Synergistic Antitumor Interaction of Human Monocyte Chemotactant Protein-1 Gene Transfer and Modulator for Tumor-Infiltrating Macrophages

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Purpose. In order to evaluate the possibility of synergistic antitumor gene therapy by the gene delivery of monocyte chemotactant protein-1 (MCP-1/MCAF/IE), the effect of a biological response modulater for macrophages on tumor progression of gene transfected tumor cells was studied.

Methods. Cachexia-inducing adenocarcinoma cells (cell line colon 26, clone 20) were transfected with either a control plasmid or MCP-1 cDNA.

Results. The production of MCP-1 reached 70-80 ng/ml *in vitro* when transfectant cells were cultured at a cell density of 1×10^5 cells/ml for 3 days. Transfection of MCP-1 cDNA did not affect the growth rate *in vitro*. Also, MCP-1-transfectants formed tumors after intra-footpad inoculation similar in size to the parental cells. The number of infiltrating macrophages in the primary tumor of the transfectant rapidly increased from the 3rd to 5th day after inoculation as revealed by immunohistochemical staining using an antibody against mouse macrophages. An earlier, greater, but no longer-lasting increase in tumor-infiltrating macrophages was induced in tumors by MCP-1 transfection was compared to that induced by the parent cells. On the 10th day after the inoculation, the tumor-infiltrating macrophages in mice inoculated MCP-1 transfectants were decreased to a level similar to that of the parent cells. Groups of mice were treated intraperitoneally with LPS at different times after the inoculation. Tumor cells producing high levels of MCP-1 were significantly lysed by macrophages treated with LPS, whereas parental or control transfected cells were not.

Conclusions. Combination immunotherapy can provide a rationale for the application of MCP-1 treatment to increase immunological responses to cancer.

KEY WORDS: gene transfer; colon 26; monocyte chemotactic and activating factor; chemokine; biological response modulater.

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ABBREVIATIONS: MCAF, monocyte chemotactic and activating factor; MCP-1, monocyte chemotactic protein-1; ELISA, enzyme-linked immunosorbent assay.

INTRODUCTION

It has been shown that gene delivery of cytokines, such as interleukin (IL)-2, IL-4, IL-6, IL-7, tumor necrosis factor (TNF), or interferon-gamma (IFN-γ) is able to induce tumor rejection if produced locally by the tumor cells after gene transfer (1). The cellular mechanisms responsible for tumor rejection with different cytokines have been associated with prominent infiltration of several immune effectors (2). However, tumor responses for cytokine have been variable as reported with the opposite effects on tumor growth also reported. The expression of TNF by different tumor cell lines demonstrates both suppression of locally growing tumor and promotion of metastasis formation (3). The pattern of susceptibility of unfractionated tumor to lysis by different effector cells was shown to be different (4).

A novel cytokine family with chemotactic activity against leukocytes has been identified over the past decade, and named chemokines (5). Monocyte chemotactant protein-1 (MCP-1/MCAF/JE) is an important mediator of monocyte recruitment to sites of chronic inflammation and neoplasia. Previously, we examined whether MCP-1 can also enhance activation of the tumoricidal capacity of monocytes. Although a potential clinical role for infused MCP-1 in patients has been pointed out (6,7), we found that MCP-1 gene transfer with relatively low level expression of MCP-1 augments the metastasis of colon 26 clone 20 cells. On the other hand, it is reported that MCP-1 can prime human monocytes to respond to LPS. Human monocytes incubated with MCP-1 and subthreshold concentrations of lipopolysaccharide (LPS) exhibited synergistic tumoricidal activity against allogeneic A375 melanoma cells (8). Another example that JE/MCP-1 can synergize with bacterial endotoxins to activate macrophages to become tumoricidal and, hence, suppress metastasis of the highly metastatic murine colon carcinoma CT-26 cells (9) again suggests the possibility of synergistic antitumor gene therapy by the gene delivery of MCP-1 with biological response modulaters for macrophages on tumor progression of gene transfected tumor cells. In the present study, cachexia-inducing adenocarcinoma cells (cell line colon 26, clone 20) were transfected with either a control plasmid or MCP-1 cDNA, and combination immunotherapy with LPS was studied.

