A Chemokine Receptor Antagonist Inhibits Experimental Breast Tumor Growth

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ABSTRACT

The leukocyte infiltrate of human and murine epithelial cancers is regulated by chemokine production in the tumor microenvironment. In this article, we tested the hypothesis that chemokine receptor antagonists may have anticancer activity by inhibiting this infiltrate. We first characterized CC chemokines, chemokine receptors, and the leukocyte infiltrate in the 410.4 murine model of breast cancer. We found that CCL5 (RANTES) was produced by the tumor cells, and its receptors, CCR1 and CCR5, were expressed by the leukocyte infiltrate. As Met-CCL5 is an antagonist of CCR1 and CCR5 with activity in models of inflammatory disease, we tested its activity against 410.4 tumors. After 5 weeks of daily treatment with Met-CCL5, the volume and weight of 410.4 tumors was significantly decreased compared with control-treated tumors. Met-CCL5 was also active against established tumors. The total cell number obtained after collagenase digestion was decreased in Met-CCL5-treated tumors as was the proportion of infiltrating macrophages. Furthermore, chemokine antagonist treatment increased stromal development and necrosis. Our results provide direct evidence that macrophages contribute to tumor development and are the first indication that chemokine receptor antagonists may provide novel strategies in cancer prevention and treatment.

INTRODUCTION

Human and murine epithelial cancers contain a leukocyte infiltrate (1, 2) and express a complex network of cytokines and chemokines (3). Leukocytes attracted into the tumor microenvironment by chemokines secrete cytokines, growth factors, angiogenic factors, and proteases. Hence, tumor associated leukocytes are just as likely to contribute to growth and spread of the malignancy than to a host antitumor response (1, 4, 5).

In human cancer, CC chemokines are the major determinants of macrophage and lymphocyte infiltration in carcinomas of the breast, ovary and cervix, and in sarcomas and gliomas (reviewed in Refs. 1, 6, 7). In breast cancer, for instance, epithelial tumor cells produce CCL5 (RANTES), and this expression correlates with disease progression (8). Human breast cancer cell-derived CCL5 promotes macrophage migration to tumor sites and matrix metalloproteinase-9 production by the tumor-associated macrophages (9, 10).

We have found that a transplantable model of breast cancer, 410.4, also produces high levels of CCL5. 410.4 murine breast carcinoma cells produced this chemokine in vitro, and when grown in BALB/c mice, 410.4 tumors developed an infiltrate of macrophages, CD8 lymphocytes, and neutrophils. These infiltrating cells expressed CCR1 and CCR5, the two major receptors for CCL5.

To obtain direct evidence for a tumor-promoting role of chemokines and tumor-infiltrating leukocytes, we studied the action of an antagonist of CCR1 and CCR5, Met-CCL5, in the 410.4 model. We show here, for the first time, that a chemokine receptor antagonist can slow tumor growth and reduce the macrophage infiltrate. We suggest that chemokine receptor antagonists may provide novel strategies in cancer treatment by modulating the relationship between host and tumor cells.

MATERIALS AND METHODS

In Vitro Culture of 410.4 Cells. 410.4 mammary carcinoma cells were grown in DMEM (Life Technologies, Inc.) supplemented with 10% FCS (Autogen Bioclear, Calne, United Kingdom) in endotoxin-free conditions in a humidified atmosphere at 37°C (5% CO2). For growth of 410.4 cells in mice, cells were trypsinized and washed twice in DMEM/10% FCS and suspended at 1 x 106 cells/ml. For in vitro experiments, 410.4 cells were trypsinized and washed twice with DMEM/10% FCS. A total of 5 x 103 cells/well was plated, left overnight, washed twice with serum-free DMEM, and then cultured in DMEM for the times indicated in the experiments.

Growth of Tumors in Mice and Treatment with Met-CCL5. Female BALB/c mice from the Special Pathogen-Free Unit, Clare Hall Laboratories (South Mimms, United Kingdom), 6–8 weeks of age were used in all experiments. The mice were housed in sterile individually ventilated cages at 20°C. All individually ventilated cage supplies were sterilized and autoclaved before entering the cage. Female BALB/c mice received injections of 410.4 cells (1 x 106 total cells) in the right flank. The growth of the 410.4 mammary tumors was evaluated by measuring the tumor every other day. Mice were killed if the tumors grew beyond an area of 1.44 cm2. Met-CCL5 was purified as previously described (11, 12) and was prepared at 100 μg/ml in 3 mg/ml mouse serum albumin (M: Sigma) made in sterile endotoxin-free PBS. Mice were injected with 0.1 ml (10 μg) i.p. every day for the duration of the treatment regime. Control mice received injections of 3 mg/ml mouse serum albumin.

