The CC Chemokine RANTES in Breast Carcinoma Progression: Regulation of Expression and Potential Mechanisms of Promalignant Activity

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ABSTRACT

Breast cancer progression may be affected by various cellular components expressed by the tumor cells and/or by microenvironmental factors. Many studies report the correlation between breast cancer progression and monocyte infiltration into the tumor site. We have identified recently the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES), a major monocyte chemoattractant expressed by breast tumor cells, as a potential contributor to breast cancer progression.

In the present study, analysis of the regulation of RANTES expression demonstrates that the expression of RANTES in breast tumor cells is elevated significantly and in a synergistic manner by IFN-γ and tumor necrosis factor-α. Identification of the mechanisms by which RANTES may contribute to breast cancer progression included the analysis of the potential ability of RANTES to act in paracrine and indirect mechanisms, as well as directly on the tumor cells, to promote disease progression. Our results suggest that breast tumor cell-derived RANTES may promote breast cancer progression by its partial contribution to monocyte migration into breast tumor sites. Moreover, RANTES promotes the expression of matrix metalloproteinase (MMP) 9 by THP-1 monocytes and elevates vascularity in chick chorioallantoic membrane assays. Tumor necrosis factor-α may be a key mediator of monocyte infiltration to the tumor site. We have identified recently the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES), a major monocyte chemoattractant expressed by breast tumor cells, as a potential contributor to breast cancer progression.

INTRODUCTION

In many of the Western hemisphere countries, breast carcinoma is a leading cause of death for women. Although the pathology of the breast has become better understood in recent years, the pathogenesis of breast cancer is not well defined. Outline by many studies, a comprehensive overview of tumor progression suggests that the process is influenced by intrinsic properties of the tumor cells, as well as by microenvironmental factors. Indeed, in breast carcinoma, high levels of infiltrating T cells and TAM are often observed. The

leukocyte infiltrates are found within the stromal tumor areas as well as in the epithelial areas that constitute the tumor mass (1–12).

Recent reports suggest that the inflammatory reaction at the breast tumor site affects tumor growth and progression. Whereas lymphocytes were shown to have divergent effects on the development of breast cancer (1, 9–12), it is widely accepted that high levels of TAM are correlated with poor prognosis (1–9). Studies on TAM in breast carcinoma demonstrated a positive relationship between macrophages and lymph node metastases, and suggested that the density of TAM is associated with clinical aggressiveness in most of the histological types of breast cancer (1–9). This may be the result of several tumor-promoting characteristics of TAM, such as the expression of growth factors for breast epithelial cells, the release of angiogenic factors, and the production of extracellular matrix-degrading enzymes (3–6, 9, 13–15).

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The potential contribution of TAM to breast cancer progression motivated researchers to identify factors that may induce the recruitment of monocytes from the circulation into breast tumor sites. Potential candidates that may support such an activity are the members of the CC subfamily of chemokines, characterized as major mediators of monocyte and T-cell migration into inflammatory loci (16, 17). Of the multiple members of the CC chemokine subfamily, several studies focused on MCP-1, a protein that potently induces monocyte migration. These studies provided evidence for the potential contribution of MCP-1 to TAM accumulation in breast tumors and to breast cancer progression (18–21).

Several studies have focused recently on another CC chemokine that attracts T cells and monocytes, namely RANTES. The potential contribution of RANTES to breast cancer progression is of major interest because: (a) human breast milk contains high concentrations of RANTES, suggesting that breast cells have the potential to produce this chemokine under specific physiological stimuli (such as hormones; Refs. 22, 23); and (b) it was demonstrated that RANTES is produced by several tumor cells including tumors that are under hormonal regulation, such as ovarian tumors (24–26).

A preliminary indication for the potential involvement of RANTES in breast cancer was provided by the constitutive expression of RANTES by the T47D and MCF-7 breast adenocarcinoma cells in vitro in physiological concentrations (27). An additional study demonstrated the inducible expression of RANTES by the human breast carcinoma MDA-MB-435S and BT-20 cells (28).

The above observations were followed in our laboratory by the analysis of the potential involvement of RANTES in breast cancer progression. RANTES expression was determined in sections of breast cancer patients diagnosed at different stages of disease (27). It was demonstrated by immunohistochemical analysis that RANTES is expressed in 74% of biopsy sections of breast carcinoma patients. On the other hand, RANTES expression was rarely detected in normal duct epithelial cells that were in proximity to the tumor cells (27). High incidence and intensity of RANTES expression were observed
in sections of most of the patients with stages II or III of disease (83%) in contrast to lower incidence and intensity of its expression in sections of patients with stage I of breast carcinoma (55%). Most importantly, the expression of RANTES was minimally detected in sections of patients diagnosed with benign breast lesions and of women that underwent reduction mammoplasty (15%; Ref. 27).

These results indicate that the high incidence and the elevated expression levels of RANTES are directly correlated with a more advanced disease, suggesting that the chemokine 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.

The potential protumorigenic contribution of RANTES to breast cancer progression is additionally supported by a recent publication demonstrating that high plasma RANTES levels are correlated with a more advanced breast carcinoma (29).

To better understand the role of RANTES in the progression of breast carcinoma, the regulation of RANTES expression and the mechanisms mediating its potential promalignant activity were investigated in the present study. On the whole, the results presented in our investigation propose that an intensive interplay between tumorderived RANTES and inflammatory cytokines may play a role in determining the metastatic spread of breast tumor cells and contribute to disease progression.

