Elevated Expression of the CC Chemokine Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) in Advanced Breast Carcinoma

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ABSTRACT

Breast carcinoma is the most common malignant disease among women and the second most lethal one. In search for a better understanding of the role of cellular mediators in the progression of this disease, we investigated the potential involvement of the CC chemokine Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) in breast carcinoma progression. To this end, RANTES expression was determined in breast tumor cell lines and in sections of breast carcinomas, followed by analysis of the incidence and intensity of its expression in different stages of the disease. Our study reveals that high and physiologically relevant levels of RANTES are constitutively produced by T47D and MCF-7 breast tumor cell lines. Analysis of RANTES expression in sections of breast carcinomas demonstrates a high incidence of RANTES expression in epithelial tumor cells; the chemokine was expressed in 74% of the sections. RANTES expression was rarely detected in normal duct epithelial cells or in epithelial cells that constitute benign breast lumps, which were located in proximity to tumor cells. High incidence and intensity of RANTES expression were detected in sections of most of the patients with stage II and stage III of the disease (expression was detected in 83 and 83.3%, respectively), whereas RANTES was expressed at a lower incidence and intensity in sections of patients with stage I of breast carcinoma (55% of the cases). Most importantly, the expression of RANTES was minimally detected in sections of patients diagnosed with benign breast disorders and of women that underwent reduction mammoplasty (15.4% of the cases). These results indicate that the expression of RANTES is directly correlated with a more advanced stage of disease, suggesting that RANTES may be involved in breast cancer progression. Moreover, it is possible that in patients diagnosed with benign breast disorders, RANTES expression may be indicative of an ongoing, but as yet undetectable, malignant process.

INTRODUCTION

Breast carcinoma is the second leading cause of cancer-related death among women in the Western Hemisphere (1). Despite advances in diagnosis and treatment, only modest improvements in survival rates have been achieved (1). This situation motivates investigators to search for new insights into the role of different cellular effectors in the progression of this disease.

Various cellular components of the mammary tissue may affect, in either paracrine and/or autocrine manners, breast cancer progression. Given their well-established roles in regulation of cellular differentiation and proliferation (2–4), cytokines may play a key role in the regulation of the malignant process. These cellular mediators may exert immunopotentiating activities that limit tumor growth but were also shown to serve as angiogenic or growth factors that support tumor development. Therefore, the characterization of cytokine expression and activities at tumor sites and their effects on the establishment of primary tumors and on metastasis formation have to be further elucidated.

Of the multiple cytokines that may affect tumor progression, the recently characterized superfamily of chemokines may be of major importance (5–8). Members of the chemokine superfamily mediate the infiltration of leukocytes to inflammatory sites. There are two major classes of chemokines, CXC and CC. The majority of CXC chemokines (such as interleukin 8) act mainly as potent chemoattractants of neutrophils. The CC chemokines (e.g., RANTES3 and monocyte chemoattractant protein-1 [MCP-1]) chemoattract primarily monocytes and T cells (9–11).

In view of their chemotactic properties, chemokines were suggested to mediate the recruitment of tumor-associated leukocytes to tumor sites (5, 6, 12), a process that was postulated to affect the progression of several malignant diseases (5, 6, 13–16). However, although primarily characterized by their chemotactic activities, chemokines have also been suggested to regulate other processes that may affect tumor establishment and metastasis formation. CXC chemokines were shown recently to have either angiogenic or angiostatic effects. It has been demonstrated that the proangiogenic activity observed during tumor progression is mediated by the biological imbalance that favors the expression of angiogenic CXC chemokines (ELR-expressing CXC chemokines) over that of the angiostatic CXC chemokines (ELR-nonexpressing CXC chemokines; Refs. 7 and 8). Moreover, in contrast to the majority of normal cells, several human and murine tumor cells were shown to constitutively produce chemokines, CXC as well as CC (17–21). In some of the cases, chemokines were demonstrated to be autocrine factors produced by tumor cells and to enhance tumor cell proliferation or survival (17, 22–25).

