Monocyte Chemoattractant Protein Inhibits the Generation of Tumor-reactive T Cells

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INTRODUCTION

T cells reactive to tumor antigens have been demonstrated to be capable of eradicating disseminated malignancy in murine animal models. T cells must be sensitized to tumor antigens in vivo to acquire antitumor reactivity. T-cell sensitization has been demonstrated to be dependent on host antigen-presenting cells. Tumor-associated macrophages are a heterogeneous population of cells that may have both inhibitory and stimulatory influences on the sensitization of naive T cells. Here we demonstrate that a weakly immunogenic tumor, the MCA 205 sarcoma, produces substantial amounts of murine monocyte chemoattractant protein 1 (MCP-1). Neutralization of MCP-1 during in vivo T-cell sensitization resulted in T cells that possessed enhanced therapeutic activity against established pulmonary metastases. These T cells sensitized during MCP-1 depletion also exhibited enhanced production of IFN-γ upon recognition of tumor targets. These results demonstrate that MCP-1 can have a potent inhibitory influence on the development of tumor-reactive T cells.

MATERIALS AND METHODS

Tumor. The MCA 205 sarcoma is a 3-methylcholanthrene-induced tumor of B6 origin, kindly provided by Dr. James Yang (Surgery Branch, National Cancer Institute, Bethesda, MD). Tumor was routinely passed in vivo in syngeneic mice and was used within the third to tenth transplantation generation. Single-cell suspensions were prepared by digestion of solid tumor with a mixture of 0.1% collagenase, 0.01% DNase, and 2.5 units/ml hyaluronidase (Sigma Chemical Co., St. Louis MO) for 3 h at room temperature. The cells were filtered through a layer of no. 100 nylon mesh, washed, and resuspended in HBSS and used in the described experiments.

Antibodies. The following monoclonal antibodies obtained from the American Type Culture Collection were used for the described experiments: CD11b (Mac-1; Ref. 20) CD11c (N418; Ref. 21), CD8 (2.43; Ref. 22), CD4 (GK1.5; Ref. 23), and F4/80 (anti-macrophage; Ref. 24). The hamster anti-murine MCP-1 was obtained from Pharmingen. Neutralizing polyclonal antisera directed at murine and rat MCP-1 was prepared in rabbits as described and macrophage growth circuit (11). Macrophages isolated from an macrophage CSF secreting tumor cell line had an unusually high S phase of 12%. Other chemokines, i.e., MCP-1, have also been isolated from tumor-cell culture supernatants, and these factors also possess potent monocyte chemoattractant ability (12–14). The methylcholanthrene-induced tumors have been demonstrated to be densely infiltrated with macrophages, yet the chemokines responsible for this infiltrate have not been defined (10). It seems likely that there are several interacting factors that will influence the overall infiltration and activation state of tumor-infiltrating macrophages.

Human MCP-1 was originally purified from the tissue culture supernatant of a malignant glioma as an Mr, 8,700 molecular weight protein that possessed substantial chemotaxis activity for monocytes (15). Subsequently, it was demonstrated to be a potent chemotaxin for T lymphocytes, with little activity for purified neutrophils (16). The molecular cloning of MCP-1 revealed that it was identical to the human JE gene (17). JE had been identified in mice as an early-response gene transcribed by platelet-derived, growth factor-stimulated fibroblasts (18). It has since been recognized that MCP-1 can be secreted by lymphocytes, macrophages, fibroblasts, endothelium, and smooth muscle cells after appropriate activation signals. MCP-1 is a member of the C-C family of chemokines, which share receptors and some biological functions (19). It appears that a wide variety of cell types are capable of secreting MCP-1 in response to inflammatory stimuli.

These studies were undertaken to explore the role of MCP-1 secretion in the sensitization of T cells to tumor antigens. We demonstrate that the MCA 205 sarcoma secretes substantial amounts of MCP-1 in vivo and in vitro. Neutralization of MCP-1 activity did not alter the degree of macrophage infiltrate into the tumor, nor did it change the growth rate of the tumor. However, neutralization of MCP-1 did lead to the generation of T cells that were substantially more active at eliminating tumor. These findings suggest that tumor cell-secreted MCP-1 exerts a negative regulatory influence on the development of tumor-reactive T cells.
was a generous gift of Dr. Theodore Standiford (University of Michigan, Ann Arbor, MI; Ref. 25).

