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Concentrations of neopterin, kynurenine and tryptophan in wound secretions of patients with breast cancer and malignant melanoma: a pilot study

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Abstract: The aim of the present pilot study was to investigate the concentrations of neopterin, kynurenine and tryptophan in wound secretion in patients undergoing surgery for breast cancer or malignant melanoma. Twenty-two patients, 16 females and 6 males, undergoing surgery for breast cancer (n = 15) or malignant melanoma (n = 7) were evaluated. Neopterin, kynurenine and tryptophan were determined using a high-performance liquid chromatography method. When the concentrations in wound secretions from the primary breast tumor and the axilla were compared, the neopterin/tryptophan ratio was significantly higher in the tumor wound secretions (0.92 ± 0.41 vs. 0.61 ± 0.14 mmol/mol; p = 0.049), but no significant differences were observed in neopterin (49.2 ± 28.6 vs. 31.5 ± 11.1 nmol/L), tryptophan (52.9 ± 13.0 vs. 51.2 ± 13.3 μmol/L) and kynurenine concentrations (5.97 ± 7.49 vs. 5.34 ± 6.25 μmol/L) and kynurenine/tryptophan ratio (108.1 ± 107.7 vs. 103.5 ± 106.7 mmol/mol). No marked differences were noted in neopterin, tryptophan and kynurenine concentrations and kynurenine/tryptophan and neopterin/tryptophan ratios in sequential samples from the axilla of breast cancer patients obtained on days 1 and 2. In conclusion, present data demonstrate that the measurement of neopterin, kynurenine and tryptophan can be used to monitor local immune response after cancer surgery.

Keywords: breast cancer; kynurenine; melanoma; neopterin; tryptophan.

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Introduction

The role of the immune response as a crucial factor determining the outcome of malignant disease is currently being increasingly recognized. In fact, tumor-promoting inflammation and escape from the immune system detection are thought to represent the key and indispensable features of malignant growth [1].

Biomarkers play an essential role in the management of cancer patients [2]. With the recognition of the role of the immune system in the outcome of cancer and the advent of immunotherapy, more attention has been focused on the biomarkers of host response against neoplasia. It has been demonstrated that tumor growth and progression is reflected in changes of the biomarkers of the immune response indicating immune dysfunction [3–7].

Current therapeutic strategy in patients with cancer is based on a multidisciplinary therapeutic approach that combines surgery, radiotherapy and systemic treatment. While the effect of radiation or systemic therapy on immune response in cancer patients has been studied extensively [8–11], the study of the effect of surgery on immune response has been more neglected.

The aim of the present pilot study was to investigate neopterin, kynurenine and tryptophan concentrations in wound secretions of patients undergoing surgery for breast cancer or malignant melanoma.

Patients and methods

Twenty-two patients, 16 females and 6 males, undergoing surgery for breast cancer (n = 15) or malignant melanoma (n = 7) were evaluated in the present study. The demographics of the patients, type of surgery and other clinical information are shown in Table 1. The present investigation was approved by the institutional ethical committee and the patients signed informed consent.

The samples were taken from light-protective drains connected to Redon drains inserted during surgery in the morning hours. If a sample of sufficient quantity was obtained, the sample was split and a part of the sample was stabilized by argon gas. The samples were frozen immediately and stored at –20°C until analysis.

Neopterin, kynurenine and tryptophan were determined using a high-performance liquid chromatography (HPLC) method as described [12]. Briefly, a monolithic C18 Chromolith high-resolution column (MERCK, Darmstadt, Germany) with dimensions of 4.6×100 mm connected to a monolithic 4.6×10 mm security guard (MERCK, Darmstadt, Germany) was used as the stationary phase. Separation was achieved using 15 mM phosphate buffer (Appplichem, Darmstadt, Germany) ($KH_2PO_4 + K_2HPO_4 \cdot 3H_2O$ at pH 3) and acetonitrile (Fluka Sigma-Aldrich, Prague, Czech Republic) in gradient mode. Five microliters of the sample was injected into the system; neopterin and tryptophan were detected using fluorescence detection at 353 nm excitation and 438 nm emission for neopterin and 254 nm excitation and 404 nm emission for tryptophan. Kynurenine was detected using diode array detection at 230 nm. Samples were prepared prior to HPLC determination by dilution with 100 µL of phosphate buffer (Appplichem, Darmstadt, Germany) (15 mM, pH 6.5) and 100 µL of cold ethanol (Fluka Sigma-Aldrich, Prague, Czech Republic) (deproteinization 10 min at –25°C), centrifugation for 10 min at 14,000× *g* and finally filtration using the 0.2 µm microtitration plate filters (Pall, Ann Arbor, MI, USA) and vacuum manifold (Phenomenex, Aschaffenburg, Germany).

