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Neutrophil Expression of ICAM1, CXCR1, and VEGFR1 in Patients with Breast Cancer Before and After Adjuvant Chemotherapy

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Abstract. *Background: Distinct populations of neutrophils have been identified based on the expression of intercellular adhesion molecule 1 (ICAM1, CD54) and chemokine receptor 1 (CXCR1, interleukin 8 receptor α). Aim: We analyzed the expression of vascular endothelial growth factor receptor 1 (VEGFR1), a physiological negative regulator of angiogenesis, on distinct populations of neutrophils from the blood of patients before and after adjuvant chemotherapy for breast cancer. Materials and Methods: Neutrophil populations were distinguished as reverse transmigrated (ICAM1^{high}/CXCR1^{low}), naïve (ICAM1^{low}/CXCR1^{high}), or tissue-resident neutrophils (ICAM1^{low}/CXCR1^{low}), and their VEGFR1 expression quantified. Results: Reverse transmigrated ICAM1^{high}/CXCR1^{low} neutrophilic granulocytes decreased significantly after chemotherapy and these were also the cells with highest mean fluorescence intensity for VEGFR1. Conclusion: Chemotherapy mainly reduces the number of reverse transmigrated long-lived ICAM1^{high}/CXCR1^{low} VEGFR1-expressing neutrophils. The decrease of antiangiogenic VEGFR1 may have a potential impact on tumour angiogenesis in patients undergoing adjuvant chemotherapy.*

Neutrophilic granulocytes constitute the most abundant sub-population of leukocytes in peripheral blood. Neutrophilic granulocyte count is very dynamic and it has been known for a long time that secondary agranulocytosis or neutropenia arises after cytotoxic chemotherapy due to impaired proliferation in bone marrow, with subsequent recovery depending on the dose of chemotherapy given (1). Maturation and expression of surface antigens of granulocytes are studied much less than other hematopoietic cells, and only for a limited spectrum of diseases (2). Early studies of granulocytic surface antigens focused on the diagnosis of certain inherited disorders, such as leukocyte adhesion molecule deficiency, in which a specific antigen is known to be missing (3).

Recent studies have demonstrated that neutrophils are not a homogenous population of cells. Phenotypically and functionally distinct populations of neutrophils have been identified based on the expression of intercellular adhesion molecule 1 (ICAM1, CD54) and chemokine receptor 1 (CXCR1, interleukin 8 receptor α) in healthy volunteers and patients with chronic inflammatory disease. Neutrophil sub-populations can be divided into three groups based on their expression of ICAM1 and CXCR1: reverse transmigrated (ICAM1^{high}/CXCR1^{low}); naïve, ICAM1^{low}/CXCR1^{high}; and tissue-resident (ICAM1^{low}/CXCR1^{low}) granulocytes (4).

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Table I. *Patients' characteristics and time of blood analysis.*

Total number of patients	15	
Median age, years	44 (range=32 to 72)	
Histology	Invasive ductal carcinoma	10 (67%)
	Invasive lobular carcinoma	5 (33%)
Tumor stage	T1	3 (20%)
	T2	9 (60%)
	T3	3 (20%)
Nodal stage	Negative	3 (20%)
	Positive	12 (80%)
ER/PR status	Positive	12 (80%)
	Negative	3 (20%)
HER2 status	Positive	3 (20%)
	Negative	12 (80%)
	ER/PR/HER2-negative	3 (20%)
Chemotherapy regimen	Doxorubicin/cyclophosphamide-paclitaxel	9 (60%)
	5-Fluorouracil/epirubicin/cyclophosphamide	5 (33%)
	Paclitaxel/carboplatin	1 (7%)
Blood analysis before/after	First cycle of chemotherapy	6 (40%)
	> First cycle of chemotherapy	9 (60%)
Median time of blood analysis, days		
Before chemotherapy	0 (range=−4 to 0)	
After chemotherapy	10 (range=7 to 54)	

Vascular endothelial growth factor receptor 1 (VEGFR1) is a physiological inhibitor of angiogenesis which is also expressed on neutrophils (5, 6). We have recently shown that after autologous stem cell transplantation a patient's serum elicits a strong antiangiogenic response, likely due to neutrophil-derived antiangiogenic-soluble VEGFR1 within the leucapheresis product, which may contribute to the therapeutic efficacy of the transplantation (7).

