Hypoxia-Inducible Erythropoietin Signaling in Squamous Dysplasia and Squamous Cell Carcinoma of the Uterine Cervix and Its Potential Role in Cervical Carcinogenesis and Tumor Progression

Geza Acs,* Paul J. Zhang,* Cindy M. McGrath,* Peter Acs,[†] John McBroom,[‡] Ahmed Mohyeldin,[§] Suzhen Liu,[§] Huasheng Lu,[§] and Ajay Verma[§]

From the Department of Pathology and Laboratory Medicine,* University of Pennsylvania Medical Center, Philadelphia, Pennsylvania; the Department of Medicine,[†] Huron Hospital, Cleveland Clinic Health System, Cleveland, Ohio; the Department of Obstetrics and Gynecology,[‡] Walter Reed Army Medical Center, Washington, D.C.; and the Department of Neurology,[§] Uniformed Services University of the Health Sciences, Bethesda, Maryland

Tissue hypoxia is a characteristic property of cervical cancers that makes tumors resistant to chemo- and radiation therapy. Erythropoietin (Epo) is a hypoxia-inducible stimulator of erythropoiesis. Acting via its receptor (EpoR), Epo up-regulates bcl-2 and inhibits apoptosis of erythroid cells and rescues neurons from hypoxic damage. In addition to human papillomavirus infection, increased bcl-2 expression and decreased apoptosis are thought to play a role in the progression of cervical neoplasia. Using reverse transcriptase-polymerase chain reaction and Western blotting we showed that HeLa and SiHa cervical carcinoma cells and human cervical carcinomas express EpoR, and that hypoxia enhances EpoR expression. Exogenous Epo stimulated tyrosine phosphorylation and inhibited the cytotoxic effect of cisplatin in HeLa cervical carcinoma cells. Using immunohistochemistry, we examined the expression of Epo, EpoR, p16, hypoxia-inducible factor (HIF)-1 α , and bcl-2 in benign and dysplastic cervical squamous epithelia and invasive squamous cell carcinomas (ISCCs). EpoR expression in benign epithelia was confined to the basal cell layers, whereas in dysplasias it increasingly appeared in more superficial cell layers and showed a significant correlation with severity of dysplasia. Diffuse EpoR expression was found in all ISCCs. Expression of Epo and HIF-1 α was increased in dysplasias compared to benign epithelia. Focal Epo and HIF-1 α expression was seen near necrotic areas in ISCCs, and showed correlation in their spatial distribution. Significant correlation was found between expression of EpoR, and p16 and bcl-2 in benign and dysplastic squamous epithelia. Our results suggest that in-

creased expression of Epo and EpoR may play a significant role in cervical carcinogenesis and tumor progression. Hypoxia-inducible Epo signaling may play a significant role in the aggressive behavior and treatment resistance of hypoxic cervical cancers. (Am J Pathol 2003, 162:1789–1806)

Carcinoma of the uterine cervix is the most common gynecological malignancy worldwide with an estimated 750,000 new cases annually, mostly occurring in under-developed countries.^{1–4} Although routine screening programs in the United States have steadily reduced the mortality rate from cervical carcinoma since the 1940s, there continues to be an under-screened, high-risk population. With an estimated 12,800 new cases and 4600 deaths in 2000, cervical cancer remains the third most common gynecological malignancy in the United States,^{3–5} and a large percentage of women with advanced stage disease continue to die of locally recurrent and metastatic disease.

Squamous cell carcinoma accounts for ~75 to 85% of cervical cancers⁶ and develops in the background of increasing grades of dysplasia (termed cervical intraepithelial neoplasia, CIN).^{6–9} High-risk human papillomavirus (HPV) DNA is detected in more than 90% of malignant cervical tumors and it is widely accepted that HPV plays an essential role in the pathogenesis of cervical cancer.^{7,8,10} The high-risk HPV oncoproteins, E6 and E7, have been shown to be necessary for immortalization and transformation of cervical keratinocytes.¹¹ E6 binds to the wild-type p53 protein and promotes its ubiquitin-dependent degradation.¹² E7 binds to the retinoblastoma protein Rb, and disrupts the complex between Rb and the E2F transcription factor family, which controls the expression of genes involved in cell-cycle progression.¹³ In

Supported by the McCabe Award (to G. A.), the National Institutes of Health (grant NS37814 to A. V.), and the Mary Kay Ash Charitable Foundation (to A. V.).

Accepted for publication February 13, 2003.

Address reprint requests to Geza Acs, M.D., Ph.D., Department of Pathology and Laboratory Medicine, University of Pennsylvania Medical Center, 6 Founders Pavilion, 3400 Spruce St., Philadelphia, PA 19104. E-mail: geza@mail.med.upenn.edu.

normal cells the activity of cyclin-dependent kinase (CDK)4 and CDK6 is tightly regulated by several cyclin-dependent kinase inhibitors including p16^{INK4a}.^{14,15} Because expression of p16^{INK4a} underlies a negative feedback control through Rb,¹⁶ the reduction or loss of Rb function (through HPV E7) results in enhanced p16^{INK4a} levels in respective cells.¹⁷ Indeed, overexpression of p16^{INK4a} has been demonstrated in cervical squamous cell carcinoma and squamous dysplasia infected with high-risk HPV types.¹⁸

Despite these associations, only a small percentage of precursor lesions infected with HPV develop into invasive carcinomas.⁷ Therefore, additional genetic and microenvironmental factors subsequent to HPV infection must play an important role in the initiation and progression of cervical neoplasia.^{7,8} The potential of tumor progression is thought to be associated with the balance between cell death and cell proliferation and apoptosis plays an important role in the maintenance of this balance.^{19,20} Several studies support an important role for apoptosis and altered expression of the anti-apoptotic protein bcl-2 in the development and progression of human cervical carcinoma.^{21–25}

Hypoxic microregions are a characteristic pathophysiological property of solid tumors^{26–29} and are thought to result from inadequate perfusion because of severe structural and functional abnormalities of the tumor microcirculation and from tumor-related anemia.^{27,30} Previous studies have shown that ~60% of locally advanced cervical squamous cell carcinomas have hypoxic tissue areas, which are heterogeneously distributed within the tumor mass.^{27–29,31,32} Tumor hypoxia makes solid tumors resistant to radiation and chemotherapy.^{27,33,34} More recent studies suggest that sustained hypoxia can additionally enhance local and systemic malignant progression and may increase aggressiveness through clonal selection and genomic and proteomic changes.^{27,31,32,35,36} Multivariate analysis has shown that tumor hypoxia is indeed the most powerful prognostic factor in cervix cancers independent of other variables.^{33,34,37,38}

Many hypoxia-regulated genes, such as vascular endothelial growth factor, are known to play a key role in carcinogenesis and tumor progression.^{39,40} The best known hypoxia-regulated gene is erythropoietin (Epo), a glycoprotein hormone stimulator of erythropoiesis.^{41–43} Epo gene expression is primarily modulated by tissue hypoxia^{44,45} and this regulation occurs mainly at the mRNA level mediated by hypoxia-inducible transcription factor-1 (HIF-1).^{41,44,46} During adult life, Epo is normally produced by the kidney and liver.^{41,43,47} The Epo receptor (EpoR) belongs to the cytokine receptor type I superfamily.^{41,48–54} The signaling mechanisms after receptor activation include the Jak/STAT and the Ras/MAP kinase pathways.^{41,46,48–59} EpoR stimulation in erythroblasts promotes their proliferation and differentiation, and leads to increased expression of the anti-apoptotic proteins bcl-2 and bcl-X_L.^{57,58} and inhibition of apoptosis.^{46,52,53,56,59}

Recently other sites of Epo production have been reported, including bone marrow macrophages,⁶⁰ brain astrocytes,^{61,62} trophoblast cells of human placenta,⁶³

and the breast.^{64–66} Epo production was also demonstrated in human female reproductive organs, including the uterus.^{67,68} In recent years it has also become clear that EpoR is expressed by a variety of cell types, including endothelial cells,⁶⁹ neurons,⁷⁰ trophoblast cells,⁷¹ and mammary epithelial cells.^{64–66} Although the specific function(s) of EpoR in these nonhematopoietic sites is not fully understood, the EpoR expressed in these tissues seems to be functional, thus suggesting a wider biological role for Epo signaling unrelated to erythropoiesis.^{72,73} There appears to be a paracrine Epo/EpoR system in the brain, where neurons express EpoR^{61,70,74} and astrocytes produce Epo.^{61,62} Evidence suggests that the signaling cascades that have been characterized in hematopoietic cells,^{48–50,52,56} are also functional in neurons and can be modulated by Epo. It has been demonstrated *in vitro* and *in vivo* that Epo is a potent inhibitor of neuronal apoptosis induced by ischemia and hypoxia.⁷⁵ EpoR mRNA is also expressed in endothelial cells and Epo stimulates proliferation and migration of human endothelial cells^{76,77} and angiogenesis.⁶⁸

Elevated Epo levels have long been recognized in patients with renal cell carcinomas, Wilms' tumors, hepatomas, and cerebellar hemangioblastomas, all tumors arising in anatomical sites in which Epo is normally expressed in low levels.⁴⁴ Moreover, ectopic Epo expression in erythroleukemia cells was found to mediate their autonomous growth.⁷⁸ EpoR expression was also reported in cases of renal cell carcinoma and a potential paracrine or autocrine role for Epo signaling for promoting growth of renal carcinomas has been suggested.⁷⁹ We have recently described that human breast cancer cell lines and human breast carcinomas express Epo and EpoR mRNA and protein and that their expression is enhanced by hypoxia.^{64,65} Furthermore, we demonstrated that Epo signaling is biologically active and stimulates tyrosine phosphorylation, DNA synthesis, and proliferation in breast cancer cells.

In the present study we report biologically active, hypoxia-inducible Epo signaling in human cervical carcinoma cells. We demonstrate that human recombinant Epo (rHu-Epo) inhibits the cytotoxic effect of the chemotherapeutic drug cisplatin in cervical carcinoma cells. Using immunohistochemistry we demonstrate the expression of Epo and EpoR in human tissue samples and cytological smears of cervical squamous dysplasia and carcinoma, and correlate their expression with that of p16^{INK4a}, HIF-1 α , and bcl-2.

Materials and Methods

Cell Culture and Treatments

All culture media were purchased from Life Technologies, Inc. (Rockville, MD). The HeLa and SiHa human cervical carcinoma, Hep3B human hepatoma, and U251 human glioblastoma cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured according to ATCC directions. Forty-eight hours before hypoxic treatment cells were switched to

serum-free medium. Hypoxic treatment of cells was performed in an enclosed chamber (Billups-Rothenberg Inc., Del Mar, CA) flushed with a premixed gas mixture (1% O₂, 5% CO₂, 94% N₂) for the times indicated. The O₂ concentration in the culture medium was confirmed to be 1% using an oxygen-sensitive electrode (World Precision Instruments, Sarasota, FL), and this oxygen concentration was maintained throughout the course of the experiments. Stimulation of tyrosine phosphorylation was performed by treating cells in phenol-free Dulbecco's modified Eagle's medium with recombinant human erythropoietin (rHu-Epo, epoetin- α , 10 U/ml; Amgen, Thousand Oaks, CA) or the Epo mimetic peptide Emp1 (AE-HCSLNENITVPDTKV, 1 μ g/ml) for 30 minutes.