Paired measurements were compared using the Wilcoxon signed rank test. Statistical analyses were performed using NCSS software (Number Cruncher Statistical Systems, Kaysville, UT, USA).

Results

The concentrations of neopterin, kynurenine and tryptophan were in the measurable range in all samples examined. The results obtained from the samples prepared by standard processing and the samples stabilized with argon gas were similar (data not shown). Therefore, standard processing without argon gas stabilization was used consistently for all samples. The concentrations of neopterin, kynurenine and tryptophan and kynurenine/tryptophan and neopterin/tryptophan ratios in the whole cohort are shown in Table 1. When the concentrations in wound secretions from the primary tumor (breast cancer in all cases) and the axilla were compared, the neopterin/tryptophan ratio was significantly higher in breast tumor wound secretions (mean ± standard deviation, 0.92 ± 0.41 vs. 0.61 ± 0.14 mmol/mol; p = 0.049), but no significant differences were observed in neopterin (49.2 ± 28.6 vs. 31.5 ± 11.1 nmol/L), tryptophan (52.9 ± 13.0 vs. 51.2 ± 13.3 µmol/L) and kynurenine concentrations (5.97 ± 7.49 vs. 5.34 ± 6.25 µmol/L) and kynurenine/tryptophan ratio (108.1 ± 107.7 vs. 103.5 ± 106.7 mmol/mol) despite the presence of some trends (Table 2). When sequential samples of wound secretion from the axilla obtained on days 1 and 2 were compared, no marked differences were noted in neopterin (35.4 ± 14.8 vs. 35.5 ± 11.1 nmol/L), tryptophan (50.4 ± 15.4 vs. 47.1 ± 14.6 µmol/L) and kynurenine concentrations (5.06 ± 6.06 vs. 7.64 ± 10.02 µmol/L) and kynurenine/tryptophan (100.3 ± 100.8 vs. 142.3 ± 166.8 mmol/mol) and neopterin/tryptophan ratios (0.70 ± 0.23 vs. 0.79 ± 0.26 mmol/mol; Table 3).

Discussion

Present data demonstrate that the measurement of neopterin, kynurenine and tryptophan can be used to monitor local immune response in wound secretions after surgery. Neopterin, kynurenine, tryptophan and ratios of these biomarkers were used to monitor the immune response. The results obtained by both normal and argon gas sample processing were similar. With the exception of high neopterin/tryptophan ratio at the breast tumor site compared to the axilla, no significant differences were observed between concentrations in the secretions from the primary site and the axilla. Similarly, no significant changes were observed in the sequential samples. However, the size of this cohort and, consequently, statistical power were limited. No comparison was made between the secretions from the primary breast tumors and melanoma because in three out of seven melanoma cases the surgery was done on lymph nodes. Future study on a larger patient cohort should be performed to compare the concentrations of neopterin, kynurenine and tryptophan from different sites and during the course of post-surgical recovery. The limited size of the present cohort also did not allow the correlation of biomarker concentrations with the clinical course and complications of surgery. The concentrations of these biomarkers in wound secretions should also be compared with simultaneously collected samples from the circulation. The neopterin/tryptophan ratio was used in the present pilot study because it could potentially correct in certain situations the dilution of wound secretions by fluid from circulation or other sources. Moreover, a number of ratios of parameters moving in opposite directions in inflammatory disorders have been introduced

Table 1: Demographics, clinical data of the patients and concentrations of neopterin, tryptophan and kynurenine in wound secretions.

