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Low TGF-β1 in Wound Exudate Predicts Surgical Site Infection After Axillary Lymph Node Dissection

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ABSTRACT

Purpose: Surgical site infection (SSI) after axillary lymph node dissection (ALND) for breast cancer increases morbidity and delays the onset of adjuvant treatment. Only a few studies have investigated the feasibility of wound exudate analysis in SSI prediction. This study assessed changes in cytokine levels in postsurgical wound exudate after ALND and examined their predictive value for the early diagnosis of SSI.

Methods: An observational prospective pilot study was conducted in 47 patients with breast cancer undergoing ALND. Wound exudate samples were collected on the first and sixth postoperative days (POD). Interleukin (IL)-1α, IL-1β, IL-4, IL-10, IL-13, tumor necrosis factor alpha (TNF-α), transforming growth factor beta1 (TGF-β1) and vascular endothelial growth factor (VEGF) C and D levels were measured by immunoassay. Patients were followed to detect SSI.

Results: SSI was diagnosed in 8/47 (17.0%) patients. Four SSI patients were hospitalized and treated with intravenous antibiotics. The concentration of TGF-β1 in wound exudate was significantly lower on POD#1 in the SSI group compared to the no SSI group (p=0.008). The receiving operator characteristics (ROC) curve for TGF-β1 showed an area under curve of 0.773 (p=0.0149) indicating good diagnostic potential. On POD#6, the concentration of TGF-β1 remained significantly lower (p=0.043) and the concentrations of IL-10 (p=0.000) and IL-1β (0.004) significantly higher in the SSI group compared to the no SSI group.

Conclusion: To our knowledge, this is the first study suggesting a predictive role of wound exudate TGF-β1 levels for SSI. Our results suggest that the risk for SSI can be detected already

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on POD#1 and that the assessment of TGF-β1 levels in the wound exudate after ALND can provide a usefull method for the early detection of SSI. The key findings of this pilot study warrant verification in a larger patient population.

Introduction

Breast cancer is the most common cancer type in women. The treatment of regional nodal metastases may include axillary lymph node dissection (ALND) [1]. The surgery and trauma lead to tissue injury and wound healing, which in the early stages is dominated by a pro-inflammatory response, followed by the proliferative and remodeling phases[2]. Surgical site infection (SSI) is a multifactorial and complex process complicating the results of surgery and delaying adjuvant treatment. Surgical operations on the breasts are considered clean procedures by the Centers for Disease Control and Prevention (CDC) wound classification system. Despite this, up to 26 % SSI rates have been reported[3-6]. Clinical symptoms of SSI include tenderness, pain, fever, pus discharge and wound dehiscence. These symptoms appear when the infection has already developed, which does not allow for early detection of an SSI. Currently, there are no clinically relevant tests available for SSI prediction.

A closed drainage suction system is inserted routinely during closure of the ALND wound. Postsurgical wound exudate provides an excellent source of information on the local wound microenvironment[7-9]. Cytokines and growth factors are produced after tissue injury on the first postoperative days (POD) and their amount is usually higher in the wound exudate than in the peripheral blood[10]. Some cytokines, such as interleukin (IL)-1 and IL-6, are acute-phase reactants that can increase soon after tissue trauma and could peak within the first 4 hours after surgery, whereas others, such as IL-10, have an anti-inflammatory action and their levels have been reported to be lower after surgery[11,12].

The aim of this study was to determine the cytokine and growth factor profile of postsurgical wound exudate after ALND in patients with and without SSI, and assess the predictive value of these profiles for the early diagnosis of SSI.

Patients and methods

Patients and samples

Permission for collecting patient samples was approved by the Ethical Committee and institutional review board of the Turku University Hospital (ETMK:11/1801/2017 and ETMK:53/180/2011). All patients signed an approval for sample collection and approved the use of their patient information in the study. Postoperative axillary wound exudate samples were collected on POD#1 and POD#6 from 47 patients undergoing axillary lymph node dissection surgery between 6/2011-1/2013 and 2/2016-8/2018. The first sample was taken while the patient was hospitalized on POD#1, and the second sample when the drainage system was removed on POD#6. Prophylactic antibiotics were not routinely used during the first data collection period. Due to the increased evidence on SSI risk factors, prophylactic cefuroxime 1.5 g i.v. was administered one hour before surgery during the second data collection period in all patients.

