Low-Level Monocyte Chemoattractant Protein-1 Stimulation of Monocytes Leads to Tumor Formation in Nontumorigenic Melanoma Cells

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Tumors commonly produce chemokines for recruitment of host cells, but the biological significance of tumor-infiltrating inflammatory cells, such as monocytes/macrophages, for disease outcome is not clear. Here, we show that all of 30 melanoma cell lines secreted monocyte chemoattractant protein-1 (MCP-1), whereas normal melanocytes did not. When low MCP-1-producing melanoma cells from a biologically early, nontumorigenic stage were transduced to overexpress the MCP-1 gene, tumor formation depended on the level of chemokine secretion and monocyte infiltration; low-level MCP-1 secretion with modest monocyte infiltration resulted in tumor formation, whereas high secretion was associated with massive monocyte/macrophage infiltration into the tumor mass, leading to its destruction within a few days after injection into mice. Tumor growth stimulated by monocytes/macrophages was due to increased angiogenesis. Vessel formation in vitro was inhibited with mAbs against TNF-α, which, when secreted by cocultures of melanoma cells with human monocytes, induced endothelial cells under collagen gels to form branching, tubular structures. These studies demonstrate that the biological effects of tumor-derived MCP-1 are biphasic, depending on the level of secretion. This correlates with the degree of monocyte cell infiltration, which results in increased tumor vascularization and TNF-α production. The Journal of Immunology, 2001, 166: 6483–6490.

Solid human tumors are often infiltrated by host immune and inflammatory cells comprised mainly of lymphocytes and cells of the mononuclear lineage (1). Whereas increased levels of lymphocyte infiltration into primary tumors decrease tumor recurrence and death rates (2), the presence of inflammatory cell infiltrates has not been clearly correlated with disease outcome. Infiltration of tumors with host cells is regulated by tumor-derived chemokines, a superfamily of proinflammatory cytokines that is responsible for the selective recruitment and activation of mononuclear cells (2). Chemokines induce directed migration of leukocytes and stimulate their adhesion and transendothelial migration (3, 4). Due to the large number of chemokines produced by human tumors and the broad spectrum of their biological functions, their precise roles in tumor development and progression remain undefined.

Monocyte-chemoattractant protein-1 (MCP-1)4 is the prototype of the CC family of chemokines (5). It can recruit monocytes (6), NK cells (7), and subpopulations of T lymphocytes (8), which all express high-affinity receptors (9, 10), predominantly CCR2 (11, 12). Because MCP-1 secretion results in tissue infiltration of monocytes and T lymphocytes, the cytokine plays a major role in autoimmune disease pathogenesis. The role of MCP-1 in tumor development and progression is less clear. Expression has been reported for melanoma (13), glioma (14, 15), sarcoma (16, 17), leukemia (18), hemangioma (19), and carcinomas of breast (20), cervix (21, 22), and ovary (23). The malignant cells express MCP-1, apparently due to the constitutive production of activating growth factors and cytokines such as IL-1 (24), TGF-β (25), and platelet-derived growth factor (26, 27). MCP-1 can be protective in some tumor models but destructive in others; murine colon carcinoma cells expressing MCP-1 fail to metastasize when injected into mice (28), whereas other carcinoma cells show enhanced metastasis (29). Overexpression of MCP-1 by tumor cells can lead to their destruction by an infiltrate of activated mononuclear cells (30–33). The potential tumoricidal activity of monocytes/macrophages has been used previously as a therapeutic strategy by enhancing their activity with muramyl dipeptides (34, 35). However, despite promising results in experimental animals, clinical studies have been disappointing. The lack of clinical success is apparently due to the potential positive effect of MCP-1 on tumor growth. MCP-1 expression results in the infiltration of macrophages that secrete stimulatory factors either for the tumor cells or the vasculature (1, 36).