Preparation of RNA from Cells and Tumor Tissue. For 410.4 cells, FACs-sortcd cells, and frozen tumor tissue, RNA was extracted using solution D as described previously (13). RNA was DNase treated with 10 units of DNase (Pharmacia Biotech, Milton Keynes, United Kingdom) following manufacturer’s instructions.

Real-Time RT-PCR. DNase treated RNA (2 μg) was reverse transcribed with Molony murine leukemia virus reverse transcriptase (Promega, Southampton, United Kingdom) according to manufacturer’s instructions and diluted to 100 μl with nucleotide-free water. Multiplex real-time analysis was performed using either CCR1 or CCR5 (FAM) and 18 s rRNA (VIC)-specific primers and probes with the ABI Prism 7700 Sequence Detection System instrument and software (PE Applied Biosysyms, Warrington, United Kingdom). Primers and probe for mouse CCR1 (NM_009912) and CCR5 (NM_009917) were designed using Primer Express 1.5 (PE Applied Biosysyms) from sequences submitted to GenBank. The sequences and concentrations of primers and probe are as follows: CCR1, forward, 5’-AGGCCCATGGGGACCTGATC-3’ (300 nm); reverse, 5’-TCCACTGCTTCAAGGCTTGT-3’ (300 nm); probe, 5’-CACCTGCTACCTGTAGCCCTCATTTCCCC-3’ (200 nm); CCR5, forward, 5’-AGGCCATGGGCAAGCAGAAG-3’ (300 nm); reverse, 5’-TCTTCCTCAAAAAGGCTATAGTGA-3’ (300 nm); and probe, 5’-CTTTGGAGAATCCACTGCTGTTAACC-3’ (200 nm).

PCR was carried out with the TaqMan Universal PCR Master Mix (PE Applied Biosysyms) using 2 μl of cDNA in a 25-μl final reaction volume. The cycling conditions were an incubation at 50°C for 2 min, followed by 10 min at 95°C and 40 cycles of 15 s at 95°C, and 1 min at 60°C. Experiments were performed in triplicate for each sample. CCR1 or CCR5 were normalized to MIP, macrophage inflammatory protein.

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Preparation of Tumors from Mice. BALB/c mice were incubated with 3 ml of digestion buffer (RPMI 1640, 5% FCS) containing Triton X-100 (1%, v/v), sodium deoxycholate (12 mM), SDS (3.5 mM), leupeptin (1 mM), pepstatin (1.5 mM), and phenylmethanesulfonyl fluoride (0.2 mM; all chemicals from Sigma). Samples were incubated on ice for 10 min. Debris was pelleted by centrifugation at 10,000 × g for 10 min. The supernatant was transferred to a fresh tube and the protein concentration determined using BCA protein assay (Sigma) and standard BSA concentrations (Sigma). The absorbance at 570 nm was measured by a Opsys MR plate reader (Dynex Technologies).

ELISA. Quantikine ELISA kits (R&D Systems, Abingdon, United Kingdom) were used. The manufacturer’s instructions were followed always. The sensitivity of the assays was as follows: murine MIP-2 (CXC2), 1.5 pg/mL; and murine RANTES, 2.0 pg/mL. For all ELISAs, the absorbance at 450 nm was measured and corrected at 570 nm in a plate reader (Opsys MR; Dynex Technologies).

FACS Analysis of Cells in the Tumors. Freshly excised tumors from BALB/c mice were incubated with 3 ml of digestion buffer (RPMI 1640, 5% FCS [Life Technologies, Inc.], 5 mg/ml collagenase D [Boehringer Mannheim], and 0.15 mg/ml DNase [Pharmacia Biotech]). Tumors were minced with a scalpel and incubated at 37°C for 40 min. Single-cell suspensions were made by passing the digested tumor pieces through a 19-g and 23-g needle and then through a cell strainer. Cells were pelleted at 1500 rpm and then resuspended in 1 ml of FACS buffer [PBS supplemented with 0.1% BSA, 0.01% NaN₃ (all from Sigma)]. Cell number and viability was assessed using a modified Neubauer (Graticules Ltd.) and trypan blue, respectively; cells were adjusted to 50–100 × 10⁵ cells/ml, depending on total cell number. Cells isolated from tumors and suspended in FACS buffer were blocked with antico tin CD16/32 FcBlock (BD Pharmingen) for 30 min on ice. Cells were then incubated with an appropriate primary antibody or an isotype-matched antibody for 30 min on ice; cells were pelleted and washed twice, then resuspended in FACS buffer. Antibodies used were CD4-FITC (H129.19), CD8b-2-PE (53-5.8), CD19-PE (1D3), Gr-1-FITC (RB6-8C5), NK1.1-PE (PK136; all from BD Pharmingen) and F4/80-FITC (Serotec). Cells were analyzed on a FACScan flow cytometer using CellQuest software (Becton Dickinson). For three-way cell sorting using the MoFlo (DakoCytomation), CD8b-2-PE, Gr-1-APC (BD Pharmingen), and F4/80-APC (Serotec) were used to isolate all three cell populations from the same cell pool recovered.