Chemokine production by tumor cells may be induced by microenvironmental stimuli or may represent an intrinsic property of the tumor cells, resulting in tumor cell-variant generation. Indeed, in our previous studies, we generated murine breast tumor cell variants and analyzed the possible contribution of CC chemokine production to the malignancy phenotype expressed by these cells. Using a unique murine model system consisting of two lines of DA3 mammary adenocarcinoma cells that were derived originally from a common ancestor but differed in their malignant potential, our results indicated that the more malignant cells expressed higher levels of CC chemokines than the less malignant cells, proposing that these mediators have promalignancy effects (26).

In view of these results, it is possible that similarly to the processes regulating murine mammary tumor progression, CC chemokines also affect the progression of human breast carcinoma. This assumption motivated us to evaluate the role of CC chemokines in breast cancer progression. Our study focused on RANTES, a CC chemokine that is

3The abbreviations used are: RANTES, Regulated on Activation, Normal T Cell Expressed and Secreted; TAM, tumor-associated macrophage.
a major chemotacticant of both monocytes and T cells for two major reasons: (a) whereas most normal adult nonhematopoietic tissues rarely constitutively express RANTES, human breast milk contains high concentrations of RANTES (27–30). These findings suggest that breast cells have the potential to produce this chemokine and that specific physiological stimuli, such as hormones, may provide the conditions for its constitutive expression; and (b) it was demonstrated that RANTES is produced by several tumor cells, including tumors that are under hormonal regulation, such as ovarian tumors (20, 21, 31).

To elucidate the role of RANTES in breast cancer progression, we first analyzed RANTES production by human breast tumor cell lines and have shown that both T47D and MCF-7 cells constitutively expressed RANTES at high and physiologically relevant concentrations. Then, experiments were designed to test whether production of RANTES by human breast tumor cell lines had in vivo relevance, and therefore, to evaluate the expression of RANTES in biopsy sections of breast carcinoma patients. This was followed by determination of the incidence and intensity of RANTES expression at different stages of the disease. The results indicate that RANTES expression was rarely observed in sections of patients with benign breast disorders and of women that underwent reduction mammoplasty, whereas high levels of RANTES expression were detected in advanced breast carcinoma. In addition, our results suggest that RANTES regulates breast carcinoma progression and may be of prognostic value for the early diagnosis of malignant processes in breast carcinoma.

**MATERIALS AND METHODS**

**Cell Cultures.** Cultured human adenocarcinoma T47D cells (clone 11 and clone 8) were established by Professor Keydar from a pleural effusion of a patient with an infiltrating ductal carcinoma of the breast (32, 33). The two clones of T47D cells differ in several characteristics, as described previously (33). MCF-7 breast adenocarcinoma cells were established from human pleural effusion and were kindly provided by Professor Kaye (Weizmann Institute of Science, Rehovot, Israel). All cell lines were grown in DMEM medium, supplemented with 10% FCS, 100 units/ml streptomycin, 12.5 units/ml nystatin, 100 units/ml penicillin, and 2 mM l-glutamine (all purchased from Biological Industries, Beit Haemek, Israel).

**Immunodot Analysis of RANTES Expression.** T47D and MCF-7 cells (1.2–1.3 × 10^6) were plated in 9-cm tissue culture plates for 24 h in DMEM medium supplemented with 10% FCS, streptomycin, nystatin, penicillin, and glutamine. The cells were washed three times with serum-free DMEM and incubated for an additional 24 h with serum-free DMEM. Then, supernatants were removed and centrifuged for 20 min at 1400 × g. Different volumes of supernatants (supplemented by serum-free DMEM to constitute an equal volume) and a standard curve of recombinant human RANTES (PeproTech/Cytolab, Rehovot, Israel) were dot blotted on nitrocellulose filter (using Manifold, Bethesda Research Laboratories, Bethesda, MD). The filter was blocked by Tris-buffered saline (TBS) supplemented by 5% nonfat milk, followed by 2 h incubation with mouse monoclonal antibodies to human RANTES (PeproTech/Cytolab; 5 μg/ml in TBS + 5% milk). After several washings in TBS + 0.5% milk, the filter was incubated for 1 h with horse- radish peroxidase-conjugated goat antibodies to mouse IgG (Sigma, Israel and Jackson ImmunoResearch Laboratories; diluted in TBS + 0.5% milk). After additional washings in TBS, chemiluminescence signal was detected using an ECL detection system (Amersham, Buckinghamshire, United Kingdom). A negative control in which the supernatants were replaced by serum-free DMEM did not show any reactivity with antibodies to RANTES. Another negative control in which no reactivity was observed included supernatants of T47D and MCF-7 cells that were exposed to the secondary antibody only.

