

The Role of Erythropoietin and Erythropoiesis-Stimulating Agents in Tumor Progression

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Abstract

Over the past few decades, understanding of the physiologic function of erythropoietin (EPO) has evolved significantly. EPO binds to erythropoietin receptors (EPOR), initiating signaling that stimulates growth, inhibits apoptosis, and induces the differentiation of erythroid progenitors to increase red blood cell mass. EPO has additionally been shown to exert tissue-protective effects on multiple tissues, suggesting a pleiotropic mechanism of action. Erythropoiesis-stimulating agents (ESA) are used clinically for treating cancer-related anemia [chemotherapy-induced anemia (CIA)]. Recent clinical trials have reported increased adverse events and/or reduced survival in ESA-treated cancer patients receiving chemotherapy, potentially related to EPO-induced cancer progression. Signaling pathways downstream of EPO/EPOR have been shown to influence numerous cellular functions in both normal and tumor cells, including proliferation, apoptosis, and drug resistance. Some studies have reported effects on proliferation, reduced chemotherapy efficacy, reduction of apoptosis, and resistance to selective therapies on cancer cell lines, whereas others have shown null effects. In addition, newer targeted cancer therapies that are directed toward specific signaling pathways may be antagonized by ESAs. This molecular interplay between anticancer agents and potential survival signals triggered by ESAs may have been underestimated and may contribute toward decreased survival seen in certain trials. As more targeted anticancer therapies become available, these types of interactions may mitigate therapeutic efficacy by allowing tumor cells to acquire drug resistance. Therefore, a more complete understanding of the complex pathways involved will allow for the rational use of ESAs for the safe treatment of CIA in oncology patients. *Clin Cancer Res*; 17(20): 6373–80. ©2011 AACR.

Background

Clinical importance of erythropoietin/erythropoietin receptor

Recombinant human erythropoietin (rHuEPO) and other erythropoiesis-stimulating agents (ESA) were synthesized after the initial cloning of the human erythropoietin (EPO) gene sequence discovered in 1985 and have provided an alternative to transfusion for increasing red blood cell mass and treating anemia. Initially produced in the fetal liver during development, in the adult, EPO is primarily produced in the kidney as a response to hypoxic induction of the *epo* gene. EPO then binds to erythropoietin

receptors (EPOR), initiating signaling that stimulates growth, inhibits apoptosis, and induces differentiation of erythroid progenitors to increase red blood cell mass (1). EPO has additionally been shown to exert tissue-protective effects on multiple tissues (2), suggesting a pleiotropic mechanism of action. Over the past few decades, our understanding of the physiologic functions of EPO has evolved significantly.

Chronic anemia and/or chemotherapy-induced anemia (CIA) is a frequent side effect in cancer patients. CIA can be due to the malignancy infiltrating the bone marrow and impairing and/or dysregulating hematopoiesis, and/or it can occur as a result of systemic therapy used to treat disease. Anemia is classified as a hemoglobin level below 13 g/dL for men or below 12 g/dL for women. Onset of anemia is associated with reduced quality of life and may also enhance the emergence of hypoxia-induced treatment resistance (3). In early studies, ESAs were shown to be a safe and effective treatment for CIA, reducing numbers of required transfusions and increasing patient quality of life (4). Anemic patients with chronic renal failure, end stage renal disease, or those undergoing anemia-inducing treatment (such as chemotherapy) have been shown to benefit from ESA administration. However, recent meta-analyses have provided conflicting data indicating that ESAs may or may

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not negatively impact overall patient survival, raising concerns about the potential for disease progression leading to increased mortality rates in cancer patients (5–8). This review provides an overview of the EPO/EPOR signaling pathway, summarizes the current preclinical and clinical data available, and discusses the resulting controversies that exist in this area of experimental and clinical oncology.

