Correlation of Tissue and Plasma RANTES Levels with Disease Course in Patients with Breast or Cervical Cancer

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ABSTRACT

The β-chemokine RANTES was measured in plasma in 43 patients with breast cancer and in 23 patients with cervical cancer, and the RANTES content in primary tumors, tumor metastatic to lymph nodes, and clinically normal skin or pelvic mucosa was measured. In addition, plasma levels were determined in all of the patients for the platelet-derived chemokine β-thromboglobulin (β-TG) and for IFN-γ, interleukin (IL)-2, IL-4, IL-5, and IL-10, along with serum IgE levels and blood eosinophils. Plasma RANTES levels were found to be higher in order of stages IV, III, II, and I of each cancer except for stage I. A marked increase in plasma RANTES level (>10,000 pg/ml) was found in 27% of patients with progressive malignancy but in none of those in clinical remission. The platelet RANTES content was correspondingly decreased in those patients with increased plasma RANTES levels. β-TG showed a pattern similar to RANTES both in plasma and platelets, but with much less dramatic differences between patients with different stages of disease. Other allergic parameters, IgE, eosinophils and plasma IFN-γ, IL-2, -5, and -10, were not elevated in the cancer patients. The RANTES content was markedly elevated in the primary tumor and metastatic lesions (lymph node or skin) from all of the patients with breast or cervical cancer, irrespective of the plasma RANTES level. In addition, in patients with progressive breast or cervical cancer, but not in patients thought to be cured of these tumors, the RANTES content was markedly increased in clinically normal tissue taken from near the operative site several months postoperatively, as well as in intact skin or mucosa taken perioperatively near the excised tumor. This study suggests an as-yet-undefined but important role played by RANTES in carcinogenesis, as well as the possibility that a RANTES assay in tissue surrounding a tumor or postoperative tumor site may help predict prognosis in these patients.

INTRODUCTION

RANTES (regulated upon activation, normal T-cell-expressed and secreted), which is one of the β (C-C) chemokines (1–12) that are chemoattractant for a variety of cells, particularly mononuclear cells, basophils, and eosinophils, is thought to be released by activated T lymphocytes and monocytes/macrophages (12), epithelial cells (7), bronchial epithelium (8, 9), and dermal fibroblast (10) and renal tubular epithelium (11). RANTES is thought to play an important role in a variety of disease states, including allergic inflammatory processes such as asthma, allergic rhinitis, and atopic dermatitis (13–17). We have found that plasma RANTES levels are significantly increased in the patients with severe, treatment-resistant atopic dermatitis (18).

Recently, it has been reported that platelets are also an important source of RANTES, and that platelet-released RANTES may play a role in allergic reaction (1–6). In this study, the levels of RANTES and another platelet-produced chemokine, β-TG,[2] and the levels of IFN-γ, IL-2, -4, -5, and -10 which are known to be produced by Th1 and Th2 T lymphocytes and increased in RANTES-relating allergic inflammatory process (5, 6, 19–27) were assessed in the patients with various malignancies. The levels of (IL-4-stimulated) IgE and blood (IL-5-stimulated) eosinophils, both of which increase with the elevation in RANTES in allergic reactions, were also assessed. RANTES and β-TG content of platelets was also assayed, and RANTES was also measured in primary and metastatic tumors, and in clinically uninvolved tissue taken peri- or postoperatively. The results indicate a strong correlation between elevated tissue RANTES levels and progressive malignancy.

PATIENTS AND METHODS

Forty-three patients with breast cancer and 23 cases of cervical cancer (all females, ages 23–56 years) were studied. The patients were classified into four stages according to 1997 TNM classification. The diagnosis of each cancer was made by tumor biopsy, imaging studies (computed tomography scan, magnetic resonance imaging) and corresponding tumor markers. "Uncontrolled or progressive stage" was diagnosed by the appearance of relapse, metastasis, or other worsening clinical findings, including an increase in tumor size on imaging studies and rise in plasma and tumor markers. Patients with inflamma-

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[2] The abbreviations used are: β-TG, β-thromboglobulin; IL, interleukin; CIS, carcinoma *in situ*.