For macrophages, it has been shown that expression of VEGFR1 depends on the activation status and maturation of the cell, with resting monocytes expressing only low levels of VEGFR1 (8). In analogy to similar findings in monocytes, VEGF-dependent neutrophil migration has been demonstrated to be mediated by VEGFR1 (5). However, it is not known if the expression of VEGFR1 in neutrophils also depends on the activation status and maturation of the cell, similarly to monocytes.

Differently from monocytes, neutrophils are especially sensitive to external perturbations and can be easily activated by the isolation process itself (9). In addition, the life-span of neutrophils *in vitro* is short (10), making it logistically almost impossible to study neutrophils *in vitro* (11). Given the dynamics of neutrophilic granulocytes in peripheral blood after chemotherapy, and neutrophilic granulocytes being a source of antiangiogenic VEGFR1, an understanding of how VEGFR1 expression is regulated as a function of the distinct neutrophilic granulocyte populations would be of interest. In addition, the effect of chemotherapy on neutrophil decrease and recovery *in vivo* could be generally exploited to overcome the limitations of cell culture and artificial stimulation and maturation of neutrophilic granulocytes *in vitro*.

The aim of our study was to analyze the expression of VEGFR1 on subpopulations of neutrophils depending on the expression of ICAM1 and CXCR1 in the blood of patients before and after adjuvant chemotherapy for early-stage breast cancer.

Materials and Methods

Patients, blood samples, and isolation of leukocytes. Patients undergoing adjuvant chemotherapy after operation for localised breast cancer were included in the study. The study protocol was approved by the local Ethics Commission (University of Zurich approval number EK-1363) and written informed consent was obtained from all patients. Patients' characteristics are shown in Table I. EDTA plasma (10 ml) and heparinised blood (20 ml) samples were obtained before the first cycle of adjuvant chemotherapy, and approximately 10 days later at the time when patients attended for nadir determinations. Due to low recruitment and logistic reasons, these rules were later expanded and patients were included at any cycle of adjuvant chemotherapy. Leukocytes were harvested after centrifugation of 20 ml heparinised blood at 400 ×g for 30 min at room temperature.

Analysis of full blood counts. Full blood counts from EDTA blood samples were analysed and leucocyte subfractions quantified using an automated blood cell analyser (ADVIA 120; Siemens, Erlangen, Germany).

Flow cytometric analysis for ICAM1, CXCR1, and VEGFR1 expression on granulocytes. Isolated leukocytes were incubated in 25 µl of FACS buffer (FB; phosphate buffered saline, 5% fetal bovine serum, 0.1%
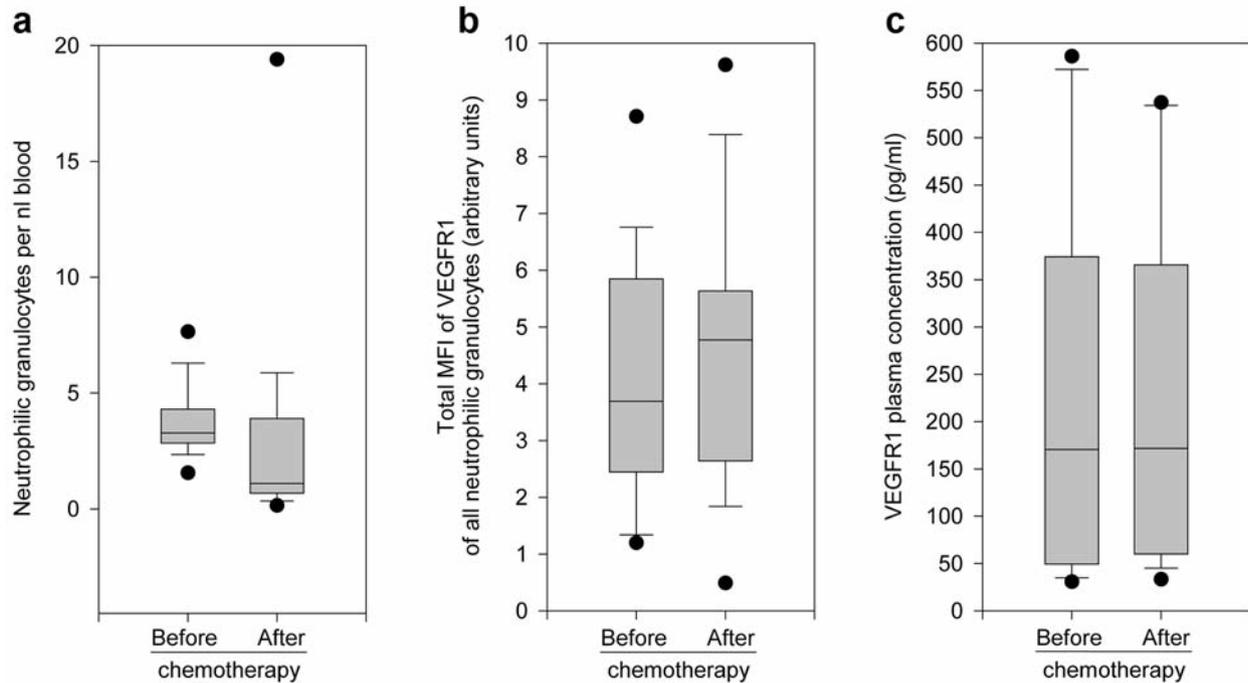
Figure 1. *Neutrophilic granulocyte counts (a), vascular endothelial growth factor receptor 1 (VEGFR1) mean fluorescence intensity (MFI) of neutrophilic granulocytes (b), and VEGFR1 plasma concentrations (c) of patients before and 12 days (median) after adjuvant chemotherapy for breast cancer (n=15). Neutrophilic granulocyte counts dropped by 66.5% from 3.28 to 1.1 per nl; this change is not statistically significant (a). Flow cytometric analysis of neutrophilic granulocytes demonstrated that VEGFR1 MFI did not change significantly (b). Median VEGFR1 plasma concentration did not change significantly (170.44 vs. 171.76 pg/ml), but there was a high variation of individual changes, ranging from 77% to +914% (c).*