Cytotoxicity Assay

For cytotoxicity studies cells were plated in 96-well plates at 3000 cells/well. The following morning cells were treated with rHuEpo (0, 25, 50, or 200 U/ml) with or without the general tyrosine kinase inhibitor genistein (10 μ mol/L) for 60 minutes. Cells were then exposed to cisplatin (10 μ g/ml) for 20 hours. Each condition was performed in quadruplicate. Viability of cells was assessed using the non-radioactive cell proliferation (MTT) assay (Promega, Madison, WI).

Annexin V Apoptosis Assay

Apoptosis was assayed by detection of membrane externalization of phosphatidylserine using the Annexin V-PE Apoptosis Detection Kit I (BD Biosciences, Palo Alto, CA). Cells were plated at 40 to 50% confluency in six-well plates and allowed to adhere overnight. Media was then removed and replaced with serum-free media with or without rHuEpo. Cells were pretreated with rHuEpo (0, 50, 100, or 200 U/ml) for 2 hours and then challenged with cisplatin (5 μ g/ml) for 48 hours. Both adherent and floating cells were harvested for the apoptosis assay. Cells were washed once with phosphate-buffered saline, resuspended in binding buffer, and stained with phycoerythrin-conjugated Annexin V and 7-amino-actinomycin (7-AAD) according to the manufacturer's protocol (5 μ l for 1×10^5 cells) for 15 minutes. Cells were analyzed using an EPICS XL-MCL (Beckman Coulter, Miami, FL) flow cytometer. Emission from Annexin V was collected through a 575-nm band-pass filter and emission from 7-AAD was collected through a 675-nm band-pass filter. A minimum of 10,000 events was collected for each sample and each treatment group was done in triplicates.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Measurement of Epo and EpoR mRNA were performed as described previously.^{64,80} RNA was isolated using the RNeasy RNA isolation kit (Qiagen, Inc., Valencia, CA) and the Superscript One-Step RT-PCR System (Life Technologies, Inc., Gaithersburg, MD) was used for RT-

PCR. Approximately 1 μ g of RNA and 10 pmol of both the forward and reverse primers were used for each sample. RT was performed at 50°C for 30 minutes. The PCR cycle protocol for the Epo primers was 42 cycles at 94°C for 20 seconds, 61°C for 45 seconds, and 72°C for 1 minute, and for EpoR was 42 cycles at 94°C for 15 seconds, 55°C for 30 seconds, 72°C for 1 minute. Primers for Epo were: sense 5'-ACTCTGCTTCGGGCTCTGGGAGCCCAGAAG-3' (corresponding to nucleic acids 581 to 610), and anti-sense 5'-AAGCAATGTTGGTGAGGGAGGTGGTGGAT-3' (corresponding to nucleic acids 786 to 814).^{64,80} Primers for EpoR were: sense 5'-GGCAGTGTGGACATAGTGGC-3' (corresponding to nucleic acids 1304 to 1323), and anti-sense 5'-AGCAGGATGGATTGGGCAGA-3' (corresponding to nucleic acids 1782 to 1801). These primer sets were used previously, with subsequent nucleotide sequencing of the 447-bp EpoR RT-PCR products obtained from MCF-7 human breast cancer and MG-U87 human glioblastoma cells demonstrating that these were identical to the normal human EpoR (BLAST NCBI search results).⁶⁴ The RT-PCR products were then run on a 1% agarose gel. The DNA standard used was the 100-bp DNA ladder from FMC Bioproducts (BioWhittaker Molecular Applications, Rockland, ME).

Western Blotting

For detection of EpoR, whole cell lysates and clinical biopsy sample protein extracts were normalized for protein. One hundred μ g of proteins from each sample were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and stained with anti-EpoR antibody (rabbit polyclonal, C20, 1:1500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in the absence or presence of EpoR blocking peptide (10:1 peptide:antibody ratio, Santa Cruz Biotechnology). Secondary antibody was goat anti-rabbit antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ). Immunoreactive bands were visualized using chemiluminescence (SuperSignal WestPico Chemiluminescence kit; Pierce, Rockford, IL). A horseradish peroxidase-conjugated monoclonal antibody (PY20, SC-508, 1:1000 dilution; Santa Cruz Biotechnology) was used to detect phosphotyrosine. Nuclear extracts and Western blot analysis for HIF-1 α were performed as previously described⁸¹ using a mouse monoclonal anti-HIF-1 α antibody (clone 54, 1:500 dilution; BD Transduction Laboratories, San Jose, CA). Secondary antibody was rabbit anti-mouse conjugated with horseradish peroxidase (Amersham Pharmacia Biotech).

Clinical Samples and Clinical Data

Study protocols involving human material were approved by the University of Pennsylvania Institutional Review Board. Fresh tissue from seven cervical squamous cell carcinomas and adjacent benign cervical squamous mucosa (in one case) was obtained at the time of surgery. A frozen section was cut from each sample and examined to confirm the presence or absence of tumor tissue: all tumor samples contained at least 80% neoplastic cells,

Table 1. Summary of Clinicopathological Features

	Benign	CIN I	CIN II	CIN III	ISCC
Main diagnosis (n)	22	17	23	20	15
Total number of samples*	85	17	23	30	15
Mean (median) age of patient	42.4 (43.5)	27.1 (26)	31.0 (31)	38.3 (36.5)	44.3 (45)
Mean size of tumor (cm, range)	N/A	N/A	N/A	N/A	4.1 (0.3–12)
Mean depth of invasion (range)					
Depth (cm)	N/A	N/A	N/A	N/A	1.45 (0.2–3)
Percent of cervical wall	N/A	N/A	N/A	N/A	81 (10–100)
Grade of tumor (n)					
Intermediate	N/A	N/A	N/A	N/A	9
High	N/A	N/A	N/A	N/A	6
FIGO stage of tumor (n)					
IA	N/A	N/A	N/A	N/A	2
IB	N/A	N/A	N/A	N/A	9
IIA	N/A	N/A	N/A	N/A	4
Nodal metastasis (n) [†]					
Absent	N/A	N/A	N/A	N/A	8
Present	N/A	N/A	N/A	N/A	6
Tumor cell keratinization (n)					
Absent	N/A	N/A	N/A	N/A	6
Present	N/A	N/A	N/A	N/A	9
Tumor cell necrosis (n)					
Absent	N/A	N/A	N/A	N/A	2
Present	N/A	N/A	N/A	N/A	13

CIN I, cervical intraepithelial neoplasia grade I; CIN II, cervical intraepithelial neoplasia grade II; CIN III, cervical intraepithelial neoplasia grade III; n, number of cases.

*In most cases more than one type of epithelium was present in a tissue specimen.

[†]Pelvic and para-aortic lymph node dissection was performed in 14 cases.

whereas no tumor cells were present in the sample of benign mucosa. Tissue samples were stored at -80°C until used for protein extraction.

Ninety-seven cases of cervical biopsies, cone biopsies, or hysterectomies performed for benign disease (leiomyomas), cervical dysplasia, or invasive squamous cell carcinoma (ISCC) were selected from the surgical pathology files of the University of Pennsylvania Medical Center. Hematoxylin and eosin (H&E)-stained slides of all cases were reviewed and the diagnoses confirmed. Grading of squamous dysplasias (CIN I to CIN III) was performed on the H&E-stained slides according to established criteria.⁹ Invasive carcinomas were also evaluated to determine the presence or absence of tumor cell keratinization and necrosis. Information regarding clinicopathological tumor features were retrieved from the pathology reports and are summarized in Table 1.

Thirty-nine SurePath (formerly known as AutoCyte PREP; Tripath Imaging, Burlington, NC) liquid-based preparations of cervicovaginal cytological samples were retrieved from the cytopathology files of the University of Pennsylvania Medical Center. All Papanicolaou-stained cytological slides were reviewed and the diagnoses confirmed. The cytological diagnoses were as follows: within normal limits, 17 cases; low-grade squamous intraepithelial lesion (HPV infection and CIN I), 5 cases; and high-grade squamous intraepithelial lesion (CIN II and CIN III), 16 cases. Because benign squamous cells were present in all cases, a total of 39 samples of benign squamous cells were analyzed.

Immunohistochemistry and Immunocytochemistry

Immunohistochemical assays were performed on formalin-fixed paraffin-embedded sections as described previously.⁶⁵ Five- μm -thick sections were cut and deparaffinized in xylene and rehydrated in graded alcohols. SurePath cytological slides were decoverslipped in xylene, rehydrated in graded alcohols, and postfixated in 10% phosphate-buffered formaldehyde for 30 minutes. All slides were steamed in 0.01 mol/L of sodium citrate buffer (pH 6.0) for 20 minutes. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in methanol for 20 minutes. Slides were incubated with the antibodies against Epo (rabbit polyclonal, H-162, 1:200 dilution; Santa Cruz Biotechnologies), EpoR (rabbit polyclonal, C-20, 1:400 dilution; Santa Cruz Biotechnologies), bcl-2 (clone 124, mouse monoclonal, 1:50 dilution; DAKO Corp., Carpinteria, CA), p16^{INK4a} (clone 16P07, mouse monoclonal, 1:100 dilution; Neomarkers, Fremont, CA), and HIF-1 α (clone H1alpha67, mouse monoclonal, 1:10,000; Neomarkers) overnight at 4°C . In the cases of Epo, EpoR, bcl-2, and p16^{INK4a} assays, the slides were then washed five times with Tris-buffered saline containing Tween 20 (TBST, pH 7.6; DAKO) and incubated for 30 minutes at room temperature with horseradish peroxidase-labeled dextran polymer coupled to anti-rabbit or anti-mouse antibody (DAKO EnVision + System HRP, DAKO). Slides were then washed three times with TBST, developed with diaminobenzidine for 10 minutes, and counterstained with hematoxylin. For HIF-1 α the DAKO

CSA signal amplification kit was used according to the manufacturer's recommendations. For Epo and EpoR, slides of fetal liver⁸² and placenta^{63,71} were used as positive controls. For bcl-2 slides of human tonsil, and for p16^{INK4a} slides of human cervical squamous cell carcinoma known to show strong p16^{INK4a} immunostaining were used as positive controls. For HIF-1 α slides of oral squamous cell carcinoma with large areas of necrosis known to show HIF-1 α overexpression were used as positive control. A negative control was done in each case by omission of the primary antibody. The specificity of the Epo and EpoR antibodies were confirmed previously.^{64,66,70,75,83} In addition, the specificity of the EpoR and Epo immunoreactivity was also evaluated by antibody absorption test: the primary antibody was preincubated with blocking peptide for EpoR (Santa Cruz Biotechnologies Inc.) or human recombinant Epo (rHuEpo; R&D Systems, Minneapolis, MN) (10:1 peptide:antibody ratio), which resulted in complete abolishment of immunohistochemical staining. The specificity of the immunostaining reaction is further supported by other experiments using a mouse monoclonal anti-Epo (clone 9C21D11, R&D Systems) and a rabbit polyclonal anti-EpoR antibody (Upstate Biotechnology, Lake Placid, NY),⁸⁴ which resulted in an immunostaining pattern similar to that obtained with the antibodies used in the current study (Acs et al, unpublished observation).

