Malignant pleural effusion (MPE), the accumulation of pleural fluid caused by cancer metastasis to the pleural space, is a common clinical condition with an annual incidence rate (500 patients/million) that equals that of lung cancer (1–5). For unknown reasons, some tumors (eg, adenocarcinomas) are more strongly associated with the condition than others (1–5). Although MPE impairs quality of life and survival of cancer patients, no specific treatment is available, and therapy has been focused on treating the primary tumor, fluid drainage, and pleural space ablation via introduction of sclerosants (pleurodesis), which often leads to morbidity and mortality (1–10).

New blood vessel formation (angiogenesis), increased permeability of existing and newly formed blood vessels (vascular hyperpermeability), and an inflammatory host response triggered by the presence of tumor cells in the pleural cavity (tumor-associated inflammation) have all been implicated in MPE pathogenesis (1–5). Recent work has highlighted the important etiologic role of intrapleural expression of angiogenic and proinflammatory mediators, new blood vessel formation (angiogenesis), increased permeability of existing and newly formed blood vessels (vascular hyperpermeability), and an inflammatory host response triggered by the presence of tumor cells in the pleural cavity (tumor-associated inflammation) have all been implicated in MPE pathogenesis (1–5). Recent work has highlighted the important etiologic role of intrapleural expression of angiogenic and proinflammatory mediators.

**Background**

Tumor cells in malignant pleural effusions (MPEs) are an important source of monocyte chemoattractant protein (MCP)-1. However, the role of tumor-derived MCP-1 in the pathogenesis and progression of MPE has not been determined.

**Methods**

B16 mouse skin melanoma cells, which are deficient in MCP-1 expression, and mouse Lewis lung cancer (LLC) cells, which express high levels of MCP-1, were engineered to stably express MCP-1 and short hairpin RNAs (shRNAs) targeting the MCP-1 transcript, respectively. Cells were injected into the pleural cavities of syngeneic immunocompetent mice, and MPE volume and pleural tumors were quantified at necropsy (day 14). MCP-1 and other mediators were determined by cytometric bead array and enzyme-linked immunosorbent assay, and mononuclear and endothelial cells were identified by immunolabeling of F4/80 and factor VIII-related antigen respectively. Mouse survival was assessed using Kaplan–Meier analysis. Vascular permeability in mice with MPE was assessed using albumin-binding Evans blue. Statistical tests were two-sided.

**Results**

LLC cells expressing shRNA against MCP-1 elaborated less than 5% of the MCP-1 level in cells expressing nonspecific shRNA (control cells), and intrapleural delivery of these cells resulted in less MPE (mean MPE volume = 86 and 585 µL, respectively; difference = 499 µL; 95% confidence interval [CI] = 331 to 669 µL; \( P < .001 \)), reduced MCP-1 levels in the pleural fluid, and lower mortality than when control cells were delivered. Overexpression of MCP-1 in intrapleurally injected B16 melanoma cells led to increased MPE and reduced survival. In mice with MPE, MCP-1 was a potent inducer of vascular permeability, mononuclear recruitment, and, in pleural tumors, of angiogenesis.

**Conclusion**

MCP-1 produced by tumor cells is an important determinant of their capacity to induce the formation of MPE and may be a useful target for the treatment of malignant pleural disease.
including vascular endothelial growth factor (VEGF), interleukin (IL)-6, and tumor necrosis factor (TNF)-α by tumor and host cells in the malignancy-affected pleura (11–16).

High levels of monocyte chemoattractant protein (MCP)-1 (also referred to as chemokine ligand 2) have been detected in human MPE. It has been proposed that the increased levels derive from tumor cells, and that the chemoattractant protein is involved in macrophage recruitment to MPE (17). In previous studies of adenocarcinoma-induced MPE in immunocompetent mice (12–14), we have demonstrated that: 1) MCP-1 is produced locally; 2) tumor cell TNF-α/nuclear factor (NF)-κB promotes MPE and chemokine expression; and 3) the therapeutic effects of zoledronic acid are associated with suppression of MCP-1 expression. Based on these observations and on the angiogenic and proinflammatory properties of MCP, which suggest that it could mediate pleural fluid accumulation (18,19), we hypothesized that the ability of cancer cells to induce MPE depends on MCP-1 secretion in the pleural cavity.

**Methods**

**Cell Lines**

Mouse Lewis lung cancer (LLC), skin melanoma (B16-F10, hereafter referred to as B16), and macrophage (RAW264.7) cell lines were purchased from the American Type Culture Collection (Manassas, VA) and were cultured as described previously (12–14). For intrapleural injections, cells were harvested from culture dishes during log phase. Cells were exposed to 0.25% trypsin, 0.02% EDTA solution for 2 minutes, washed with calcium and magnesium-free phosphate-buffered saline (PBS), resuspended in PBS, and titrated using a hemocytometer. Only cell populations in which more than 90% of cells were viable as assessed by Trypan blue exclusion were used.

**Short Hairpin (sh)RNA Constructs, MCP-1 Overexpression Plasmids, and Transfection**

The cDNA sequence of the mRNA of the murine MCP-1 gene (accession number: NM011333) was retrieved from Entrez Nucleotide (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=141803162), and three 19-bp target sequences were manually identified within the 584-bp sequence using established empiric rules (26). Using the identified target sequences and their complementary sequences, 64-bp double-stranded cDNAs that included a 9-bp hinge region and nonoverlapping overhangs were designed to target MCP-1 mRNA (Supplementary Figure 1, available online). These constructs were cloned into the pSUPER.retro.puro expression vector (Oligogene, Seattle, WA), and correct insertion was verified by restriction analysis according to the manufacturer’s instructions. The original unprocessed control containing a 976-bp random sequence was used as the control. The vectors expressing anti–MCP-1 shRNA constructs were designated sh166, sh401, and sh436, respectively, according to the position of the target sequences within the MCP-1 transcript. Only the sh166 and sh436 vectors were used for experiments because the sh401 vector did not prove to be a stable construct. Control, sh166, and sh436 vectors (1.5 µg each) were transfection into LLC cells using linear polyethylenimine (Polyplus Transfection, San Marcos, CA), at a negative/positive charge ratio of 10:1. Several LLC cell clones stably expressing the random sequence, sh166, or sh436 were selected using puromycin (1 µg/mL medium for 10 days) and were screened for MCP-1 expression by enzyme-linked immunosorbent assay (ELISA, Carlsbad, CA) of cell-free supernatants. One clone from each of control-, sh166 plasmid-, and sh436 plasmid-transfected LLC cells were selected based on MCP-1 expression and tested for expression of mouse macrophage inflammatory protein (MIP)-2, VEGF, TNF-α, interferon (IFN)-γ, IL-6, IL-10, and IL-12p70 by cytometric bead array (CBA)/ELISA, Carlsbad, CA, to rule out off-target effects of shRNA-mediated silencing of MCP-1. The stable transfected LLC clones were passaged several times, and the cytokine measurements were repeated to ensure sustained inhibition of MCP-1 expression with minimal off-target effects.