MATERIALS AND METHODS

Cell Cultures. Cultured human adenocarcinoma T47D cells were established from a pleural effusion of a patient with an infiltrating ductal carcinoma of the breast (30). MCF-7 breast adenocarcinoma cells were established from human pleural effusion. Both cell lines were maintained in DMEM, supplemented as described previously (27). Human monocytic THP-1 cells were maintained in RPMI 1640 supplemented as described previously (31).

FACS Analysis. For indirect immunofluorescence, 5 × 10^5 MCF-7 or T47D cells were incubated for 45 min at 4°C with monoclonal antibodies specific for human IFN-γ receptors or human TNF-α receptors (1 μg/ml × 10^5 cells; Serotec, Oxford, England). After two washes with FACS medium (PBS supplemented with 1% FCS, 0.02% sodium azide, and 25 mM of HEPEs), the cells were incubated with FITC-conjugated secondary antibodies (goat anti-mouse IgG; Jackson ImmunoResearch Laboratories, West Grove, PA) for 60 min at 4°C. After one more wash, the cells were resuspended in PBS and 0.02% sodium azide. Control cells were incubated with FACS medium instead of primary antibodies. The analysis was performed with and/or without the addition of 70 μg of aggregated human IgG (Sigma Chemical Co., St. Louis, MO) before the addition of the primary antibody. Receptor expression was determined in 5000 living cells with a Becton Dickinson FACSort (Mountain View, CA) using the CellQuest software.

Detection of RANTES Expression by ELISA Assays. The expression of RANTES by T47D cells was determined in serum-free supernatants derived after 48 h incubation in the absence or in the presence of rhIFN-γ and/or rhTNF-α (PeproTech, Rocky Hill, NJ). The conditioned medium of the cells were analyzed after concentration with Vivavisin (cutoff 5 kDa; Vivasience, Binbrook, United Kingdom). Determination of RANTES expression was performed by ELISA assays in which the coating antibodies were mouse monoclonal antibodies against human RANTES (4 μg/ml; PeproTech), and detection antibodies were biotinylated goat antihuman RANTES antibodies (5 ng/ml; R&D Systems, Minneapolis, MN). After the addition of streptavidin-HRP (Jackson ImmunoResearch Laboratories), the substrate TMB/E solution (Chemicon International, Temecula, CA) was added. The reaction was stopped by the addition of 0.18 M H_2SO_4 and was measured at 450 nm.

Determination of Migratory Activity. T47D cells (1.3 × 10^5) were plated for 24 h in growth (DMEM) medium in a 9-cm tissue culture plate. The cells were washed three times with serum-free DMEM and incubated for an additional 24 h in serum-free medium. Supernatants were removed and centrifuged for 20 min at 1050 × g. The resulting conditioned medium was concentrated by Vivaspin 4 (as described previously).

The ability of the concentrated conditioned medium to induce the migration of THP-1 cells was assessed by a 48-well microchemotaxis chamber technique, as described previously (32). The lower compartment of the chamber was loaded with 30-μl aliquots of control BSA medium (RPMM 1640 containing 1% BSA and 25 mM HEPES buffer), with rhRANTES, rhMCP-1, recombinant murine MCP-1 (PeproTech), or with dilutions of concentrated T47D-derived serum-free conditioned medium. All of the reagents were diluted in BSA medium. The upper compartment of the chamber was loaded with a 50-μl cell suspension of THP-1 cells (4 × 10^5/ml), which were washed twice previously in BSA medium. The two compartments were separated by a polycarbonate polystyreneuloidine free filter (8 μm pore size; Osmonics, Livermore, CA). The chamber was incubated for 2 h at 37°C in humidified air with 5% CO_2_. At the end of the incubation period, the filter was removed, fixed, and stained with a Diff-Quik kit (Dade Behring, Düren, Switzerland). Cells migrating through to the underside of the filter were counted in 3–5 fields (×160 to ×400) by light microscopy, in triplicate. Statistical analysis was performed using Student’s t test.

To determine the contribution of T47D-derived RANTES or MCP-1 to THP-1 migration, aliquots of conditioned medium of T47D cells were incubated with neutralizing antibodies against human RANTES or human MCP-1 (PeproTech; for concentrations, see figure legends) for 10 min at 37°C.

Gelatin Substrate Zymography and Western Blot Analysis for MMPs. THP-1 cells (10^6/well) were plated in 96-well plates in serum-free RPMI 1640 for 48 h in the absence or in the presence of different concentrations of rhRANTES (PeproTech). MCF-7 cells were plated in 24-well plates (5 × 10^5/ml) for 48 h in growth medium. After overnight incubation the cells were washed twice in serum-free DMEM and incubated for an additional 48 h in serum-free medium in the absence or in the presence of rhRANTES, rhIFN-γ, or rhTNF-α. T47D cells were plated in 24-well plates (9 × 10^5/well) in growth medium overnight, followed by two washes in phenol red and serum-free DMEM (Biological Industries, Beit Haemek, Israel). After an overnight incubation in phenol red-free medium containing 1% dialyzed FCS and 10^-8 M 17-β-estradiol (Sigma Chemical Co., St. Louis, MO), the process of washing and incubation of T47D cells with 17-β-estradiol was repeated. The cells were similarly washed and incubated for 48 h in the absence or the presence of rhTNF-α, in phenol red-free, and in serum-free DMEM containing 10^-8 M progesterone (Sigma Chemical Co.).