Double Immunohistochemistry for HIF-1 α and Epo

For double-immunohistochemical staining slides were immunostained for HIF-1 α using the DAKO CSA kit as described above. After incubation with diaminobenzidine, the slides were washed three times in deionized water and three times in TBST. Slides were then incubated with the primary Epo antibody as described above, washed five times with TBST, and incubated for 30 minutes at room temperature with alkaline phosphatase-labeled dextran polymer coupled to anti-rabbit antibody (DAKO EnVision + System AP, DAKO). Slides were then washed three times with TBST, developed with fast red chromogen for 10 minutes, and counterstained with hematoxylin.

Interpretation of Immunohistochemical Stains

Immunohistochemical stains for Epo, EpoR, p16^{INK4a}, and HIF-1 α were interpreted semiquantitatively by assessing the intensity and extent of staining on the entire tissue sections present on the slides according to a four-tiered (0 to 3) scale. For Epo cytoplasmic, and for EpoR cytoplasmic and/or membrane immunoreactivity was considered positive. For HIF-1 α nuclear staining, and for p16^{INK4a} nuclear and/or cytoplasmic staining was considered positive. In the case of dysplasias or *in situ* carcinomas, first, the percentage of total epithelial thickness showing positive staining was determined (eg, 50% if the basal half or 75% if the basal three-fourths of the squamous epithelium showed positive immunostaining, and

so forth). In the case of invasive tumors, first the total percentage of positively staining tumor cells was determined. Then the percentage of weakly (1), moderately (2), and strongly (3) staining cells was determined, so that the sum of these categories equated with the overall percentage of positivity. A staining score was then calculated as follows: score (out of maximum of 300) = sum of 1 $\times$ percentage of weak, 2 $\times$ percentage of moderate, and 3 $\times$ percentage of strong staining. To assess the immunostaining for Epo and HIF-1 α adjacent to areas of tumor cell necrosis and keratinization, immunostaining was scored as described above in at least 10 high-power fields of viable tumor cells within 1 mm of necrotic or keratinized areas, and in tumor regions away from such areas. Bcl-2-stained slides were interpreted by determining the percentage of squamous epithelial cells showing cytoplasmic immunoreactivity of any intensity. Immunohistochemical stains were evaluated independently by two pathologists (GA and PJZ). Slight differences in interpretation were resolved by simultaneous viewing.

Immunofluorescence

For immunofluorescence staining slides were pretreated and incubated with the primary antibody against p16^{INK4a} or EpoR as described above. Slides were then washed five times with TBST and incubated with a fluorescein isothiocyanate-conjugated rabbit anti-mouse secondary antibody (1:20 dilution, DAKO) or a tetramethyl-rhodamine isothiocyanate-conjugated swine anti-rabbit secondary antibody (1:40 dilution, DAKO) for 30 minutes on 37°C in the case of p16^{INK4a} and EpoR, respectively. Slides were then coverslipped with 4,6-diamidino-2-phenylindole and examined using a Leica DMRA Research Microscope equipped with a Megapixel charge-coupled device camera. For simultaneous immunostaining of tissue sections and cytological preparations for p16^{INK4a} and EpoR, slides were first stained for EpoR as described above. Slides were then washed five times with TBST and immunostained for p16^{INK4a} as described above.

Statistical Analysis

The Wilcoxon signed rank test was used for the comparison of median p16^{INK4a}, EpoR, Epo, HIF-1 α , and bcl-2 immunohistochemical expression levels in dysplastic epithelia and the adjacent benign squamous epithelium, and for the comparison of Epo and HIF-1 α immunostaining in tumor areas adjacent to or away from necrosis and keratinization. Median p16^{INK4a}, EpoR, Epo, HIF-1 α , and bcl-2 immunohistochemical expression levels in benign epithelia, CIN I, CIN II, CIN III, and ISCC were compared using the Kruskal-Wallis one-way analysis of variance by ranks followed by Dunn's multiple comparison test, when appropriate. The correlation between the levels of Epo and EpoR staining and p16, bcl-2, and HIF-1 α staining was estimated using the Spearman rank correlation test. The normal *versus* abnormal distribution of HIF-1 α expression in benign and dysplastic epithelia was com-

Figure 1. RT-PCR analysis of Epo and EpoR expression in HeLa and SiHa cervical carcinoma cells. **A:** HeLa and SiHa cells cultured under normoxia (N, 21% O₂) or hypoxia (H, 1% O₂) were analyzed for Epo and EpoR mRNA expression. Although abundant expression of EpoR mRNA was detected in HeLa cells under both conditions, SiHa cervical carcinoma cells showed a prominent increase in EPOR mRNA expression after hypoxia treatment. Epo mRNA expression could not be detected in either cell lines. **B:** In contrast, human hepatoma Hep3B and glioblastoma U251 cells expressed abundant Epo mRNA.

pared using the Fisher's exact test. Statistical significance was determined if the two-sided *P* value of a test was less than 0.05.

Results

Hypoxia-Inducible Expression of Functional EpoR in Human Cervical Carcinoma Cell Lines and Human Cervical Carcinomas

Using RT-PCR, HeLa and SiHa human cervical carcinoma cells cultured under normoxia (21% O₂) or hypoxia (1% O₂) were analyzed for Epo and EpoR mRNA expression. Abundant expression of EpoR mRNA was detected in HeLa cells under both conditions, whereas SiHa cells showed a prominent increase in EpoR mRNA after hypoxia treatment (Figure 1A). No Epo mRNA expression could be detected in HeLa and SiHa cervical carcinoma cells (Figure 1A). In contrast, human hepatoma Hep3B and glioblastoma U251 cells expressed abundant Epo mRNA (Figure 1B), as reported previously.⁶⁴

Western blot analysis using EpoR antibodies demonstrated that HeLa cervical carcinoma cells express a prominent and specific immunoreactive band at *M_r* ~66,000 corresponding to the EpoR protein (Figure 2A). The immunostaining was specifically abolished when the antibody was preincubated with the EpoR peptide against which the antiserum was raised (not shown). Hypoxia treatment induced a marked increase in the expression of both HIF-1 α and EpoR in the cells (Figure 2A). Functional EpoR signaling in HeLa cells was evidenced by induction of tyrosine phosphorylation after

Figure 2. HeLa cervical carcinoma cells express hypoxia-inducible and functional EpoR. **A:** Hypoxia activates HIF-1 α and stimulates EpoR expression in cervical carcinoma cells. HeLa cells were cultured under normoxia (N, 21% O₂) or hypoxia (H, 1% O₂) for 3 hours and then their nuclear extracts examined by Western blotting for HIF-1 α (HIF) immunoreactivity. For evaluation of EpoR expression, membranes from HeLa cells cultured under normoxia (N) or hypoxia (H) for 24 hours were used. HeLa cells cultured under normoxia (N) express a strongly immunoreactive band at *M_r* ~66,000 identified with the antibody. Under normoxic culture conditions HeLa cells show very low levels of HIF-1 α expression. Hypoxia (H) for 3 hours dramatically increased the level of HIF-1 α protein expression and resulted in an increase in EpoR protein expression after 24 hours. **B:** EpoR-mediated activation of tyrosine phosphorylation in HeLa cells. HeLa cells were treated with either 10 U/ml of rHuEpo (Epo) or 1 μ g/ml of Epo-mimetic peptide (Emp1) for 30 minutes and then analyzed for tyrosine phosphorylation by Western blotting. Phosphotyrosine immunoreactivity was dramatically enhanced by both Epo and Emp1 with the most prominent effect being at a molecular weight of 120 to 130 kD. **C:** Western blot analysis of cervical tissue samples (1, benign squamous mucosa; 2 to 8, cervical carcinoma; 9, HeLa cervical carcinoma cells) in the absence (-pep) or presence (+ pep) of EpoR-blocking peptide. In the absence of the blocking peptide a prominent immunoreactive band at *M_r* ~66,000 corresponds to the EpoR protein in the tumor samples. No EpoR expression is seen in the sample of benign mucosa.¹ Note the absence of the immunoreactive band at *M_r* ~66,000 in the presence of the blocking peptide.

treatment of the cells with rHuEpo or Epo mimetic peptide (Figure 2B).

Next, we examined the expression of EpoR protein using Western blotting in clinical samples of cervical squamous cell carcinoma. All seven samples of carcinoma examined showed expression of a specific immunoreactive band at *M_r* ~66,000 corresponding to the EpoR protein (Figure 2C). Pretreatment of the antibody with the EpoR-blocking peptide completely abolished the immunostaining. No EpoR expression was seen in the sample of benign squamous mucosa.

The majority of chemotherapy regimens used to treat patients with cervical carcinoma includes cisplatin.⁸⁵⁻⁸⁷ Given the potent anti-apoptotic effect of Epo^{46,52,53,56,59}, we examined whether EpoR signaling may inhibit cispla-

Figure 3. rHuEpo reverses toxicity of the chemotherapeutic drug *cis*-platin in cervical carcinoma cells. **A:** HeLa cells were treated with rHuEpo in the presence or absence of the general tyrosine kinase inhibitor genistein (10 μ mol/L) for 1 hour. Cells were then exposed to *cis*-platin (10 μ g/ml) for 20 hours. Cell viability was assessed using the MTT assay. Results show the fraction of surviving cells *versus* cells not treated with *cis*-platin. Epoetin α dose dependently inhibited *cis*-platin cytotoxicity and this effect was significantly diminished by genistein. **B:** *Cis*-platin-induced apoptosis in HeLa cells was examined by detection of membrane externalization of phosphatidylserine using the Annexin V assay. HeLa cells were pretreated with rHuEpo for 2 hours and then challenged with *cis*-platin (5 μ g/ml) for 48 hours. Results are expressed as percentage of the apoptotic cell population compared to the control. *Cis*-platin-induced apoptosis was dose dependently reversed by rHuEpo (*, $P < 0.05$).

tin-induced cytotoxicity and apoptosis in cervical carcinoma cells. We treated HeLa cells with cisplatin in the absence and presence of rHuEpo. Pretreatment of HeLa cells with rHuEpo showed a dose-dependent protective effect on HeLa cells treated with the chemotherapeutic agent cisplatin, as determined by the MTT assay (Figure 3A). Using the annexin V assay, ~10% of the cell killing caused by cisplatin was purely apoptotic, and rHuEpo dose dependently inhibited this apoptosis (Figure 3B).

