Erythropoietin activates cell survival pathways in breast cancer stem-like cells to protect them from chemotherapy

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Abstract

Recombinant erythropoietin (EPO) analogs (ESAs, erythropoiesis-stimulating agents) are clinically used to treat anemia in cancer patients receiving chemotherapy. After clinical trials reporting increased adverse events and/or reduced survival in ESAs-treated patients, concerns have raised about the potential role of ESAs in promoting tumor progression, possibly through tumor cell stimulation. However, evidence is lacking on the ability of EPO to directly affect cancer stem-like cells, which are thought to be responsible for tumor progression and relapse. We found that breast cancer stem-like cells (BCSC) isolated from patient tumors express the EPO receptor and respond to EPO treatment with increased proliferation and self-renewal. Importantly, EPO stimulation increased BCSC resistance to chemotherapeutic agents and activated cellular pathways responsible for survival and drug resistance. Specifically, the Akt and ERK pathways were activated in BCSC at early time points following EPO treatment, while Bcl-xL levels increased at later times. In vivo, EPO administration counteracted the effects of chemotherapeutic agents on BCSC-derived orthotopic tumor xenografts and promoted metastatic progression both in the presence and in the absence of chemotherapy treatment. Altogether, these results indicate that Epo acts directly on BCSC by activating specific survival pathways, resulting in BCSC protection from chemotherapy and enhanced tumor progression.

Introduction

Erythropoiesis-stimulating agents (ESAs) have been used for two decades in the supportive therapy of cancer patients, due to their ability to increase red blood cell production and to reduce the need of transfusions (1). In 2002, ESAs were administered to approximately 45% of all cancer patients (2). However, following clinical trials reporting a shorter progression-free survival and/or overall survival in ESAs-treated patients, ESAs were suspected to increase the risks of thromboembolic events and to enhance tumor progression (3-6). Consequently, in 2008 the Food and Drug Administration (FDA) limited the indication for ESAs administration to cancer patients with...
hemoglobin less than 10 g/dL receiving chemotherapy for palliative intent (7). Since then, the use of ESAs in cancer patients progressively declined, and recently the FDA released new guidelines ensuring that ESAs access is strictly controlled and that patients are fully informed about ESAs-related risks (8). Despite clinical observations suggesting a possible association between ESAs and tumor progression, the effect of EPO on neoplastic cells remains a matter of debate. In particular, experimental studies on the effect of EPO on cancer cells yielded controversial results, likely due to variable methodologic approaches. Recent in vivo studies, however, provided important clues on tumors’ response to EPO. Specifically, EPO was shown to antagonize the effect of trastuzumab on breast cancer xenografts and to decrease the effect of chemotherapy in a mouse model of metastatic breast cancer (9, 10). Such studies suggest a direct influence of EPO on breast tumors and highlight the importance of reliable in vivo models to elucidate the interactions between EPO and tumor cells.

The existence of cancer stem cells (CSC) in solid tumors was demonstrated for the first time in breast cancer, where CSC were isolated as a CD44+/CD24−/low population able to initiate tumors with as few as 200 cells (11). Lately, breast tumorigenic cells were identified either by distinctive phenotypes such as ALDH+, CD24high/CD49fhigh/delta-like 1 (DLL1)high, CD24high/CD49fhigh /Delta-notch like epidermal growth factor repeat-containing transmembrane (DNER)high, or through functional characteristics such as enhanced PKH26 dye-retaining capacity or low proteasome activity (reviewed in (12)). More recently, breast cancer stem cells (BCSC) were identified as a ganglioside GD2+ population able to form tumors with as few as 10 cells (13). BCSC have been shown to increase after chemotherapy treatment (14) and to be quantitatively associated with chemotherapy resistance (15). Moreover, BCSC have been shown to mediate invasion and metastasis both in vitro and in mouse models (16). Elucidating the effect of EPO on BCSC is therefore crucial to fully understand the effects of ESAs treatment in breast cancer patients. As ESAs administration is reserved to patients with metastatic breast cancer receiving chemotherapy, it is particularly important to understand whether they may influence BCSC response to anticancer drugs and metastasis progression. Here, we employed human BCSC-derived orthotopic/metastatic...
xenografts to show that BCSC response to EPO *in vivo* results in increased chemotherapy resistance of primary tumors and metastases, resulting in enhanced tumor progression.

