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Natural Killer Cell-dependent Suppression of Systemic Spread of Human Lung Adenocarcinoma Cells by Monocyte Chemoattractant Protein-1 Gene Transfection in Severe Combined Immunodeficient Mice[1]

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ABSTRACT

Monocyte chemoattractant protein-1 (MCP-1) is a chemokine with various biological activities, including augmentation of cytotoxic activity of monocytes and natural killer (NK) cells. The present study was undertaken to determine whether transfection of the *MCP-1* gene into lung cancer cells affected their tumorigenicity and metastatic potential by the NK cell-mediated mechanism. The human *MCP-1* gene inserted into an expression vector (BCMGSNeo) was transfected into human lung adenocarcinoma (PC-14) cells. There was no difference in *in vitro* proliferation between *MCP-1* gene-transfected PC-14 cells and the parent cells or mock-transfected cells. The tumorigenicity and *in vivo* tumor growth of *MCP-1* gene-transfected PC-14 cells were similar to those of the parent cells or mock-transfected cells when tumor cells were injected into the s.c. space of NK cell-intact severe combined immunodeficient (SCID) mice. Although parent cells and mock-transfected cells inoculated i.v. formed lung metastatic colonies and pleural effusion, *MCP-1* gene transfectants reduced the systemic spread in NK cell-intact SCID mice. Interestingly, these modulations in a systemic spread by *MCP-1* gene transfection were not observed in NK cell-depleted SCID mice. Decreased survival of *MCP-1* gene transfectants in the lung was observed in NK cell-intact SCID mice but not in NK cell-depleted SCID mice. Recombinant MCP-1 or the supernatant of *MCP-1* gene transfectants enhanced the cytotoxicity of human $CD56^+$ NK cells and spleen cells of SCID mice against PC-14 cells. These findings suggest that locally produced MCP-1 suppresses tumor progression by a NK cell-mediated mechanism, depending on organ microenvironment.

INTRODUCTION

MCP-1,[3] also known as MCAF, is a member of the CC (or β) chemokine family of cytokines (1) and has been reported as a selective chemoattractant protein for monocytes but not for neutrophils (2, 3). It stimulates the cytotoxic activity of monocytes and enhances the expression of adhesion molecules, such as CD11b and CD11c. In addition to the activation of monocyte functions, the enhancing activity of NK cells by MCP-1 on cytotoxicity and migration has been reported (4–7).

Transfection of cytokine genes into tumor cells has been investigated widely for analysis of cytokine-mediated biological effects on tumor growth and is very important in successful cancer immunotherapy to attract effector cells such as monocyte-macrophages and lymphocytes into the tumor growth site (8, 9). Therefore, we examined the effect of *MCP-1* gene transfection on tumor progression. Our recent reports (10, 11) demonstrated that *MCP-1* gene transfection into a human small cell lung cancer cell line (H69/VP) did not affect tumorigenicity or growth rates in the s.c. space of nude mice or SCID mice, although it caused greater macrophage infiltration into the tumors. Moreover, to evaluate the monocyte-mediated effect of MCP-1 more selectively, we used NK cell-depleted SCID mice pretreated with anti-IL-2 receptor β chain Ab (TM-β1) and showed that *MCP-1* gene transfection into H69/VP cells again had no effect on metastasis formation in NK cell-depleted SCID mice (11). However, *MCP-1* gene transfection into H69/VP cells caused a remarkable enhancement of Ab-dependent cellular cytotoxicity reaction by augmentation of macrophage infiltration into metastatic lesions and hence augmented the therapeutic efficacy of anti-P-glycoprotein Ab on metastasis by P-glycoprotein-expressing H69/VP cells in multiple organs such as the liver, kidneys, and systemic lymph nodes (11).

On the other hand, the NK cell-mediated effect of MCP-1 on metastasis has not been examined thus far. We developed a model for lung metastasis and PEs of human lung adenocarcinoma cells in NK cell-intact SCID mice (12). To directly analyze the contribution of NK cells, we examined the metastatic potential of *MCP-1* gene-transfected lung adenocarcinoma cells in NK cell-intact and NK cell-depleted SCID mice in this study.

MATERIALS AND METHODS

Reagents. FBS and G418 were purchased from Life Technologies (Rockville, MD). Recombinant human MCP-1 was produced in a murine myeloma cell line, X5563, transfected with cDNA for human MCP-1, and purified to homogeneity by carboxymethyl high-performance liquid chromatography (13). Recombinant human IL-2 (specific activity, 1.14×10^7 units/mg protein as assayed on IL-2-dependent murine NKC3 cells) was a gift from Takeda Pharmaceutical Co. (Osaka, Japan). None of these materials contained endotoxins, as judged by Limulus amebocyte assay (Seikagaku Kogyo, Tokyo, Japan). [^{125}I]IdUrd (specific activity, 5 μCi/mg) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, England).

Cell Cultures. Human lung adenocarcinoma PC-14 cells were kindly supplied by Dr. N. Saijo (National Cancer Institute, Tokyo, Japan), and K562 cells were obtained from the American Type Culture Collection (Rockville, MD). These cell cultures were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS (CRPMI 1640) and gentamicin at 37°C in a humidified atmosphere of 5% CO_2 in air. YAC-1 cells were generously given by W. Chambers (University of Pittsburgh, Pittsburgh, PA) and were maintained in CRPMI 1640 and 2 mM glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 5×10^{-5} M 2-mercaptoethanol (Life Technologies) at 37°C in a humidified atmosphere of 5% CO_2 in air.