Patient	Age, years	Sex	Histology	Prior therapy, comorbidities or complications	Number of LN positive/LN examined	Location	Procedure	Complications of healing	Interval from surgery, days	Neopterin, nmol/L	Tryptophan, µmol/L	Kynurenine, µmol/L	Kynurenine/ tryptophan ratio, mmol/mol	Neopterin/ tryptophan ratio, mmol/mol
1	50	F	BC	Neoadjuvant chemotherapy; lymphedema	0/3	Breast	ME	None	2	31.9	48.3	2.42	50.0	0.66
						Axilla	Exenteration	None	1	53.4	73.6	1.82	24.7	0.73
2	67	F	BC	None	0/2	Breast	Lumpectomy	None	1	27.4	50.9	2.27	44.7	0.54
						Axilla	SLNB	None	1	41.9	60.6	1.59	26.3	0.69
3	67	F	BC	Rhabdomyosarcoma	0/10	Breast	ME	None	1	44.5	45.7	1.60	35.0	0.97
						Axilla	Exenteration	None	1	33.4	36.8	1.49	40.6	0.91
									2	38.1	35.6	1.27	35.8	1.07
									4	42.5	47.4	4.50	94.9	0.90
4	74	F	BC	Chronic venous ulceration	0/10	Breast	Lumpectomy	None	1	77.0	45.1	1.50	33.3	1.71
						Axilla	Exenteration	None	1	18.5	33.7	2.07	61.4	0.55
									2	25.1	26.7	1.43	53.6	0.94
									8	10.2	33.9	4.81	142.0	0.30
5	37	F	BC	Multicentric BC, breast implants	4/12	Breast	ME	None	1	120.3	77.4	NE	NE	1.55
									2	24.8	62.6	0.71	11.4	0.40
						Axilla	Exenteration	None	1	32.4	62.6	0.89	14.2	0.52
6	68	F	BC	None	0/7	Breast	Lumpectomy	None	1	36.0	51.9	0.55	10.6	0.69
						Axilla	Sampling	None	1	20.9	40.4	0.82	20.2	0.52
7	69	F	BC	Triple negative BC	0/4	Breast	ME	None	1	46.1	75.0	22.9	305.1	0.62
									2	29.3	50.7	25.8	508.9	0.58
						Axilla	SLNB	None	1	35.0	58.4	17.2	294.0	0.60
									2	30.0	58.2	26.2	450.2	0.52
8	76	F	BC	Endometrial carcinoma	0/11	Axilla	Exenteration	None	1	45.0	77.0	1.58	20.5	0.58
									2	46.3	68.7	1.13	16.4	0.67
9	53	F	BC	Anaphylactic shock	0/1	Breast	Lumpectomy	None	1	69.3	44.7	NE	NE	1.55
									2	35.0	43.0	4.13	96.2	0.81
10	66	F	BC	None	0/1	Breast	Lumpectomy	None	1	27.1	51.6	0.95	18.4	0.52
11	66	F	BC	None	0/1	Axilla	SLNB	None	1	62.1	60.7	12.4	204.8	1.02
12	56	F	BC	None	0/2	Breast	Lumpectomy	None	1	41.1	36.8	7.41	201.0	1.12
									2	40.7	47.9	9.53	198.9	0.85
						Axilla	SLNB	Purulent secretion on day 10	1	18.9	37.8	4.60	122.0	0.50

Table 1 (continued)

Patient	Age, years	Sex	Histology	Prior therapy, comorbidities or complications	Number of LN positive/LN examined	Location	Procedure	Complications of healing	Interval from surgery, days	Neopterin, nmol/L	Tryptophan, µmol/L	Kynurenine, µmol/L	Kynurenine/ tryptophan ratio, mmol/mol	Neopterin/ tryptophan ratio, mmol/mol
									2	38.3	56.1	12.1	216.6	0.68
13	76	F	BC	None	0/1	Breast	Lumpectomy	None	1	36.9	49.6	9.17	185.0	0.74
						Axilla	SLNB	None	1	35.9	55.0	13.1	238.6	0.65
									2	20.0	39.6	NE	NE	0.51
14	73	F	BC	Multifocal BC	0/1	Breast	Lumpectomy	None	1	29.1	56.6	12.0	211.7	0.51
15	69	F	BC	None	0/1	Breast	Lumpectomy	None	1	31.1	48.7	NE	NE	0.64
						Axilla	SLNB	None	1	24.8	53.2	NE	NE	0.47
16	87	M	MM	None	NE	Knee	Re-excision	None	2	26.1	56.8	1.46	25.8	0.46
17	67	F	MM	None	NE	Back	Radical excision	None	1	66.2	33.5	0.93	27.9	1.98
18	71	M	MM	Relapse	1/2	Axilla	Re-exenteration	None	1	61.2	54.4	3.45	63.4	1.12
									2	50.6	45.0	3.65	81.1	1.12
									4	42.2	44.5	2.74	61.6	0.95
19	38	M	MM	None	0/1	Groin	SLNB	None	1	17.7	64.6	1.51	23.4	0.27
20	66	M	MM	Liver and lung metastases	NE	Back	Radical excision	Wound dehiscence	1	75.9	59.1	1.91	32.3	1.29
									2	120.0	75.9	2.85	37.6	1.58
21	69	M	MM	None	0/10	Axilla	Exenteration	None	2	21.2	63.6	3.71	58.4	0.33
22	52	M	MM	None	NE	Back	Re-excision	None	1	23.1	46.1	1.80	39.0	0.50
									2	31.6	52.5	1.52	29.0	0.60
						Back	Re-excision	None	2	45.9	57.5	NE	NE	0.80

BC, breast cancer; F, female; LN, lymph node; M, male; ME, mastectomy; MM, malignant melanoma; NE, not examined; SLNB, sentinel lymph node biopsy.

Table 2: Differences in biomarker concentrations between the wound secretions of primary tumor and axilla.