**Materials and Methods**

**Antibodies and reagents**

Primary antibodies were: mouse monoclonal anti-EPOR MAB307 (R&D Systems) (10), rabbit polyclonal anti-EPOR M20 (Santa Cruz Biotechnology) (9), rabbit polyclonal anti-Akt and rabbit polyclonal anti-phospho-Akt Ser 473 (9272 and 9271, Cell Signaling Technology), mouse monoclonal anti-phospho Erk1/2 Tyr 204 (E4, Santa Cruz), rabbit polyclonal anti-Erk1 (K23, Santa Cruz), mouse monoclonal anti-Bcl-xL (H-5, Santa Cruz), mouse monoclonal anti-β-actin (JLA20, Calbiochem), mouse monoclonal anti-CD44 (BU75, Ancell), mouse monoclonal anti-CD24 (HIS50, BD Biosciences), mouse monoclonal anti-p63 (4A4, Santa Cruz), mouse monoclonal CK8-18 (5D3), mouse monoclonal CK14 (LL002) and mouse monoclonal CK5 (XM26) (all from Novocastra), mouse monoclonal anti-Ki67 (MIB-1, Dako), mouse monoclonal anti-CD49f (MP4F10, R&D Systems). Secondary antibodies were: horseradish peroxidase (HRP)-conjugated anti mouse antibody (Pierce), HRP-conjugated anti rabbit antibody (Thermo Scientific), mouse FITC- and Rhodamine red-conjugated antibodies (Invitrogen Molecular Probes), and mouse R-Phycoerythrin (PE) antibody (Sigma). Recombinant human erythropoietin (EPO) was purchased from R&D Systems.

**BCSC isolation and culture**

Human breast cancer tissues were obtained from patients undergoing surgery in accordance with the ethical standards of the institutional Committee on human experimentation (authorization no. CE-
ISS 09/282). Tumor tissues were mechanically and enzymatically digested with collagenase (1.5 mg/ml, Gibco) and hyaluronidase (20 mg/ml; Sigma-Aldrich) in DMEM (Gibco), shaking for 1 hour at 37°C. The resulting cell suspension was plated in ultra-low attachment flasks (Corning) in serum-free medium supplemented with basic fibroblast growth factor (bFGF, 10 ng/ml) and epidermal growth factor (EGF, 20 ng/ml) as previously described (17). This procedure yielded BCSC lines that were subjected to genotyping in order to validate each cell line individuality and were further tested for their ability to generate tumor xenografts that replicated the histology of the parental tumor.

**Viability, proliferation and clonogenic assays**

For viability assays, BCSC untreated or pretreated 24 hours with 3U/ml EPO were cultured for the indicated times in presence of doxorubicin (1 µM), 5-fluorouracil (25 µM) or taxol (5 µM). The number of viable cells was detected by CellTiter Aqueous Assay Kit (Promega). Cell death was also assessed by acridine orange (50 µg/ml)/ethidium bromide (1 µg/ml) staining and fluorescence microscopy detection. Colony forming assays were performed on soft agar (Seaplaque, Lonza) with 0.4% base agar and 0.3% top-layer agar. After 21 days, colonies were stained with 0.01% crystal violet and visualized under a light microscope.