Erythropoietin/erythropoietin receptor signaling

Direct influences of EPO on normal cell function and/or tumor progression require a functional cell surface EPOR to be present to activate downstream signaling pathways. EPOR is a *trans*-membrane receptor consisting of an extracellular domain that changes conformation upon ligand binding and a cytoplasmic domain with multiple phosphorylation residues (8 in total), serving as docking sites for proteins involved in downstream signal transduction. In erythroid cells, this receptor is present as a preformed EPOR homodimer (1); however, heterodimers formed between EPOR and the β common receptor have also been reported in erythroid and normal nonerythroid cells (9–11). The homodimer is thought to be responsible for proliferative effects in erythroid and nonerythroid cells, whereas the heterodimer is postulated to be responsible for EPO's tissue-protective effects (10, 12). The mechanisms and intracellular signaling pathways of the EPOR have been investigated in experimental models of both normal and pathologic conditions.

Downstream of EPOR, 4 main signaling pathways have been defined, including the Janus-activated kinase 2 (JAK2)/STAT5; mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK); phosphoinositide 3-kinase (PI3K)/Akt; and protein kinase C (PKC) pathways. Experimental evidence supports the involvement of additional pathways related to apoptosis and hypoxia. Fig. 1 depicts these key signaling pathways and their potential interaction with other pathways targeted in cancer therapy.

JAK/STAT pathway. The first of these is the JAK/STAT5 pathway. The STAT family plays a necessary role in signaling of ligand binding to multiple receptors. STATs have been shown to be activated by cytokines, numerous growth factors, as well as EPO, and have been implicated in carcinogenesis (reviewed in ref. 13). STAT5 is membrane bound via the EPOR cytoplasmic tail, and upon phosphorylation of EPOR by ligand binding, it, in turn, is phosphorylated by pJAK2 (14). The STAT protein becomes activated through the formation of a homodimer, upon which the complex translocates to the nucleus. This dimer interacts directly with DNA through STAT-specific binding domains, activating genes for cyclin D1 and BCL-XL and resulting in downstream effects on cell cycle control and resistance to apoptosis.

MAPK/ERK and PI3K pathways. The MAPK/ERK pathway has also been shown to be induced by EPOR phosphorylation through adaptor proteins (i.e., SOS and GRB2, also bound to the cytoplasmic tail of EPOR), which in turn activate RAS by GDP removal (15). Activated RAS then

induces a kinase cascade, activating both the MAPK/ERK and PI3K/Akt pathways. Studies investigating EPO induction of both pathways have shown activation of *c-Myc* expression, resulting in the production of the nuclear protein myc. Myc forms heterodimers with numerous other proteins in the nucleus, where functionality is dimer specific. The wide range of factors that can be modulated by myc expression includes cell cycle kinase inhibitors, protein synthesis regulators, and microRNAs (16).

PKC pathway. Finally, the PKC pathway is activated by hydrolysis of membrane-bound glycoposphatidylinositol (GPI)-linked proteins. EPO binding to EPOR phosphorylates one or more tyrosine residues on phospholipase C-gamma (PLC- γ), inducing hydrolysis of GPI (17). The final element in this cascade is translocation of regulatory factors to the nucleus such as ERK, which ultimately influences gene transcription and alters cell behavior (e.g., protection from apoptosis and DNA synthesis).

Independently, each of these pathways has been shown to influence numerous cellular functions in both normal and tumor cells, including proliferation, apoptosis, and drug resistance (13, 18–21). EPOR signaling in erythropoiesis may be downregulated by the production of inhibitory proteins (SOCS family of proteins or certain tyrosine phosphatases, e.g., Shp-1 or CD45), ubiquitination, and/or by dephosphorylation of key sites in the EPOR dimer, all of which influence the pathways described above. It is speculated that similar inhibitory mechanisms may be impaired in cancer cells (22).

