

Erythropoietin and Erythropoietin Receptor Expression in Human Cancer¹

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Abstract

Erythropoietin (EPO) stimulates the growth of erythroblasts in the bone marrow (C. Lacombe and P. Mayeux, *Nephrol. Dial. Transplant.*, 14 [Suppl. 2]: 22–28, 1999). We report basal and hypoxia-stimulated expression of EPO and its receptor, EPOR, in human breast cancer cells, and we demonstrate EPO-stimulated tyrosine phosphorylation and the proliferation of these cells *in vitro*. In 50 clinical specimens of breast carcinoma, we report high levels of EPO and EPOR associated with malignant cells and tumor vasculature but not with normal breast, benign papilloma, or fibrocystic tissue. Hypoxic tumor regions display the highest levels of EPO and EPOR expression. Enhanced EPO signaling may contribute to the promotion of human cancer by tissue hypoxia.

Introduction

EPO,⁴ a glycoprotein hormone normally produced by the kidney and fetal liver, acts via EPORs to stimulate growth, prevent apoptosis, and induce differentiation of RBC precursors (1). Expression of EPO and EPOR has recently been demonstrated in several nonhematopoietic tissues (2–4), which suggests broader roles for EPO signaling in regulating cell growth, cell survival, and angiogenesis (4, 5). Because autonomous EPO expression can mediate autocrine growth of EPOR-bearing erythrocytic leukemia cells (6), expression of EPO and EPOR by tumors of nonhematopoietic tissues may also stimulate cancer. EPOR is a member of the cytokine receptor family. Although EPOR has no intrinsic kinase activity, it binds and activates intracellular tyrosine kinases to elicit its mitogenic signals (1, 7). EPOR expression has occasionally been observed in cancers arising from the kidney (8), and because this tissue normally produces EPO, a potential paracrine or autocrine role for EPO signaling in promoting the growth of renal cancers has recently been suggested (8). Brain (2), breast (3), and female genital tract tissues (9) have recently been shown to express EPO and EPOR. To investigate whether EPO signaling may play a role in the development and progression of malignancies arising from these tissues, we determined whether EPOR was expressed in human breast cancer cell lines in culture and in biopsies of breast carcinoma.

Materials and Methods

Cell Culture and Treatments. All culture media were purchased from Life Technologies, Inc. (Rockville, MD). All of the cell lines were obtained from American Type Culture Collection (ATCC; Manassas, VA) and cultured

according to ATCC directions. Hypoxic treatment of cells was performed in an enclosed chamber (Billups-Rothenberg Inc., Del Mar, CA) flushed with pre-mixed gas mixture (1% O₂, 5% CO₂, 94% N₂) for the times indicated. Stimulation of tyrosine phosphorylation was performed by treating cells in phenol-free DMEM with rhEPO (250 units/ml; R&D Systems, Minneapolis, MN) or the cytotrophic EPO mimetic peptide AEHCSLNENITVPDTKV (5) for 5 min. For DNA synthesis and cell proliferation experiments, cells plated in six-well plates in DMEM with 20% FBS for 16 h were switched to 1 ml of phenol-free DMEM at the time of treatments. rhEPO (10 units) was added directly to this medium, and the cells were cultured for another 5 days. Thymidine labeling was performed by adding 1 μCi of [³H]thymidine (20 mCi/ml; NEN Life Science Products, Inc., Boston, MA) per well for 1 h; DNA-associated counts were then determined by liquid scintillation as described previously (8). For cell proliferation studies, the vital dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the medium after the 5-day rhEPO treatment, and cell viability was determined 4 h later by measuring reduced MTT absorbance after its extraction.

RT-PCR and Sequencing. Measurements of EPO and EPOR mRNA were performed as described previously (10). RNA was isolated using the Qiagen RNA isolation kit (Qiagen, Inc., Valencia, CA), and the two-step GeneAmp RT-PCR kit (PE-Applied Biosystems, Foster City, CA) was used for RT-PCR. Approximately 2 μg of RNA and 10 μmol of both the forward and reverse primers were used for each sample. The PCR cycle protocol for the EPO and EPOR primers is 35 cycles at 94°C for 1 min, at 56°C for 1 min, and at 72°C for 2 min. The RT-PCR products were then run on a 2% agarose gel. The DNA standard used was the 100-bp DNA ladder from FMC Bioproducts (BioWhittaker Molecular Applications, Rockland, ME). The 449-bp RT-PCR product was purified with Qiagen's PCR purification kit and was sequenced using ABI Prism.

