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Erythropoietin Receptors in Endometrial Carcinoma as Related to HIF1α and VEGF Expression

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Abstract. *Erythropoietin receptors (EpoRs) are expressed in a large percentage of cells in many human malignancies, including endometrial adenocarcinoma. In such tumors, administration of recombinant human erythropoietin (rhEpo) during radiotherapy and chemotherapy may oppose tumor progression by interfering with growth and invasion pathways. In the present study, a strong EpoR expression was demonstrated in 58.8% of 72 stage I endometrial adenocarcinomas, and this pattern was linked with a high degree of tumor differentiation (p=0.01), deep myometrial invasion (p=0.04) and, marginally, with poor prognosis (p=0.06). In addition, a strong association with the immunohistochemical expression of hypoxia-inducible factor 1α (HIF1α) and the downstream angiogenic protein vascular endothelial growth factor (VEGF) was noted. In multivariate analysis, HIF1α, but not EpoR, was associated with the depth of myometrial invasion (p=0.04) and marginally with prognosis (p=0.07). It is concluded that EpoR are common constituents of endometrial adenocarcinomas and are related to tumor aggressiveness, although this is probably a result of their involvement in an active HIF pathway.*

The rationale for investigating the role of erythropoietin receptors (EpoR) in cancer biology has been raised recently after a report on patients with head and neck cancer (HNC) receiving radiotherapy concurrently with recombinant human erythropoietin (rhEpo) (1). In that study, hemoglobin levels improved after administration of rhEpo but the prognosis of patients decreased, despite the expected good tumor response to radiation following the increased tumor oxygenation. Yet others failed to confirm these results (2). Further analysis of EpoR expression in HNC suggested that the adverse effect of rhEPO administration was, by and large, confined to patients with EpoR-positive tumors (3).

EpoRs are essential for erythroid progenitors in bone marrow as they provide the means by which these cells respond to erythropoietin and maintain a steady number of circulating erythrocytes. The levels of erythropoietin are regulated by hypoxia through a direct control of the hypoxia-inducible factors (HIFs) residing on the hypoxia response element of the Epo gene (4). Epo binds to EpoR, inducing conformational changes and phosphorylation of its intracellular domain; this, in turn, triggers a cascade of molecular events, such as Janus kinase-2 signaling, which stimulate proliferation and differentiation of the erythroid progenitor cells (5). The presence of EpoR in cancer cells, an apparent consequence of cellular dedifferentiation, may therefore serve similar pathways of growth and survival that may eventually enhance tumor aggressiveness.

In the present study, we investigated the expression of EpoR in human endometrial adenocarcinomas and assessed their possible association with various histopathological features, angiogenesis and the expression of vascular endothelial growth factor (VEGF) and the HIF1α gene. Moreover, the association between EpoR expression and prognosis was evaluated.

Materials and Methods

Formalin-fixed paraffin-embedded tissues from 72 patients with stage I endometrial adenocarcinoma of the endometrioid cell type and 20 samples from normally cycling endometrium of both proliferative and secretory phase were retrieved from the archives of the Department of Pathology, Democritus University of Thrace Medical School, Alexandroupolis, Greece. All patients had been treated surgically with total abdominal hysterectomy and bilateral salpingo-oophorectomy. No lymph node sampling of the iliac nodes was performed and N-staging was based on pelvic and abdominal computed tomography.

Histological typing and grading of the endometrial tumors (grade 1 *vs.* grade 2 and 3) and the depth of myometrial invasion (<1/2 *vs.* >1/2) were assessed on hematoxylin-eosin sections, using standard criteria. The follow-up of patients ranged from 6-161 months with a mean of 78 months.

