Limitations of a Murine Transgenic Breast Cancer Model for Studies of Erythropoietin-Induced Tumor Progression

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Abstract
Adverse effects of erythropoietin (EPO) on tumor progression and survival were observed in recent phase 3 oncology trials. However, mechanisms remain poorly understood. We tested the effects of exogenous EPO on murine B16F10 melanoma growth in a subcutaneous tumor transplant model, and for the first time, in a model of spontaneous tumor formation within autochthonous epithelial tissues using murine mammary tumor virus promoter polyoma virus middle T antigen (MMTV-PyMT) transgenic mice. EPO receptor (EPOR) messenger RNA (mRNA) was detectable in both B16F10 tumors and mammary tumors from MMTV-PyMT mice but was 0.12 ± 0.02% and 1.3 ± 0.91% of the EPOR mRNA level in murine erythroid HCD-57 cells, respectively. B16F10 tumor growth rates in mice treated for 3 weeks with 30 μg/kg per week of darbepoetin α, 0.41 inverse days (range, 0.05-0.69 inverse days; n = 16), were similar to tumor growth rates observed in mice treated with PBS, 0.42 inverse days (range, 0.10-0.69 inverse days; n = 17). In contrast, darbepoetin α raised hematocrit levels to 0.593 (maximum, 0.729) compared with 0.448 (maximum, 0.532) in PBS-treated mice (P = .0004). In MMTV-PyMT mice, the weights of tumor-bearing mammary glands in mice treated for 6 weeks with 30 μg/kg per week of darbepoetin α, 3.37 g (range, 1.94-5.81 g; n = 27), did not significantly differ from the weights in PBS-treated mice, 3.76 g (range, 2.30-6.33 g; n = 26). In contrast, darbepoetin α raised hematocrit levels to 0.441 (maximum, 0.606) compared with 0.405 (maximum, 0.492) in PBS-treated mice (P = .05). Thus, effects of exogenous EPO on tumor growth were not recapitulated in these murine tumor models.

Introduction
Recombinant erythropoietin (EPO) revolutionized the treatment of chemotherapy-induced anemia by reducing red cell transfusions, but recent clinical trials indicate that EPO may worsen cancer survival [1–8]. Whereas venous thromboembolism associated with EPO therapy is a well-recognized risk, the extent to which EPO may stimulate tumor progression through effects on tumor cells, tumor blood vessels, and alternate mechanisms remains unclear. The notion that EPO exerts off-target effects is supported by the observed activity of this growth factor in a variety of nonhematopoietic tissues including the endothelium, uterus, heart, brain, retina, muscle, and kidney [9]. Binding sites for EPO in malignant lung cancer cells were discovered more than 15 years ago [10], and EPO receptor (EPOR) mRNA and protein expression has since been detected in diverse cancer types [11]. Whereas the potential for EPO to promote tumor growth was raised almost 50 years ago [12], concern was elevated by the adverse effects of EPO on tumor progression and survival observed in the aforementioned oncology trials.

While definitive determination of the risk of EPO-induced tumor progression must come from clinical investigations, studies in cell lines and animal models may yield insights into mechanisms of EPO-induced off-target effects. In vitro studies using cancer cell lines, although disparate, suggest that EPO/EPOR signal transduction can
stimulate the proliferation, survival, and/or migration of at least certain malignant cell lines [11]. Animal models allow the opportunity to investigate the effects of EPO on tumors in a physiologic context. In at least eight independent studies of both syngeneic and xenogeneic subcutaneous tumor implantation, exogenous EPO administration at doses that significantly increased hematocrit levels did not alter tumor growth, angiogenesis, chemosensitivity, or radiosensitivity [11,13]. Furthermore, in at least 10 studies, exogenous EPO actually enhanced the effects of chemotherapy, radiotherapy, or photodynamic therapy on tumor ablation, and in two studies, EPO alone seemed to stimulate tumor regression [13]. In contrast, EPO increased tumor growth in a syngeneic tumor implant model and in a methylcholanthrene-induced fibrosarcoma model, effects that were attributed to increased tumor microvessel density [14]. Similarly, coadministration of EPO along with syngeneic breast tumor implants in a rat skin-fold window chamber model increased neovascularization and tumor growth [15]. In addition, EPO was reported to enhance the growth of human tumor xenografts after mock surgical trauma [16]. Finally, several studies have investigated the effects of local EPO blockade on tumor growth. Local injections of soluble EPOR, anti-EPO antibodies, and antagonistic EPO mimetic peptides resulted in tumor cell destruction and reduction of vascularity in human tumor xenografts [17,18]. Similarly, soluble EPOR, anti-EPO antibodies, and a nonfunctional mutant ligand (EPO103A) reduced angiogenesis and delayed growth of syngeneic tumors in the aforementioned rat skin-fold window chamber model [15].

