Human Recombinant Erythropoietin Significantly Improves Tumor Oxygenation Independent of Its Effects on Hemoglobin

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Abstract

Tumor oxygenation is known to be an important predictive/prognostic marker in a variety of tumors, including cervix, head/neck, sarcoma, non-small cell of the lung, and breast. Tumor oxygenation is influenced by many interactions, including oxygen delivery (angiogenesis, permeability, and Hgb) and consumption (metabolic and growth rates). This study randomized 30 nonanemic, female Fischer 344 rats into three treatment arms to examine the effects of recombinant human erythropoietin (EPO) on R3230 rodent mammary carcinoma oxygenation. The three treatment arms were: (a) placebo; (b) EPO after tumor implantation (2000 units/kg/SQ dose, M/W/F for six doses); and (c) EPO before tumor implantation (2000 units/kg/SQ dose, M/U/F for six doses). Tumors were implanted in the hindflank, and in vivo oxygenation was measured at day 22 after implantation using the Oxylite system (Oxford Optronix, Oxford, England). An average of 180 measurements/animal were performed. On day 22, median tumor volume was 399 mm³ (range: 65–950 mm³), and no differences in tumor volume were seen between treatment arms. Mean hematocrit was equal between arms at therapy initiation but were significantly higher for both arms receiving EPO at day 22 (placebo versus Arm B versus Arm C; Wilcoxon P = 0.052). EPO-treated tumors had significantly less hypoxic measurements when compared with either the placebo or those receiving EPO before implantation. These data confirm that tumor oxygenation in nonanemic individuals may be improved through the administration of EPO, and this improvement appears to be independent of Hgb effects.

Introduction

For many years, tumor hypoxia has been the subject of investigation for physiologists and radiation oncologists. Currently, its impact on factors important in tumor development, progression, and therapy responsiveness is being studied in both preclinical and clinical settings (1, 2). Tumor oxygenation plays an important role in altered gene expression, multidrug resistance, tumor cell invasiveness, angiogenesis, and metastasis (1, 3, 4). Tumor hypoxia has been shown to be of prognostic and predictive value in several clinical trials involving radiation, chemotherapy, and surgery for various tumor types (5–20).

Many different approaches to improve tumor oxygenation have been attempted. Most of these approaches have tried to improve oxygen delivery by increasing the oxygen content of the blood, decreasing tumor metabolism, or by altering blood flow. Examples of methods that have been tried in the past include hyperbaric oxygen, carbogen breathing with and without nicotinamide, infusion of artificial blood substitutes, glucose infusion to reduce oxygen consumption rates, and agents that right shift the Hgb saturation curve (21–23). Despite the publication of dozens of papers on this subject, there is yet to be an established method that reliably and reproducibly improves tumor oxygenation enough to be clinically relevant (24). Blood transfusions in anemic patients have also been used in an attempt to improve tumor oxygenation. However, these studies have failed to document consistent improvement in therapy efficacy, even in the setting of complete resolution of the underlying anemia (25, 26).

EPO is a glycoprotein hormone produced by the liver and kidney that acts to stimulate growth and prevent apoptosis of RBC precursors (27). EPO is currently approved by the FDA for the treatment of anemia related to chronic renal disease, malignancy, HIV, and surgery. EPO and its effects on tumor oxygenation have been studied in anemic animal models using a rodent sarcoma, which is known to be extremely hypoxic. These studies have shown that EPO partially improves tumor oxygenation in anemic animals as well as improves the efficacy of both radiotherapy and cyclophosphamide therapy (26, 28–30). Recently, there have been several reports of the effects of both EPO and its receptor on both normal and neoplastic tissues (31–37). As EPO and its receptor are present on many cell types, including cancer and endothelial cells, this study examined the effects of EPO treatment on the oxygenation of breast carcinoma in a non-anemic, clinically relevant animal model.

Materials and Methods

Animals. Female Fischer 344 rats (Charles River Laboratories, Raleigh, NC; body weight 150–200 grams) were used for all experiments. R3230 mammary adenocarcinomas were taken from tumor-bearing donor animals, cut into 1–2-mm pieces, and transplanted into the subcutis of the left lateral quadriceps muscle, using methods described previously (38). After tumor implantation, animals were provided continuous access to food and water. All experiments were approved by the Duke Institutional Animal Care and Use Committee.

EPO Administration. EPO was administered in the form of Procrit (Ortho-Biotech, Raritan, NJ). All drug volumes were equivalent between the arms (150–200 µl) depending on body weight. Animals were placed in one of three groups. Twenty animals were randomized and treated in Arms A and B, and 10 animals were directly treated in Arm C. EPO was used at a concentration of 2 units/µl. The EPO dosing for each arm is as follows: Arm A: placebo group; depending on body weight, 150–200 µl of saline were administered s.c. three times/week for a total of six doses during the experimental treatment period of 18 days. Arm B: EPO three times/week; EPO, 2000 units/kg, s.c. was administered three times/week for a total of six doses during the experimental treatment period of 18 days. Arm C: EPO pretreatment; EPO, 2000 units/kg, s.c. was administered three times/week for a total of six doses before the implantation of the tumors. The EPO dosing is similar to a EPO dose of 60,000–100,000 IU/dose in humans based on pharmacokinetic/pharmacodynam-
namic modeling in healthy human volunteers (Johnson and Johnson Pharmaceutical Research and Development, Raritan, NJ). All animals received six doses of saline EPO.