Apoptotic pathways. Many studies have focused on the antiapoptotic effects of EPO therapy in models of cancer (23–25) and neuroprotection (26, 27). Some studies have shown upregulation of antiapoptotic factors, such as BCL-X_L and other BCL-2-related proteins, whereas work from other groups has conversely shown downregulation of proapoptotic proteins, such as BAX, BAD, or caspases (28–30), suggesting that EPO may play a role in mitigating the effect of apoptotic signaling. Another protein that has been shown to be associated with EPO-mediated apoptotic resistance has been NF- κ B (31, 32). Numerous anticancer agents use NF- κ B activation to kill tumor cells; therefore, ESA therapy has the potential to negatively impact cancer therapy that is dependent on NF- κ B signaling.

Hypoxia pathways. In normal physiology, the EPO gene is regulated by hypoxia through the transcriptional regulator family of hypoxia-inducible factors (HIF) and can be upregulated more than 1,000-fold in the case of severe hypoxia. Endogenous EPO is essential in embryonic development, and absence of EPO in the brain results in neural defects (33) and is required for neuronal survival (34). Studies with *epo*-null mice are embryonic lethal (35), due to systemic hypoxia resulting from severe anemia induction because of the lack of EPO production. Ischemia studies with pre- and postocclusion administration of EPO have shown a reduction in infarct size and neuronal apoptosis in EPO-treated animals (36–40), suggesting EPO-mediated neuroprotective effects in the brain. HIF-1 is one member of this family of transcription factors that has a binding site