Immunohistochemistry

To further investigate the potential role of Epo signaling in cervical squamous dysplasia and carcinoma we examined the expression of Epo and EpoR by immunohistochemistry in a series of clinical samples of cervical dysplasia and carcinoma and compared their expression with those of p16^{INK4a}, HIF-1 α , and bcl-2. The results of

the immunohistochemical assays are summarized in Table 2 and illustrated in Figures 4 and 5.

None of the 85 benign squamous epithelia showed expression of p16^{INK4a}, whereas strong nuclear and cytoplasmic immunostaining was seen in 11 of 17 CIN I, all CIN II and CIN III, and 14 of 15 ISCCs (Table 1 and Figures 4A and 5). The difference in p16^{INK4a} expression between benign squamous epithelia, CIN I, CIN II, CIN III, and ISCCs was highly significant (see Table 2). Immunofluorescence staining for p16^{INK4a} showed a similar staining pattern as seen for immunoperoxidase staining.

Cytoplasmic and/or membrane immunostaining for EpoR was present in all samples (Table 2, Figures 4B and 5). In benign squamous epithelia EpoR expression was confined to the basal and parabasal cell layers. In contrast, in squamous dysplasia EpoR expression increasingly appeared in the more superficial cell layers and was significantly increased compared to the adjacent benign epithelia ($P = 0.0002$ for CIN I, $P = 0.0003$ for CIN II, and $P < 0.0001$ for CIN III; Wilcoxon signed rank test). In CIN I and CIN II cases EpoR expression was seen in the basal 50% and 70% of the epithelial thickness, whereas in CIN III the full thickness of the dysplastic epithelia showed EpoR expression (Figure 6). The mean EpoR staining score increased with increasing grade of dysplasia (slope 51.72 ± 4.61 , $r^2 = 0.9844$, $P = 0.0078$, linear regression). Prominent EpoR expression was also present in the endothelial and smooth muscle cells of blood vessels (not shown). Immunofluorescence staining for EpoR resulted in a similar immunostaining pattern, including prominent labeling of endothelial and smooth muscle cells of blood vessels (Figure 6C).

Cytoplasmic granular Epo immunostaining was seen in 20 of 85 benign squamous epithelia and was restricted to the basal cell layer (Table 2, Figures 4C and 5). Epo immunostaining was significantly increased in squamous dysplasias compared to adjacent benign squamous epithelia ($P = 0.0313$ for CIN I, $P = 0.0469$ for CIN II, and $P = 0.0105$ for CIN III; Wilcoxon signed rank test). Epo immunostaining was present in 14 of 15 ISCCs, and was usually localized to small areas adjacent to tumor cell necrosis and keratinization, and to the infiltrating edge of tumors (Figure 7, A and B). Epo immunostaining was significantly increased adjacent to necrotic regions ($P = 0.0002$, Wilcoxon signed rank test) and areas of tumor cell keratinization ($P = 0.0039$, Wilcoxon signed rank test) (Table 3).

HIF-1 α immunostaining was predominantly nuclear, although in a few cases weak cytoplasmic reactivity was also observed. In benign squamous epithelia, HIF-1 α immunostaining was seen in the parabasal cells and in the middle third of the epithelium in 72 of 85 cases (Table 2 and Figures 4 and 5). Although overall HIF-1 α immunostaining was not significantly different in benign squamous epithelia, dysplasias, and ISCCs (Table 2), when compared to the respective benign squamous epithelia, HIF-1 α expression was significantly increased in all grades of dysplasia ($P = 0.0156$ for CIN I, $P = 0.0215$ for CIN II, and $P = 0.0139$ for CIN III; Wilcoxon signed rank test). In addition, in dysplastic epithelia HIF-1 α immunostaining was not restricted to the middle third of the

Table 2. Expression of p16, EpoR, Epo, HIF-1 α , and bcl-2 in Benign Squamous Epithelium, Squamous Dysplasia, and Invasive Squamous Cell Carcinoma

	Benign (n = 85)	CIN I (n = 17)	CIN II (n = 23)	CIN III (n = 30)	ISCC (n = 15)
P16*					
No. pos	0	11	23	30	14
Median	0	120	188	300	300
Mean $\pm$ SEM	0 $\pm$ 0	105.0 $\pm$ 32.3	186.8 $\pm$ 14.5	262.5 $\pm$ 9.4	245.4 $\pm$ 27.2
P†	<0.0001				
EpoR*					
No. pos	85	17	23	30	15
Median	30	80	115	180	175
Mean $\pm$ SEM	27.7 $\pm$ 1.3	90.7 $\pm$ 1.2	123 $\pm$ 11.4	189.3 $\pm$ 8.6	171.8 $\pm$ 5.5
P†	<0.0001				
Epo*					
No. pos	20	10	9	16	14
Median	0	5	0	11	40
Mean $\pm$ SEM	4.3 $\pm$ 2.2	18.2 $\pm$ 6.9	12.4 $\pm$ 4.8	31.6 $\pm$ 8.0	52.8 $\pm$ 14.1
P†	<0.0001				
HIF-1 α *					
No. pos	72	14	19	26	12
Median	45	55	40	60	65
Mean $\pm$ SEM	48.5 $\pm$ 4.6	80.5 $\pm$ 17.2	66.4 $\pm$ 14.6	81.6 $\pm$ 13.2	62.9 $\pm$ 16.3
P†	0.2885				
Bcl-2†					
No. pos	81	13	18	28	7
Median	5	8	4	20	0
Mean $\pm$ SEM	4.4 $\pm$ 0.3	11.1 $\pm$ 2.7	8.9 $\pm$ 3.5	26.8 $\pm$ 4.6	11.9 $\pm$ 7.8
P†	<0.0001				

EpoR, erythropoietin receptor; Epo, erythropoietin; HIF-1 α , hypoxia inducible factor-1 α .

*Results for p16, EpoR, Epo, and HIF-1 α are expressed as immunostaining score.

†Results for bcl-2 are expressed as percentage of positively staining cells.

*Kruskal-Wallis test followed by Dunn's post-test.

epithelium as in benign cases (normal staining), but it was also present in the basal and/or the superficial portions (abnormal staining) (Figure 8). The difference in the distribution of immunostaining (normal *versus* abnormal) in benign and dysplastic epithelia was highly significant ($P < 0.0001$, Fisher's exact test). In invasive carcinomas, HIF-1 α expression was seen in 12 of 15 cases: expression was focal (less than 50% of tumor cells) in 10 cases, whereas in 2 cases it was expressed diffusely in the majority of the tumor cells. When focal, HIF-1 α immunostaining was concentrated to areas surrounding necrotic regions, tumor cell keratinization and the infiltrating edge of tumors (Figure 7, C and D). HIF-1 α immunostaining was significantly increased adjacent to necrotic regions ($P = 0.001$, Wilcoxon signed rank test) and areas of tumor cell keratinization ($P = 0.0156$, Wilcoxon signed rank test) (Table 3).

In benign squamous epithelia, cytoplasmic bcl-2 staining was found in 81 of 85 cases and was sharply restricted to the basal cell layer (Table 2, Figure 5). In CIN I and CIN II, cytoplasmic bcl-2 staining appeared in the more superficial cell layers, however, the percentage of positively staining cells did not differ significantly compared to benign epithelia ($P = 0.125$ for CIN I and $P = 0.1094$ for CIN II, Wilcoxon signed rank test). In contrast, in cases of CIN III bcl-2 immunostaining not only extended to the superficial cell layers, but the percentage of positively staining cells was significantly increased as well ($P = 0.0007$, Wilcoxon signed rank test). Bcl-2 ex-

pression was present in 7 of 15 ISCCs, whereas no bcl-2 staining was seen in the rest of the cases.

EpoR expression showed a highly significant correlation with the expression of p16^{INK4a} ($r = 0.8644$, $P < 0.0001$, Spearman's test) (Figure 9A), Epo ($r = 0.4801$, $P < 0.0001$, Spearman's test, not shown), and bcl-2 ($r = 0.4632$, $P < 0.0001$, Spearman's test) (Figure 9B). Double-immunofluorescence staining showed co-expression of EpoR and p16^{INK4a} in squamous dysplasias and invasive carcinomas (Figure 10). Double-immunohistochemical staining also showed co-expression of Epo and HIF-1 α in invasive carcinomas (Figure 7, E and F).

Cytological Samples of Cervical Squamous Dysplasia

Cytoplasmic EpoR immunostaining was seen in benign squamous cells in 2 of 39 cases. In contrast, dysplastic squamous cells showed strong cytoplasmic EpoR expression in all 16 high-grade squamous intraepithelial lesion and 4 of 5 low-grade squamous intraepithelial lesion cases (Figure 11). The difference in EpoR expression between benign and dysplastic squamous cells was highly significant ($P < 0.0001$, Fisher's exact test). Double-immunofluorescence staining showed co-expression of EpoR and p16^{INK4a} in dysplastic squamous cells, whereas benign squamous cells showed no staining with either antibody (Figure 11).

Figure 4. Expression of p16^{INK4a} (p16) (A), EpoR (B), Epo (C), and HIF-1 α (D) in benign cervical squamous epithelium, cervical dysplasia (CIN, grades I to III), and ISCC, determined by immunohistochemistry. Bars indicate median immunostaining score values.

Discussion

The major finding of the current study is the demonstration of hypoxia-inducible, functional Epo signaling in human cervical squamous cell carcinomas. We have shown that HeLa and SiHa human cervical carcinoma cells express Epo receptor (EpoR) mRNA and protein. Stimulation of the tumor cells with rHuEpo resulted in activation of tyrosine phosphorylation, indicating that EpoR expressed by the cells is biologically active. In clinical cytological and biopsy samples of cervical squamous dysplasia and ISCC we found significantly increased immunohistochemical EpoR expression compared to benign squamous epithelium. In squamous dysplasias EpoR expression increased with increasing severity of the lesions.

We found a highly significant correlation between the expression of EpoR and overexpression of p16^{INK4a} in the samples. Previous studies by Klaes and colleagues¹⁷ indicate that diffuse and strong overexpression of the p16^{INK4a} protein is observed in almost 100% of high-grade dysplastic cervical lesions, whereas normal cervical epithelium or inflammatory and metaplastic lesions were not stained. It was suggested that p16^{INK4a} is a specific marker for cells that express high-risk HPV E6/E7 oncogenes and retain the capacity to replicate. In our

study, the sensitivity, specificity, positive and negative predictive value of p16^{INK4a} overexpression for detecting squamous dysplasia (all grades combined) were 92.9%, 100%, 100%, and 93.4%, respectively. The corresponding values for the detection of high-grade squamous dysplasia (CIN II and CIN III) were 100%, 100%, 100%, and 100%, respectively. These results are in excellent agreement with those of Klaes and colleagues,¹⁷ and indicate that p16^{INK4a} overexpression can be used as a useful diagnostic tool in the detection of cervical dysplasia. The co-expression of EpoR and p16^{INK4a} in neoplastic squamous cells, also demonstrated by double-immunofluorescence staining, suggests that detection of increased EpoR expression may also serve as a useful diagnostic marker for neoplastic cervical squamous cells.