Table 1 The levels of RANTES and β-TG in plasma and platelets in patients with breast and cervical cancers according to clinical stage and in progression or in remission[a]

Subjects	Stage	Plasma RANTES pg/ml mean ± SD	No. of cases with RANTES levels >10,000	No. of cases with RANTES levels <4000	Platelet RANTES pg/10^6 platelets mean ± SD	Plasma β-TG IU/ml mean ± SD	Platelet β-TG IU/10^6 platelets mean ± SD
Breast cancer patients (43)	I (5)	2,781 ± 315	0	4	1,706 ± 183	1,196 ± 104	136 ± 12
	II (11)	4,263 ± 604[b]	1	5	1,498 ± 124[b]	1,251 ± 106	124 ± 11
	III (12)	7,489 ± 1,234[c]	2	5	1,409 ± 128[b]	1,395 ± 120[b]	108 ± 12[b]
	IV (15)	8,769 ± 1,527[c]	4	8	1,112 ± 126[b]	1,496 ± 138[c]	98 ± 11[b]
Cervical cancer patients (23)	I (3)	2,743 ± 306	0	2	1,698 ± 176	1,203 ± 116	137 ± 10
	II (4)	3,929 ± 346	2	2	1,550 ± 162	1,229 ± 121	126 ± 13
	III (6)	7,046 ± 1,149[c]	2	3	1,369 ± 115[b]	1,346 ± 113[b]	114 ± 13[b]
	IV (10)	8,272 ± 1,564[c]	3	5	1,204 ± 141[b]	1,428 ± 145[c]	108 ± 11[b]
Patients with breast and cervical cancers (66)	Progressive (45)	8,472 ± 1,432[c]	12	22	1,125 ± 138[b]	1,462 ± 130[c]	103 ± 12[b]
	In remission (21)	4,123 ± 623[b]	2	12	1,684 ± 181[b]	1,114 ± 112	128 ± 12
Healthy volunteers (12)		2,916 ± 348	2	8	1,866 ± 175	909 ± 82	140 ± 12

[a] Parentheses denotes the number of the cases tested.
[b] $0.01 < P < 0.05$ *versus* healthy volunteers.
[c] $P < 0.01$.

tory states, *e.g.*, allergy and infections, were excluded from this study; those who had bronchial asthma, rhinitis, and atopic dermatitis (13–18) and those with serum IgE levels above 1000 IU/l and/or a percentage of eosinophils above 10% in peripheral bloods leukocytes were also excluded. Also excluded were patients with positive blood cultures, and those who showed both CRP above 3 mg/dl and peripheral leukocyte counts above $10^4/mm^3$ were also excluded because of the possibility of bacterial infection.

Because of the potential effect of chemotherapy or radiation on these parameters, only patients who were treated with natural products were studied, and patients with thrombocytopenia were excluded. Patients were tested for plasma levels of RANTES, β-TG (another chemokine produced by platelets), IFN-γ, IL-2, -4, -5, -8, and -10, serum IgE, and blood eosinophils. Platelet RANTES and β-TG content was also measured.

RANTES levels were also assayed in tumor tissue. This included primary tumors and/or tumors metastatic to lymph nodes or skin from 22 breast cancer patients. In an additional 18 breast cancer patients, 8 with progressive disease and 10 in remission, RANTES content was measured in skin biopsies taken near the operative site from 1–3 months after tumor excision (postoperative skin). RANTES content was examined in resected tumor from 17 cervical cancer patients, including 10 with invasive disease and 7 with CIS. In addition, RANTES content was determined in the cervical mucosa near the operative site from six patients in remission and six patients with progressive malignancy, as assessed by rectal and vaginal examination and/or biopsy. For control values, plasma RANTES and skin were measured in 12 healthy controls and in biopsies of normal cervical mucosa from 11 women attending a gynecological clinic for routine pap test. Informed consent was obtained from each subject.