Western Blotting. Cell lysates or clinical biopsy samples were normalized for protein, subjected to SDS-PAGE, transferred to nitrocellulose, and stained with polyclonal anti-EPOR antibody (C20, affinity purified antibody, 1:1500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or EPO antibody (rabbit polyclonal, H-162; Santa Cruz Biotechnology) in the presence and absence of blocking peptide (10:1 peptide:antibody ratio; Santa Cruz Biotechnology) or rhEPO (250 units/ml). Secondary antibody was goat antirabbit antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ). Immunoreactive bands (*M_r* 66,000 for EPOR and 34,000 for EPO) were visualized using chemiluminescence (SuperSignal WestPico Chemiluminescence kit; Pierce, Rockford, IL). A horseradish peroxidase-conjugated monoclonal antibody (PY20, SC-508; Santa Cruz Biotechnology) was used to detect phosphotyrosine.

Immunohistochemistry and Immunocytochemistry. Fifty cases of primary resections of breast carcinomas were retrieved from the Surgical Pathology files of the University of Pennsylvania Medical Center. Four resections of human medulloblastoma were retrieved from the Neuropathology files of The Armed Forces Institute of Pathology. None of these cases had received any treatment at the time of biopsy. Immunohistochemical assays were performed on formalin-fixed paraffin-embedded sections. Five-μm-thick sections, deparaffinized in xylene and rehydrated in graded alcohol, were steamed in 0.01 M sodium citrate buffer (pH 6.0) for 20 min. Cells grown on chamber slides were fixed in 95% ethanol for 15 min and then washed twice in Automation Buffer (pH 7.0; Biomedica Corp, Foster City, CA). Endogenous peroxidase was blocked by 3% hydrogen peroxide in methanol for 20 min. Endogenous biotin was blocked by DAKO's Biotin blocking system (DAKO Corp., Carpinteria, CA), according to the manufacturer's specifications. After blocking with 1.5% normal goat serum (Vector Laboratories, Inc., Burlingame, CA) in Automation

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⁴ The abbreviations used are: EPO, erythropoietin; EPOR, EPO receptor; rhEPO, recombinant human EPO; RT-PCR, reverse transcription-PCR; TUNEL, terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling; DCIS, ductal carcinoma(s) *in situ*; HIF-1, hypoxia-inducible factor-1.

Buffer, slides were incubated with the primary antibodies against EPO (rabbit polyclonal, H-162; Santa Cruz Biotechnology) and EPOR (rabbit polyclonal, C-20; Santa Cruz Biotechnology; 1: 200 dilution, Santa Cruz Biotechnology) overnight at 4°C. Slides were then washed three times with Automation Buffer and incubated for 30 min at 37°C with biotinylated goat antirabbit IgG (heavy and light chains) secondary antibody (1:200 dilution; Vector Laboratories, Inc.). After incubation with horseradish peroxidase-conjugated streptavidin (Streptavidin HP detection system; Research Genetics, Huntsville, AL) for 40 min at 37°C, slides were developed with diaminobenzidine chromogen (Research Genetics) for 10 min and counterstained with hematoxylin. Negative controls included the omission of the primary antibody and primary antibody preincubated with the blocking peptide (1:10 dilution) or rhEpo (1:10; R&D Systems). Slides of human fetal liver and placenta were used as positive controls.

Double Staining for Apoptosis and EPO or EPOR. TUNEL stain was performed using the ApopTag Peroxidase *In Situ* Apoptosis detection system (Intergen, Purchase, NY), according to the manufacturer's specifications. Sections of reactive lymph nodes with numerous apoptotic bodies in germinal centers were used as positive controls. After developing the slides with diaminobenzidine, slides were washed five times in Automation Buffer, blocked with normal goat serum and stained for EPO and EPOR as above. After incubation with alkaline phosphatase-conjugated streptavidin (Streptavidin AP detection system; Research Genetics) for 40 min at 37°C, slides were developed with stable Fast Red chromogen (Research Genetics) for 10 min and counterstained with hematoxylin.