In contrast to breast^{64,65} and head and neck squamous cell carcinoma cells (Cohen et al, unpublished observations), HeLa and SiHa cervical carcinoma cells did not show Epo expression by RT-PCR analysis. However, focal Epo expression was detected by immunohistochemistry in a proportion of benign and neoplastic squamous epithelia. Epo immunostaining was focal and restricted to a small minority of the cells in most cases. In

Figure 5. Immunohistochemical expression of p16^{INK4a} (p16), EpoR, Epo, HIF-1 α , and bcl-2 in benign cervical squamous epithelium (benign), low-grade squamous dysplasia (CIN I), high-grade squamous dysplasia (CIN III), and ISCC (immunohistochemical stains with hematoxylin counterstain; H&E).

benign squamous epithelia, when present, Epo staining was strictly restricted to the basal cell layer. Epo expression was significantly increased in squamous dysplasia compared to benign epithelia. Focal Epo expression was found in most ISCC cases, with the immunostaining localized to small areas surrounding necrotic regions, areas of tumor cell keratinization, and to the infiltrating edge of tumors.

Many hypoxia-inducible genes, including that of Epo, are controlled by the heterodimeric transcription factor HIF-1. Distribution of Epo immunostaining was very similar to that of the HIF-1 α subunit in the cervical tumors and co-expression of Epo and HIF-1 α was also demonstrated by double-immunohistochemical stains, consistent with the well-known regulation of Epo expression by HIF-1.^{39,43,44,46,88} It is thought that the principal determinant of HIF-1 α accumulation in tumor cells is tissue hypoxia.⁸⁹ In human cervical carcinoma xenografts a positive correlation between HIF1- α expression and oxygenation status (as determined by EF-5

binding) was found,⁹⁰ suggesting that HIF-1 α expression can be used as a surrogate marker in paraffin-embedded tissue specimens for the detection of hypoxic tumor areas. Indeed, immunostaining for this protein was mainly encountered adjacent to necrotic regions at some distance from tumor vessels and at the infiltrating edge of cancers, areas thought to represent the most hypoxic parts of solid tumors.^{30,91,92}

However, in two cases HIF-1 α immunoreactivity was observed diffusely throughout the entire tumor tissue, a finding that may be because of alternative regulatory modes of HIF-1 α expression. It has been demonstrated that HIF-1 α expression can in fact be induced by signals other than hypoxia. Mutations that activate oncogenes (v-src, ras) and those that inactivate tumor suppressor genes (PTEN, VHL, p53) result in increased HIF-1 α expression.⁹³ Thus, although increased HIF-1 α can be attributed in part to tumor hypoxia, the basal level of HIF-1 α expression in tumor cells may also be markedly in-

Figure 6. Expression of EpoR increases with increasing severity of dysplasia in cervical squamous epithelia. **A:** Immunohistochemical expression of EpoR in benign squamous epithelium and CIN grades I, II and III. In benign squamous epithelium EpoR expression is seen only in the basal one or two cell layers. With increasing severity of dysplasia EpoR immunostaining appears in the more superficial cell layers, and in CIN III, EpoR immunostaining is present in full thickness of the epithelium (immunohistochemical stains for EpoR with hematoxylin counterstain; H&E). **B:** EpoR expression increases with increasing severity of dysplasia in cervical squamous epithelia. Data are mean values $\pm$ SEM of percent epithelium thickness showing EpoR expression. **C:** Strong EpoR expression in endothelial and smooth muscle cells of a small artery and a lymphatic channel (arrowhead) (immunofluorescence staining for EpoR, tetramethyl-rhodamine isothiocyanate, red; nuclei are stained with 4,6-diamidino-2-phenylindole, blue; original magnification, $\times 400$).

creased as a consequence of somatic mutations that activate oncogene products or inactivate tumor suppressors.⁹³ The increased and abnormal HIF-1 α expression observed in squamous dysplasias may also be because of similar mechanisms, such as inactivation of p53 by the HPV E6 protein.^{10,12} Our results and previous reports of increased HIF-1 α expression in other early neoplastic lesions (such as ductal carcinoma *in situ* of the breast, colonic adenoma, and prostatic intraepithelial neoplasia),⁹² suggest that increased HIF-1 α expression can occur early in tumor progression.

In contrast to the focal and heterogeneous staining for Epo, EpoR immunostaining was diffuse and uniform throughout the tumor tissue, with further accentuation near necrotic areas. These findings are similar to those we previously described in human breast carcinomas,⁶⁵ and suggest that although hypoxia induces EpoR expression, other oncogenic mechanisms are likely to play a role in increased EpoR expression in neoplastic cervical squamous cells.

Recently, binding of the hypoxia marker pimonidazole to suprabasal cells in normal epithelia was described and

it was hypothesized that hypoxia may act as a morphogen, inducing terminal differentiation of cells.⁹⁴ Our observation of prominent HIF-1 α expression in the suprabasal cells of benign squamous epithelia also supports this hypothesis. This phenomenon appears to have a counterpart in squamous cell carcinoma, in which squamous differentiation was constantly seen in tumor areas several cell layers remote from adjacent vessels.³¹ Our observation, that in squamous cell carcinomas HIF-1 α expression can be consistently found adjacent to areas of squamous differentiation with keratinization, also suggest a potential role for HIF-1 α in the differentiation of neoplastic squamous cells. In dysplastic squamous epithelia HIF-1 α expression was not only increased, but also showed an abnormal distribution compared to benign epithelia, suggesting a potential role in the disturbed maturation of dysplastic squamous cells. At present, the molecular mechanisms of hypoxia induced terminal differentiation are primarily unknown.

Although the fundamental role of high-risk HPV infection in the pathogenesis of cervical carcinoma is well established, other (both genetic and microenvironmental)

Figure 7. Expression of Epo and HIF-1 α in cervical squamous cell carcinomas show correlation in spatial distribution. Prominent Epo (A) and HIF-1 α (C) expression are present in tumor cells adjacent to necrotic areas (asterisk). Epo (B) and HIF-1 α (D) expression is also present adjacent to tumor areas showing tumor cell keratinization. Double-immunohistochemical stains (E and F) show co-expression of Epo (cytoplasmic staining, red) and HIF-1 α (nuclear staining, brown) in cervical carcinomas adjacent to necrotic areas (asterisk) (immunohistochemical stains with hematoxylin counterstain).

factors are thought to play a role in cervical carcinogenesis.^{7,10} The anti-apoptotic protein bcl-2 is expressed in the basal cells of cervical squamous epithelia and is thought to reflect the stem cell function of this compartment. Previous studies have demonstrated increased bcl-2 expression and decreased apoptosis in cervical

squamous dysplasias. With the progression of CIN, increasing levels of bcl-2 immunoreactivity were observed: the number of bcl-2-positive cells increased and immunostaining became increasingly apparent in the higher cell compartments.^{23–25,95} Our current findings of increased bcl-2 expression in dysplastic squamous epithe-

Table 3. Erythropoietin (Epo) and Hypoxia-Inducible Factor 1- α (HIF) Immunostaining in Invasive Squamous Cell Carcinoma of the Cervix in Areas of Tumor Cell Necrosis and Keratinization

	Epo immunostaining			HIF immunostaining		
	Mean $\pm$ SEM	Median	<i>P</i> *	Mean $\pm$ SEM	Median	<i>P</i> *
Necrosis						
+	60.4 $\pm$ 6.7	60.0	0.0002	106.3 $\pm$ 24.3	107.5	0.001
-	10.0 $\pm$ 2.3	5.0		19.2 $\pm$ 12.0	10.0	
Keratinization						
+	45.0 $\pm$ 5.0	50.0	0.0039	133.1 $\pm$ 32.8	137.5	0.0156
-	5.0 $\pm$ 1.2	5.0		31.9 $\pm$ 25.5	10.0	

*Wilcoxon signed rank test.

lia are in agreement with the results of these previous studies.

In erythroblasts EpoR activation results in the stimulation of proliferation, leads to increased expression of the anti-apoptotic proteins bcl-2 and bcl-X_L,^{57,58} and inhibition of apoptosis.^{46,52-54,56,59,96} Because EpoR expressed by human cervical carcinoma cells seems to be functional, our results suggest that via similar mechanisms, activation of EpoR expressed in dysplastic and neoplastic cervical squamous cells may up-regulate bcl-2 expression and reduce apoptotic potential. This hypothesis is also supported by the significant positive correlation between EpoR and bcl-2 expression in cervical squamous dysplasias.

Experimental data suggest that one of the major modes of action of most anti-cancer treatment methods, including radiation and chemotherapy, may be via activation of apoptosis in sensitive cells,^{19,97-104} and the tendency of a cancer cell to undergo apoptosis may have important implications for tumor progression and response to treatment.^{19,99,100} Bcl-2 overexpression protects cancer cells from apoptotic cell death induced by a variety of stimuli, including radiation and most cytotoxic drugs.¹⁰⁴⁻¹¹⁰

In erythroblasts and neurons the major action of Epo is inhibition of apoptosis.^{46,52-54,56,59,75,96} We have shown that Epo dose dependently inhibits the cytotoxic effect and apoptosis induced by the chemotherapeutic drug

cisplatin in cervical carcinoma cells, providing further evidence that Epo signaling is biologically active in cervical cancer cells. Because human cervical carcinoma cells express functional EpoR, we hypothesize that the action of Epo in cervical cancer cells is likely mediated by mechanisms similar to those described in erythroblasts and neurons. Although we have found increased bcl-2 expression in approximately half of the cervical carcinomas examined, the number of ISCC cases included in this study did not allow a meaningful analysis of a possi-

Figure 8. Distribution of HIF-1 α immunostaining in benign and dysplastic cervical squamous epithelia. Basal, mid, and upper designate the basal, mid, and upper thirds of the squamous epithelium. Each bar represents one case and indicates the presence of immunostaining in the respective portion of the squamous epithelium. Note that in benign squamous epithelia HIF-1 α immunostaining is primarily restricted to the suprabasal, middle portion of the epithelium. In contrast, in most cases of dysplasia HIF-1 α immunostaining showed an abnormal distribution and was present in the basal and/or more superficial epithelial cells as well.

Figure 9. Correlation of immunohistochemical expression of EpoR with p16^{INK4a} (p16) and bcl-2 in benign and dysplastic cervical squamous epithelia. **A:** The level of EpoR expression shows positive correlation with the expression of p16 ($r = 0.8644$, $P < 0.0001$, Spearman's test). The line represents the calculated regression line. **B:** The level of EpoR expression shows positive correlation with the expression of bcl-2 ($r = 0.4632$, $P < 0.0001$, Spearman's test). The line represents the calculated regression line.

