[Frontiers in Bioscience E3, 227-232, January 1, 2011]

Relationship between RANTES and dendritic cells in ovarian cancer patients

Iwona Wertel [1], Rafal Tarkowski[1], Wieslawa Bednarek[1], Jan Kotarski [1]

[1] 1st Department of Gynaecology, University School of Medicine, Lublin, Poland

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1. ABSTRACT

This study was undertaken to evaluate RANTES levels in the peritoneal fluid (PF) and plasma of patients with ovarian cancer (n=73), serous cystadenoma (n=32) or normal controls (n=9). RANTES levels were correlated to myeloid and lymphoid dendritic cells (DCs). RANTES levels were evaluated using the ELISA assay. DCs were quantified using flow cytometry. The PF and plasma RANTES concentrations were elevated in the ovarian cancer (OVC) patients when compared to the patients with benign tumor (the reference group). Plasma levels of RANTES were higher in OVC patients compared with the reference group and with the controls. There were no significant differences in the plasma RANTES levels based on tumor stage, grade or histology. Women with serous cystadenocarcinoma, clear cell carcinoma and endometrioid cystadenocarcinoma had significantly higher PF RANTES levels than patients with undifferentiated carcinoma. Women with clear cell carcinoma and patients with endometrioid cystadenocarcinoma had higher PF RANTES levels than women with mucinous cystadenocarcinoma. We concluded that RANTES production in the peritoneal cavities of OVC patients depends on the histological type of the tumor cells.

2. INTRODUCTION

Ovarian cancer (OVC) differs from other female genital tract malignancies because it is typically spread throughout the peritoneal cavity. Serous ovarian cancer spreads along the peritoneum even in case of relatively small tumors of the ovary. It has been shown that the CC chemokines and their receptors expression are implicated in the migration of ovarian cancer cells (1). Negus *et al.* (2) have reported the production of MCP-1/CCL2 by human ovarian cancer cells and demonstrated the presence of this chemokine gradient between ascites and plasma. Other findings suggest that a specific chemokine, RANTES/CCL5 (Regulated on Activation, Normal T cell Expressed and Secreted) may be useful in differentiating benign ovarian tumors from malignancy, and may correlate with the extent of the disorder (3).

The 8 kDa protein RANTES is produced by activated T lymphocytes (4), platelets (5,6), monocytes/macrophages (7), epithelial and bronchial epithelium (8,9), dermal fibroblasts (10) and renal tubular epithelium (11). Recently, it has been reported that several types of tumor cells (12), including breast tumor cells, release RANTES (13).

Table 1. Patient's characteristics

Parameters	Value
Patient age, n (min.-max.)	
Ovarian cancer	57 (22-85)
Serous cystadenoma	27 (18-76)
Histology, n (%)	
Serous cystadenocarcinoma	37 (50.68)
Undifferentiated carcinoma	19 (26.02)
Mucinous cystadenocarcinoma	11 (15.06)
Endometrioid cystadenocarcinoma	4 (5.48)
Clear cell carcinoma	2 (2.74)
Serous cystadenoma	32 (100%)
Grading, n (%)	
G II	33 (45.20)
G III	40 (54.80)
FIGO Stage, n (%)	
I	21 (28.76)
II	6 (8.21)
III	42 (57.53)
IV	4 (5.48)

RANTES plays an important role in a variety of diseases, including the allergic inflammatory diseases asthma, allergic rhinitis, and atopic dermatitis (14). Niwa *et al.* (15) have demonstrated that high plasma RANTES levels are correlated with advanced breast cancer. The investigators also found that breast tumor cell-derived RANTES may promote breast cancer progression and metastasis. RANTES may be also important for the immunology of ovarian cancer: -it influences migration of CD3 T cells (16), CD8 T cells (17), monocytes (3) and Th17 cells (18) into the ovarian tumor microenvironment. It was shown *in vitro* that RANTES is also a chemoattractant for myeloid (19) and lymphoid (20) dendritic cells.

In this study we investigated the concentrations of RANTES in the peritoneal fluid and plasma of patients with malignant and benign ovarian disease. RANTES levels were correlated with PF and peripheral blood (PB) myeloid and lymphoid dendritic cells. To our knowledge there are no previous reports of the peritoneal fluid RANTES levels in patients with different histological types of advanced ovarian cancer.

