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ORIGINAL

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) AND INTERLEUKIN (IL)- 6 IN SERUM AND SEROMA AFTER BREAST CANCER SURGERY

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Due to better understanding complex immune-inflammatory responses following surgical injury, the aim of present study was to investigate the changes of VEGF and IL-6 levels in serum and seroma of invasive breast cancer patients after surgery and their correlation with breast cancer tumour expression of VEGF Samples from 20 breast cancer patients and 15 benign breast disease patients were included in the study. Immunohistochemical staining was used for determining VEGF expression in tissue samples from tumour, stromal and normal breast. Measuring VEGF and IL-6 levels was conducted by ELISA. Differences in VEGF expression between tumour and stroma (p=0,007) and between tumour and normal breast tissue were significant (p=0,0001), as well as differences in VEGF expression between stromal and normal breast tissue (p=0,004). Serum level of VEGF was higher in patients with breast cancer then in patients with benign breast disease, before surgery (p=0,023).VEGF was significantly elevated postoperatively in serum (p=0,009) and seroma (p=0,0001) in patients with breast cancer. Level of IL-6 was elevated after surgery in serum (p=0,015) and seroma (p=0,0001) in patients with breast cancer, as well as in serum of patients with benign breast disease (p=0,018). Significant correlation was found between seroma levels of VEGF and IL-6 in breast cancer patients (p=0,009). The findings suggest involvement of VEGF and IL-6 in physiological changes after breast cancer surgery.

Key words: Breast cancer, VEGF, IL-6, surgery, wound healing

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INTRODUCTION

Breast cancer represents the most common malignancy among women worldwide [1]. The surgical treatment includes either modified radical mastectomy or breast preservation depending upon stage of the disease and the most common postoperative complication is seroma formation. Seroma is defined as a serous fluid collection that develops under the skin flaps during mastectomy or in the axillary's dead space after axillary dissection [2].

The wound healing after surgery is a chain of processes necessary for the removal of damaged tissues or invading pathogens from the body and for subsequent tissue remodelling. These processes require interactions between inflammatory cells, biochemical mediators including cytokines, matrix molecules and microenvironment cells [3]. Angiogenesis plays a major role in both wound healing and the ability of a cancer to survive and grow[4]. Normal wound healing generates an angiogenic response in order to deliver nutrients and inflammatory cells to injured tissue enabling the removal of debris [5]. Major mediator of wound angiogenesis is soluble form of vascular endothelial growth factor (VEGF) which was identified in several wound models [6,7]. Wound healing after surgery generates inflammatory response. The inflammatory phase of wound healing is characterised by the presence of inflammatory cells, such as neutrophils, macrophages and lymphocytes, which participate in this phase of wound healing releasing inflammatory cytokines interleukin 6 [IL-6], tumour necrosis factor α (TNF-α) and IL -1β [8, 9, 10]. Proangiogenic cytokines, such as VEGF and inflammatory cytokines, such as interleukin 6 (IL-6), accompanying wound healing, remodelling and regenerations and were previously detected in seroma obtained through surgical suction drains [9, 10, 11, 12]. Recent reports showed that wounds in IL-6 deficient mice showed delays in macrophage infiltration, fibrin clearance and wound contraction [13]. IL-6 modulates immune response and is essential for the wound healing processes [13]. In some circumstances, inflammatory cytokines may be applied as a therapeutic agent for the improvement of surgical wounds [14]. Thus, over the past decades, cytokines have gained more attention in the understanding of physiological changes after trauma or surgery [14]. Present study investigates alterations of proangiogenic cytokine VEGF and inflammatory cytokine

IL-6 after breast cancer surgery and presence of these proteins in seroma which can contribute to better understanding of physiological changes and complex interaction after surgery.

METHODS

Patients

This study was approved by the Ethic committee of the University Clinical Centre Tuzla. Informed consent was obtained from all of the patients. It was prospective case-control study with well-defined including criteria: histologically proven invasive breast cancer; no distant metastases; no previous adjuvant therapy; currently under no treatment; no other major illnesses.

