. Of 4 cohorts (n = 12 per group), the first group received weekly subcutaneous injections of saline, the second received 300 U/kg/wk
rHuEPO, the third received 10 mg/kg/wk paclitaxel, and the fourth received 300 U/kg/wk rHuEPO in combination with 10 mg/kg/wk paclitaxel. Treatments were initiated 3 weeks posttumor injection (rHuEPO) and 5 weeks post-injection (paclitaxel) when tumors reached their exponential growth phase.

At endpoint, lungs were collected and fixed in 10% neutral-buffered formalin and embedded in paraffin such that all 5 lung lobes could be sectioned in one plane. Four-micrometer thick sections (n = 2 per mouse) were subjected to standard hematoxylin and eosin staining. The entire cross-sectional area of the tissues was captured by light microscopy, and metastatic tumor burden and lesion size was quantified in a blinded manner using ImageJ software as previously described (26).

**Statistical analysis**

*In vitro* assays were conducted at least in triplicate. *In vivo* studies were carried out using multiple animals (n = 4–12 per group). All data were compiled for analysis. Statistical analysis was conducted using GraphPad Prism 4.0 Software using Student's t tests (between 2 groups) or ANOVA (between ≥3 groups) with Tukey's post hoc tests for normally distributed data and Dunn's post tests for nonnormal data. In all cases, differences were considered statistically significant when P ≤ 0.05.

**Results**

**EPOR mRNA and protein expression levels**

We first investigated the expression of EPOR and related molecules in a panel of normal and malignant cell lines using RT-PCR, immunoblotting, and flow cytometry. At the mRNA level, EPOR amplicons of 288 bp (murine) and 290 bp (human) were observed in all samples, with the highest intensities seen in the expected positive controls (mBM, UT7/EPO leukemia cells, and EPO-treated H838 lung cancer cells; ref. 27). We also investigated expression of Janus-activated kinase 2 (JAK2) as an important downstream tyrosine kinase in the EPO signaling cascade (28). With the exception of the H838 cell line, JAK2 mRNA was present in all cell lines tested at the expected sizes of 342 bp (murine) and 396 bp (human; Fig. 1A and Supplementary Fig. S1).

Western blot analysis using MAB307 (R&D Systems) revealed multiple bands, notably a band at approximately 75 kDa in the positive control (UT7/EPO) and another at approximately 55 to 60 kDa in both the UT7/EPO and mBM controls (Fig. 1B). The 75-kDa band was also observed in 3T3 and 4T1 murine cell lines. The 55- to 60-kDa band was additionally seen in untreated H838 and MDA-MB-231 samples but was absent in rHuEPO-treated samples of the same cell lines. Interestingly, when the anti-EPOR antibody was preincubated with a 2-fold excess of HuEPO, the 55- to 60-kDa band was no longer detected (Fig. 1C), indicating that MAB307 was specifically binding to EPOR at this size.

Flow cytometric analysis using the MAB307 antibody showed that UT7/EPO and H838 cells expressed cell surface EPO, whereas MDA-MB-435, MDA-MB-231, MDA-MB-468, and 4T1 cell lines produced negative results relative to the IgG isotype control (Fig. 1D). Pretreatment of cells with rHuEPO did not result in any change in EPO cell surface expression relative to untreated cells (data not shown).

To determine any potential autocrine effect, an ELISA assay was used to investigate the level of endogenous EPO secreted by breast cancer cell lines. None of the cell lines tested produced enough endogenous EPO to surpass the sensitivity threshold of the assay (1.6 U/L of EPO; dashed line, Fig. 1E).

**rHuEPO does not influence *in vitro* growth of human breast cancer cell lines**

We next evaluated the influence of rHuEPO on breast cancer cell function using *in vitro* assays that are representative of cell behaviors important to the metastatic process. Two human breast cancer cell lines were used, moderately metastatic MDA-MB-231 cells and highly metastatic MDA-MB-435 cells. These cell lines were chosen for further functional assays because of their differing metastatic abilities and their expression of EPOR at the mRNA and/or protein level.