**Immunoblotting**

Cells were growth factor-starved for 24 hours and treated with 3U/ml EPO for 10, 30, 120 min and 48 hours. Protein extracts were obtained in ice-cold T-PER buffer (Thermo Scientific) with protease inhibitors (Sigma). Equal amounts of proteins were loaded on SDS-PAGE gels and transferred to nitrocellulose membranes, subsequently blocked with 5% nonfat dry milk in TBS containing 0.1% Tween 20 and probed with primary and HRP-linked secondary antibodies. Immunoreactive bands were visualized with SuperSignal West Dura Substrate (Pierce). Image acquisition was performed with a ChemiDoc Imaging system (UVP Advanced Imaging Systems).
**Immunohistochemistry**

Apoptotic cells on paraffin-embedded breast cancer xenograft sections were visualized by the TUNEL (TdT-mediated dUTP-X nick end labeling) reaction with the In Situ AP Cell Death Detection Kit (Roche). Immunohistochemical analyses were performed on 5 µm-thick paraffin-embedded sections of breast cancer tissue and xenografts. Tissues were heated for antigen retrieval and stained with specific antibodies against Bcl-xL, CK 8-18, CK 14, CK 5, p63, Ki67, EPOR or isotype-matched controls overnight at 4°C. Sections were incubated with biotinylated anti-mouse or anti-rabbit immunoglobulins and subjected to streptavidin-peroxidase (Dako). Stainings were revealed using 3-amino-9-ethylcarbazole substrate (AEC, Dako) substrate and cells counterstained with aqueous hematoxylin. Slides were mounted with synthetic resin.

**Immunofluorescence and flow cytometry**

Immunofluorescence was performed on cytospins of cultured BCSC fixed with 2% paraformaldehyde for 20 min at 37°C, blocked with 0,5% BSA for 30 min and exposed overnight at 4°C to antibodies against EPOR, CD44 and CD24. Stained slides were treated with Rhodamine Red or FITC conjugated anti-mouse antibodies with the addition of 200 ng/ml RNAse (Sigma). Nuclei were counterstained with TOTO-3 iodide (Invitrogen Molecular Probes) and images were acquired using an Olympus FV1000 confocal microscope. For FACS staining, BCSC were fixed with 2% paraformaldehyde and stained with primary antibodies against CD44, CD24, EPOR or isotype-matched controls and then with fluorochrome-conjugated secondary antibodies. Samples were analyzed with a FACS Calibur equipped with CellQuest Software (BD Biosciences).

**Mice treatment**

Animal studies were performed according to the institutional guidelines under the Italian Ministry of Health authorization (DM 23/2011-B). BCSC (3x10^5) were suspended in 100 µl of 1:6 Matrigel (BD) and orthotopically injected in five week-old NOD/SCID mice (Charles River Laboratories).
Tumor size was measured weekly with an electronic caliper and volume was calculated using the formula: \( \pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2 \). After 4 weeks, mice were treated intraperitoneally either with doxorubicin (2 mg/Kg, on day 2 and day 5 every week for 4 weeks) or 5-fluorouracil (150 mg/Kg, on day 1 every week for 4 weeks), alone or in combination with EPO (150 U/Kg on day 1 and day 4 every week for 4 weeks). PBS was used as control. At the end of the treatment, mice were sacrificed and tumors collected for histological analyses. In order to determine the \textit{in vivo} effects of EPO on a metastatic breast cancer model, NOD/SCID mice were orthotopically injected with \( 4.5 \times 10^5 \) BCSC carrying a Tween Luciferase - GFP lentiviral vector. After cell inoculation, mice received a subcutaneous injection of D-luciferin (150 mg/kg, Promega) and were analyzed by \textit{in vivo} imaging (Biospace Laboratories). Five weeks later, primary tumors of mice showing lung metastases (as measured by luciferase intensity) were removed and mice (\( n=4 \) per group) received respectively i.p. injections of PBS, paclitaxel (10mg/Kg, on day 1 every week for 3 weeks), EPO (300 U/Kg on day 2 every week for 3 weeks) or paclitaxel + EPO. Three weeks later, mice were euthanized and lungs were analyzed for luciferase expression. Data were quantified with Biospace Lab M3 Vision software.