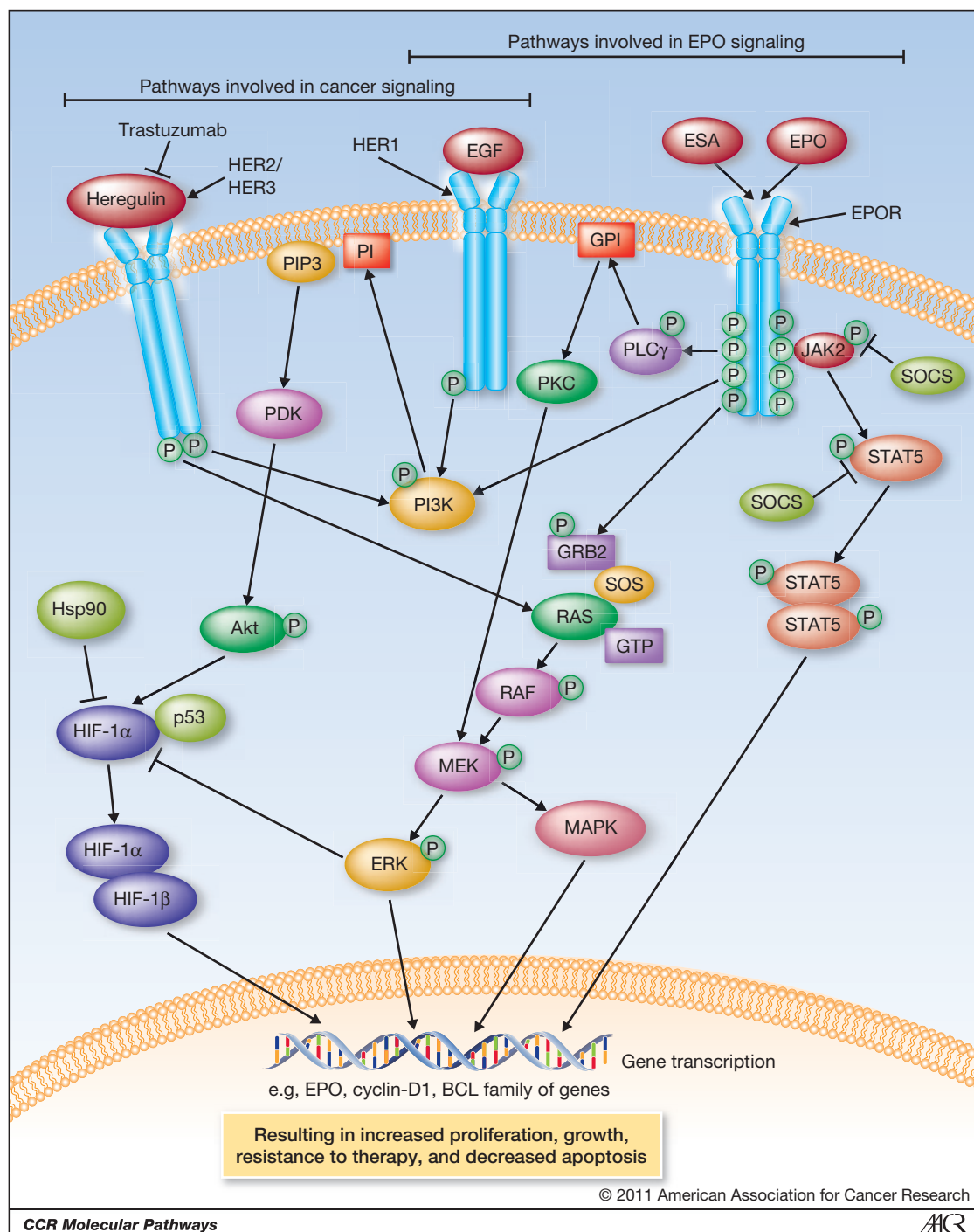


Figure 1. EPO-associated signaling pathways. The interplay between the EPOR signaling pathway(s) from studies done with hematopoietic cells, the EPO production pathway through HER1 and HIF-1 α expression, and the signaling pathway for HER2 and the targeted therapy trastuzumab. EPOR signaling: EPOR is present as a preformed EPOR homodimer. Downstream of EPOR, 4 main signaling pathways have been defined, including the JAK2/STAT5, MAPK/ERK, PI3K/Akt, and PKC pathways, which ultimately influence gene transcription and alter cell behavior. EPO production: the transcription factor HIF-1 is primarily responsible for the transcription of hypoxia-regulated genes, including EPO. Induction of the PI3K/Akt pathway by oxygen sensing and signaling results in increased half-life of HIF-1 α (the rate-limiting subunit of the HIF-1 heterodimer) and permits the formation of functionally active heterodimers. This pathway is regulated by specific inhibitors (e.g., Hsp-90, Erk), protein stability, and other factors, such as phosphorylation, redox chemistry, and nuclear localization. EPO and HER2 signaling: heterodimer formation of the Her family of receptors leads to intracellular phosphorylation that induces multiple signaling cascades, including the MAPK and PI3K/Akt pathways. Trastuzumab is a humanized monoclonal antibody that binds to the extracellular segment of the HER2 receptor and induces disruption of receptor dimerization and signaling through the downstream PI3K cascade. EGF, epidermal growth factor; GRB2, growth factor receptor binding protein 2; MEK, MAP/ERK kinase.

on the 3' end of the *epo* gene. The HIF-1 heterodimer is composed of 2 subunits (α and β), where HIF-1 α protein is only detected after hypoxic insult (41). Under normoxic conditions, HIF-1 α is rapidly ubiquitinated; however, specific inhibitors (e.g., Hsp-90 and transition metals such as Co^{2+} , Ni^{2+} , or Mn^{2+}) of this ubiquitin-proteasome pathway increase the abundance of HIF-1 α protein (41, 42). The resulting increased half-life of HIF-1 α permits formation of functionally active heterodimers, and this and other factors, such as phosphorylation (43), redox chemistry (41), and nuclear localization (44), have been shown to modulate activation of HIF-1.