Gene Transfection and Cloning of Transfected Cell Lines. PC-14 cells were transfected with BCMGSNeoMCAF (13) containing a 400-bp *Pst*I fragment of human MCP-1 cDNA using Lipofectin Reagents (Life Technologies), as described in detail elsewhere (14). After 18 h, the medium was changed to

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[3] The abbreviations used are: MCP, monocyte chemoattractant protein; rMCP-1, recombinant MCP-1; NK, natural killer; IL, interleukin; SCID, severe combined immunodeficient; PE, pleural effusion; Ab, antibody; FBS, fetal bovine serum; ICAM-1, intercellular adhesion molecule-1; Neo, neomycin resistant; MCAF, monocyte chemotactic and activating factor; MACS, magnetic cell sorting; CSF, colony-stimulating factor; VEGF, vascular endothelial growth factor; IdUrd, iododeoxyuridine.

fresh CRPMI 1640 medium containing 800 μg/ml of G418 in 24-well plates. Cell subclones resistant to G418 were assayed for the expression of MCP-1 mRNA by Northern blotting. For the establishment of cell lines that stably expressed MCP-1, the subclones positive for MCP-1 mRNA were cloned by limiting dilution. Stable clones transfected with BCMGSNeo were established by a similar method.

Preparation of Total Cellular RNA and Northern Blot Analysis. Tumor cells were lysed in ISOGEN (Nippon Gene, Tokyo, Japan), a mixture of guanidinium isothiocyanate and phenol. Total cellular RNA was then extracted with chloroform and precipitated with isopropanol, as described elsewhere (11). The denatured RNA (5 μg) was electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde, transferred to a Hybond-N^+ nylon membrane, and hybridized with ^{32}P-labeled probes. The filters were washed twice with 0.1 × saline-sodium phosphate-EDTA and 0.1% SDS for 1 h at 60°C. The membranes were autoradiographed using a Fujix bioimaging analyzer BAS 1500 (Fuji Photofilm Co., Tokyo, Japan). A cDNA probe of human *MCP-1* was obtained by digesting BCMGSNeo-MCAF plasmid vector with *Xho*I and *Not*I. cDNA fragments of human *MCP-1* and β-actin (Wako, Osaka, Japan) were labeled using a random primer labeling kit (Takara Shuzo, Kyoto, Japan) with [α-^{32}P]dCTP (Amersham Pharmacia Biotech; 110 TBq/mmol).

Enzyme Immunoassay of Human MCP-1. Tumor cells were plated at 5×10^5 cells/1 ml/well in 24-well plates. After 24 h, the supernatants were harvested and used for measuring MCP-1 protein. An ELISA of human MCP-1 was performed essentially as described previously (15). Briefly, microtiter plates were coated overnight with antihuman MCP-1 monoclonal Ab (ME61, 1 μg/ml) in 100 μl/well of 0.05 M carbonate buffer (pH 9.6) at 4°C. The plates were then washed three times with PBS containing 0.05% Tween 20 (buffer A), blocked with a solution of 1% BSA in buffer A (buffer B) at 37°C for 1 h, and washed again with buffer A. The standards and samples diluted in buffer B were then incubated in the wells overnight at 4°C. The wells were then washed three times and incubated with 100 μl of rabbit antihuman MCP-1 Ab at 37°C for 2 h. They were then washed at least 10 times and incubated with 100 μl of alkaline phosphatase-conjugated antirabbit IgG (Tago code 6500; diluted 1:10,000 with buffer B) at 37°C for 2 h. Finally, 100 μl of enzyme substrate solution [1 mg/ml *p*-nitrophenylphosphate (Sigma Chemical Co., St. Louis, MO) in 1 M diethanolamine (pH 9.8) supplemented with 0.5 mM $MgCl_2$] were added to each well, and the plates were incubated at room temperature for 30 min. The enzyme reaction was stopped by adding 100 μl 1 M NaOH, and A_{405} was determined using an ELISA plate reader (Titertek Multiscan; Flow, Meckenheim, Germany). All samples were assayed in triplicate at least.

Analysis by Flow Cytometry. Expression of surface antigens was measured by flow microfluorometry as described in detail previously (11). Briefly, tumor cells (1×10^6) were washed once with PBS supplemented with 2% FBS and 0.05% sodium azide (2% FBS-PBS). The washed cells were incubated for 30 min at 4°C in 2% FBS-PBS with anti-CD44 (Cosmo Bio Co., Tokyo, Japan), anti-MHC class I (Immunotech, Marseilles, France), anti-ICAM-1 Abs (Becton Dickinson, San Jose, CA), or normal mouse serum as a negative control. They were then washed twice with 2% FBS-PBS and treated with FITC-conjugated goat antimouse IgG (H+L; Immunotech) for 30 min at 4°C. The cells were washed again, and their fluorescence intensity was measured with a FACScan (Becton Dickinson, Mountain View, CA).