Immunohistochemistry and Quantitative Microscopy. The goat anti-mouse CCL5 antibody (AF478) was used at 25 μg/ml and was purchased from R&D Systems; the rat anti-mouse F4/80 antibody (MCA497R) was used at a 1:200 dilution and was from Serotec (Oxon, United Kingdom). The rat anti-mouse CCR5 (MC-68) was a kind gift from Matthias Mack (14). Both CCL5 and F4/80 were detected in paraffin-embedded sections with either pressure cooker antigen retrieval (CCL5) or trypsin digestion (F4/80). Mouse CCR5 was detected on cryostat sections. Sections were incubated with the appropriate dilution of primary antibody followed by a secondary biotinylated antibody and streptavidin-HRP (using either Vectastain Elite ABC goat or rat IgG kits; Vector Labs). Controls were performed using either the secondary antibody alone, diluted serum from the species of the primary antibody, or isotype-matched antibodies. The immune complexes were visualized using 3,3′-diaminobenzidine substrate kit (Vector Labs). Area counts were carried out using a modified Chalkley array (Graticules Ltd). The modified Chalkley array consists of 25 randomly arranged points; the proportion of points coincident with any tissue component is proportional to its area and volume fractions, which was inserted into one eyepiece of the Nikon Labophot II microscope (Nikon). Three components within the tumors were assessed: tumor cell islands; stroma; and areas of necrosis. Areas of necrosis were defined as having cell debris or apoptotic bodies. For each section, nine areas were assessed with the modified Chalkley point arranged in a 3 × 3-pattern to give a total area of 2.3 mm².

Statistical Analysis. For comparisons between control and treated tumor volumes, the AUC for each individual tumor was calculated using GraphPad Prism software. Statistical significance between control and treated AUC was calculated using the nonparametric Mann-Whitney U test. For comparisons between control and treated tumors weights and also with Chalkley point analysis, the Mann-Whitney U test was also used.

RESULTS

410.4 Breast Cancer Cells Produce CCL5 in Vitro. Because the expression of specific chemokines has been correlated with disease progression in human breast cancer (9), we characterized chemokine expression in murine 410.4 breast carcinoma cells. RNA was isolated from 410.4 cells in vitro and analyzed for a subset of chemokines using RPA that measured six CC, two XC, and one C chemokines. As shown in Fig. 1A, 410.4 cells only expressed mRNA for the CC chemokine CCL5 and the CXC chemokine CXCL2 (MIP-2). CCL5 and CXCL2 protein secretion by 410.4 cells in vitro was confirmed by ELISA. CCL5 protein could be measured after 2 h, and at 24 h, 35 pg/ml was detected (Fig. 1B). Similarly, CXCL2 protein was secreted with 65 pg/mL measured in the medium at 24 h (data not shown).

410.4 Tumors Develop a Chemokine Network in Vivo. 410.4 cells develop as a solid tumor in BALB/c mice when injected s.c. (15). To investigate the chemokine profile of the 410.4 in vivo, tumors were removed at weekly intervals after 410.4 cell injection. In agreement with the in vitro observations, CCL5 and CXCL2 mRNA were expressed at all stages of tumor growth (Fig. 2A). In addition, mRNA for CCL2 (MCP-1) was also expressed at weeks 1–4 of tumor growth. Several other chemokines were detected as the tumor developed in vivo. CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL11 (Eotaxin) were expressed at weeks 3 and 4 (Fig. 2A).

As CCL5 mRNA was expressed in vitro and in vivo, we measured the level of CCL5 protein in 410.4 tumors. CCL5 protein was present at 3.4, and 5 weeks after tumor cell injection (Fig. 2B). The level of CCL5 protein was similar at each time point with a range of 600–1200 pg/mg total protein (Fig. 2B). To confirm the identity of CCL5-producing cells, tumors were collected at 5 weeks, and paraffin
sections were analyzed using immunohistochemistry with an anti-CCL5 antibody. The majority of CCL5 positive cells were epithelial 410.4 cells [Fig. 2](a).

