Erythropoietin and Erythropoietin Receptor Expression in Head and Neck Cancer: Relationship to Tumor Hypoxia

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ABSTRACT

Purpose: Erythropoietin, an oxygen-regulated glycoprotein hormone, is a hematopoietic cytokine that stimulates erythropoiesis by binding to its cellular receptor [erythropoietin receptor (EPOR)]. The recombinant form of human erythropoietin is used to prevent or treat anemia in cancer patients. However, in a recent randomized, placebo-controlled trial involving patients receiving curative radiotherapy for squamous cell carcinoma of the head and neck, erythropoietin treatment was associated with poorer locoregional progression-free survival. The purpose of our study was to determine whether EPOR and its ligand erythropoietin are expressed in primary head and neck cancer. We also investigated the hypothesis that erythropoietin expression in malignant cells may be associated with the presence of tumor hypoxia, an important factor involved in resistance to radiation treatment, tumor aggressiveness, and poor prognosis.

Experimental Design: Twenty-one patients received an i.v. infusion of the hypoxia marker pimonidazole hydrochloride before multiple tumor biopsies. Contiguous sections from 74 biopsies were analyzed by immunohistochemistry for EPOR and erythropoietin expression and pimonidazole binding.

Results: EPOR expression was present in tumor cells in 97% of the biopsies. Coexpression of erythropoietin was observed in 90% of biopsies. Erythropoietin and pimonidazole adduct staining did not always colocalize within tumors, but there was a significant positive correlation between levels of microregional erythropoietin expression and pimonidazole binding.

Conclusions: The coexpression of erythropoietin and EPOR in tumor cells suggests that erythropoietin may potentially function as an autocrine or paracrine factor in head and neck cancer. The expression of the hypoxia-inducible protein erythropoietin in tumor cells correlates with levels of tumor hypoxia.

INTRODUCTION

Erythropoietin, a 34-kDa glycoprotein, regulates RBC production by stimulating the proliferation, survival, and terminal differentiation of erythroid progenitor cells in the bone marrow. In contrast to other hematopoietic cytokines, erythropoietin behaves like a hormone and its physiologic production in the kidney is under the feedback control of an oxygen-sensing mechanism regulated by the binding of hypoxia-inducible factor-1 (1). The biological effects of erythropoietin are mediated by the specific binding of erythropoietin to its cognate transmembrane receptor [erythropoietin receptor (EPOR)] expressed on the surface of erythroid progenitor cells in the bone marrow (2–4) as well as several nonhematopoietic cell types, including vascular endothelial cells (5), cardiac myocytes (6), neurons (7, 8), and macrophages (9). Distinct from its regulatory function in erythropoiesis, erythropoietin exhibits diverse nonhematopoietic biological activities, such as the protection against anoxic injury in the brain (10) and cardiovascular system (6, 11) and the stimulation of physiologic angiogenesis in the female reproductive tract (12) and during wound healing (9).

The recombinant form of human erythropoietin (rHuEPO) has been widely used in clinical practice for the prevention or treatment of anemia associated with cancer and chemoradiation therapy (13). The benefits and safety of rHuEPO in anemic cancer patients leading to increased hemoglobin levels, decreased RBC transfusions, and improved quality of life have been documented in several large studies (14–16) as well as in a randomized, placebo-controlled study that showed a trend in overall survival favoring rHuEPO (17). In patients with head and neck cancer receiving chemotherapy, rHuEPO improved hemoglobin levels and reduced transfusion requirements (18–20). Previous studies have explored the incorporation of rHuEPO in curative treatment regimens to improve outcome of head and neck cancer patients. A retrospective clinical study found that rHuEPO administration during neoadjuvant chemoradiotherapy may improve tumor control and survival (21), consistent with the findings of several preclinical studies where erythropoietin was shown to modulate tumor hypoxia and increase radiation sensitivity in various animal models of human cancer (22–24). However, a recent double-blind, randomized, placebo-controlled multicenter trial found poorer locoregional progression-free survival in rHuEPO-treated patients with head and neck cancer compared with placebo despite correction of anemia (25). The latter prospective study was specifically designed to investigate the effect of rHuEPO on outcome for patients with head and neck cancer treated with radiotherapy only. It was noted, however, that the adverse outcomes may be associated with the study design that
allowed higher than recommended increases in hemoglobin in erythropoietin-treated patients (26). It was also speculated that underlying biological factors, such as potential direct effects of erythropoietin on tumors, may underlie the unexpected, unfavorable clinical results observed in the erythropoietin arm (25).