For forced overexpression of MCP-1, the mouse MCP-1 cDNA (bp 89–535) was cloned into the TOPO expression vector according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA) using primers encompassing the MCP-1 transcript (Supplementary Figure 1, available online), and correct insertion was verified by restriction analysis. A control vector containing a gene encoding β-gal was used as the control. MCP-1– and β-gal–expressing plasmids were transfected into B16 cells using linear polyethylenimine as described above; stable clones were selected on the basis of blasticidin resistance (10 µg/mL medium for 10 days) and were assessed for MCP-1, MIP-2, VEGF, TNF-α, IFN-γ, IL-6, IL-10, and IL-12p70 expression using ELISA/CBA as described above. In all experiments, three different MCP-1– or β-gal–expressing clones were pooled to rule out nonspecific effects.

**Animal Models**

Wild-type C57BL/6 mice were purchased from the Hellenic Pasteur Institute in Athens, Greece, and the Alexander Fleming Pasteur Institute in Athens, Greece, and the Alexander Fleming Institute in Athens, Greece.
Biomedical Research Center in Vari, Greece, and inbred at the Animal Care facilities of the Evangelismos General Hospital (Athens, Greece). Animal experiments were approved by the Veterinary Administration Bureau of the Prefecture of Athens, Greece, and were conducted according to International Animal Care and Use Guidelines (http://grants.nih.gov/grants/olaw/GuideBook.pdf). Mice used for experiments were sex, weight (20–25 g), and age (8–12 weeks) matched.

In total, 267 C57BL/6 mice were used for this study, and the mice were treated as follows: In two separate pilot experiments, 16 C57BL/6 mice received intrapleural injections of 50 µL PBS (n = 8) or 1.5 x 10^5 tumor cells in 50 µL PBS (n = 8). These mice were sacrificed by CO2 asphyxiation after 14 days for determination of MCP-1 levels in pleural fluid and blood. In subsequent experiments, C57BL/6 mice (n = 108) received intrapleural injections of 1.5 x 10^5 tumor cells expressing varying amounts of MCP-1 in three separate experiments. These mice were injected with wild-type LLC cells (n = 31 mice), LLC cells expressing stuffer control (ie, a random sequence) (n = 13), LLC cells expressing anti-MCP-1 shRNA sh166 (n = 13), LLC cells expressing anti-MCP-1 shRNA sh436 (n = 13), wild-type B16 cells (n = 10), B16 cells expressing ß-galactosidase (ß-gal) (control, n = 15), and B16 cells expressing MCP-1 (n = 13), respectively. All of these mice were killed after 14 days for determination of body mass, pleural effusions and tumors, inflammatory cells, cytokines, pleural vascular permeability, pleural tumor proliferation, apoptosis, and angiogenesis. In two separate experiments, an additional 101 C57BL/6 mice received intrapleural injections of 1.5 x 10^5 tumor cells expressing varying amounts of MCP-1 and were monitored for survival. In these experiments, mice were injected with wild-type LLC cells (n = 35), LLC cells expressing RNA of random sequence (control, n = 7), LLC cells expressing anti-MCP-1 shRNA sh166 (n = 7), LLC cells expressing anti-MCP-1 shRNA sh436 (n = 7), wild-type B16 cells (n = 15), B16 cells expressing ß-gal (control, n = 14), and B16 cells expressing MCP-1 (n = 16), respectively. Another 15 C57BL/7 mice were used for three different Miles (skin vascular permeability) assays (n = 5 for each). Finally, 27 C57BL/6 mice received intrapleural injections of bovine serum albumin (BSA, n = 9), recombinant mouse VEGF (n = 9), or recombinant mouse MCP-1 (n = 9). These mice were killed after various time intervals for determination of pleural fluid exudation, vascular permeability, and number of inflammatory cells.

Intrapleural injections were performed as described previously, using 1.5 x 10^5 cells per 50 µL PBS per mouse (12–14). After 14 days, mice were killed by CO2 exposure, and pleural fluid, blood, and tumors were collected (12–14). When an MPE was not present, the pleural fluid volume was set to 20 µL, the amount of pleural fluid present in healthy mice (12) and retrieved by pleural lavage with 1 mL of normal saline, as described previously (20). Measurements in MPE or pleural lavage were corrected for protein content. For survival studies, mice were observed daily and sacrificed when moribund.

Cytokine Determinations

Mouse and human MCP-1 (detection limits: 31.3 and 5.0 pg/mL, respectively), MIP-2 (detection limit: 15.6 pg/mL), and mouse VEGF (detection limit: 31.3 pg/mL) were determined by ELISA (R&D Systems, Minneapolis, MN and Peprotech EC, London, UK). Mouse TNF-α, IFN-γ, MCP-1, and IL-6, IL-10, and IL-12p70 were measured using a CBA (BD Biosciences, San Jose, CA) (detection limits: 7.3, 2.5, 52.7, 5.0, 17.5, and 10.7 pg/mL, respectively) (21).

Human MPE

MPE fluid and matched serum were obtained during the initial diagnostic thoracenteses performed in 24 consecutive patients with MPE, for whom the causative neoplasms were non–small-cell lung cancer (n = 12 patients), breast adenocarcinoma (n = 5), mesothelioma, (n = 4), thyroid cancer (n = 1), large-cell neuroendocrine lung cancer (n = 1), and adenocarcinoma of unknown primary (n = 1), and 8 patients with congestive heart failure who were treated at the Evangelismos General Hospital, Athens, Greece, from September 2006 through May 2007. The study was approved by the ethics committee of the hospital, and all patients gave written informed consent. Human MPE fluids and matched sera were handled like mouse samples (12–14, 22). MPE was always diagnosed based on positive pleural fluid cytology or pleural tissue histology (1,2). Diagnostic criteria for pleural effusion due to congestive heart failure were 1) transudative effusion, 2) echocardiographic evidence of left ventricular dysfunction, 3) serum pro-brain natriuretic peptide levels above 1500 pg/mL, 4) increased heart size on the chest x-ray, 5) negative pleural fluid bacteriology and cytology, and 6) improvement with diuretic and/or inotropic drugs (1,23).

RNA Isolation and Northern Blots

RNA was extracted from frozen tissue using Trizol (Invitrogen, Carlsbad, CA). Complementary DNA (cDNA) probes were generated by polymerase chain reaction using primers for MCP-1 (forward: CACCATGCAGGTCCCTGT; reverse: CACTAGTTCACTGTCACACTGGTCA; see Supplementary Figure 1, A, available online). cDNA corresponding to 18S and 28S ribosomal RNA (Ambion, Austin, TX) was used as the loading control. Probes were labeled with Strip-EZ DNA (Ambion). Twenty micrograms of RNA was loaded into individual lanes of an agarose gel, separated by electrophoresis, transferred to the membrane, and probed as described previously (24).