**Tissues.** Formalin-fixed, paraffin-embedded tissues from 87 patients were obtained from the pathology departments of hospitals in the Tel-Aviv area, Israel. The tumors were staged according to Union International Contre Cancer/Tumor-Node-Metastasis Classifications of Malignant Tumors (34). Included in the study was a group of 23 patients diagnosed with benign breast disorders and of 3 women that underwent reduction mammoplasty (the “Benign/Mammoplasty” group), as well as 61 patients with breast carcinoma, diagnosed at different stages of the disease (stages I, II, and III). Approximately 85% of the breast carcinoma patients were diagnosed with invasive ductal carcinoma. Most of the breast carcinoma patients were in the post-menopausal age group.

**Determination of RANTES Expression by Immunohistochemistry.** Serial sections (5 μm thick) were prepared from paraffin-embedded blocks and stored at room temperature. Sections were deparaffinized, dehydrated in xylene and graded alcohols, rinsed in PBS, and incubated with hyaluronidase at 37°C for 1 h. After rinsing in PBS, the sections were treated with 1% H2O2 for 20 min at room temperature. After additional rinsing in PBS, nonspecific binding was blocked by incubating the sections with normal goat serum at 37°C for 30 min. Then, the sections were incubated with mouse antibodies to human RANTES (60 μg/ml; PeproTech/Cytolab) at 4°C overnight. Sections were washed thoroughly in PBS, stained with biotinylated goat anti-mouse IgG (1:8) and ExtrAvidin Peroxidase (1:20; Mab ExtrAvidin Peroxidase staining kit; Sigma), washed in PBS; and incubated with 0.04% diaminobenzidine (Sigma). After rinsing in PBS, counterstaining was performed with 1% mylene blue, followed by dehydration and mounting with Merckoglas (Merck, Darmstadt, Germany). According to several standard procedures (35, 36), the methylene blue counterstaining was used to facilitate determination of diaminobenzidine brown reaction product, giving rise to the blue-color background that is observed in stainings of all sections.

All stained slides were submitted to light microscopy, and the staining pattern in tumor cells, in morphologically nontransformed epithelial cells and in leukocytes, was evaluated. Intracellular immunoreactivity was evaluated on a qualitative basis by evaluating each section in its entirety for brown reaction product and graded according to the following criteria: −, no staining; +, low staining intensity; ++, moderate staining intensity; and ++++, high staining intensity. For evaluation of the results, staining intensities of +, ++, and ++++ were all considered to be positive for RANTES expression. The evaluation of staining was confirmed by two independent pathologists that graded the sections in a double-blind manner. The consistency of the grading was assured by including in the study a number of breast carcinoma patients from which biopsy sections were prepared from different blocks that contained a considerable amount of tumor tissue. These sections were stained with antibodies directed against human RANTES, and in these cases, the different blocks that were derived from the same tumor gave a similar degree of RANTES expression.

Dot blot analysis indicated that the antibodies to human RANTES bind this chemokine in a dose-dependent manner. For all of the cases included in this study, a negative control for RANTES staining was included in which the primary antibody to RANTES was substituted by PBS. All these control slides were negative and showed no evidence of staining, indicating that the positive staining in either epithelial cells or leukocytes did not result from expression of endogenous peroxidase. Additional negative control included staining with irrelevant mouse monoclonal IgG antibodies. Controls for the specificity of immunostaining also included sections stained with antibodies to RANTES that were immunoabsorbed with recombinant human RANTES, resulting in elimination of the cytoplasmic RANTES staining in epithelial cells.