Age, weight and height variables, primary tumor size and characteristics, surgical treatment, prior history of diabetes and smoking, length of hospital stay, and the presence of SSI were recorded. A diagnosis of SSI entailed one of the following CDC criteria within 30 days of operation as previously described[6]: (1) purulent drainage from the incision; (2) organisms isolated from an aseptically obtained culture of fluid or tissue; (3) deliberate opening of the incision by a surgeon in patients having either tenderness, localized swelling, redness, or warmth; or (4) diagnosis of SSI by the surgeon or health care center physician. Patients clinically diagnosed and documented with cellulitis of the operation area were also categorized as having an SSI. Wound exudate levels of IL-1 α, IL-1 β, IL-4, IL-10, IL-13, tumor necrosis factor alpha (TNF-α), transforming growth factor beta1 (TGF-β1), vascular endothelial growth factor (VEGF) C and D were determined on POD#1 and POD#6. The presence or absence of SSI was the main variable.

Evaluation of wound exudate cytokines

A protease inhibitor (cOmplete EDTA-free Protease Inhibitor Cocktail Tablets; Roche Diagnostics, Mannheim, Germany) was added to the wound exudate samples, followed by centrifugation to separate the supernatant and cell pellet. Supernatant cytokine protein concentrations of IL-1 α, IL-1 β, IL-4, IL-10, IL-13, TNF-α, TGF-β1 and VEGF-D were measured using ProcartaPlex multiplex immunoassay panels (Thermo Fisher Scientific, Bender MedSystems GmbH, Austria) and Luminex 200 (Thermo Fisher Scientific) according to the manufacturer's instructions. Luminex results were obtained by the 4PL algorithm and analyzed according to the operation manual (Luminex200) and ProcartaPlex Analyst Software. Supernatant VEGF-C concentration was measured using a commercial ELISA kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Absorbance was measured using a microplate reader (Thermo Scientific, Multiskan FC) at 450 nm wavelength and converted to pg/ml based on a standard curve.

Statistical analysis

Statistical analysis was performed using SPSS 18.0.2 for Windows (SPSS, Inc, Chicago IL). Mean values for normally

Table 1 – – Patient demographics, tumor variables, and surgical characters of the cohort separated by SSI and no SSI groups.

Variable	SSI (*n*=8)	no SSI (*n*=39)	P value
Age (y)	57.5(±9.8)	57.6(±13.9)	0.978
Height (cm)	164.9(±5.9)	163.8(±5.7)	0.640
Weight (kg)	74.0(±11.5)	73.9(±11.8)	0.980
Diabetes	0 (0)	4 (10.2)	0.461
Smoking	1 (12.5)	3 (7.7)	0.539
Neoadjuvant treatment	1 (12.5)	4 (10.2)	0.625
Breast tumor size (mm)	18.9(±8.4)	36.6(±21.9)	0.042*
Breast surgery *lumpectomy*	1 (12.5)	12 (30.8)	0.271
Breast surgery *mastectomy*	6 (75.0)	26 (66.7)	0.271
Breast surgery *none (only ALND)*	1 (12.5)	1 (2.6)	0.271
Tumor grade *1 or 2*	3 (37.5)	23 (59.0)	0.267
Tumor grade *3*	5 (62.5)	15 (38.4)	0.267
Number of axillary metastasis	5.1(±6.0)	4.0(±7.0)	0.654
HER2 positive	3 (37.5)	8 (20.5)	0.367
Estrogen receptor (%)	54.4(±45.6)	77.6(±33.8)	0.103
Progesterone receptor (%)	47.1(±45.6)	63.5(±38.8)	0.297
Triple negative	2 (25.0)	2 (5.1)	0.129
Prolonged seroma	1 (12.5)	7 (17.9)	0.660

