Erythropoietin Promotes MCF-7 Breast Cancer Cell Migration by an ERK/Mitogen-activated Protein Kinase-dependent Pathway and Is Primarily Responsible for the Increase in Migration Observed in Hypoxia

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Recent studies indicate that cancer cells express erythropoietin receptor (EpoR). In this study, we have shown that erythropoietin (Epo) activates the mitogen-activated protein kinase, extracellular signal-regulated kinase (ERK), and promotes migration in MCF-7 breast cancer cells. Epo-stimulated MCF-7 cell migration was blocked by the MEK inhibitor PD098059 and by dominant negative MEK-1, indicating an essential role for ERK. When MCF-7 cells were exposed to hypoxia (1.0% O2) for 3 h, the Epo mRNA level increased 2.4 ± 0.5-fold, the basal level of ERK activation increased, and cell migration increased 2.0 ± 0.1-fold. Soluble EpoR and Epo-neutralizing antibody significantly inhibited hypoxia-induced MCF-7 cell migration, suggesting a major role for autocrine EpoR cell signaling. MCF-7 cell migration under hypoxic conditions was also inhibited by PD098059. These experiments identify a novel pathway by which exogenously administered Epo, and Epo that is produced locally by cancer cells under hypoxic conditions, may stimulate cancer cell migration.

Erythropoietin (Epo) is a 34-kDa member of the class I cytokine family that initiates cell signaling by forming a trimeric complex with two molecules of the Epo receptor (EpoR) (1). Epo binding induces a conformational change in EpoR so that receptor-associated Janus kinase-2 is activated (2). This leads to phosphorylation of tyrosine residues in EpoR and recruitment of Src homology 2 domain-containing proteins. Signaling proteins activated downstream of EpoR and Janus kinase-2 include phosphatidylinositol 3-kinase, protein kinase C, and the mitogen-activated protein kinase, extracellular signal-regulated kinase (ERK) (3, 4).

In adults, Epo is produced mainly by peritubular fibroblasts in the kidney; however, diverse cells in multiple organs also express Epo (5). Epo expression is stimulated by hypoxia as a result of activation of the transcription factor, hypoxia-inducible factor-1 (HIF-1α) (6). Epo is well characterized for its ability to promote proliferation and differentiation of erythroid progenitor cells and also inhibit apoptosis in these cells (7); however, Epo is active outside the hematopoietic system as well. In the nervous system, Epo inhibits neuronal apoptosis resulting from hypoxia or ischemia (8–10) and stimulates Schwann cell proliferation (11). Epo also regulates angiogenesis by multiple mechanisms, including stimulation of endothelial cell proliferation and migration (12).

The function of Epo in cancer is not well understood. EpoR is expressed by several human tumor types, including breast carcinoma, renal cell carcinoma, melanoma, endometrial carcinoma, and gastric carcinoma (13–17). In breast cancer, Epo is observed at high levels by immunohistochemistry, whereas normal mammary tissue is typically EpoR negative (18). Many tumors are also strongly Epo positive (13). Furthermore, in at least one study, Epo and EpoR were observed at increased levels in hypoxic regions of breast cancers (13).

Lai et al. (19) demonstrated increased Matrigel invasion by squamous cell carcinoma cells after treatment with Epo and attributed this increase to activation of the Janus kinase-2–STAT signaling pathway. In a separate study, reagents that inhibit Epo binding to EpoR, or that block Janus kinase-2 signaling, reduced growth of breast cancer cells in tumor Z-chambers implanted subcutaneously in rats (20). Cancer cells may be exposed to Epo in patients not only due to local synthesis but also as a result of Epo therapy, which is used to treat anemia secondary to chemotherapy and radiation therapy (21). Possible problems associated with Epo therapy in cancer were highlighted by a recent clinical trial in which patients with metastatic breast cancer received chemotherapy, with or without Epo. The trial was terminated early because of increased mortality in the Epo-treated patients (22).