Pearson χ² and Fisher Exact tests were used to determine the statistical significance of the differences in percentages of patients expressing RANTES between the four different groups included in the study [the Benign/Mammo-plasty group and the three groups of patients in different stages of the disease (stage I, stage II and stage III)]. Mann-Whitney U test was used to determine the statistical significance of the differences in intensities of RANTES staining between these four different groups of patients.

**RESULTS**

**Detection of RANTES Expression by Human Breast Tumor Cell Lines and in Biopsy Sections of the Patients Included in the Study.** RANTES expression was determined at the protein level in supernatants of human breast tumor cell lines, T47D (clone 11) and MCF-7. Dot blot analysis demonstrated the constitutive expression of RANTES by both cell lines (Fig. 1). Although smaller quantities of the chemokine were produced by MCF-7 than by T47D cells, both
types of cells produced notable amounts of the chemokine, at estimated quantities of 5–35 ng/ml supernatant by MCF-7 and 1.5 times more by T47D cells (as determined by ImageMaster Densitometry of dot blots, in comparison with recombinant human RANTES). In addition, considerable levels of RANTES were detected also in supernatants of another clone of T47D cells (clone 8; data not shown). Because RANTES was shown to induce an optimal chemotactic activity at the concentration range of 1–100 ng/ml (37, 38), these results indicate that RANTES is expressed at physiologically relevant levels by human breast tumor cells lines.

Because tumor cell lines are kept in culture and are thus remote from the original in vivo situation, we analyzed the expression of RANTES in biopsy sections of breast carcinoma patients. The study included paraffin biopsy sections from 87 patients: 61 patients with breast carcinoma and 26 patients diagnosed with benign breast disorders or women that underwent reduction mammoplasty (the Benign/Mammoplasty group). Our study focused on RANTES expression in epithelial cells, constituting either tumors, normal ducts, or benign lumps.

A high incidence of RANTES expression (74%) was observed in breast cancer patients (Figs. 2D, 3, and 4). Whereas RANTES expression in breast cancer patients was detected in malignant epithelial cells (Fig. 2D), normal mammary ducts and benign lumps that were adjacent to the malignant ones, rarely expressed RANTES (Fig. 2C). On the other hand, positive staining of RANTES was observed in only 15.4% of the patients included in the Benign/Mammoplasty group (staining was observed in three cases of benign breast disorders and in one case of reduction mammoplasty; Fig. 4A). As shown in Fig. 2, normal mammary ducts (Fig. 2A) or benign lumps (Fig. 2B) of these patients only rarely stained for RANTES. The difference between the
incidence of RANTES expression in sections of breast carcinoma (stages I, II, and III combined) and the incidence of its expression in sections of the Benign/Mammoplasty group was highly significant ($P < 0.001$). Because high incidence of RANTES expression was observed in sections of breast carcinoma patients and because this chemokine was only rarely detected in morphologically nontransformed epithelial cells of the breast, detection of its expression may be indicative of transformation events occurring in the breast tissue.

When observed, RANTES expression was primarily intracellular (Fig. 2D and Fig. 3). In most cases, the staining was coarsely granular, but in some cases, a diffuse staining pattern was observed. Moreover, RANTES expression was observed not only in epithelial tumor cells but also in leukocyte infiltrates in close proximity to the tumor mass (leukocyte infiltrates were detected in 43 of the 61 breast carcinoma patients; 74.4% of these 43 cases were positive for RANTES expression in leukocytes).