\textbf{Statistical analysis}

Data were expressed as mean ± standard deviation (SD). The statistical significance of results was determined by Bonferroni’s multiple comparison tests. Results were considered significant when \( P \) values were less than 0.05 (\(* P<0.05; **P<0.01; ***P<0.001\) ).
Results and discussion

Breast cancer stem cells (BCSC) can be isolated from tumor specimens by selective culture in medium containing EGF and bFGF (18), resulting in a majority of CD44+/CD24−/low cells that form progressively expanding tumor spheres (Fig. 1A and Supplementary Fig. S1A). According to this method, five BCSC lines were isolated from infiltrating breast tumors (Supplementary Table S1). Cells isolated in such conditions fulfilled the functional characteristics of cancer stem cells, as they were able to produce tumors in immunocompromised mice that replicate the original patient tumor in terms of histological structure and marker expression (Fig. 1B). As a first step to investigate the potential BCSC sensitivity to EPO, we assessed whether the EPO receptor was detectable on cultured CSC and on tissue sections of different breast tumor subtypes, whose BCSC content was reportedly related to increasing malignancy (19). Due to previous controversies about the specificity of anti-EPO receptor antibodies, we used only antibodies that were validated by recent authoritative studies (see Methods). The specificity of the anti-EPO receptor antibody used for immunofluorescence and flow cytometry was further validated by assessing its ability to detect EPO receptor increase in leukemic UT-7 EPO cells upon growth factor starvation (20) (Supplementary Fig. S1B). Staining of intact BCSC with anti-EPOR antibody revealed substantial (31-99%) EPOR expression on all the BCSC lines examined (Fig. 1C), indicating a potential role of EPO in the regulation of BCSC proliferation and survival. EPO receptor was also detected on cultured BCSC stained with CD49f antibodies (Supplementary Fig. S1C), indicating its presence on BCSC populations identified with different stem cell-associated markers. In tissue sections, strong EPO receptor expression was detected on the positive control (placenta) and on basal-like tumors. EPO receptor was also present, at a lesser extent, on HER2+ and luminal B tumors, while a faint expression was detectable on luminal A tumors and on normal breast, the latter showing a positivity at the limit of detection (Fig. 2A and related Supplementary Table S2). Treatment of BCSC cultures with recombinant human EPO resulted in increased cell proliferation, indicating that EPOR
expressed on BCSC surface is functional and delivers signals that modulate cell growth (Fig. 2B). To determine whether EPO was able to affect BCSC self-renewal, cells derived from dissociated mammospheres were plated in soft agar in the presence or in the absence of EPO. Colony scoring after three weeks showed a significantly higher number of colonies in EPO-treated samples in 3/5 cases, indicating that EPO can increase BCSC self-renewal in vitro (Fig. 2C).

BCSC were previously shown to be more resistant than bulk tumor cells to chemotherapeutic drugs (14). Since EPO is used almost exclusively in cancer patients undergoing chemotherapy, it is essential to clarify whether EPO can further enhance BCSC resistance to chemotherapy, therefore favoring drug resistance and tumor relapse. Upon treatment of mammospheres with EPO and with chemotherapeutic agents commonly used for breast cancer therapy, we found that the presence of EPO resulted in increased BCSC survival in the presence of cytotoxic drugs (Fig. 3A-B). This observation indicates that EPO activates survival signals in BCSC that are responsible for chemoresistance. To identify pathways downstream of EPOR that may be responsible for apoptosis resistance in BCSC, we analyzed levels of phospho-Akt, phospho-Erk and Bcl-xL at different time points upon EPO stimulation (Fig. 3C and Supplementary Figure S2). Phosphorylation/activation of Erk and Akt was maximal respectively 10 minutes and 2 hours after EPO stimulation in 5/5 and 4/5 BCSC lines (Fig. 3C). Differently, an increase in Bcl-xL levels was not apparent at early time points of EPO stimulation (Supplementary Figure S2), but became clear in 5/5 BCSC lines after 48 hours of treatment (Fig. 3C). Increase in pErk, pAkt and Bcl-xL upon EPO stimulation was apparent also in intact spheres (Fig. 3D).