3. MATERIALS AND METHODS

3.1. Patients

A total of 73 women with histologically confirmed ovarian cancer were enrolled in this study. The clinical characteristics of the OVC patients are summarized in Table 1. No patients received chemotherapy before surgery. The reference group consisted of 32 women with serous cystadenoma, with no evidence of malignancies or pelvic adhesions. As a control, peripheral blood of nine healthy donors was taken. Patients gave written, informed consent in accordance with the Declaration of Helsinki. The study was approved by the Lublin University School of Medicine Ethics Committee.

3.2. Methods

Peripheral venous blood samples from each women were collected into heparinised tubes (sodium heparin) before the surgery. Peritoneal fluid specimens were obtained at the time of surgery and also stored in heparinised tubes. Plasma and PF samples were rendered cell-free by centrifugation at 1500 rpm for 10 minutes, and stored at -80°C before being tested by ELISA (enzyme-linked immunosorbent assay). RANTES concentrations in plasma and PF were determined by the Immunoassay kit (Research and Diagnostic Systems, Minneapolis, Minnesota, USA) following the manufacturer's protocol. Concentrations of RANTES were calculated by interpolation from a standard curve. The sensitivity of the RANTES ELISA was 16 pg/ml. All samples were assayed in duplicate.

3.3. Cell preparation

In the non-cancer group, all visible PF was aspirated during surgery from the anterior and posterior cul-de-sacs, under direct vision to avoid blood contamination. PF and PB mononuclear cells were isolated by density-gradient centrifugation on Lymphoprep (Nycomed, Norway) for 25 minutes at 600 g at room temperature. Interface cells were collected and washed twice in phosphate buffered saline (PBS). The cell surface antigens were determined on fresh cells at the time of sample submission. Isolated cells (1×10^6) were incubated for 20 minutes at 4^oC with monoclonal antibodies (mAbs) specified against DC surface antigens and washed twice in PBS containing 0.2 mM ethylene-diaminetetraacetic acid (EDTA) and 0.5% bovine serum albumin (BSA). The following directly conjugated mAbs were used: anti-BDCA-1 (CD1c) FITC, anti-BDCA-2 (CD303) FITC (Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-CD19 CyChrome, anti-CD123 PE (Pharmingen, San Diego, California, USA). Mouse anti-human IgG_{2a} isotype control was used for anti-CD1c staining. Mouse anti-human IgG_1 isotype control was used for anti-BDCA-2 staining.

3.4. Flow cytometric analysis

Flow cytometric analysis of stained samples was performed with a FacsCanto flow cytometer (Becton Dickinson, San Jose, California, USA). A total of 300.000 events were acquired and analysed using FacsDiva software. Cell debris and dead cells were excluded from the analysis based on scatter signals. We identified immature myeloid DCs as BDCA-1 $(CD1c)^+$ $CD19^-$ cells, as previously described (21). Next, the mononuclear cell analysis region was analysed for BDCA-2 and CD123 antigens. BDCA-2^+CD123^+ cells were counted as immature lymphoid DCs. Results are expressed as a percentage of myeloid and lymphoid DC in mononuclear cells.

3.4. Statistical analysis

Data were presented as medians with the interquartile ranges. The Wilcoxon paired test was used to compare results in peritoneal fluid and plasma. The Mann-Whitney U test was applied to the results of statistical comparison between the studied groups. Spearman's rank test was used to assess the relationship between concentrations of RANTES and dendritic cells numbers. A p value of less than 0.05 was considered statistically significant.

