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Granulocyte-Colony Stimulating Factor Receptor, Tissue Factor, and VEGF-R Bound VEGF in Human Breast Cancer *In Loco*

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Abstract

Background. Doxorubicin and docetaxel-based chemotherapy regimens used in breast cancer patients are associated with high risk of febrile neutropenia (FN). Granulocyte colony-stimulating factors (G-CSF) are recommended for both treating and preventing chemotherapy-induced neutropenia. Increased thrombosis incidence in G-CSF treated patients was reported; however, the underlying mechanisms remain unclear. The principal activator of blood coagulation in cancer is tissue factor (TF). It additionally contributes to cancer progression and stimulates angiogenesis. The main proangiogenic factor is vascular endothelial growth factor (VEGF).

Objectives. The aim of the study was to evaluate granulocyte-colony stimulating factor receptor (G-CSFR), tissue factor (TF) expression and vascular endothelial growth factor receptor (VEGF-R) bound VEGF in human breast cancer *in loco*.

Material and Methods. G-CSFR, TF and VEGFR bound VEGF (VEGF: VEGFR) were assessed in 28 breast cancer tissue samples. Immunohistochemical (IHC) methodologies according to ABC technique and double staining IHC procedure were employed utilizing antibodies against G-CSFR, TF and VEGF associated with VEGFR (VEGF: VEGFR).

Results. Expression of G-CSFR was demonstrated in 20 breast cancer tissue specimens (71%). In 6 cases (21%) the expression was strong (IRS 9–12). Strong expression of TF was observed in all investigated cases (100%). Moreover, expression of VEGF: VEGFR was visualized in cancer cells (IRS 5–8). No presence of G-CSFR, TF or VEGF: VEGFR was detected on healthy breast cells. Double staining IHC studies revealed co-localization of G-CSFR and TF, G-CSFR and VEGF: VEGFR, as well as TF and VEGF: VEGFR on breast cancer cells and ECs.

Conclusions. The results of the study indicate that GCSFR, TF and VEGF: VEGFR expression as well as their co-expression might influence breast cancer biology, and may increase thromboembolic adverse events incidence (Adv Clin Exp Med 2016, 25, 3, 505–511).

Key words: breast cancer, neutropenia, granulocyte colony stimulating factor, tissue factor, vascular endothelial growth factor receptor.

Breast cancer is the most frequent malignant neoplasm affecting women in Poland [1]. Both adjuvant and palliative chemotherapy play an important role in disease management. Unfortunately, the introduction of these cancer treatment methods is associated with numerous adverse events

(AE), among which neutropenia of different grade is one of the most common. Decreasing the total neutrophils blood count, especially below the critical value of 500 cells/microliter, multiplies the risk of severe infections and febrile neutropenia (FN). The latter, when it is present, significantly

decreases the quality of a patient's life due to frequent hospitalizations and intensified medication. Furthermore, FN increases the risk of early death and increases the total mortality rate [2]. Moreover, FN might become a reason for less intensified treatment *via* reduced doses of cytostatics and prolonged intervals between cycles. Disturbances in the rhythm of chemotherapy decrease the effectiveness of oncological treatment and may even lead to its termination. It has been proven, in both prospective and retrospective studies, that all those factors have a direct negative impact on the overall survival (OS) [3, 4]. Granulocyte colony-stimulating factors (G-CSF) have been introduced to clinical practice to prevent or to treat neutropenia. G-CSF has proven to be highly effective in decreasing the number of FN incidents and neutropenia-related infections as well as in shortening the duration of neutropenia [5]. As a result, antibiotics usage is decreased and sub-optimal dosing of chemotherapy is avoided. G-CSF is now highly recommended, as it significantly reduces the early mortality rate [5]. G-CSF administration is currently strongly advised when patients receive chemotherapy associated with high (estimated as greater than 20%) risk of FN. Multinational Association of Supportive Care in Cancer (MASCC) highlights the significance of G-CSF administration also when applied chemotherapy regimen is associated with FN risk between 10–20% and patients present certain characteristics that may result in higher susceptibility to FN. Two chemotherapy regimens administered to breast cancer patients, TAC [6] (docetaxel, doxorubicin and cyclofosfamide) and AT [7] (doxorubicin and docetaxel), are associated with high risk of FN. Typically breast cancer patients receive G-CSFs injections after each cycle of chemotherapy, though some studies have shown that exposure to GCSFs is associated with an elevated risk of venous and arterial thrombosis [8]. Mechanisms underlying this effect are still under investigation. An elevated expression of adhesive particles on the surface of granulocytes result in intensified interaction between G-CSFs and endothelial cells (ECs), is suspected to be the cause of uncontrolled intravascular coagulation activation. Granulocyte colony stimulating factor receptor (G-CSFR) is expressed on various cells. It has been found not only on hematopoietic cells, but also on platelets, placental cells, neurons, endothelial and myocardial cells [9, 10]. It is well known that among cancer patients coagulation cascade is primed for activation on blood vessels and in the tumor [11]. Coagulation factors and their receptors are not only essential players in the coagulation process, but they play an important role in the tumor biology [12]. Tissue factor (TF) is the main