***In Vitro* Proliferation Assays.** Tumor cells (1×10^5) were plated into each well of six-well plates. After 12, 24, 48, 72, and 96 h incubation at 37°C, the cells were trypsinized, and the number of viable cells was counted using trypan blue stain (Life Technologies). Triplicate wells were counted per time point. Cell proliferation was estimated as the doubling time.

Animals. Male SCID mice, 6–8 weeks of age, were obtained from Charles River (Yokohama, Japan) and maintained under specific pathogen-free conditions. Experiments were performed according to the guidelines of our university (University of Tokushima).

Tumorigenicity and Experimental Metastasis. For the production of tumors, parental PC-14 and PC-14 clones transfected with the *MCP-1* gene or *Neo* gene (5×10^6 cells/200 μl PBS/mouse) were injected s.c. into the flanks of SCID mice. The formation and size of the tumors were monitored every 3–4 days until day 49. Tumor volume (V) was calculated by the formula $V = 1/2ab^2$, in which a is the longest diameter and b is the shortest diameter of the tumor.

For experimental metastases of human lung cancer cells, viable tumor cells suspended in 300 μl of PBS were injected into the lateral tail vein of SCID mice (12, 16). In some experiments, SCID mice received injections i.p. with TM-β1 Ab (1 mg/mouse) 2 days before tumor inoculation to deplete NK cells (16). After the indicated periods, the mice were sacrificed, and the numbers of metastatic lymph nodes were counted. Nodules in the liver, kidneys, and lungs were counted with the aid of a dissecting microscope.

Isolation of Human $CD56^+$ NK Cells. Leukocyte concentrates from healthy donors were separated into peripheral blood mononuclear cells by density gradient centrifugation in lymphocyte separation medium (10). Subsequently, peripheral blood mononuclear cells were separated into lymphocytes and monocytes by counterflow centrifugal elutriation in a Beckman JE-5.0 rotor (Beckman Instruments, Inc., Fullerton, CA) as reported previously (10). The lymphocyte-rich fraction was collected at flow rates of 12–16 ml/min at 2000 rpm. The purity of the lymphocyte fraction, determined by morphological examination and nonspecific esterase staining, was >99%. In these lymphocytes, highly purified $CD56^+$ cells were obtained by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions as described previously (17). Briefly, the purified lymphocytes were incubated with MACS CD56 MicroBeads on ice for 15 min. After washing, these cells were applied for the positive selection column MiniMACS MS^+/RS^+. The column was washed five times, and $CD56^+$ cells were flushed out. The purity of $CD56^+$ NK cells was >94%, as determined by flow cytometry after staining with PE-conjugated antihuman $CD56^+$ Ab (Immunotech).

The Cytotoxicity Assay. As effector cells, the spleen cells of SCID mice were also prepared as described elsewhere (18). The purified human $CD56^+$ NK cells or murine spleen cells were cultured in medium with various concentrations of rMCP-1, IL-2 (500 units/ml), or culture supernatants of tumor of PC-14neo13 or PC-14MCP2 cells. The cytotoxicity was determined using a standard ^{51}Cr release assay as described previously (10). The K562, YAC-1, or PC-14 cells were used as targets to evaluate NK activity. In brief, target cells were labeled with 100 μCi of ^{51}Cr for 1 h. After washing twice, these effector and target cells were plated at an appropriate E:T ratio in 96-well round-bottomed plates. The supernatant (100 μl) was collected after a 4-h (human $CD56^+$ NK cells) or an 8-h (murine spleen cells) incubation, and the radioactivity was counted using a gamma counter. Determinations were carried out in triplicate. The percentage of specific cytolysis was calculated from the release of ^{51}Cr from test samples and control samples, as follows:

$$\%\ \text{specific lysis} = \frac{(E - S)}{(M - S)} \times 100$$

in which E is the release in the test sample (cpm in the supernatant from target cells incubated with test effector cells), S is the spontaneous release (cpm in the supernatant from target cells incubated in medium alone), and M is the maximum release (cpm released from target cells lysed with 1 N HCl).

Distribution and Fate of [^{125}I]IdUrd-labeled Tumor Cells *in Vivo*. To investigate the distribution and fate of mock-transfected cells or *MCP-1* gene-transfected cells, we compared the organ distribution of these cells after tumor injection with a method reported earlier (19). In brief, cells were labeled with [^{125}I]-IdUrd for 24 h. After washing twice, [^{125}I]IdUrd-labeled cells (1×10^6 cells/300 μl of PBS/mouse) were injected into the lateral tail vein of SCID mice. After the indicated periods, the mice were sacrificed, and the radioactivity of each organ was counted using a gamma counter.

Statistical Analysis. The significance of differences of *in vitro* data were analyzed by Student's t test (two-tailed), and the significance of differences of *in vivo* data were analyzed by Mann-Whitney U test or Wilcoxon test.