All of the above studies involved the formation of subcutaneous tumors by transplanting cancer cell lines or chemical induced carcinogenesis. For the first time, we sought to test the effects of EPO on tumor growth using tumors formed spontaneously within endogenous epithelial tissues, a process that more closely reflects the complexity of human tumorigenesis and tumor progression including interactions with endogenous tissue-specific stroma. For this initial study, we focused on measurements of tumor growth using the murine mammary tumor virus promoter polyoma virus middle T antigen (MMTV-PyMT) transgenic model on the FVB background. These mice reproducibly develop pregnancy-independent multifocal mammary tumors with a mean latency of only 53 days in 100% of females [19,20]. PyMT is a potent oncogene that targets several signal transduction pathways that are altered in human breast cancer [21]. Tumors in MMTV-PyMT mice progress through stages of hyperplasia, adenoma/mammary intraepithelial neoplasia, early and late carcinoma, and metastasis that are comparable to human breast cancer [22]. As in the human disease, loss of estrogen receptor, progesterone receptor, and integrin-β and sustained expression of erbB2 and cyclin D1 are all associated with poor prognosis [22]. In an effort to establish a preclinical animal model for studies of EPO-induced tumor progression, we compared the effects of EPO on tumor growth in a syngeneic tumor transplantation model versus a transgenic model of spontaneous cancer within endogenous mammary epithelium. We found that EPO did not accelerate tumor formation or growth in these models. The limitations of these murine tumor models for studies of EPO-induced tumor progression are discussed.

Materials and Methods

Mice

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Washington. C57BL/6 and FVB mice were purchased from Taconic (Hudson, NY) and were maintained under specific pathogen-free conditions at the University of Washington. MMTV-PyMT mice on the FVB background were generously provided by Dr Sandra Gendler and were genotyped as described [23].

B16F10 Tumor Formation in Syngeneic C57BL/6 Mice

Murine B16F10 melanoma cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco's modified Eagle medium, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, 0.10 mg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B. Cells were washed three times in PBS and were resuspended in PBS at a concentration of 1 million cells per 300 μl. On day 0, C57BL/6 mice (aged 9-10 weeks) were anesthetized using an isoflurane/oxygen vaporizer, their flanks were shaved, and they received a subcutaneous injection of 1 million cells. Mice were stratified by sex and were randomly assigned to receive subcutaneous injections of darbepoetin α (30 μg/kg per week; Amgen, Thousand Oaks, CA) or an equal volume of PBS starting on the day of tumor injection. Equal numbers of mice were assigned to darbepoetin α versus PBS for each cell preparation. To ensure that sex did not confound results, males and females were equally distributed to EPO and PBS groups. Tumor measurements were obtained in anesthetized mice in the cranial/caudal (length), superior/inferior (height), and medial/lateral (width) directions using Vernier calipers. In experiment 1 (n = 8), darbepoetin α or PBS was injected on days 0 and 7, tumors were measured on days 10 and 14, and hematocrit levels were not determined. In experiments 2 to 4, darbepoetin α or PBS was injected on days 0, 7, and 14; tumors were measured on days 7, 14, 17, and 20; and hematocrit levels were determined on day 20. Tumor-bearing mice were monitored daily (no morbidity was observed) and were humanely killed on the last day of tumor measurement or sooner on excessive tumor burden in accordance with local animal ethics regulations. Tumor volume was calculated using the following formula: 0.5 × length × width × height. Tumor growth was determined using the following formula: 1 / doubling time, where doubling time = (T − T₀) × log2 / (log V − log V₀), and T − T₀ indicates the difference in time between tumor measurements and V and V₀ indicate the tumor volume at the two times of measurement.

Analysis of Tumor Growth in MMTV-PyMT Mice

Heterozygous MMTV-PyMT males were bred to wild-type FVB females, and heterozygous female offspring without palpable mammary tumors were accrued to the study when they reached 48 to 51 days old. On day 0, mice were randomly assigned to subcutaneous injections of darbepoetin α (30 μg/kg per week) or PBS continuing weekly. Three mice each received a total of five injections of darbepoetin α or PBS and were humanely killed on day 31, whereas all remaining mice (n = 48) received a total of six injections and were humanely killed on day 37. Mice were inspected weekly by an investigator blinded to treatment assignment to determine the time to first visible tumor. Tumor-bearing mice were monitored daily (no morbidity was observed) and were humanely killed at the study end point or sooner on excessive tumor burden. Hematocrit levels were determined at the study end point using heparinized microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA) and a microhematocrit centrifuge (Damon/IEC, now ThermoScientific, Waltham, MA). All mammary glands (including thoracic and inguinal) were collected on sacrifice and weighed, and their volume was determined by PBS displacement. Mammary gland
weights were not obtained for one mouse in the PBS group that was removed from the study early to provide tissue for assay development.

Quantitative Reverse Transcription—Polymerase Chain Reaction

Total RNA was extracted from B16F10 tumors and MMTV-PyMT tumor-bearing mammary glands using the miRNeasy Mini Kit (Qiagen, Valencia, CA) with on-column DnaseI digestion to remove genomic DNA. First-strand complementary DNA was synthesized using random hexamers and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Triplicate quantitative polymerase chain reactions (PCRs) were performed for each sample using TaqMan assays for EPOR (assay identification number Mm00833882_m1), the reference gene PGK1 (Mm00435617_m1), and standard thermal cycling conditions (Applied Biosystems, Foster City, CA). The coefficient of variation for triplicate PCRs was less than 1.6% for all samples. Relative quantification was determined using the comparative cycle threshold (Ct) method, \(2^{-\Delta\Delta Ct}\) where \(\Delta Ct = \text{mean } C_t \text{ for target gene} - \text{mean } C_t \text{ for reference gene}.