MATERIALS AND METHODS

Mice

Female Balb/c mice were obtained from Clea, Tokyo, Japan. They were maintained under pathogen-free conditions. Body weight was measured twice a week between 9:00–11:00 a.m. All animal experiments were approved by the Institutional Animal Care and Use Committee and complied with the standards set out in the Guideline for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University.

Tumor Cells

Colon 26 clone 20 adenocarcinoma cells were grown as monolayer cultures in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine at 37°C with 5% CO_2 (10).

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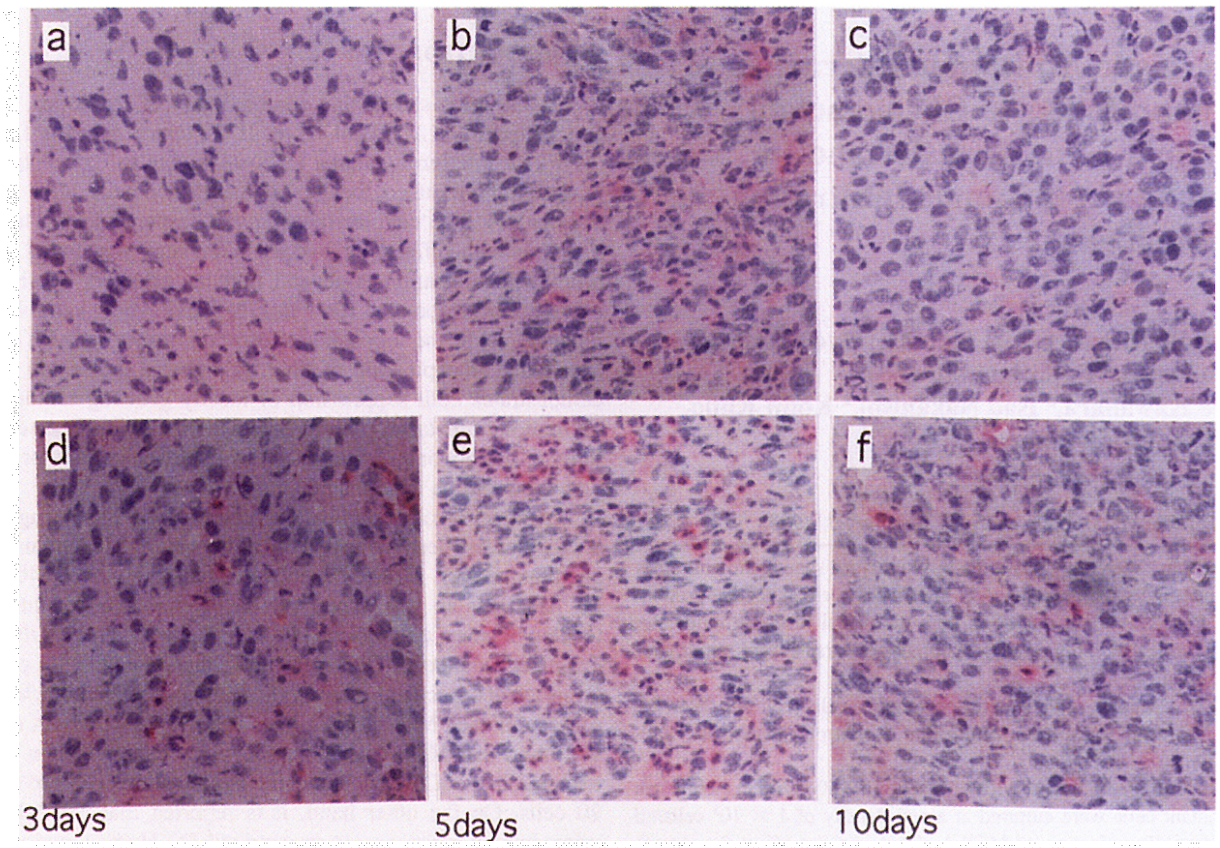

Fig. 1. Immunohistochemical stain for mouse macrophage in primary tumors of Colon 26 clone 20 carcinoma (a, b, c) and MCP-1-secreting(d, e, f) cells, × 200. Macrophage in tumors was determined using an antibody to mouse macrophage for the tumors 3 (a, d), 5 (b, e) and 10 (c, f) days after footpad inoculation.