Total of 35 patients (20 breast cancer patients and 15 benign breast disease patients) were hospitalized in the Department of Surgery, University Clinical Centre Tuzla during 2011.

Samples

Tumour, stroma and unchanged breast tissue samples that have been obtained during surgery were sent to the Department of pathology, University Clinical Centre Tuzla, to the pathological examination and pathohistological diagnosis. Pathohistological examination was performed with hematoxylin-eosin staining. Tumours were classified according to the criteria of the World Health Organization [18]. Histological grade was determined in accordance to modified Scarff-Bloom-Richardson grading system. Tumour size was graded in three categories: I (0,1-2cm or ≤ 2cm), II (2-5cm), III (> 5cm). Tumour tissue samples and the stromal tissue from patients with invasive breast cancer were used, as well as normal breast tissue samples from patients with benign breast diseases.

Samples for ELISA were collected at the Department of Surgery, University Clinical Centre. Venous blood samples were obtained 24 hours before surgery and five days after surgery from patients with malignant disease and 24 hours after surgery from patients with benign disease. Samples for ELISA from patients with malignant disease are collected on the fifth day after surgery in order to avoid surgical stress and to be sure that wound healing processes are activated. Samples from patients with benign disease are collected 24 hours after surgery because that kind of patients are not hospitalised longer than that period and the surgical stress is negligible due to less aggressive type of surgery. Seroma samples were collected on the fifth day after surgery. Blood samples were collected by venipuncture (7ml of blood for analysis from each patient). After half an hour samples were processed at room temperature by centrifuging at 3000 rpm for 10 minutes. Serum and seroma samples were stored in plastic microtubes (0,5 mL/microtube) in freezer at -20°C. Extracting and sample storing were carried out in Polyclinic for Laboratory Diagnostics, University Clinical Centre Tuzla.

Immunohistochemistry

Three-step immunohistochemical procedure for VEGF was performed. Deparaffinization and rehydration of 4 μm thick formalin-fixed paraffin embedded sections were performed in xylene and ethanol solutions (decreasing concentration 96-70%). To block endogenous peroxydase activity sections were incubated in H2O2 solution (1,5% H2O2 in methanol) for 15 minutes. Antigen retrieval were performed in procedure with the buffer (pH=9.0, TRIS 20 mmol/L, EDTA 0.05 mmol/L, 0.05% Tween 20) in a microwave oven by heating the slides for 15 minutes. After rinsing with the PBS buffer, Protein block (Mouse and rabbit specific HRP plus detection IHC kit, Abcam,plc, Cambridge, UK) were applied for 15 minutes at room temperatures to block non-specific antibody binding. Sections were than incubated for an hour with primary antibody at 37°C. After rinsing with the PBS buffer, Biotinylated Goat-polyvalent Plus (Mouse and rabbit specific HRP plus detection IHC kit, Abcam,plc, Cambridge, UK) was applied for 30 minutes at room temperature. After rinsing with the PBS buffer Streptavidin Peroxidase Plus (Mouse and rabbit specific HRP plus detection IHC kit, Abcam,plc, Cambridge, UK) was applied for 30 minutes at room temperature. As chromogenwasused diaminobenzidine (FlukaChemie, Switzerland). For counterstaining we used hematoxylin (FlukaChemie GmbH, Buchs; Switzerland). Slide preservation was performed in Canada balsam. Used primary antibody and its dilution was as follows: a mouse antihuman monoclonal antibody against VEGF that detects the 121, 165 and 189 VEGF isoforms in routinely fixed specimens, clone VG-1 (Abcam,plc, Cambridge, UK) dilution 1:200 in the PBS/BSA with NaN3 buffer;

Endothelial cells of breast tissue were used as positive control for VEGF. Sections from the same paraffin blocks were also used as negative control but during immunohistochemical staining specific primary antibody was replaced with normal species (the same species as the primary antibody).

Immunohistochemical staining evaluation

Due to some universally accepted criteria, the final immunohistochemical staining evaluation scoring for VEGF expression was developed by authors. The expression of VEGF was scored as 0 for no immunoreactivity staining, +1 for poorly staining, +2 for moderately staining and +3 for strongly staining.

