

ORIGINAL ARTICLE

Erythropoietin receptor and tissue factor are coexpressed in human breast cancer cells

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Summary

Purpose: Erythropoiesis-stimulating agents (ESAs) are recommended for treating chemotherapy-induced anemia in breast cancer patients. Reduced survival rates in ESAs-treated patients have been reported, possibly due to thromboembolic complications, however the exact mechanism remains obscure. The principal activator of blood coagulation in cancer is tissue factor (TF). There are data that erythropoietin receptor (EPO-R) is expressed in tumor cells. The purpose of this study was to evaluate the expression of EPO-R and TF in loco in breast cancer.

Methods: The expression of EPO-R and TF was investigated in 24 invasive breast carcinoma specimens. Immunohistochemical (IHC) methodologies according to ABC technique and double-staining IHC procedure were employed utilizing antibodies against EPO-R and TF.

Results: Expression of EPO-R and TF was demonstrated

in the tumor cells in all breast cancer specimens. No staining for EPO-R and TF was visualized in normal breast tissue. Double staining studies revealed co-expression of both EPO-R and TF in breast cancer cells and endothelial cells.

Conclusions: EPO-R and TF expression and their coexpression in breast cancer cells suggest a possibility that EPO-R might be responsible for some adverse effects and reduced survival observed in ESAs-treated breast cancer patients with anemia, possibly due to the interaction with TF. Further experimental studies are warranted to determine the role of both EPO-R and TF in the treatment with ESAs of breast cancer patients with chemotherapy-induced anemia.

Key words: anemia, breast cancer, erythropoietin, erythropoietin receptor, tissue factor, thromboembolic complications

Introduction

Breast cancer is the most frequent malignancy in women worldwide [1]. Anemia is one of the most common complications observed during disease progression which is also caused by chemotherapy administration, and affects both the patients' quality of life and compliance with the antineoplastic treatment [2]. Transfusion of packed red blood cells (RBCs) improves the immediately hematological parameters and allows

chemotherapy continuation. Administration of ESAs is another method of increasing hemoglobin level in anemic patients with cancer. ESAs encompass recombinant erythropoietins and their analogs introduced in the treatment following erythropoietin (EPO) gene cloning in the early 1980s [3]. EPO is a glycoprotein hormone synthesized in the kidneys (70-80%). It stimulates proliferation and differentiation of erythroid progenitor cells as

well as inhibition of their apoptosis. EPO binds to EPO-R expressed on the surface of erythroid progenitor cells, which results in its activation and leads to formation of hemoglobin-containing RBCs. EPO-R expression in tumor cells may be related to the alarming data from recent clinical trials, which demonstrated that treatment of anemia in cancer patients with recombinant human EPO (rHuEpo) or other ESAs has been associated with severe adverse effects, mainly thromboembolic complications leading to decreased locoregional control and/or shortened survival of the patients with head and neck, breast and lung cancer [4-14]. However, the exact pathogenetic mechanisms of the thromboembolic complications remain obscure. There are data that EPO-R is expressed in cancer cells of several tumor types and may contribute to cancer biological behavior [15-24]. Thus, the nonhematopoietic effects of ESAs may be associated with direct activation of EPO-R in malignant cells. On the other hand, activation of blood coagulation is a phenomenon frequently observed in breast cancer patients [25,26]. The risk of thrombotic complications during chemotherapy or hormone therapy is relatively high and amounts to 5% and 0.8-7.1%, respectively [27]. The risk is profoundly increased and reaches up to 13.6% when these modalities are administered concomitantly [26,28,29]. Surgical procedures also enhance the risk of complications (mastectomy 2.3%, tumor-ectomy 0.7%) [28]. The tendency to hypercoagulability in breast cancer patients is reflected by the abnormalities in the laboratory hemostatic parameters, e.g. increased levels of F1+2, thrombin-antithrombin complex, fibrinogen, fibrinogen degradation products (FDP), observed even in patients without overt clinical thromboembolic symptoms [30,31]. The principal initiator of blood coagulation activation in cancer patients is TF, the presence of which was documented in breast cancer tissues [32]. The constitutively active TF on malignant cell surface activates the coagulation cascade, leading to thrombin generation, fibrin deposition and platelets recruitment, all of which contribute to cancer progression [25,33].

In this study we attempted to test the possibility of coexpression of EPO-R and TF *in loco* in human primary breast cancers.