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Table I. *Details of the antibodies, dilutions and antigen retrieval methods used in this study.*

Primary Antibody	Dilution (incubation time)	Antigen retrieval	Specificity	Source	Ref.
EpoR H-194	1:20 (Overnight)	MW	EpoR	Santa Cruz Biotechnology, USA	
ESEE 122	1:20 (90 min[a])	MW	HIF-1α	Oxford University	(11)
VG1	1:4 (90 min[a])	MW	VEGF	Oxford University	(12)
JC70 (CD31)	1:50 (60 min[a])	Protease XXIV	Endothelium	Dako, Denmark	(12)

[a]At room temperature; MW, microwave heating.

Immunohistochemistry. The EpoR status was detected immunohistochemically after using EpoR H-194 rabbit polyclonal antobody (sc-5624; Santa Cruz Biotechnology, Santa Cruz, CA, USA) raised against a recombinant protein corresponding to amino acids 21-214 mapping near the amino terminus of EpoR of human origin. The primary antibody was applied at 10 μg/ml overnight. Following washing with TBS, sections were incubated with a secondary mouse anti-rabbit antibody (Kwik Biotinylated Secondary, 0.69A; Shandon-Upshaw, Pittsburgh, PA, USA) for 15 min and washed in tris-buffered saline (TBS). Kwik Streptavidin peroxidase reagent (039A; Shandon-Upshaw) was applied for 15 min and sections were again washed in TBS. The color was developed by 15 min incubation with diaminobenzidine (DAB) solution and sections were weakly counterstained with hematoxylin. Normal immunoglobulin-G was substituted for the primary antibody as negative control at the same concentration as the primary antibody.

The same immunohistochemical procedure was also employed for investigating hypoxia and VEGF. Table I shows the primary antibodies used, with details on antigen retrieval and incubation time. Angiogenesis was assessed applying the alkaline-phosphatase/anti-alkaline-phosphatase staining protocol, as described elsewhere (6, 7).

EpoR and HIF1α were scored according to a grading system reported previously, which takes into account both the cytoplasmic and nuclear staining patterns (6). Thus, strong cytoplasmic reactivity in more than 50% of cancer cells and/or nuclear reactivity in more than 10% of cancer cells was scored as 'high' EpoR expression, while all other cases (including weak cytoplasmic or strong cytoplasmic in <50% of cells) was scored as 'low'.

Scoring for VEGF expression was based on assessing the percentage of cancer cells with cytoplasmic expression, following examination of the whole tumor area at ×200 magnification. The median value was used to score cases with low or high cytoplasmic reactivity (7).

Vessel counting was used for assessing vascular density (VD) on CD31-stained slides. Sections were first scanned at low power and subsequently at ×200 magnification in order to group cases into low, medium and high categories. Areas of highest vascularization were chosen along the invading tumor edge at low power field (×100), followed by vessel counting in three chosen fields (×200); the VD was the mean of the vessel counts obtained in these three fields. Vessels with a clearly defined lumen or well defined linear vessel shape, but not single endothelial cells, were taken into account for microvessel counting. The median value was used to define two groups of tumors: those with low *vs.* high VD.

Statistical analysis. Statistical analysis and graphic presentation were performed using the GraphPad Prism® 4.0 and the Instat® 3.0 packages (GraphPad, San Diego CA, USA, www.graphpad.com). Fisher's exact test or Yates' continuity-corrected chi-square test was used for testing relationships between categorical variables, as appropriate. Survival curves of patents were plotted using the method of Kaplan-Meier, and the log-rank test was used to determine statistical differences between life tables. A Cox proportional hazard model was used to assess the effects of patient and tumor variables on overall survival. A p-value ≤0.05 was considered significant.

Results

In the normal endometrium, EpoRs were expressed mainly in the cytoplasm of tumor cells and only occasionally in their nuclei, particularly during the secretory phase of the menstrual cycle. Cancer cases, however, showed a mixed cytoplasmic/nuclear pattern of staining (Figure 1), with the percentage of cancer cells revealing a strong cytoplasmic EpoR expression ranging from 0-90% (66th percentile 50%) and that of nuclear EpoR staining from 0-70% (66th percentile 30%). Using the aforementioned grading system that combines cytoplasmic and nuclear expression, 42/72 (58.3%) of endometrial adenocarcinoma cases had high EpoR reactivity.