sodium azide) with an allophycocyanin (APC)-labelled monoclonal mouse antibody to human VEGFR1 (R&D Systems, Abingdon, Oxford, UK), followed by incubation with a fluorescein isothiocyanate (FITC)-labelled mouse antibody to human CXCR1 (CD181) (BD Biosciences-Pharmingen, San Diego, CA, USA) and a phycoerythrin (PE)-labelled mouse antibody to human ICAM1 (CD54) (BD Biosciences-Pharmingen). As a control for the scatter pattern of neutrophilic granulocytes, an aliquot of leukocytes incubated with mouse antibody to human VEGFR1 were incubated with a FITC-labelled mouse antibody to human CD16 (BD Biosciences-Pharmingen). After incubation for 20 min at 4°C in the dark, leukocytes were washed once with 2 ml FB, then 1 ml FACS lysing solution (BD Biosciences-Pharmingen) was added to lyse erythrocytes and fix the cells. When a clear solution was reached (3-5 min), cells were washed with 2 ml FB, resuspended in 300 μl FB, and subjected to flow cytometry. Flow cytometry was performed using a FACSCalibur® flow cytometer running with CellQuestPro software (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA). Neutrophilic granulocytes were first identified according to their typical light-scattering pattern (12), then divided into three groups dependent upon their expression of ICAM1 and CXCR1: reverse transmigrated, ICAM1^{high}/CXCR1^{low}; naïve, ICAM1^{low}/CXCR1^{high}; and tissue-resident, ICAM1low/CXCR1^{low}. Each of the three different subgroups was further analysed according to the mean fluorescence intensity (MFI) of VEGFR1, using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Quantification of VEGFR1 protein expression by enzyme-linked immunosorbent assay (ELISA). ELISA for the quantification of VEGFR1 was performed using the immunoassay from R&D Systems (Abingdon, Oxford, UK) according to the manufacturer's instructions. VEGFR1 concentrations in EDTA-plasma were expressed in pg/ml.

Statistics. The non-parametric Wilcoxon signed rank and Mann Whitney *U*-tests were applied to determine statistical significance of the difference between two groups for paired, and unpaired data, respectively. A value of *p*<0.05 was considered significant.