Results

Western blot analysis using anti-EPOR antibodies demonstrated that many breast carcinoma cell lines including MCF-7, BT-549, T47D, MDA-134, and MDA-231 (Fig. 1a) express a prominent and specific immunoreactive band at $M_r \sim 66,000$ corresponding to the EPOR protein. Functional EPO signaling in these cells was evidenced by strong enhancement of phosphotyrosine levels in MCF-7 cells on stimulation with either rhEPO or an EPO-mimetic peptide (Ref. 5; Fig. 1b). When added to serum-deprived cells, low amounts of rhEPO were able to stimulate DNA synthesis (Fig. 1c) and cell proliferation

(Fig. 1d) in both MCF-7 and BT549 cells. These findings demonstrate that cell lines arising from solid human cancers, such as breast carcinoma, express functional EPOR and can proliferate in response to EPO.

To investigate whether EPOR expression was preferentially associated with malignant tissue, we examined the expression of EPOR protein in clinical samples of untreated breast carcinomas using Western blotting and immunohistochemistry. EPOR was identified in Western blot analysis of clinical breast biopsy specimens containing cancerous tissue but was not seen in normal breast tissue from regions immediately adjacent to the tumors (Fig. 2a). Immunohistochemistry revealed high levels of both EPO and EPOR expression in the 15 lobular and 35 ductal carcinomas that we examined (Fig. 2, b-l). Invasive lobular (Fig. 2, b-d) and invasive ductal (Fig. 2, e and f) breast carcinomas showed prominent staining for both EPO and EPOR, whereas normal breast tissue in all of the specimens examined displayed much lower immunoreactivity for EPO as well as for EPOR. Most specimens, in fact, had no detectable EPO or EPOR staining associated with the normal ductal, lobular, or stromal breast elements. In a few samples, low levels of EPO staining were seen associated with the luminal aspect of normal duct cells (Fig. 2e). Even in these cases, the far more intense staining of cancer cells could easily distinguish neoplastic from normal tissue. Cells from invasive ductal carcinoma and DCIS (Fig. 2, g-i) typically displayed prominent staining for EPOR throughout the lesion (Fig. 2, c and h), whereas EPO expression was typically heterogeneous (Fig. 2, b and g). In samples containing malignant as well as benign pathology (Fig. 2, j-l), we observed intense EPOR staining only in malignant tissue with minimal staining seen in fibrocystic disease (not shown), benign papilloma (Fig. 2, k and l), and hyperplasia.

In solid neoplasias, adaptive responses to hypoxia are correlated with angiogenesis, enhanced aggressiveness, reduced apoptosis, and poor responses to therapy (11, 12). The well-known regulation of EPO expression by hypoxia (1) and the recognized roles of EPO signaling in promoting cell proliferation (1, 4-8) and angiogenesis (4, 9, 13) as well as in the inhibition of apoptosis (14, 15), led us to examine whether EPO and EPOR were regulated by hypoxia in breast cancer cells. Using RT-PCR, we identified EPO and EPOR mRNA in each of the breast cancer cell lines that we examined. Nucleotide sequencing of the 449-bp EPOR RT-PCR products obtained from MCF-7 and also from MG-U87 glioblastoma cells demonstrated that these were identical to the normal human EPOR (BLAST National Center for Biotechnology Information search results). Exposure to hypoxia stimulated EPO- and, to a lesser extent, EPOR mRNA expression in both MCF-7 and BT549 cell lines within 4 h (Fig. 3a). A prominent increase in EPO protein as well as EPOR protein (Fig. 3b) was seen in both of the cell lines after 8 h of hypoxia. EPO and EPOR protein expression was markedly up-regulated by hypoxia in the glioblastoma MG-U87 cell line as well (data not shown). Immunocytochemical analysis of MCF-7 cells in culture revealed low basal levels of EPO and EPOR expression in most cells with some heterogeneously distributed cells having high levels (Fig. 3c). After 24 h of hypoxia, there was a marked increase both in the numbers of positively stained cells and in the amount of staining per cell for both EPO and EPOR. These data demonstrate that, in cells derived from solid human tumors, such as breast carcinomas, hypoxia stimulates expression of not only the EPO but also the EPOR protein. Clinical samples of high-grade DCIS (Fig. 3, d, e, g, and h) and invasive ductal carcinomas frequently showed necrotic areas with viable rims of cancer cells. In these regions, which correspond to the most hypoxic parts of experimental tumors (11), viable cancer cells demonstrated the highest levels of staining for both EPO and EPOR (Fig. 3, d and g). Higher magnification revealed a large number of apoptotic nuclei with condensed