A series of recent studies from our laboratories and others have reported EPOR expression in various types of malignant human tumors and continuous cancer cell lines, including breast cancer (27–30), melanoma (31), renal cell carcinoma (32), gastric cancer (33), pediatric tumors (34), and uterine and ovarian carcinomas (35). In breast cancer cells and squamous cell carcinomas of the uterine cervix, coexpression of EPOR and its ligand erythropoietin was detected in primary tumors, suggesting that erythropoietin-EPOR may potentially play a role as an autocrine or paracrine growth factor in some malignancies (29, 36). In our studies of erythropoietin-EPOR expression and its biology in human cancer, we explored the expression of erythropoietin and EPOR protein in squamous cell carcinomas of the head and neck using immunohistochemistry. Furthermore, we tested the hypothesis that the expression of erythropoietin, an oxygen-regulated protein, in malignant head and neck tumors may be associated with the presence of tumor hypoxia, a factor that is associated with tumor aggressiveness, treatment resistance, and poor prognosis in squamous cell carcinomas of the head and neck (37–40).

Table 1  Clinical characteristics and mean weighed staining intensity scores for EPOR and erythropoietin expression in squamous cell carcinomas of the head and neck

<table>
<thead>
<tr>
<th>Tumor Site</th>
<th>Stage</th>
<th>Grade</th>
<th>Hemoglobin (g/dL)</th>
<th>Hematocrit (%)</th>
<th>EPOR</th>
<th>Erythropoietin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oropharynx</td>
<td>T1N0</td>
<td>2</td>
<td>12.2</td>
<td>38.5</td>
<td>150</td>
<td>10</td>
</tr>
<tr>
<td>Larynx</td>
<td>T3N0</td>
<td>3</td>
<td>13.6</td>
<td>41.7</td>
<td>62</td>
<td>20</td>
</tr>
<tr>
<td>Oropharynx</td>
<td>T1N0</td>
<td>2</td>
<td>15.4</td>
<td>43.3</td>
<td>23 ± 6</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Oral cavity</td>
<td>T1N2b</td>
<td>2</td>
<td>11.6</td>
<td>33</td>
<td>230 ± 11</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Oropharynx</td>
<td>T2N1</td>
<td>2</td>
<td>12.8</td>
<td>38</td>
<td>245</td>
<td>15</td>
</tr>
<tr>
<td>Larynx</td>
<td>T2N0</td>
<td>2</td>
<td>12.5</td>
<td>39.4</td>
<td>283 ± 3</td>
<td>32 ± 15</td>
</tr>
<tr>
<td>Hypopharynx</td>
<td>T3N1</td>
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<td>12.3</td>
<td>40</td>
<td>255 ± 17</td>
<td>46 ± 26</td>
</tr>
<tr>
<td>Oral cavity</td>
<td>T3N2</td>
<td>2</td>
<td>15.5</td>
<td>46.8</td>
<td>235 ± 3</td>
<td>46 ± 12</td>
</tr>
<tr>
<td>Larynx</td>
<td>T3N2</td>
<td>2</td>
<td>15.4</td>
<td>41</td>
<td>270 ± 4</td>
<td>17 ± 3</td>
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<td>Oral cavity</td>
<td>T2N1</td>
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<td>13.5</td>
<td>41.1</td>
<td>274 ± 10</td>
<td>88 ± 5</td>
</tr>
<tr>
<td>Larynx</td>
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<td>12.4</td>
<td>39</td>
<td>275 ± 3</td>
<td>45 ± 6</td>
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<td>Larynx</td>
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<td>13.7</td>
<td>40.3</td>
<td>275 ± 2</td>
<td>50 ± 13</td>
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<tr>
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<td>13.7</td>
<td>40.3</td>
<td>275 ± 2</td>
<td>50 ± 13</td>
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<td>46.8</td>
<td>235 ± 3</td>
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<td>Oropharynx</td>
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<td>15.4</td>
<td>41</td>
<td>270 ± 4</td>
<td>17 ± 3</td>
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<tr>
<td>Oral cavity</td>
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<td>13.5</td>
<td>41.1</td>
<td>274 ± 10</td>
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<td>50 ± 13</td>
</tr>
<tr>
<td>Larynx</td>
<td>T3N2</td>
<td>2</td>
<td>13.7</td>
<td>40.3</td>
<td>275 ± 2</td>
<td>50 ± 13</td>
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Table 2  Pimonidazole binding and erythropoietin expression in head and neck carcinomas