**In Vivo Depletion of MCP-1.** On the day of tumor inoculation to sensitize T cells, mice were injected i.p. with 0.5 ml of MCP-1 neutralizing sera or with normal rabbit sera. Mice were then injected with 0.25 ml i.p. every other day for four more doses. None of the mice exhibited any signs of serum sickness during the treatment with MCP-1 antisera or normal rabbit sera.

**Tumor-draining LN Cells.** MCA 205 cells (1.5 × 10⁶) in 0.1 ml HBSS were inoculated intradermally in the flanks of B6 mice. Tumor-draining inguinal LN were harvested under sterile conditions 9 days following tumor inoculation. Lymphocytes were dissociated mechanically and prepared into a single-cell suspension by teasing apart the LNs using 20-gauge needles and then pressing the tissue fragments with the blunt end of a 10-ml plastic syringe.

**In Vitro Anti-CD3/IL-2 T-Cell Activation.** Single-cell suspensions of LN cells were activated for 2 days with an anti-CD3 mAb (145-2C11, American Type Culture Collection; Ref. 26) immobilized on 24-well tissue culture plates at 4 × 10⁶ cells/well as described previously (9). Anti-CD3 activation, the cells were harvested, washed, and resuspended at a concentration of 1.5 × 10⁵ cells/ml of CM containing 4 units/ml of IL-2 and cultured in LIFecell tissue culture flasks (Fenwall Division, Baxter Corp., Deerfield, IL). CM consisted of RPMI 1640 supplemented with 10% heat-inactivated FCS, 0.1 mM nonessential amino acids, 1 µM sodium pyruvate, 2 mM fresh L-glutamine, 100 µg/ml streptomycin, 1000 units/ml penicillin, 50 µg/ml gentamicin, 0.5 µg/ml fungizone (all from Life Technologies, Inc., Grand Island, New York) and 5 × 10⁻⁵ M 2-mercaptoethanol. Three days later, the cells were harvested, washed, and resuspended in HBSS for adoptive immunotherapy.

**Procedure for Adoptive Immunotherapy of Pulmonary Metastases.** Three-day lung metastases were established by injecting mice with 3 × 10⁵ MCA 205 cells in 1 ml HBSS i.v. Three days after tumor establishment, the indicated populations of effector cells, suspended in 1 ml of HBSS, were administered i.v. to each mouse. Approximately 18 days after tumor inoculation, the mice were sacrificed, and the lungs were insufflated with India ink and then fixed in Fekete’s solution. Pulmonary metastases were counted without knowledge of the treatment. For all experiments, lungs with pulmonary nodules too numerous to count were arbitrarily assigned a value of 250 for statistical analysis.

**IL-2.** Recombinant human IL-2 was kindly supplied by Chiron Therapeutic (Emeryville, CA) with a specific activity of 3 × 10⁷ units/mg protein. In this report, all units of IL-2 are expressed in Cetus units, where 1 Cetus unit is equivalent to 6 IU.

**Immunohistochemical Staining.** Tumors were harvested from animals, placed in OCT media, and flash frozen in liquid nitrogen. Tumor sections 5 µm thick were obtained using a cryostat microtome. Sections were placed onto charged glass slides and allowed to dry for 1 h. The slides were blocked with avidin biotin complexes, washed, and then incubated for 1 h with the primary antibody or a control antibody of the same species. The slides were washed and incubated with mouse adsorbed, biotinylated goat anti-rat IgG or biotinylated goat anti-hamster IgG for 1 h. Slides were washed and incubated with performed avidin/peroxidase complexes (Vector Labs) and then stained with 3-amino-9-ethyl-carbazole.