The results for the remaining three immunohistochemical parameters were as follows: HIF1α expression: 34/72 (47.2%) endometrial adenocarcinomas examined were of high reactivity; VEGF expression: 28/72 (38.8%) tumors were of high VEGF reactivity; VD: 38/72 (52.7%) endometrial neoplasms in the series exhibited increased vascularity.

Analysis, according to histopathological variables, showed an inverse association of EpoR with histological grade (p=0.01). Thus, 10/30 (33.3%) tumors with low EpoR expression were grade 3 *vs.* 4/42 (9.5%) neoplasms with high EpoR reactivity. Moreover, a direct association between EpoR expression and deep myometrial invasion was noted (p=0.04): 14/30 (46.6%) carcinoma cases with low EpoR expression infiltrated beyond the inner half of the myometrium *vs.* 30/42 (71%) of malignancies with high EpoR expression. In multivariate analysis tumor grade, HIF1α and VEGF expression were independently linked with the depth of myometrial invasion (Table II).

Table III shows the association among immunohistochemical features. High EpoR reactivity was linked with high HIF1α (p=0.05) and high VEGF expression (p<0.0001). HIF1α and high VEGF expression were also linked (p=0.02). In addition, high VEGF expression was strongly linked with high VD (p=0.0006).

Kaplan-Meier disease-specific overall survival showed that all cancer patients with low EpoR expression were alive at 5 years after surgery *vs*. 81% of carcinoma cases with high EpoR expression (p=0.06). In a multivariate analysis, taking into account histological grade, depth of myometrial invasion and all the immunohistochemical parameters analyzed (Table II), high HIF1α expression was the only variable that approached independent prognostic significance (p=0.07, t-ratio=1.8).

Discussion

Erythropoietin, a 193 amino acid glycoprotein hormone released by the kidneys, regulates erythropoiesis by stimulating growth, preventing apoptosis and inducing differentiation of erythroid cell precursors (8). It exerts its activity on type-1 specific single-transmembrane 507 amino acid EpoRs mainly expressed in progenitor red blood cells, but also in megakaryocytes and endothelial cells (9). These receptors, whether of the full length or the truncated form, sustain erythopoiesis, in contrast to the soluble form which has a rather antagonist action competing with the full-length EpoR for binding to Epo. Erythropoietin, after binding to its receptors, induces homodimerization of EpoR on the cell surface and initiates the Janus kinase (JAK)2/signal transducer and activator of transcription 5 (STAT5) signal transduction cascade. Phosphorylation of EpoRs lead to their activation which results in nuclear translocation, binding to specific regulatory sites and activation of the transcription of target genes, leading to erythroid proliferation and differentiation (10). It is expected, therefore, that EpoR can be identified on the membrane, cytoplasm and the nuclei of normal and malignant cells having an activated Epo/EpoR pathway.

Given that EpoRs have a clear function in suppressing growth and apoptosis of hemopoietic progenitors and endothelial cells, it is reasonable to hypothesize that a similar function may bear cancer cells overexpressing EpoR. However, the studies of Henke *et al*. (1, 3) raised concerns as to whether tumor growth and/or tumor resistance to radiotherapy is induced by the exogenous administration of rhEpo, at least in HNC with high EpoR reactivity. Expression of Epo/EpoRs in such malignancies and their association with hypoxia pathways has been also investigated by Winters *et al*. (11). Indeed, EpoRs are widely expressed in a large percentage of human carcinomas. Pelekanou *et al*. found EpoR expression in 84% of breast carcinomas which were further linked with poor prognosis (12). Inhibition of apoptosis and promotion of tumor cell migration has also been reported in breast cancer (13, 14). Interestingly, a study by Acs *et al*. showed a hypoxia-stimulated expression of Epo/EpoRs in breast cancer cell lines, suggestive of a hypoxia-induced autocrine pathway with an anti-apoptotic function (15). That EpoR expression is associated with poor prognosis has been also reported for esophageal (16) and non-small cell lung cancer (17).