procoagulant, both under physiological conditions and in the state of neoplastic disease [13, 14]. Additionally, it was documented that TF activity contributes to cancer progression and stimulation of new vessel formation at the tumor-host interface [14]. Angiogenesis is an important process during cancer growth and metastatic dissemination [10, 15, 16]. Newly formed blood vessels are irregular and highly permeable, with turbulent blood flow, which further contributes to the activation of blood coagulation [17]. The main proangiogenic factor is vascular endothelial growth factor (VEGF) [16, 18, 19] which exerts its biological effect through binding with receptors: VEGFR-1 and VEGFR-2 [20–22]. The aim of the study was the assessment of G-CSFR expression in relation to the localization of TF as well as to VEGF bound to its receptor VEGFR-2 (indicating VEGFR-2 activation) in human breast cancer tumor samples.

Material and Methods

G-CSFR, TF and VEGFR bound VEGF (VEGF: VEGFR) were assessed in 28 breast cancer samples obtained during surgeries. All tumors were ductal invasive adenocarcinomas at T1-2N0M0 clinical stage of the disease. The control group consisted of samples of neoplastic infiltration-free breast tissue from surgical margins obtained from the same patients. Exclusion criteria involved previous anti-cancer treatment, any abnormalities in the results of routine laboratory tests, clinically overt thromboembolic complications, and disseminated disease (M1). Fragments of tumors were first formalin-fixed followed by paraffin embedding. Paraffin used during the study was characterized by low melting temperature. Cancer tissue sections and respective normal tissues were stained using immunohistochemistry (IHC) according to ABC method using Vectastain Kits, Vector Laboratories, Burlingame, USA. This method is based on formation of avidine-biotine complexes, using diaminobenzine as a substrate. Expression assessment was carried out by using highly specific antibodies: anti-G-CSFR antibody (a complete set produced by USBiological, USA, catalogue number G8950-50D) and an antibody against human recombinant tissue factor (anti-TF antibody received from prof. Walter Kisiel from the University of New Mexico, Department of Pathology, School of Medicine, Albuquerque, USA). Furthermore, a specific monoclonal antibody (GV39M) binding to VEGF exclusively when it is associated with its receptor – VEGFR (VEGF: VEGFR) [23, 24] was utilized in the IHC procedures. Antibodies were tested at concentrations that provided maximum staining intensity

with minimal background staining. Controls consisted of omission of the primary antibodies from the procedure. The results of staining the breast cancer tissues were compared with respective healthy tissues and were processed simultaneously. In ABC IHC technique the examined antigens were visualized as dark brown reaction products. The expression level of the receptors was measured using semiquantitative scale by Remmale and Stegner in our own modification [25]. Numerical results were grouped by the percentage of stained cells (A) and intensiveness of this staining (B). Immunoreactive score (IRS) used to present those results comes from multiplying A and B parameters. IRS values for both proteins expression ranged from 0 to 12.0 meant no expression of the receptor, 1–4–weak expression, whereas values from 5–8 and 9–12 were interpreted as mild and strong expression, respectively. Immunohistochemical staining studies according to Dako EnVision™ (Dako, Carpinteria USA) protocol provided by the manufacturer using commercially available Dako Envision™ Kit (Dako, Carpinteria, USA) were used to assess potential co-expression of:

- G-CSFR and TF (TF – brown staining, G-CSFR – a red reaction product)
- G-CSFR and VEGF: VEGFR (G-CSFR – brown staining, whereas VEGF: VEGFR – a red reaction product)
- TF and VEGF: VEGFR (TF – brown staining, whereas VEGF: VEGFR – a red reaction product)

The proteins' expression assessment was performed in 10 random high-power fields. The specimens were assessed by two independent blinded observers. The study protocol was approved by the local Ethics Committee of the Medical University in Białystok, Poland. Informed consent was obtained from all patients.