In normal (2 dimensional) culture, we observed that doubling times were not significantly influenced by rHuEPO treatment for either cell line (Fig. 2A), nor was plating efficiency/colony formation (Fig. 2B). Assessment of anchorage-independent (3 dimensional) growth in soft agar also showed no significant difference in either the number (Fig. 2C) or diameter (Fig. 2D) of colonies formed (P > 0.05).

**rHuEPO exerts a partial protective effect on human breast cancer cells in response to specific *in vitro* cytotoxic treatments**

Given the conflicting literature on whether or not EPO can exert cell-protective effects on tumor cells (29), we investigated this question *in vitro* using clinically relevant treatment modalities in combination with rHuEPO. Following radiation therapy, a significant difference was seen in cell survival for both MDA-MB-231 and MDA-MB-435 cell lines (P < 0.005; Fig. 3A). Although irradiated MDA-MB-231 cells exhibited no significant difference in survival in the presence of rHuEPO (P = 0.06), irradiated MDA-MB-435 cells did show a small but significant increase in survival upon rHuEPO treatment (P = 0.05). Treatment with the commonly used chemotherapeutic paclitaxel (30) induced a significant decrease in survival relative to control for both MDA-MB-231 and MDA-MB-435 cell lines (P < 0.01; Fig. 3B). Treatment of both cell lines with rHuEPO in conjunction with paclitaxel had no significant protective effect on survival (P > 0.05). Under hypoxic conditions, we observed that both MDA-MB-231 and MDA-MB-435 cell survival significantly
decreased relative to untreated controls ($P < 0.01$) and that rHuEPO exerted no hypoxia-protective effect ($P > 0.05$; Fig. 3C).

**rHuEPO reduces the efficacy of chemotherapy on metastasis and enhances the inherent metastatic growth potential of human breast cancer cells**

Mimicking specific steps in metastasis in vitro allows for controlled experiments; however, only in vivo experiments allow us to evaluate the effects of rHuEPO on the entire process of breast cancer disease progression. Thus, we next examined the effect of rHuEPO on the tumorigenic and metastatic behavior of breast cancer cells in vivo.

Initial pilot dose-finding experiments were carried out in which MDA-MB-231 or MDA-MB-435 cells were injected into the mammary fat pad of NOD/SCID or nude mice, respectively ($n = 4$ per group). Three weeks postinjection, treatment with HuEPO was initiated using a dose range of rHuEPO (300, 600, or 1,200 U/kg; Supplementary Fig. S2). Doses were chosen to reflect the dose ranges used for clinical treatment of CIA (300–600 U/kg; ref. 31) and for tissue protection in experimental models (1,200 U/kg; ref. 2). rHuEPO had no significant influence on primary tumor growth regardless of dose level (Supplementary Fig. S2A and E). However, assessment of spontaneous metastasis to the lung

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**Figure 1. EPO-related molecular characteristics in cell lines.** UT7/EPO (human erythroleukemia cells), NIH 3T3 (3T3; mouse fibroblast cells), NCI-H838 (H838; human non–small cell lung cancer cells), MDA-MB-231, MDA-MB-435, MDA-MB-468 (231, 435, 468; human breast cancer cells), and 4T1 (mouse mammary cancer cells) were incubated in the absence or presence (*) of 10 U/mL rHuEPO for 5 minutes prior to sample collection.

A, RT-PCR analysis of EPOR and JAK2 mRNA expression in mBM and cell lines. B, Western blot analysis of EPOR protein levels in 30 μg of total protein using the anti-EPOR antibody, MAB307 (R&D Systems). Expected size of EPOR is between 52 and 60 kDa (19). C, the specificity of the MAB307 antibody was determined by carrying out an overnight blocking step using a 2-fold excess concentration of rHuEPOR (R&D Systems). D, flow cytometric analysis of UT7/EPO, NCI-H838, MDA-MB-435, MDA-MB-231, MDA-MB-468, and 4T1 cell lines for EPOR cell surface protein expression using the MAB307 primary antibody and a phycoerythrin (PE)-conjugated secondary antibody (black profiles). An IgG isotype control (white profiles) indicates the baseline fluorescence levels for each cell line. E, ELISA-based assessment of cell-secreted endogenous EPO levels for the UT7/EPO, MDA-MB-435, MDA-MB-231, MDA-MB-468, and 4T1 cell lines. The dashed line indicates the minimum sensitivity of the assay; all results below the line are considered negative. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Supplementary Fig. S2B–D and F–H) suggested a potential metastasis-promoting effect of rHuEPO, particularly with regard to the metastatic lesion size ($P = 0.043$) and lesion number ($P = 0.002$) in mice injected with MDA-MB-231 cells and treated with 300 U/kg rHuEPO (Supplementary Fig. S2C and D). Hb was monitored weekly to determine whether dosing at these levels would elevate Hb and/or cause VTE. All groups showed a dose-dependent increase in mean Hb after 9 weeks of treatment (Supplementary Table S4) and no unexplained morbidity or mortality was observed. On the basis of these results and the fact that a dose of 300 U/kg is within the clinical dose range used to treat CIA in cancer patients (31), 300 U/kg was chosen for the subsequent combination treatment experiments.