Infiltration of macrophages/monocytes into cutaneous malignant melanomas may be critical for progression of melanomas toward an aggressive phenotype (37). Most melanomas from primary and metastatic lesions produce MCP-1 (38), and macrophage infiltration appears to correlate with tumor stage and angiogenesis (39). We hypothesized that monocyte recruitment depends on the level of MCP-1 secretion by melanoma cells and that the effect of monocytes on tumor growth depends on their level of infiltration. We constructed a replication-defective adenoviral vector for MCP-1 overexpression and established a MCP-1 gradient before injection into SCID mice. We demonstrate that intermediate levels of MCP-1 elicit an angiogenic effect mediated through monocyte activation that results in tumor growth, whereas high levels of 3MCP-1 lead to massive monocyte/macrophage accumulation and tumor destruction. Monocytes/macrophages activated by tumor cells that secrete MCP-1 release TNF-α, which may induce angiogenesis. Thus, there is a delicate,

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4 Abbreviations used in this paper: MCP-1, monocyte chemoattractant protein-1; pAb, polyclonal Ab; PECAM-1, platelet endothelial cell adhesion molecule-1; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; Ad, adenovirus.
concentration-dependent balance for the biological function of MCP-1, which may result in either tumor enhancement or destruction by infiltrating monocytes/macrophages.

Materials and Methods

Cells
SBcl2 cells (obtained from Dr. B. Giovanella, St. Joseph’s Hospital Cancer Center, Houston, TX) were isolated from a primary cutaneous melanoma. These cells are nonmalignant in immunodeficient mice, grow poorly in soft agar, and require exogenous growth factors for proliferation (40). All other melanoma cell lines were isolated and are maintained at the Wistar Institute (Philadelphia, PA) (41). They were grown in melanoma growth medium W489, consisting of MCDB 153 medium (Sigma, St. Louis, MO) and LeibovitzL-15 medium (Sigma) at a 4:1 (v/v) ratio (40) and supplemented with insulin at 5 μg/ml (Sigma) and 2% heat-inactivated FCS (Irvin Scientific, Irvine, CA) unless otherwise stated. Normal human melanocytes were obtained from newborn foreskin as described (42). They were cultured in medium W489, supplemented with 2 mM CaCl2, 2% FCS and 5 μg/ml insulin (Sigma), 10 ng/ml epidermal growth factor, 140 μg/ml bovine pituitary extract, and 10 ng/ml 12-O-tetradecanoylphorbol-13-acetate (TPA; Tocris Cookson, Ellisville, MO). HUVECs were grown on gelatin-coated plastic dishes in M199 medium (Life Technologies, Carlsbad, CA) supplemented with 10% FCS, endothelial cell growth factor (150 μg/ml), and heparin (5 U/ml) as previously described (43). Cells were used between the second and eighth passages. The 293 E1A-transformed human embryonic kidney cells (American Type Culture Collection, Manassas, VA) were grown in DMEM supplemented with 10% FCS.

Human peripheral blood monocytes were isolated essentially as described (44) using only endotoxin-free reagents. Briefly, human peripheral blood monocytes from the blood of healthy volunteers were separated on a Ficol-Paque (Pharmacia Biotech, Uppsala, Sweden) gradient and resuspended in RPMI 1640 medium (Sigma) supplemented with 10% human AB serum and polymyxin-B (10 μg/ml) at 4 × 10^6 cells/ml. Tissue culture dishes (150 mm; Corning Glass, Corning, NY) were coated with 5 μl of 2% gelatin in physiological saline and incubated for 2 h at 37°C, after which the gelatin was aspirated and the dishes were left to dry. Autologous serum (10 ml) was added, and the dishes were incubated for 60 min at 37°C. After removal of the serum, dishes were rinsed with Mg2+- and Ca2+-free PBS, 30 ml of mononuclear cell suspension was added per dish, and they were incubated for 45 min at 37°C. Nonadherent cells were aspirated, and adherent cells were rinsed with prewarmed (37°C) RPMI 1640 medium. A 10-ml mixture (1:1) of 10 mM EDTA and PBS (Mg2+ and Ca2+-free) was added for 15 min to remove adherent cells. Cells were centrifuged and resuspended in RPMI 1640 with 10% FCS and analyzed by flow cytometry. Monocyte yields were calculated to be >70% with >90% cell viability.