In this study, we have demonstrated that Epo activates ERK in MCF-7 breast cancer cells in vitro. Epo also promotes MCF-7 cell migration, and this effect is ERK dependent. Under hypoxic conditions, the Epo mRNA level and the basal level of ERK activation are increased. These changes are accompanied by an increase in the rate of MCF-7 cell migration, which is significantly inhibited by Epo-neutralizing antibody, soluble EpoR, and by the MEK inhibitor, PD098059. Our results demonstrate that Epo may promote breast cancer cell motility by activating ERK under normoxic or hypoxic conditions. In hypoxia, EpoR autocrine signaling assumes a dominant role and determines the basal level of MCF-7 cell migration.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Recombinant human Epo (287-TC), recombinant human EpoR (963ER), and Epo-neutralizing antibody 287 were obtained from R&D Systems, Inc. (Minneapolis, MN). Phospho-Epo-specific antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific for phosphorylated ERK and total EK were from Cell Signaling Technology (Beverly, MA) and Upstate Biotechnology (Waltham, MA), respectively. The MEK inhibitor,
Erythropoietin Promotes Breast Cancer Cell Migration

PD098059, and the Janus kinase-2 inhibitor, AG490, were from EMD Biosciences (San Diego, CA). Non-specific murine IgG was from Sigma.

Cell Culture and Transfection Methods—Low passage MCF-7 cells were kindly provided by Dr. Sarah J. Parsons (University of Virginia, Charlottesville, VA) and cultured in RPMI (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO2. The expression construct encoding dominant negative MEK1 (S217→A) (dn-MEK1) in pBABE is previously described (23). In some experiments, MCF-7 cells (105) were co-transfected with dn-MEK1 (3.2 μg) and pEGFP (0.8 μg) using FuGENE 6 (Roche Applied Science). Transfection efficiencies were 20–30% as determined by fluorescence microscopy to detect green fluorescent protein (GFP). Co-transfection efficiencies were determined as previously described (24) and were nearly 100%.

Immunoblot Analysis of ERK Activation—MCF-7 cells were seeded into 6-well plates (3 × 10⁵ cells/well) and cultured in phenol red-free, serum-free medium for 4 h before treatment with Epo (1.7 nM) for up to 120 min. In some experiments, the Janus kinase-2 inhibitor, AG490 (50 μM), or vehicle was added to cultures for 2 h prior to adding Epo for 15 min. Cell extracts were prepared in 50 mM Hepes, 100 mM NaCl, 2 mM EDTA, and 1% (v/v) Triton X-100, pH 7.5, containing protease inhibitor mixture and 1 mM sodium orthovanadate. To assess the effects of hypoxia on the basal level of MCF-7 cell ERK activation, cells were transferred to an incubator equilibrated at 1% O2 for 3 h or kept under normoxic conditions as a control. The protein concentration in each extract was determined by bicinchoninic acid assay (Sigma). Equal amounts of cell extracts were subjected to SDS-PAGE on 10% slabs, electrotransferred to polyvinylidene difluoride membranes (Bio-Rad), and probed with antibodies specific for phosphorylated ERK and total ERK.

EpoR Phosphorylation—MCF-7 cells (5 × 10⁵) were seeded into 6-well plates and cultured in phenol red-free, serum-free medium for 4 h prior to treatment with Epo (1.7 nM) for 1 min. Cell extracts were prepared in radioimmune precipitation assay buffer containing 0.1% SDS, 0.5% sodium deoxycholate, 1% (v/v) Triton X-100, protease inhibitor mixture, and 1 mM sodium orthovanadate. Equal amounts of cell extracts were subjected to immunoblot analysis, using phospho-EpoR-specific antibody. Membranes were reprobed for total ERK as a loading control.