RESULTS

Transfer of the *MCP-1* Gene to Human Lung Adenocarcinoma Cells. In the first set of experiments, we examined whether parental PC-14 cells produced endogenous human MCP-1 (Fig. 1; Table 1). We found no production of MCP-1 at mRNA or protein level in parental PC-14 cells. After transfection with BCMGSNeoMCAF or BCMGSNeo, we selected three clones that produce MCP-1 (PC-14MCP2, PC-14MCP10, and PC-14MCP12) and two neo control clones (PC-14neo7 and PC-14neo13) for the following assays (Fig. 1). These transfectants (PC-14MCP2, PC-14NCP10, and PC-14MCP12)

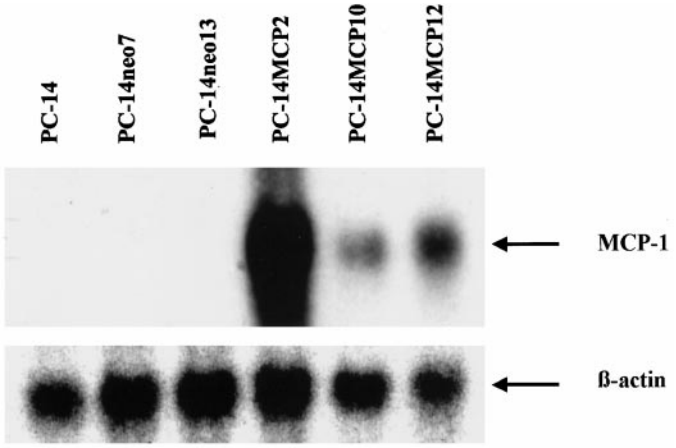

Fig. 1. Expression of MCP-1 mRNA in established PC-14 variant cell lines transfected with the *MCP-1* gene. Total RNA (5 μg) was separated in a 1% agarose gel containing 6% formaldehyde. It was then transferred to a nitrocellulose filter and hybridized with either ^{32}P-labeled human *MCP-1* or *β-actin* probes.

secreted MCP-1 stably for at least 15 months *in vitro*. Transfection of the *MCP-1* gene into PC-14 cells did not cause any change in the expression of surface antigens (MHC class I, CD44, and ICAM-1) or *in vitro* proliferation (Table 1). Moreover, we examined production of other cytokines, such as IL-1α, IL-1β, IL-6, IL-8, granulocyte-CSF, granulocyte/macrophage-CSF, macrophage-CSF, tumor necrosis factor-α, IFN-γ, or VEGF. PC-14 cells or transfectants produced IL-6, IL-8, M-CSF, and VEGF, and there was no difference in production of these cytokines (data not shown).

Tumorigenicity and *in Vivo* Growth of *MCP-1* Gene-transfected Human Lung Adenocarcinoma Cells in NK Cell-intact SCID Mice. To evaluate the effect of MCP-1 production on tumorigenicity and *in vivo* growth, we inoculated parental, neo control, and *MCP-1* gene-transfected PC-14 cells (5×10^6 cells) s.c. into the flank of SCID mice and measured tumor diameters every 3–4 days until day 42. All seven cell lines tested showed 90–100% tumor uptake on day 42, irrespective of MCP-1 production (Table 1). There was no significant difference statistically in the *in vivo* growth rate between parental tumors and transfectants (Fig. 2).

Metastasis of *MCP-1* Gene-transfected Human Lung Adenocarcinoma Cells in NK Cell-intact SCID Mice. We established previously (12) a model of lung metastasis and malignant PEs by PC-14 cells in NK cell-intact SCID mice. Next, we examined the metastatic potential of *MCP-1* gene-transfected PC-14 cells in this model. PC-14 cells with or without *MCP-1* gene transfection (1×10^6) were inoculated i.v. into SCID mice, and the mice were sacrificed on day 56. Parent and neo control cells formed numerous metastases mainly in the lungs, and a high incidence in the formation of PE was observed (Table 2). In contrast, *MCP-1* gene transfectants formed fewer lung metastases, and the incidence in the formation of PE was remarkably less.

Metastasis of *MCP-1* Gene-transfected Human Lung Adenocarcinoma Cells in NK Cell-depleted SCID Mice. To directly analyze the possible contribution of NK cells in the antimetastatic effect of *MCP-1* gene transfection to PC-14 cells, PC-14 cells with or without *MCP-1* gene transfection were injected i.v. into NK cell-depleted SCID mice (pretreated with TM-β1 Ab; Refs. 16 and 20). Parent and neo control cells formed numerous metastases in the liver and kidneys as well as in the lungs, which indicated that NK cell depletion facilitated metastasis formation into multiple organs by PC-14 cells consistent with previous report (12). Under the same experimental conditions, no significant differences between parent or neo control cells and *MCP-1* gene transfectants were observed in metastatic formation (Table 3), which suggests that NK cells play a critical role in metastatic formation by *MCP-1* gene transfectants.

Effect of *MCP-1* Gene Transfection on Survival of NK Cell-intact SCID Mice Inoculated with Tumor Cells. We examined the effect of *MCP-1* gene transfection on the survival of NK cell-intact