Adenovirus (Ad) vector

A plasmid containing the 741-bp human MCP-1 cDNA was used to construct the adenoviral vector MCP-1-Ad5 using previously described techniques (45). Briefly, the open reading frame of MCP-1 cDNA (~400 bp) was subcloned into a modified pSL301 vector (Vector Core, Institute for Human Gene Therapy, University of Pennsylvania, Philadelphia, PA) using EcoRI and PsI digestion of both MCP-1 cDNA and pSL301. The plasmid containing the MCP-1 cDNA was excised with NotI, pAdCMV (Vector Core), linearized with NotI at the unique restriction site, and ligated. Sense orientation of the insert was determined by restriction analysis using EcoRI and sequencing. MCP-1 cDNA was under the control of the CMV immediate/early enhancer-promoter element and the SV40 polyadenylation signal. Recombination was done in 293 cells, and the rAd was plaque-purified, expanded in 293 cells, and purified by cesium chloride gradient centrifugation. The adenoviral control vector LacZ-Ad5 expressing β-galactosidase (45) was produced using the same techniques.

Production of rMCP-1, mAb, and polyclonal Ab (pAb)

The rMCP-1 was produced from Escherichia coli as a GST fusion protein and affinity-purified on glutathione-Sepharose beads (Pharmacia, Piscataway, NJ). The cDNA was cloned into the PGEX-2T vector (Pharmacia). Western analysis indicated a 13-kDa protein with mouse pAb specific for MCP-1 (AB-PharMingen) at 25 μg/ml to detect mouse macrophages, anti-mouse TNF-α (BD PharMingen) at 10 μg/ml, and anti-mouse CD31 (platelet endothelial cell adhesion molecule-1 (PECAM-1); BD PharMingen) at 25 μg/ml to show vessel formation. For immunofluorescence, an FITC-conjugated goat anti-rat IgG Ab (Jackson ImmunoResearch) was used.

Chemotaxis assay

Chemotaxis assays were conducted using filter inserts with 3-μm pores (Millipore) in triplate-24-well plates. SCb12 melanoma cells were transduced with MCP-1 or LacZ and, 24 h later, the growth medium was changed to serum- and growth factor-free medium for 72 h, after which supernatants were collected. Freshly isolated monocytes (3 × 10^6 cells/liter) preincubated with human IgG (1 mg/ml per 10^6 monocytes) for 15 min were placed in the upper chambers of inserts, and supernatants of cocultures or recombinant human MCP-1 (50 ng/ml) were added to the lower chamber. After 3 h, monocytes, which had migrated through the filter to the lower chamber, were collected and viable cells confirmed by trypan blue exclusion were counted. Chemotaxis was inhibited by adding mAb MCP-1-D10-1 (5 μg/ml) or a rabbit pAb against MCP-1 (50 μg/ml) to the lower chamber.

MCP-1 EITHER STIMULATES OR INHIBITS MELANOMAS

To demonstrate MCP-1 production from rAd, 5 × 10^5 infected 293 cells in 20 ml of DMEM with 10% FCS were left to develop to 50% cytopathic effects. Aliquots (50 μl) of the supernatants were heated to 100°C for 10 min in the same amount of SDS sample buffer (100 μl SDS, 100 mM Tris (pH 6.8), 1% glycerol, 125 mg/ml bromophenol blue, with or without 5% (v/v) 2-ME (12.5 M)), separated on 12% SDS-polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA) overnight at 4°C with 20 V constant voltage. Nonpecific binding to the polyvinylidene difluoride membranes was blocked with PBS containing 3% BSA for 60 min at room temperature. Between incubations, membranes were washed three times for 5 min each with PBS containing 0.05% Tween 20. Membranes were probed with mouse mAb MCP-1-D10-1 (60.8 μg/ml; 1:20) or rabbit pAb against MCP-1 (1:200) for 1 h. Membranes were then incubated with IgG goat anti-mouse phosphate (Jackson ImmunoResearch Laboratories, West Grove, PA) or donkey IgG anti-rabbit phosphate (Jackson ImmunoResearch Laboratories) as secondary Abs for 2 h at room temperature. Immunoreactive bands were visualized using 3-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Promega, Madison, WI) in alkaline phosphate buffer.