Data are presented as mean (±SD) or number of patients (percentages). SSI=wound infection. ALND=axillary lymph node dissection. *$P \leq 0.05$

distributed continuous variables were compared by independent samples Student t-test and by Wilcoxon test for non-normally distributed data. Categorical data were analyzed by Fisher's exact test. Univariate logistic regression analysis was performed for significant variables to calculate Odds ratios (ORs) with 95% confidence intervals (CIs). Due to the low number of events of the dependent variable (8 SSI), multivariate logistic regression was not performed. Receiver operating characteristic (ROC) curves were constructed by plotting sensitivity% against 100%- specificity%. The area under the curve (AUC with 95% CI) was calculated to ascertain the optimal diagnostic cutoff of each cytokine biomarker for early diagnosis of SSI.

Results

Patients

Forty-seven female patients were enrolled in the study. Their ages ranged from 27 to 86 years (mean 56.6). The main variables related to patients are listed in Table 1. Two patients underwent ALND only without surgery of the breast, 13 had lumpectomy and 32 had mastectomy as the surgical method for treating the breast tumor. Eight (8/47, 17.0%) patients presented with an SSI. 4 of 8 patients were treated with peroral antibiotics, and 4 of 8 patients required intravenous antibiotics and hospitalization. 2 of 8 (25%) patients required bedside procedures for wound revision and 1 of 8 (12.5%) required surgical revision of the wound in the operating room. On the Clavien-Dindo scale, there were six Grade II and two Grade III complications. Excessive seroma formation that required drainage with a syringe and needle after drainage system removal (removed on POD#6) was present in 7 of 8 (87.5%) of the SSI patients and in 33 of 38 (86.8%) of the no SSI patients. Other non-infectious complications such as postoperative hematoma were noted in 2 of 47 patients (one in SSI and one in no SSI group). Primary breast tumor size differed significantly between groups (SSI 18.9±8.4mm and no SSI 36.6±21.9mm, P=0.04, OR 0.928, 95% CI 0.865-0.995). Patient-related factors such as older age, higher weight, diabetes and smoking were not risk-factors for SSI in our patient cohort. No statistical association was found between operation type, tumor grade, estrogen and progesterone receptor status, HER2 positivity, number of axillary metastases, prolonged seroma accumulation, and SSI. There were no significant differences between groups regarding prior neoadjuvant treatment and further adjuvant treatment.

Cytokine profiles

Wound exudate samples were collected from the closed suction drainage system on POD#1 and POD#6. IL-1 α, IL-1β, IL-4, IL-10, IL-13, TNF-α, TGF-β1, VEGF-D and VEGF-C levels were measured. The concentrations of IL-10, IL-1α and TNF-α were elevated in the SSI group on POD#1 but these results were not statistically significant (Table 2). However, the concentration of TGF-β1 on POD#1 was significantly lower in the SSI group compared to no SSI group (SSI 258.7±119.4 pg/ml, SSI 999.1 ±740.8 pg/ml, P=0.008. (Table 2, Fig. 1A). The receiving operator characteristics (ROC) curve for TGF-β1 was performed and the area under curve was 0.773 (P=0.0149) indicating good potential for diagnostic profitability (Fig. 1B). On POD#6 the concentration of TGF-β1 was still significantly lower (P=0.043) (Table 2,