For zymography assays, the conditioned medium of THP-1 and MCF-7 cells were analyzed without additional concentration. Conditioned medium of T47D cells were concentrated before analysis by Vivavisin 4 (as described previously). All of the conditioned medium were analyzed for the expression of MMPs by the addition of sample buffer (sample buffer ×5 contains Tris HCl 0.2 M, SDS 4%, Glycerol 40%, Bromophenol blue 0.004%) that did not contain β-mercaptoethanol, and separation on 7.5% SDS-polyacrylamide gels containing 0.1% gelatin substrate. After electrophoresis, the gels were washed in 50 mM Tris-HCl (pH 7.4) containing 2.5% Triton X 100. The gels were then washed in 50 mM Tris-HCl (pH 7.4) buffer, followed by incubation in buffer containing 50 mM Tris-HCl (pH 7.4), 0.02% sodium azide and 10 mM CaCl_2, for 18 (THP-1 and MCF-7 cells) to 43 h (T47D cells). After washes in double distilled H_2O, the gels were stained with 0.25% Coomassie Blue, destained in 20% methanol containing 10% glacial acetic acid, and clear bands of protein degradation were visualized. Bands seen on gelatin gels were confirmed as MMPs by the addition of 10 mM EDTA to the incubation buffer to inhibit enzyme activity.

For MMPs characterization by Western blot analysis, conditioned medium of the THP-1, MCF-7, and T47D cells were concentrated before analysis by Vivavisin 4 (as described previously). Sample buffer ×5 (0.2 M Tris-HCl 4%, SDS 4% glycerol, 0.004% bromphenol blue, and β-mercaptoethanol 25%) was added to samples of the conditioned medium to reach a final concentration of 1×. The samples were treated for 10 min at 95°C, centrifuged at 15,800 × g for 4 min, run in 10% SDS-PAGE, and transferred to nitrocellulose. After blocking of the membrane with TTBS (TWEEN 20-Tris-buffered saline) (1.5 M NaCl, 3% Tris base, 7% Tris-HCl, and 0.5% Tween 20) containing 5% dry milk for 1 h at room temperature, primary antibodies against MMP1, MMP2 (1 μg/ml; Oncogene Research Products, Cambridge, MA), or MMP9 (3 μg/ml; R&D Systems) were added in TTBS containing 5% dry milk to the membrane. After an overnight incubation at 4°C, the membrane was washed in wash buffer (TTBS containing 1% dry milk) and incubated for 1 h at room temperature with 1:5000 dilution of HRP-conjugated sheep antismouse IgG (Amer sham, Buckinghamshire, United Kingdom) in wash buffer. After additional...
washes, bands were detected by using enhanced chemiluminescence (Amersham).

Markers from different companies were used for determination of MWs: For zymography, Precision protein standards (Bio-Rad, Hercules, CA); for Western blot analysis, marker no. 1 was a Rainbow colored protein molecular weight marker (Amersham) and marker no. 2 was Bench Mark prestrained protein ladder (Life Technologies, Inc., Inchinnan, Scotland).

Chick Chorioallantoic Membrane Assay. The assay was performed on 3-day-old chicken embryos. Eggs were paired according to the embryonic stage of the embryo, its location under the CAM, and the thickness/density of the blood vessels (5–8 pairs/experiment). Agarose beads (1%) at identical size (~1.5 mm³) were incubated with RANTES (5 μg/ml; dissolved in PBS) or with PBS for 3 h at 4°C. The agarose beads were then placed on the CAM of each of the pair members for 24 h at 37°C in a humidified atmosphere. The thickness and the number of the blood vessels were graded in eggs incubated with RANTES agarose beads as positive or negative for modified vascularity, as compared with eggs treated with PBS agarose pieces. The evaluation was performed independently by two to three researchers using BX12 Olympus dissecting microscope.

Immunohistochemistry. Serial sections (5-μm thick) were prepared from paraffin-embedded blocks derived from breast tumors of three patients. Sections were deparaffinized, dehydrated in xylene and graded alcohols, rinsed in PBS, and microwaved for 10 min in buffer citrate (pH 6.0; Zymed, San Francisco, CA). After quick cooling the sections were rinsed in PBS and treated with 3% H₂O₂ for 10 min at room temperature. From this stage and on, all of the procedures were performed at room temperature. The sections were washed in Wash buffer (Biogenics, San Ramon, CA). Nonspecific binding was blocked by incubating the sections with normal rabbit serum for 10 min. Then, the sections were incubated with 60 μg/ml polyclonal goat antibodies against CCR5 (Santa Cruz Biotechnology, Santa Cruz, CA). Two controls were included in the study: (a) biopsy sections in which the primary antibodies were substituted by PBS; and (b) biopsy sections in which the primary polyclonal goat antibodies against CCR5 were substituted by polyclonal goat antibodies directed against ERβ (20 μg/ml, as determined for optimal activity in preliminary experiments; Santa Cruz Biotechnology). In contrast with CCR5, ERβ staining is localized in the nucleus. The incubation with the primary antibodies was performed for 30 min, and sections were washed in wash buffer and stained with HRP-conjugated rabbit antigoat polyclonal antibodies (7.5 μg/ml; Zymed). After rinsing in wash buffer, the sections were incubated for 3 min with diaminobenzidine (Zymed; according to manufacturer’s instructions), washed and counterstained by incubation with hematoxylin (Zymed) for 3 min, followed by wash in H₂O₂, dehydration in graded alcohols, and mounting with Mercoglas (Merck, Darmstadt, Germany). All of the stained slides were submitted to light microscopy, and the staining pattern in tumor cells was independently evaluated by two researchers and a pathologist.