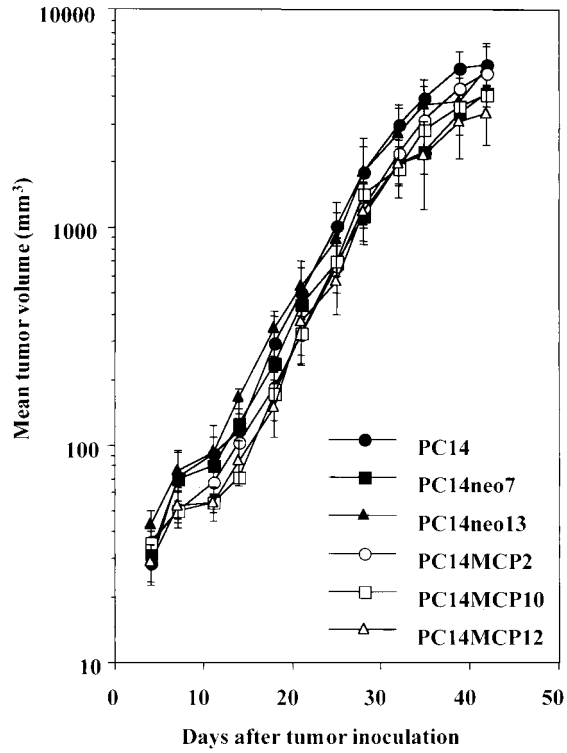

Fig. 2. *In vivo* growth of *MCP-1* gene-transfected PC-14 cells. SCID mice were inoculated s.c. in the flank with PC-14 or PC-14 variant cells (5×10^6). Tumor volumes were measured every 3–4 days until day 42. *Bars*, SD of values in five mice.

Table 1 *In vitro characterization of MCP-1 gene-transfected human lung adenocarcinoma cells*

Cell line	MCP-1[a] (ng/ml)	Surface antigens[b] Class I (control)	CD44	ICAM-1	*In vitro* proliferation[c] (doubling time, h)	Tumorigenicity[d] (mice with tumor/total)
PC-14	<0.05	70.4 (2.8)	487.9	3.0	22.9	9/9
PC-14neo7	<0.05	65.7 (2.9)	500.3	3.3	23.4	8/9
PC-14neo13	<0.05	76.1 (3.0)	477.0	3.1	22.6	9/9
PC-14MCP2	325.0	77.5 (2.9)	455.9	3.2	21.9	9/9
PC-14MCP10	89.4	63.9 (2.8)	522.5	2.9	22.7	8/9
PC-14MCP12	107.4	73.8 (3.0)	581.9	3.2	22.3	9/9

[a] PC-14 or PC-14 variant cells (5×10^5) were cultured for 24 h. The MCP-1 concentration in culture supernatants was determined by ELISA.
[b] Surface antigen expression was measured by flow cytometry. Values are expressed as mean fluorescence.
[c] *In vitro* proliferation of tumor cells was counted using trypan blue stain.
[d] PC-14 or PC-14 variant cells (5×10^6) were inoculated s.c. into SCID mice, and tumorigenicity was determined on day 42.

Table 2 *Metastatic formation of MCP-1 gene-transfected human lung adenocarcinoma cells in NK cell-intact SCID mice*[a]

Cell line	No. of metastatic colonies (mean ± SD)				Incidence of PE (mice with PE/total)
	Liver	Kidneys	Lymph nodes	Lungs	
Experiment 1					
PC-14	0	0	0	20 ± 7	5/5
PC-14neo7	0	0	0	16 ± 7	4/5
PC-14neo13	0	0	0	19 ± 7	5/5
PC-14MCP2	0	0	0	2 ± 3[b]	0/5
PC-14MCP10	0	0	0	7 ± 3[b]	1/5
PC-14MCP12	0	0	0	5 ± 4[b]	1/5
Experiment 2					
PC-14	0	0	0	20 ± 9	4/5
PC-14neo13	0	0	0	25 ± 8	5/5
PC-14MCP2	0	0	0	1 ± 1[b]	0/5
Experiment 3					
PC-14	0	0	0	23 ± 11	4/5
PC-14neo7	0	0	0	13 ± 6	3/5
PC-14MCP2	0	0	0	1 ± 1[b]	0/5
PC-14MCP10	0	0	0	6 ± 2[b]	0/5

[a] SCID mice received injections i.v. with PC-14 or PC-14 variant cells (1×10^6) on day 0 and were sacrificed on day 56. Metastatic colonies were counted as described in "Materials and Methods."

[b] $P < 0.05$ *versus* value for PC-14neo7 or PC-14neo13.

SCID mice inoculated i.v. with tumor cells (5×10^6). The survival of mice inoculated with PC-14MCP2 cells was significantly longer than that of mice inoculated with PC-14neo13 cells ($P < 0.05$; Fig. 3).

Effect of MCP-1 on the Activation of NK Cells *in Vitro*. To explore the mechanism of the antimetastatic effect of MCP-1 in the NK cell-intact model, we examined the potential of MCP-1 to enhance the killer activity of NK cells against K562 (human NK-sensitive cells), YAC-1 (murine NK-sensitive cells), and PC-14 cells *in vitro*. We used human $CD56^+$ NK cells and murine spleen cells of SCID mice as effector cells. Human rMCP-1 enhanced the cytotoxicity of human $CD56^+$ NK cells against K562 and PC-14 cells (Fig. 4). In addition, pretreatment of NK cells with the supernatant of PC-14neo13 cells also stimulated NK activity. Because PC-14 cells produced various cytokines, such as IL-6, IL-8, macrophage-CSF, and VEGF (data not shown), and IL-6 has been reported to stimulate NK activity (21), IL-6 (and/or other NK cell-activating molecules) secreted in the supernatants might stimulate NK activity. Under these experimental conditions, culture supernatants of PC-14MCP2 cells further augmented NK activity compared with those of PC-14neo13 cells. Furthermore, human rMCP-1 or culture supernatants of PC-14MCP2 cells also enhanced the killer activity of murine spleen cells against YAC-1 or PC-14 cells (Fig. 5). The cytotoxicity of murine spleen cells cultured with recombinant human IL-2 against YAC-1 or PC-14 cells was 73.2% or 26.4%, respectively.