<table>
<thead>
<tr>
<th>Tumor Site</th>
<th>No. biopsies</th>
<th>Erythropoietin %*</th>
<th>Pimo %*</th>
<th>Erythropoietin and hypoxia†</th>
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<tbody>
<tr>
<td>Oropharynx</td>
<td>2</td>
<td>7.5</td>
<td>0</td>
<td>na, n</td>
</tr>
<tr>
<td>Oropharynx</td>
<td>2</td>
<td>15.5</td>
<td>1.5</td>
<td>m, m</td>
</tr>
<tr>
<td>Larynx</td>
<td>2</td>
<td>32 ± 15</td>
<td>2 ± 1</td>
<td>n, y, m</td>
</tr>
<tr>
<td>Larynx</td>
<td>2</td>
<td>36 ± 20</td>
<td>4.5 ± 1.8</td>
<td>n, n, n, n</td>
</tr>
<tr>
<td>Larynx</td>
<td>3</td>
<td>30 ± 5.8</td>
<td>5 ± 0</td>
<td>m, y, y</td>
</tr>
<tr>
<td>Larynx</td>
<td>4</td>
<td>5 ± 0.6</td>
<td>5 ± 0.6</td>
<td>m, m, m, m</td>
</tr>
<tr>
<td>Larynx</td>
<td>5</td>
<td>8 ± 5.6</td>
<td>7.5 ± 1.4</td>
<td>m, m, m, y</td>
</tr>
<tr>
<td>Larynx</td>
<td>6</td>
<td>42.5 ± 7</td>
<td>9.5 ± 2.7</td>
<td>m, m, m, m</td>
</tr>
<tr>
<td>Larynx</td>
<td>7</td>
<td>35 ± 8.7</td>
<td>10 ± 3.5</td>
<td>y, m, y, y, n</td>
</tr>
<tr>
<td>Larynx</td>
<td>8</td>
<td>47 ± 10</td>
<td>12.3 ± 1.6</td>
<td>m, y, y</td>
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<tr>
<td>Larynx</td>
<td>9</td>
<td>48.7 ± 8</td>
<td>14.5 ± 4.9</td>
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</tr>
<tr>
<td>Larynx</td>
<td>10</td>
<td>38.7 ± 11</td>
<td>15 ± 2</td>
<td>y, m, m, y</td>
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<tr>
<td>Larynx</td>
<td>11</td>
<td>48.8 ± 9</td>
<td>15 ± 2</td>
<td>m, m, m, m</td>
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<tr>
<td>Larynx</td>
<td>12</td>
<td>35 ± 5.8</td>
<td>15.7 ± 2.9</td>
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<tr>
<td>Larynx</td>
<td>13</td>
<td>58.7 ± 5</td>
<td>25 ± 2</td>
<td>m, m, m, m</td>
</tr>
<tr>
<td>Larynx</td>
<td>14</td>
<td>42 ± 4</td>
<td>29 ± 10</td>
<td>m, m, m, m</td>
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</tbody>
</table>

*% Fraction of tumor area staining positive for erythropoietin immunoreactivity or pimonidazole binding. Data are mean ± SE for each tumor.
†Regional comparisons of erythropoietin expression with tumor hypoxia as determined by pimonidazole binding in each tumor biopsy.

Abbreviations: y, overlap of erythropoietin expression and pimo binding; n, no overlap of erythropoietin and pimo; m, mix of overlap and no overlap in same biopsy section; na, not applicable due to absence of both erythropoietin expression and pimonidazole binding.
MATERIALS AND METHODS

Patients. Twenty-one patients with squamous cell carcinoma of the head and neck were enrolled in a tumor hypoxia study in accordance with a research protocol approved by the Institutional Review Board at the University of North Carolina Hospitals. All patients provided signed informed consent before their participation in the study. The primary tumors were localized to the larynx in nine patients, oropharynx in six patients (four base of tongue and two tonsillar region), oral cavity in five patients (three floor of mouth, one tongue, and one alveolar ridge), and hypopharynx in one patient. The tissue specimen from a patient with laryngeal cancer was excluded from the studies because of the availability of only a single, small, and fragmented biopsy. Two or more biopsy specimens were available from the tumors of the remaining 20 patients, and 74 biopsies in total were analyzed in the studies. The site, stage, and grade of each patient’s tumor and the baseline hemoglobin and hematocrit values are illustrated in Table 1, and the number of biopsies obtained from each tumor are illustrated in Table 2.