**Tumors.** Four days after transplant (to verify tumorigenicity), 20 animals had their tumors measured bidirectionally and were randomized into two groups, placebo (Arm A) or EPO 3X (Arm B). Tumor volume was determined using the formula: \(V = \frac{4}{3}\pi r^3\). One animal died after transplant but before randomization. A third group of 10 animals (Arm C) was anesthetized before the final EPO administration for Hct determination and also at the time of tumor implantation. All tumors were in place for 22 days before oxygenation determination, and therefore, all animals were exposed to the same dose of anesthetic at the same time interval before oxygenation determination.

**Blood Parameter Measurements.** At the time of transplant and at tumor oxygenation measurement, anesthetized (Nembutal, Abbott Laboratories, Chicago, IL; 50 mg/kg, administered i.p.) animals had a blood sample (~250 μl) taken from the ventral tail artery for Hct determination. Hcts were determined by the staff of Division of Laboratory Animal Resources at Duke University using an ABX Pentra 60 blood analyzer (ABX diagnostics; Montpellier, Cedex, France).

**Tumor Oxygenation Assessments.** All tumor oxygenation measurements were made on day 22 after tumor implantation. Animals were anesthetized with pentobarbital (Nembutal; 50 mg/kg, administered i.p.) and placed on a heated circulating water blanket to maintain a core body temperature of 37°C (Baxter K-module K20; Baxter Healthcare, Deerfield, IL). Once appropriate depth of anesthesia was reached (assessed by toe pinch reflex), measurements of tumor tissue pO2 and Hct took place. pO2 was measured using the Oxford Optronix Oxylite pO2 sensor (Oxford Optronix, Ltd., Oxford, United Kingdom; Ref. 39). The microelectrode was inserted by use of a micromanipulator (model MO102E; Narashigie, Inc., Japan) starting in the subcutis space and advancing over-the-needle Quick Cath (Baxter) was used to pierce the tumor and create a small track and facilitate penetration of the probe through the tumor to the opposite side of the tumor. The Quick Cath was then removed, and the microdrive retracted the probe back through the track at 50-μm intervals. The probe remained stationary for 5–10 s at each interval, and the pO2 was recorded. The probe was repeatedly retracted in this fashion until a total of 90 measurements had been made. The probe was then carefully removed, the animal rotated 90°, and the procedure repeated with care taken to make sure the probe was inserted into a different tumor plane to not bisect the first track. This gave two separate perpendicular tracks for a total of 180 pO2 measurements per tumor. The animal was then humanely euthanized with an overdose of sodium pentobarbital (Nembutal; 150 mg/kg, i.p.).

**Statistical Analysis.** Comparisons of tumor volume, Hct, and pO2 between the three arms of the study were made using the Kruskal-Wallis method. Each animal’s oxygen measurements were analyzed first for that individual animal, and then the means/medians across the group of animals was determined for each treatment group. Median 25th percentile values were determined using the median value of the lowest 45 readings per animal. Experiments in which \(P < 0.05\) were considered statistically significant.

**Results**

The Hcts of the three groups of animals were the same before treatment with EPO (Arm A: 38%; Arm B: 40%; and Arm C: 39%; \(P = 0.91\)). After administration of EPO for six doses in Arms B and C, Hcts were significantly increased compared with the placebo group (Arm A: placebo group: 41% versus Arm B; EPO, 3X/week group: 51% and Arm C; EPO, pretreat group: 49%; Wilcoxon \(P = 0.052\)). The tumor volumes between all three study arms were equivalent at day 22 (Arm A: 371 mm³, Arm B: 442 mm³, and Arm C: 411 mm³; \(P = 0.95\)).

There is a clear difference in pO2 distributions between the tumors treated with EPO compared with the placebo tumors or tumors treated with EPO before implantation as evaluated by tumor pO2 histography (Fig. 1). There was no difference in oxygen distribution between the placebo tumors and the tumors treated with EPO before implantation (data not shown). The group that received EPO starting at the time of tumor transplant had virtually no hypoxia (median 25th percentile = 18 mmHg), whereas the median 25th percentiles in the placebo group and EPO pretreated group were both <10 mm Hg (9 and 7 mmHg, respectively; \(P = 0.015\); Table 1). In addition, the EPO-treated tumors had almost no measurements at levels <5 mmHg, which was significantly different from the other two groups (0% of measurements compared with 11.9% for the placebo group and 18.2% for the EPO-pretreated group; \(P = 0.006\)). There were no significant differences in either median 25th-percentile pO2 or the fraction of pO2 measurements <5 mmHg or <10 mmHg between the placebo and the EPO-pretreated group (Fig. 2).