Results

Blood samples from 15 patients undergoing adjuvant chemotherapy for breast cancer were analyzed in this study. For six patients, blood samples were taken before and after the first cycle of chemotherapy, nine patients were more advanced in their chemotherapy cycles. The median time of blood sampling was 0 (range=4-0) days before, and 10 (range=7-54) days after chemotherapy. Patients' characteristics are shown in Table I. Table II summarizes the results of neutrophilic granulocyte analysis. Automated blood cell analysis showed that neutrophilic granulocyte counts dropped by 66.5% (median) after chemotherapy, from 3.28 to 1.1 cells/nl, however, this change was not statistically significant (Figure 1a). FACS analysis of neutrophilic granulocytes demonstrated that MFI of VEGFR1 did not change significantly after chemotherapy (Figure 1b).
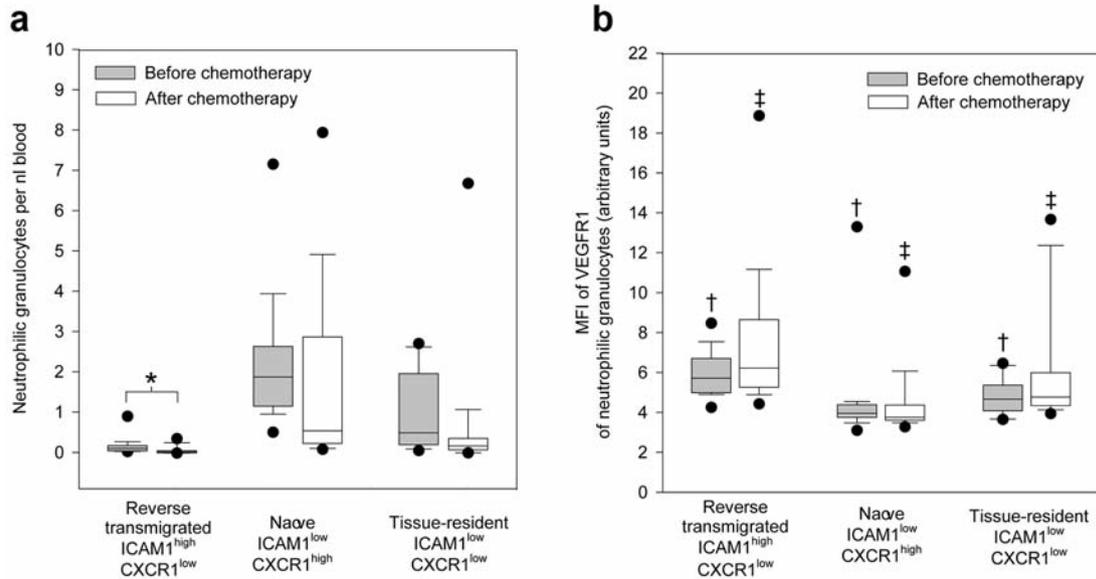
Figure 2. *Fluorescence-activated cell sorting (FACS) characterisation of neutrophilic granulocytes from blood of patients (n=15) before and 10 days (median) after adjuvant chemotherapy for breast cancer based on the expression of inter-cellular adhesion molecule 1 (ICAM1, CD54), chemokine receptor 1 (CXCR1, interleukin 8 receptor α), and vascular endothelial growth factor receptor 1 (VEGFR1). Reverse transmigrated ICAM1^{high}/CXCR1^{low} neutrophilic granulocytes decreased significantly after chemotherapy from 98 to 31/µl. The decrease in naïve ICAM1^{low}/CXCR1^{high} and in tissue-resident ICAM1^{low}/CXCR1^{low} neutrophilic granulocytes (from 1871 to 555, and 485 to 179/µl, respectively) were not statistically significant (a). Mean fluorescence intensity (MFI) of VEGFR1 was significantly different for the three neutrophilic granulocyte populations, with highest units for reverse transmigrated neutrophils, followed by tissue-resident, and naïve neutrophilic granulocytes. The MFIs of all three neutrophilic granulocyte populations did not change significantly after chemotherapy and the significantly different MFIs between the three neutrophilic granulocyte populations were maintained (b). *p<0.05. p<0.05 (reverse transmigrated vs. naïve, and vs. tissue-resident and naïve vs. tissue-resident neutrophilic granulocytes, respectively) †before, and ‡after chemotherapy, respectively.*

ELISA of patient EDTA-treated plasma showed that the median VEGFR1 concentration did not change significantly (170.4 before *vs.* 171.76 pg/ml after chemotherapy). However, there was a high variation between individual changes, ranging from −77% to 914% (Figure 1c).