Flow Cytometry Analysis. Cells were analyzed by indirect immunofluorescence using FITC-conjugated mouse anti-rat immunoglobulin (PharMingen, San Diego, CA). Primary mAb directed at the following murine epitopes were used: Thy 1.2 (30-H12; Becton-Dickinson Labware, Bedford, MA); CD4 (L3T4); and CD 8 (2.43). Membrane fluorescence was analyzed on a FACSscan (Becton-Dickinson, San Jose, CA). Routinely, 5 × 10⁶ cells were stained, and 10⁵ cells in each sample were analyzed. The percentage of positive cells was calculated by subtracting the background staining of the negative control.

**Chemokine and Cytokine ELISA.** MCP-1 was quantified using an ELISA specific for murine MCP-1 (Fig. 2). Routinely, the MCA 205 sarcoma has been demonstrated to be densely infiltrated with macrophages (27). To explore which chemokines might be responsible for this infiltrate, immunochemistry was performed on 1-week-old tumor with an antibody directed at MCP-1 (Fig. 1). Modest levels of MCP-1 were observed throughout the tumor bed, with higher amounts noted in the area of the pseudocapsule surrounding the tumor. This area has a much higher concentration of macrophages, particularly those that express F4/80 antigen. The secretion of MCP-1 by isolated tumor cells were confirmed by performing an ELISA specific for murine MCP-1 (Fig. 2). Routinely, the MCA sarcomas are passaged in vivo to maintain stable cell growth and immunogenicity characteristics. Tissue culture media conditioned by a single-cell suspension of the fresh isolated in vivo passaged tumor cell line, MCA 205, revealed 6 ng/ml of MCP-1. A similar amount of MCP-1 was secreted by the two in vitro passaged cell lines 20SH12 and 207G11. These results suggest that the majority of the chemokine detected in vivo by immunochemistry is secreted by the tumor cells and not by the tumor-associated macrophages.

The ability of neutralization of tumor-derived MCP-1 to alter tumor growth and LN hyperplasia was tested. Groups of five mice each were inoculated bilaterally with tumor and treated with anti-MCP-1 antibodies or normal rabbit serum every other day for 9 days (Table 1). The primary tumor size was slightly smaller in the MCP-1-treated mice than in control mice, but this size difference did not reach statistical significance. No animals were excluded from the statistical analysis.

**RESULTS**

**MCP-1 Is Produced by Tumor Cells in Situ and in Vitro.** The MCA 205 sarcoma has been demonstrated to be densely infiltrated with macrophages (27). To explore which chemokines might be responsible for this infiltrate, immunochemistry was performed on 1-week-old tumor with an antibody directed at MCP-1 (Fig. 1). Modest levels of MCP-1 were observed throughout the tumor bed, with higher amounts noted in the area of the pseudocapsule surrounding the tumor. This area has a much higher concentration of macrophages, particularly those that express F4/80 antigen. The secretion of MCP-1 by isolated tumor cells were confirmed by performing an ELISA specific for murine MCP-1 (Fig. 2). Routinely, the MCA sarcomas are passaged in vivo to maintain stable cell growth and immunogenicity characteristics. Tissue culture media conditioned by a single-cell suspension of the fresh isolated in vivo passaged tumor cell line, MCA 205, revealed 6 ng/ml of MCP-1. A similar amount of MCP-1 was secreted by the two in vitro passaged cell lines 20SH12 and 207G11. These results suggest that the majority of the chemokine detected in vivo by immunochemistry is secreted by the tumor cells and not by the tumor-associated macrophages.

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**Statistical Analysis.** The significance of differences in the numbers of pulmonary metastases between groups was determined on the raw data of pulmonary metastases number by the Kruskal-Wallis ANOVA of ranks, using SigmaStat software (Jandel Scientific, San Rafael, CA). Two-sided Ps of <0.05 were considered statistically significant. No animals were excluded from the statistical analysis.
within the tumor bed were also not different between the two groups (data not shown). Tumors from mice treated with MCP-1 antiserum demonstrated equivalent levels of infiltration of CD4 cells and CD8 cells (Fig. 3). These results suggest that other chemotactic cytokines may be important for the infiltration of these cells into the growing MCA 205 sarcoma. Of note, the MCA 205 sarcoma does not secrete RANTES, another potent chemoattractant of T cells.