To investigate whether EPO treatment affected tumor response to chemotherapy in vivo, we produced orthotopic breast tumors by injecting BCSC in the mammary fat pad of NOD/SCID mice. Tumors were allowed to grow until they reached the size of 50 mm³, then mice were treated with EPO and/or chemotherapeutic agents doxorubicin (Fig. 4A-B) or 5-fluorouracil (Supplementary Fig. S3A-B) for four weeks, during which tumor volume was constantly monitored with an electronic caliper. While vehicle-treated tumors grew exponentially, chemotherapy-treated tumors
were significantly inhibited. By contrast, the growth of tumors treated with EPO + chemotherapy was similar to controls, indicating a chemoprotective effect of EPO in vivo (Fig. 4A-B and Supplementary Fig. S3A). Staining of xenograft sections at the end of the treatment revealed an increased rate of apoptosis and lower levels of Bcl-xL expression in chemotherapy-treated tumors but not in tumors treated with chemotherapy and EPO in combination (Fig. 4B and Supplementary Fig. S3B). These results indicate that EPO reduces the efficacy of chemotherapy in vivo by promoting BCSC apoptosis resistance. In order to investigate whether EPO could influence the growth of metastatic tumors, we injected luciferase-transduced BCSC in the mammary fat pad of NOD/SCID mice and awaited for the formation of spontaneous lung metastases. Five weeks after BCSC injection, when metastases started to be detectable, the primary tumor was removed in order to observe the effect of subsequent treatments solely on metastatic sites. Mice were then treated for three weeks with EPO alone, with paclitaxel or with the EPO + paclitaxel combination. At the end of the treatment mice were sacrificed and lungs were analyzed for luciferase expression. Tumor burden in lungs of mice treated with EPO alone was strongly increased as compared to that of control mice (Fig. 4C-D). Enhanced metastatic progression was also found in the lungs of mice treated with the paclitaxel + EPO combination as compared to mice treated with paclitaxel alone, indicating that EPO exerted a chemoprotective effect on metastatic tumors (Fig. 4C-D).

Since the disclosure of clinical trials showing that ESAs treatment had an adverse influence on patient survival, the effect of EPO on tumor cells has been the subject of an intense debate. While ESAs likely influence patient survival through multiple mechanisms, few in vivo studies have specifically addressed the question of whether EPO modifies tumor response to therapy. We have shown for the first time that EPO can bind and stimulate BCSC, resulting in increased tumor growth and chemoresistance. These results confirm and expand previous observations performed by Hedley et al. on xenografts obtained with breast cancer cell lines (10). In breast cancer patients, EPO-mediated BCSC stimulation may not result in immediate effects on tumor growth or response to
chemotherapy, as BCSC represent a minority of cells, but may favor subsequent tumor relapse. Further clinical studies that evaluate rates of relapse in ESAs-treated patients would be required to clarify this issue.

Authors’ Contribution:

Conception and design: A. Zeuner, R. De Maria.

Acquisition of data: M. Todaro, A. Turdo, M. Bartucci, F. Iovino, R. Dattilo, G. Federici.

Supply of clinical samples: M. Biffoni.

Analysis and interpretation of data: M. Todaro, G. Federici.

Writing, review, and/or revision of the manuscript: A. Zeuner, R. De Maria, G. Stassi.

Study supervision: A. Zeuner, M. Todaro, R. De Maria.

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References


Legend to the figures

**Figure 1.** Characterization of BCSC lines and BCSC-derived xenografts. A, Microscopic imaging of progressive sphere formation by a single BCSC (line 308). Bar, 25 μm. B, Immunohistochemical staining with hematoxylin/eosin (H&E) or antibodies against cytokeratins 5 (CK5), 14 (CK14), 8-18 (CK8-18), Ki67, p63 of sections derived from breast carcinoma (Parental) and from a mouse xenograft (Xenograft) generated with BCSC derived from the same tumor (BCSC line 308). Bar, 40 μm (inset 30 μm). C, EPO receptor (EPOR) expression on BCSC lines detected by fluorescence microscopy (upper panels. Bar, 25 μm) or flow cytometry (lower panels).