Tumor formation in SCID mice and immunohistochemistry

SBcl2 melanoma cells were infected with MCP-1-Ad5 or LacZ-Ad5 at defined PFU per cell. At 48 h after transduction, 2 × 10^6 SCb12 cells in 100 μl of PBS were injected s.c. into five SCID mice per group. To inhibit tumor growth after injection of SBcl2 transduced cells at 0.5 PFU/cell, SBcl2 cells were treated daily i.p. with 150 μg of a neutralizing rabbit pAb against MCP-1 starting 1 day before s.c. injection of transduced SBcl2 cells until day 4. Tumor growth was evaluated 4, 8, and 14 days later. For histological examination, tumor lesions were fixed in formalin, dehydrated through graded alcohol and xylene, and embedded in paraffin. Fresh frozen sections were embedded in OCT embedding medium (Sakura Finetek, Torrance, CA). Serial 5-μm sections were cut and stained with hematoxylin and eosin. Immunohistochemistry was performed on serial cryosections by an immunoperoxidase technique using an avidin-biotin-peroxidase complex system (Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine as chromagen. Tissue sections were aceton-fixed for 10 min at 4°C, incubated with primary Ab overnight at 4°C, thoroughly rinsed with PBS, and overlaid with biotinylated anti-mouse or anti-rabbit IgG for 30 min at room temperature. After rinsing, avidin-biotin-peroxidase complex was added for 45 min. Slides were rinsed well with PBS, developed with 3,3′-diaminobenzidine, and counterstained lightly with hematoxylin.

The following mAbs were used: anti-human Ki67 proliferation marker (Immunootech, Westbrook, ME) at 10 μg/ml, anti-mouse CD11b (Mac-1) (BD PharMingen, San Diego, CA) at 25 μg/ml to detect mouse macrophages, anti-mouse TNF-α (BD PharMingen) at 10 μg/ml, and anti-mouse CD31 (platelet endothelial cell adhesion molecule-1 (PECAM-1); BD PharMingen) at 25 μg/ml to show vessel formation. For immunofluorescence, an FITC-conjugated goat anti-rat IgG Ab (Jackson ImmunoResearch) was used.

Immunoblotting

Immunoblotting indicated a 13-kDa protein with mouse pAb specific for MCP-1 (AB-PharMingen) at 25 μg/ml to detect mouse macrophages, anti-mouse TNF-α (BD PharMingen) at 10 μg/ml, and anti-mouse CD31 (platelet endothelial cell adhesion molecule-1 (PECAM-1); BD PharMingen) at 25 μg/ml to show vessel formation. For immunofluorescence, an FITC-conjugated goat anti-rat IgG Ab (Jackson ImmunoResearch) was used.
Coculture assays, ELISA, and radioimmunoassay

SBcl2 melanoma cells were infected with the adenoviral vectors for MCP-1 or LacZ 36 h before coculture with freshly isolated human monocytes in DMEM with 5% FCS for 18 h. Supernatants were tested for IL-4, IL-8, IL-10, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), GM-CSF, and MCP-1 using ELISA kits (Quantikine, obtained from R&D Systems; and Endogen, Woburn, MA). TNF-α was quantitated by radioimmunoassay as previously described (46). Briefly, mAb-coated plates were washed four times with PBS-Tween 20, and 50 µl of sample in replicates or standard was added to each plate (detection limit ≥ 1 pg/ml). The assay was repeated three times. Chemotaxis was measured as migration of human monocytes in the upper chamber through filter inserts into the lower chamber containing 72-h supernatants of MCP-1-Ad5-transduced SBcl2 cells. R, Chemotaxis in response to recombinant human MCP-1 at 50 ng/ml. Supernatants of SBcl2 cells, which were not transduced, served as control. Chemotaxis is given as percentage of control (mean ± SD) of triplicate assays performed twice. E, Same as D, except that culture supernatants contained mAb MCP-1-D10-1 (5 µg/ml). *p < 0.05, significant inhibition of chemotaxis as compared with supernatant without Ab.

Modulation of HUVEC phenotype

Changes in endothelial morphology were assessed as previously described (43) with modifications. HUVECs were removed from plastic flasks with trypsin and EDTA, which were neutralized with FCS and M199 medium. Cells were washed and seeded in quadruplicate in 96-well plates at 2 × 10⁴ cells/well. When confluent, cells were washed with HBSS, incubated for 4 h with conditioned or control medium, and overlaid with an acellular collagen mix of M199 medium supplemented with heparin, glutamine, endothelial cell growth factor, sodium bicarbonate, and bovine collagen (collagen type I; Organogenesis, Canton, MA) (final concentration of 1 mg/ml). The mixture was left to gel, 200 µl of conditioned or control medium was added, and cultures were incubated at 37°C for 18 h. Morphological changes in cells were evaluated microscopically.