Figure 10. Cells of cervical squamous dysplasia (CIN III) and ISCC show co-expression of EpoR (red) and p16^{INK4a} (p16, green). Note that benign squamous epithelium (asterisk) shows weak EpoR expression in the basal cell layers, but no expression of p16^{INK4a}. (Double-immunofluorescence stain for EpoR, tetramethyl-rhodamine isothiocyanate, red, and p16^{INK4a}, fluorescein isothiocyanate, green; nuclei are stained with 4,6-diamidino-2-phenylindole, blue; H&E.)

ble correlation between Epo and EpoR, and bcl-2 expression in the tumors.

A recent study by Westphal and colleagues¹¹¹ suggested that although various cancer cell lines expressed

EpoR, Epo signaling was not biologically active, and Epo was not essential for the growth of the cells in culture. The findings reported in this study are in contrast with several other reports of biologically active Epo signaling in ma-

Figure 11. Expression of EpoR and p16^{INK4a} in dysplastic cervical squamous cells in SurePath liquid-based cervicovaginal cytological preparations. **A, B, and E:** Papanicolaou-stained preparations showing high-grade squamous intraepithelial lesion and background benign squamous cells. **C and D:** Cells of high-grade squamous intraepithelial lesion show prominent EpoR expression, whereas no immunostaining is seen in benign squamous cells (immunohistochemical stain for EpoR with hematoxylin counterstain). **F to J:** Cells of high-grade squamous intraepithelial lesion co-express p16^{INK4a} (**F and I**, green), and EpoR (**G and J**, red). **H:** Triple band pass filter image showing the co-expression of both proteins in the same cells (yellow). (Double-immunofluorescence stain for EpoR, tetramethyl-rhodamine isothiocyanate, red, and p16^{INK4a}, fluorescein isothiocyanate, green; nuclei are stained with 4,6-diamidino-2-phenylindole, blue.)

lignant cells, including cells of erythroleukemia,⁷⁸ renal cell carcinoma,⁷⁹ breast cancer,^{64,112} and cancers of the female genital tract.¹¹³ The lack of biological activity of Epo in the study of Westphal and colleagues¹¹¹ may be because of methodological differences or differences in the cell lines used.

In solid tumors heterogeneously distributed areas with very low-oxygen partial pressures exist, predominantly because of a deteriorating diffusion geometry, severe structural abnormalities of tumor microvessels, disturbed microcirculation, and tumor-related anemia.^{30,32} Tumor hypoxia has been traditionally considered to be a therapeutic problem because it makes solid tumors resistant to radiation and chemotherapy.²⁷ Patients with hypoxic tumors have a significantly shorter recurrence-free and overall survival, and tumor oxygenation has been shown to be the strongest independent prognostic factor in cervix cancers followed by International Federation of Gynecology and Obstetrics (FIGO) stage.^{33,34,37,38} Importantly, disadvantage in outcome for hypoxic tumors is independent of the mode of primary treatment (radiation or radical surgery). Thus, it was suggested that tumor hypoxia may not just counteract oxygen-dependent therapy forms, but, through clonal selection and genomic and proteomic changes, it may also increase aggressiveness and advance tumor progression *per se*.^{31–33}

One insight into why low-oxygen conditions may affect the aggressiveness of cervical carcinomas is that *in vitro* hypoxia initiates the induction of apoptosis in HPV-infected, oncogenically transformed cells¹¹⁴ and acts to select for populations of cells with reduced apoptotic sensitivity.¹¹⁵ Kim and colleagues¹¹⁴ showed that *in vitro*, low-oxygen conditions can select for populations of HPV-infected cervical epithelial cells that have lost their sensitivity to hypoxia-induced apoptosis, a situation that may be reflected in the malignant progression of cervical carcinomas. This hypoxia-induced selection hypothesis is also consistent with the lack of apoptotic sensitivity of cervical carcinoma cell lines to low-oxygen conditions.¹¹⁴ Although at present, the mechanism of hypoxia-induced loss of apoptotic sensitivity during cell culture is unclear, evidence suggests that transformed cells that possess mutations in their apoptotic program because of inactivation of p53 or overexpression of anti-apoptotic genes have a survival advantage in a low-oxygen environment.¹¹⁴ The secondary alterations in the cellular apoptotic programs that make HPV-infected cervical epithelial cells refractory to hypoxia-induced apoptosis appear to be independent or downstream of p53, and it was suggested that genes such as bcl-2 may play a more important role in the development of their reduced apoptotic sensitivity.¹¹⁴ In fact, studies indicate that bcl-2 protein levels are increased in both CIN²⁴ and in cervical carcinoma cell lines.¹¹⁶ Our current results suggest that stimulation of EpoR signaling by hypoxia may be an important mechanism leading to increased bcl-2 expression and decreased apoptotic potential in cervical cancer cells. Further studies are needed to confirm this hypothesis.

Anemia has been a well-recognized complication of cancer and cancer treatment.¹¹⁷ One important consequence of anemia is the resulting tumor hypoxia.¹¹⁸ Al-

though the relationship among anemia, hypoxia, transfusion, and treatment outcome is complex, anemia has traditionally been considered to be one of the most powerful prognostic factors in patients with cancer of the cervix.¹¹⁹ rHuEpo has been shown to effectively increase hemoglobin levels and is often used in patients receiving curative radio- and chemotherapy.^{120,121} However, Epo is a potent growth factor that may stimulate proliferation and inhibit apoptosis of EpoR-bearing tumor cells.^{64,79} Epo also stimulates proliferation and migration of vascular endothelial cells^{69,77} and augments angiogenesis.^{68,122–124} We have shown previously⁶⁴ and in the current study that the vasculature of solid tumors express EpoR and thus the possible detrimental effect of Epo on tumor growth may be further aggravated by its known angiogenic activity.^{69,77,122,123} Previous studies in renal cell carcinoma patients suggested that increased serum Epo levels in the absence of polycythemia carried a worse prognosis and indicated a higher incidence of progressive metastatic disease.¹²⁵ Furthermore, Epo administration to a patient with multiple myeloma may have caused further malignant transformation resulting in plasma cell leukemia called into question the safety of Epo treatment for patients with EpoR-expressing myeloma cells.¹²⁶

Although previous studies have indicated that patients treated by rHuEpo respond better to treatment and have better prognosis compared to untreated control patients with significantly lower hemoglobin levels,¹¹⁷ these studies only suggested that raising hemoglobin concentration and decreasing anemia probably have some beneficial effect in cancer patients. In addition, most of the studies have not been well controlled, and their results have recently been challenged.¹¹⁹ We are not aware of any study in the literature that compared response to curative radio/chemotherapy and outcome in cancer patients treated with rHuEpo with a control group of patients with tumors of similar type, size, grade, and treatment, whose hemoglobin levels were maintained at comparable levels by other means, such as transfusion. Currently, an international, multicenter, randomized trial is being developed to evaluate the effect of raising hemoglobin with rHuEpo *versus* blood transfusions in anemic patients with advanced cervical carcinoma who are receiving concurrent platinum/radiotherapy.¹²⁷

The cellular responses to Epo may collectively promote growth of EpoR-bearing tumors, and these actions may be further enhanced by either high endogenous Epo production or by exogenous Epo administration. Thus, until it is demonstrated that pharmacological doses of Epo lacks such trophic effects *in vivo*, we suggest that treatment of cancer patients with rHuEpo should probably be performed with some degree of caution.

In summary, we have shown that dysplastic and neoplastic human cervical squamous cells show increased expression of functional EpoR, which can be further enhanced by hypoxia. Increased expression of EpoR may lead to elevated levels of bcl-2 and decreased apoptosis in the tumor cells. Administration of Epo inhibited cisplatin-induced cytotoxicity and apoptosis in cervical cancer cells. Our results suggest that increased EpoR signaling may play an impor-

tant role in cervical carcinogenesis, tumor progression, and the aggressive behavior and therapy resistance of hypoxic cervical cancers.