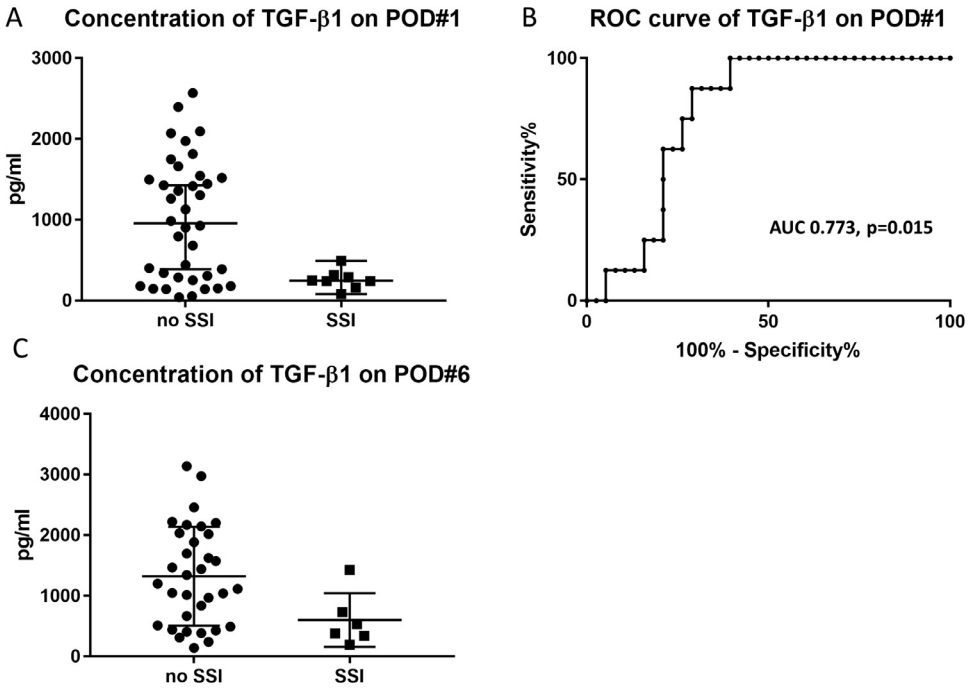

Fig. 1 – Low concentration of TGF-β1 in wound exudate on the first postoperative day predicts surgical site infection (SSI). (A) Scatter plot of TGF-β1 concentration (pg/ml; mean±sd; *n*=46) on POD#1 in SSI and non SSI groups. (B) Receiving operator characteristics (ROC) curve for TGF-β1 on POD#1, AUC=area under curve. (C) Scatter plot of TGF-β1 concentration (pg/ml;mean±sd, *n*=40) on POD#6 in SSI and non SSI groups.

Table 2 – – Wound exudate cytokine and growth factor concentrations of the cohort separated by SSI and no SSI groups.

Cytokine/ growth factor	SSI (*n*=8)	no SSI (*n*=39)	*P* value
IL-10 POD#1	17.3 (±9.2)	14.2 (±14.8)	0.568
IL-10 POD#6	51.5 (±59.4)	4.3 (±3.7)	0.000*
IL-13 POD#1	2.2 (±0.4)	2.5 (±1.3)	0.490
IL-13 POD#6	2.3 (±0.0)	2.7 (±1.8)	0.680
IL-1α POD#1	3.8 (±2.9)	4.8 (±7.6)	0.735
IL-1α POD#6	6.7 (±6.7)	6.4 (±16.4)	0.960
IL-1β POD#1	38.7 (±71.8)	23.1 (±24.1)	0.281
IL-1β POD#6	450.5 (±851.4)	24.9 (±36.1)	0.004*
IL-4 POD#1	8.5 (±0.0)	10.2 (±7.3)	0.524
IL-4 POD#6	8.5 (±0.0)	9.3 (±5.3)	0.712
TNF-α POD#1	13.3 (±32.4)	6.2 (±5.9)	0.232
TNF-α POD#6	19.5 (±24.5)	16.9 (±41.7)	0.884
VEGF-D POD#1	4.1 (±1.7)	4.1 (±2.9)	0.977
VEGF-D POD#6	5.9 (±3.7)	6.6 (±6.8)	0.824
TGF-β1 POD#1	258.7 (±119.4)	999.1 (±740.8)	0.008*
TGF-β1 POD#6	601.5 (±444.8)	1323.0 (±815.0)	0.043*
VEGF-C POD#1	1825.1 (±147.4)	1730.4 (±198.6)	0.209
VEGF-C POD#6	1810.6 (±85.0)	1703.3 (±144.1)	0.087

Data are presented as mean (± SD) pg/ml. POD=postoperative day, SSI=wound infection,
*$P \leq 0.05$

Fig. 1C) and the concentrations of IL-10 (P=0.000) and IL-1β (0.004) significantly higher in the SSI group compared to the no SSI group. The concentration of VEGF-C was also higher in the SSI group on POD#6 but this did not reach statistical significance (P=0.087). As some of the patients had an ongoing SSI on POD#6, the predictive role of day 6 cytokine measurements was not analyzed.