In NOD/SCID mice injected with MDA-MB-231 human breast cancer cells, we observed that rHuEPO treatment alone significantly increased the mean primary tumor volume at endpoint relative to untreated mice ($P < 0.001$; Fig. 4A). However, when MDA-MB-435 human breast cancer cells were injected into nude mice, no significant difference was observed in primary tumor volume between the 4 treatment groups (Fig. 4B). In both cell line models, the tumor growth rate over time was not significantly different between groups. In addition, rHuEPO did not exert any chemoprotective effect on the primary tumor volume in vivo at any time point ($P > 0.05$).

Having established the effects of rHuEPO in the presence or absence of chemotherapy on the primary tumor, we next investigated the influence of rHuEPO on spontaneous metastasis to the lung. We first assessed the incidence of lung metastases (number of mice per group showing histologic metastases). With the exception of mice injected with MDA-MB-231 cells and treated with paclitaxel (who developed no metastases; $P < 0.005$), there was no significant difference in metastatic incidence between treatment groups (Fig. 5A and D). However, strikingly, in both cell line models, we observed that rHuEPO treatment combined with paclitaxel resulted in a significant increase in lung metastasis size relative to paclitaxel treatment alone ($P < 0.001$; Fig. 5C and F), suggesting a chemoprotective effect at the metastatic site. With regard to lung tumor burden (percentage of lung area occupied by tumor), it was observed that mice injected with MDA-MB-231 cells and treated with rHuEPO in combination with paclitaxel had a significant

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Figure 2. Effect of rHuEPO on in vitro growth characteristics of MDA-MB-231 (231) and MDA-MB-435 (435) human breast cancer cells. Cells were cultured in the absence (white bars) or presence (black bars) of rHuEPO (10 U/mL) for the duration of the assays. A, doubling time of cells grown under normal anchorage-dependent (2 dimensional) culture conditions. A total of $5.0 \times 10^6$ cells per 60-mm dish ($n = 3$ per time point) were cultured and counted by hemocytometer every 48 hours for 14 days. B, plating efficiency of cells in anchorage-dependent (2 dimensional) culture. A total of 100 cells per 60-mm dish ($n = 3$) were plated and allowed to grow for 2 weeks. Colonies were stained and counted as described in the Materials and Methods section. Data are expressed as a percentage of expected (100) colonies formed. C and D, anchorage-independent (3 dimensional) growth. Cells ($4 \times 10^5$ cells per 60-mm plate, $n = 4$ plates per treatment) were grown in soft agar (0.3%) for 3 to 4 weeks. Plates were scanned at 100X magnification and 5 fields of view (FOV) were counted for each dish. C, average number of colonies per FOV. D, average diameter of colonies ($\mu$m). All data are presented as the mean ± SEM. No statistical differences were observed in any of the assays ($P > 0.05$).
increase in lung tumor burden relative to mice treated with paclitaxel alone ($P < 0.001$), with a similar trend observed in mice injected with MDA-MB-435 cells (Fig. 5B and E).

**Discussion**

Although EPO and EPOR have a well-established functional role in erythropoiesis and tissue protection, their influence on tumor progression remains controversial (2). EPOR has been found to be expressed in several normal and malignant cell lines, various normal tissues, and many types of cancer (17). However, conflicting reports have shown the nonspecificity of many anti-EPOR antibodies, raising questions about the validity of some of these expression studies (18). In addition, in vitro and in vivo studies using tumor cell lines have yielded conflicting reports with regard to the influence of ESAs on tumor cell behavior and the potential association with risk versus benefit of using ESAs to treat clinical cancer-related anemia (22–25).