VEGF and IL-6 ELISA

VEGF concentrations in serum and seroma of 20 breast cancer patients and 15 benign breast disease patients were determined using a commercial sandwich enzyme immunoassay technique that detects the soluble isoforms of human VEGF (Quantikine Human VEGF Immunoassay; R&D Systems, Inc. Minneapolis, USA). A monoclonal antibody raised against human VEGF was loaded onto a microtiter plate. 100 μL of diluting solution, 100 μL of standard solution, serum and seroma from patients and 100 μL of serum from patients from control group were used for each analyse.

Plate was coated by adhesive foil and incubated for two hours at room temperature. During the incubation the soluble VEGF165 has bound to antibodies adsorbed on the plate. The unbound material was washed for four times in WASH buffer on washer (Columbus, Tecan, Austria). The plate was additionally dried by patting with paper towels. Afterwards, 200µL anti-VEGF pAB conjugated by horseradish peroxidise was added in each plate well, and the plate was incubated for two hours at room temperature. After the incubation,the plate was again washed for four times and 200 µL of substrate solution was added into each well (TMB with H2O2). After 25 minutes of incubation at room temperature the reaction was stopped by adding 50 µL of 2mol/L H2SO4. The absorbance of the coloured product was measured at 450 nm (TECAN, Sunrise). The VEGF165 concentrations, expressed in pg/mL of serum or seroma, were read from the calibration curve.

IL-6 levels were determined likewise in samples from 20 breast cancer patients and 15 benign breast disease patients. The determining procedure was equivalent to VEGF procedure and in accordance to the manufacturer instructions (Quantikine Human IL-6 Immunoassay; R&D Systems, Inc. Minneapolis, USA).

Statistical analysis

The results were evaluated by Wilcoxon test for dependent sample, Mann-Whitney U test for independent sample and with Spearman's correlation. For all performed tests, $P < 0.05$ was considered as statistically significant. For statistical analyses we used SPSS 17.0 software (SPSS Inc., USA).

RESULTS

VEGF expression was determined in tumour tissue and stromal tissue of 20 patients with breast cancer and in the normal breast tissue of 15 patients with benign breast disease. Differences in VEGF expression between tumour and stroma were significant (p=0,007) (Table 1) (Figure 1 and Figure 2).

Table 1. VEGF expression in the breast cancer tumour and the stromal tissue with patohistological parameters

Sample No	HG	pTNM	Tumour size	VEGF expression in tumour	VEGF expression in stroma
1	III	pT1N2M0	<2cm	+1	0
2	II	pT2N1M0	2-5cm	+3	0
3	III	pT1miLV+pN1	≤2cm	+2	+1
4	III	pT3N3	>5cm	+1	+1
5	III	pT2N1a	2-5cm	+2	+2
6	II	pT1cpN1micro	1-2cm	+1	+1
7	II	pT4N2	>5cm	+1	+1
8	II	pT2N2	2-5cm	+2	+2
9	III	pT2N2	2-5cm	+1	+1
10	II	pT2pN1	2-5cm	+1	+1
11	II	pT2[R,m], N1	2-5cm	+2	+1
12	III	pT2N0	2-5cm	+1	+1
13	II	pT2N0	2-5cm	+1	+1
14	III	pT[2]1N0	2-5cm	+1	+1
15	III	pT2N0	2-5cm	+2	+1
16	II	pT2N0M0	2-5cm	+1	+1
17	II	pT2N0	2-5cm	+1	+1
18	II	pT1N0	<2cm	+1	0
19	II	pT2N0	2-5cm	+2	+1
20	II	pT1N1M0	<2cm	+1	0

- Differences in VEGF expression between tumour and stroma (Wilcoxon test), p=0,007