Pleural Tumor Counting and Processing

Pleural tumor nodules were counted under a dissecting microscope by three independent and blinded readers (25). Parietal pleural tumors were dissected avoiding adjacent normal tissue, snap frozen in liquid nitrogen, and stored at −80°C. Tumor tissue was suspended in 1 mL of protein extraction buffer containing protease inhibitors, and cytoplasmic protein extracts were collected after Dounce homogenization and centrifugation at 16000 g for 5 minutes as described previously (24). All measurements in tumor cytoplasmic protein extracts were corrected for protein content.

In Vivo Vascular Permeability Assays

To determine pleural vascular permeability in mice with MPE, the mice were injected intravenously with 200 µL 4 mg/mL Evans blue dye (Sigma, St Louis, MO; total dose = 0.8 mg) 14 days after LLC cell injection and were killed 1 hour later. Evans blue levels in pleural fluid were determined by measuring absorbance at 630 nm
in comparison to standards of known Evans blue concentrations (12–14).

To evaluate the contribution of MCP-1 to the vascular permeability induced by mouse MPE, we used three modified Miles vascular permeability assays (13,15,27). In the first series of experiments, PBS (control) or MPE supernatants from seven mice that received random sequence–expressing LLC cells (volume = 50 µL) were injected at different sites of the shaved dorsal skin of five C57BL/6 mice. Six spots on each mouse (n = 5) received premixed BSA (Peprotech EC, London; 150 pg) + mouse IgG subclass 2a (Peprotech EC; 50 ng), MCP-1 (Peprotech EC; 150 pg), MPE + IgG (50 ng), MPE + neutralizing anti–MCP-1 antibody (Peprotech EC; 50 ng), MPE + neutralizing anti-VEGF antibody (R&D Systems, Minneapolis, MN; 50 ng), or MPE + anti–MCP-1 antibody + anti-VEGF antibody (50 ng antibodies) at final injection volumes of 50 µL. In a second series of experiments, five spots on each of five mice received BSA (1.5 ng), recombinant mouse VEGF (150 pg or 1.5 ng), or recombinant mouse MCP-1 (150 pg or 1.5 ng) at final injection volumes of 50 µL. In a third Miles assay, seven spots on each mouse (n = 5) received BSA (1.5 ng) + mouse IgG2a (50 ng), recombinant mouse MCP-1 (1.5 ng) + mouse IgG2a (50 ng), recombinant mouse MCP-1 (1.5 ng) + neutralizing anti–MCP-1 antibody (50 ng), recombinant mouse MCP-1 (1.5 ng) + neutralizing anti-VEGF antibody (50 ng), recombinant mouse VEGF (1.5 ng) + mouse IgG2a (50 ng), recombinant mouse VEGF (1.5 ng) + neutralizing anti-VEGF antibody (50 ng), or recombinant mouse VEGF (1.5 ng) + neutralizing anti–MCP-1 antibody (50 ng) at final injection volumes of 50 µL. Immediately after dermal injections, the mice received 200 µL of 4 mg/mL Evans blue (total dose = 0.8 mg) intravenously and were killed 30 minutes later. The skin was removed, the test sites were photographed, and the area of Evans blue extravasation was determined using ImageJ freeware (Rasband 1997–2008; http://rsb.info.nih.gov/ij).

To determine the direct effects of MCP-1 on the pleural cavity, 30 ng BSA, 30 ng recombinant mouse MCP-1, or 30 ng recombinant mouse VEGF in 100 µL PBS were injected directly into the pleural cavity. Immediately thereafter or 3 or 16 hours later, mice were given an intravenous injection of 0.8 mg Evans blue and were killed 1 hour later. Pleural lavage was performed as described and the retrieved fluid volume, Evans blue concentration, and cellular content and type were determined.

**Cytology**

Blood was smeared on glass slides. Pleural fluid cells (n = 50000) were used for cytocentrifugal specimen (cytospin) preparation. The slides were air-dried, fixed in methanol for 10 seconds, and stained with May–Gruenwald–Giemsa. Distinct cell types were enumerated as a percentage of 500 cells on the slide. Alternatively, pleural cell cytospins were stained for macrophage-specific F4/80 antigen (Hycult, Uden, The Netherlands; dilution 1:50), as described previously (14). Antibody binding was detected using horseradish peroxidase (HRP)–conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:200) and diaminobenzidine substrate. Myeloid monocyte/macrophage, mesothelial, and cancer cells were grouped as mononuclear cells in the analyses, whereas lymphocytes were counted separately. F4/80+ mononuclear cells are of the myeloid monocyte/macrophage lineage, whereas F4/80− mononuclear cells are either cancer or mesothelial cells (14).

**Histology**

Mouse lungs bearing pleural tumors were fixed in 10% neutral-buffered formalin (24 hours) and 70% ethanol (3 days). Tumors were dissected and embedded in paraffin. Sections (5-µm thick) were cut, mounted on glass slides, and stained with hematoxylin–eosin. Alternatively, tissue sections were immunostained using antibodies against proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology; dilution 1:200), terminal deoxynucleotidyl nick-end labeling (TUNEL; Roche Molecular Biochemicals, Penzberg, Germany) induced DNA strand breaks, or factor VIII–related antigen (fVIIIra; Invitrogen, San Francisco, CA; dilution 1:50), as described previously (13,14). Antibody binding was detected using HRP-conjugated secondary antibodies (Santa Cruz Biotechnology; dilution 1:200) and diaminobenzidine substrate. Immunodetection of PCNA, TUNEL-induced DNA strand breaks, and fVIIIra labeling of tumor tissue sections was quantified by two blinded readers as described previously (13,14). The results were averaged for each tumor, and then for each mouse.

**Biochemical and Cellular Assays**

Protein was determined using the Bio-Rad protein assay (Hercules, CA). A 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) assay was used to assess cell viability according to the manufacturers’ instructions (Promega, Madison, WI). For cell experiments, LLC, B16, and RAW264.7 cells were plated at equal densities in 12- or 96-well culture dishes. All cell experiments were done in triplicate.

**Macrophage Migration Assay**

RAW264.7 cells were seeded onto the top compartment and LLC cells containing stuffer (ie, a random sequence), sh166, or sh436 or B16 cells that overexpressed β-gal and MCP-1 were seeded into the bottom compartments of Boyden chambers (Corning, Schiphol-Rijk, The Netherlands). The compartments were divided by a membrane with 8-µm pores. At various times, the membrane was removed, inverted, air-dried, fixed in methanol for 5 seconds, and stained with May–Gruenwald–Giemsa. Migrated macrophages were counted in five high-power (600 Å) fields, and these five counts were averaged for analyses.

**Statistical Analysis**

For quantitative comparison of MPE volume, mice without effusion were considered to have pleural fluid volume of 20 µL, equivalent to the normal amount of fluid present in healthy mice (12). All values were calculated as means and 95% confidence intervals (CIs). The Student t test was used to test for differences in the means between two groups. One-way analysis of variance with least-square difference post hoc tests was used to test for differences in the means between multiple groups. The Fisher exact test was used to compare the proportion of mice developing an MPE among all experimental groups. For survival studies, Kaplan–Meier curves were plotted and compared using the log-rank test. Correlation of MPE MCP-1 levels with MPE mononuclear cells was calculated using Pearson analysis. All P values were derived.
from two-sided tests; *P < .05* was considered as the threshold for statistical significance. All statistical analyses were performed using the Statistical Package for the Social Sciences v.13.0.0 (SPSS, Chicago, IL).