The expression of Epo/EpoR in normal and/or malignant endometrium was investigated in two previous studies. Our findings are in accordance with those of Yokomizo *et al*. who showed that Epo/EpoR expression is present in normal endometrium, reaching higher levels during the secretory phase, suggesting a role in the differentiation of the cycling endometrium (18). Acs *et al*. not only confirmed these findings but, in addition, showed an Epo/EpoR expression in the majority of endometrial carcinomas which was often intense and linked with adverse prognostic factors, such as advanced stage of disease, lymphovascular invasion, lymph node metastasis, loss of estrogen receptor (ER) expression and poor prognosis (19). In the present study, a strong EpoR expression in the cytoplasm and/or the nuclei of tumor cells was a feature in more than half of endometrial adenocarcinomas examined. This series of patients was homogeneous in the sense that it comprised only stage I endometrial tumors of the endometrioid cell type. Analysis according to the histopathological variables revealed that although EpoRs were associated with adenocarcinomas of high differentiation, in accordance with EpoR expression in the normal endometrium, their presence was associated with deep myometrial invasion. This makes EpoR an adverse prognostic indicator in endometrial carcinoma, as was further indicated by the trend for EpoRs to be associated with disease-specific death.

Epo and EpoR genes are under the direct control of hypoxia through stabilization of the HIF1α transcription factor that binds to the hypoxia-responsive element of the Epo gene (20, 21). Indeed, in our study a strong and direct association of EpoR with HIF1α and the expression of downstream HIF-regulated VEGF protein was noted. Its association, however, with tumor VD was only marginal, in contrast to the strong association noted between VEGF and tumor angiogenic activity. A multivariate analysis investigating the parameters linked to myometrial invasion and survival revealed an association with HIF1α, but not with EpoR, expression. This may indicate that the biological aggressiveness associated with EpoR-expressing carcinomas may not be a direct effect of the receptors themselves but rather an effect of other HIF-regulated downstream genes. Multivariate models of analysis which include the Epo/EpoR and the downstream genes may help to clarify the role of the Epo/EpoR pathway in tumor biology and answer the question of whether or not rhEpo administration is hazardous for patients undergoing radical radiotherapy or chemotherapy.