Experimental and translational challenges

Determination of EPOR expression remains problematic owing to splice variants at the mRNA level and poor and/or nonspecific antibodies at the protein level (2). Antibody specificity remains one of the biggest obstacles for EPOR research, as several antibodies used for clinical studies have since been shown to bind nonspecifically to proteins of a similar size to EPOR, but not to EPOR itself (45). The C-20 antibody was widely used prior to 2006, but subsequently, it was found to be unsuitable for immunohistochemistry and immunoblotting, leading to questionable results from studies using this antibody (46). Recent work by Swift and colleagues showed minimal protein expression of EPOR in 66 commonly used cell lines using a rigorously tested custom-made antibody (47). Other studies have reported conflicting results to this work and have shown EPOR expression in tumor cell lines, as well as differences of effects on EPO-associated signaling pathways (48), leading to further confusion over whether tumor cells, at least at the cell-line level, express functional EPO receptors.

Clinical-Translational Advances

Bedside to bench: erythropoiesis-stimulating agent treatment in clinical trials

The original rationale for using ESAs in cancer patients arose from studies designed to determine the benefit of ESA treatment that highlighted improved quality of life with ESA treatment, reduction in transfusion dependency, and potential improved treatment efficacy because of increased tumor oxygenation. However, several publications in the last 5 years have suggested that ESA treatment may, in fact, have adverse effects on patient survival (5–8, 49, 50). Due to diversity in trial design, comparison between studies is difficult; multiple interstudy differences include variability in disease stage, patient treatment history, control groups, reporting parameters, and most frequently, variation in target hemoglobin (Hb) levels. Not surprisingly, this large variation in study design translates to varying responses to ESA treatment, depending on endpoints, and the demonstration of both adverse and beneficial effects on overall survival, with a large number of trials showing no effect of ESA administration (6–8).

Recent meta-analyses have included analyses of single patient data and suggest an increased mortality with ESA

use for certain subgroups of patients and target Hb levels (51). Recent trials, such as the ENHANCE (head and neck cancer), EPO-CAN 20 (non-small cell lung cancer), GOG 191 (cervical cancer), and trials in breast cancer have raised concerns over ESA treatment by reporting shorter progression-free survival and/or overall survival in patients treated with ESAs (52). In breast cancer, multiple trials have evaluated ESA effects (4, 49, 53–57), and of these, 2 studies (BRAVE and BEST) included only patients with metastatic disease (53, 54). The BRAVE study detected no difference in overall survival (53), whereas the BEST study was prematurely terminated because of a higher mortality rate at 12 months in the ESA-treated arm (54). It should be noted that ESA-treated patients in the BEST trial had higher Hb levels at endpoint than those in the BRAVE trial; thus, it cannot be determined if the higher Hb levels contributed to the adverse outcomes or if ESAs can directly promote tumor progression. It has been speculated that the negative outcomes reported may be due to either direct or indirect actions on the tumor. For example, direct interactions might include EPO/EPOR-mediated effects on growth, survival, or apoptosis of cancer cells, as is seen in erythroid cells (reviewed in ref. 1). Indirect influences of EPO may include factors related to the metastatic niche [i.e., host-tumor cell mobilization, angiogenesis, increased thrombosis, matrix metalloproteinase (MMP) production], which in turn promote tumor progression and metastasis and/or may be a result of interactions with cancer therapies given to treat patients. Taken together with data from preclinical studies that show evidence of EPOR expression on tumor cells, biological effects on cancer cells after ESA treatment, and the potential to mitigate curative cancer therapy, these trial results have raised concerns over using ESAs in clinical oncology (58).

Ultimately, the important clinical question is whether ESA-tumor interactions (direct or indirect) may lead to disease progression. Because of this uncertainty, the use of ESAs in oncology has recently been limited. Clinicians are currently faced with determining the risk-to-benefit ratio of ESA therapy in individual patients with different tumor types and disease stages. Because clinical trials have not been able to provide answers to these biological questions owing to difficulty in determining EPOR expression in tissue tumor, poor study design (measurement of the right parameters), and endpoints that do not measure tumor progression, preclinical models may be the best approach for answering some of these questions and providing a mechanistic basis to help develop informed clinical application of ESA therapy.