**Evaluation of the Incidence of RANTES Expression in Different Pathological Stages of Breast Carcinoma.** Further analysis of RANTES expression in breast tumors, according to the stage of disease, indicated that the incidence of RANTES expression was higher in the advanced stages of the disease (stages II and III), suggesting a direct correlation between the severity of the disease and detectability of the protein in tumor cells. Positive staining was observed in 83 and 83.3% of the patients in stages II and III of the disease, respectively, whereas 55% of the patients with stage I of the disease were positive for RANTES expression (Fig. 4B). Statistical analysis indicated that a direct and a significant correlation exists between high incidence of RANTES expression and a more advanced stage of disease.

Regional lymph nodes into which tumor cells have infiltrated were obtained from four patients with stage II of the disease. The expression of the chemokine was observed in all these cases in tumor cells of the primary tumor, in metastatic tumor cells invading the lymph node, and in lymphocytes that constitute the lymph node (Fig. 3, B and C).

**Determination of the Intensity of RANTES Expression in Biopsy Sections of the Patients Included in the Study.** As mentioned above, RANTES expression was detected only in a small percentage of the patients that were included in the Benign/Mammoplasty group (Fig. 4A). If detected in these cases, RANTES was expressed at low levels (+) in normal mammary duct cells or benign lumps (Table 1). Similar analysis of RANTES expression levels in breast carcinoma patients revealed a shift from lack or low intensity of staining in tumor cells of the majority of patients with stage I of the disease to moderate or high staining intensities in 75% of the patients with stage III of the disease (Table 2). In most of the patients classified as stage I, the expression of RANTES was either not detected (−) or at low levels of expression (+) (45 and 20%, respectively). Moderate (++) or high levels (+++) of RANTES expression in tumor cells were observed in only 20 and 15%, respectively, of the patients with stage I of the disease. On the other hand, high intensity levels (+++) of RANTES expression were observed in 33.3% of the stage III tumors, and an
Our study of RANTES expression in breast tumors is the first to demonstrate RANTES production in physiologically relevant quantities by human breast tumor cell lines. Most importantly, both T47D and MCF-7 cells expressed high levels of the chemokine in a constitutive manner, suggesting that the expression of this chemokine may contribute to tumor growth. This possibility was supported by our observations on the expression of RANTES in biopsy sections of breast cancer patients, providing novel findings that may shed light on the mechanisms involved in the progression of this disease:

(a) A substantial expression of RANTES in epithelial cells that constitute the tumors in breast carcinoma patients was detected. In almost all of these patients, RANTES expression was not detected in normal duct epithelial cells or in epithelial cells that constitute benign breast lumps that were located in proximity to tumor cells. Moreover, based on morphological parameters, RANTES was detected also in leukocytes that reside in proximity to the tumor mass.

(b) Also observed in our studies was RANTES expression in local tumors, as well as in tumor metastases into regional lymph nodes. In lymph node metastases, RANTES expression was noted in epithelial tumor cells that infiltrated the lymph node and in lymphocytes that constitute the node.

(c) High incidence and elevated levels of RANTES expression were directly correlated with a more advanced stage of disease. High incidence and intensity of RANTES expression were detected in stage II and stage III tumors, in contrast to low incidence and intensity of its expression in sections of patients with stage II or stage III of disease.

Additional 41.7% of these tumors expressed moderate levels (+ +) of RANTES. Only a small percentage of the patients with stage III of the disease (16.7%) was negative for the expression of this chemokine. The difference between the staining intensities of sections derived from patients with carcinoma (stages I, II, and III combined) and RANTES staining intensity in sections of patients included in the Benign/Mammoplasty group was highly significant (P < 0.001). Moreover, statistical analysis indicated that the intensity of RANTES expression in sections of patients with stage II or stage III of disease was significantly different from that observed in sections of patients with stage I of disease (P < 0.05), and that a direct and a significant correlation exists between a high intensity of RANTES expression and a more advanced stage of disease. These findings indicate that RANTES expression levels are correlated with a more progressed stage of the disease and suggest that RANTES may contribute to the progression of the disease.
expression in patients devoid of diagnosed carcinoma. Interestingly, the incidence and intensity of RANTES expression in patients with stage I of breast carcinoma was higher than in patients of the Benign/Mammoplasty group but lower than in patients with stage II or stage III of the disease, suggesting that the incidence and intensity of expression of this chemokine may be indicative of processes involved in disease progression.