Table 2. Levels of RANTES (pg/ml) in the peritoneal fluid (PF) and plasma of patients with ovarian tumors and normal donors

Group of patients	PLASMA		PF	
	Median	Interquartile range	Median	Interquartile range
Ovarian cancer (n=73)	12087.35*	8418.51-17407.75	148.87**	63.88-722.65
Serous cystadenoma (n=32)	8957.28	6588.61-9916.14	10.40	5.943-32.686
Normal donors (n=9)	7891.26	5926.21-10299.00		

* $p<0.001$ in relation to serous cystadenoma and control group, ** $p<0.001$ in relation to serous cystadenoma

Table 3. Characteristics of cancer patients and associated plasma and peritoneal fluid (PF) RANTES levels

Ovarian cancer (n=73)		PLASMA		PF	
		Median	Interquartile range	Median	Interquartile range
FIGO stage	I, II	9617.09	7699.0-15932.0	151.0	78.743-660.16
	III, IV	13281.60	9018.99-18048.5	144.63	57.2-784.17
Grading	G1,2	10320.4	7727.85-18194.20	148.88	65.371-697.27
	G3	13165.0	9018.99-16262.10	148.91	58.051-784.18
Histology	Serous cystadenocarcinoma	13106.80	8686.71-17883.5	203.45*	89.14-845.70
	Undifferentiated carcinoma	12038.80	8302.22-17893.20	74.28	32.68-278.77
	Mucinous cystadenocarcinoma	10837.94	7699.03-15213.60	115.88	37.14-434.21
	Endometrioid cystadenocarcinoma	16262.10	9768.99-18194.20	1734.96**	742.68-3672.92
	Clear cell carcinoma	9925.63	6370.25-12135.90	3021.91**	677.73-5366.09

* $p<0.05$ in relation to PF in patients with undifferentiated carcinoma, ** $p<0.05$ in relation to PF in patients with mucinous cystadenocarcinoma and undifferentiated carcinoma

4. RESULTS

4.1. Concentration of RANTES in the peritoneal fluid and plasma of women with ovarian cancer and serous cystadenoma

The concentrations of RANTES in the peritoneal fluid and plasma of patients with ovarian tumors and normal control group are presented in Table 2.

RANTES levels detected in the peritoneal fluid of women with OVC were found to be significantly higher ($p<0.001$) than those with benign ovarian disease. Additionally, plasma samples from women with ovarian cancer contained significantly higher ($p<0.001$) concentrations of RANTES compared with samples from women with serous cystadenoma and the control group. In the OVC group such as in the benign tumor group RANTES levels were significantly higher in the plasma ($p<0.001$) than in the peritoneal fluid (Table 2).

4.2. Concentration of RANTES in patients with different stage, grade and histologic type of ovarian cancer

There was a significant difference ($p<0.001$) between the plasma and peritoneal fluid RANTES levels between the benign ovarian tumor group and the ovarian cancer stage I FIGO (International Federation of Gynecologists and Obstetricians), stage II, stage III or stage IV groups. The plasma and the PF RANTES levels did not differ significantly between different FIGO stages of ovarian cancer (Table 3). There were no significant differences in the plasma RANTES levels based on tumor grade or histology. However, women with serous cystadenocarcinoma, clear cell carcinoma or endometrioid cystadenocarcinoma had significantly higher ($p<0.05$) PF RANTES levels than patients with undifferentiated carcinoma. Women with clear cell carcinoma and patients with endometrioid cystadenocarcinoma had significantly higher ($p<0.05$) PF RANTES levels than women with mucinous cystadenocarcinoma. There were no significant differences in the PF RANTES levels based on tumor grade. Table 3 shows descriptive statistics for RANTES levels classified by patient characteristics among those with invasive ovarian cancer.

4.3. Concentration of RANTES in the plasma of women with ovarian cancer, serous cystadenoma and in the control group

Plasma levels of RANTES in ovarian cancer patients were significantly higher ($p<0.001$) compared with the reference and with the control group. There were no significant differences between plasma RANTES levels in women with serous cystadenoma and the controls (Table 2).

4.4. The percentage of DCs in PF and PB of women with ovarian cancer

The percentage of myeloid DCs in the PF was 0.64% of mononuclear cells (interquartile ranges 0.24% to 1.24%) and was significantly higher than in the PB (0.12%; interquartile ranges 0.08% to 0.26%). Also the percentage of lymphoid DCs was significantly higher in the PF (0.66%; interquartile ranges 0.37% to 1.72%) compared with PB (0.18%; interquartile ranges 0.08% to 0.27%).

4.5. The percentage of DCs in PF and PB of women with serous cystadenoma

The percentage of myeloid DCs in the PF was 7.76% (interquartile ranges 3.24% to 15.25%) and was significantly higher than in the PB (0.24%; interquartile ranges 0.16% to 0.40%). However, the percentage of lymphoid DCs was significantly higher in the PB (0.30%; interquartile ranges 0.17% to 0.45%) than in the PF (0.20%; interquartile ranges 0.11% to 0.25%).