Labeling of Tumor Hypoxia. The hypoxia marker pimonidazole hydrochloride (Hypoxyprobe-1) was obtained from NPI, Inc. (Belmont, MA). Before tumor biopsy, the patients received pimonidazole hydrochloride (0.5 g/m²) diluted in 100 mL of 0.9% NaCl infused i.v. >20 minutes. Between 16 to 24 hours later, multiple incisional biopsies were obtained from the primary tumors. After the biopsies, the fresh tumor samples were placed in cold 10% neutral-buffered formalin held at 4°C for 12 to 24 hours and processed into paraffin blocks. Four-μm-thick contiguous sections were cut and mounted on glass slides before immunohistochemical staining. One slide per block was stained with H&E for pathologic review to confirm the presence of tumor.

Immunohistochemical Staining. Immunostaining for erythropoietin and EPOR was carried out on contiguous sections using procedures described previously with some modifications (29). The primary antibodies were monoclonal anti-erythropoietin (mAb-287, 1:25 dilution) from R&D Systems (Minneapolis, MN) and polyclonal anti-EPOR (C-20, 1:50 dilution) from Santa Cruz Biotechnology (Santa Cruz, CA). EPOR expression was confirmed using a second monoclonal anti-EPOR (mh2er 16.5.1, 1:50 dilution) generously provided by Genetics Institute (Cambridge, MA). Negative controls from which primary antibodies were omitted from the staining procedure were free of nonspecific background staining. The sections were deparaffinized in Hemo-D, rehydrated in graded alcohols, and subjected to endogenous peroxidase block in 3% H₂O₂. For EPOR immunostaining, antigen retrieval was carried out by boiling the sections in antigen retrieval solution (Innovex Biosciences, Richmond, CA), whereas sections for erythropoietin immunostaining were boiled in 10% citrate buffer for antigen retrieval. This was followed by blocking with 5% donkey serum (Jackson ImmunoResearch Labs, West Grove, PA). Slides were incubated with anti-erythropoietin or anti-EPOR antibodies overnight at 4°C and then washed and incubated with either biotinylated donkey anti-mouse (1:1,000) or anti-rabbit (1:2,000) secondary antibodies (Jackson ImmunoResearch Labs) at 37°C for 25 minutes followed by another incubation for 25 minutes with avidin-biotin peroxidase complex (ABC Kit, Vector Laboratories, Burlingame, CA). Finally, the sections were developed with...
diaminobenzidine tetrahydrochloride chromogen and counterstained with hematoxylin. Sections contiguous to those stained for erythropoietin and EPOR were stained for pimonidazole binding using a mouse monoclonal antibody against pimonidazole adducts (clone 4.3.11.3, 70 μg/mL, IgG1, diluted 1:50) as described in detail previously (41).

Semiquantitative Analysis of Erythropoietin, EPOR Staining, and Tumor Hypoxia. Immunohistochemistry results were evaluated by two pathologists (K.A. and Z.A.H.) in a blinded fashion, and differences between the two investigators were resolved by consensus. Cytoplasmic or membrane staining for erythropoietin and EPOR were considered positive. Immunohistochemical detection of tumor hypoxia using immunostaining for pimonidazole binding has been described previously (42–44). To assess the percentage of tumor cells staining for erythropoietin, EPOR, or pimonidazole adducts, the entire tumor section (regardless of the number of fields) was examined at low magnification (×25-50) without taking into account areas of acellularity, necrosis, and stroma. For assessment of erythropoietin and EPOR expression, we also evaluated the intensity of the staining in tumor cells. The percentage of tumor cells that exhibited no erythropoietin or EPOR staining (intensity score = 0) or weak (score = 1), moderate (score = 2), or intense (score = 3) staining was determined by microscopic examination at higher magnification (×100-200). The semiquantitative immunoreactivity score was derived from the product of the percentage of tumor cells staining for erythropoietin or EPOR and the intensity score of that staining using the formula: Weighed staining intensity = Σ Intensity score × Percentage of cells. The maximum weighed staining intensity score was 300, and the expression data was presented as means ± SE, except for tumors 1, 2 and 5, which had only two biopsies available. The intensity of pimonidazole binding was not factored as discussed previously (44). Although the extent of 2-nitroimidazole immunostaining measures the extent of oxygen tensions ≤10 mm Hg, the intensity of immunostaining is affected by pharmacokinetic and pharmacodynamic factors besides hypoxia. Microregional comparison of erythropoietin expression and tumor hypoxia in tumor fields was carried out at higher magnification (×100-200) for each biopsy on a section-by-section basis to assess individual microscopic fields with respect to (a) overlap, (b) no overlap, and (c) a mix of overlap and no overlap with pimonidazole immunostaining (44).