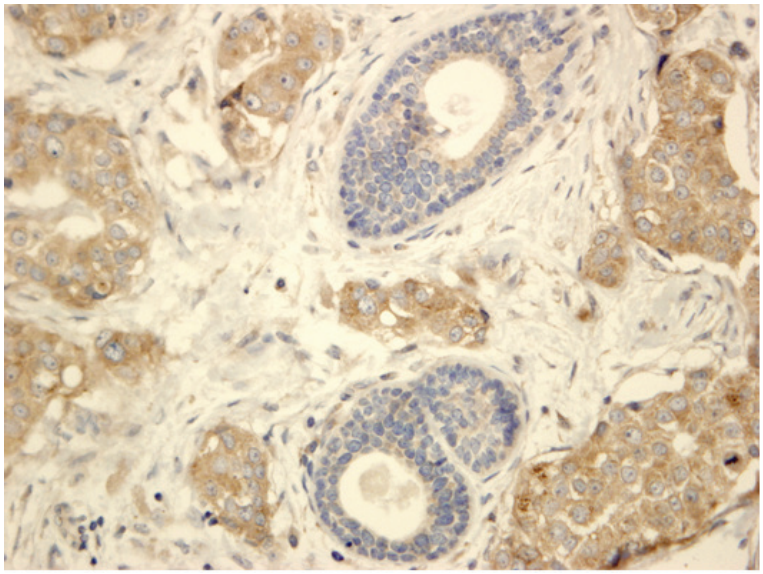

Figure 1. Microscopic appearance of breast cancer tumour tissue with strong expression of VEGF and stromal breast tissue with very discrete immunohistochemical staining showed as brown staining (HE; x40)

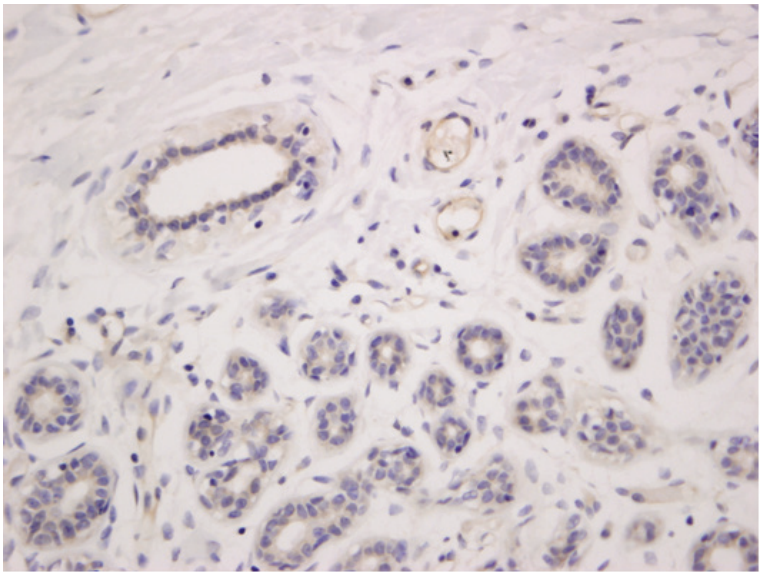

Figure 2. Microscopic appearance of normal breast tissue of benign breast disease patients with very discrete immunohistochemical staining for VEGF showed as brown staining (HE; x40)

Differences in VEGF expression between breast cancer tumour and normal breast tissue were also significant (p=0,0001) (Table 2) as well as differences in VEGF expression between stromal tissue and normal breast tissue (p=0,004) (Table 2).

Table 2. VEGF expression in normal breast tissue of benign breast cancer patients

Sample No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
VEGF expression	0	+1	0	0	0	0	0	0	+1	+1	0	+1	0	0	0

- Differences in VEGF expression between breast cancer tumour and normal breast tissue (Mann-Whitney U test) p=0,0001
- Differences in VEGF expression between breast cancer stromal tissue and normal breast tissue **(**Mann-Whitney U test**)** p=0,004.

Circulating VEGF and IL-6 levels were determined in 20 patients with breast cancer and 15 patients with benign breast disease (Table 3 and Table 4). Circulating VEGF before surgery was significantly higher in serum samples from patients with breast cancer than in patients with benign breast disease (p=0,023) (Table 3). After surgery VEGF level was elevated in serum (p=0,009) and in seroma (p=0,0001) compared to VEGF level in serum before surgery (Table 3).