4.6. The percentage of DCs in PF and PB of women with ovarian cancer and benign tumors

The percentage of myeloid DCs was significantly lower in the PF of patients with ovarian cancer (0.64%; interquartile ranges 0.24% to 1.24%) than in women with benign tumors (7.76%; interquartile ranges 3.24% to 15.25%). In contrary, the percentage of lymphoid DCs was higher in the PF of patients with malignant disease (0.66%;

Table 4. Correlations between RANTES and dendritic cell populations in patients with ovarian tumors

Patients	Correlation between	R Spearman rank correlation coefficient	t (N-2)	p
Ovarian cancer n=73)	PF RANTES and MDC	-0.343	-3.059	<0.05
	PF RANTES and LDC	0.597	4.656	NS
	Plasma RANTES and MDC	0.066	0.403	NS
	Plasma RANTES and LDC	-0.056	-0.345	NS
Serous cystadenoma (n=32)	PF RANTES and MDC	-0.328	-2.026	<0.05
	PF RANTES and LDC	0.268	0.186	NS
	Plasma RANTES and MDC	0.066	0.403	NS
	Plasma RANTES and LDC	-0.056	-0.345	NS

NS-non significant

interquartile ranges 0.37% to 1.72%) than in the reference group (0.20%; interquartile ranges 0.11% to 0.25%).

4.7. Correlation between concentration of RANTES and dendritic cell subsets

Significant correlations were found between the PF and plasma RANTES levels and myeloid DCs. The statistical data are detailed in Table 4.

4.8. Correlation between serum Ca-125 and RANTES concentrations

The preoperative Ca-125 marker levels in the ovarian cancer patients ranged from 5.05 U/ml to 23935 U/ml with the median 570 U/ml. Ca-125 serum levels were significantly higher ($p<0.05$) in the group with grade III (1420 U/ml) ovarian cancer compared with the grades I-II (216 U/ml). Serum levels of Ca-125 were also significantly higher ($p<0.005$ and $p<0.05$) in the group with stage III (1425 U/ml) or IV (946 U/ml) ovarian cancer compared with the stage I-II group (79 U/ml). No significant correlation between serum Ca-125 and RANTES was observed in the ovarian cancer patients ($R=-0.0252$; $t(N-2)=-0.192$; $p=0.847$).

5. DISCUSSION

In this study, for the first time, we have carefully analysed the concentration of RANTES in the peritoneal fluid of women with different histological types of ovarian cancer. In view of currently insurmountable difficulties in obtaining normal peritoneal fluid, we acquired PF from women with serous cystadenoma as a reference fluid developing in the absence of malignant process.

We found concentrations of RANTES in the peritoneal fluid to be significantly elevated in women with ovarian cancer compared to benign ovarian cyst patients. Our results suggest that -a specific chemokine, RANTES, may be produced locally by ovarian tumor cells. This is in agreement with findings by Burke *et al.* (22) who demonstrated expression of RANTES in ovarian cancer cells. Furthermore, our data suggest that malignant and benign tumors induce significantly different levels of RANTES production.

Moreover, levels of RANTES in plasma samples were consistently higher in women suffering from OVC than in patients with serous cystadenoma or normal donor controls. Additionally, levels of RANTES in the plasma samples were nearly ten-fold higher than in peritoneal fluid samples. Although only limited data are available on the circulating levels of RANTES in patients with advanced-stage tumors, our results are consistent with a previous report showing that a large percentage of such patients have abnormal plasma levels of RANTES (16,3). The reason for this finding is not well-understood. It is worth noting that not only tumor cells, but also other cells including activated T lymphocytes (4), platelets (5) and monocytes/macrophages (7,6) secrete RANTES *in vivo*. This raises the possibility that elevation of RANTES in plasma from ovarian cancer patients could be attributed to RANTES released from activated mononuclear cells and/or platelets in circulation. Because no methods are available to determine the origin of RANTES circulating in the plasma, future studies will be necessary to examine this hypothesis.