Plasmid Construction

A *Pst-I-Pst-I* fragment (0.4 kb) containing the entire coding region for human MCP-1 (11) was subcloned into a digested mammalian expression vector pHbAPr-3p-neo (pHbAPr-3p-neo/MCP-1) (12) which has a neomycin resistance gene. pHbAPr-3p-neo/MCP-1 can express a foreign gene under control of the human β-actin promoter.

Transfection with MCP-1 cDNA

Colon 26 clone 20 cells were transfected with control- or MCP-1-cDNA by a modified calcium phosphate co-precipitation method (13). After transfection, the cells were cultured in medium supplemented with G418 (antibiotic) at a final concentration of 300 mg/ml. Three weeks later, G418-resistant clones were isolated. The MCP-1 producing cells (> 0.4 ng/ml) were used for the animal experiment.

In Vitro Cell Growth and the Secreted MCP-1 Detection Assay

Cells were plated in 10 cm plates with 20 ml standard medium without G418, at a density of 10^6 cells per plate. Three days later, supernatants were collected and tested for MCP-1 levels using the ELISA (14). Briefly, each well of a 96 well microtiter plate was coated with 100 ml of rabbit polyclonal antibodies against human MCP-1 (2.0 mg/ml) in 0.1 M carbonate buffer (pH 9.6) at 4°C overnight. Unbound sites were blocked by adding 150 ml/well of 1% BSA-PBS at 37°C for 1h. After washing with 0.05% Tween-PBS (washing buffer), aliquots of standards and samples diluted in 1% BSA-PBS were added and incubated at 37°C for 1h. The wells were washed five times with washing buffer, then 100 ml of biotin-conjugated rabbit polyclonal anti-MCP-1 antibody (1 mg/ml) in 1% BSA-PBS was added, and incubated at 37°C for 1h. After washing, 100 ml of alkaline phosphatase-conjugated streptoavidin diluted at 1:1000 in 1% BSA-PBS (BRL-GIBCO, Gaithersburg, MD) was added, and incubated at 37°C for 1h. Finally, 100 ml of *p*-nitrophenylphosphate disodium salt (1 mg/ml) in 1M-diethanolamine (pH 9.0) supplemented with 10 mM $MgCl_2$ was added, and incubated at room temperature for 1h. The enzyme reaction was stopped by the addition of 0.1 M NaOH, and the optical density at 405 nm was measured. The concentrations were determined against the recombinant human MCP-1 expressed using an animal cell (15). The detection limit of the assay was 185 pg/ml.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis for mRNA in Primary and Metastatic Tumors

MCP-1 mRNA expression was evaluated by RT-PCR analysis. Two mg total RNA prepared using RNAzol B (Biotex Laboratory Inc. Houston, Texas), was reverse transcribed in 20

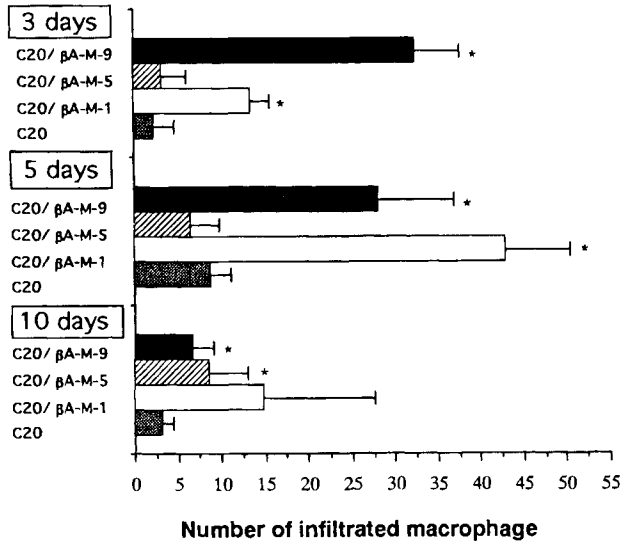

Fig. 2. Macrophage infiltration into the primary tumor in the tumor-bearing mice 3, 5, and 10 days after inoculation. Macrophage in tumors was determined using an antibody to mouse macrophage for the tumors.