Methods

The expression of EPO-R and TF was analyzed in T2N1-2M0 invasive breast carcinoma specimens obtained during surgical treatment of 24 previously untreated patients who underwent mastectomy. The con-

trol fragments of normal breast tissues derived from neoplasm-free surgical margins.

IHC studies utilizing avidin-biotin complex (ABC) technique were performed (Vectastain Kits, Vector Laboratories, Burlingame, CA, USA), employing a polyclonal antibody against EPO-R (R&D System, USA, MAB307) and a polyclonal antibody directed to human recombinant TF (American Diagnostica, Greenwich, CT, USA) [34]. Controls consisted of omission of the primary antibody from the procedure. Antigen staining was detected by the dark brown reaction product. EPO-R and TF IHC expressions were analyzed using a semiquantitative method according to the Remmale and Stegner scale with our own modification [35]. Briefly, numerical values were assigned in relation to the percentage of cancer cells expressing EPO-R or TF positive staining (A) and the intensity of the staining (B). The immunoreactive score (IRS) used for presentation of the results was a product of multiplication of both values ($IRS=A \times B$). The results ranged from 0 to 12, where "0" meant lack of any specific staining. The IRS values of 1-4, 5-8 and 9-12 were interpreted as weak, medium and strong EPO-R or TF expression, respectively. Visual assessment of the proteins' expression was performed in 10 random high-power fields. The specimens were assessed by two independent blinded observers.

EPO-R and TF coexpression was assessed by IHC staining studies according to Dako EnVision™ (Dako, Carpinteria CA, USA) protocol provided by the manufacturer employing commercially available Dako Envision™ Kit (Dako, Carpinteria CA, USA). In the above technique, EPO-R was visualized as a brown staining, whereas TF – as a red reaction product.

The study protocol was approved by Ethics Committee of the Medical University, Bialystok, Poland.

Results

Expression of EPO-R was demonstrated in all breast cancer specimens, in the tumor cells. In most cases (17/24 specimens) a strong expression of EPO-R (IRS 9-12) was observed in all cancer cells (Figure 1A). Medium (IRS 5-8) and weak (IRS 1-4) EPO-R expression was reported in 4/24 and 3/24 breast cancer specimens, respectively. Malignant cells localized in small foci demonstrated particularly strong expression (IRS 11-12) of EPO-R. The receptor was detected in the endothelial cells (ECs) forming tumor vessels but was not visualized in the normal breast tissue (Figure 1B).

Strong expression of TF (IRS 9-12) in breast cancer cells as well as in ECs was observed in all examined specimens (Figure 1C). No staining for TF was visualized in the normal breast tissue (Figure 1D).

Double staining studies revealed coexpression of both EPO-R and TF in breast cancer cells