Table 3. VEGF level alterations after breast surgery

Group	N	VEGF levels mean±st.error, min-max. [pg/mL]			Wilcoxon Z; p≤0,05*
		A I sample	B II sample	C Seroma	
1. Patients with breast cancer	20	439,854±52,02 71,02-922,54	557,221±77,177 121,59-1290,9	3371,43±393,05 557,81-7000,00	A vs B z=-2,613, **p=0,009** A vs C z=-3,883, **p=0,0001** B vs C z=-3,962, **p=0,0001**
2. Patients with benign breast disease	15	281,08±53,95 90,33-916,83	288,187±55,83 0,00-836,67	—	A vs B z=-1,363, p=0,173
Mann-Whitney U; p≤0,05		A1 vs A2 U=82,00 **p=0,023***			

Legend: A **(**I sample**)** – serum sample before surgery, B **(**II sample**)** – serum sample after surgery, C – seroma sample

Circulating IL-6 was elevated after surgery in serum (p=0,015) and in seroma (p=0,0001) (Table 4). IL-6 level was significantly elevated after partial reconstruction surgery of patients with benign breast disease patients (p=0,018) (Table 4).

Table 4. IL-6 level alterations after breast surgery

Group	N	IL-6 levels mean±st.error, min-max. [pg/mL]			Wilcoxon Z; p≤0,05
		A I sample	B II sample	C Seroma	
1. Patients with breast cancer	20	3,175±1,74 0,00-29,86	8,207±2,21 0,00-41,155	1603,894±112,8 3,39-1939,90	A vs B z=-2,443, **p=0,015*** A vs C z=-3,883, **p=0,0001** B vs C z=-3,92, **p=0,0001**
2. Patients with benign breast disease	15	0,235±0,235 0,00-3,52	3,197±1,47 0,00-21,981	—	A vs B z=-2,731, **p=0,018***
Mann-Whitney U; p≤0,05*		A1 vs A2 U=121,00 p=0,347			

Legend: A **(**I sample**)** – serum sample before surgery, B **(**II sample**)** – serum sample after surgery, C – seroma sample

Significant correlation was found only between VEGF and IL-6 levels in seroma samples from patients with breast cancer (rho=0,567, p=0,009) (Figure 3).

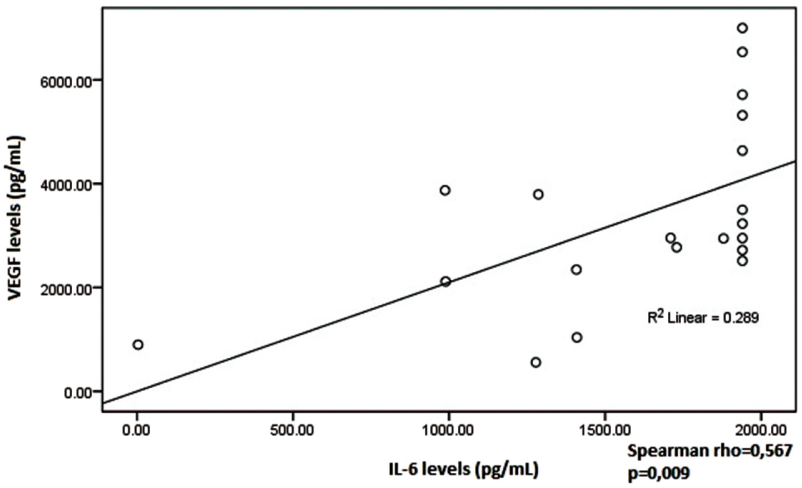

Figure 3. Spearman's correlation between VEGF and IL-6 levels (pg/ml) in seroma occurring after breast cancer surgery

No significant correlations between VEGF expression in tumour or stromal tissue of breast cancer patients and VEGF and IL-6 in serum samples of those patients were found.