Distribution and Fate of *MCP-1* Gene Transfectants *in Vivo*. We examined distribution (by 4 h) and fate (up to 72 h) of MCP-1 gene transfectants compared with neo control cells in SCID mice with or without NK cell depletion. For this purpose, radiolabeled neo control cells or *MCP-1* gene transfectants were injected i.v. into NK cell-intact or -depleted SCID mice, and the percentage of [^{125}I]IdUrd-labeled tumor cells in various organs (including the lungs) was determined as described in "Materials and Methods." Neo control cells and *MCP-1* gene transfectants were distributed equally to the lung by 4 h after tumor cell injection, irrespective of NK cell depletion from SCID mice (Table 4). However, at 24 h after tumor cell injection, six times more viable neo control cells were detected in the lungs of NK cell-depleted SCID mice compared with NK cell-intact SCID mice, which suggests that NK cells play an important role in tumor cell eradication at this phase. In NK cell-depleted SCID mice, there was no difference between neo control cells and *MCP-1* gene transfectants on viability in the lungs up to 72 h after tumor cell injection. In contrast, a marked difference in cell viability in the lung was observed at 24 and 72 h after tumor cell injection in NK cell-intact SCID mice. The viability of *MCP-1* gene transfectants in the lung was much lower than that of neo control cells in NK cell-intact SCID mice.

DISCUSSION

In the present study, we demonstrated that *MCP-1* gene transfection into human lung adenocarcinoma (PC-14) cells suppressed metastatic spread in NK cell-intact SCID mice but not in NK cell-depleted SCID mice. MCP-1 produced by *MCP-1* gene transfectants significantly augmented the cytotoxic activity of human NK cells and murine spleen cells, which suggests that the antimetastatic effect of *MCP-1* gene transfection is mediated predominantly by the activation of NK cells with MCP-1 secreted by tumor cells.

Because lymphocytes (T and B cells) are lacking in SCID mice, NK

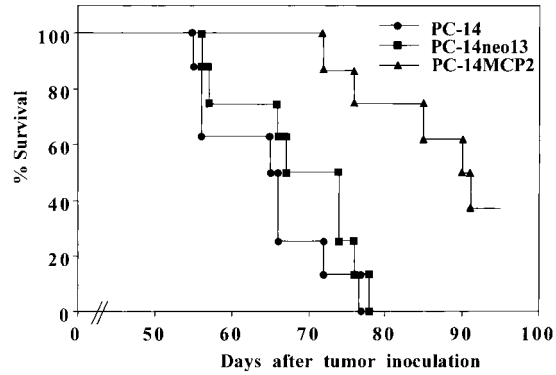

Fig. 3. Effect of *MCP-1* gene transfection into PC-14 cells on survival of SCID mice. SCID mice received injections i.v. with PC-14 or PC-14 variant cells (1×10^6). Eight mice were included in each group.

Table 3 *Metastatic formation of MCP-1 gene-transfected human lung adenocarcinoma cells in NK cell-depleted SCID mice*[a]

Cell line	Number of metastatic colonies (mean ± SD)				Incidence of PE (mice with PE/total)
	Liver	Kidneys	Lymph nodes	Lungs	
Experiment 1					
PC-14	7 ± 4	8 ± 2	4 ± 1	174 ± 55	1/5
PC-14neo7	5 ± 3	9 ± 3	5 ± 1	161 ± 56	1/5
PC-14neo13	8 ± 6	8 ± 2	5 ± 4	167 ± 47	2/5
PC-14MCP2	5 ± 2	7 ± 3	5 ± 1	146 ± 59	1/5
PC-14MCP10	7 ± 4	10 ± 4	4 ± 1	170 ± 54	2/5
PC-14MCP12	6 ± 3	8 ± 2	5 ± 2	142 ± 55	1/5
Experiment 2					
PC-14	5 ± 2	8 ± 3	1 ± 1	198 ± 54	1/5
PC-14neo13	8 ± 4	9 ± 2	2 ± 2	173 ± 33	2/5
PC-14MCP2	5 ± 4	10 ± 4	2 ± 2	148 ± 42	1/5
PC-14MCP10	5 ± 2	7 ± 3	2 ± 1	163 ± 52	1/5

[a] SCID mice were pretreated with TM-β1 Ab (1 mg) for NK cell depletion and received injections i.v. with 1×10^6 cells of PC-14 cells or PC-14 variant cell lines on day 0 and were sacrificed on day 21. Metastatic colonies were counted as described in "Materials and Methods."