Parameter	Breast primary	Axilla	p-Value
Neopterin, nmol/L	49.2 ± 28.6	31.5 ± 11.1	0.131
Tryptophan, μmol/L	52.9 ± 13.0	51.2 ± 13.3	0.557
Kynurenine, μmol/L	5.97 ± 7.49	5.34 ± 6.25	0.461
Kynurenine/tryptophan ratio, mmol/mol	108.1 ± 107.7	103.5 ± 106.7	0.844
Neopterin/tryptophan ratio, mmol/mol	0.92 ± 0.41	0.61 ± 0.14	0.049

Shown are the means ± standard deviations of wound secretions from the breast and axilla in 10 breast cancer patients.

Table 3: Differences in biomarker concentrations in wound secretions from the axilla in breast cancer patients after the surgery.

Parameter	Day 1	Day 2	p-Value
Neopterin, nmol/L	35.4 ± 14.8	35.5 ± 11.1	1.000
Tryptophan, μmol/L	50.4 ± 15.4	47.1 ± 14.6	0.297
Kynurenine, μmol/L	5.06 ± 6.06	7.64 ± 10.02	0.844
Kynurenine/tryptophan ratio, mmol/mol	100.3 ± 100.8	142.3 ± 166.8	0.438
Neopterin/tryptophan ratio, mmol/mol	0.70 ± 0.23	0.79 ± 0.26	0.297

Shown are the means ± standard deviations of wound secretions from the axilla collected on two consecutive days in seven breast cancer patients.

recently, e.g. ratios of peripheral blood cell components to lymphocytes, and the neopterin/tryptophan ratio could represent another new biomarker of inflammatory response that could be investigated in future studies.

Biomarkers of the immune response have been studied mostly systemically in the circulation as sampling of the tumor microenvironment is inherently difficult. Repetitive sampling is possible only in some situations, e.g. in patients with malignant effusions. For example, a number of studies have addressed the biomarkers of immune response, both immune cell populations and molecular biomarkers, in malignant ascites [5, 6, 13]. However, the data on malignant ascites are restricted solely to tumors involving the peritoneal cavity [14]. Moreover, the presence of malignant effusions is associated with advanced disease and the findings obtained in this setting cannot be extrapolated to patients with early tumors. Breast cancer and malignant melanoma are two tumors that are diagnosed predominantly at an early stage, can be effectively treated with surgery and the long-term prognosis of most patients is excellent. At the same time, it is evident that systemic therapy improves prognosis even in patients with localized breast cancer or malignant melanoma of the skin. There is also increasing evidence that the immune response determines the outcome of patients with breast cancer as well as melanoma [15, 16]. Moreover, malignant melanoma represents a model for the introduction of immunotherapy into the treatment of cancer [17].

Neopterin is produced by macrophages after the activation with interferon-gamma. Increased neopterin concentrations have been reported across a range of disorders [3, 18, 19]. In cancer patients, systemic (serum or urinary) neopterin concentrations are increased across a range of different primary tumors [3, 20, 21], including breast cancer [22–26] or melanoma [20, 21], and predict poor prognosis. Increased neopterin concentrations have been associated with the dysfunction of the immune system both in the tumor microenvironment [5, 6] and at the systemic level [4, 7].

In addition to the synthesis of neopterin, interferon-gamma induces catabolism of tryptophan to kynurenine that is catalyzed by indoleamine 2,3-dioxygenase (IDO). IDO is thought to represent one of the major factors responsible for the cancer escape from the immune response [27], one of the hallmarks of cancer. On the other hand, IDO induction has also been postulated to represent one of the mechanisms of the antitumor activity of interferon-gamma [28, 29]. High kynurenine concentrations have also been shown to inhibit the growth of tumor cells *in vitro* [30], but its relevance for the *in vivo* situation is questionable. In an experimental study, IDO activity has been shown to negatively affect wound healing [31]. It remains to be determined on a larger cohort of patients whether high kynurenine/tryptophan ratio in wound secretions would also be associated with impaired wound healing in clinical practice.

Biomarkers are used not only to predict prognosis or response to therapy, but may also be of value in the monitoring and prediction of complications of the therapy, e.g. toxicity of cytotoxic agents [32, 33]. For example, it was

recently demonstrated that increased neopterin concentrations predict the complications of neoadjuvant chemoradiation in patients with rectal cancer [34]. It may be hypothesized that the concentrations of neopterin, kynurenine and tryptophan in wound secretions could serve as biomarkers of the local complications or even predict subsequent adverse events. This hypothesis should be tested in a large patient cohort. Future studies on a larger cohort of patients in a more homogeneous patient population should also investigate the potential prognostic role of neopterin, kynurenine and tryptophan in wound secretion as well as whether these parameters could represent predictive biomarkers of surgical complications.

In conclusion, present data demonstrate that the measurement of neopterin, kynurenine and tryptophan in wound secretions can also be used to monitor local immune response after cancer surgery.

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