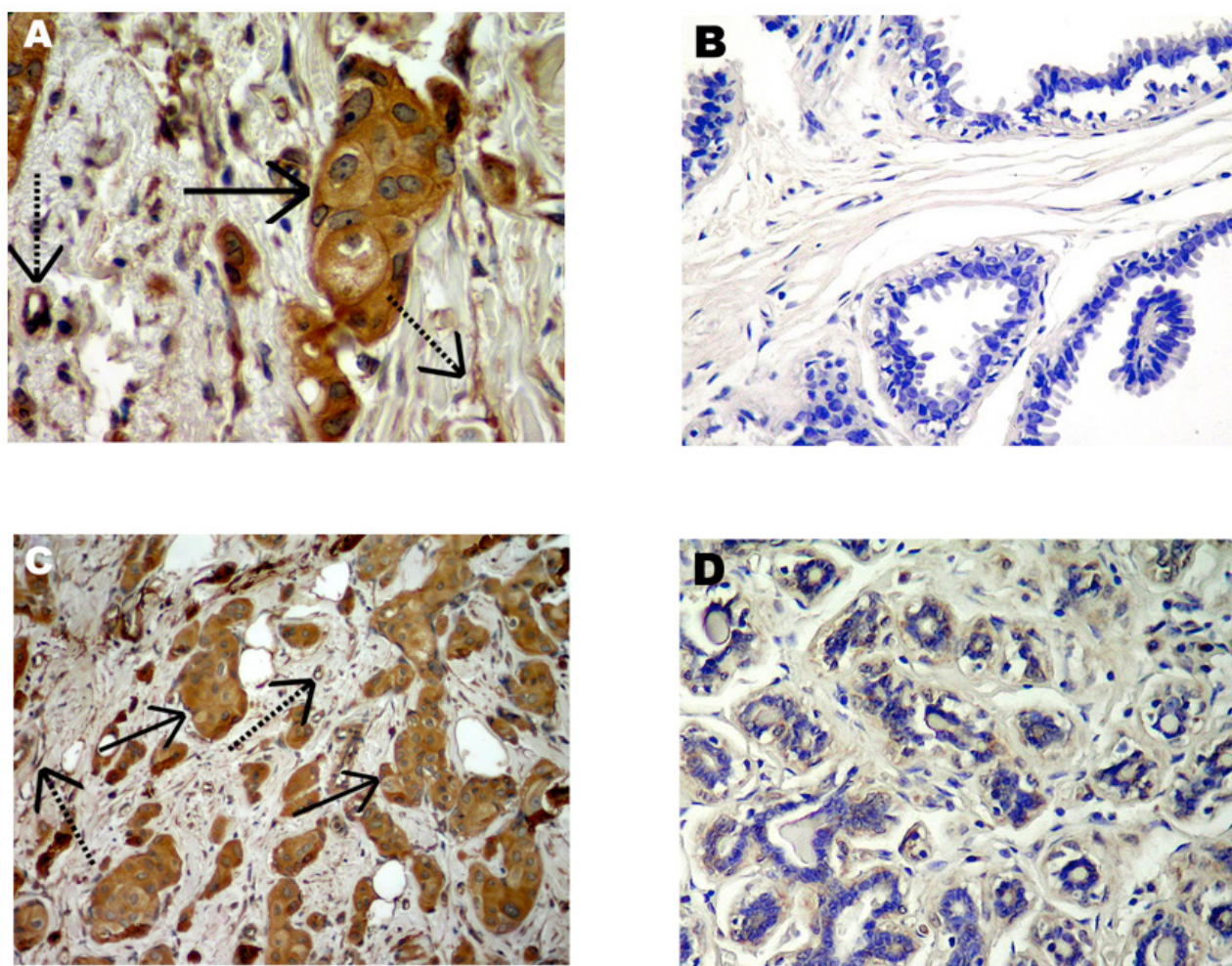


Figure 1. Breast cancer. Specific staining (brown reaction product) for erythropoietin receptor (EPO-R) (A) or tissue factor (TF) (C) by the ABC peroxidase technique. Solid arrows show staining of breast cancer cells, dotted arrows indicate endothelial cells. No staining for EPO-R or TF was observed in normal breast tissue (B,D). Hematoxylin counterstain, magnification 400x (A,B) or 200x (C,D).

and endothelial cells of small blood vessel supplying the tumor (Figure 2).

Discussion

ESAs revolutionized the treatment of anemia in cancer patients as an alternative to blood transfusions [36,37]. Unfortunately, recent clinical trials provide alarming information that such a treatment is associated with thromboembolic events and decreased antineoplastic treatment efficacy [8-14]. EPO, a regulator of erythropoiesis, stimulates also proliferation, migration and invasion of cancer cells [38-40]. It was suggested that interaction between rHuEPO and EPO-R expressed on the surface of malignant cells may contribute to the observed complications, however the potential mechanism of rHuEPO-induced thromboembolic events in cancer patients remains poorly un-

derstood [15-18,41,42]. That is why determination whether EPO-R is coexpressed with known cancer coagulant (TF) in breast cancer cells, is of great importance.

In the present study the expression of EPO-R in breast cancer cells was detected. Interestingly, the immunostaining was observed in all specimens, almost in 100% of breast cancer cells. Our data are in accordance with results described by other authors [43]. The expression of EPO-R in breast cancer cells has been investigated by immunohistochemistry [43,44], Western Blot [24,45] and RT-PCR [22,46]. Similar to our observations, the expression of EPO-R has not been detected in healthy breast tissue in other studies [18,44]. The presence of EPO-R in cancer cells, but not in the normal breast cells, might suggest its role in breast cancer biology. The EPO-R expression has been detected in many other cancers, e.g. head