RESULTS

The Regulation of RANTES Expression by Proinflammatory Cytokines. To evaluate the possibility that cytokines that are produced by the tumor cells, or by adjacent stroma cells and/or inflammatory cells, may regulate RANTES expression by the tumor cells, we determined the ability of IFN-γ and TNF-α to affect RANTES expression by the T47D and MCF-7 breast adenocarcinoma cells. These two cytokines were shown to promote RANTES expression in fibroblasts, as well as in epithelial and endothelial cells (33–36). First, we determined the expression of receptors for these cytokines by the T47D and MCF-7 cells. As demonstrated in Fig. 1, A and B, both cell lines expressed receptors for IFN-γ and TNF-α.

Previous analysis performed in our laboratory has indicated that RANTES is constitutively expressed by T47D and MCF-7 cells (27). To allow for a more comprehensible analysis of the effects of the two cytokines on RANTES expression, cell variants that had reduced constitutive expression levels of RANTES were used for these assays. As shown in Fig. 1C, the expression of RANTES by T47D cells was highly elevated by rhIFN-γ and rhTNF-α. The ability of the cytokines to up-regulate RANTES expression was noted also in MCF-7 cells (data not shown). Of major interest is the fact that the two cytokines synergised in their ability to promote RANTES expression. As mentioned, the results shown are of T47D cells that originated spontaneously in the laboratory and expressed a low ability to constitutively express RANTES; however, IFN-γ and TNF-α were shown to promote RANTES expression also by cells that expressed much higher constitutive levels of RANTES (data not shown). In all, the above results provide evidence for the coordinated activity of IFN-γ and TNF-α in promoting RANTES expression by breast tumor cells.

Paracrine Activities of RANTES That May Promote Breast Cancer Progression. To elucidate the possibility that RANTES may contribute to breast cancer progression by inducing the infiltration of monocytes, the ability of breast tumor cells to express factors that induce monocyte migration was determined. To this end, the migration of THP-1 monocyctic cells in response to serum-free supernatants derived from T47D and MCF-7 breast adenocarcinoma cells was determined. The results shown in Fig. 2A demonstrate that T47D cells express factors that potently induce monocyte migration. Expression of such factors was observed also in MCF-7 breast adenocarcinoma cells, although at lower potency of activity (data not shown).

Monocyte migration, similarly to the recruitment of monocytes to inflammatory loci, may be mediated by a variety of chemokines. Because THP-1 migration was shown to be induced by RANTES (37), and RANTES was demonstrated to be expressed by T47D cells (27), we determined the contribution of T47D-derived RANTES to THP-1 migration (using cells that express constitutively high levels of RANTES). To this end, serum-free supernatants of T47D cells were
incubated with neutralizing antibodies against human RANTES, then analyzed for their ability to induce THP-1 migration. To ensure the neutralizing activity of these antibodies, the range of antibody concentration that inhibits rhRANTES-induced migration of THP-1 cells was first determined. According to this analysis, different concentrations of antibodies were analyzed for inhibition of THP-1 migration induced by T47D-derived supernatants. As shown in Fig. 2B, inhibition of tumor cell-derived RANTES activity by neutralizing antibodies resulted in a partial decrease of $31.2 \pm 10.6\%$ (mean $\pm$ SD of different experiments) in the migration of THP-1 cells. The inhibition by the antibodies to RANTES was significant in all of the experiments performed, being in the range of 18.7–46.9% inhibition in seven independent experiments.

Because the breast tumor cell-derived RANTES contributed only partially to the migratory activity observed in the supernatants of T47D cells, assays were set to determine whether MCP-1 was involved in this activity. MCP-1 is a highly potent attractant of monocytes, and high levels of its expression were shown to be correlated with advanced breast carcinoma (18–20). Our preliminary experiments indicated that T47D cells express high levels of MCP-1 (data not shown). To determine the contribution of T47D-derived MCP-1 to THP-1 migration, serum-free supernatants of T47D cells were incubated with neutralizing antibodies against human MCP-1, then analyzed for their potential to induce THP-1 migration. The conditions for inhibition of MCP-1-induced activities were determined by experiments in which the migratory activity of THP-1 cells to rhMCP-1 was inhibited by different concentrations of antibodies to human MCP-1. By the use of neutralizing antibodies against human MCP-1, a significant decrease of $79 \pm 20.2\%$ (mean $\pm$ SD of different experiments) in the T47D-induced migration of THP-1 cells was observed (Fig. 2C). The inhibition of migration induced by the antibodies against MCP-1 ranged between 43.5% and 100% (in five independent experiments), indicating that T47D-derived MCP-1 is a major contributor to the monocyte migratory activity expressed by the T47D breast adenocarcinoma cells. Therefore, our results suggest that RANTES and MCP-1 contribute concomitantly to the monocyte-migratory activity expressed by T47D cells.

Monocytes that migrated to breast tumors may express promalignant properties such as the secretion of MMPs (15, 38, 39). Because active MMPs are major contributors to degradation of the basement membrane and to metastasis formation in breast carcinoma (39–42), we determined the possibility that RANTES promotes the expression of MMPs by monocytes. To this end, monotypic THP-1 cells were grown in the presence of rhRANTES in a serum-free medium. Because many of the MMPs break down gelatin, gelatin substrate zymography was used to determine this issue. On the basis of the fact...
that MMPs require Ca$^{2+}$ for their activity (39, 43), the bands seen on the gels were confirmed as resulting from MMPs activity by treatment with EDTA, as described in “Materials and Methods.”

Before stimulation with rhRANTES, the expression of gelatinases by the monocytic THP-1 cells was determined. The results demonstrated in Fig. 3A indicate that THP-1 cells express three different proteolytic enzymes that degrade gelatin, having the MWs of 190 kDa (band no. 1), 83 kDa (band no. 2), and 50 kDa (band no. 3). The 83 kDa gelatinase was the most prominent of these three enzymes. The addition of EDTA to the incubation solution inhibited the activity of these enzymes, indicating that the proteolytic activity expressed in the THP-1-derived supernatants resulted from the activity of MMPs.