μl reaction containing AMV reverse transcriptase (Seikagaku Corp., Tokyo) and the antisense primer. After 1 hr incubation at 42°C, 2 μl of cDNA product was amplified in 20 μl reaction containing 0.2 mM of dNTP, 10% glycerol, 100 ng each of the sense and antisense primers and 0.75 U of Taq polymerase by using a DNA thermal cycler (Perkin Elmer Cetus Corp., Norwalk, CT). The cycler program consisted of denaturation at 94°C for 30 sec, annealing at 57°C for 1 min, and extension at 72°C for 1 min 30 sec. Fragment size by PCR is 284 bps (MCP-1, 70-353). cDNA samples were size-fractionated on agarose gel. mRNA of MCP-1 was clearly detected in both primary and metastatic tumors of the MCP-1-transfected clones.

Tumor Inoculation

The adherent cells were collected after brief trypsinization and counted. Nine-week-old female Balb/c mice were inoculated into the footpad of the right hind leg with 10^6 tumor cells suspended in 0.04 ml of sterilized endotoxin-free PBS per mouse. Tumor size at the injected site was determined by measuring the footpad height with calipers.

Histological Analyses

Tissue from the site of tumor cell inoculation was fixed with OCT compound (Miles, IN, USA) and frozen in liquid N_2. Immunohistochemical analyses were performed on the tumors at the injected footpad using a monoclonal antibody against mouse macrophages (U-RM2800, Caltag Lab. Inc., CA, USA). For immunoperoxidase, sections were incubated with the antibody before indirect immunoperoxidase staining by the avidin-biotin complex(ABC) method.

Statistical Analysis

The means and SDs were calculated on all of the parameters determined in this study. Statistical analysis was performed using two-way ANOVA, followed by a multiple comparison according to the Tukey-Krammer procedure and Wilcoxon single-rank sum test. A p value of 0.05 or less was used to indicate a significant difference between the sets of data.

RESULTS

Characteristics of Transfected Colon 26 Clone 20 *In Vitro*

Three clones (designated C20/βA-M-1, C20/βA-M-5, and C20/βA-M-9) were obtained after the transfection of pHβAPr-