From a clinical perspective, multiple trials have been carried out to evaluate the effect of ESAs on patients with breast cancer (5, 10–13, 15, 32, 33), although only...
In light of unanswered questions about the safety of ESAs and the limitations of previous published experimental studies, there remained a need for a well-controlled and comprehensive assessment of the functional influence that rHuEPO has on cancer disease progression using clinically relevant model systems (17). The goal of this study was therefore to test the hypothesis that rHuEPO can influence metastatic cell behavior and/or the metastatic process in preclinical models of breast cancer. We first examined tumor cell lines for EPOR using multiple approaches, then assessed the effects of rHuEPO on malignant cell behaviors in vitro, and finally used clinically relevant in vivo models of breast cancer disease progression to determine the role of rHuEPO in metastasis.

Our in vitro results indicate that determining protein expression of EPOR by Western blot and flow cytometric analyses varies considerably depending on the antibody used. All 3 commonly used metastatic breast cancer cell lines that we assessed (MDA-MB-231, MDA-MB-435, and MDA-MB-468) expressed EPOR mRNA, although at much lower levels than known EPOR-positive cell lines. In addition, all breast cancer cell lines expressed JAK2, an important downstream signaling component of the EPO/EPOR pathway (34). However, analysis of EPOR expression by immunoblotting and flow cytometry in the same panel of breast cancer cell lines showed discordance between mRNA and protein expression, with only MDA-MB-231 cells expressing EPOR protein (as assessed by immunoblotting) and no cell line showing positive cell surface expression of EPOR (as assessed by flow cytometry). Our results are in agreement with a recent study by Swift and colleagues that showed little expression of EPOR in 66 commonly used cell lines from various tumor types (35). Our evaluation of 4 anti-human EPOR antibodies found varying results between antibodies, leading us to have concerns about the specificity of these antibodies also raised by other studies (18, 25, 36). Our results also highlight the need for positive and negative controls when assessing EPOR expression. UT7/EPO and H838 cell lines were used as positive controls for PCR, immunoblotting, and flow cytometric experiments (27). These cell lines revealed a positive mRNA signal, an appropriately sized band in Western blot (analysis which disappeared with pretreatment with rHuEPO), and positive cell surface expression by flow cytometry. With a known EPOR-positive cell line, H838, we observed discordant results with mRNA expression absence in untreated cells but protein present by Western blot and the converse seen when H838 cells were treated with rHuEPO. The only breast cancer cell line to show EPOR protein expression was also positive for EPOR mRNA, however, both signals disappeared upon treatment with rHuEPO (because of rapid internalization of the receptor). Having used multiple controls (blocking human EPOR antibodies found varying results between antibodies, leading us to have concerns about the specificity of these antibodies also raised by other studies (18, 25, 36). Our results also highlight the need for positive and negative controls when assessing EPOR expression. UT7/EPO and H838 cell lines were used as positive controls for PCR, immunoblotting, and flow cytometric experiments (27). These cell lines revealed a positive mRNA signal, an appropriately sized band in Western blot (analysis which disappeared with pretreatment with rHuEPO), and positive cell surface expression by flow cytometry. With a known EPOR-positive cell line, H838, we observed discordant results with mRNA expression absence in untreated cells but protein present by Western blot and the converse seen when H838 cells were treated with rHuEPO. The only breast cancer cell line to show EPOR protein expression was also positive for EPOR mRNA, however, both signals disappeared upon treatment with rHuEPO (because of rapid internalization of the receptor). Having used multiple controls (blocking with rHuEPO, UT7 cells, mBM, H838, and 3T3 cells) to determine specificity to EPOR, these discordant results suggest that MAB307 may have limited specificity to EPOR. The limitations may be due to the EPOR protein

2 of these published studies (BRAVE and BEST) have been limited to patients with metastatic disease (10, 11). However, these studies contradict each other in their findings, with the BRAVE study detecting no difference in overall survival between treatment arms (10), whereas the BEST study was ended early because of a higher mortality rate in the rHuEPO-treated versus placebo arm (11). Taken together, these studies raise the question of whether there is an underlying EPO–tumor cell interaction that may lead to disease progression. However, previous preclinical studies have limited their investigations to the effect of rHuEPO on the primary tumor or on cell lines in vitro and have not investigated the role of rHuEPO in the setting of metastatic disease.
being differently posttranslationally modified, thus obscuring the protein to the monoclonal antibody resulting in the discordant results seen in Figure 1A–D. Taken together, we believe that the MAB307 anti-EPOR antibody that we used in our final expression studies may show variable results and lack specifically in detecting all variants of human EPOR. To accurately determine EPOR protein expression, new antibodies must be generated.