**Results**

**MCP-1 Expression and Macrophages in MPE**

Determination of MCP-1 levels yielded locally elevated expression of the chemokine in LLC-induced mouse MPE and in human MPE but not in pleural fluid from sham-treated mice or in transudes resulting from congestive heart failure (Supplementary Figure 2, available online). As reported previously, MCP-1 levels were correlated with relative macrophage abundance in mouse and human MPE (Supplementary Figure 2, available online) (12,17).

**Tumor-Derived MCP-1 and MPE in Mice: Two Contrasting Models**

Because human MPE is often associated with adenocarcinoma but rarely with melanoma, we studied MCP-1 expression by lung cancer (LLC) and melanoma (B16) cells syngeneic to C57BL/6 mice, in which we developed our previously reported model of MPE (12–14). LLC cells produced abundant MCP-1, as reflected by levels of RNA and protein, in contrast to B16 cells (Figure 1). MCP-1 levels secreted by LLC and B16 cells in 24 hours = 16 and 0.3 µg/g protein, respectively; difference = 15.7 µg/g; 95% CI = 14.9 to 16.5 µg/g; *P < .001*. However, LLC and B16 cells secreted other inflammatory and angiogenic mediators at indistinguishable levels (Table 1). The difference in MCP-1 expression allowed us to test the hypothesis that MCP-1 is critical for MPE development and cancer progression in the mouse model. Upon intrapleural injection to C57BL/6 mice, LLC cells were more potent inducers than B16 cells of cachexia (mean body weight, day 14 = 21.8 and 24.3 g, respectively; difference = 2.5 g; 95% CI = 0.8 to 4.1 g; *P = .004*) and MPE (mean MPE volume = 555 and 62 µL, respectively; difference = 493 µL; 95% CI = 333 to 651 µL; *P < .001*, Figure 2). The differences were not explained by differences in intrapleural tumor dissemination, which was greater in mice injected with B16 cells (Table 2). Intrapleural LLC injection also led to earlier animal death compared with injection of B16 cells (Figure 3, A).

MCP-1 levels were higher in LLC-induced MPE and pleural tumors, compared with those induced by B16 cells: mean MCP-1 content in MPE = 65 and 8 ng/g protein, respectively (difference = 57 ng/g; 95% CI = 20 to 95 ng/g; *P = .003*; Figure 4, A); MCP-1 content in pleural tumors = 785 and 206 ng/g protein, respectively (difference = 579 ng/g; 95% CI = 254 to 903 ng/g; *P = .004*). Moreover, intrapleural LLC injection led to increased prevalence of F4/80+ macrophages in MPEs and of monocytes in blood compared with B16 injection (Figure 4, B and C). In vitro, LLC cells elicited strong chemotaxis of syngeneic RAW264.7 macrophages that was not observed with medium or B16 cells (Figure 3, A).

For assessment of pleural vascular leakiness, mice injected with LLC or B16 cells were administered 0.8 mg intravenous Evans blue on day 14 and killed 1 hour later (12–14). LLC-induced MPEs had increased albumin-binding dye content compared with B16-induced MPEs (Figure 5, A). We next analyzed LLC- and B16-induced pleural tumors for proliferation, apoptosis, and angiogenesis by immunodetection of PCNA (nuclear protein expressed during mitosis), TUNEL (labeling of DNA strand breaks during apoptosis), and IVIIIra (endothelial surface marker), respectively. LLC- and B16-induced pleural tumors displayed similar proliferation and apoptosis rates (Supplementary Figure 3, available online), but the former exhibited greater angiogenesis (mean IVIIIra+ endothelial cell clusters/high-power field in LLC- and B16-induced pleural tumors = 10.3 and 5.5, respectively; difference = 4.8; 95% CI = 2.6 to 7.1; *P < .001*; Figure 5, B). These results were consistent with the involvement of tumor-derived MCP-1 in MPE formation.

**Failure of MCP-1–Deficient LLC Cells to Cause MPE in Mice**

To confirm a critical role for MCP-1 in MPE and other aspects of cancer growth, we constructed two LLC cell lines that expressed shRNAs (sh166, sh436) targeting MCP-1 mRNA and one that expressed a random (stuffer) RNA sequence as the control. MCP-1
Table 1. Viability and mediator elaboration of cells expressing varying amounts of monocyte chemoattractant protein (MCP-1)*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>LLC Untransformed</th>
<th>Stuffer</th>
<th>sh166</th>
<th>sh436</th>
<th>B16 Untransformed</th>
<th>β-gal</th>
<th>MCP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean MTS reduction relative to untransformed LLC cells (95% CI)</td>
<td>1.00 (0.95 to 1.05)</td>
<td>1.04 (1.00 to 1.08)</td>
<td>1.10 (0.82 to 1.38)</td>
<td>1.22 (0.79 to 1.65)</td>
<td>1.00 (0.97 to 1.03)</td>
<td>1.08 (0.91 to 1.25)</td>
<td>1.08 (1.01 to 1.15)</td>
</tr>
<tr>
<td>Mean mediator level, µg/g protein (95% CI)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>15.96 (15.57 to 16.34)</td>
<td>17.29 (15.60 to 19.18)</td>
<td>0.35 (0.31 to 0.40)†</td>
<td>0.67 (0.56 to 0.77)†</td>
<td>0.25 (0.25 to 0.26)†</td>
<td>0.45 (0.38 to 0.53)</td>
<td>17.59 (16.34 to 18.85)‡</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0.05 (0.03 to 0.07)</td>
<td>0.05 (0.04 to 0.06)</td>
<td>0.05 (0.02 to 0.08)</td>
<td>0.05 (0.03 to 0.08)</td>
<td>0.08 (0.05 to 0.10)</td>
<td>0.07 (0.05 to 0.09)</td>
<td>0.07 (0.06 to 0.09)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.05 (0.02 to 0.08)</td>
<td>0.04 (0.03 to 0.05)</td>
<td>0.04 (0.02 to 0.07)</td>
<td>0.05 (0.03 to 0.06)</td>
<td>0.06 (0.04 to 0.08)</td>
<td>0.05 (0.04 to 0.06)</td>
<td>0.05 (0.05 to 0.06)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.02 (0.02 to 0.03)</td>
<td>0.03 (0.02 to 0.04)</td>
<td>0.03 (0.01 to 0.04)</td>
<td>0.03 (0.02 to 0.04)</td>
<td>0.03 (0.02 to 0.04)</td>
<td>0.03 (0.02 to 0.04)</td>
<td>0.02 (0.02 to 0.03)</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.04 (0.02 to 0.06)</td>
<td>0.04 (0.01 to 0.06)</td>
<td>0.04 (0.02 to 0.06)</td>
<td>0.04 (0.00 to 0.08)</td>
<td>0.07 (0.06 to 0.08)</td>
<td>0.06 (0.04 to 0.07)</td>
<td>0.05 (0.03 to 0.07)</td>
</tr>
<tr>
<td>MIP-2</td>
<td>0.02 (0.01 to 0.03)</td>
<td>0.03 (0.00 to 0.06)</td>
<td>0.03 (0.00 to 0.07)</td>
<td>0.04 (0.00 to 0.08)</td>
<td>0.04 (0.03 to 0.04)</td>
<td>0.03 (0.01 to 0.05)</td>
<td>0.03 (0.03 to 0.04)</td>
</tr>
<tr>
<td>VEGF</td>
<td>1.72 (1.06 to 2.39)</td>
<td>1.58 (1.09 to 2.08)</td>
<td>1.64 (1.11 to 2.17)</td>
<td>1.63 (0.89 to 2.37)</td>
<td>1.96 (1.34 to 2.56)</td>
<td>1.76 (1.53 to 1.99)</td>
<td>1.84 (1.48 to 2.21)</td>
</tr>
</tbody>
</table>