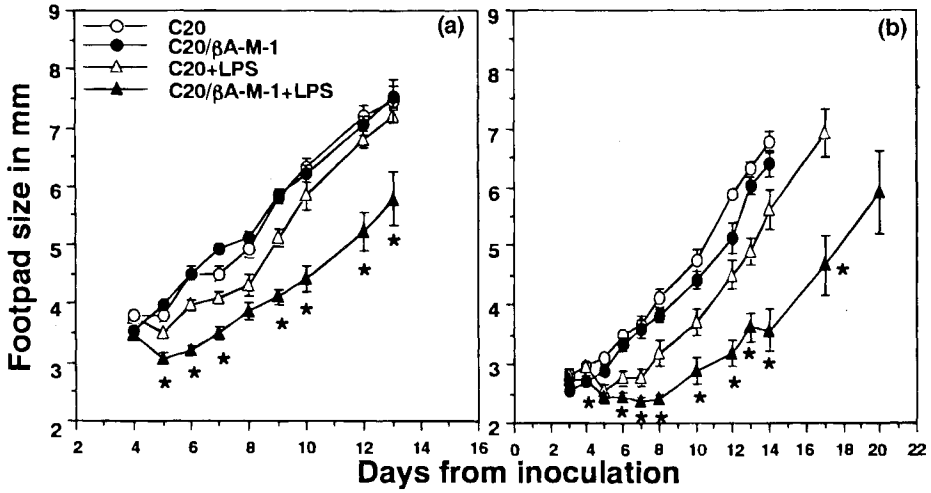

Fig. 3. Effects of multiple administration of LPS on local tumor growth. Groups of four BALB/c mice were inoculated with 10^6 (a) or 10^5 (b) tumor cells into the footpad. LPS (30 μg) was injected intraperitoneally 4, 6, and 8 days after the tumor inoculation. The combined results of three experiments with three to five mice per group are shown for C20 (o), C20/βA-M-1 (o), C20 treated with LPS (Δ), and C20/βA-M-1 treated with LPS (Δ). Data presented are mean with SEM of at least 9 mice. Statistical differences between clones 20 and MCP-1 transfectant treated with LPS were determined using two-way ANOVA, followed by a multiple comparison according to the Turkey-Krammer procedure; *, significance with $P < 0.05$.

3p-neo/βA-M into parental colon 26 clone 20. The production of MCP-1 of C20/βA-M-1 and C20/βA-M-9 reached 70–80 ng/ml *in vitro* when transfectant cells were cultured at a cell density of 1×10^5 cells/ml for 3 days. The stable expression was confirmed by measuring the amount in supernatant over 30 days and 6th seeding.

Transfection of MCP-1 cDNA did not reduce the growth rates *in vitro* (Table I). To study the effects of MCP-1 gene transfer on the tumorigenicity of the cells, we injected parental and transfected clones into the footpads of syngeneic Balb/c mice. Both parental and transfected clones readily formed tumors within 1–2 days after the inoculation. No tumor suppression effects were observed in any of the mice innoculated.

Histological Analyses of the Tumor Tissues at the Inoculation Sites

Immunohistochemical analysis using a monoclonal antibody to mouse macrophages demonstrated that the number of macrophages progressively increased in the footpad on the 3rd day after mice were injected with MCP-1-transfected clones (Fig. 1). An earlier, greater, but no longer-lasting increase in tumor-infiltrating macrophages was induced in tumors by MCP-1 transfection as compared to that induced by the parent cells. The number of macrophages tended to be higher in the footpads of mice inoculated with highly producing clone C20/βA-M-1 and C20/βA-M-9 transfectants than those inoculated with parental clones on 3 days and 5 days after the inoculation. On the 10th day after the inoculation, the tumor-infiltrating macrophages in mice inoculated MCP-1 transfectants were decreased to a level similar to that of parent cells (Fig. 2).

Synergistic Treatment with Macrophage Modulator

Since LPS is a super antigen which primes macrophages at low concentrations, we examined the possibility of increasing the tumor suppressing effect of MCP-1 transfer. Groups of mice were treated intraperitoneally with LPS at different times after the inoculation. Tumor cells producing high levels of MCP-1 were significantly lysed by macrophages treated with LPS, whereas parental or control transfected cells were not (Fig. 3). When the number of cells was reduced to one tenth, the synergistic effects of LPS and gene transfer on tumor growth were more obvious as shown in Fig. 3(b). Neither group of mice showed enhanced lung metastasis (data not shown).

Table I. Growth Rate, Doubling Time, and Production of MCP-1 *In Vitro*

Cell type (day^{-1})[a]	Growth rate (hr)[a]	Doubling time ($ng/10^5$ cells/3 days)[b]	Production of MCP-1
C20	0.719	23.2	—
C20/βA-M-V	0.765	21.8	—
C20/βA-M-1	0.789	21.1	69.1
C20/βA-M-5	0.765	22.1	>3.5
C20/βA-M-9	0.824	20.2	81.4

[a] Each clone was seeded at 1×10^6 cells and after 3 days, the cells were harvested by trypsinize and counted.
[b] Each clone was seeded at 3.5×10^5 cells/7 ml culture medium and after 3 days, the culture supernatants were collected and the amount of MCP-1 in culture supernatants was measured by ELISA.