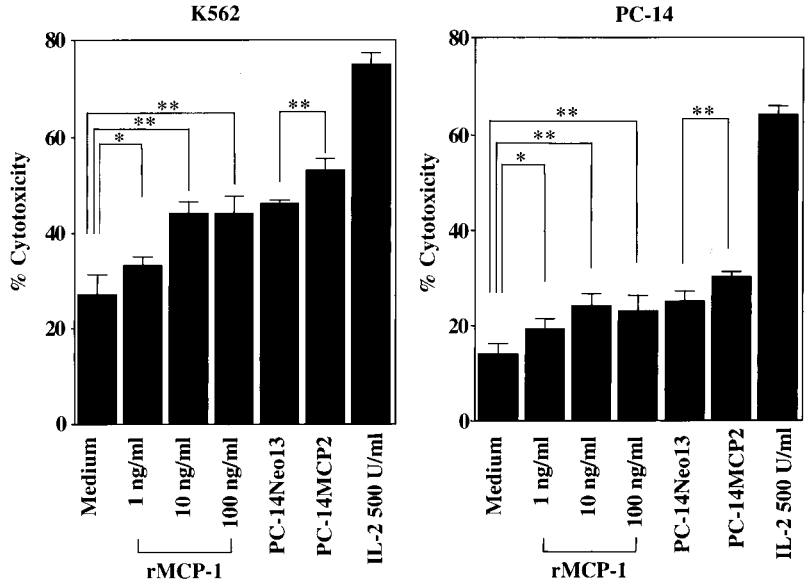

Fig. 4. Effect of MCP-1 on the activation of human $CD56^+$ NK cells *in vitro*. The culture supernatants of PC-14neo13 or PC-14MCP2 cells were harvested after incubation for 24 h. The purified $CD56^+$ NK cells were cultured for 18 h in medium with or without rMCP-1 or culture supernatants (1:4 dilution) of tumor of PC-14neo13 or PC-14MCP2 cells. The resultant cells were incubated with ^{51}Cr-labeled K562 cells or PC-14 cells (E:T ratio, 10) for 4 h. *, $P < 0.1$; **, $P < 0.05$.

cells or monocyte-macrophages can be considered as mechanisms of the antimetastatic effect observed in SCID mice. NK cells, especially, have been reported to play an important role in metastasis formation (22). We demonstrated previously that NK cell depletion promoted metastasis formation by human lung cancer cell lines (H69/VP small cell carcinoma and RERF-LC-AI squamous cell carcinoma) into multiple organs, and we extended this phenomenon using PC-14 adenocarcinoma cells in this study. To deplete NK cells, we pretreated SCID mice with an i.p. injection of antimouse IL-2 receptor β chain Ab TM-β1. This treatment successfully and selectively depletes NK cells in SCID mice at least for 6 weeks, irrespective of tumor cell injection (16, 23).

Although MCP-1 was discovered originally as a chemotactic and activating factor for monocytes, the chemotactic activity of MCP-1 for T lymphocytes was documented later. In addition, recent reports (4–7) have shown that CC chemokines, including MCP-1, potentiate the function of NK cells. Loetscher *et al.* (6) found that CC chemokines induced chemotaxis, Ca^{2+} changes, and the release of granule enzymes from both cloned and blood NK cells. Consistent with these reports, we show in this study that MCP-1 enhanced the killer activity of human $CD56^+$ NK cells or spleen cells of SCID mice against K562, YAC-1, or PC-14 cells *in vitro*. Moreover, *MCP-1* gene transfection into PC-14 cells resulted in inhibition of lung metastasis only in NK cell-intact SICD mice but not in NK cell-depleted SCID mice. MCP-1 was reported to augment not only killer activity of NK cells but also migration of NK cells (4–7). These findings strongly suggest that MCP-1 secreted by transfectants augments the migration and killer activity of NK cells, hence inhibiting the lung metastasis of PC-14 cells.

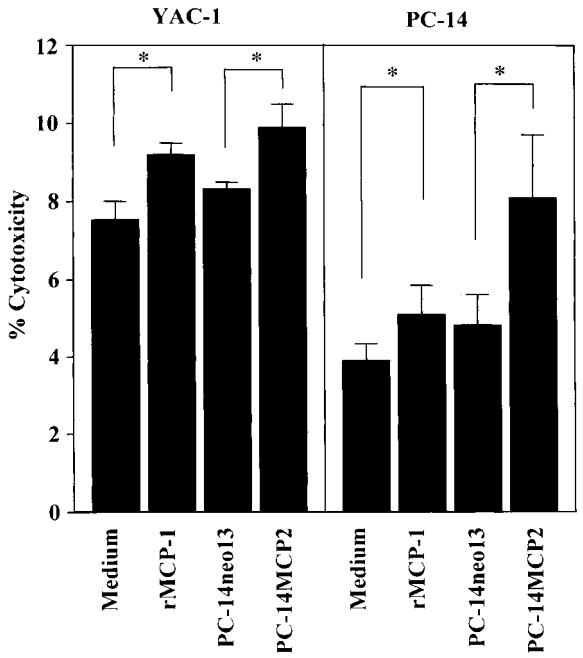

Fig. 5. Effect of MCP-1 on the activation of mouse spleen cells *in vitro*. The culture supernatants of PC-14neo13 or PC-14MCP2 cells were harvested after incubation for 24 h. Mouse spleen cells were cultured for 18 h in medium with or without rMCP-1 (100 ng/ml) or culture supernatants (1:2 dilution) of tumor of PC-14neo13 or PC-14MCP2 cells. The resultant cells were incubated with ^{51}Cr-labeled YAC-1 cells or PC-14 cells (E:T ratio, 100) for 8 h. *, $P < 0.05$.