In the plasma RANTES assay, citrated blood was drawn from each subject. RANTES levels were assessed by ELISA (28), as follows. One hundred μl of a 1:10 dilution of plasma were added to microtiter wells coated with a mouse monoclonal antibody to human RANTES. After mixing, the plate was incubated for 2 h at 25°C; the wells were then washed six times with 300 μl of 0.9% NaCl and 0.05% Tween 20, and then 100 μl alkaline phosphatase-labeled RANTES monoclonal antibody were added to each well, and plate was incubated for 1 h at 25°C. After another six washings, 100 μl of paranitrophenylphosphate (final concentration, 5.4 mM) were added, and the reaction was terminated after 1 h at 25°C by the addition of 50 μl of EDTA. Absorbance was measured at 450 and 595 nm by spectrophotometer (Hitachi, U-3200, Tokyo, Japan), and concentration was determined from a standard calibration curve.

For tissue RANTES assay, biopsy specimens, lymph node, skin, or mucosa, from the tumor were homogenized in a 10-fold (wt/vol) volume of PBS with 0.1% Triton X-100 in a homogenizer (KONTES; Scientific Glassware Instruments, Vineland, New Jersey). After centrifugation of the homogenates at 5500 × *g* for 10 min, supernatants were assayed for RANTES as described above for plasma.

Plasma IFN-γ, IL-2, 4, 5, 8, and 10, and IgE were assessed by ELISA (29–31) as described elsewhere (32), and eosinophils were assessed by routine clinical laboratory methods.

The results were expressed as the mean ± SD of replicate assays. For statistical analysis, the Student *t* test was applied for each plasma or blood assay level for comparisons between patients with progressive disease, patients in remission, and healthy volunteers, and for RANTES content between tumor or metastatic lesion and improved or normal skin or mucosa.

RESULTS

RANTES Levels in Plasma. Plasma RANTES levels were found to be statistically significantly elevated in patients with stage II, III or IV breast cancer or stage III or IV cervical cancer (stage IV, $P < 0.001$; stage III, $P < 0.01$; stage II, $0.01 < P < 0.05$; stage I, $P > 0.05$; Table 1). Moreover, as

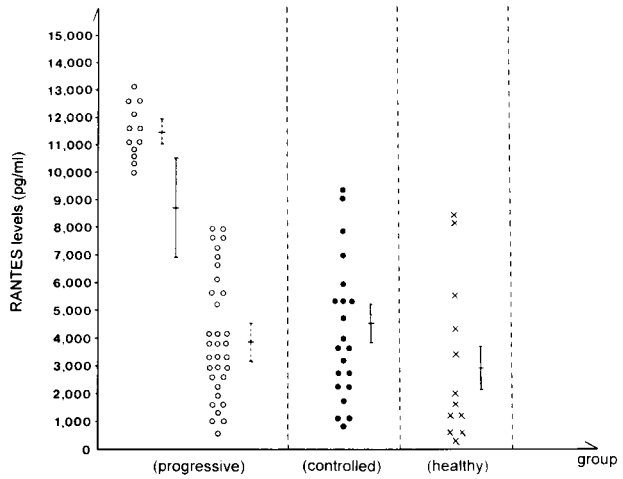

Fig. 1 Distribution of plasma RANTES levels of patients with breast or cervical cancer, classified according to those with controlled disease (cured or in remission) and those with progressive disease. ○, patients with progressive disease; ●, patients with controlled disease; ×, healthy controls. —, mean ± SD of each group (progressive, controlled, and healthy groups); - - - - , mean ± SD of each bimodal phase of the patients with progressive disease.

shown in Table 1, plasma RANTES levels correlated directly with clinical stage for both breast and cervical cancer.