Bench to bedside: erythropoiesis-stimulating agent treatment in preclinical models

As with clinical studies, accurate detection of EPOR (mRNA and protein) expression levels is required to determine if EPO can directly mediate changes in signaling, growth, and survival of tumor cells. Preclinical studies also allow indirect effects of ESA treatment to be monitored in ways that cannot be done in patients.

In vitro studies have shown EPOR to be expressed in both normal and malignant cells (59). As discussed, many of these studies have relied on anti-EPOR antibodies that have since been shown to be nonspecific (45, 46). Preclinical studies have used a wide spectrum of cell lines and therapies currently used to treat cancer patients. Results from these studies have been mixed about tumor cell proliferation, chemoprotection, and/or treatment resistance in response to rHuEPO (60–62). Work published by Liu and colleagues showed no chemoprotective effect of rHuEPO (61), whereas studies by Belenkov and colleagues showed a survival benefit with rHuEPO in combination with radiotherapy (62). *In vitro* work done by our group has shown no growth enhancement or protective effect from various treatments in combination with ESA treatment (63). Variable methodologic approaches were used in these conflicting studies, often limited to histopathologic, biochemical, or *in vitro* evaluation, thus highlighting the need for a more complete functional assessment of the influence of ESAs on tumor progression in any given model (59).

In vivo studies are also contradictory, in which ESA treatment has shown increased primary tumor growth of Lewis lung carcinoma cells (cells lacking EPOR; ref. 64), and other studies have shown no growth-enhancing effect of ESAs (65–67). ESA-induced increases in chemotherapeutic response (65, 67) and increased radiosensitivity in response to rHuEPO have been observed (66). However, none of these studies evaluated metastasis, and doses of rHuEPO used were mostly considerably higher [from 1,000 U/kg biweekly (65) to 1,000 U/kg 3 times a week (66)] than the normal clinical dose range of 300 to 600 U/kg weekly used to treat CIA (68). Additional recent work by Liang and colleagues (with dosing in the clinical range) has shown an EPO-mediated reversal of the effects of the HER2 monoclonal antibody trastuzumab on primary tumor growth, indicating that the signaling pathway inhibition mediated by trastuzumab (PI3K/Akt and ERK) may be disrupted by rHuEPO (48).

With the exception of our studies (63), *in vivo* preclinical ESA studies to date have been limited to assessment of primary tumor growth with variable results. *In vivo* work by our group has shown EPO-mediated (300 U/kg weekly) reversal of paclitaxel treatment in 2 animal models of metastasis. Immune-compromised mice were injected with either MDA-MB-231 or MDA-MB-435 breast cancer cells and left untreated or treated with chemotherapy alone, ESA alone, or combination chemotherapy and ESA. Primary tumor growth remained unaffected in our experiments; however, the incidence and burden of metastases was increased when ESA treatment was given with chemotherapy versus chemotherapy alone ($P < 0.05$; ref. 63). Our results are in agreement with those from Liang and colleagues showing no effect of ESA therapy alone (48). Clinical disease progression in solid tumors requires evolution from localized disease to metastatic disease, which often is the precipitating cause of patient death. Investigating rHuEPO effects on cell lines *in vitro* or on primary tumor growth does not accurately mimic systemic tumor progres-

sion seen clinically; therefore, future preclinical studies should investigate ESA treatment effects on metastasis *in vivo* in more detail. The *in vivo* effects on metastasis that we have observed could involve rHuEPO binding to its receptor on host cells in the surrounding microenvironment, and subsequent activation of signaling could result in numerous host effects, including vasculogenesis, increased MMP production, endothelial progenitor mobilization, and activation of the coagulation cascade leading to increased thrombin deposition (69, 70). Importantly, these parameters are not usually monitored clinically after rHuEPO administration (68), although experimental studies have shown that activation of these processes can also enhance metastasis (71). In the case of thrombosis (a known adverse effect of ESA therapy), increased microthrombi formation may facilitate local vascular invasion and increase metastatic burden (72).