FACS characterisation of neutrophilic granulocytes from blood of patients before and at a median of 10 days after adjuvant chemotherapy for breast cancer, based on the expression of ICAM1, CXCR1 and VEGFR1, showed that reverse transmigrated neutrophilic granulocytes decreased significantly after chemotherapy from 98 to 31/µl. The decrease in naïve and in tissue-resident phenotype neutrophilic granulocytes (from 1871 to 555, and 485 to 179/µl, respectively) was not statistically significant (Figure 2a).

Of note, VEGFR1 expression levels were significantly different between the three neutrophilic granulocyte populations, with the MFI being highest for reverse transmigrated neutrophils, followed by tissue-resident, and naïve neutrophilic granulocytes. The MFIs of all three neutrophilic granulocyte populations did not change significantly after chemotherapy and the significant difference in MFI between the three neutrophilic granulocyte populations was maintained (Figure 2b).

An example of FACS characterisation of neutrophilic granulocytes, depending on the scatter pattern (cell size and complexity), expression of ICAM1, CXCR1, and VEGFR1, from a patient before and after adjuvant chemotherapy for breast cancer is shown in Figure 3.

Discussion

Only little is known about the expression of VEGFR1 in neutrophils in relation to activation status and maturation of the cell. Buckley *et al.* identified a subset of human neutrophils with a distinct profile of cell-surface receptors which represent cells that have migrated through an endothelial monolayer and then re-emerged by reverse transmigration (ICAM1^{high}/CXCR1^{low}). These neutrophils were rescued from apoptosis, demonstrated functional priming, and were rheologically distinct from neutrophils that had not undergone transendothelial migration, such as naïve circulatory neutrophils and tissue-resident neutrophils (4).

In this study, we analyzed the expression of VEGFR1 on sub-populations of neutrophils depending on the expression of ICAM1 and CXCR1 in the blood of patients before and after adjuvant chemotherapy for early-stage breast cancer.
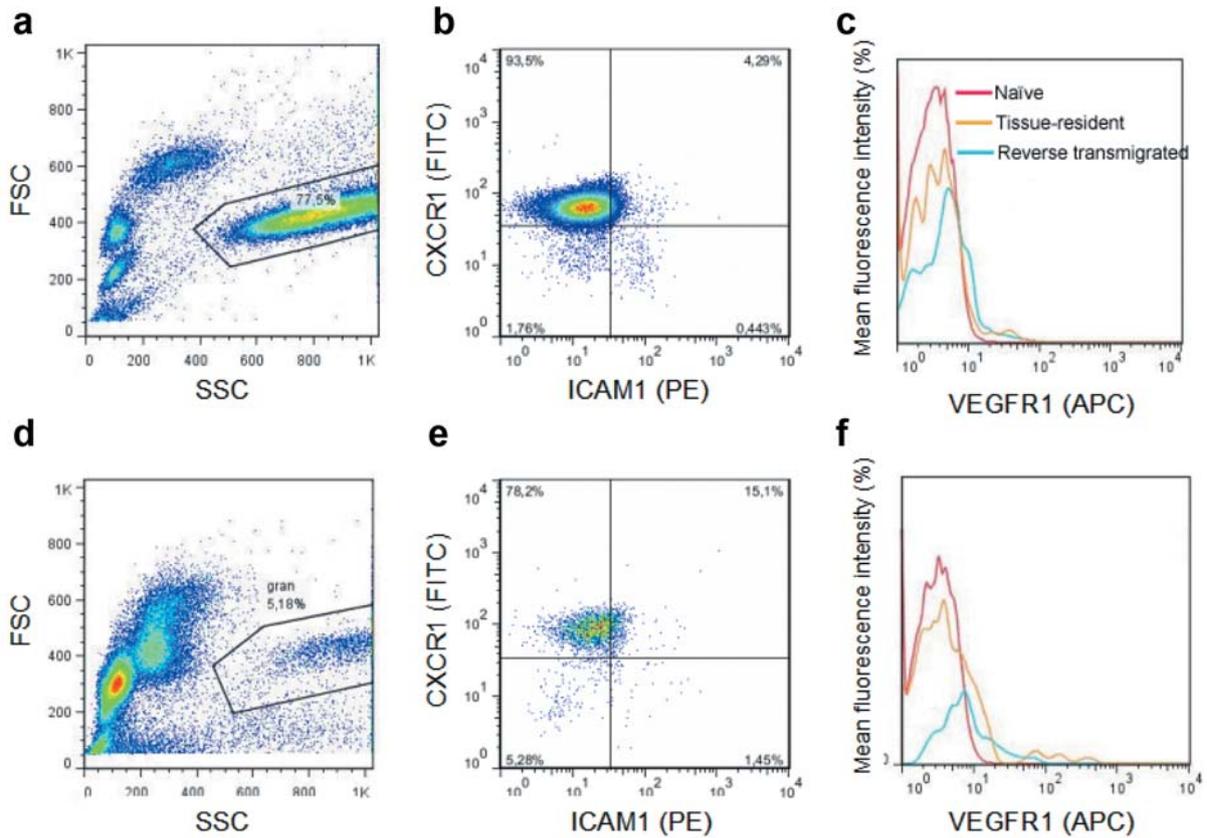
Figure 3. *Example of fluorescence-activated cell sorting (FACS) characterisation of neutrophilic granulocytes, depending on the scatter pattern (cell size and complexity), expression of inter-cellular adhesion molecule 1 (ICAM1, CD54), chemokine receptor 1 (CXCR1, interleukin 8 receptor α), and vascular endothelial growth factor receptor 1 (VEGFR1) of a patient before (a,b,c) and after (d,e,f) adjuvant chemotherapy for breast cancer (pT2 pN1, estrogen receptor/progesterone receptor-positive, HER2-negative invasive-lobular carcinoma). Blood was taken on the day of, and 15 days after chemotherapy, respectively (1st cycle doxorubicin/cyclophosphamide). Neutrophilic granulocyte count after chemotherapy (a, d) and proportion of reverse transmigrated ICAM1^high/CXCR1^low neutrophilic granulocytes after chemotherapy (b, e). Mean fluorescence intensity (MFI) of VEGFR1 in the three types of neutrophilic granulocytes before and after chemotherapy (c, f).*