This correlation is also seen when patients were classified either as having progressive disease or as being in remission. As indicated in Table 1, mean plasma RANTES levels were significantly elevated in the patients with progressive and uncontrolled malignancy, compared with those in remission, and both groups showed elevated levels compared with healthy individuals. Moreover, in contrast to patients in remission and healthy volunteers, the distribution of RANTES levels in cancer patients with progressive disease showed a bimodal distribution. As shown in Fig. 1, the mean RANTES level in the first group was 11,435 pg/ml and that of the second group was 3,864 pg/ml, which is similar to that of the patients in remission. Of the patients with progress disease, 27% of the patients in progression had a plasma RANTES level of >10,000 pg/ml, compared with none in either the group of cancer patients (the second group of progressive patients and the controlled group) or the healthy control group.

Because RANTES is produced from platelets, we measured levels of another platelet-secreted cytokine, β-TG, as a specificity control. The plasma levels of β-TG showed a modest correlation with cancer stage, but were much less dramatically elevated than those of RANTES (Table 1). The platelet content of both RANTES and β-TG were decreased in the patients with stage III or IV cancer, compared with healthy volunteers ($0.01 < P < 0.05$; Table 1), but, again, the decrease in RANTES content was more pronounced.

Interestingly, the other parameters that were measured, including IgE, eosinophils, IFN-γ, and IL-2, -4, -5, -8, and -10, were not significantly elevated in the cancer patients in whom plasma RANTES levels were found to be increased, nor in the cancer patients as a whole, compared with the healthy controls (data not shown).

RANTES Content in Tissues. The mean RANTES content of primary or metastatic tumor from patients with breast or cervical cancer was 50-fold greater than that of normal skin of healthy volunteers (breast cancer, $P < 0.0001$; invasive cervical cancer, $P < 0.00001$) and at least 5-fold greater than that of postoperative skin (as defined in "Patients and Methods") or normal skin or mucosa taken at surgery near the site of the original tumor (breast cancer, $P < 0.001$; invasive cervical cancer, $P < 0.01$; Table 2). Both invasive cervical cancer and CIS of the cervix showed markedly elevated RANTES levels, although somewhat lower than in primary breast tumors. An increased RANTES content was found in all of the breast and cervical tumors examined, although only one-fourth of these patients showed elevated plasma RANTES levels. The RANTES content in the clinically normal skin or mucosa, taken perioperatively near original tumor or metastatic lesions from the patients with progression, relapse, and/or metastasis, was also markedly (7–10 times) increased compared with those from the biopsy specimens from patients in remission and from healthy controls (breast cancer, $P < 0.001$; invasive cervical cancer, $P < 0.0001$; Table 2), whereas there was no significant difference in the RANTES content of specimens from the latter two groups.

DISCUSSION

The β-chemokine RANTES is now known to be chemoattractant for monocytes and eosinophils and to play a major role in allergic inflammatory processes (13–17, 19), and investigation on a receptor for IgE on platelet and the correlation of allergic reaction-inducing IgE (5, 6, 13, 14, 32) and eosinophils (2, 3, 33) or basophils (34) to RANTES has been reported. RANTES expression in atopic dermatitis skin, and allergen-induced transcription of mRNA for RANTES and subfamily of RANTES, MCP3, and an increase in plasma RANTES levels have been reported. More recently, we have again observed a marked increase in plasma RANTES levels accompanied by a marked decrease in IL-10 levels in one-third of patients with severe, treatment-resistant atopic dermatitis, as well as a marked increase in the RANTES content of skin lesions and in improved or normal skin from these patients (18). In this study, we have observed that RANTES levels were markedly increased in the plasma of one-fourth of patients with advanced breast or cervical cancer. Moreover, the RANTES content in all of the specimens of tumor, metastases-containing lymph nodes, or tumor-involved skin or mucosa was markedly increased. We also have observed similar patterns of plasma RANTES levels in patients with many other malignancies.[3] Markedly elevated levels of plasma RANTES (>10,000 pg/ml) were found only in 27% of patients with progressive cancer, whereas those in remission had normal levels. However, because only 27% of the patients with progressive cancer had elevated levels, the role of RANTES in cancer progression, if any, remains to be determined. It is, of course, possible that RANTES plays a key role in this process in some patients but not in others.