Cell Migration Assays—Migration of MCF-7 cells was studied using 6.5-mm Transwell chambers with 8-μm pores (Corning Costar, Corning, NY) as previously described (25). The bottom surface of each membrane was coated with 20% fetal bovine serum for 2 h. Under these conditions, the major protein that adsorbs to the membrane is vitronectin (26). MCF-7 cells in serum-free, phenol red-free medium were transferred to an incubator equilibrated at 1% O2 for 3 h or kept under normoxic conditions as a control. The protein concentration in each extract was determined by bicinchoninic acid assay (Sigma). Equal amounts of cell extracts were subjected to SDS-PAGE and immunoblot analysis to detect phosphorylated EpoR and, as a control for load, total ERK.

RESULTS

Epo Activates ERK in Breast Cancer Cells—MCF-7 breast cancer cells typically demonstrate limited motility; however, agents that have been implicated in breast cancer progression, including urokinase-type plasminogen activator (uPA), epidermal growth factor, and heregulin activate ERK in MCF-7 cells and promote MCF-7 cell migration (25, 27, 28). MCF-7 cells express EpoR (29). Thus, to begin, we examined cell signaling in response to exogenously added Epo in MCF-7 cells. Phosphorylated EpoR was increased within 1 min of exposure to Epo (1.7 nM), as determined by immunoblot analysis (Fig. 1A). Within 2 min, ERK also was phosphorylated (Fig. 1B). Both isoforms of ERK (p42 and p44) were observed with phospho-ERK-specific antibody. The largest signal was observed at 15–60 min; however, ERK activation was transient. Phosphorylated ERK returned to baseline by 120 min. The Janus kinase-2 inhibitor, AG490, inhibited ERK phosphorylation in response to Epo in MCF-7 cells by 63 ± 12% (p < 0.05, n = 5). These results suggest that ERK activation occurs, in part, downstream of Janus kinase-2 (Fig. 1C). Equivalent results were reported previously by us in studies with primary Schwann cell cultures (11).
Erythropoietin Promotes Breast Cancer Cell Migration

**Epo Promotes Cell Migration**—MCF-7 cells that were treated with 1.7 nM Epo migrated across serum-coated Transwell membranes at a rate that was increased by 2.1 ± 0.1-fold (p < 0.005, n = 7) compared with untreated cells (Fig. 2A). We previously reported a similar increase in MCF-7 cell migration in response to uPA (25). The effects of uPA on MCF-7 cell migration reflected activation of ERK and downstream of ERK, myosin light chain kinase (24, 25).

To test the role of ERK in Epo-stimulated MCF-7 cell migration, we treated cells with the highly specific MEK inhibitor, PD098059. In the absence of Epo, PD098059 had no effect on MCF-7 cell migration (Fig. 2A); however, PD098059 inhibited the Epo-induced increase in migration by 80 ± 10% (p < 0.005, n = 7).

To confirm the role of ERK as a critical effector of Epo-stimulated MCF-7 cell migration, cells were transfected to express dn-MEK1 and GFP or GFP alone. Cell migration was studied using Biocat cell culture inserts, which allowed selective monitoring of fluorescent cells. As shown in Fig. 2B, dn-MEK1 had no effect on the basal level of MCF-7 cell migration but inhibited the pro-migratory activity of Epo by 90 ± 18% (p < 0.05, n = 9), confirming the results obtained with PD098059.

The K<sub>D</sub> for Epo binding to EpoR dimer is ~0.1 nM in erythrocyte precursors (30), although binding affinities for Epo are different in various cell types. In concentration dependence experiments, 13 pm Epo was sufficient to significantly increase MCF-7 cell migration (Fig. 2C); however, the curve was bimodal. Epo concentrations exceeding 10 nM were less efficacious. The reason for the bimodal response is unclear; however, similar results have been observed with uPA receptor-derived chemokinetic peptides, which also stimulate MCF-7 cell migration by activating ERK (31).

**Hypoxia Activates ERK and Promotes MCF-7 Cell Migration**—When MCF-7 cells were exposed to 1.0% O<sub>2</sub> for 3 h, Epo mRNA increased 2.4 ± 0.5-fold, as determined by real-time qPCR (p < 0.05, n = 9) (Fig. 3A). A modest but statistically significant increase in the basal level of phosphorylated ERK was also observed (p < 0.005, n = 6) (Fig. 3B). Based on these results, we examined the effects of hypoxia on MCF-7 cell migration.