**Figure 2.** EPO receptor expression in breast cancer subtypes and EPO response of cultured BCSC. A, EPO receptor (EPOR) staining of tissue sections derived from human placenta, normal breast (upper panels) and breast tumors of different subtypes (lower panels). Black arrows indicate EPOR-positive cells. Bar, 45 μm (inset 25 μm). B, Number of cells obtained after 72 hours of culture in the absence (control) or in the presence of EPO 3U/ml (EPO). Results shown are the mean ± SD of experiments performed in triplicate with five BCSC lines. *, P < 0.05. C, Left: number of colonies generated in semisolid culture conditions by BCSC lines in the absence (Control) or in the presence (EPO) of EPO 3U/ml. Right: representative picture of the plates (BCSC line 308). *, P < 0.05.
**Figure 3.** EPO increases BCSC resistance to chemotherapy *in vitro* by stimulating cell survival pathways. A, BCSC untreated (Control), treated with EPO 3U/ml (EPO), with chemotherapeutic agents doxorubicin (Doxo, 1µM), 5-fluorouracil (5FU, 25 µM) or taxol (Taxol, 5 µM) and with the combination EPO-chemotherapy were assessed for cell viability after 24, 48 and 72 hours. Results shown are the mean ± SD of experiments performed in triplicate with five BCSC lines. *, P < 0.05; **, P < 0.01; ***, P < 0.001. B, ethidium bromide/acridine orange staining of tumor spheres (BCSC line 308) treated as above. C, levels of phospho-Erk (pErk), phospho-Akt (pAkt) and Bcl-xL in BCSC untreated (Control) or growth factor-starved and stimulated with 3U/ml EPO respectively for 10 minutes, 2 hours and 48 hours. D, cells treated as in C (BCSC line 208) were stained with the indicated antibodies and visualized by fluorescence microscopy. Bar, 25 µm.

**Figure 4.** EPO protects primary and metastatic tumors from chemotherapy *in vivo*. A, upper panel: growth of BCSC-derived tumor xenografts (BCSC line 308) vehicle-treated (Control), treated with doxorubicin (Doxo) alone or in combination with EPO (Doxo+EPO) as described in Materials and Methods. Results shown are the mean ± SD of three experiments performed with groups of three mice each. *, P < 0.05; ***, P < 0.001. Lower panels: representative pictures of the tumors. B, immunohistochemical staining of xenograft sections obtained at the end of the experiment shown in A and stained with hematoxylin/eosin (H&E), with anti-cytokeratin 14 (CK14), anti-Bcl-xL (Bcl-xL) or TUNEL. Bar, 30 µm. C, whole body imaging of tumors at different time points after injection (Day 0) of 5 x 10^5 BCSC transduced with Tween-LUC GFP in the mammary fat pad of NOD/SCID mice, as described in the Methods section. Five weeks after injection, when lung metastases (and in some cases peritoneal metastases) were visible, the primary tumor was removed and the treatment with EPO, paclitaxel or paclitaxel + EPO was started (red arrow). After three weeks of treatment (8 weeks post-injection), mice were sacrificed and lungs were subjected to bioimaging in order to detect metastatic tumors (Lung metastases). The black square on the left side of the mice was positioned in order to shield luciferase signals emitted from residual cells that...
remained after primary tumor removal. One representative experiment of four mice per group is shown. D, photon counts emitted from mice lungs derived in the experiment described in C. Photon/s/sr, photons per second per steradian. *, P < 0.05; **, P < 0.01.
Figure 1 Todaro et al.
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Erythropoietin activates cell survival pathways in breast cancer stem-like cells to protect them from chemotherapy

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