To characterize the type of MMPs that are expressed by THP-1 cells, Western blot analysis was performed on THP-1-derived supernatants with antibodies that are directed against two of the MMPs that have the relevant MWs and that were shown to highly contribute to malignant processes (39–42), namely MMP1 and MMP9. Several of the MMPs were described to vary in their MWs in different studies (e.g., Refs. 39, 42–44). To comply with the possibility that some of the MW differences resulted of the use of different MW markers, the MW of the proteins that were recognized by the antibodies against MMP1 and MMP9 were determined by two different MW markers (different from the one used for zymography, as indicated in “Materials and Methods”). When the differences between the two MW markers were subtle (≈ 5 kDa), the MW provided is that of higher similarity to the MW indicated by the different marker that was used for the zymography analysis. However, when more prominent differences were observed, mostly for proteins at the 75–100 kDa range, two MWs are provided for every protein that was detected in the Western blot analysis: (a) without parentheses the MW according to marker no. 1 (as described in “Materials and Methods”); and (b) with parentheses the MW according to marker no. 2 (as described in “Materials and Methods”).

The results of the Western blot analysis performed with antibodies directed against MMP1 indicated the expression of a protein with a MW of 60 kDa (band no. 1) and another protein of 48 kDa (band no. 2; Fig. 3B). On the basis of the MWs of MMP1 that are described by other researchers (42, 43), these results suggest that THP-1 cells express the active form of MMP1 (the 48 kDa band) as well as the pro-MMP1 (the 60 kDa band). In addition, few lower MW proteins (e.g., band no. 3, which is of the highest MW of these lower MW proteins) that may be degradation products of MMP1 were recognized by the antibodies against MMP-1.

Additional characterization of the proteolytic enzymes that were expressed by THP-1 cells by the use of antibodies directed against MMP9 resulted in the identification of a pronounced band having the MW of 97 kDa (82 kDa; band no. 1). Combined with the results of the zymography, this protein is presumably the active form of MMP9 (as based on Refs. 42–44). Three additional bands of lower MW (the highest of which is shown as band no. 2; Fig. 3B) were recognized by the antibodies against MMP9 and may result of degradation of this enzyme.

When combined, the results of the zymography and the Western blot analyses suggest that THP-1 cells express several MMPs: the proteolytic activity at 50 kDa that was identified by zymography assays may result in the activity of MMP1, whereas the degradative activity at 83 kDa may result of the activity of MMP9. The 190 kDa enzyme expressed by THP-1 cells in zymography assays may be the result of the activity of a MMP9 dimer (maybe in a glycosylated form), shown previously to be expressed in neutrophils (42, 44), dissociating in the Western blot analysis performed in the presence of β-mercaptoethanol.

After the characterization of the MMPs that are expressed by THP-1 cells, the expression of these enzymes was determined after the exposure of the cells to rhRANTES for 48 h. Zymography analysis did not provide evidence for any RANTES-mediated effects on the expression of MMP1. In contrast, a marked elevation in the expression of MMP9 was noted by the rhRANTES treatment in a dose-dependent manner (Fig. 3C). Therefore, these results indicate that RANTES promotes the expression of MMPs by monocytic cells.

An additional biological process of major importance for tumor development and progression is the level of vascularization within the primary tumor (45, 46). Because several chemokines, including MCP-1, were shown to be major regulators of angiogenesis (21, 47), we determined the ability of RANTES to affect vascularization, using chick CAM assays. When compared with the PBS negative control in many of the RANTES/PBS-treated embryo pairs, the exposure to RANTES resulted in thickening of the blood vessels and partially in elevated numbers of blood vessels radiating from the chick embryo (Fig. 4). The scores of positive responses to RANTES ranged between 57% and 100% in five independent experiments (mean ± SD of the experiments was 79.6 ± 19.7%). For each of the RANTES versus PBS-treated embryo pairs in which a positive effect of RANTES was noted, the potency of the effect was estimated. In the five experiments performed, high potency of RANTES-mediated effects were observed in 65–100% of the positive pairs: 65% with high potency in one experiment; 75% in the second; and 80%, 100%, and 100% in the others. Altogether, these results indicate that RANTES promotes...
vascularization and point to a potential mechanism by which it may contribute to breast cancer progression.

**Indirect Activities by Which RANTES May Induce Tumor-promoting Properties in Breast Tumor Cells.** To evaluate the possibility that cytokines that are produced by infiltrating T cells and monocytes (or by the tumor cells) may contribute to tumor progression, we analyzed the ability of IFN-γ and TNF-α to affect the expression of MMPs by MCF-7 and T47D cells. Before analyzing the effects of the cytokines on MMPs expression by MCF-7 cells, the expression of proteolytic enzymes that degrade gelatin by these cells was determined by gelatin-based zymography assays. This analysis provided evidence for the expression of one gelatinase by MCF-7 cells, having a MW of 83 kDa (band no. 1; Fig. 5A). This enzyme was identified as a member of the MMPs family by the ability of EDTA to inhibit its activity (Fig. 5A). The expression level of this enzyme was too low to enable its characterization by Western blot analysis.