MCF-7 cells were added to Transwell chambers, which were immediately transferred to a 1.0% O<sub>2</sub> environment. Migration was measured 6 h later. Fig. 4 shows that cell migration was increased by 2.0 ± 0.1-fold (p < 0.001, n = 9) in the low oxygen atmosphere compared with cells that were maintained under normoxic conditions. The increase in cell migration was inhibited by >70% by PD098059 (p < 0.001, n = 11), again suggesting an essential role for ERK.

The hypoxia-induced increase in endogenously produced Epo suggested that autocrine EpoR signaling may be responsible for the increase in the basal level of MCF-7 cell ERK activation and migration in 1% O<sub>2</sub>. Hypoxia also has been reported to increase autocrine signaling through other receptors capable of activating ERK and promoting migration in cancer cells, including uPA receptor and c-Met (32, 33). To test the role of autocrine EpoR signaling, MCF-7 cell migration was studied in 1% O<sub>2</sub>. Hypoxia also has been reported to increase autocrine signaling through other receptors capable of activating ERK and promoting migration in cancer cells, including uPA receptor and c-Met (32, 33). To test the role of autocrine EpoR signaling, MCF-7 cell migration was studied in 1% O<sub>2</sub> in the presence of soluble EpoR (2.5 μg/ml). The increase in cell migration associated with hypoxia was inhibited by 53 ± 5% (p < 0.005, n = 9) (Fig. 5). A number of experiments were performed with higher concentrations of EpoR (up to 8 μg/ml); however, the degree of inhibition was not increased. Soluble EpoR had no effect on MCF-7 cell migration under normoxic conditions.

Epo-neutralizing antibody also blocked the hypoxia-induced increase in MCF-7 cell migration. In this case, the degree of inhibition was 98 ± 18% (p < 0.005, n = 5). Epo-neutralizing antibody did not affect MCF-7 cell migration under normoxic conditions. Furthermore, nonspecific
hypoxia, these processes are inhibited and HIF-1
PO2 used in cell culture (150 torr) (34). There is considerable evidence
the nucleus where it is available to dimerize with HIF-1
exposed to 1.0% O2, as reported here and elsewhere (29).

In this study, we show that Epo has similar effects on MCF-7 cells. Under normoxic conditions, exogenously added Epo activated ERK and promoted cell migration by a pathway that was dependent on ERK, as demonstrated in experiments with PD098059 and dn-MEK1. ERK activation by Epo was transient (<2 h); however, the response was actually sustained for a longer time than what was previously observed with uPA (25).

Under hypoxic conditions, the basal level of MCF-7 cell ERK activation and cell migration was increased, suggesting the establishment of autocrine signaling pathways that support these processes. To identify the responsible autocrine signaling pathway, it was necessary to consider a number of receptor systems. Hypoxia increases EpoR expression in cancer cells (32). Furthermore, the Met tyrosine kinase is up-regulated and activated in cancer (33). Nevertheless, our results with Epo-neutralizing antibody indicate that EpoR autocrine signaling plays a principal role in stimulating MCF-7 cell migration in hypoxia. Supporting results were obtained in experiments with soluble EpoR. The degree of inhibition of cell migration achieved with soluble EpoR was >50% but still less than that observed with Epo-neutralizing antibody. The reason for this is unclear but under investigation.

From these studies, a model emerges in which multiple extracellular factors contribute to activation of common signaling pathways involved in cancer cell migration and perhaps other processes that support cancer cell invasion and metastasis. Our results demonstrate that Epo is a potentially important signal for promoting cell migration. The relative importance of Epo, compared with other factors such as uPA and EGF receptor ligands, may depend on the PO2 and thus vary within different locations of a single tumor.

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Erythropoietin Promotes Breast Cancer Cell Migration

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