Statistical Analyses. The analyses were done using GraphPad Prism version 3.0 for Windows software. The relationships among tumor hypoxia, erythropoietin-EPOR expression, and other variables were examined by two-tailed Spearman’s rank correlation analysis and when appropriate using Kruskal-Wallis ANOVA and Dunn multiple comparisons post hoc test. P < 0.05 was considered significant.

RESULTS

Expression of EPOR and Erythropoietin in Squamous Cell Carcinomas of the Head and Neck. To determine whether primary squamous cell carcinomas of the head and neck express EPOR and its ligand erythropoietin, we carried out immunohistochemical analysis on a total of 74 tumor biopsies from 20 patients. Photomicrographs in Fig. 1 are representative of the immunostaining patterns for EPOR (A, B, and C) and erythropoietin (D). EPOR was expressed in tumor cells in 72 of 74 (97%) biopsies and exhibited predominantly a cytoplasmic pattern of immunostaining (Fig. 1A and B). EPOR expression was also observed in vascular endothelial cells (Fig. 1B). EPOR expression
immunoreactivity localized to the membrane of tumor cells in some sections (Fig. 1C). The pattern and high frequency of EPOR staining is comparable with what our laboratory and others have observed in other types of neoplasms that express EPOR such as breast cancer and squamous cell carcinomas of the uterine cervix (27–29). Erythropoietin expression in tumor cells was observed in 67 of 74 (90%) biopsies and exhibited focal immunoreactivity of varying intensity in many tumors (Figs. 1D and 2). We found coexpression of EPOR and its ligand erythropoietin in most (90%) of biopsies with overlap of immunoreactivity in many cases. The results of immunohistochemistry analysis for EPOR and erythropoietin expression are summarized in Table 1. There was no significant difference in erythropoietin or EPOR expression scores between different primary tumor sites ($P > 0.2$, Kruskal-Wallis test). There was no correlation between baseline hemoglobin or hematocrit levels and tumor erythropoietin ($P > 0.2$) or EPOR expression scores ($P > 0.7$, Spearman rank correlation).

### Detection of Tumor Hypoxia by Pimonidazole Binding

Qualitative assessment of tumor hypoxia detected by pimonidazole binding revealed the presence of tumor hypoxia in 17 of 20 patients with head and neck carcinoma, with immunostaining for pimonidazole adducts observed in 63 of 74 (85%) total tumor biopsy sections. Representative photomicrographs illustrating pimonidazole immunostaining of hypoxic tumor cells are illustrated in Fig. 2B, D, F, H, and J. Semiquantitative assessment of the percentage tumor area labeled for hypoxia based on immunoreactivity for pimonidazole binding was carried out for each biopsy section and the mean fraction of tumor area was determined for each tumor (Table 2). In hypoxic tumors, the mean area fractions labeled with pimonidazole exhibited a wide range from 0.7% to 29% of the total tumor area. These results are consistent with the findings of previous studies evaluating tumor hypoxia in squamous cell carcinomas (40, 43–46). In this cohort of patients, there was no correlation between tumor hypoxia and baseline hemoglobin or hematocrit levels ($P > 0.9$ and $P > 0.2$, respectively, Spearman rank correlation). There was no significant difference in hypoxia status between different primary tumor sites ($P > 0.9$; Kruskal-Wallis test).