Figure 5. Effect of rHuEPO on in vivo metastatic capability of MDA-MB-231 and MDA-MB-435 human breast cancer cells. Cells (2 × 10^6) were injected into the right thoracic mammary fat pad of 6- to 7-week-old female NOD/SCID (MDA-MB-231 cells) or nude (nu/nu; MDA-MB-435 cells) mice (n = 12 per group) and allowed to grow for 12 weeks. Mice were then sacrificed and assessed by histopathology for metastasis to distant organs. A–C, NOD/SCID mice injected with MDA-MB-231 cells. D–F, nude mice injected with MDA-MB-435 cells. A and D, incidence of lung metastasis (percentage of mice in each treatment group that developed metastases). C and F, size (diameter) of lung metastases scored. Data are presented as the mean ± SEM. B and E, lung metastatic tumor burden (percentage of total lung volume occupied by tumor). Data are presented as the mean ± SEM. #, a statistically significant difference relative to the untreated control cohort (P < 0.01); *, a statistically significant difference relative to the paclitaxel-only treatment cohort (P < 0.001).
that have been rigorously tested and accurately and sensitively detect EPOR such as in the work described by Elliott and colleagues (21).

To determine whether even very low expression of EPOR could influence tumor cell ability to complete individual steps in the metastatic process, we carried out in vitro assays mimicking various steps in metastasis in the presence or absence of rHuEPO, including the initiation and maintenance of growth and survival under stress. Two- and 3-dimensional growth in the presence or absence of rHuEPO was unaffected, indicating that rHuEPO does not directly increase the growth of either MDA-MB-231 or MDA-MB-435 cells. However, direct effects have been seen with breast cancer cells that express functional surface EPOR as seen in the work by Shi and colleagues (37). Because recent clinical studies have shown decreased survival of patients treated with rHuEPO when treated concurrently with chemotherapy or radiotherapy (11, 38), we decided to investigate whether in vitro dosing of rHuEPO could increase tumor cell survival after cytotoxic insult. Our results indicate that, with the exception of a small protective effect on 1 cell line (MDA-MB-435) treated with radiation, rHuEPO treatment alone or combined with cytotoxic therapy does not cause increased cell survival. Previous studies have shown mixed results with regard to tumor cell proliferation, chemoprotection, and/or treatment resistance in response to EPO (39–42).

In line with the results of our study, work by Liu and colleagues showed no chemoprotective effect of EPO (40), whereas studies by Belenkov and colleagues showed a survival benefit with EPO in combination with radiotherapy (41) and Liang and colleagues showed unfavorable drug interactions between EPO and trastuzumab (42).

Although mimicking specific steps in metastasis in vitro allows for controlled experiments, there are 2 main drawbacks to these types of assays. First, assays are often carried out using individual immortalized cancer cell lines which may or may not have the same characteristics of heterogeneous clinical disease. Second, and more importantly, in vitro studies are limited to studying the effect of a factor on tumor cells alone, rather than being able to also take into account the influence of the tumor microenvironment. Interactions between cancer cells and their immediate microenvironment as well as the organ microenvironment in distant metastatic sites have been shown to be critically important for influencing metastatic behavior (43). Thus, the complexity of the metastatic process is such that the only way to comprehensively study metastasis is to use in vivo model systems.