To determine the effects of IFN-γ and TNF-α on the expression of MMPs by the breast adenocarcinoma MCF-7 cells, the cells were grown in the absence or in the presence of the cytokines for 48 h in serum-free medium. The two cytokines were used in concentrations that were shown to be of major competence for induction of RANTES expression in these cells, namely 500 ng/ml rhIFN-γ and 2000 units/ml rhTNF-α (Fig. 1C). The zymography analysis indicated that IFN-γ did not affect the expression of the MMP that was already shown to be expressed by the MCF-7 cells (83 kDa; Fig. 5A). On the other hand, exposure of the cells to 2000 units/ml TNF-α resulted in a marked increase in the expression of this enzyme (Fig. 5B).

The very prominent increase in the expression level of the enzyme after TNF-α treatment enabled us to identify it by Western blot analysis using antibodies against MMP9. As shown in Fig. 5C, a protein with a MW characteristic of MMP9 was identified, at 97 kDa (82 kDa; band no. 1; one more low MW protein was also observed before and after stimulation with TNF-α, not shown). As based on the described MW of this enzyme (42–44) combined with its MW in the zymography analysis (provided by the use of a different marker), the above results suggest that TNF-α induces the expression of active MMP9 in MCF-7 cells. To determine the dose response of the TNF-α-induced promotion of MMP9, MCF-7 cells were exposed to different concentrations of the cytokine, ranging from 100–2000 units/ml. The results demonstrated in Fig. 5D indicate that the TNF-α-induced expression of MMP9 was dose dependent, starting at concentration as low as 100 units/ml and reaching maximal effect at 2000 units/ml.

Similar to the analysis performed on MCF-7 cells, the effects of IFN-γ and TNF-α on the expression of MMPs were also determined in T47D cells. First, the expression of gelatinases by these cells was analyzed, demonstrating the expression of one enzyme, with the MW of 59 kDa (band no. 1; Fig. 6A). The activity of the enzyme was inhibited by EDTA, indicating that the T47D-derived proteolytic enzyme is a member of the MMP family. Western blot analysis performed by the use of antibodies against MMP2 resulted in characterization of a 60 kDa protein (band no. 1; Fig. 6B). In addition, in different assays, one to two additional low MW proteins that may be degradation products of MMP2 were observed (not shown). Combined with the zymography analysis and by indication of a 62 kDa MW for MMP2 (42), these results suggest that T47D cells express the active form of MMP2.

To determine the effects of IFN-γ and TNF-α on MMPs expression by T47D cells, the cells were exposed to the cytokines for 48 h followed by zymography analysis of their supernatants. Although the receptors for IFN-γ are expressed by these cells (Fig. 1A), the exposure of the cells to 500 ng/ml rhIFN-γ did not give rise to a reproducible effect on the expression of MMP2 (data not shown). On the other hand, the exposure of T47D cells to 2000 units/ml rhTNF-α resulted in a significant increase in the expression of MMP2 (band no. 2; Fig. 6C). In addition, the *de novo* expression of an additional enzyme, of 83 kDa, was noted after the treatment of the cells by rhTNF-α (band no. 1; Fig. 6C). The expression level of this enzyme was too low to allow its characterization by Western blot analysis. However, based on the characterization of similar gelatinases that were expressed by the THP-1 and MCF-7 cells, it is possible that this enzyme is the active form of MMP9. To determine the dose dependency of the effects of TNF-α on MMPs expression by T47D cells, the cells were exposed to 500 units/ml and 2000 units/ml TNF-α. As demonstrated in Fig. 6D, the TNF-α-induced expression of MMP2 expression were observed already after exposure of the cells to 500 units/ml TNF-α. On the other hand, the *de novo* expression of the 83 kDa gelatinase after TNF-α treatment was noted only after stimulation of the cells with 2000 units/ml TNF-α. In all, these results provide...
RANTES Promotes the Expression of MMPs by Breast Tumor Cells. An additional mechanism by which RANTES may affect tumor progression is by acting directly on the tumor cells. Such an activity would require the expression of specific receptors for RANTES by the tumor cells. To determine the expression of RANTES receptors on breast tumor cells, we analyzed the expression of CCR5, a well-characterized receptor for RANTES (16), by breast tumor cells in sections of three breast cancer patients. In all three of the cases, a positive expression of CCR5 was determined (Fig. 7, A and A'; sections of two of the three patients are demonstrated), whereas no staining was observed in the PBS negative control (Fig. 7, B and B', respectively). In agreement with the well-described heterogeneity of tumor cells, the expression of CCR5 was not detected in all of the tumor cells. An additional control used for staining of the biopsy sections of the three patients consisted of antibodies against ERβ. In similarity to the antibodies against CCR5, the antibodies against ERβ were goat polyclonal antibodies. However, in contrast to CCR5, the antibodies against ERβ gave rise to either no staining (in one patient, Fig. 7C) or to nuclear staining only (in the other two patients, one shown in Fig. 7C'), as expected for positive staining of ERβ.

A mechanism by which RANTES may act as a promalignancy factor is its ability to promote the expression of factors that may facilitate metastasis formation in the tumor cells, such as MMPs. To evaluate this possibility we determined by gelatin-based zymography the ability of RANTES to induce MMPs expression by MCF-7 cells. Studies on the T47D and MCF-7 cells indicated that receptors for RANTES are expressed by these cells (48, 49). Analysis of the expression of MMPs by the MCF-7 cells provided evidence for the ability of rhRANTES to induce the production of a 83 kDa gelatinase by the breast tumor cells (Fig. 8). The results shown in Fig. 5, A and C, combined with the results showing the RANTES-induced up-regulation of a 83 kDa gelatinase by the MCF-7 cells, suggest that RANTES promoted the expression of MMP9 in MCF-7 cells. The addition of RANTES to MCF-7 cells promoted in a dose-dependent manner the expression of this enzyme (Fig. 8). Combined with the expression of receptors for RANTES by breast tumor cells, these results indicate that RANTES acts on the tumor cells to promote their ability to express protumorigenic properties such as MMPs.