Statistics

Comparisons between groups were made by the Student t test. A difference between groups of p < 0.05 was considered significant.

Results

MCP-1 production by melanoma cells

ELISA screening of normal melanocytes and melanoma cells revealed constitutive production of MCP-1 in all 30 melanoma cell lines but in none of five melanocyte cultures (Fig. 1A). About half
of the melanoma cell lines produced 5–100 ng MCP-1/ml per 10^6 cells in 72 h, with three of these cell lines producing between 200 and 400 ng/ml. A low-producer primary melanoma cell line, SBC12, representing a biologically early radial growth-phase primary melanoma, was selected for MCP-1 transduction with an adenoviral vector. As shown in Fig. 1B, nontransduced SBC12 cells secreted ~4–9 ng/ml over a 72-h period, and LacZ-transduced cells secreted only marginally more compared with MCP-1-transduced cells. In cells transduced with the viral vector MCP-1-Ad5, MCP-1 production increased with increasing PFU, reaching a maximum of 6000 ng/ml at a dose of 50 PFU/cell.

Western blotting using mAb MCP-1-D101-1 (Fig. 1C) confirmed the production of MCP-1 protein by transduced SBC12 cells (lane 1), whereas MCP-1 was not detected in LacZ-transduced cells (lane 2). The mature protein migrated at 14.5 kDa, with a smaller band apparently the result of incomplete glycosylation. The MCP-1-GST fusion protein was detected at 34 kDa (lanes 3 and 4). Chemotaxis assays confirmed the biological activity of adenoviral vector-induced MCP-1 (Fig. 1D). The migration of human monocytes in response to the culture supernatants of transduced SBC12 cells depended on the dose of MCP-1-Ad5 used for transduction. Supernatants of melanoma cells transduced with MCP-1 at 50 PFU/cell increased chemotaxis >4-fold compared with control, whereas rMCP-1 was 2-fold more chemotactic, similar to the response to supernatants of LacZ-transduced cells at 50 PFU/cell, which was due to the increase in MCP-1 production after LacZ transduction (see Fig. 1B). There was a significant difference in chemotaxis between 50 PFU/cell and 0.5 or 0.05 PFU/cell (p < 0.05) as well as LacZ-transduced cells and 0.05 PFU/cell (p < 0.05), but there was not a significant difference with rMCP-1 and 0.05 PFU/cell. Chemotaxis in response to supernatants of MCP-1-Ad5-transduced SBC12 cells was significantly inhibited with mAb against MCP-1, except when 50 PFU/cell of MCP-1-Ad5 was used for transduction (Fig. 1E). No inhibition was observed with a nonspecific murine IgG (data not shown).

**In vivo survival and growth of SBC12 melanoma cells is dependent on low-level MCP-1 production**

SBC12 cells injected into SCID mice (2 × 10^6 cells/mouse) did not survive and grow; after 4 days, the tumor nodule was no longer visible at the injection site, nor were viable tumor cells seen in histological sections of tumor cell debris at the injection site. The same results were obtained with SBC12 cells transduced with MCP-1-Ad5 at 0.005 PFU/cell. Following transduction of SBC12 cells with LacZ-Ad5 at 50 PFU/cell, the tumor remained palpable, and histochemical analysis indicated a moderate inflammatory reaction, necrotic cells, and only a few surviving melanoma cells at day 4 (Fig. 2C). At day 14, the tumor had disappeared. SBC12 cells transduced with MCP-1-Ad5 at 50 PFU/cell underwent rapid necrosis, with large infiltrates of inflammatory cells, presumably mononuclear cells, in tumor sections (Fig. 2D). Lesions had disappeared by day 14. In contrast, tumor growth and survival was obtained using SBC12 cells transduced with MCP-1-Ad5 at 0.5 PFU/cell. After 4 days, the lesions were very well circumscribed with a mild inflammatory reaction and a small necrotic area in the middle of the tumor (Fig. 2A). Viable cells and mitotic figures were abundant. Tumor growth continued, and, by day 14, the lesion was highly vascularized (Fig. 2B). Transduction of SBC12 cells at 0.05 and 5 PFU/cell resulted in a somewhat intermediate tumor phenotype. Growth of tumors formed by SBC12 cells transduced with MCP-1-Ad5 at 0.5 PFU/cell was almost completely inhibited by daily i.p. injection of a rabbit pAb against MCP-1, with tumor sections revealing some inflammatory reaction but little growth by day 10 (Fig. 2E). Fig. 2F shows a lesion from a mouse treated without Ab. Injection of mice with a nonspecific rabbit IgG showed the same results.