Discussion

In the present study we analyzed the concentration of cytokines and growth factors involved in wound healing after ALND from wound exudate samples. We found that the concentration of TGF-β1 was lower in patients with SSI, which is a completely novel finding. The ROC curve showed an area under curve of 0.773 which indicates good diagnostic potential.

We found tumor size to be smaller in SSI patients. However, this cannot be explained by the surgical method (lumpectomy or mastectomy) as it did not differ between groups. Other tumor related variables and prior neoadjuvant treatment did not differ significantly between groups.

TGF-β1 belongs to the TGB-β family of growth factors and has multiple roles in regulation of proliferation, migration, differentiation, and extracellular matrix production during tissue repair and wound healing[13,14]. It is secreted by platelets, monocytes, endothelial cells, fibroblasts and keratinocytes[15]. Although the crucial role of TGF-β1 in wound healing is well known, its role in the development of wound infection has not been previously investigated in a clinical setting. The active

and mature form of TGF-β is a disulfide-linked homodimer. This form is kept inactive through non-covalent association with a latency-associated peptide homodimer (LAP). LAP removal, which can be achieved in the extracellular space through diverse mechanisms ranging from conformational changes promoted by interaction with integrins to enzymatic digestion by matrix metalloproteinases[15], constitutes a critical regulatory event. The ProcartaPlex assay used in this study detects only the active form of TGF-β1 and therefore we cannot rule out the possibility of TGF-β1 residing predominantly in the inactive latent form in SSI patients. In that case, differences in TGF-β1 activation and LAP removal patterns between SSI and non SSI patients could exist.

Upon skin injury, regional induction of TGF-β in the wound has been found to result in a double-peak availability pattern. In the case of TGF-β1, peaks can be seen on day 1 and day 7 post surgery[16]. This pattern emerges from the initial quick and abundant platelet release of TGF-β1, and the later build-up of the aggregated contributions from endothelial cells, monocytes, fibroblast, and keratinocytes over time[16]. The role of TGF-β1 in chronic wounds is well established, but its role in acute SSI has not been studied previously. TGF-β1 is known to induce chemotaxis of neutrophils[17], that are important players of the immune system against early bacterial infections. Our results show a reduced level of TGF-β1 secretion in SSI patients already on POD#1. The early decrease in TGF-β1 levels could potentially be linked to differences in platelet functions, and the release of active TGF-β from platelets has previously been shown to suppress T cell-mediated immunity[18]. Therefore, increased incidence of SSI might be associated with altered T cell and neutrophil responses in low TGF-β1 environment. However, this hypothesis remains to be investigated.

The risk for wound infection can be predicted from preoperative parameters, using for example the scales of the Study on the Efficacy of Nosocomial Infection Control (SENIC) [19], and the National Nosocomial Infection Surveillance System (NNISS) [20]. Govinda et al. investigated the role of tissue oxygen measurements in the prediction of SSI, and found that upper arm tissue oxygen measurements 75 min after surgery may have a predictive role (sensitivity 71% and specificity 60%) after colorectal surgery, but further research on the utilization of this method is needed[21]. The role of wound cytokine production in the prediction of SSI in head and neck cancer patients was previously analyzed by Candau-Alvarez et al[22]. Although this study consisted of a limited number of patients (n=39), they found that TNF-α served as the best predictive value for SSI on POD#1 and IL-1 β on POD#3. On the contrary, IL-6 and TNF-α have been shown to be important in normal postoperative wound healing, and IL-4 and interferon-γ are associated with postoperative necrosis and seroma after mastectomy[23]. We also found that patients with SSI had higher IL-1 β concentrations on POD#6, which is in line with previous data[22]. In addition, we found that patients with infection had higher IL-10 and VEGF-C levels, and lower TGF-β1 levels on POD#6. Pro-inflammatory cytokines have previously been shown to increase VEGF-C concentration and inflammation induced lymphangiogenesis[24].