DISCUSSION

The evolvement of breast cancer depends on multiple factors: genetic, environmental, and others. The interplay between the developing tumor cells and the stroma cells may be a major contributor to evidence for the ability of TNF-α to up-regulate the expression of MMPs by the MCF-7 and T47D breast adenocarcinoma cells.

Fig. 5. TNF-α up-regulates MMPs expression by MCF-7 breast adenocarcinoma cells. A, the expression of MMPs by MCF-7 cells, as determined by gelatin substrate zymography assays, in the absence or in the presence of EDTA. A representative experiment of two to five independent experiments performed is demonstrated. B, the expression of MMPs by MCF-7 cells after incubation in the absence or presence of rhIFN-γ or rhTNF-α for 48 h in serum-free medium. The expression of MMPs was determined by gelatin substrate zymography. A representative experiment of six independent experiments performed is demonstrated. C, characterization of the MMPs expressed by MCF-7 cells after treatment with 2000 units/ml TNF-α, as determined by Western blot analysis with antibodies against MMP9. A representative experiment of three independent experiments performed is demonstrated. D, dose response of the TNF-α-induced elevation in MMPs expression by MCF-7 cells, as determined by gelatin substrate zymography. A representative experiment of two to three independent experiments performed is demonstrated.

Fig. 6. TNF-α up-regulates MMPs expression by T47D breast adenocarcinoma cells. A, the expression of MMPs by T47D cells, as determined by gelatin substrate zymography assays, in the absence or in the presence of EDTA. A representative experiment of two to five independent experiments performed is demonstrated. B, characterization of the MMPs expressed by T47D cells, as determined by Western blot analysis with antibodies against MMP2. A representative experiment of two independent experiments performed is demonstrated. C, the expression of MMPs by T47D cells after incubation in the absence or in the presence of rhTNF-α for 48 h in serum-free medium. The expression of MMPs was determined by gelatin substrate zymography. A representative experiment of four independent experiments performed is demonstrated. D, dose response of the TNF-α-induced elevation in MMPs expression by T47D cells, as determined by gelatin substrate zymography. A representative experiment of five independent experiments performed is demonstrated.

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the determination of disease progression. Factors that are expressed by the tumor cells may affect microenvironmental properties and vice versa. The high correlation between monocyte infiltration and advanced breast carcinoma (1–9, 18–20) suggests that monocytes contribute to disease progression and illustrates the need for a better understanding of their function after infiltration into breast tumors. Studies aimed at the elucidation of such processes suggest the involvement of CC chemokines, which are expressed by the breast tumor cells and/or by adjacent stroma cells, in this process. Several investigations have provided evidence recently for the potential contribution of monocyte chemoattractants, the CC chemokines RANTES and MCP-1, to breast cancer progression (18–20, 27, 29). However, the elucidation of the mechanisms by which these chemokines act is only at its beginning. In that respect, our study sheds light on the regulation of RANTES expression in breast tumor cells and on the potential mechanisms by which it may contribute to breast cancer progression.

First, the results of our study demonstrate that the expression of RANTES by breast tumor cells is synergistically promoted by the proinflammatory cytokines IFN-γ and TNF-α. Our observation on the up-regulation of RANTES expression in breast tumor cells induced synergistically by the proinflammatory cytokines IFN-γ and TNF-α suggests that the existence of a cytokine network at the tumor site may give rise to elevated levels of RANTES expression by the tumor cells. Such cytokines may be expressed by the tumor cells themselves or by adjacent stroma cells including lymphocytes and/or monocytes that infiltrated the tumor. It is possible that one of the primary events in breast cancer progression is the constitutive expression of RANTES and MCP-1 by the tumor cells because of modifications in gene

**Fig. 7.** The expression of CCR5 in biopsy sections of breast cancer patients. The expression of CCR5 was determined in biopsy sections of breast cancer patients. Results of two of three patients are demonstrated. A–C, serial sections of one patient diagnosed with infiltrating ductal carcinoma of the breast, grade II. A′–C′, sections of another patient, diagnosed with lobular carcinoma of the breast, multicentric in situ, and with extensive areas of infiltrative lobular carcinoma, stage I. A and A′, serial sections stained by polyclonal goat antibodies against CCR5. B and B′, serial sections from the same tumors (respectively) in which the antibody against CCR5 was replaced by PBS. C and C′, serial sections from the same tumors (respectively) in which the antibody against CCR5 was replaced by polyclonal goat antibodies against ERβ.

**Fig. 8.** RANTES induces MMPs expression by MCF-7 breast adenocarcinoma cells. MCF-7 cells were incubated in the absence or presence of different concentrations of rhRANTES for 48 h in serum-free medium. The expression of MMPs was determined by gelatin substrate zymography as described in “Materials and Methods.” A representative experiment of six independent experiments performed is demonstrated.
expression patterns. This prime event may be followed by a promoted migration of immune cells (T cells and monocytes) into the tumor, resulting thereafter in their ability to secrete cytokines that further stimulate the expression of RANTES. The end point of such a process is a high level of RANTES expression by the tumor cells, which may then mediate processes that contribute to tumor progression.