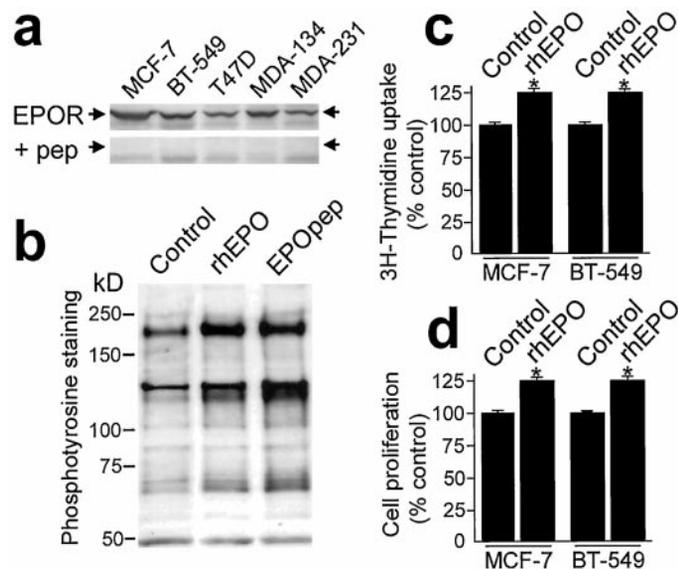


Fig. 1. Human breast cancer cell lines express functional EPOR. *a*, MCF-7, BT-549, T47D, MDA-134, and MDA-231 cells all express a strongly immunoreactive band at $M_r \sim 66,000$ identified with the anti-EPOR antibody (EPOR; arrow). Staining was specifically abolished when the antibody was preincubated with the EPOR peptide (+ pep) against which the antiserum was raised. *b*, basal levels (Control) of phosphotyrosine-containing proteins in MCF-7 cells were markedly enhanced after 5-min incubation with either 250 units/ml rhEPO or 10 μ M EPO mimetic peptide (EPOpep; AEHCSLNEN-ITVPDTKV). *c*, [3 H]thymidine incorporation and *d*, cell proliferation in both the MCF-7 and the BT-549 cell lines were enhanced by $\sim 25\%$ on the addition of 10 units/ml exogenous rhEPO to the media. *, $P < 0.01$ (one tailed *t* test).