For these reasons, we conducted in vivo assays using 2 different human breast cancer cell lines (MDA-MB-231 and MDA-MB-435) of differing genetic backgrounds, metastatic abilities, and EPOR protein expression together with a clinically relevant mouse model in which breast cancer cells can be injected orthotopically into the mammary fat pad and allowed to spontaneously metastasize to distant organs (44). In striking contrast to our in vitro findings, our in vivo results indicate that rHuEPO can reduce the efficacy of chemotherapy in the metastatic setting, and in some cases, enhance the inherent metastatic growth potential of human breast cancer cells. Previously, rHuEPO has been shown to increase primary tumor growth of Lewis lung carcinoma cells (cells lacking demonstrable cell surface EPOR; ref. 45); however, in other studies investigating different types of tumors, no growth-enhancing effect of rHuEPO was seen (46–49). Increased chemotherapeutic response of the primary tumor has been shown (46, 48) and in one case, an in vivo increase in radiosensitivity in response to rHuEPO was observed (47). Additional recent work by Liang and colleagues has shown an EPO-mediated reversal of the effects of the anti-HER2 therapy trastuzumab on primary tumor growth, indicating that the signaling pathways inhibited by trastuzumab (PI3K/Akt and extracellular signal–regulated kinase) may be in turn disrupted by rHuEPO (42). However, none of these studies evaluated metastasis, and in many cases, doses of rHuEPO were considerably higher [from 1,000 U/kg biweekly (ref. 46) to 1,000 U/kg 3 times a week (ref. 47) than the normal clinical dose range of 300 to 600 U/kg used in cancer patients (31).

Interestingly, in this study, we observed that the growth-promoting influence of rHuEPO and chemoprotective effects at the metastatic site were more pronounced in mice injected with the moderately metastatic MDA-MB-231 cell line which expressed EPOR protein. However, mice injected with the highly metastatic MDA-MB-435 (which did not express detectable levels of EPOR protein) also showed additional enhancement of metastatic growth in the presence of rHuEPO alone, suggesting that the influence of rHuEPO may either act directly on tumor cells through both EPOR-dependent and -independent pathways. However, taken together with the observed minimal in vitro effect of rHuEPO on breast cancer cell behavior, we believe another more likely explanation is that in our model rHuEPO is acting via EPOR-related signaling directly on host microenvironmental cells, affecting their functionality and, in turn, promoting tumor cell survival. This could involve rHuEPO binding to its receptor on microenvironmental cells and initiating signaling that results in numerous host effects including vasculogenesis, increased matrix metalloproteinase production, endothelial progenitor mobilization, and increased thrombosis (50, 51). Importantly, these parameters are not usually monitored clinically after rHuEPO is given (31), although studies have shown that activation of these processes can also enhance metastasis (52). In the case of thrombosis, an increase in the formation of microthrombi may increase the metastatic burden (53) and could account for the results seen in this study, although this was not investigated because of technical challenges associated with accurately assessing coagulation parameters in mice (53). Interestingly, multiple rHuEPO trials have reported increases in thrombotic events in the EPO arm (6, 8, 11, 54), and taken together with our preclinical data showing effects on...
metastasis without any observed direct effects on cancer cells, it is possible that this may be a factor leading to disease progression and decreased patient survival.

In summary, there have been a large number of trials investigating ESAs for the treatment of CIA in cancer patients (5, 10–15, 32). In several trials, an increase in thrombotic events was seen in the ESA arm (6, 8, 11, 54). More importantly, meta-analyses show a decrease in overall survival in the ESA arm of some studies (6, 8). This has prompted concern about the use of rHuEPO in cancer patients with anemia because, although the cause of this decreased survival is unknown, it has been speculated that the administration of rHuEPO may cause cancer progression (8, 10). This preclinical study aimed to shed some light on the observed clinical effect of rHuEPO by investigating the expression and function of EPO in preclinical model systems of human breast cancer metastasis. Our novel findings indicate that rHuEPO can reduce the efficacy of chemotherapy in the metastatic setting, and in some cases, enhance the inherent metastatic growth potential of human breast cancer cells, potentially because of indirect effects of HuEPO on the host rather than just direct effects on tumor cells. These studies highlight the importance of using clinically relevant in vivo models to investigate disease progression and may help to explain the underlying functional cause of the observed EPO-related adverse events and decreased survival in ESA-treated metastatic breast cancer patients undergoing chemotherapy.

Disclosure of Potential Conflicts of Interest

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Benjamin D. Hedley, Jenny E. Chu, D. George Ormond, et al.