**Measurement of Chemokine Receptor Expression in Vitro and in Vivo.** To determine which chemokine receptors were present in the 410.4 tumor, we analyzed mRNA from tumors removed at weekly intervals. Chemokine receptor expression was determined by RPA. Three CC chemokine receptors were detected at all stages of tumor growth; CCR1 and CCR5 were expressed and are receptors that bind CCL5 (Fig. 3 A). The other chemokine receptor CCR2, which binds CCL2, was also detected during tumor development (Fig. 3 A). Chemokine receptors can also be expressed by tumor cells (16, 17); however, the 410.4 cell line did not express any CC chemokine receptors in [vitro]( Fig. 3 A).

**The Leukocyte Infiltrate of 410.4 Tumors.** Chemokines secreted by tumors correlate with the type and extent of leukocyte infiltrate (7). The leukocyte infiltrate in the 410.4 tumor was assessed using collagenase D-digested tumors, which were taken at weekly intervals, and the various cell populations were analyzed by FACS.

As shown in Fig. 3B, a leukocyte infiltrate was present in the tumors. Three cell populations were present in the tumors at weeks 1–5. CD8<sup>+</sup> lymphocytes, F4/80<sup>+</sup> macrophages, and Gr-1<sup>+</sup> neutrophils (Fig. 3B). The cell number for all three leukocyte subsets increased with each week of tumor growth. NK1.1<sup>+</sup> NK/NKT cells and CD19<sup>+</sup> B cells were only detected at weeks 1 and 2 (Fig. 3B). CD4<sup>+</sup> lymphocytes were not present in the tumors at any time point (Fig. 3B).

**CCR1 and CCR5 Chemokine Receptors Are Expressed by the Tumor Leukocyte Infiltrate.** To determine whether CD8<sup>+</sup>, F4/80<sup>+</sup>, and Gr-1<sup>+</sup> leukocyte subsets expressed the CCL5 receptors CCR1 and CCR5, tumors were disaggregated, cells isolated by FACS sorting, and RNA was extracted. Expression of the CCL5 receptors CCR1 and CCR5 was investigated using real-time RT-PCR. All three populations of infiltrating leukocytes expressed both CCR1 and CCR5 (Fig. 3C). As the tumor cells did not express CCR1 or CCR5 in [vitro](, we concluded that the majority of CCR1 and CCR5 mRNA detected in tumor extracts was derived from the infiltrating cells.

To determine whether neoplastic cells expressed CCR5 in [vitro](, cryostat sections from tumors were stained for CCL5 using immunohistochemistry. CCR5 was detected on infiltrating cells. However, we could not detect this receptor on neoplastic cells in any sections tested (Fig. 3D).

**A CCR1/5 Antagonist Slows 410.4 Tumor Growth in Vivo.** Because 410.4 cells produced CCL5 and tumor-infiltrating leukocytes expressed CCR1 and CCR5, we studied the action of an antagonist against these receptors in [vitro](. Met-CCL5 is a well-characterized antagonist for CCR1 and CCR5 and has therapeutic activity in murine models of inflammation (11, 12). In four separate experiments, mice were treated with a daily i.p. injection of control diluent or Met-CCL5 after tumor cell injection. Tumor volume was measured at regular intervals. The dose and schedule of Met-CCL5 was determined by activity of this compound in models of multiple sclerosis and renal transplant rejection (18, 19).

Treatment of the 410.4 tumor with Met-CCL5 significantly slowed tumor growth (Fig. 4A). The AUC, calculated for the tumor volume of each individual mouse, was significantly reduced in Met-CCL5 (1.7533)-treated mice compared with control (3.0749, P = 0.001, Fig. 4B).

Tumors were also excised and weighed after 5 weeks of growth; tumor weights were significantly different (median control versus Met-CCL5, 166 and 71 mg, respectively, P = 0.03; Fig. 4C). In a subsequent experiment, mice were treated with Met-CCL5 after the tumor had been growing in [vitro]( for 14 days. This regime also resulted in a significant decrease in tumor weight (median control versus Met-CCL5, 166 and 68 mg, respectively, P = 0.01; Fig. 4D). On macroscopic examination, Met-CCL5-treated tumors were smaller and considerably paler than control tumors (Fig. 4E).

In addition, treatment of 410.4 cells with Met-CCL5 in [vitro]( had no effect on cell growth when doses from 1 to 100 μg/ml were tested (data not shown). This suggests that treatment with Met-CCL5 did not directly inhibit 410.4 cell proliferation in [vitro](.