* Endogenous MCP-1 expression is high in LLC cells and low in B16 cells and was modulated with short hairpin RNAs targeting MCP-1 or using an MCP-1 expression vector. MTS = 3-(4,5-dimethylthiazol-2-y)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt; LLC = Lewis lung cancer; B16 = mouse skin melanoma; sh = short hairpin; β-gal = β-galactosidase; MCP = monocyte chemoattractant protein; IL = interleukin; TNF = tumor necrosis factor; IFN = interferon; MIP = macrophage inflammatory protein; VEGF = vascular endothelial growth factor.

† P < .001 compared with untransformed and control-transformed LLC cells.
‡ P < .001 compared with untransformed and β-gal-expressing B16 cells.

Table 2. Pleural tumors, malignant pleural effusions, and malignant pleural effusion arising in C57BL/6 mice by intrapleural injection of cells expressing varying amounts of MCP-1*

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Untransformed</th>
<th>Stuffer</th>
<th>sh166</th>
<th>sh436</th>
<th>Untransformed</th>
<th>β-gal</th>
<th>MCP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice with pleural tumors/Total No. of mice (%)</td>
<td>31/31 (100)</td>
<td>13/13 (100)</td>
<td>13/13 (100)</td>
<td>13/13 (100)</td>
<td>10/10 (100)</td>
<td>15/15 (100)</td>
<td>13/13 (100)</td>
</tr>
<tr>
<td>Pleural tumor number (mean [95% CI])</td>
<td></td>
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</tr>
<tr>
<td>Visceral</td>
<td>6.1 (5.2 to 7.0)</td>
<td>4.6 (3.4 to 5.8)</td>
<td>1.8 (0.9 to 2.7)†</td>
<td>2.7 (1.3 to 4.1)†‡</td>
<td>9.9 (6.4 to 13.4)</td>
<td>8.3 (4.7 to 11.9)</td>
<td>13.0 (7.2 to 18.8)‡</td>
</tr>
<tr>
<td>Parietal</td>
<td>8.2 (7.2 to 9.2)</td>
<td>7.1 (5.3 to 8.9)</td>
<td>3.1 (2.2 to 4.0)</td>
<td>4.7 (2.7 to 6.7)</td>
<td>20.4 (13.4 to 27.4)</td>
<td>27.9 (19.6 to 36.2)</td>
<td>29.5 (19.5 to 39.5)‡</td>
</tr>
<tr>
<td>Total</td>
<td>14.3 (12.5 to 16.1)</td>
<td>11.7 (9.1 to 14.3)</td>
<td>4.9 (3.4 to 6.4)†</td>
<td>7.4 (4.4 to 10.4)</td>
<td>30.3 (21.6 to 39.0)</td>
<td>36.2 (28.2 to 44.2)</td>
<td>42.6 (29.3 to 55.9)‡</td>
</tr>
<tr>
<td>No. of mice with MPE/Total No. of mice (%)¶</td>
<td>31/31 (100)</td>
<td>12/13 (92)</td>
<td>7/13 (54)†‡</td>
<td>6/13 (46)‡</td>
<td>6/10 (60)†</td>
<td>6/15 (40)</td>
<td>13/13 (100)¶</td>
</tr>
</tbody>
</table>

* MCP-1 expression is high in LLC cells and low in B16 cells and was modulated with short hairpin RNAs targeting MCP-1 or using an MCP-1 expression vector. LLC = Lewis lung cancer; B16 = mouse skin melanoma; sh = short hairpin; β-gal = β-galactosidase; MCP = monocyte chemoattractant protein; MPE = malignant pleural effusion.

† P < .05 compared with untransformed LLC cells.
‡ P < .05 compared with stuffer LLC cells.
¶ P < .05 compared with untransformed B16 cells.
|| P < .05 compared with β-gal B16 cells.
†† Pearson χ² P < .001 and Fisher exact P < .001.
Nal fluid accumulation
RNA sequence. Means and 95% confidence intervals are from three RNAs correspond to MCP-1 sequence and stuffer designates a random sh, short hairpin; separate experiments. MPE, malignant pleural effusion; wt, wild-type; 1470 

10 5 cancer cells and were sacrificed at day 14. Body weight sion with shRNA. C57BL/6 mice received intrapleural injections of 1.5 × engineered to produce various amounts of monocyte chemoattractant (LLC) and mouse skin melanoma (B16) cells that were in some cases mice 14 days after intrapleural injection of mouse Lewis lung cancer (Figure 3, available online). Pleural tumors induced by sh166- and sh436-expressing LLC cells, we observed reductions in MPE F4/80+ macrophages (% F4/80+ pleural cells in mice injected with LLC cells expressing random sequence, sh166, and sh436 = 8.3, 3.5%, and 3.3%, respectively; P = .002 for comparisons of mice injected with cells expressing sh166 or sh436 to control mice). Similar reductions were observed in blood monocytes (% monocytes/white blood cells in mice injected with LLC cells expressing random sequence, sh166, and sh436 = 8.6, 3.5%, and 3.3%, respectively; P = .002 for comparisons of sh166 or sh436 mice to controls). However, there were no statistically significant differences in lymphocytes, neutrophils, or eosinophils (Figure 4, B and C). In vitro, sh166 and sh436 LLC cells were defective in eliciting Boyden chamber chemotaxis of RAW264.7 macrophages (mean RAW264.7 cell number/high-power field migrated toward LLC cell expressing random sequence, sh166, and sh436 LLC after 24 hours = 95, 39, and 51), and the differences relative to controls were statistically significant (P < .001) (Figure 4, D).