The chemokine RANTES is known to be a chemoattractant for activated T cells (4), monocytes, and eosinophils, and to play a role in allergic inflammatory processes (14). Studies by Tsukishiro *et al.* (3) suggest that high serum RANTES levels could chemoattract monocytes into ovarian cancer and in this manner could contribute to the disease. They found that the preoperative serum RANTES concentration was significantly elevated in the ovarian cancer patients compared to the benign group values, and that the RANTES levels correlated with the stage of disease and the extent of residual tumor mass. The authors suggested that RANTES plays a role in the process of cancer progression. Previous studies have demonstrated that RANTES is also a chemoattractant for myeloid (19) and lymphoid (20) dendritic cells.

The results from our group (21) showed that the PF environment in women with malignant ovarian tumors contains considerably more dendritic cells than the peripheral blood does. This observation appears to support the hypothesis that peripheral blood DCs are specifically recruited into the peritoneal cavity. The reason for the accumulation of DCs in the PF has not been clearly explained yet. Our previous studies, which identified lymphoid DCs as the most abundant pelvic mononuclear cells in women with OVC, led us to examine the relationship between RANTES and the presence of lymphoid and myeloid DCs. However, we did not find a correlation between PF and plasma RANTES levels and the percentage of PF and PB lymphoid DC. It is possible that significantly lower RANTES levels in PF than in plasma, could affect formation of a gradient for lymphoid DC migration. Multiple chemokines likely cooperate to attract this particular DC population into the peritoneal cavity. However, we detected a negative correlation between PF RANTES levels and PF myeloid DC in patients with ovarian tumors. This is in agreement with findings by

Milliken and co-workers (16) who detected the negative correlation between RANTES and monocytes in ovarian cancer ascites. This observation may suggest that the chemokine RANTES may have an antagonistic effect under certain circumstances.

The studies by Su *et al.*(18) have demonstrated that RANTES chemoattracts Th17 cells into tumor sites and might be involved in the immune response to cancer. Recently Kryczek *et al.* (23) showed that Th17 infiltrates correlated with improved overall survival in ovarian cancer patients. Consequently, it is possible that elevated RANTES levels correlate with improved patient survival. However, it is also possible that RANTES attracts other T cell populations, including regulatory T cells (Treg), which predict reduced patient survival (24). Future studies will be necessary to examine this interesting hypothesis.

In our study we show that the plasma and the PF RANTES levels did not differ significantly among patients with FIGO stage I, II, III, and stage IV of ovarian cancer. However, there were significant differences in the peritoneal fluid RANTES levels based on tumor histology. These data led us to surmise that RANTES production in the peritoneal cavities of ovarian cancer patients depends on the histological type of the tumor cells. We conclude that the increased level of chemokine RANTES in the PF and plasma of women suffering from OVC may have an important role in the pathogenesis of this disease.

6. ACKNOWLEDGMENTS

All authors listed on the Title page have made substantive intellectual contributions to the content of this paper: Iwona Wertel designed and performed research, analyzed data, and wrote the paper, Rafal Tarkowski contributed to statistical analysis and analyzed data, Wieslawa Bednarek provided specimen and clinical information, and Jan Kotarski reviewed the paper and interpreted data. The study was partially supported by the State Committee for Scientific Research, Warsaw, Poland, Grant #N N407 114036.

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Abbreviations: RANTES: Regulated on Activation, Normal T-cell Expressed and Secreted, PF: peritoneal fluid,PB: peripheral blood, DCs: dendritic cells, OVC: ovarian carcinoma, MCP-1/CCL2: monocyte chemo-attractant protein-1, ELISA: enzyme-linked immunosorbent assay, PBS: phosphate buffered saline, mAbs: monoclonal antibodies, EDTA: ethylene-diaminetetraacetic acid, FIGO:International Federation of Gynecologists and Obstetricians, Ca-125: antigen 125, Tregs: regulatory T cells

Key Words: RANTES, Peritoneal Fluid, Dendritic Cells, Ovarian Cancer

Send correspondence to: Iwona Wertel, 1st Department of Gynecology, University School of Medicine, Staszica 16, 20-081 Lublin, Poland. Tel: 4881-5327847, Fax: 4881-5320608; E-mail: iwonawertel@wp.pl

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