DISCUSSION

In the present study, we investigated the antitumor effects of combined immunotherapy with MCP-1 gene transfer and an immunomodulator in the treatment of primary tumor of cachexia-inducing colon 26 clone 20 cells (Fig. 3). Human MCP-1 is an equally potent chemoattractant with mouse MCP-1/JE for mouse macrophage *in vitro* (16), and we observed an increased infiltration of macrophages into primary tumors of MCP-1 transfectants producing relatively high levels of MCP-1 in culture medium, on the 3 and 5th days after the inoculation.

Since, the MCP-1 transfectant, C20/βA-M-5, producing low level of MCP-1 could not recruite the macrophages into the primary tumor site more than the parental cells did, it is suggested that the effectiveness of MCP-1 is dose-dependent. However, neither highly producing transfectants C20/βA-M-1 nor C20/βA-M-9 showed a long duration of infiltrating macrophages. The number of macrophages was similar to that of the parental cells on the 10th day in *in vitro* primary tumors. MCP-1 is capable of acting as a potent chemoattractant to T-lymphocytes, as well as monocytes, at a concentration of 3.3 nM *in vitro* (17). It is still difficult to estimate the concentration gradient of chemokine in primary tumor sites of MCP-1 transfectants; however, a high level of MCP-1 in plasma may cause a decreased concentration gradient inducing monocytes in the blood stream or down regulation of MCP-1 receptor on the surface of monocytes (18). Thus the number of infiltrated macrophages decreased 10 days after the inoculation of C20/βA-1 or -9.

Transfection of MCP-1 expression vector did not affect the growth rate *in vitro* or *in vivo* as we previously reported (1). Moreover, the growth rate of parental tumor cells was not affected by the treatment with LPS. However, sequential administration of LPS showed synergistic tumoricidal activity against MCP-1 transfectant as shown in Figure 3, suggesting that MCP-1 alone is not sufficient to cause macrophage activation *in vivo*. Singh reported that human monocytes incubated with MCP-1 and subthreshold concentrations of LPS exhibited synergistic tumoricidal activity against allogeneic A375 melanoma cells *in vitro*, whereas monocytes first treated with LPS and then with MCP-1 did not (8). Pretreatment of macrophages with MCP-1 significantly enhanced their response to low concentrations of LPS as measured by lysis of murine melanoma cells *in situ* (19). Our results and the reported data suggest that MCP-1 can prime macrophages to respond to LPS and therefore may regulate the macrophage-tumor interactions *in vivo*. These cells may be useful as a tumor vaccination. In order to perform a clinical study, it would be necessary to modify the transfected cells so as not to grow, i.e. by irradiation or treatment of antibiotics.

These results demonstrate that combination immunotherapy can provide a rationale for the application of MCP-1 treatment to increase immunological responses to cancer.