**MCP-1-induced macrophage migration leads to increased vessel formation and production of TNF-α**

To further characterize the inflammatory infiltrate observed at sites surrounding the tumor and to show an increase in vessel formation, melanoma tissue sections were analyzed immunohistochemically (Fig. 3). After injection of SBC12 cells transduced with MCP-1-Ad5 at 50 PFU/cell, a strong infiltration of macrophages, as detected with mAb Mac-1, was observed within and around the lesions on day 4 (Fig. 3A). With MCP-1-Ad5 at decreasing PFU/cell, fewer macrophages were seen. Tissue sections from SBC12 cells transduced with LacZ-Ad5 at 50 PFU/cell revealed only a few macrophages (Fig. 3B). Tumor growth 14 days after injection of SBC12 cells transduced with MCP-1-Ad5 at 0.5 PFU/cell was confirmed by staining with the proliferation marker Ki67 (Fig. 3C), and tumor vasculature within the tumor area had increased, as indicated by immunofluorescence analysis for mouse PECAM-1 (CD31) (Fig. 3D). No such increase in vessel formation was observed after injection of SBC12 cells transduced with MCP-1-Ad5 at higher PFU per cell or with LacZ-Ad5-transduced cells. Nontransduced cells could not be evaluated because they did not survive. A significant increase in tumor vessels in lesions of SBC12 cells transduced with MCP-1 at 0.5 PFU/cell on day 14 (5.5 ± 1.2/mm²) was observed if compared with LacZ at 50 PFU/cell (1.8 ± 0.53/mm²) or MCP-1 at 50 PFU/cell (2.5 ± 0.87/mm²) on day 4. Murine TNF-α was produced on day 4 at sites of infiltration with macrophages in sections of MCP-1-Ad5-transduced SBC12 cells, as shown for 50 PFU/cell (Fig. 3E), whereas, at sites of LacZ-Ad5-transduced cells, hardly any positive cells were found (Fig. 3F).

**Production of TNF-α in cocultures of SBC12 melanoma cells and human monocytes**

Conditioned medium of SBC12 cells transduced at different PFU per cell and cocultured overnight with freshly isolated human monocytes was analyzed for production of cytokines and growth factors. Levels of IL-4, IL-8, IL-10, GM-CSF, bFGF, and VEGF remained unchanged in supernatants of transduced SBC12 cells alone or under coculture conditions. However, TNF-α levels were increased 6-fold in MCP-1-transduced SBcl2 cells cocultured with human monocytes (Fig. 4A). No TNF-α production was found in supernatants of transduced SBC12 cells alone or in supernatants of monocytes activated overnight with recombinant human MCP-1 (100 ng/ml). Screening of 20 melanoma cell lines revealed no constitutive TNF-α production.