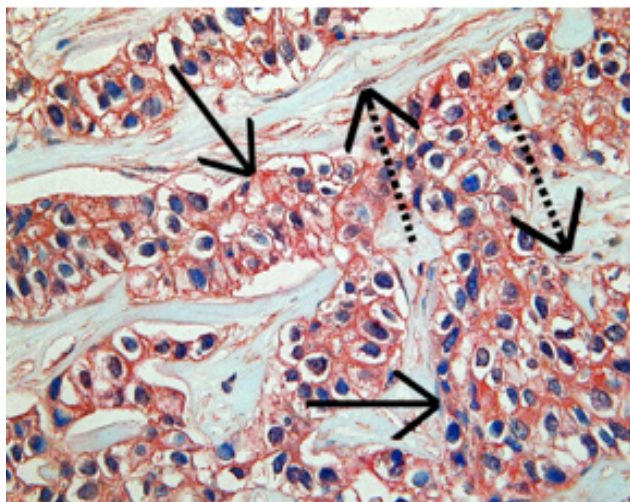


Figure 2. Breast cancer. Specific staining for erythropoietin receptor (brown reaction product) and tissue factor (red reaction product) by double staining Dako EnVision™ immunohistochemical technique. The two colors are overlapping indicating colocalization of erythropoietin receptor and tissue factor. Solid arrows show staining of breast cancer cells, whereas dotted arrows indicate endothelial cells. Hematoxylin counterstain, magnification 300x.

and neck cancer, cervical cancer, endometrial cancer, gastric cancer, renal cancer and melanoma [15-20]. In contrast, in other tumors, e.g. lung, colorectal, and prostate cancer, the EPO-R expression has been described both in malignant cells as well as in the surrounding normal tissue [17]. Strong EPO-R expression may be observed in hypoxic areas of the tumor [17,47,48], however in the present study such localization was not revealed. What is more, some authors observed EPO-R expression in ECs of small vessels in pediatric tumors [48]. In our study the EPO-R expression was also detected in the endothelium.

Intriguingly, there are some reports, which failed to demonstrate EPO-R expression in tumor cells [49,50]. Elliott et al. [50] have shown that EpoR protein is not detectable in normal human and matching cancer tissues from breast, lung, colon, ovary and skin. It seems that the differences in the sensitivity of the antibodies used in different studies impede the interpretation of the results [15,17,41,50,51]. In addition, immunohistochemistry may evaluate also the intracellular EPO-R, apart from the receptor localized on the cell surface, which is ready to be activated. The concentration of EPO-R in the cellular membrane

is lower in nonhematopoietic cells as compared to erythroid cell line which additionally makes the EPO-R detection a challenge. Moreover, multiple isoforms of EPO-R caused by differential splicing have been reported [24,52]. The presence of EPO-R gene and mRNA detection by RT-PCR were proved in many malignant tumors, such as breast, renal, colorectal, gastric, pancreatic, liver and lung cancer [18,53]. Of note, an association between the level of mRNA transcript and poor antineoplastic treatment outcome was also described [46]. Unfortunately, there was no evidence for transcripts translation into EPO-R protein [18]. It is vital to point out that EPO-R transport to the cell surface is inefficient – less than 10% reaches the membrane due to its degradation into smaller fragments in the endoplasmic reticulum, Golgi, and endosome-like structures [18,41]. Widespread availability of novel, well characterised antibodies directed against the human EPO-R should increase the understanding of the role of EPO-R in cancer [52].

It should be mentioned that thromboembolic events, observed in the trials with rHuEpo, led to premature termination of some of them [5]. Furthermore, the increased incidence of thromboembolic complications, also during chemotherapy or radiotherapy resulting in reduced survival in breast cancer patients has been widely described [25,26,28,29]. Expression of TF – the principal activator of hypercoagulability in cancer patients, was documented in breast cancer cells in other studies [32,54]. The coexpression of EPO-R and TF in breast cancer cells, demonstrated in the present study, might suggest potential interactions between EPO-R and TF, which might lead to the observed thrombotic events in breast cancer patients (also ESAs-treated breast cancer patients) as well as influence breast cancer biology.

Conclusions

The results of this study indicate that EPO-R and TF expression, as well as their co-expression, might influence breast cancer biology. They may suggest a possible explanation of thromboembolic complications of breast cancer patients with anemia of chronic disease treated with ESAs. However, further studies are warranted to determine the precise role of the factors in the development of the complications in breast cancer patients requiring ESAs for the treatment of anemia.

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