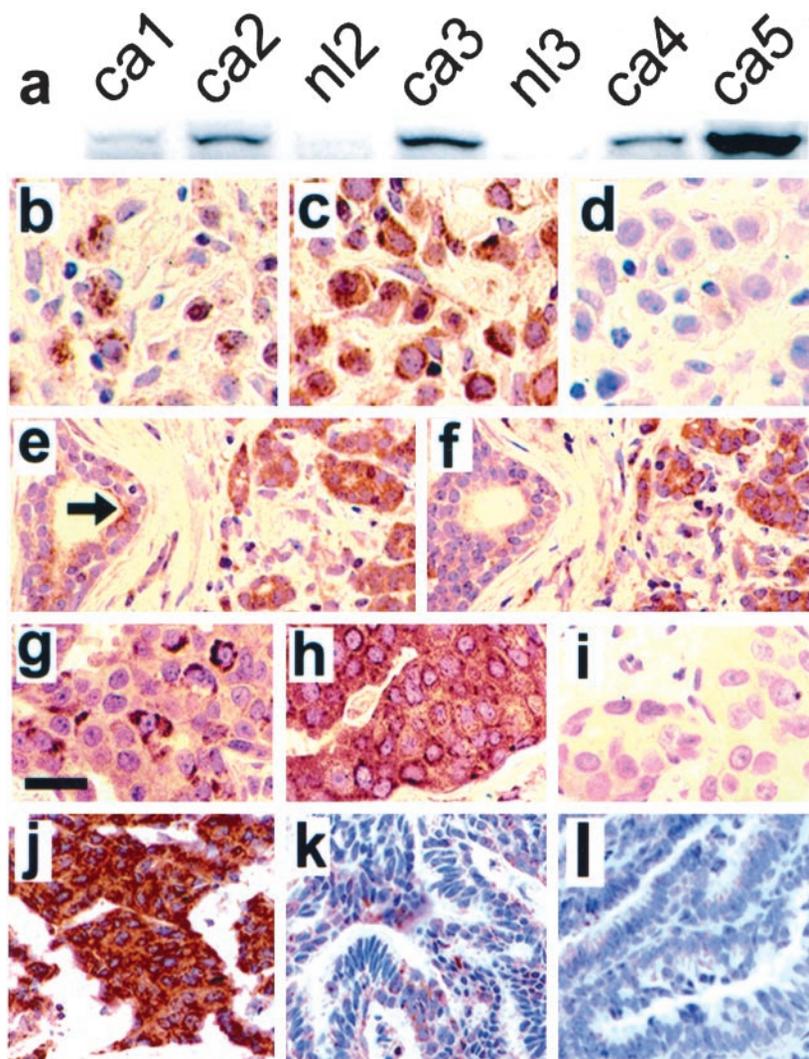


Fig. 2. High EPO and EPOR expression in human breast cancer biopsies distinguishes malignant from benign tissue. *a*, Western blot of frozen biopsy samples (all normalized for protein) from five cases (*ca1–ca5*) shows specific EPOR immunoreactivity. Normal, noncancerous breast tissue (*nl2, nl3*), biopsied adjacent to samples *ca2* and *ca3*, does not show EPOR immunoreactivity. *b–l*, immunohistochemistry of EPO (*b, e, and g*) and EPOR (*c, f, h, and j–l*) in human breast biopsies. Sections of invasive lobular carcinoma (*b–d*), invasive ductal carcinoma (*e and f*), and DCIS (*g–i*) show the intense staining (brown peroxidase reaction product) of EPO and EPOR associated with breast cancer cells and the minimal staining of the normal breast tissue (*e, arrow*). *j and k*, marked enrichment of EPOR in ductal carcinoma cells (*j*) as compared with benign papilloma cells (*k*) stained in the same specimen on the same slide. *l*, another example of benign papilloma with undetectable EPOR staining. All of the staining for EPOR was abolished when the anti-EPOR antibodies were preincubated with the control peptide against which the serum was raised (*d and i*). Preincubation of anti-EPO antibodies with rhEPO abolished similarly all of the staining for EPO (data not shown). Scale bar, 25 μm for *b–d* and *g–i* and 60 μm for *e, f, and j–l*.

chromatin and DNA strand breaks in the tumor regions bordering necrotic and viable areas (Fig. 3, *e and h*). Apoptotic cells were rarely seen among the EPO- and EPOR-expressing tumor cells. Strong staining for EPO in the tumor vasculature (Fig. 3*f*) was commonly seen associated with the endothelium, whereas strong EPOR staining was associated with both the endothelium and the smooth muscle layers (Fig. 3*i*). We also observed prominent EPOR expression in several other human cancer cell lines and malignant tissue samples originating from the brain and female genital tract (Fig. 4*a*). Of 4 clinical cases of medulloblastoma analyzed, one was found to have prominent expression of both EPO (Fig. 4, *b and c*) and EPOR (Fig. 4, *d and e*) with strong EPOR expression in the tumor vessel endothelium (Fig. 4*e*).

Discussion

Our findings have significant implications for theories regarding multistage carcinogenesis, as well as for the diagnosis, treatment, and prevention of cancer. Our data suggest that EPO and EPOR expression may contribute to the survival of hypoxic solid tumors. Hypoxia is known to select for aggressive cancer phenotypes and to promote tumor neovascularization (12). Our finding of high EPO and EPOR levels in human solid tumors, as well as our demonstration of hypoxic up-regulation of both of these proteins, suggests novel ways by which hypoxia may contribute to cancer promotion. EPO and EPOR proteins are up-regulated after ischemic stroke in the rat brain (16) and EPO