Current American clinical guidelines recommend a single dose of preoperative antibiotics for breast procedures with postoperative duration of antimicrobial prophylaxis being limited to less than 24 h, regardless of the presence of surgical drains[25]. The data concerning the usage of postoperative prophylactic antibiotics is controversial: a study by Edwards reported that patients prescribed postoperative prophylactic antibiotics during surgical drain presence were significantly less likely to develop SSI compared to those receiving only perioperative antibiotics (3.4 and 14 %, respectively) [26]. This differs from the study by Throckmorton which did not observe a significant reduction in SSI rate among patients receiving postoperative antibiotic prophylaxis during drain usage[4]. In the era of worsening bacterial antibiotic resistance, it is important to reserve prophylactic antibiotic treatment to those that are expected to benefit from it, and to develop better diagnostic tools for the prediction of infections to target the treatment to patients at risk.

Day surgery is a common practice in many hospitals minimizing the time spent at the hospital. When the patient is recovering at home, they are left with the task of identifying the signs of infection. In our patient cohort material, half of the infections required hospitalization and intravenous antibiotics immediately after the diagnosis was made by their treating physician. If these infections would have been predicted earlier, a peroral antibiotics course and smaller surgical interventions might have been sufficient treatment methods. A delay in the onset of adjuvant treatment can be crucial for a breast cancer patient. A simple test from the wound exudate that could predict infection would be an optimal solution to predict the risk for SSI as early as on POD#1. If a risk for infection was detected, one could either arrange an additional control visit or choose to treat these patients with postoperative prophylactic antibiotics.

One of the limitations of this study was the number of patients enrolled. Due to the low number of subjects, multivariate analyses could not be performed and the association of tumor size, SSI and TGF-β1 could not be analyzed. Another limitation of the study was that the cytokine concentrations were not normalized based on drain output as we did not collect data on wound exudate volumes. Data on excessive seroma formation after drainage system removal was recorded categorically without recording the actual amount of times seroma was removed using a needle and syringe. Thus, we could not analyze the SSI risk related to multiple punctures. As this was a pilot study, further studies enrolling more patients based on a power analysis will be performed. Comprehensive data will be gathered on drainage output volumes and seroma formation. In future studies, comprehensive analysis of cytokine kinetics, including their levels in blood circulation, will be performed.

Currently, there are no clinically relevant tests available for SSI risk prediction. Our results show that SSI can be predicted on POD#1 through identification of changes in cytokine and growth factor levels in postsurgical wound exudate. We found that TGF-β1 has an exceptionally good predictive value on POD#1. Because of these encouraging results, further studies addressing our novel finding have already been initialized in a larger patient population. In the future, validation of the findings in other areas of surgery will also be performed.

Author contributions

I-M L: sample analysis, data collection, writing ER: sample gathering, data analysis, writing, ML: sample analysis, data analysis, writing EP: study design, data interpreting, writing RV: study design, sample gathering, patient recruitment, writing TV: study design, sample gathering, data interpreting, writing IK: study design, data interpreting, writing PH: study design, data analysis and interpreting, writing

Ethical statement and informed consent

All procedures were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Permission for collecting patient samples was approved by the Ethical Committee and institutional review board of the Turku University Hospital (ETMK:11/1801/2017 and ETMK:53/180/2011). All patients signed an approval for sample collection and approved the use of their patient information in the study.

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Disclosure

PH and TV are research scientists involved in a clinical trial of Herantis Pharma (Lymfactin) and PH and EP received honoraria for participating in data monitoring committee meetings and advisory boards of Herantis Pharma. Authors I-ML, ER, ML, EP, RV and IK declare no conflict of interest.

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