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REFERENCES

1. E. Nakashima, N. Mukaida, Y. Kubota, K. Kuno, K. Yasumoto, F. Ichimura, I. Nakanishi, M. Miyasaka, and K. Matsushima. Human MCAF gene transfer enhances the metastatic capacity of a mouse cachectic adenocarcinoma cell line *in vivo*. *Pharm Res.* **12**(11):1598–1604 (1995).
2. H. Hock, M. Dorsch, U. Kunzendorf, Z. Qin, T. Diamantstein, and T. Blankenstein. Mechanisms of rejection induced by tumor cell-targeted gene transfer of interleukin 2, interleukin 4, interleukin 7, tumor necrosis factor, or interferon gamma. *Proc Natl Acad Sci USA* **90**(7):2774–8 (1993).
3. Z. Qin, K. S. Kruger, U. Kunzendorf, H. Hock, T. Diamantstein, and T. Blankenstein. Expression of tumor necrosis factor by different tumor cell lines results either in tumor suppression or augmented metastasis. *J Exp Med.* **178**(1):355–60 (1993).
4. D. A. Clark, H. W. Hirte, and R. N. Buick. Human ovarian carcinoma: evidence for patient-related differences in susceptibility to cytotoxic effectors that attack different cellular subpopulations within a tumour. *Br J Cancer.* **58**(4):415–8 (1988).
5. D. D. Taub, P. Proost, W. J. Murphy, M. Anver, D. L. Longo, D. J. Van, and J. J. Oppenheim. Monocyte chemotactic protein-1 (MCP-1), -2, and -3 are chemotactic for human T lymphocytes. *J Clin Invest.* **95**(3):1370–6 (1995).
6. B. J. Rollins and M. E. Sunday. Suppression of tumor formation *in vivo* by expression of the JE gene in malignant cells. *Mol Cell Biol.* **11**(6):3125–31 (1991).
7. B. J. Rollins. JE/MCP-1: an early-response gene encodes a monocyte-specific cytokine. *Cancer Cells* **3**(12):517–24 (1991).
8. R. K. Singh and I. J. Fidler. Synergism between human recombinant monocyte chemotactic and activating factor and lipopolysaccharide for activation of antitumor properties in human blood monocytes. *Lymphokine Cytokine Res.* **12**(5):285–91 (1993).
9. S. Huang, R. K. Singh, K. Xie, M. Gutman, K. K. Berry, C. D. Bucana, I. J. Fidler, and E. M. Bar. Expression of the JE/MCP-1 gene suppresses metastatic potential in murine colon carcinoma cells. *Cancer Immunol Immunother.* **39**(4):231–8 (1994).
10. Y. Tanaka, H. Eda, K. Fujimoto, T. Tanaka, T. Ishikawa, and H. Ishitsuka. Anticachectic activity of 5′-deoxy-5-fluorouridine in a murine tumor cachexia model, colon 26 adenocarcinoma. *Cancer Res.* **50**(15):4528–32 (1990).
11. Y. Furutani, H. Nomura, M. Notake, Y. Oyamada, T. Fukui, M. Yamada, C. G. Larsen, J. J. Oppenheim, and K. Matsushima. Cloning and sequencing of the cDNA for human monocyte chemotactic and activating factor (MCAF). *Biochem Biophys Res Commun.* **159**(1):249–55 (1989).
12. P. Gunning, J. Leavitt, G. Muscat, S. Y. Ng, and L. Kedes. A human beta-actin expression vector system directs high-level accumulation of antisense transcripts. *Proc Natl Acad Sci USA.* **84**(14):4831–5 (1987).
13. C. Chen and H. Okayama. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol.* **7**(8): 2745–52 (1987).
14. Y. Ko, N. Mukaida, A. Panyutich, N. N. Voitenok, K. Matsushima, T. Kawai, and T. Kasahara. A sensitive enzyme-linked immunosorbent assay for human interleukin-8. *J Immunol Methods* **149**(2):227–35 (1992).
15. Y. Nakano, T. Kasahara, N. Mukaida, Y. C. Ko, M. Nakano, and K. Matsushima. Protection against lethal bacterial infection in mice by monocyte-chemotactic and -activating factor. *Infect Immun.* **62**(2):377–83 (1994).
16. K. Matsushima, C. G. Larsen, G. C. DuBois, and J. J. Oppenheim. Purification and characterization of a novel monocyte chemotactic and activating factor produced by a human myelomonocytic cell line. *J Exp Med.* **169**(4):1485–90 (1989).
17. M. W. Carr, S. J. Roth, E. Luther, S. S. Rose, and T. A. Springer. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc Natl Acad Sci USA* **91**(9):3652–6 (1994).
18. J. M. Wang, A. Hishinuma, J. J. Oppenheim, and K. Matsushima. Studies of binding and internalization of human recombinant monocyte chemotactic and activating factor (MCAF) by monocytic cells. *Cytokine.* **5**(3):264–75 (1993).
19. R. K. Singh, K. Berry, K. Matsushima, K. Yasumoto, and I. J. Fidler. Synergism between human monocyte chemotactic and activating factor and bacterial products for activation of tumoricidal properties in murine macrophages. *J Immunol.* **151**(5): 2786–93 (1993).