Second, additional novel insights were provided in the present study in regard to the mechanisms by which RANTES may contribute to breast cancer progression. Our results suggest that RANTES may act in paracrine and indirect manners, and also directly on the tumor cells, to support breast cancer progression. A key mechanism by which RANTES may contribute to breast cancer progression is the ability of breast tumor cell-derived RANTES to contribute to monocyte recruitment into breast tumors. Migration assays performed in vitro with supernatants derived from breast tumor cells indicate that these cells indeed secrete factors that induce monocyte migration. MCP-1 was characterized as the prime inducer of the monocyte migration expressed by T47D breast adenocarcinoma cells; however our findings propose that RANTES partially contributes to this activity. Our results suggest that the expression of RANTES and MCP-1 by breast tumor cells results in their combined activity, inducing monocyte migration to tumor areas. Monocytes that have migrated into the tumors may now exert protumorigenic activities that support disease progression (3–6, 9, 13–15, 38, 39).

Another mechanism by which RANTES may affect breast tumor progression is its ability to up-regulate the expression of MMPs in monocytes. Our results suggest that the RANTES- and MCP-1-induced infiltration of monocytes to breast tumor sites may be followed by RANTES-induced up-regulation of the expression of promalignant properties in the infiltrating monocytes, such as the expression of MMPs.

In addition, RANTES may act in another paracrine manner to support the metastatic process by its ability to increase vascularity. This observation is in line with previous studies, demonstrating the potent angiogenic effects of ELR+–CXCL chemokines and of the CC chemokine MCP-1 (21, 47). Therefore, tumor-derived chemokines such as RANTES may be major regulators of angiogenic processes that facilitate tumor progression.

In addition to paracrine manners by which RANTES may act to promote breast cancer progression, the results of our study propose that the promalignant activities of RANTES may be the indirect result of its ability to induce monocyte migration to breast tumor sites. As previously mentioned, TAM were described to exert promalignant activities such as the expression of growth factors for breast epithelial cells, the secretion of angiogenic factors, and the release of MMPs (3–6, 9, 13–15, 38, 39). The results of the present study suggest that tumor-infiltrating monocytes may assist the progression process by their ability to express TNF-α. On the basis of our results, TNF-α-mediated activity on the breast tumor cells may result in elevated expression of MMPs by the tumor cells, thus facilitating breast cancer progression. TNF-α, of which the cellular source may be not only the infiltrating monocytes but also the tumor cells and/or other stroma cells, may therefore be a key protumorigenic factor. Indeed, recent findings (50–52) raise the possibility that TNF-α, which is usually considered as an antimalignant cytokine (50), may serve under certain circumstances as a powerful promalignant factor in breast cancer.

The ability of RANTES to act in paracrine and/or indirect manners to promote breast cancer progression may be complemented by its possible activity on the tumor cells, resulting in the elevated expression of promalignant activities by the cancer cells. Our study demonstrates the expression of CCR5, a well-characterized receptor for RANTES (16), by breast tumor cells in biopsy sections of breast cancer patients. The expression of RANTES receptors by the tumor cells may enable RANTES, which may be derived from the tumor cells, from adjacent stroma cells or from inflammatory cells, to induce tumor-promoting properties in the cancer cells. This is evidenced by the RANTES-promoted expression of MMP9 by the MCF-7 breast adenocarcinoma cells.

In all, based on the results of our study as well as on additional studies on the potential contribution of RANTES and MCP-1 to breast cancer progression, a hypothetical model may be suggested, illustrating the complex set of interactions that are initiated by RANTES and MCP-1 expression by the tumor cells, interactions that may give rise to breast cancer progression. According to this model, a prime event that takes place in early stages of breast carcinoma development may be the ability of transformed breast cells to constitutively express RANTES and MCP-1. The two chemokines may act concomitantly to induce monocyte and T-cell migration into the tumor site. Thereafter, as suggested by our analysis on the regulation of RANTES expression, proinflammatory cytokines that are secreted by the infiltrating leukocytes or by the tumor cells may up-regulate RANTES expression by the tumor cells. The result of such a promoted RANTES expression is the extensive additional migration of monocytes and T cells to the tumor site, giving rise to TAM-induced tumor-promoting activities that may result in additional growth of the tumor (e.g., secretion of growth factors, mediators of angiogenesis, and MMPs). This process may eventually result in increased cellular mass of the primary tumor.

In compliance with the above hypothesis, it is possible that metastasis formation and breast cancer progression are additionally supported by activities mediated by RANTES and by inflammatory cytokines at the tumor site. It is possible that at the tumor site, RANTES may induce MMP expression by acting on the monocytes as well as on the tumor cells. The ability of RANTES to promote vascularity may act in conjunction with the elevated expression of MMPs to result in facilitated metastasis formation. Furthermore, the infiltrating monocytes, other adjacent stroma cells, or the tumor cells may secrete TNF-α, resulting in overexpression of MMPs by the tumor cells. On the basis of numerous studies, the elevated expression of MMPs and promoted vascularity may be key events in disease progression, facilitating tissue degradation, and metastasis formation.

The model suggested above is based on an in vitro experimental system that allowed us to dissect the metastatic process into different mediators that act in cooperation, therefore contributing to breast cancer metastasis. Such an analysis leads the way to studies that could be performed in situ on biopsy sections of breast cancer patients, as well as to an in vivo experimental approach in which the role of each of these mediators may be investigated. In all, our results suggest that RANTES and the cellular products that may be induced by its indirect effects may have a significant role in breast cancer progression. The ability of RANTES to induce processes that facilitate metastasis formation is in full agreement with our observation on its expression in breast cancer patients (27), correlating high levels of RANTES expression with advanced disease. This demonstrates the complex regulation of breast cancer development as illustrated by the combined expression of many different factors at the tumor site, each having its specific role and contribution to disease progression.

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The CC Chemokine RANTES in Breast Carcinoma Progression: Regulation of Expression and Potential Mechanisms of Promalignant Activity

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