Table II. *Median neutrophilic granulocyte count, mean fluorescence intensity (MFI) of vascular endothelial growth factor receptor 1 (VEGFR1), and plasma VEGFR1 concentration before and after adjuvant chemotherapy for breast cancer.*

	Chemotherapy		Change (%)	*p*-Value*
	Before	After		
Median count (range) (×10³/ul)				
Neutrophilic granulocytes	3.28 (1.56-7.65)	1.10 (0.15-19.4)	−66.5	0.11
Reverse transmigrated	0.098 (0.021-0.895)	0.031 (0.002-0.359)	−68.1	0.031
Naïve	1.871 (0.501-7.153)	0.555 (0.096-7.954)	−70.3	0.46
Tissue-resident	0.485 (0.045-2.701)	0.179 (0.008-6.693)	−63.0	0.16
Median (range) MFI of VEGFR1				
Neutrophilic granulocytes	3.69 (0.02-0.43)	4.77 (0.00-1.63)	29.3	0.51
Reverse transmigrated	5.720 (0.078-0.504)	6.250 (0.017-1.853)	9.3	0.22
Naïve	3.960 (0.048-0.837)	3.790 (0.005-2.153)	−4.3	0.46
Tissue-resident	4.660 (0.057-0.414)	4.800 (0.016-2.406)	3.0	0.14
Median (range) plasma VEGFR1 (pg/ml)	170.4 (30.8-586.1)	171.76 (33.4-537.3)	0.8	0.75

Reverse transmigrated: Inter-cellular adhesion molecule 1 (ICAM1)^high/chemokine receptor 1 (CXCR1)^low; naïve : ICAM1^low/CXCR1^high; tissue-resident: ICAM1^low/CXCR1^low. *Wilcoxon signed rank test.

Reverse transmigrated neutrophilic granulocytes decreased significantly after chemotherapy. The decrease in naïve and tissue-resident neutrophilic granulocytes were not statistically significant. The percentage of reverse transmigrated neutrophilic granulocytes in our study was comparable with the one found by Buckley *et al*. (*i.e.* 0.25% in healthy donors and 1-2% in patients with systemic inflammation (4)).