enhances ischemic and hypoxic brain cell survival (16, 17). The high expression of EPO and EPOR in solid tumors could similarly improve the hypoxic or ischemic survival of cancer cells. Hypoxia induces transcription of the EPO gene via activation of the transcription factor HIF-1 (1, 11, 12). Overexpression of HIF-1 is common in human cancers and is correlated with increased cell proliferation, angiogenesis, and a malignant phenotype (11, 12). However, HIF-1 regulates the expression of several genes that are known to confer growth advantages to hypoxic cancers (11, 12). HIF-1-mediated EPO expression is, thus, unlikely to be an exclusive mechanism for hypoxic cell survival. Sustained activation of the EPOR by EPO is known to block apoptosis in hematopoietic progenitors (14, 15). If EPO signaling has a similar function in human cancer cells, then up-regulation of functional EPOR along with EPO may contribute to the hypoxia-mediated selection of cells with diminished apoptotic potential and relative resistance to therapy (12). Indeed, we only rarely observed apoptotic nuclei among the EPO- and EPOR-expressing breast cancer cells in clinical samples. These observations suggest several experimental studies for the definitive implication of EPO signaling in the hypoxic survival of cancer cells. The recently realized effects of EPO on angiogenesis (3, 9, 13) as well as the ability of EPO to increase vascular endothelial growth factor levels in humans (18) are also highly relevant to hypoxic tumor survival. Although we have not yet determined whether the EPO staining that was observed in endothelial cells originated from the tumor cells, the prominent expression of

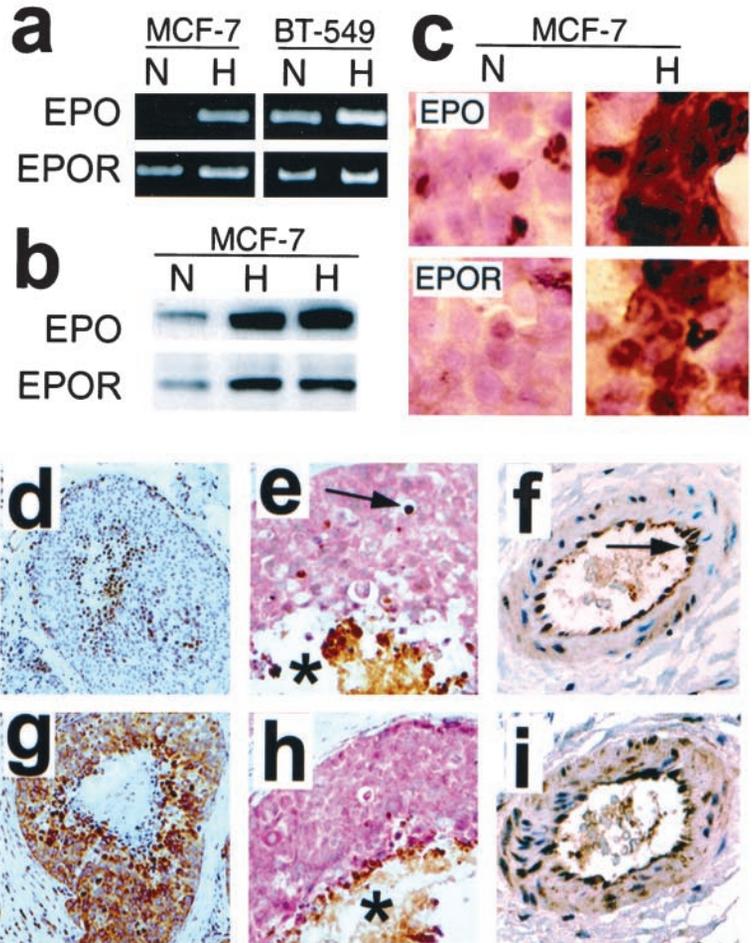


Fig. 3. Hypoxia stimulates EPO and EPOR expression in breast cancer. *a*, both the MCF-7 and the BT-549 cell lines demonstrated stimulation of EPO mRNA expression via RT-PCR analysis after exposure to hypoxia (1% oxygen) for 4 h (*H*) when compared with normoxic cultures (*N*). EPOR mRNA changed less prominently in hypoxic cells. *b*, hypoxia (8 h) enhanced expression of both EPO (M_r 34,000 band) and EPOR (M_r 66,000) at the protein level. Two independent hypoxic MCF-7 samples (*H*) are compared with a normoxic sample (*N*) in this figure. *c*, the basal expression of EPO and EPOR in MCF-7 cultures was localized to a fraction of the cells via immunoreactivity, whereas hypoxia induced prominent staining of nearly all of the cells. Similar results were seen with BT-549 cells (data not shown). *d* and *g*, DCIS specimens with areas of comedo-like necrosis showed markedly enhanced EPO (*d*) and EPOR (*g*) staining in the cells forming the viable rim around the necrotic center. *e* and *h*, double staining of DCIS samples for EPO (*e*) or EPOR (*h*; both stained red in these panels) and apoptotic nuclei via the TUNEL method (brown). TUNEL positive apoptotic nuclei are prominent in the border zone between necrotic (*) and viable areas of DCIS specimens and occur rarely among the viable cells (arrow), which stain strongly for EPO (*e*) and EPOR (*h*). *f* and *i*, EPO and EPOR staining of tumor vasculature. Blood vessels in the tumor samples showed immunoreactivity for EPO (*f*) associated predominantly with the endothelium (arrow), whereas EPOR staining (*i*) was seen strongly in endothelial and smooth muscle cells. All of the staining for EPOR and EPO was abolished on preincubation of the respective antiserum with the control EPOR peptide or rhEPO (data not shown).