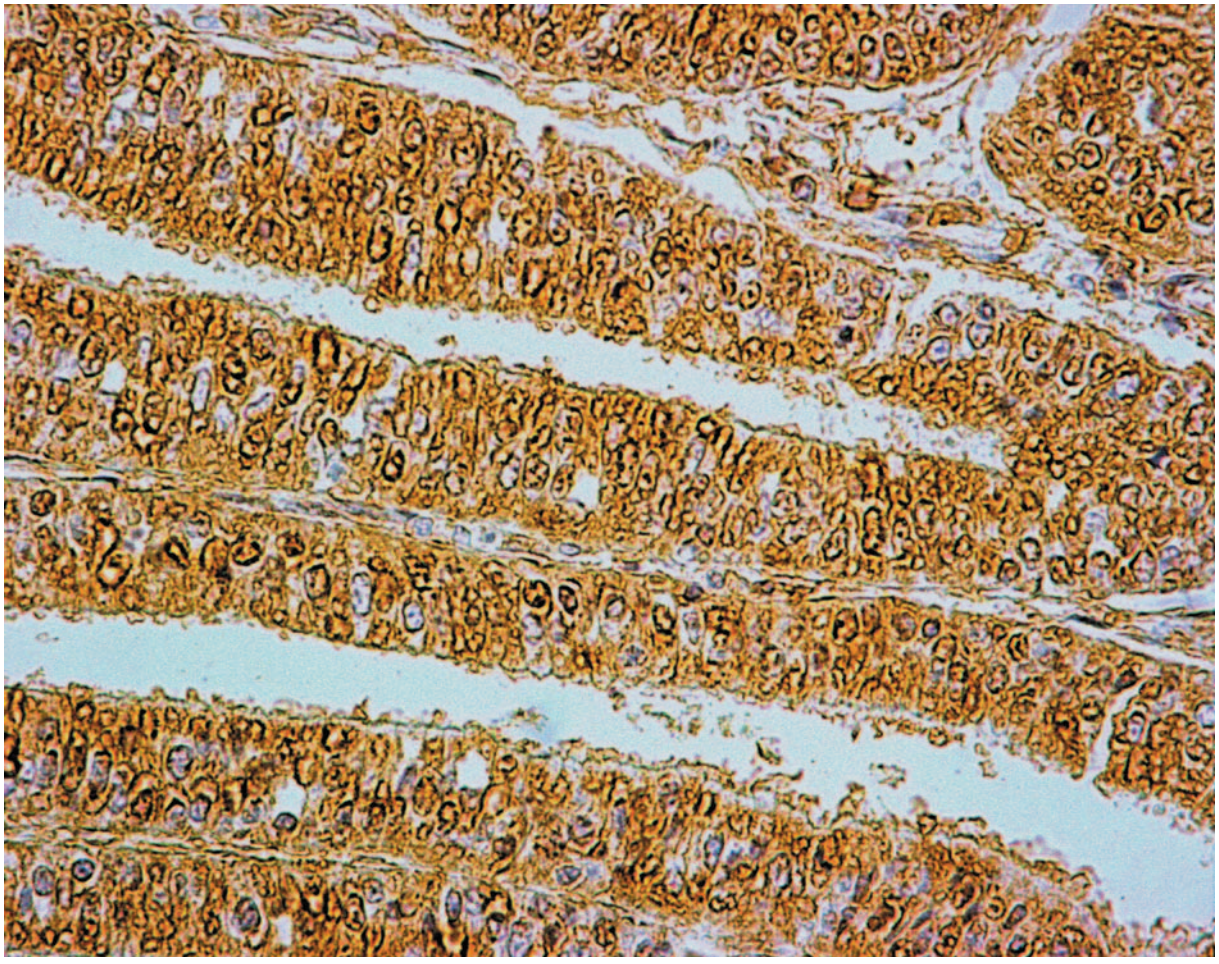

Figure 1. *Mixed nuclear/cytoplasmic pattern of EpoR expression in endometrial cancer.*

It is concluded that EpoRs are common constituents of stage I endometrial adenocarcinomas of the endometrioid cell type, and that EpoR-bearing endometrioid adenocarcinomas are clinically aggressive, even though this is probably related to an active HIF pathway rather than to EpoRs themselves.

Table II. *Multivariate analysis of the depth of myometrial invasion, histological grade and molecular variables as related to death events.*

	Invasion		Death events	
Variable	*t*-ratio	*P*-value	*t*-ratio	*P*-value
EpoR	1.05	0.29	0.25	0.79
VEGF	2.17	0.03	1.60	0.11
HIF1α	2.03	0.04	1.80	0.07
VD	0.71	0.47	1.16	0.24
Histological grade	2.81	0.006	0.64	0.52
Depth of myoinvasion	-	-	0.85	0.39

EpoR, Erythropoietin receptor; VEGF, vascular endothelial growth factor; HIF1α, hypoxia-inducible factor 1α; VD, vascular density.

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Table III. *Association among immunohistrochemical variables*.

	EpoR			HIF1α			VEGF		
Parameter	Low	High	*p*-Value	Low	High	*p*-Value	Low	High	*p*-Value
HIF1α									
Low	20	18	0.05						
High	10	24							
VEGF									
Low	28	16	<0.0001	28	16	0.02			
High	2	26		10	18				
VD									
Low	18	16	0.09	22	12	0.06	28	6	0.0006
High	12	26		16	22		16	22	

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