[3] Unpublished data.

Table 2 RANTES content[a] in biopsy specimens from primary tumor or metastatic lesions of the lymph node or skin and from postoperative or perioperative skin or mucosa from patients with breast cancer or cervical cancer, either progressive or in remission[b]

Tumor lesions (29)			Metastatic lymph node or skin from breast cancer (11)	Postoperative skin or mucosa and normal perioperative skin or mucosa (30)				Normal level of healthy controls[e] (15)	
	Cervical cancer (17)			Breast cancer		Cervical cancer			
Breast cancer (12)	Invasive (10)	CIS (7)		Progressive[c] (8)	In remission[c] (10)	Progressive[d] (6)	In remission[d] (6)	Skin (7)	Cervical mucosa (11)[e]
1,032 ± 120[f]	450 ± 61[g]	300 ± 42[h]	984 ± 115[f]	201 ± 24[i]	27.8 ± 3.5	152 ± 22[f]	3.7 ± 0.5	22.6 ± 3.1	3.4 ± 0.4

[a] RANTES levels in the skin, mucosa, lymph node, and tumor are expressed as pg/mg protein.
[b] Parentheses denotes the number of the cases tested.
[c] Disease activity of breast cancer was diagnosed by tumor markers, progression of the tumor itself, or appearance of metastasis.
[d] Disease activity of cervical cancer was diagnosed by relapse of malignancy by rectal and vaginal examination, and/or biopsy.
[e] The women undergoing cervical biopsy that was diagnosed as normal.
[f] $P < 0.0001$ *versus* normal level of healthy controls.
[g] $P < 0.000001$ *versus* normal level of healthy controls.
[h] $P < 0.00001$ *versus* normal level of healthy controls.
[i] $P < 0.001$ *versus* normal level of healthy controls.

Although RANTES is known to be released by a variety of cells, including activated T lymphocytes, monocytes, and fibroblasts, there is a suggestion that the elevated plasma RANTES levels seen in some of the patients in this study were derived from platelets. RANTES is known to be produced by platelets, with strong effects on allergic processes (1–6). Moreover, the platelet-secreted chemokine β-TG was also increased in the plasma of the patients with elevated plasma RANTES levels, and these same patients had decreased RANTES and β-TG content in platelets. We have previously observed a similar pattern in patients with severe atopic dermatitis (18). From the above findings, it is not unlikely that elevated plasma RANTES in the patients with severe atopic dermatitis and malignancies derives from platelets.

The patients with elevated RANTES levels in plasma had normal blood levels of IgE, eosinophils, IFN-γ, and IL-2, -4, -5, and -10. The stimulus for RANTES secretion in association with malignancy is thus not clear.

Regarding the literature on the association of chemokines with malignancy, Iantorno *et al.* (35) reported that monocytes from the patients with bladder carcinoma undergoing BCG immunotherapy were stimulated in the presence of the β-chemokine MCP-1. Youngs *et al.* (36) reported that β-chemokines induced migrational responses in human breast carcinoma cells. Expression of RANTES and of MIP-1 α and β in ovarian cancer (37) and the loss of tumorigenicity *in vivo* by RANTES secretion by gene-modified tumor cells (38) have also been demonstrated. However, there are no previous reports of RANTES levels in plasma, tumor, or perioperative tissue in cancer patients, nor has the correlation between RANTES levels and cancer disease course been previously described.

It is noteworthy that RANTES content was markedly increased in both perioperative and postoperative skin from patients with progressive breast or cervical cancer, whereas this was not the case in any of the patients thought to be cured or in remission. The RANTES content of peri- or postoperative biopsy sites would thus appear to have a prognostic value with regard to subsequent tumor relapse. The correlation of elevated RANTES content in diseased tissue seems to be much stronger than the correlation of elevated plasma RANTES, which suggests some fundamental role for RANTES in the lesions.

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