The therapeutic reactivity of T cells from the draining LNs of mice treated with MCP-1 antiserum was tested. The draining LNs were harvested, prepared into a single-cell suspension, and activated by the anti-CD3/IL-2 method. Total cell number increased 1.81–2.10-fold in 5-day culture, representing a net T-cell expansion of approximately 6-fold (Table 1). T-cell growth rates were not diminished nor augmented in the T cells derived from the anti-MCP-1-treated mice. After the 5-day culture, the cells were all T cells, approximately 20% CD4 T cells and 60% CD8 T cells, which is similar to previous results with this T-cell culture system (6). In two independent experiments, T cells derived from the anti-MCP-1-treated mice were substantially more active at eliminating pulmonary metastases than the control serum-treated mice (Table 2). Increased numbers of T cells from either group were able to completely eliminate pulmonary metastases (data not shown). These results suggest that endogenous MCP-1 production exerts a negative regulatory influence on the development of tumor-sensitized T cells.

### Table 1  Tumor growth and LN characteristics in mice subjected to MCP-1 neutralization

<table>
<thead>
<tr>
<th>Antibody treatment</th>
<th>Tumor size (mm²)</th>
<th>Cell no. per LN</th>
<th>Overall cell expansion</th>
<th>Cultured cell phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
<td>Exp. 2</td>
<td>Thyl.2</td>
<td>CD4</td>
</tr>
<tr>
<td>Anti-MCP-1</td>
<td>28.8 ± 4.4</td>
<td>21.6</td>
<td>22.5</td>
<td>2.31</td>
</tr>
<tr>
<td>NRS</td>
<td>39.3 ± 6.7</td>
<td>24.5</td>
<td>22.0</td>
<td>1.87</td>
</tr>
</tbody>
</table>

*Exp., experiment; NRS, normal rabbit serum.*
Tumor-sensitized T cells release IFN-γ in response to recognition of tumor antigen (7). T cells derived from anti-MCP-1-treated mice secreted twice as much IFN-γ as T cells isolated from nonimmune sera-treated mice (Fig. 4). The IFN-γ production correlates well with the specific activity of the T cells at eliminating pulmonary metastases. No antigen-specific release of IL-4 or IL-10 was detected (data not shown), suggesting that MCP-1 production by the tumor is inhibiting the generation of a TH1-type T-cell response, rather than promoting a switch to a TH2-type T-cell response.

**Table 2. Therapeutic reactivity of T cells isolated from MCP-1-neutralized mice**

<table>
<thead>
<tr>
<th>Source of T cells</th>
<th>Mean no. of pulmonary metastases (SE)</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>250 (0)</td>
<td>250 (0)</td>
<td></td>
</tr>
<tr>
<td>NRS</td>
<td>86 (21)</td>
<td>122 (17)</td>
<td></td>
</tr>
<tr>
<td>Anti-MCP-1</td>
<td>20 (6)*</td>
<td>12 (5)*</td>
<td></td>
</tr>
</tbody>
</table>

* Mice (four to five/group) bearing 3-day MCA 205 sarcomas were treated with adoptively transferred T cells isolated from mice treated with normal rabbit serum (NRS) or anti-MCP-1 serum, as described in “Materials and Methods.” Fourteen days after adoptive transfer of T cells, the lungs were harvested, and the number of pulmonary metastases was enumerated. In experiment (Exp.) 1, mice were treated with $10 \times 10^6$ T cells, whereas in experiment 2, mice were treated with $5 \times 10^6$ T cells.

DISCUSSION

These results demonstrate that neutralization of tumor-produced MCP-1 can augment T-cell sensitization to tumor antigens, resulting in a T-cell population with substantially augmented antitumor reactivity. These T cells also demonstrate substantially increased amounts of IFN-γ production in response to tumor targets. Antagonism of MCP-1 did not decrease the number of tumor-associated macrophages, nor did it alter the growth rate of the tumor. This suggests that endogenously produced MCP-1 has a negative impact on the generation of a therapeutic T-cell response to tumors and implies that MCP-1 is not required for the generation of a T-cell response to tumor antigens.