Results

G-CSFR expression was observed in the majority (20/28–71%) of assessed cases. Expression of the antigens was weak and irregular (IRS 1–4) (Fig. 1a, Table 1). In 6 cases (21%) high expression (IRS 9–12) of G-CSFR antigens was noticed (especially in small focal infiltrations of breast cancer). On the other hand, high (IRS 9–12) TF expression was confirmed in all investigated breast cancer tissue samples. Similarly, mild expression of VEGF: VEGFR was visualized in breast cancer cells (IRS 5–8) (Fig. 1e). No presence of G-CSFR, TF or VEGF: VEGFR was detected on normal breast cells (Fig. 1 b, d, f). In turn, G-CSFR, TF and VEGF: VEGFR were expressed in ECs of small blood vessels infiltrating the tumor (Fig. 1 a, c, e).

Double staining IHC studies revealed co-localization of G-CSFR and TF (Fig. 2a), G-CSFR and VEGF: VEGFR (Fig. 2b) as well as TF and VEGF: VEGFR (Fig. 2c) in breast cancer cells and ECs.

Discussion

Introduction of G-CSFs into clinical practice revolutionized oncological therapy and enabled effective prevention of FN associated with chemo- and radiotherapy [26]. It has been proven that G-CSFR is localized not only to bone marrow stem cells, but also to numerous types of normal tissue cells. The significance of its presence on cells other than hematopoietic ones is unclear. In our study we confirmed the expression of G-CSFR on ECs of small blood vessels supplying breast cancer. More interestingly, results of experimental studies indicate that G-CSFR activation can stimulate proliferation and migration of ECs and may lead to intensification of angiogenesis [16]. This multistage process is essential for cancer progression above 2–3 mm³ [27]. Furthermore, the invasiveness of cancer depends on forming new vessels inside the tumor [28]. The proangiogenic effect of the main angiogenesis stimulating factor – VEGF results from VEGFR-2 activation, whereas the role of VEGFR-1 is not fully recognized yet [9, 10, 12]. The current study revealed the expression of VEGF bound to VEGFR-2 in association with ECs. Newly formed vessels are abnormal, highly permeable, they differ in diameter and blood flow direction. Additionally, the vessel walls are characterized by irregular ECs coverage [29]. Such disorganization of blood flow facilitates the activation of blood coagulation. Experimental studies demonstrated that VEGF induces TF expression in ECs, increasing procoagulant properties of the vessel wall [30]. Interestingly, the study revealed the presence of the main procoagulant – TF in association with ECs of small vessels in the breast cancer tissue. Of note, TF, apart from its essential role in the coagulation process, exerts a role in angiogenesis [31]. Namely, expression of TF correlates with increased vessel density in non-small-cell lung cancer (NSCLC) and prostate cancer as well as with high VEGF concentration in NSCLC [32, 33]. Furthermore, TF induces transcription of VEGF, whereas it inhibits the synthesis of thrombospondin an endogenous inhibitor of angiogenesis [34]. Additionally, TF activity leads to thrombin generation and fibrin formation, the activity of which contributes to increased angiogenesis [35–37]. In this context, double staining demonstration of co-localization of G-CSFR, VEGF: VEGFR and TF in ECs in breast cancer tissue is of interest and might suggest an in-

Fig. 1. Breast cancer. Specific staining (brown reaction product) for G-CSFR expression (a), VEGF: VEGFR (e) by the ABC peroxidase technique. Solid arrows show staining of breast cancer cells, dotted arrows indicate endothelial cells. No staining for G-CSFR, TF, VEGF: VEGFR was observed in normal breast tissue (b, d, f). Hematoxylin counterstain, magnification about $\times 300$ (a, c) or $\times 200$ (e, b, d, f)

terplay between the receptors in the process of angiogenesis. Further functional studies aimed to explore the issue are needed. It appears that G-CSFR presence has been observed in many cancer cells, e.g. NSCLC, ovarian cancer, bladder cancer, nasopharyngeal cancer and astrocytoma [9, 10, 19]. Recent studies show G-CSFR expression on colon cancer cells, and, more interestingly, G-CSFRs on colorectal cells outnumber the same type of receptors on healthy large intestine tissue [38]. Clinical

observations indicate potential meaning of G-CSFR activation in cancer progression. Expression of GCSFR on nasopharyngeal, oral cavity and ovarian cancer cells is associated with poorer prognosis [39]. In the current study G-CSFR presence has been proven on breast cancer cells. It is thus possible that this protein influences cancer biology. Of note, VEGF was described primarily as the angiogenesis specific factor [40]. However, further studies demonstrated VEGFR expression on many