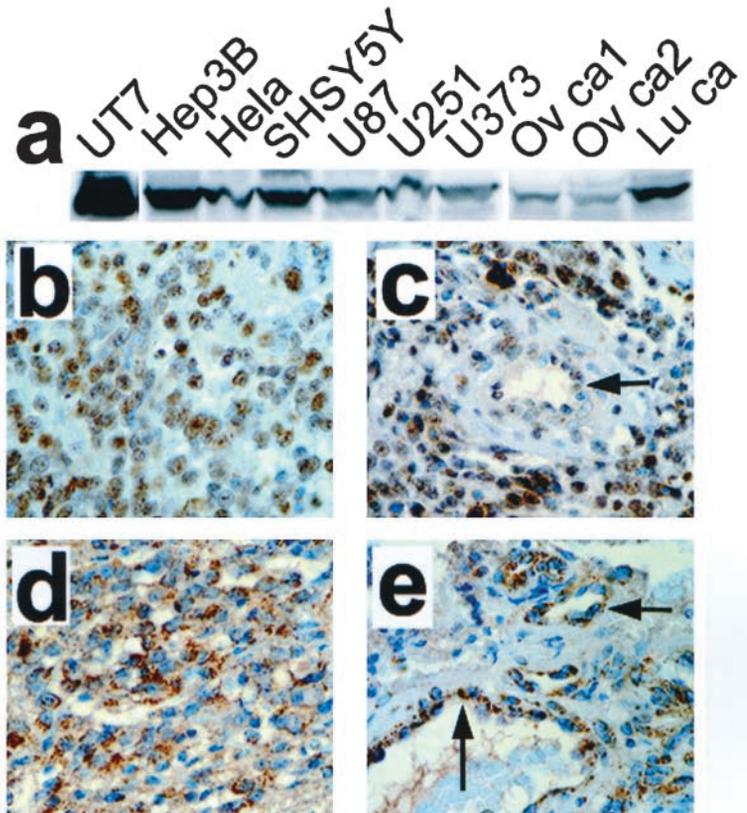


Fig. 4. Human solid cancers express EPO and EPOR. *a*, Western blot analysis showing strong expression of EPOR in several human cell lines: UT-7 erythrocytic leukemia cells, HEP3B hepatoma cells, HeLa cervical carcinoma cells, SHSY5Y neuroblastoma cells, U87 glioblastoma cells, U251 glioma cells, and U373 glioma cells. Biopsy specimen from two ovarian cancers (*Ov ca1* and *Ov ca2*) and one lung cancer (*Lu ca*) were also positive for EPOR. *b-e*, expression of EPO (*b* and *c*) and EPOR (*d* and *e*) in a human medulloblastoma biopsy specimen. In the tumor vasculature, EPO staining (*c*) is only faintly seen in the endothelium, whereas strong staining for EPOR (*e*) is associated with endothelium of arteriole (vertical arrow) and capillaries (horizontal arrow).

EPOR that we observed in tumor vasculature suggests an important role for EPO signaling in tumor angiogenesis. Neoplastic cells in each of the 50 cases of breast carcinoma examined immunohistochemically by us were strongly positive for EPO and EPOR, and normal breast tissue was minimally reactive in all of the specimens, which suggests that an examination of clinical biopsies for EPO and EPOR may aid in cancer diagnosis. Either endogenously produced or exogenously administered EPO could conceivably promote proliferation and survival of EPOR-expressing cancer cells. In light of our findings, the use of rhEPO for treating the anemia that results from chemotherapy should perhaps be preceded by an examination of biopsy material for EPOR expression. The most common reason for elevated EPO levels and secondary erythrocytosis in humans is hypoxia caused by cigarette smoking (19). Our findings, thus, also suggest another mechanism that links smoking with cancer promotion (20).

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