Do erythropoiesis-stimulating agents affect the efficacy of anticancer therapies?

Four main modalities of anticancer therapy are being investigated in preclinical and clinical ESA studies: hormone, chemotherapy, radiation, and targeted therapies. In clinical trials, treatment resistance directly attributable to ESA administration has not been reported; however, disease progression has been reported (7, 8, 50). In the majority of cases, ESA treatment alone has shown no effect on tumor cells, although effects on control cells were not presented in all published work. Differences in response may reflect biological differences in cell lines, experimental design, ESA dose administered, or anticancer agent used. Often therapeutic resistance in preclinical models has been observed in studies in which the ESA dosing was much higher than doses used in patients.

It is reasonable to hypothesize that directed treatments targeting common pathways involved in EPO/EPOR signaling may be modulated by ESAs. In erythropoiesis, ESAs exert their effect primarily by inhibiting apoptosis of erythroblast precursors and, hence, increasing red blood cell mass. Thus, the efficacy of chemotherapy regimes designed to target antiapoptotic pathways may be diminished by ESAs that have been shown to increase protein levels of antiapoptotic genes, such as *BCL-XL*, and decrease protein levels of proapoptotic genes, such as *BAX* (24). Liang and colleagues used both *in vitro* and *in vivo* studies with HER2-positive, EPOR-expressing breast cancer cell lines to show that a functional EPOR in breast cancer cell lines can stimulate downstream signaling pathways (PI3K/Akt and ERK) and block anti-Her2 treatment (trastuzumab) designed to act on these same pathways (48). *In vivo* ESA administration combined with trastuzumab in mice with HER2-positive tumors suppressed the effects of trastuzumab, allowing primary tumor growth. These effects may be limited to a single therapy, and combination therapy may be able to overcome this type of resistance. However, treatment resistance with ESAs may also be due, in part, to changes in the microenvironment in response to

ESA influence on host cells. Platelet aggregation and protection of circulating tumor cells, increased growth factor production, and host endothelial cell mobilization have all been shown to be enhanced by ESA treatment (69, 70) and, separately, to enhance tumor progression (72).

Conclusions

In summary, the role of ESAs in cancer patients with CIA has been investigated in a large number of trials, which include patients with multiple disease types and different stages from early to metastatic disease (4, 49, 53–57, 73). In several trials, an increase in thrombotic events was seen in the ESA-treated arm (5, 7, 54, 74). More importantly, meta-analyses show a decrease in overall survival in some studies in patients treated with ESAs (5, 7), prompting concern about ESA use in cancer patients with anemia. Although the cause of this decreased survival is unknown, it has been speculated that administration of ESAs may cause cancer progression (7, 53). However, to date preclinical studies have not been informative because of poor reagents and the fact that most *in vivo* studies analyzed primary tumor growth alone.

The present American Society of Hematology and the American Society of Clinical Oncology guidelines, updated in 2010, state that ESA use should be limited in oncology and patients monitored more closely (58). Patients with CIA who experience a drop in Hb levels below 10 g/L and are being treated without curative intent are the only patients to whom ESAs should be administered. Paradoxically, patients in this group (i.e., those with bulky and/or late-stage disease) are also at much

higher risk of venous thromboembolism (5, 57). Additionally, current guidelines suggest that ESA treatment continue for the minimum period necessary to minimize any adverse effects of ESA therapy. Future work in this area must also examine the role of possible drug interactions with common signaling pathways triggered by both ESA and cancer therapeutics. A complete understanding of the pathways involved and their molecular interplay will allow proper clinical management of patients with this class of drug and determine how future ESAs can be used to treat CIA safely and effectively.

Disclosure of Potential Conflicts of Interest

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