The process of metastasis consists of several sequentially linked steps (24). Although tumor cells bypassed the initial steps of metastasis (separation from the primary tumor, invasion, and release into blood vessels) in our experimental metastasis model, all subsequent steps (arrest at a distant site, extravasation, and proliferation as a secondary colony with neovascularization) must have occurred. To clarify further the mechanism of inhibition of lung metastasis of *MCP-1* gene transfectants in NK cell-intact SCID mice, we determined at which steps this inhibition occurred. Our results show that *MCP-1* gene transfectants and neo control cells were equally arrested at the capillary of the lung at 4 h after tumor cell injection and that a marked difference in cell viability between the transfectants and neo control was observed at 24–72 h after injection. Within 6 h after injection, extravasation can occur from the capillary plexus (25), although it was not determined when PC-14 cells extravasated in this model. Therefore, it is possible that NK cells activated with MCP-1 secreted by transfectants eradicated these tumor cells just before or after extravasation from the lung capillary (at least after the arrest at the lung capillary).

An interesting point of this study is that *MCP-1* gene transfection did not result in the inhibition of tumorigenicity or growth when inoculated into the s.c. space even in NK cell-intact SCID mice. The same phenomenon has been well documented by Hirose *et al.* (26) and ourselves (10, 11). Hirose *et al.* (26) demonstrated that *MCP-1* gene transfection into Chinese hamster ovary cells had no effect on tumorigenicity in nude mice. We reported also that there was no effect of *MCP-1* gene transfection on the tumorigenicity of human small cell

Table 4 *The distribution and fate of MCP-1 gene-transfected human lung adenocarcinoma cells in NK cell-intact and NK cell-depleted SCID mice*[a]

	Distribution of cells (%)			
	NK cell-intact SCID mice		NK cell-depleted SCID mice	
	PC-14neo13	PC-14MCP2	PC-14neo13	PC-14MCP2
4 h				
Blood[b]	0.288 ± 0.058	0.339 ± 0.046	0.255 ± 0.050	0.312 ± 0.033
Lungs	20.348 ± 3.146	18.544 ± 2.114	23.075 ± 2.810	20.638 ± 4.332
Liver	1.044 ± 0.125	1.211 ± 0.214	1.073 ± 0.081	1.266 ± 0.196
Kidneys	0.066 ± 0.006	0.082 ± 0.012	0.077 ± 0.010	0.100 ± 0.027
Brain	0.014 ± 0.004	0.022 ± 0.010	0.016 ± 0.004	0.032 ± 0.012
24 h				
Blood[b]	0.018 ± 0.008	0.012 ± 0.001	0.027 ± 0.003	0.021 ± 0.005
Lungs	0.520 ± 0.028	0.058 ± 0.003[c]	3.388 ± 0.592	2.128 ± 0.581
Liver	0.107 ± 0.013	0.073 ± 0.008[c]	0.132 ± 0.008	0.110 ± 0.015
Kidneys	0.035 ± 0.002	0.028 ± 0.007	0.046 ± 0.008	0.037 ± 0.004
Brain	0.004 ± 0.003	0.003 ± 0.001	0.005 ± 0.002	0.003 ± 0.001
72 h				
Blood[b]	0.013 ± 0.003	0.011 ± 0.002	0.018 ± 0.005	0.012 ± 0.004
Lungs	0.024 ± 0.005	0.013 ± 0.001[c]	0.275 ± 0.047	0.201 ± 0.063
Liver	0.067 ± 0.005	0.061 ± 0.001	0.075 ± 0.015	0.057 ± 0.003
Kidneys	0.060 ± 0.016	0.081 ± 0.006	0.092 ± 0.011	0.091 ± 0.029
Brain	0.000 ± 0.000	0.001 ± 0.001	0.003 ± 0.003	0.002 ± 0.002

[a] SCID mice were pretreated with TM-β1 Ab (1 mg) for NK cell depletion and received injections i.v. with ^{125}I-Urd-labeled cells (1×10^6) of PC-14neo13 or PC-14MCP2. Mice were sacrificed at 4, 24, or 72 h after tumor injection. The radioactivity of each organ was counted using a gamma counter.

[b] 0.5 ml of blood was counted.

[c] $P < 0.05$ *versus* value for PC-14neo13.

lung cancer (H69/VP) cells in nude and SCID mice. As we proposed previously (20), antitumor effects by cytokine gene modification can be regulated differentially by various organ microenvironments (including the lung and skin). In fact, NK cells are found predominantly in the blood and spleen (27). Therefore, the microenvironments of the lungs and skin might differ especially in the accumulation or activation of NK cells induced by MCP-1.

In summary, *MCP-1* gene transfection into human lung adenocarcinoma cells resulted in an inhibition of metastatic formation presumably via the activation of NK cells. Locally secreted MCP-1 can augment Ab-dependent cellular cytotoxicity reaction and the therapeutic efficacy of Ab through the accumulation of monocyte-macrophages into the tumors (11). Therefore, an approach to facilitate the recruitment and activation of immuno-effector cells such as NK cells and monocyte-macrophages may be crucial for the development of a novel immunotherapy for lung cancer patients.

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