Fig. 2. Breast cancer. Specific double staining for TF (brown reaction product) and G-CSFR (red reaction product) (a), G-CSFR (brown staining) and VEGF: VEGFR (red reaction product) (b) and TF (brown staining) and VEGF:VEGFR (red reaction product) (c) by the double staining Dako EnVision™ immunohistochemical technique. The two colors are overlapping indicating co-localization of TF, G-CSFR and VEGF: VEGFR. Solid arrows show staining of breast cancer cells, whereas dotted arrows indicate endothelial cells. Hematoxylin counterstain, magnification about $\times 300$ (a, c) or $\times 400$ (b)

normal cell types (e.g. macrophages, neurons, etc.) as well as on cancer cells (e.g. prostate and colorectal cancer cells) apart from ECs [19, 39]. Experimental studies demonstrated that GV39M binds mainly to VEGF:VEGFR localized on ECs and, to a lesser extent, on cancer cells [23, 24]. The present study performed on human-derived breast tissues demonstrated VEGF:VEGFR presence not only on ECs, but on breast cancer cells as well. Demonstration of VEGF:VEGFR on breast cancer cells, which indicates that VEGFR signaling is present on breast tumor cell, although its function in tumor progression is unclear. In this regard prosurvival and mitogenic activities of VEGF [19, 40] are of interest in terms of breast cancer progression. Furthermore, the present study demonstrated TF expression on breast cancer cells as well. Presence of G-CSFR was documented on the cells of many solid tumors: astrocytomas, melanomas, pancreatic, gastric, lung, colon and breast cancers [38]. In breast cancer and NSCLC TF expression on cancer cells correlates with an increased risk of liver metastases formation [41]. It correlates with worse prognosis in breast or prostate cancer and NSCLC patients [42]. Activation of coagulation cascade dependable on TF results in thrombin generation and fibrin formation. Thrombin affects cancer progression not only due to fibrinogenesis, but it has been prov-

en that it stimulates angiogenesis inside the tumor [43]. Furthermore, cancer cells induce platelets aggregation mediated by thrombin, which, in return, intensifies their adhesive features. Thrombin stimulates intracellular signals transduction as well as mitosis and DNA synthesis [35]. It has been demonstrated that the presence of fibrin inside the tumor influences cancer progression due to mechanical support of malignant cells and ECs, creating a mechanical barrier that reduces the effectiveness of the host's immune system. Finally, it stimulates the growth of the tumor by masking the cancer cells' antigen epitops [36]. Demonstration of each of G-CSFR, TF and VEGF: VEGFR per se as well as their co-expression in cancer cells does not seem to be coincidental and calls for subsequent functional studies to resolve the problem. The progression of cancer disturbs the balance between coagulation and fibrinolytic cascades, which leads to increased risk of thrombotic incidents, mainly thromboembolism, which are the second leading cause of death in oncological patients [36]. It might suggest mutual interaction between TF, G-CSFR and VEGFR on the level of intracellular signaling pathways dependable on those receptors. It cannot be ruled out that exogenic G-CSF, while interacting with its receptor (G-CSFR), may also interfere with TF's and/or VEGFR's activity, which

may result in initializing angiogenesis and in activation of coagulation cascade with all its consequences. Because of the demonstrated role of both TF and G-CSF in stimulation of angiogenesis and described impact of those proteins on poorer prognosis of cancer patients it can be suspected that they can also collaborate in cancer progression. Taking into account the frequent use of G-CSFs in clinical practice combined with a substantial number of episodes of thrombosis among breast can-

cer patients, an interplay between G-CSFR, TF and VEGFR should be closely studied. Further investigation of their interactions is needed.

The co-existence of G-CSFR, TF and VEGFR on breast cancer cells and on ECs might suggest their mutual functional interaction, possible influence on the biology of the tumor and thrombotic adverse events occurrence. This implies a need for further investigation to precisely clarify potential mechanisms underlying the observed dependency.

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