For assessment of pleural vascular leakiness, mice injected with LLC cells expressing random sequence, sh166, or sh436 LLC were administered Evans blue on day 14 (12–14). Sh166 or sh436 LLC-induced MPEs had markedly reduced dye content compared with control MPEs (MPE Evans blue in control, sh166, and sh436 LLC-injected mice = 476, 29, and 109 µg, respectively; P < .001 for comparisons of dye content of sh166 or sh436 LLC-induced MPEs with that of MPEs in mice who received LLCs expressing random sequence; Figure 5, A).

Pleural tumor analysis by immunodetection of PCNA, TUNEL, and IVIIIa showed that tumor cell proliferation was not influenced by expression of sh166 and sh436, although expression of these constructs tended to increase apoptotic rates (Supplementary Figure 3, available online). Pleural tumors induced by sh166- and sh436-LLC displayed impaired angiogenesis (mean IVIIIa+ endothelial cell clusters/high-power field in pleural tumors induced by LLC cells expressing random sequence, sh166, and sh436 = 8.3, 4.9, and 5.7, respectively; P = .009 and P = .042 for comparison of angiogenesis in tumors formed by cells expressing sh166 and sh436.

Involvement of Tumor-Derived MCP-1 in Promotion of Monocyte/Macrophage Recruitment, Pleural Vascular Hyperpermeability, and Neoangiogenesis

We next assessed the involvement of MCP-1 in inflammatory cell recruitment, pleural vascular leakiness, and angiogenesis, all phenomena previously implicated in MPE pathobiology (1–4,11–15). In mice that received sh166- or sh436-expressing LLC cells, we observed reductions in MCP-1 levels in MPEs generated by sh166- and sh436-expressing LLC were 10-fold reduced (mean MPE MCP-1 in control, sh166, and sh436 = 51, 4, and 5 ng/g protein, respectively; difference relative to control = 47 ng/g protein [95% CI = 7 to 86; P = .017] and 46 ng/g protein [95% CI = 7 to 85; P = .021], respectively), whereas the levels of other mediators were not different (Figure 4, A). These results suggest that tumor cells are the source of approximately 90% of the MCP-1 in experimental MPE and that this tumor-derived chemokine contributes to MPE formation.

secretion by sh166- or sh436-expressing LLC cells was 2%–4% of that displayed by control cells, although in vitro cell growth and expression of other soluble factors was not affected (Figure 1, C, Table 1). Treatment of mice with LLC expressing random RNA caused weight loss similar to that observed in wild-type LLC-treated mice, but sh166- or sh436 LLC-treated mice grew modestly (mean body weight, day 14 = 21.2, 25.3, and 24.6 g, respectively; difference between random RNA and sh166 or sh436 = 4.1 [95% CI = 2.3 to 5.9] and 3.3 [95% CI = 1.6 to 5.1] g, respectively; P < .001 for both comparisons; Figure 2, A). The mean volume of MPE in mice injected with LLC cells expressing random RNA, sh166, and sh436 (n = 13 per group) was 585, 48, and 123 µL, respectively; difference (95% CI) between random RNA and sh166 and sh436 = 537 µL (95% CI = 365 to 709) and 463 µL (95% CI = 291 to 634), respectively; P < .001 for both comparisons (Figure 2, B). Cells expressing sh166 and sh436 had fewer tumors in both visceral and parietal pleurae (Table 2). Mice that received intrapleural sh166 and sh436 LLC displayed prolonged survival compared with controls (Figure 3, A). Even when moribund (30 days after LLC injection), mice had large pleural tumors with minimal effusions (Figure 3, B). Compared with MPEs in control mice injected with LLC expressing random RNA sequence, MCP-1 levels in MPEs generated by sh166- and sh436-expressing LLC were 10-fold reduced (mean MPE MCP-1 in control, sh166, and sh436 = 51, 4, and 5 ng/g protein, respectively; difference relative to control = 47 ng/g protein [95% CI = 7 to 86; P = .017] and 46 ng/g protein [95% CI = 7 to 85; P = .021], respectively), whereas the levels of other mediators were not different (Figure 4, A). These results suggest that tumor cells are the source of approximately 90% of the MCP-1 in experimental MPE and that this tumor-derived chemokine contributes to MPE formation.
with those formed by control cells, respectively; Figure 5, B). In vitro, incubation of serum-deprived LLC cells with mouse 2.5 nM MCP-1 and/or isomolar amounts of neutralizing anti–MCP-1 antibody did not affect cell growth determined by MTS reduction (data not shown). These data and the comparable growth rates of LLC cells expressing random sequence, sh166, and sh436 (Table 1) indicated that tumor-derived MCP-1 functions to induce enhanced vascular permeability, mononuclear chemotaxis, and neoangiogenesis in MPE, rather than affecting tumor cell proliferation/apoptosis.

**Effect of MCP-1 Overexpression in B16 Cells on MPE Generation**

We next reconstituted B16 cells with MCP-1 by transforming them with a plasmid encoding mouse MCP-1. MCP-1–overexpressing B16 cells displayed proliferation and mediator secretion that was comparable to that of β-gal–overexpressing B16 cells, and MCP-1 secretion was increased to levels similar to those observed in LLC cells (Figure 1, C, Table 1). Compared with what was observed with injection of β-gal–expressing B16 cells, intra-pleural injection of B16 cells expressing MCP-1 resulted in enhanced weight loss (mean body weight, day 14 = 22.7 and 20.3 g, respectively; difference = 2.4 g; 95% CI = 0.7 to 4.1 g; P = .007), and MPE formation (mean MPE volume = 162 and 424 µL, respectively; difference = 263 µL; 95% CI = 97 to 429 µL; P = .002), and unchanged tumor dissemination (Figure 2, Table 2). Mice injected intrapleurally with MCP-1-B16 cells had shortened survival compared with mice injected with B16 cells that expressed β-gal (Figure 3, A). Even when moribund (30 days after B16 inoculation), mice that received β-gal–expressing B16 cells had large pleural tumors with minimal effusions, in contrast to mice injected with B16 cells expressing MCP-1, which developed large effusions earlier (Figure 3, C). MCP-1 levels in MPEs from mice injected with B16 cells expressing MCP-1 were eightfold higher than those observed in mice injected with B16 cells expressing β-gal (mean MPE MCP-1 = 93 and 12 ng/g protein, respectively; difference = 81 ng/g; 95% CI = 43 to 118 ng/g; P < .001), whereas no differences were detected in other mediators (Figure 4, A). These