Our observations of the higher frequency of RANTES expression in patients that are in advanced stage of the disease (stages II and III) suggest that RANTES is a potential marker for disease severity. Whereas RANTES expression was detected in sections of >80% of the stage II or stage III carcinomas, positive detection of the chemokine was demonstrated in only 15.4% of the sections derived from patients diagnosed with benign breast disorders and women that underwent reduction mammoplasty. One should recall that biopsies of patients included in the latter group (Benign/Mammoplasty) were characterized as negative for a malignant disease based on histological characteristics. However, despite the normal histomorphological appearance of these tissues, the possibility exists that transformation events have already occurred in cells constituting the apparently “normal” tissue. In that respect, it is tempting to propose that RANTES expression in these patients may be indicative of an ongoing, but as yet undetectable, malignant process. A retrospective study of those benign breast disorders in which RANTES expression could be observed will allow us to determine whether the detectability of this chemokine has a prognostic value.

The direct correlation between the progressively increased expression of RANTES and the severity of the disease suggests that RANTES may have a key role in the regulation of breast carcinoma progression. Tumor-derived as well as stroma-derived RANTES may contribute under a specific set of conditions to the etiology of the disease and to its progression to a more advanced stage. In similarity to the growth-stimulatory activities of other chemokines (17, 22–25), it is possible that RANTES acts in an autocrine and/or paracrine manner to enhance and support the proliferation and/or survival of breast epithelial cells that have undergone the first stage of transformation, thus promoting tumor establishment. This possibility is supported by our observations on constitutive RANTES expression by T47D and MCF-7 breast tumor cell lines and by a study demonstrating the expression of high-affinity receptors for RANTES by these cells (39), suggesting that RANTES may act as an autocrine growth factor for breast tumor cells.

Moreover, RANTES may contribute to breast cancer progression due to its chemotactic properties, acting mainly as a migratory factor for monocytes and T cells. This possibility is supported by previous observations correlating high frequency of monocyte infiltration to breast tumors with poor prognosis (14–16, 40–44). Breast tumors, similar to many other solid tumors of epithelial origin, are infiltrated by host leukocytes, primarily TAMs and T cells (14–16, 40–42, 45–50). In breast carcinomas, TAMs were found within the stromal tumor areas, as well as in the epithelial areas that constitute the tumor mass (14, 41, 42). The density of stromal macrophage infiltrates was shown to be associated with clinical aggressiveness, and a positive relationship between macrophages and lymph node metastases was observed (14–16, 40–44). This may be the consequence of interactions occurring between TAMs and tumor cells. TAMs were suggested to promote tumor growth by virtue of their abilities to produce growth factors for breast epithelial cells, such as epidermal growth factor, and to highly express angiogenic cytokines, e.g., vascular endothelial growth factor and basic fibroblast growth factor (5, 6, 14, 16, 40, 42, 43). During tumor growth, the content of TAMs in each tumor is usually maintained as a constant property, requiring the recruitment of monocytes from the circulation (6). The possibility exists that TAMs are attracted to tumor sites by tumor-derived “professional” chemotactic molecules, such as RANTES. Studies are now in progress to determine the mechanisms by which RANTES may contribute to breast tumor progression, acting either directly as an autocrine growth factor or indirectly by mediating recruitment of tumor-associated leukocytes to tumor sites. To complement these studies and to determine the contribution of leukocyte infiltrates to RANTES expression, experiments are designed to determine the incidence of leukocyte infiltration in tumor tissues and the phenotype of the chemokine-expressing leukocytes.

To conclude, our findings identify RANTES as a potential regulatory and prognostic factor in breast carcinoma. The understanding of the contribution of RANTES to processes involved in the progression of breast cancer may lead the way for the identification of a powerful prognostic tool and to the application of novel tumor-limiting modalities (such as antagonists to RANTES) to be used in therapy of this neoplasm.

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