#### Relationship between Erythropoietin Expression and Pimonidazole Binding

The physiologic expression of erythropoietin in the kidneys is under the control of an oxygen-sensing mechanism. To test the hypothesis that in vivo tumor hypoxia and erythropoietin expression may be coupled in squamous carcinoma cells, we compared the microregional pattern and the extent of erythropoietin expression and hypoxia in tumor biopsies using contiguous tissue sections. In 4 of 74 biopsies, neither erythropoietin expression nor pimonidazole binding was present. Figure 2 illustrates representative immunostaining patterns for erythropoietin (A, C, E, G, and I) and pimonidazole adducts (B, D, F, H, and J) in contiguous sections. Figure 2A and C shows two tumors exhibiting erythropoietin immunoreactivity overlapping with regions of pimonidazole binding in the contiguous sections in Fig. 2B and D, respectively. Although this pattern of pimonidazole and erythropoietin overlap suggests that erythropoietin may serve as an endogenous hypoxia marker, the overlap pattern was observed in only 19% (13 of 70) of the biopsies. The most common pattern of hypoxia and erythropoietin expression was observed in 36 (51%) biopsies in which a mixture of overlap and no overlap of erythropoietin and hypoxia occurred in the same biopsy (Table 2). An example of this pattern is illustrated in Fig. 2E and F in which an area of overlap for erythropoietin staining and pimonidazole binding is adjacent to a hypoxic tumor zone with pimonidazole binding that does not exhibit erythropoietin immunoreactivity. A third pattern of erythropoietin expression and pimonidazole binding was characterized by staining for pimonidazole adducts in tumor zones different from those for erythropoietin staining, observed in 21 of 70 (30%) biopsies. In 6 of these biopsy sections, lack of overlap was attributable to expression of erythropoietin in the absence of pimonidazole binding (Fig. 2, compare G and H). In 2 biopsy sections, the lack of overlap was due to presence of tumor hypoxia and the absence of erythropoietin expression (Fig. 2, compare I and J). In the remaining 13 biopsies, both
erythropoietin staining and pimonidazole binding were observed but without any regions of overlap.

Quantitative comparisons between microregional erythropoietin expression and tumor hypoxia were done initially on a tumor-by-tumor basis (Fig. 3). The percentage fraction of tumor area staining positive for erythropoietin protein and pimonidazole binding was assessed for each biopsy section and the mean values for each tumor were calculated (Table 2). The data were organized in ascending order of tumor hypoxia. Hypoxia was present in all but three tumors. Areas immunostained for erythropoietin were measurable in all tumors with a wide range from 1.5% to 86%. On a tumor-by-tumor basis, there was a significant positive correlation between erythropoietin expression and pimonidazole binding as shown in Fig. 4A (P < 0.001).

Figure 4B illustrates the results of the analysis on a biopsy-by-biopsy basis, demonstrating that the correlation between erythropoietin expression and hypoxia was weaker but remained significant (P < 0.002). In contrast to the correlation between pimonidazole binding and erythropoietin, there was no correlation between pimonidazole binding and EPOR expression (P > 0.2, Spearman rank correlation). We also compared the levels of pimonidazole binding to the mean weighed staining intensity erythropoietin score that was determined for each tumor. The weighed staining intensity score (Table 1) takes into account not only the percentage of tumor cells that exhibit staining but also the intensity of erythropoietin staining in the tumor cells. There was a significant positive correlation between pimonidazole binding and the erythropoietin expression score (r = 0.678, P < 0.002, Spearman rank correlation). Figure 4C illustrates the results of comparisons of the extent of pimonidazole binding in tumor biopsies stratified into absent, low, moderate, or high erythropoietin expression groups according to the erythropoietin expression scores. Tumor biopsies with moderate or high erythropoietin scores showed significantly higher pimonidazole binding compared with tumors with low erythropoietin scores or no erythropoietin expression (P < 0.01).

DISCUSSION

In the present study, we show the expression of EPOR in primary squamous cell carcinomas of the head and neck. EPOR expression in tumor cells is consistent with the possibility that rHuEPO may exert direct effects in the malignant cells that may modulate cellular proliferation, apoptosis, or responsiveness to chemoradiation therapy (25). However, further studies using both in vitro and in vivo experimental models are required to characterize the direct effects, if any, of exogenous erythropoietin in tumor cells that express EPOR. The biology of erythropoietin-EPOR in squamous cell carcinomas of the head and neck is likely to be complex in view of our findings that tumor cells express not only EPOR but also endogenous erythropoietin protein. Furthermore, EPOR expression is not restricted to tumor cells, as it is also expressed in vascular endothelial cells and tumor vasculature (5, 12, 35, 47–49). EPOR expression in vascular endothelial cells is associated with the ability of erythropoietin to stimulate physiologic in vivo angiogenesis in the female genital tract (12), chick embryo chorioallantoic membrane (49), and during wound healing (9). It remains to be determined whether erythropoietin-EPOR signaling may directly modulate pathologic tumor