To test for the biological significance of TNF-α produced during cocultures of human monocytes and melanoma cells, HUVECs were cultured under collagen type I in the presence of conditioned medium from cocultures (Fig. 4, B–D). Only culture supernatants containing TNF-α induced a branching-like network resembling tubule formation in endothelial cells (23 ± 4.3 vessel-like structures per 27 mm²) (Fig. 4G). This network was inhibited with a neutralizing Ab against TNF-α (7 ± 2.1 vessel-like structures per 27 mm²) (Fig. 4H). No inhibition was observed with an unspecific human IgG (data not shown). The rTNF-α also induced the same morphologic changes (12 ± 2.8 vessel-like structures per 27 mm²) (Fig. 4I), which were inhibited with the same neutralizing Ab (5 ± 3.2 vessel-like structures per 27 mm²) (Fig. 4J) but not with a control Ab (data not shown). No other culture conditions tested (Fig. 4, B–F) induced circular structures, suggesting that TNF-α secreted under coculture conditions is most likely responsible for the angiogenic activity of MCP-1-induced tumors.
The contribution of tumor-derived chemokines in either supporting tumor growth or suppressing it is still controversial. Transfection of tumor cells with MCP-1 can prevent tumor formation (30) and decrease metastasis (28) but can also increase tumorigenicity and lung metastasis if fewer cells are injected into animals (47). The adenoviral vector-mediated transfer of human MCP-1 into a melanoma cell line that is immortalized but nontumorigenic (40) is ideally suited to establish a gradient-dependent expression system. SBcl2 cells are derived from an early, primary cutaneous melanoma and do not grow upon injection into SCID mice at $2 \times 10^6$ cells. When infected at 0.5 PFU/cell with the rAd, melanoma cells showed a 10-fold increase in MCP-1 production as compared with noninfected controls. The higher production levels after 0.5 PFU/cell transduction corresponded to the constitutive levels in about half of the 30 melanoma cell lines tested. These data suggest that MCP-1-mediated recruitment of monocytes into melanoma lesions may be important in many cases for the critical progression step, when the melanoma cells begin to proliferate to form an expanding vertical growth-phase tumor (48). However, experiments in transgenic mice have shown that MCP-1 expression alone does not cause inflammatory activation of cells (49). Thus, the stimulation of monocytes is crucial for the observed biological effects. Lower MCP-1 concentrations have little effect, whereas higher production levels appear to lead to massive infiltration of monocytes/macrophages capable of tumor destruction. To prove the concept of tumor destruction by macrophages at high levels of MCP-1, we transduced an aggressive human melanoma cell line (WM9), which is metastatic in SCID mice, at 50 PFU/cell. As expected, these cells did not grow in vivo (data not shown). Because human MCP-1 binds to the murine receptor with $\sim 50\%$ affinity (50), the production levels by patients' tumors to attract the critical number of monocytes for tumor growth stimulation may be lower.

In mice injected with SBcl2 cells that were transduced with MCP-1-Ad5 at 0.5 PFU/cell, tumor-associated murine macrophages were found mainly as a peritumoral infiltration, whereas the 50 PFU/cell

**FIGURE 2.** MCP-1-dependent growth of SBcl2 cell tumors in vivo. SBcl2 cells were infected with MCP-1-Ad5 or LacZ-Ad5 and 2 days later were injected s.c. into SCID mice ($2 \times 10^6$ cells/mouse). Sections were stained with hematoxylin and eosin. A, SBcl2 cells 4 days after injection of MCP-1-transduced cells at 0.5 PFU/cell (magnification, $\times 40$). At higher magnification (A1; magnification, $\times 600$) mitosis is visible (indicated by arrowhead). The bar represents 0.5 mm. B, Same as A, except 14 days after injection (magnification, $\times 40$). Higher magnification (B1; magnification, $\times 400$) reveals abundant blood vessels (indicated by arrowhead), with a single vessel shown in B2 (magnification, $\times 600$). C, SBcl2 cells transduced with LacZ at 50 PFU/cell. No tumor growth 4 days after injection (magnification, $\times 40$). Higher magnifications show only few surviving tumor cells (C1; magnification, $\times 400$) and apoptosis (C2; magnification, $\times 600$). D, SBcl2 cells transduced with MCP-1-Ad5 at 50 PFU/cell 4 days after injection. A strong cellular infiltrate and tumor necrosis are visible (magnification, $\times 40$). Higher magnifications show tumor necrosis (D1; magnification, $\times 600$) and a mononuclear cell infiltrate (D2; magnification, $\times 600$). E, Inhibition of tumor growth of SBcl2-transduced cells at 0.5 PFU/cell at day 10 after daily i.p. injection with 150 µg of rabbit pAb against MCP-1 starting 1 day before s.c. injection of transduced SBcl2 cells until day 4 (magnification, $\times 80$), with some surviving tumor cells (E1; magnification, $\times 160$). F, Same as E but without Ab against MCP-1 (magnification, $\times 80$), showing tumor growth (F1; magnification, $\times 160$). The same results were obtained with nonspecific rabbit IgG.
infection rate resulted in intratumoral as well as peritumoral infiltration patterns. Recruitment of peritumoral macrophages was likely beneficial, whereas intratumoral infiltration led to macrophage-mediated cytotoxicity. Due to the transient nature of adenoviral-mediated gene transfer and the decline in MCP-1 levels when tumor cells divide, the observation period in our experiments was 2 wk, which restricts overall conclusions for a longer period of time, especially with respect to sustained tumor growth and progression. After 3 wk, when Ad-driven MCP-1 production had ceased, tumors disappeared, suggesting that continuous stimulation by mouse macrophages is necessary to maintain tumor survival and growth.