DISCUSSION

Over recent decades, cytokines have gained more attention in the understanding of physiological and pathophysiological changes after trauma or surgery [14]. Patients with surgical injury induce endogenous mediators that alter hemodynamic, metabolic and immune response which is initiated immediately following injury [15]. Major mediator of wound angiogenesis is soluble form of VEGF which was identified in several wound models [6, 7]. VEGF also plays a major role in cancer angiogenesis [4] and it was identified in breast cancer tumour tissue [16]. Results from present study showed significant elevation of VEGF in serum of breast cancer patients after surgery (p=0,009) and in seroma as well (p=0,0001) (Table 3) which implicates that angiogenic response was initiated. Breast cancer tumour tissue express VEGF [16]. Presented results confirmed significant expression of VEGF in primary breast cancer tumour in relation to unchanged breast tissue of benign breast disease patients and stromal tissue of breast cancer patients (p=0,0001, p=0,004 respectively) (Table 2). VEGF in serum of breast cancer patients before surgery was significantly higher than in serum from benign breast disease patients (Table 3) which suggests that serum VEGF might be in relation to VEGF expression in tumour. In that case it is expected that serum level of VEGF drops after surgery, as some authors previously have shown on colon [17] and gastric [18] cancer surgery. Still, results obtained in present study and previous studies [4, 6, 7, 19] suggest that elevation of serum and seroma VEGF after surgery is in relation with angiogenic response to surgical injury. Significant change in VEGF level has not been found after surgery in patients with benign breast disease (Table 3) which can be explained by less aggressive partial reconstruction surgery compared to the radical mastectomy, surgery that cancer patients undergo (Table 3). Therefore, obtained results suggest that type of surgery and the wound size may influence the alteration of VEGF level which would be interesting for further investigation. Wound healing after surgery generates inflammatory response. The inflammatory phase of wound healing is characterised by the presence of well-studied inflammatory cytokine IL-6 [8-10]. Its contribution to tumour angiogenesis has been also previously reported [20]. Present study showed elevation of serum IL-6 in breast cancer patients after surgery (p=0,015) (Table 4) as well as in patients with benign breast disease (p=0,018) (Table 4). Determined statistically significant correlation between VEGF and IL-6 levels in seroma samples after breast cancer surgery (p=0,009) (Table 4) confirmed previously reported data, which suggests contribution of IL-6 to angiogenesis [20]. Although, involvement of IL-6 in immuno-inflammatory processes induced by surgical injury is well studied [8, 9, 10, 20], due to complexity of immune-inflamatory response that involves a complex crosstalk between several hormones (glucagon, cortisol, cateholamines, eiocosanoides, adrenocorticotropic hormone) and cytokines [21] , there is opposite results of clinical implication of inflammation after surgery. Inflammatory cascade, induced after cardiac surgery with cardiopulmonary bypass that includes release of inflammatory cytokines such as IL-6, contributes to the development of postoperative complications including respiratory failure, renal dysfunction, bleeding disorders, neurologic dysfunction and multiple organ failure [22 - 25]. Therefore, it would be interesting to determine level of IL-6 in breast cancer patients with postoperative complications. However, studies on mice showed that IL-6 modulates immune response and it is essential in wound healing [13]. Furthermore, recent studies showed that inflammatory cytokine IL-19

induces release of IL-1β, IL-6, TGF- β, MMP2, MMP9 and CXR4 which contribute to cutaneous wound healing [26]. Applied IL-19 protein on surgical wounds in mice can promote a cutaneous wound healing process [27]. In present study, very high levels of VEGF and IL-6 in seroma were found (Table 3 and Table 4) which implicates that surgical wound healing, including angiogenic and inflammatory immune response, is activated and includes VEGF and IL-6 expression.

CONCLUSION

Obtained results suggest involvement of VEGF and IL-6 in physiological changes after breast cancer surgery. Still, influence of surgery on cytokine production and physiological and pathophysiological responses needs to be further investigated.

List of abbreviations

CXR4	Chemokine Receptor Type 4
IL-1β	Interleukin-1β
IL-6	Interleukin -6
MMP2	Metalloproteinase 2
MMP9	Metalloproteinase
pAb	Polyclonal Antibody
TGFβ	Tumour Growth Factro β
TNFα	Tumour Necrosis Factor α
VEGF	Vascular Endothelial Growth Factor

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