VEGFR1 expression levels were significantly different between the three neutrophilic granulocyte populations, with highest MFI for reverse transmigrated neutrophils, followed by tissue resident, and naïve neutrophilic granulocytes. This pattern was retained as the MFIs of all three neutrophilic granulocyte populations did not change significantly after chemotherapy. This finding suggests that VEGFR1 expression by neutrophilic granulocytes may follow the same pattern as monocytes, since naïve neutrophilic granulocytes seem to express VEGFR1 less intensely than the more mature, activated tissue resident and transmigrated neutrophilic granulocytes, as shown for monocytes previously.

These differences could be explained by the fact that granulocytes have intracytoplasmic storage pools of numerous surface antigens that can be translocated to the surface following granulocytic activation or excessive manipulation (13). Given the abundance of VEGFR1 expression on neutrophilic granulocytes of leukapheresis products (7), which seems higher than seen in this study, one might speculate if the mechanically excessive manipulation of neutrophilic granulocytes (and other blood cells) during the collection of bone marrow-derived stem cells from peripheral blood, involving vigorous centrifugation, might induce a stress response and trigger VEGFR1 expression. However, the subpopulation of reverse transmigrated neutrophilic granulocytes is also the smallest, and therefore it seems unlike that the difference might result in biological relevant changes in VEGFR1 density.

Overall, MFI of VEGFR1 of neutrophilic granulocytes did not change significantly. Similarly, serum VEGFR1 concentrations did not change significantly, although there was a high variation between individual changes, ranging from −77% to 914%.

VEGFR1 is not only expressed on neutrophils (5, 6), but also on other cell types such as tumor cells (14), bone marrow-derived progenitor cells (15), monocytes/ macrophages (8), basophils (16), and platelets (17). Several different isoforms of VEGFR1 are known to arise through alternative splicing: classically, a full-length transmembrane protein, containing an intracellular tyrosine kinase domain, is distinguished from a truncated soluble form, lacking the transmembrane and tyrosine kinase domains (sVEGFR1, also known as sFLT1) (18-22). However, evidence is emerging that proteolytic cleavage of VEGFR1 may also be involved in the generation of sVEGFR (23, 24). VEGFR1 and sVEGFR1 both exert antiangiogenic effects by sequestration of VEGF, and by forming heterodimers with VEGFR1 and -2, respectively (25, 26).

We have previously shown that in neutrophilic granulocytes undergoing massive manipulation during apheresis for stem cell collection, *VEGFR1* mRNA expression is shifted towards the soluble isoform, sVEGFR1 (7). However, the sVEGFR1:VEGFR1 ratio of unprocessed neutrophilic granulocytes is not known. The fact that plasma concentrations of sVEGFR1 are not different after chemotherapy in this study are in line with the finding that overall MFI of VEGFR1 of neutrophilic granulocytes is also unchanged. However, one should bear in mind that the expression of VEGFR1 of endothelial cells, for example, is one order of magnitude higher (7) and thus the main source of sVEGFR1 in plasma might not necessarily be neutrophilic granulocytes.

There are several limitations affecting this study: the relatively small number of patients and especially the inconsistency of therapy cycles and heterogeneity of chemotherapy regimens may have affected the results. In further studies, one should therefore stratify for the type of substances used for chemotherapy and for patients with significant neutropenia. Multiple measurements over time would allow kinetics of changes to be seen. Given the sometimes large individual variations of parameter changes seen in this study, there may still be unknown factors influencing dynamics of VEGFR1-expressing subpopulations of neutrophilic granulocytes.

In summary, subpopulations of neutrophilic granulocytes in patients before adjuvant chemotherapy for breast cancer have similar distribution patterns to those previously reported for healthy donors and patients with systemic inflammation (4). Chemotherapy alters this distribution, with the population of reverse transmigrated neutrophilic granulocytes being significantly reduced; this is also the subpopulation with the highest MFI for VEGFR1. However, reverse transmigrated neutrophilic granulocytes only account for a very small subpopulation of neutrophilic granulocytes and consequently, expression and plasma concentration of VEGFR1 do not change significantly after chemotherapy.

Studies including more homogenous patient populations and consistent treatment regimens should further investigate the potential impact on angiogenesis of decreasing numbers of neutrophils expressing antiangiogenic VEGFR1 after chemotherapy.

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