Results and Discussion

EPO Does Not Increase the Growth of B16F10 Subcutaneous Implants

We first tested the effects of exogenous EPO administration in a syngeneic subcutaneous tumor implant model. We used B16F10 murine melanoma cells because they are known to develop as tumors when injected subcutaneously in syngeneic C57BL/6 hosts, and no previous studies have examined the effects of EPO on B16F10 melanoma growth. Tumor take rates in mice treated with darbepoetin \(\alpha\) (14/18 on day 10 and 16/18 on day 17) were comparable to those in the PBS group (14/19 on day 10 and 17/19 on day 17). Likewise, the tumors in mice treated with darbepoetin \(\alpha\) neither grew faster than tumors in mice treated with PBS (Figure 1). The biologic activity of darbepoetin \(\alpha\) in this setting was confirmed by significantly increased hematocrit levels to a mean of 0.593 (maximum, 0.729) compared with 0.448 (maximum, 0.532) in PBS-treated mice (\(P = .0004\)).

EPO mRNA was detectable in B16F10 tumors but was less than 0.12 ± 0.02 of the EPO mRNA level in control murine EPO-dependent HCD-57 cells (24) (n = 15). The lack of effects of EPO on tumor growth in this model may be related to this low level of EPO in the B16F10 tumor cells. In addition, the rapid growth rate of B16F10 cells in this syngeneic tumor transplant system provided only a limited window for EPO dosing. Although we did not assess the possibility that EPO may have subtly increased tumor microvessel density, such an effect is unlikely because there was no effect of EPO on tumor growth.

EPO Does Not Increase the Growth of Mammary Tumors in MMTV-PyMT Mice

No previous studies have examined the effects of EPO on tumors formed spontaneously within endogenous epithelial tissues. We examined whether exogenous EPO administration influences mammary tumor growth using MMTV-PyMT mice. This model provided the opportunity to administer EPO before the onset of tumors and during
tumor growth. Mice without palpable tumors at days 48 to 51 of age were randomly assigned to darbepoetin α or PBS. Visible tumors were detected in the mammary glands of darbepoetin α–treated mice 3.8 ± 1.1 weeks later (n = 27) and in PBS-treated mice 3.6 ± 1.1 weeks later (n = 27; Figure 3). Thus, darbepoetin α did not accelerate the rate of initial tumor formation. Similarly, the weights of tumor-bearing mammary glands at the study end point in darbepoetin α–treated mice, 3.37 g (range, 1.94–5.81 g), did not significantly differ from the weights in PBS-treated mice, 3.76 g (range, 2.30–6.33 g; Figure 4). Similar results were obtained when tumor volumes were measured by liquid displacement (data not shown). In contrast, darbepoetin α significantly raised hematocrit levels to 0.441 (maximum, 0.606) compared with 0.405 (maximum, 0.492) in PBS-treated mice (P = .05). Of note, the hematocrit value for one mouse in the darbepoetin α group could not be determined due to clotting in the microhematocrit tube. When three additional mice with hematocrit determinations less than 0.30 were excluded from the darbepoetin α group, the significance of the difference in hematocrit levels between the darbepoetin α and PBS groups was increased (P = .0006).

The absence of an effect of EPO on tumor growth may be related to the low EPOR mRNA levels in MMTV-PyMT tumors. Although detectable, EPOR mRNA levels in tumor-bearing mammary glands from MMTV-PyMT mice (n = 7) were only 1.13 ± 0.91% of the EPOR mRNA level in control murine erythroid HCD-57 cells. Another limitation of the MMTV-PyMT-FVB model is the short window available for EPO administration during the tumor growth phase. Less than 6 weeks were available before animals had to be humanely killed owing to the rapid growth of tumors. Of note, the expression of the MMTV-PyMT transgene on the C57BL/6 background has been shown to result in significantly delayed tumor latency of 92 days compared with 53 days in the FVB strain [20].

In several investigations, EPO’s tumor growth–promoting activity was attributed to proangiogenic effects [14,15,17,18]. Because EPO did not promote tumor growth in the MMTV-PyMT-FVB mice, we did not undertake secondary studies to uncover possible mechanisms of action of EPO such as quantification of tumor microvessel density. Such studies are better suited to tumor models that may be more sensitive to the effects of exogenous factors on tumor angiogenesis. For example, mammary tumors from (MMTV-PyMT-FVB × I/LnJ) F1 hybrids exhibit a 20% reduction in tumor growth and a five-fold reduction in interior tumor microvessel density compared with tumors from the FVB strain [25]. Future studies using the MMTV-PyMT mice of alternate strains and other transgenic models with relatively slower tumor growth rates, higher tumor EPOR expression levels, or sensitivity to the effects of EPO on tumor angiogenesis may be needed to detect effects of EPO on tumor growth.

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References