References

- Rose PG: Chemoradiotherapy for cervical cancer. *Eur J Cancer* 2002, 38:270–278
- Advances in the treatment of cervical cancer. International Network for Cancer Treatment and Research, Brussels, Belgium, 20 April, 2001 Meeting
- Parkin DM, Pisani P, Ferlay J: Global cancer statistics. *CA Cancer J Clin* 1999, 49:31–64
- Parkin DM, Pisani P, Ferlay J: Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 1999, 80:827–841
- Greenlee RT, Murray T, Bolden S, Wingo PA: Cancer statistics, 2000. *CA Cancer J Clin* 2000, 50:7–33
- Wright TC, Ferenczy A, Kurman RJ: Carcinoma and other tumors of the cervix. *Blaustein's Pathology of the Female Genital Tract*. Edited by RJ Kurman. New York, Springer, 2002, pp 325–381
- Milde-Langosch K, Riethdorf S, Loning T: Association of human papillomavirus infection with carcinoma of the cervix uteri and its precursor lesions: theoretical and practical implications. *Virchows Arch* 2000, 437:227–233
- Schiffman MH, Brinton LA: The epidemiology of cervical carcinogenesis. *Cancer* 1995, 76:1888–1901
- Kurman RJ, Norris HJ, Wilkinson EJ: Tumors of the Cervix, Vagina, and Vulva. Washington DC, Armed Forces Institute of Pathology, 1992
- Park TW, Fujiwara H, Wright TC: Molecular biology of cervical cancer and its precursors. *Cancer* 1995, 76:1902–1913
- Munger K, Phelps WC, Bubbs V, Howley PM, Schlegel R: The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J Virol* 1989, 63:4417–4421
- Huibregtse JM, Scheffner M, Howley PM: A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. *EMBO J* 1991, 10:4129–4135
- Dyson N, Guida P, Munger K, Harlow E: Homologous sequences in adenovirus E1A and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins. *J Virol* 1992, 66:6893–6902
- Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day III RS, Johnson BE, Skolnick MH: A cell cycle regulator potentially involved in genesis of many tumor types. *Science* 1994, 264:436–440
- Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA: Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* 1994, 368:753–756
- Li Y, Nichols MA, Shay JW, Xiong Y: Transcriptional repression of the D-type cyclin-dependent kinase inhibitor p16 by the retinoblastoma susceptibility gene product pRb. *Cancer Res* 1994, 54:6078–6082
- Klaes R, Friedrich T, Spitkovsky D, Ridder R, Rudy W, Petry U, Dallenbach-Hellweg G, Schmidt D, von Knebel Doeberitz M: Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. *Int J Cancer* 2001, 92:276–284
- Sano T, Oyama T, Kashiwabara K, Fukuda T, Nakajima T: Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions. *Am J Pathol* 1998, 153:1741–1748
- Kerr JF, Winterford CM, Harmon BV: Apoptosis. Its significance in cancer and cancer therapy. *Cancer* 1994, 73:2013–2026
- Williams GT: Programmed cell death: apoptosis and oncogenesis. *Cell* 1991, 65:1097–1098
- Levine EL, Renehan A, Gossiel R, Davidson SE, Roberts SA, Chadwick C, Wilks DP, Potten CS, Hendry JH, Hunter RD: Apoptosis, intrinsic radiosensitivity and prediction of radiotherapy response in cervical carcinoma. *Radiother Oncol* 1995, 37:1–9
- Sheets EE, Yeh J: The role of apoptosis in gynaecological malignancies. *Ann Med* 1997, 29:121–126
- Dellas A, Schultheiss E, Holzgreve W, Oberholzer M, Torhorst J, Gudat F: Investigation of the Bcl-2 and C-myc expression in relationship to the Ki-67 labelling index in cervical intraepithelial neoplasia. *Int J Gynecol Pathol* 1997, 16:212–218
- Saegusa M, Takano Y, Hashimura M, Shoji Y, Okayasu I: The possible role of bcl-2 expression in the progression of tumors of the uterine cervix. *Cancer* 1995, 76:2297–2303
- ter Harmsel B, Kuijpers J, Smedts F, Jeunink M, Trimbos B, Ramaekers F: Progressing imbalance between proliferation and apoptosis with increasing severity of cervical intraepithelial neoplasia. *Int J Gynecol Pathol* 1997, 16:205–211
- Höckel M, Schlenger K, Knoop C, Vaupel P: Oxygenation of carcinomas of the uterine cervix: evaluation by computerized O2 tension measurements. *Cancer Res* 1991, 51:6098–6102
- Vaupel P, Kelleher DK, Höckel M: Oxygen status of malignant tumors: pathogenesis of hypoxia and significance for tumor therapy. *Semin Oncol* 2001, 28:29–35
- Vaupel P, Schlenger K, Knoop C, Höckel M: Oxygenation of human tumors: evaluation of tissue oxygen distribution in breast cancers by computerized O2 tension measurements. *Cancer Res* 1991, 51:3316–3322
- Moulder JE, Rockwell S: Hypoxic fractions of solid tumors: experimental techniques, methods of analysis, and a survey of existing data. *Int J Rad Oncol Biol Phys* 1984, 10:695–712
- Vaupel P, Kallinowski F, Okunieff P: Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer Res* 1989, 49:6449–6465
- Höckel M, Vaupel P: Biological consequences of tumor hypoxia. *Semin Oncol* 2001, 28:36–41
- Höckel M, Vaupel P: Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst* 2001, 93:266–276
- Höckel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P: Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res* 1996, 56:4509–4515
- Fyles AW, Milosevic M, Wong R, Kavanagh MC, Pintilie M, Sun A, Chapman W, Levin W, Manchul L, Keane TJ, Hill RP: Oxygenation predicts radiation response and survival in patients with cervix cancer. *Radiother Oncol* 1998, 48:149–156
- Brown JM, Giaccia AJ: The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. *Cancer Res* 1998, 58:1408–1416
- Dachs GU, Chaplin DJ: Microenvironmental control of gene expression: implications for tumor angiogenesis, progression, and metastasis. *Semin Rad Oncol* 1998, 8:208–216
- Höckel M, Knoop C, Schlenger K, Vorndran B, Bausmann E, Mitze M, Knapstein PG, Vaupel P: Intratumoral pO2 predicts survival in advanced cancer of the uterine cervix. *Radiother Oncol* 1993, 26:45–50
- Höckel M, Schlenger K, Höckel S, Vaupel P: Hypoxic cervical cancers with low apoptotic index are highly aggressive. *Cancer Res* 1999, 59:4525–4528
- Semenza GL: Hypoxia-inducible factor 1: control of oxygen homeostasis in health and disease. *Pediatr Res* 2001, 49:614–617
- Shweiki D, Itin A, Soffer D, Keshet E: Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 1992, 359:843–845
- Moritz KM, Lim GB, Wintour EM: Developmental regulation of erythropoietin and erythropoiesis. *Am J Physiol* 1997, 273:R1829–R1844
- Stopka T, Zivny JH, Stopkova P, Prchal JF, Prchal JT: Human hematopoietic progenitors express erythropoietin. *Blood* 1998, 91:3766–3772
- Jelkmann W: Biology of erythropoietin. *Clin Invest* 1994, 72:S3–S10
- Ebert BL, Bunn HF: Regulation of the erythropoietin gene. *Blood* 1999, 94:1864–1877
- Ratcliffe PJ, Ebert BL, Firth JD, Gleadle JM, Maxwell PH, Nagao M, O'Rourke JF, Pugh CW, Wood SM: Oxygen regulated gene expression: erythropoietin as a model system. *Kidney Int* 1997, 51:514–526
- Lacombe C, Mayeux P: The molecular biology of erythropoietin. *Nephrol Dial Transplant* 1999, 14:22–28
- Koury MJ, Bondurant MC: The molecular mechanism of erythropoietin action. *Eur J Biochem* 1992, 210:649–663
- Ilhe JN, Thierfelder W, Teglund S, Stravapodis D, Wang D, Feng J, Parganas E: Signaling by the cytokine receptor superfamily. *Ann NY Acad Sci* 1998, 865:1–9
- Ilhe JN, Witthuhn BA, Quelle FW, Yamamoto K, Silvennoinen O:

- Signaling through the hematopoietic cytokine receptors. *Annu Rev Immunol* 1995, 13:369–398
50. Ihle JN, Witthuhn BA, Quelle FW, Yamamoto K, Thierfelder WE, Kreider B, Silvennoinen O: Signaling by the cytokine receptor superfamily: JAKs and STATs. *Trends Biochem Sci* 1994, 19:222–227
51. Klingmuller U: The role of tyrosine phosphorylation in proliferation and maturation of erythroid progenitor cells—signals emanating from the erythropoietin receptor. *Eur J Biochem* 1997, 249:637–647
52. Wojchowski DM, Gregory RC, Miller CP, Pandit AK, Pircher TJ: Signal transduction in the erythropoietin receptor system. *Exp Cell Res* 1999, 253:143–156
53. Tilbrook PA, Klinken SP: The erythropoietin receptor. *Int J Biochem Cell Biol* 1999, 31:1001–1005
54. Tilbrook PA, Klinken SP: Erythropoietin and erythropoietin receptor. *Growth Factors* 1999, 17:25–35
55. Chin H, Nakamura N, Kamiyama R, Miyasaka N, Ihle JN, Miura O: Physical and functional interactions between Stat5 and the tyrosine-phosphorylated receptors for erythropoietin and interleukin-3. *Blood* 1996, 88:4415–4425
56. Miura Y, Miura O, Ihle JN, Aoki N: Activation of the mitogen-activated protein kinase pathway by the erythropoietin receptor. *J Biol Chem* 1994, 269:29962–29969
57. Silva M, Benito A, Sanz C, Prosper F, Ekhterae D, Nunez G, Fernandez-Luna JL: Erythropoietin can induce the expression of bcl-x(L) through Stat5 in erythropoietin-dependent progenitor cell lines. *J Biol Chem* 1999, 274:22165–22169
58. Silva M, Grillot D, Benito A, Richard C, Nunez G, Fernandez-Luna JL: Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-XL and Bcl-2. *Blood* 1996, 88:1576–1582
59. Gobert S, Duprez V, Lacombe C, Gisselbrecht S, Mayeux P: The signal transduction pathway of erythropoietin involves three forms of mitogen-activated protein (MAP) kinase in UT7 erythroleukemia cells. *Eur J Biochem* 1995, 234:75–83
60. Vogt C, Pentz S, Rich IN: A role for the macrophage in normal hemopoiesis: III. In vitro and in vivo erythropoietin gene expression in macrophages detected by in situ hybridization. *Exp Hematol* 1989, 17:391–397
61. Masuda S, Okano M, Yamagishi K, Nagao M, Ueda M, Sasaki R: A novel site of erythropoietin production. Oxygen-dependent production in cultured rat astrocytes. *J Biol Chem* 1994, 269:19488–19493
62. Marti HH, Wenger RH, Rivas LA, Straumann U, Digicaylioglu M, Henn V, Yonekawa Y, Bauer C, Gassmann M: Erythropoietin gene expression in human, monkey and murine brain. *Eur J Neurosci* 1996, 8:666–676
63. Conrad KP, Benyo DF, Westerhausen-Larsen A, Miles TM: Expression of erythropoietin by the human placenta. *EMBO J* 1996, 10:760–768
64. Acs G, Acs P, Beckwith SM, Pitts RL, Clements E, Wong K, Verma A: Erythropoietin and erythropoietin receptor expression in human cancer. *Cancer Res* 2001, 61:3561–3565
65. Acs G, Zhang PJ, Rebbeck TR, Acs P, Verma A: Immunohistochemical expression of erythropoietin and erythropoietin receptor in breast carcinoma. *Cancer* 2002, 95:969–981
66. Juul SE, Zhao Y, Dame JB, Du Y, Hutson AD, Christensen RD: Origin and fate of erythropoietin in human milk. *Pediatr Res* 2000, 48:660–667
67. Yasuda Y, Fujita Y, Musha T, Tanaka H, Shiokawa S, Nakamatsu K, Mori S, Matsuo T, Nakamura Y: Expression of erythropoietin in human female reproductive organs. *Ital J Anat Embryol* 2001, 106: 215–222
68. Yasuda Y, Masuda S, Chikuma M, Inoue K, Nagao M, Sasaki R: Estrogen-dependent production of erythropoietin in uterus and its implication in uterine angiogenesis. *J Biol Chem* 1998, 273:25381–25387
69. Anagnostou A, Liu Z, Steiner M, Chin K, Lee ES, Kessimian N, Noguchi CT: Erythropoietin receptor mRNA expression in human endothelial cells. *Proc Natl Acad Sci USA* 1994, 91:3974–3978
70. Juul SE, Anderson DK, Li Y, Christensen RD: Erythropoietin and erythropoietin receptor in the developing human central nervous system. *Pediatr Res* 1998, 43:40–49
71. Fairchild Benyo D, Conrad KP: Expression of the erythropoietin receptor by trophoblast cells in the human placenta. *Biol Reprod* 1999, 60:861–870
72. Masuda S, Nagao M, Sasaki R: Erythropoietic, neurotrophic, and angiogenic functions of erythropoietin and regulation of erythropoietin production. *Int J Hematol* 1999, 70:1–6
73. Sasaki R, Masuda S, Nagao M: Erythropoietin: multiple physiological functions and regulation of biosynthesis. *Biosci Biotechnol Biochem* 2000, 64:1775–1793
74. Morishita E, Masuda S, Nagao M, Yasuda Y, Sasaki R: Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. *Neurosci* 1997, 76:105–116
75. Siren AL, Knerlich F, Poser W, Gleiter CH, Bruck W, Ehrenreich H: Erythropoietin and erythropoietin receptor in human ischemic/hypoxic brain. *Acta Neuropathol* 2001, 101:271–276
76. Yamaji R, Okada T, Moriya M, Naito M, Tsuruo T, Miyatake K, Nakano Y: Brain capillary endothelial cells express two forms of erythropoietin receptor mRNA. *Eur J Biochem* 1996, 239:494–500
77. Anagnostou A, Lee ES, Kessimian N, Levinson R, Steiner M: Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. *Proc Natl Acad Sci USA* 1990, 87:5978–5982
78. Mitjavila MT, Le Couedic JP, Casadevall N, Navarro S, Villeval JL, Dubart A, Vainchenker W: Autocrine stimulation by erythropoietin and autonomous growth of human erythroid leukemic cells in vitro. *J Clin Invest* 1991, 88:789–797
79. Westenfelder C, Baranowski RL: Erythropoietin stimulates proliferation of human renal carcinoma cells. *Kidney Int* 2000, 58:647–657
80. Assandri R, Egger M, Gassmann M, Niggli E, Bauer C, Forster I, Gorlach A: Erythropoietin modulates intracellular calcium in a human neuroblastoma cell line. *J Physiol* 1999, 516:343–352
81. Wang GL, Semenza GL: Purification and characterization of hypoxia-inducible factor 1. *J Biol Chem* 1995, 270:1230–1237
82. Juul SE, Yachnis AT, Christensen RD: Tissue distribution of erythropoietin and erythropoietin receptor in the developing human fetus. *Early Hum Dev* 1998, 52:235–249
83. Kim MJ, Bogie L, Cheung CY, Brace RA: Placental expression of erythropoietin mRNA, protein and receptor in sheep. *Placenta* 2001, 22:484–489
84. Ikegaya N, Yamamoto T, Takeshita A, Watanabe T, Yonemura K, Miyaji T, Ohishi K, Furuhashi M, Maruyama Y, Hishida A: Elevated erythropoietin receptor and transforming growth factor-beta1 expression in stenotic arteriovenous fistulae used for hemodialysis. *J Am Soc Nephrol* 2000, 11:928–935
85. Basil JB, Horowitz IR: Cervical carcinoma: contemporary management. *Obstet Gynecol Clin North Am* 2001, 28:727–742
86. Grigsby PW, Herzog TJ: Current management of patients with invasive cervical carcinoma. *Clin Obstet Gynecol* 2001, 44:531–537
87. Keys HM, Bundy BN, Stehman FB, Muddersbach LI, Chafe WE, Suggs III CL, Walker JL, Gersell D: Cisplatin, radiation, and adjuvant hysterectomy compared with radiation and adjuvant hysterectomy for bulky stage IB cervical carcinoma. *N Engl J Med* 1999, 340: 1154–1161
88. Wang GL, Semenza GL: Molecular basis of hypoxia-induced erythropoietin expression. *Curr Opin Hematol* 1996, 3:156–162
89. Aebbersold DM, Burri P, Beer KT, Laissue J, Djonov V, Greiner RH, Semenza GL: Expression of hypoxia-inducible factor-1alpha: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. *Cancer Res* 2001, 61:2911–2916
90. Vukovic V, Haugland HK, Nicklee T, Morrison AJ, Hedley DW: Hypoxia-inducible factor-1alpha is an intrinsic marker for hypoxia in cervical cancer xenografts. *Cancer Res* 2001, 61:7394–7398
91. Colpaert C, Vermeulen P, van Beest P, Goovaerts G, Weyler J, Van Dam P, Dirix L, Van Marck E: Intratumoral hypoxia resulting in the presence of a fibrotic focus is an independent predictor of early distant relapse in lymph node-negative breast cancer patients. *Histopathol* 2001, 39:416–425
92. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, Simons JW: Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res* 1999, 59:5830–5835
93. Semenza GL: Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. *Crit Rev Biochem Mol Biol* 2000, 35:71–103
94. Haroon ZA, Raleigh JA, Greenberg CS, Dewhirst MW: Early wound healing exhibits cytokine surge without evidence of hypoxia. *Ann Surg* 2000, 231:137–147
95. Ter Harsel B, Smedts F, Kuijpers J, Jeunink M, Trimbos B, Ramaekers F: BCL-2 immunoreactivity increases with severity of CIN: a study of normal cervical epithelia, CIN, and cervical carcinoma. *J Pathol* 1996, 179:26–30
96. Pallard C, Gouilleux F, Charon M, Groner B, Gisselbrecht S, Dus-