Tumor-associated macrophages are most likely a heterogeneous collection of cells with diverse function (8). It has been demonstrated conclusively that host antigen-presenting cells, derived from bone marrow, are responsible for presenting antigens to T cells during the development of a cytolytic T-cell response to tumor antigens (28). Likely candidate cells for this antigen-presenting cell function would be dendritic cells or macrophages. Recent reports that show that blood macrophages can be differentiated to dendritic cells by culture in IL-4/granulocyte-macrophage-CSF raises the possibility that tumor-associated macrophages may have contained some subpopulations.
with this characteristic (29). MCP-1 has also been demonstrated to be a potent chemoattractant for dendritic cells (30). It is, therefore, conceivable that influx of a subset of tumor-associated macrophages with potent antigen-presenting capability would have immunostimulating properties for the T-cell response to tumors. The results presented here suggest that macrophages that are infiltrating the tumor in response to MCP-1 are inhibitory to the efficient sensitization of tumor-reactive T cells.

In human malignancies, gliomas that secrete large amounts of MCP-1 have been demonstrated to have a more substantial infiltrate of macrophages (31). Similar features have been observed among clones of a murine sarcoma that secreted various amounts of MCP-1 (32). However, even in the murine tumor cell line that did not secrete MCP-1, there was still 15% macrophages, suggesting that other chemoattractant factors for macrophages can be secreted by tumors. Murine tumors transfected with the MCP-1 gene were substantially less tumorigenic and had a higher proportion of F4/80-positive cells than nontransfected controls (33). The MCA 205 sarcoma studied here is only minimally infiltrated with F4/80-positive cells, and this was not substantially moderated by neutralizing MCP-1 activity.

MCP-1 is critical to the development of an immune response to inhaled fungus, Cryptococcus neoforms (34). Mice treated with MCP-1 neutralizing antibodies demonstrated reduced macrophages in their bronchoalveolar lavage fluid and reduced granuloma formation in their lungs. This reduced inflammatory infiltrate correlated with an increased burden of the fungus in the lungs (34). In contrast, granulomas caused by schistosoma, MCP-1 is much less important in primary granuloma formation (35). In this infection, MCP-1 neutralization does not reduce primary granulomas but does attenuate the late inflammatory phase of the immune response. Development of the primary granulomas is more dependent on a TH1 T-cell response, whereas the development of the secondary granulomas is dependent on a TH2 response. This, combined with our results would suggest that MCP-1 production may be inhibiting to a TH1-type cytokine response. In support of this observation, mice that overproduced MCP-1 have a decreased ability to eliminate Leishmania and tuberculosis, both of which depend upon TH1-type responses to control the infection (36).

There are two leading hypotheses for the mechanism through which MCP-1 neutralization would enhance the sensitization of tumor-reactive T cells. The first would be the inhibition of infiltration of a minor percentage of macrophages that possess the inhibitory effect. However, immunohistochemistry of the tumors with several different macrophage markers failed to reveal a substantial difference in the tumor-associated macrophages derived from the anti-MCP-1-treated mice. Formal quantification of tumor-associated macrophages is not feasible; therefore, we cannot rule out a small difference in macrophage numbers with MCP-1 neutralization. Alternatively, neutralization of MCP-1 during tumor growth could be altering the phenotype of the tumor-associated macrophages, such as the inhibitory phenotype to TH1 cells does not develop. MCP-1 has been reported to increase CD11b and CD11c on monocytes; however, we did not observe diminished levels of either of these antigens on the tumor-associated macrophages in the anti-MCP-1-treated mice (37). This would implicate MCP-1 in other, yet undefined, pathways that either promote the generation and expansion of TH2 cells or inhibit the generation of TH1 cells. Furthermore, these results would suggest that other chemokines are important in causing the infiltrate of tumor-associated macrophages and perhaps in determining the immunogenicity of the methylcholanthrene-induced sarcomas.

REFERENCES


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