**Discussion**

The finding in our study that EPO significantly improved tumor oxygenation in the absence of anemia has far-reaching effects for the treatment of many types of cancer. Other investigators have examined the connection between anemia and tumor oxygenation (40) and whether or not EPO can improve tumor oxygenation by improving Hgb levels (41, 42). However, no investigations have been made on the direct effects of EPO when anemia is not present. In addition, the animal models that have been used involve the artificial induction of anemia, either through carboplatin administration or the creation of hemorrhagic ascites. The tumor types that have been studied are

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4 Johnson and Johnson Pharmaceutical Research and Development, unpublished data.
increases tumor lactate. These changes have been associated with breast cancer patients (46, 47). Hypoxia also lowers tumor pH and these angiogenic proteins have been associated with poorer prognosis likelihood for metastasis, this up-regulation may lead to increased angiogenesis and greater vascular endothelial growth factor, basic fibroblastic growth factor, tumor-1-mediated up-regulation of proangiogenic cytokines, such as HIF-1α. Hypoxia inducible factor-1 has also been shown to down-regulate transcription of estrogen receptor α, which could lead to hormonal resistance in breast cancer (52).

This study also offers a possible explanation to the survival advantage seen on retrospective analysis of the clinical trials of EPO for the prevention of treatment-related anemia. Several of these studies demonstrated a survival advantage for those patients receiving EPO treatment concurrent with their radiation, chemotherapy, or combination therapy (53, 54). Perhaps the improvement in tumor oxygenation seen in our preclinical model can help explain this survival advantage. Increases in tumor oxygenation have been shown to improve responses to chemotherapy (30, 55, 56) as well as radiotherapy (57). Therefore, improving tumor oxygenation with EPO as seen in our study, even in nonanemic patients, needs further investigation and might offer increased efficacy for a variety of therapeutic modalities.

Improvements in tumor oxygenation caused by EPO might lead to the down-regulation of a number of signaling pathways, which are associated with a poorer prognosis for a hypoxic compared with well-oxygenated tumors. Hypoxia causes the hypoxia inducible factor-1-mediated up-regulation of proangiogenic cytokines, such as vascular endothelial growth factor, basic fibroblastic growth factor, tumor growth factor β, and platelet-derived growth factor (44, 45). This up-regulation may lead to increased angiogenesis and greater likelihood for metastasis, e.g., elevated tissue and systemic levels of these angiogenic proteins have been associated with poorer prognosis in breast cancer patients (46, 47). Hypoxia also lowers tumor pH and increases tumor lactate. These changes have been associated with greater cellular mobility in melanoma (48) and breast tumor cell lines (49). In clinical studies, elevated lactate levels are associated with higher likelihood for development of metastases in cervix and head/neck human carcinoma (50, 51). Hypoxia inducible factor-1 has also been shown to down-regulate transcription of estrogen receptor α, which could lead to hormonal resistance in breast cancer (52).

Our study demonstrated that the improvement in tumor oxygenation seen with EPO administration is independent of the increase seen in Hgb. We used a third experimental arm where the animals received EPO before tumor implantation; therefore, the tumors experienced Hgb levels similar to those tumors where the EPO was given after tumor implantation. Although the Hgb levels were similar between the groups at the time of tumor oxygenation determination, the group that received the EPO before implantation would no longer have elevated levels of EPO during the tumor’s growth given EPO’s short half-life. Therefore, the increases in tumor oxygenation seen with the EPO administration concurrent with tumor growth are probably directly dependent on EPO’s tumor effects at the time the measurements were made. These nonhematological effects are consistent with other recent reports of EPO receptors being present on many different cell types (31–37). These effects could also be mediated through direct and indirect effects on angiogenesis, tyrosine kinase activation, and other transcriptional processes (27, 32, 36, 43).

Table 1  *Tumors treated with EPO show a significant reduction in pO2 measurements <10 mmHg compared with tumors treated with either placebo or treated with EPO before implantation*.

<table>
<thead>
<tr>
<th>Group</th>
<th>Median percentage of measurements ≤5 mmHg (%)</th>
<th>Median percentage of measurements ≤10 mmHg (%)</th>
<th>Median 25th percentile (mmHg)</th>
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<tr>
<td>EPO treated (n = 9)</td>
<td>0.0%</td>
<td>9.73%</td>
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<td>Placebo treated (n = 10)</td>
<td>11.9%</td>
<td>27.8%</td>
<td>8.98</td>
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<td>EPO Pretreat (n = 10)</td>
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<td>33.7%</td>
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<td>Kruskal-Wallis P</td>
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<td>0.006</td>
<td>0.015</td>
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</tbody>
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