- anter-Fourt I: Interleukin-3, erythropoietin, and prolactin activate a STAT5-like factor in lymphoid cells. *J Biol Chem* 1995, 270:15942–15945
97. Hickman JA: Apoptosis induced by anticancer drugs. *Cancer Met Rev* 1992, 11:121–139
98. Makin G, Hickman JA: Apoptosis and cancer chemotherapy. *Cell Tissue Res* 2000, 301:143–152
99. Stephens LC, Ang KK, Schultheiss TE, Milas L, Meyn RE: Apoptosis in irradiated murine tumors. *Radiat Res* 1991, 127:308–316
100. Levine EL, Davidson SE, Roberts SA, Chadwick CA, Potten CS, West CM: Apoptosis as predictor of response to radiotherapy in cervical carcinoma. *Lancet* 1994, 344:472
101. Makin G: Targeting apoptosis in cancer chemotherapy. *Expert Opin Ther Targets* 2002, 6:73–84
102. Makin G, Dive C: Apoptosis and cancer chemotherapy. *Trends Cell Biol* 2001, 11:S22–S26
103. Zhivotovsky B, Joseph B, Orrenius S: Tumor radiosensitivity and apoptosis. *Exp Cell Res* 1999, 248:10–17
104. Verheij M, Bartelink H: Radiation-induced apoptosis. *Cell Tissue Res* 2000, 301:133–142
105. Sakakura C, Sweeney EA, Shirahama T, Igarashi Y, Hakomori S, Nakatani H, Tsujimoto H, Imanishi T, Ohgaki M, Ohyama T, Yamazaki J, Hagiwara A, Yamaguchi T, Sawai K, Takahashi T: Overexpression of bax sensitizes human breast cancer MCF-7 cells to radiation-induced apoptosis. *Int J Cancer* 1996, 67:101–105
106. Reed JC, Miyashita T, Takayama S, Wang HG, Sato T, Krajewski S, Aime-Sempe C, Bodrug S, Kitada S, Hanada M: BCL-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. *J Cell Biochem* 1996, 60:23–32
107. Kitada S, Krajewski S, Miyashita T, Krajewska M, Reed JC: Gamma-radiation induces upregulation of Bax protein and apoptosis in radiosensitive cells in vivo. *Oncogene* 1996, 12:187–192
108. Kokawa K, Shikone T, Otani T, Nakano R: Transient increases of apoptosis and Bax expression occurring during radiotherapy in patients with invasive cervical carcinoma. *Cancer* 1999, 86:79–87
109. Krajewski S, Blomqvist C, Franssila K, Krajewska M, Wasenius VM, Niskanen E, Nordling S, Reed JC: Reduced expression of proapoptotic gene BAX is associated with poor response rates to combination chemotherapy and shorter survival in women with metastatic breast adenocarcinoma. *Cancer Res* 1995, 55:4471–4478
110. Datta R, Manome Y, Taneja N, Boise LH, Weichselbaum R, Thompson CB, Slapak CA, Kufe D: Overexpression of Bcl-XL by cytotoxic drug exposure confers resistance to ionizing radiation-induced internucleosomal DNA fragmentation. *Cell Growth Differ* 1995, 6:363–370
111. Westphal G, Niederberger E, Blum C, Wollman Y, Knoch TA, Rebel W, Debus J, Friedrich E: Erythropoietin and G-CSF receptors in human tumor cells: expression and aspects regarding functionality. *Tumori* 2002, 88:150–159
112. Arcasoy MO, Amin K, Karayal AF, Chou SC, Raleigh JA, Varia MA, Haroon ZA: Functional significance of erythropoietin receptor expression in breast cancer. *Lab Invest* 2002, 82:911–918
113. Yasuda Y, Musha T, Tanaka H, Fujita Y, Fujita H, Utsumi H, Matsuo T, Masuda S, Nagao M, Sasaki R, Nakamura Y: Inhibition of erythropoietin signalling destroys xenografts of ovarian and uterine cancers in nude mice. *Br J Cancer* 2001, 84:836–843
114. Kim CY, Tsai MH, Osmanian C, Graeber TG, Lee JE, Giffard RG, DiPaolo JA, Peehl DM, Giaccia AJ: Selection of human cervical epithelial cells that possess reduced apoptotic potential to low-oxygen conditions. *Cancer Res* 1997, 57:4200–4204
115. Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ: Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* 1996, 379:88–91
116. Liang XH, Mungal S, Ayscue A, Meissner JD, Wodnicki P, Hockenbery D, Lockett S, Herman B: Bcl-2 protooncogene expression in cervical carcinoma cell lines containing inactive p53. *J Cell Biochem* 1995, 57:509–521
117. Littlewood TJ: The impact of hemoglobin levels on treatment outcomes in patients with cancer. *Semin Oncol* 2001, 28:49–53
118. Kelleher DK, Matthiensen U, Thews O, Vaupel P: Blood flow, oxygenation, and bioenergetic status of tumors after erythropoietin treatment in normal and anemic rats. *Cancer Res* 1996, 56:4728–4734
119. Fyles AW, Milosevic M, Pintilie M, Syed A, Hill RP: Anemia, hypoxia and transfusion in patients with cervix cancer: a review. *Radiother Oncol* 2000, 57:13–19
120. Littlewood TJ, Bajetta E, Nortier JW, Vercammen E, Rapoport B: Epoetin Alfa Study G: effects of epoetin alfa on hematologic parameters and quality of life in cancer patients receiving nonplatinum chemotherapy: results of a randomized, double-blind, placebo-controlled trial. *J Clin Oncol* 2001, 19:2865–2874
121. Thatcher N: Management of chemotherapy-induced anemia in solid tumors. *Semin Oncol* 1998, 25:23–26
122. Carlini RG, Alonzo EJ, Dominguez J, Blanca I, Weisinger JR, Rothstein M, Bellorin-Font E: Effect of recombinant human erythropoietin on endothelial cell apoptosis. *Kidney Int* 1999, 55:546–553
123. Carlini RG, Reyes AA, Rothstein M: Recombinant human erythropoietin stimulates angiogenesis in vitro. *Kidney Int* 1995, 47:740–745
124. Jaquet K, Krause K, Tawakol-Khodai M, Geidel S, Kuck K: Erythropoietin and VEGF exhibit equal angiogenic potential. *Microvasc Res* 2002, 64:326–333
125. Ljungberg B, Rasmuson T, Grankvist K: Erythropoietin in renal cell carcinoma: evaluation of its usefulness as a tumor marker. *Eur Urol* 1992, 21:160–163
126. Olujohungbe A, Handa S, Holmes J: Does erythropoietin accelerate malignant transformation in multiple myeloma? *Postgrad Med J* 1997, 73:163–164
127. Thomas G: The effect of hemoglobin level on radiotherapy outcomes: the Canadian experience. *Semin Oncol* 2001, 28:60–65