**Figure 3.** Survival of C57BL/6 mice after intrapleural injection of mouse Lewis lung cancer (LLC) and mouse skin melanoma (B16) cells that were in some cases engineered to produce various amounts of monocyte chemoattractant protein (MCP)-1 by overexpression or targeting endogenous expression with short hairpin (sh)RNA. C57BL/6 mice received intrapleural injections of 1.5 × 10⁶ cancer cells and were observed until they became moribund. A) Kaplan–Meier survival curves (top) and life tables (bottom). Sh166 and sh436 RNAs correspond to MCP-1 sequence and stuffer designates a random RNA sequence. Data are given as median and 95% confidence interval. P values were derived from log-rank test. Shown are pooled data from two separate experiments wt, wild-type; β-gal, β-galactosidase. B) Malignant pleural effusion (MPE; dashed lines) in mice after intrapleural injection of LLC cells expressing different shRNAs. Top: subdiaphragmatic view of C57BL/6 mouse 17 days after intrapleural injection of LLC cells expressing RNA of random sequence (control); the pleural fluid volume was 830 µL. Middle: subdiaphragmatic view of C57BL/6 mouse 30 days after intrapleural injection of LLC cells expressing sh166; the pleural fluid volume was 350 µL. White arrows indicate large pleural tumors. Bottom: subdiaphragmatic view of C57BL/6 mouse 30 days after intrapleural injection of LLC cells expressing sh436 targeting MCP-1; the pleural fluid volume was 150 µL. C) MPE in mice after intrapleural injection of LLC cells producing different amounts of MCP-1. Top: subdiaphragmatic view of C57BL/6 mouse 30 days after intrapleural injection of B16 cells expressing β-galactosidase (control); the pleural fluid volume was <20 µL. Asterisks denote lungs that are visible due to lack of fluid. Bottom: subdiaphragmatic view of C57BL/6 mouse 14 days after intrapleural injection of B16 cells expressing MCP-1; the pleural fluid volume was 250 µL.
results confirmed that tumor-derived MCP-1 enhances MPE formation, leading to increased morbidity and mortality in a mouse model.

**Monocyte/Macrophage Recruitment, Pleural Vascular Permeability, and Neoangiogenesis by MCP-1—Overexpressing B16 Cells**

During subsequent cytological and histological examinations of MPE fluid, blood, and tumor tissues, we observed increased F4/80+ macrophages in MPE and increased monocytes in blood in mice that received MCP-1-B16 cells (Figure 4, B and C). In vitro, MCP-1-B16 cells elicited increased RAW264.7 chemotaxis compared with β-gal-B16 cells, an effect abrogated by MCP-1 neutralization (Figure 4, D; mean RAW264.7 number/high-power field migrated toward β-gal- and MCP-1-B16 after 24 hours = 9 and 43, respectively; difference = 34; 95% CI = 16 to 52; P = .001). For vessel permeability determinations, mice bearing β-gal- or MCP-1-B16 cell–induced MPEs received 0.8 mg intravenous Evans blue (day 14), 1 hour before sacrifice. MCP-1-B16–induced MPEs had increased dye content compared with control MPEs (Figure 5, A). Finally, overexpression of MCP-1 in B16 melanoma cells did not affect tumor cell proliferation and survival (Supplementary Figure 3, available online) but induced angiogenesis (Figure 5, B). Incubation of serum-deprived β-gal- and MCP-1-B16 cells with mouse MCP-1 (2.5 nM) and/or isomolar amounts of neutralizing anti-MCP-1 antibody did not impact cell growth in vitro (data not shown). These data suggested that MCP-1 does not increase cell growth but exerts strong

![Figure 4. Mononuclear/macrophage chemoattraction to malignant pleural effusion (MPE) by tumor-derived monocyte chemoattractant protein (MCP)-1. C57BL/6 mice received intrapleural injections of 1.5 × 10^5 mouse Lewis lung cancer (LLC) or mouse skin melanoma (B16) cells that were in some cases engineered to produce various amounts of MCP-1 (by overexpression or targeting endogenous expression with short hairpin [sh]RNA) and were sacrificed at day 14. A Level of proinflammatory and angiogenic mediators in MPEs in mice that were injected with cancer cells expressing varying amounts of monocyte chemoattractant protein (MCP)-1. Sh166 and sh436 RNAs correspond to MCP-1 sequence, and stuffer designates a random RNA sequence. B Levels of F4/80+ mononuclear cells (macrophages), F4/80− mononuclear cells (mesothelial and cancer cells), lymphoocytes, neutrophils, and eosinophils as a percentage of pleural cells in MPEs of mice that were injected with cancer cells engineered to express varying amounts of MCP-1. C Level of monocytes and other inflammatory cells as percentage of white blood cells (WBC) in peripheral blood of mice that received tumor cells expressing varying amounts of MCP-1. D Attraction of RAW264.7 macrophages by cancer cells engineered to express varying amounts of MCP-1. MCP-1 expressing B16 cells were treated or not treated with neutralizing anti–MCP-1 antibody. Means, confidence intervals, and P values derive from three separate experiments. hpf, high-power field; wt, wild-type; β-gal, β-galactosidase; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; MIP, macrophage inflammatory protein; VEGF, vascular endothelial growth factor.](image-url)
effects on tumor-associated vascular and inflammatory cell milieus.

Direct Induction of Vascular Hyperpermeability and Inflammation by MCP-1

We next determined the direct effects of MCP-1 on healthy skin and pleura. Intradermal delivery of mouse MCP-1 at the levels found in LLC-generated MPE (3 ng/mL) induced vascular hyperpermeability in skin, and neutralizing anti-MCP-1 antibody reduced dermal hyperpermeability induced by MPE (Figure 6, A and B). The effects of MCP-1 on skin vascular leakiness were specific and comparable to those of VEGF (Supplementary Figure 4, available online). When 30 ng BSA, 30 ng VEGF, or 30 ng MCP-1 were delivered into the pleural cavities of naive mice, more fluid exudation (pleural lavage volume = 1000, 1005, and 1175 µL, respectively; difference [95% CI] between BSA and VEGF or MCP-1 = 5 µL [95% CI = 57 to 67] and 175 µL [95% CI = 113 to 237], respectively; \( P < .001 \) for the latter comparison), Evans blue extravasation (pleural lavage Evans blue = 12, 55, and 95 µg, respectively; difference [95% CI] between BSA and VEGF or MCP-1 = 40 µg [95% CI = 10 to 91] and 83 µg [95% CI = 32 to 134], respectively; \( P = .007 \) for the latter comparison), and macrophage accumulation (pleural lavage macrophages \( \times 10^3 \) = 526, 468, and 1029, respectively; difference [95% CI] between BSA and VEGF or MCP-1 = 70 [95% CI = 427 to 569] and 516 [95% CI = 18 to 1014], respectively; \( P = .044 \) for the latter comparison) occurred (Figure 6, C–E). These results indicate that MCP-1 affects vascular permeability and mononuclear recruitment, mechanisms that are involved in MPE formation.

Discussion

We have evaluated the role of tumor-derived MCP-1 in MPE pathogenesis. Based on earlier observations of locally elevated chemokine levels in human and experimental MPE (12–14,17), we hypothesized that tumor cells produce MCP-1 when homing to the pleural cavity, where the chemokine contributes to MPE formation. We found that mouse lung cancer (LLC) cells produced MCP-1 and induced MPE in syngeneic mice, whereas B16 melanoma cells were deficient in MCP-1 secretion and MPE induction. We generated MCP-1–deficient LLC cells using RNA interference and observed that they were unable to induce MPE. Conversely, overexpression of the chemokine in B16 cells led to enhanced MPE formation. This modulation of MCP-1 expression in these two contrasting models (ie, elimination of MCP-1 in highly expressing LLC cells and induction of MCP-1 in nonexpressing B16 cells) did not affect tumor cell proliferation or apoptosis but was associated with changes in mononuclear/macrophage recruitment, pleural vascular permeability, and angiogenesis. These observations support the hypothesis that tumor-derived MCP-1 is important for MPE formation in mice. Because MCP-1 is produced by human adenocarcinoma cells and is found at high levels in human MPE (17), this chemokine may be a prime mediator of malignant fluid accumulation in human cancer.