MCP-1-mediated macrophage attraction appears to be essential for tumor growth at 0.5 PFU/cell because i.p. injection of tumor-bearing mice with a neutralizing pAb against MCP-1 inhibited the recruitment of macrophages and abrogated tumor growth. Similar results have been obtained in a tumorigenic melanoma cell line, which recruited large numbers of macrophages within the tumor mass. Treatment with a neutralizing Ab against MCP-1 resulted in reduced numbers of intratumoral macrophages and, subsequently, in significantly higher tumor growth (33).

Tumor-associated macrophages play a pivotal role in tumor angiogenesis (51), thus enabling tumor cells to survive and proliferate. Activated macrophages can release growth factors (VEGF, platelet-derived growth factor, insulin-like growth factor-1, bFGF, GM-CSF) and cytokines (IL-1, IL-6, IL-8, and TNF-α), some of which are candidates for melanoma growth stimulation in the 0.5 PFU/cell transduction group. However, MCP-1 overexpression in melanoma cells did not increase production of VEGF and bFGF, the two most likely candidates inducing tumorigenicity in biologically early melanoma cells (45 and our unpublished observations), nor was their production increased in cocultures of human monocytes and melanoma cells. MCP-1 can also trigger adhesion of monocytes to vascular endothelium (3) and can regulate expression of adhesion molecules and cytokines, in particular, the α-chains of two members of the β2 integrin family (52, 53). MCP-1 stimulates monocytes to produce IL-1 and IL-6 but not TNF-α (52). In our investigations, TNF-α appears to be the pivotal cytokine in inducing tumor growth; production of TNF-α was increased up to 6-fold in cocultures of monocytes and melanoma cells, depending on the batch of isolated monocytes, whereas production in either melanoma cells or monocytes alone was unchanged. TNF-α is expressed at low levels in nevi and primary and metastatic melanoma in situ throughout the progression of melanocytic lesions (54). In vitro, only a few melanoma cell lines express TNF-α RNA transcripts (55). Therefore, the source of the TNF-α increase observed in this study may be due to the activation of monocytes through contact with Sbcl2 cells or indirectly through soluble factors. Staining for murine TNF-α in sections revealed that TNF-α is indeed produced by macrophages infiltrating (Fig. 3E) or surrounding tumor lesions. This is in accordance with recent findings that describe the production of murine TNF-α after stimulation with melanoma-conditioned medium (56) or IL-18 (57). TNF-α production by monocytes/macrophages after activation is well known (51). The reasons for the dramatic increase in TNF-α production in cocultures of melanoma cells and monocytes are unclear. It is possible that MCP-1-stimulated monocytes produce IL-6, and melanoma cells in vitro express functional IL-6 receptors (54, 58). However, biologically early melanoma cells are inhibited by IL-6, and only metastatic melanoma cells are stimulated (58).

The reasons for the increase in the vascularization of the tumors observed at intermediate concentrations of MCP-1 remain speculative. In the rabbit corneal angiogenesis assay, MCP-1 was capable of inducing neovascularization, although the angiogenic process was linked to the recruitment of macrophages (59), suggesting that macrophages were a prerequisite for this process and that the effect of MCP-1 was only indirect. However, a direct involvement of MCP-1 in angiogenesis has been reported recently (60). In our study, no changes in HUVEC morphology were observed when rMCP-1 of 100 ng/ml was added to the medium, whereas conditioned medium from cocultures or rTNF-α induced a branching-like network. The phenotypic alterations were partially inhibited by a TNF-α neutralizing Ab. Thus, it seems likely that the angiogenic phenotype is initiated
through activated macrophages producing TNF-α. In turn, activated macrophages may produce proangiogenic factors (61–63). Although TNF-α was shown to inhibit the growth of endothelial cells (64), low doses of TNF-α stimulated migration of endothelial cells and induced tubule-like structures (65). The dual role of TNF-α in angiogenesis has been demonstrated by Fajardo et al. (66) through direct stimulation and modulation of angiogenic factors such as IL-8, VEGF, and bFGF (63). Together, our results point to the pivotal role of tumor-infiltrating inflammatory cells for melanoma progression at a stage when the tumor cells are still susceptible to cytotoxicity by host cells.

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References