**Met-CCL5 Inhibits Leukocyte Infiltration into the 410.4 Tumor.** To determine whether Met-CCL5 treatment decreased leukocyte infiltration, the proportion of infiltrating leukocytes was determined by FACS analysis in disaggregated tumors at 4 weeks of tumor growth. In Met-CCL5-treated tumors, the percentage of F4/80<sup>+</sup> macrophages was significantly decreased from 17.91 to 9.6% (P = 0.04, Table 1). F4/80<sup>+</sup> macrophages were also analyzed by immunohistochemistry. F4/80<sup>+</sup> macrophages were present around the periphery of both control and Met-CCL5-treated tumors (Fig. 5, C and D, respectively). However, Met-CCL5 appeared to prevent macrophage migration into the central tumor areas compared with control (Fig. 5, B and A, respectively). There was also a small increase in the percentage of CD8<sup>+</sup> lymphocytes in Met-CCL5-treated tumors com-
pared with control tumors (19.61% compared with 16.63%, \( P = 0.02 \); Table 1). Numbers of Gr-1\(^+\) neutrophils were not significantly different in control and Met-CCL5 tumors. The total number of cells from Met-CCL5-treated tumors (11.29 ± 3.39 \( \times 10^6 \) cells/tumor) was also significantly decreased compared with control tumors (27.48 ± 2.06 \( \times 10^6 \) cells/tumor, \( P = 0.02 \); data not shown).

**Met-CCL5 Reduces Tumor Cell Islands and Increases Necrosis.**

H&E sections from control and Met-CCL5-treated tumors were scored for areas of tumor cell islands, stroma, and necrosis using a 25-point modified Chalkley point array (20). The percentage of the area occupied by tumor cell islands, stroma, and necrosis was calculated. In Met-CCL5-treated tumors, there was a significant decrease in the area occupied by tumor cell islands when compared with control treated tumors (47.00% compared with 56.35%, respectively, \( P = 0.002 \); Table 2). The areas occupied by stromal cells and necrosis were increased in Met-CCL5-treated tumors compared with control tumors. Considered together, there was a significant increase in stromal and necrotic regions in Met-CCL5-treated tumors compared with control tumors (52.94% compared with 43.65%, respectively, \( P = 0.002 \); Table 2).

**DISCUSSION**

To our knowledge, this is the first demonstration that a chemokine antagonist, with documented activity in models of inflammatory disease, inhibits the growth of an experimental cancer. Our data also provide direct evidence for the tumor-promoting activity of the macrophage infiltrate in this experimental breast cancer.

The 410.4 model was originally derived from a spontaneous breast tumor in a BALB/cfC3H mouse (21). The leukocyte infiltrate that develops in this tumor correlates well with that reported for human breast cancer (22). The major infiltrating leukocyte populations were
secreted CXCL2, a potent neutrophil chemoattractant, and the tumor expresses CCL2. Neither of these chemokines are affected by Met-CCL5.

There was a concomitant decrease in the percentage of epithelial tumor islands in Met-CCL5-treated tumors. Met-CCL5-treated tumors were also paler in appearance and showed increased areas of necrosis.
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compared with control-treated tumors. This would suggest an additional effect on the tumor vasculature. Indeed, there is a study that endothelial cells express CCR5 (27). However, endothelial cells did not express CCR5 in our experimental conditions.

Preliminary data (not shown) using RNA from control- and Met-CCL5-treated tumors and a gene expression profiling system suggest that treatment with Met-CCL5 may promote apoptosis and decrease angiogenesis within the tumor. Several antiapoptotic genes, namely Bcl-x, inhibitor of apoptosis survivin, and an inhibitor of tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis (caspase-c-FLIP), were all down-regulated in Met-CCL5-treated tumors. Similarly, proteins that have been shown to play roles in angiogenesis, fibroblast growth factor receptor 2, urokinase-type plasminogen activator, and tissue inhibitor of metalloproteinase-1 were all down-regulated in Met-CCL5-treated tumors. These results may help to explain the increased areas of necrosis and the macroscopic decrease in vasculature observed in Met-CCL5-treated tumors. This suggests that macrophages contribute factors that are important for growth, angiogenesis, and survival of the tumor.

Currently, there are some cancer therapies that target endothelial cells, but little attention is paid to inhibiting the leukocyte infiltrate. We believe there is justification to extend these observations to a range of murine tumors, assessing a number of chemokine receptor antagonists. In developing this novel approach, it will also be important to investigate combinations of chemokine antagonists that can inhibit the influx of different leukocyte populations within a tumor. In the model described in this article, it would be especially interesting to combine Met-CCL5 with inhibitors that prevent binding of CCL2 and CXCL2 to their receptors.

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