Fig. 4 Comparisons between erythropoietin protein expression and pimonidazole binding. A, percentage fraction of tumor area staining for erythropoietin (% EPO) and pimonidazole binding (% pimonidazole) was determined for all biopsy sections as described in MATERIALS AND METHODS. A significant correlation was observed between levels of erythropoietin and pimonidazole labeling on a tumor-by-tumor basis using mean expression levels for each tumor (r = 0.736, P < 0.001, Spearman rank correlation). B, comparison of percentage fraction tumor area staining for erythropoietin and pimonidazole binding on a biopsy-by-biopsy basis (r = 0.365, P < 0.002, Spearman rank correlation). C, comparison between pimonidazole binding and weighed mean erythropoietin expression score. Tumor biopsies were stratified according to their erythropoietin scores into absent (0), low (1-20), moderate (21-40), or high (>40) erythropoietin expression groups. Bars, median values of pimonidazole binding in each group. Tumors with moderate or high erythropoietin scores showed significantly higher pimonidazole binding compared with tumors with absent or low erythropoietin scores (*, P < 0.01, Kruskal-Wallis and Dunn multiple comparisons post hoc test).
angiogenesis, a process that is essential for tumor formation and progression.

We found expression of erythropoietin protein in the majority of primary head and neck cancers and investigated the hypothesis that erythropoietin expression in tumor cells may be associated with the presence of hypoxia, an indicator of poor outcome in some types of cancer (50). In patients with head and neck cancer, tumor hypoxia has been associated with aggressive course and resistance to radiation therapy (37–39) and high levels of pimonidazole binding in hypoxic tumors have been associated with poor locoregional control (40). Hypoxia-inducible erythropoietin expression has been reported in nonrenal sites, including brain astrocytes (51), and the female genital tract where erythropoietin expression was also inducible by estrogen (12, 52). Our studies addressed the question of whether erythropoietin may serve as an endogenous hypoxia marker in head and neck tumors. We found that erythropoietin expression did not always colocalize with regional tumor hypoxia as determined by pimonidazole binding. However, there was a significant positive correlation between levels of erythropoietin expression and tumor hypoxia as determined by pimonidazole binding.

The findings of previous preclinical studies of erythropoietin-EPOR biology in cancer support the possibility that expression of erythropoietin and EPOR in squamous cell carcinomas of the head and neck is likely to be functionally significant. For instance, rHuEPO treatment of monolayer cultures of uterine cervix squamous carcinoma cells was associated with diminished cytotoxic effect and apoptosis induced by the chemotherapeutic agent cisplatin in vitro (36). In animal studies using xenografts of human female genital tract cancers, gastric chorocarcinomas or melanoma, it was shown that administration of inhibitors of erythropoietin-EPOR signaling resulted in significant antitumor effects, characterized by the destruction of xenografts as well as tumor vasculature (35, 53). In studies in our laboratory using a syngeneic breast cancer model, administration of erythropoietin-EPOR inhibitors resulted in significant tumor growth delay (29). Thus, a growing body of preclinical experimental evidence suggests that functional erythropoietin-EPOR signaling may represent an emerging therapeutic target in several different types of cancer.

A recent randomized study involving patients with locally advanced head and neck cancer treated with concomitant chemoradiotherapy did not find a significant effect of rHuEPO on progression-free or overall survival (20). Thus, further studies are required to determine whether EPOR expression in malignant head and neck tumors may have contributed to the reported unfavorable effect of systemic rHuEPO on treatment response and outcome in patients treated with radiation therapy alone (25). Irrespective of potential direct effects, if any, of exogenous rHuEPO in tumor cells, the coexpression of EPOR and its ligand erythropoietin in squamous cell carcinomas of the head and neck suggests that endogenous erythropoietin may play a role as a potential autocrine or paracrine growth factor in cancer cells. The expression of EPOR in tumor cells needs to be taken into consideration in the design of future clinical trials investigating the role of rHuEPO in head and neck cancer. Further preclinical studies investigating the biology of erythropoietin-EPOR signaling in squamous cell carcinoma of the head and neck are warranted.

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Erythropoietin and Erythropoietin Receptor Expression in Head and Neck Cancer: Relationship to Tumor Hypoxia

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