We examined possible mechanisms of MPE promotion by MCP-1, such as enhanced tumor cell proliferation (pro-growth effect), evasion of programmed tumor cell death (anti-apoptotic effect), sustained new vessel formation (angiogenic effect), increased vascular leakiness (hyperpermeability effect), and increased recruitment of macrophages (paracrine effect). These mechanisms have been linked with the pathogenesis of human and mouse MPE (1–5,11–15). MCP-1 has been found to act as an autocrine growth signal for prostate cancer cells (28,29), but we observed that the chemokine did not influence tumor cell proliferation or apoptosis in our model, implying that the chemokine promotes MPE via alternate pathways.

Angiogenesis is critical for tumor progression, has been reported to be associated with MPE pathogenesis (13,14), and is promoted by VEGF and other molecules, such as angiopoietins (30–33). Recent evidence suggests that VEGF acts synergistically with proinflammatory mediators to promote angiogenesis (32,33). Our study demonstrates that one of the main functions of MCP-1 is induction of new vessel formation, adding to the growing number of examples of inflammatory angiogenesis (18,28,29,34,35).

Figure 5. Pleural vascular permeability and new vessel formation in malignant pleural effusions (MPE) and pleural tumors induced in C57BL/6 mice 14 days after intrapleural injection of mouse Lewis lung cancer (LLC) and mouse skin melanoma (B16) cells that in some cases were engineered to produce various amounts of MCP-1 (by overexpression or targeting endogenous expression with short hairpin [sh]RNAs). C57BL/6 mice received intrapleural injections of 1.5 × 10⁶ cancer cells and were sacrificed at day 14. A) One hour prior to sacrifice, mice received an intravenous injection of 0.8 mg albumin-binding Evans blue, with subsequent measurement of the dye levels in MPE fluid. B) After sacrifice, pleural tumor tissue was fixed in formalin, embedded in paraffin, cut into 5-µm sections, applied to glass slides, and immunolabeled for factor VIII–related antigen (fVIIIra), an endothelial cell marker. Sh166 and sh436 RNAs correspond to MCP-1 sequence and stuffer designates a random RNA sequence. Means, 95% confidence intervals, and \( P \) values derive from three separate experiments. wt, wild-type; sh, short hairpin; β-gal, β-galactosidase.
The angiogenic activity of MCP-1 may be direct (via promotion of endothelial proliferation/migration and vessel stabilization) or indirect (through chemoattraction of inflammatory cells that contribute to angiogenesis). In this regard, MCP-1 binds to chemokine receptor (CCR)2 expressed on endothelial cells to promote angiogenesis without involvement of leukocyte products (18) and via recruitment of neutrophils and macrophages (19, 34, 35). Both direct and indirect mechanisms of MCP-1–induced angiogenesis may also occur in the context of MPE in that direct stimulation of neovascularization may be accompanied by mononuclear recruitment. In support of the latter possibility, we observed that tumor-derived MCP-1 contributed to mononuclear cell trafficking to MPE.

In addition to contributing to angiogenesis, MCP-1 increased pleural vascular permeability, which is absolutely required for MPE formation in humans and mice (1–5,12–15). MCP-1 also induced vascular leakiness in mouse skin and pleura, and MCP-1 neutralization by addition of an antibody to the chemokine reduced the vascular leakiness induced by MPE. The mechanisms by which MCP-1 induces vascular hyperpermeability are likely direct because vascular leakage began shortly after topical administration to the skin or the pleural cavity in vivo (Figure 6). Our findings support previous work that indicates that proinflammatory mediators assist angiogenic molecules in enhancing vascular permeability in various diseases, including cancer (12–15,18,27).

The involvement of inflammation in cancer progression is well documented (36–38). Proinflammatory pathways (these include NF-κB and Ras pathways) are activated in at least some cancers, and this signaling generates a host milieu that facilitates tumor progression (39–42). We have shown that the proinflammatory TNF-α/NF-κB pathway in cancer cells promotes MPE (12,13). In the present study, MCP-1 exerted both angiogenic and proinflammatory functions, suggesting that MCP-1 may be an effector of NF-κB in the inflammatory cascade of MPE pathogenesis. It was recently shown that genetic alterations in tumor cells affect not only cancer cell turnover but also the paracrine expression of soluble factors that help shape a pro-tumor host microenvironment (37,39–42).

Macrophages are attracted to tumor sites as a result of alterations in cancer cells that lead to changes in paracrine expression of mediators that have potent tumor-promoting effects (43). In this study, we have demonstrated that tumor-derived MCP-1 is important for the recruitment of mononuclear cells to MPE. These cells may contribute to the angiogenic and permeability-inducing effects of MCP-1 in MPE. Our results and those of others (34,35) strengthen the role of MCP-1 as a focal effector of mononuclear
recruitment to cancer-involved sites, and it may have application in the development of therapeutic approaches that block mononuclear trafficking to tumor sites.

Our study had several limitations. We did not analyze expression of MCP-1 or other soluble factors or the effect of interventions that altered MCP-1 levels in other human and mouse cancers, in which alternative mechanisms may be responsible for fluid exudation. Furthermore, we did not assess the therapeutic effects of MCP-1 antagonists in the treatment of MPE. The question as to why MCP-1 expression is different in LLC and B16 cells was not resolved in our study.

In conclusion, our results demonstrate that MCP-1 is a powerful inducer of MPE when secreted by cancer cells in the pleural cavity. MPE is an important complication for millions of cancer patients worldwide, and no specific treatment is available (1–5). Therapeutic trials of treatments that interfere with MCP-1 signaling may soon become possible, as CCR2 antagonists and methods to accomplish in vivo RNA interference are being developed (44, 45). The results described here may lay the groundwork for future strategies that use inhibition of MCP-1 and/or its receptor CCR2 to block or prevent pleural fluid accumulation in patients with cancer.

References


6. Sugiyura S, Ando Y, Minami H, Ando M, Sakai S, Shimokata K. Prognostic value of pleural fluid exudation. Furthermore, we did not assess the therapeutic effects of MCP-1 antagonists in the treatment of MPE. The question as to why MCP-1 expression is different in LLC and B16 cells was not resolved in our study.


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Notes

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Conflict of Interest: The authors of this study declare no conflict of interest with the study matter.

The authors take full responsibility for the study design, data collection, analysis and interpretation of the data, the decision to submit the manuscript for publication, and the writing of the manuscript.

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