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Human MCAF Gene Transfer Enhances the Metastatic Capacity of a Mouse Cachectic Adenocarcinoma Cell Line *in Vivo*

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Purpose. To evaluate the effect of monocyte chemotactic and activating factor (MCAF/MCP-1/JE) on tumor progression and metastasis.
Methods. Cachexia-inducing adenocarcinoma cells (cell line colon 26, clone 20) were transfected with either a control plasmid or MCAF expression vector. Spontaneous lung metastases were determined in mouse.
Results. The production of MCAF reached 0.4 ng/ml in vitro when transfectant cells were cultured at a cell density of 5×10^4 cells/ml for 3 days. Transfection of MCAF expression vector did not affect the growth rate in vitro. Also, after MCAF-transfection, the size of tumors after intra-footpad inoculation was similar to that of the parental cells. When the primary tumors were resected on the 10th day after inoculation, the incidence of spontaneous lung metastasis was less than 20% in both cells. The number of endothelial cells in the primary tumor rapidly increased from the 10th to the 14th day after inoculation, as revealed by immunohistochemical staining. In accordance with enhanced angiogenesis, the incidence rates of spontaneous metastasis increased when the primary tumors were resected on the 14th day after inoculation. Moreover, the spontaneous lung metastases were augmented in the animals injected with MCAF-transfectants compared to those injected with parental cells with a concomitant increase of angiogenesis.
Conclusions. These results suggest that MCAF may augment the metastatic potential by modulating tumor associated angiogenesis.

KEY WORDS: gene transfer; metastasis; colon 26; monocyte chemotactic and activating factor; chemokine.

INTRODUCTION

Delivery of cytokine genes to tumors is a potent candidate for cancer gene therapy to stimulate host immune responses that could reduce tumor size and metastasis. A novel cytokine family with chemotactic activity against leukocytes has been identified over the past decade, and named chemokines (1). The monocyte chemotactic and activating factor (MCAF), alternative acronym monocyte chemotactic protein-1 (MCP-1/JE), is thought to regulate the recruitment of monocytes/macrophages to inflammatory sites and neoplastic tissues as well as their activation, including lysosomal enzyme release and cytocidal activities against tumor cells, and is active in both mice and humans (2). Since MCAF is shown to regulate the chemotaxis and tumoricidal activation of blood monocytes, it might be an important mediator of tumor regression (3). When MCAF gene was transferred into tumor cells, the tumor-derived MCAF could recruit the monocytes in neoplastic tissues (4,5). The mechanisms underlying chemokine-induced tumor rejection are of interest as a contribution to a rational basis for future immunotherapy. Parameters such as the mouse strain, the level of cytokine expression, or tumor cell line are still unclear at present.

Multiple cytokines are capable of stimulating an angiogenetic response involved in the metastatic process (reviewed in reference (6) as a review). Although the direct effect of MCAF on angiogenesis has not been clarified, the angiogenetic activity of IL-8, a C-X-C chemokine, has been demonstrated by several laboratories; moreover, the expression of IL-8 has also been shown to correlate with the metastatic potential of human melanoma cells in nude mice (7). Hence, these findings raised the possibility that MCAF might also enhance the metastatic potential of tumor cells through increased angiogenesis. We examined here the effect of human MCAF cDNA transfection on the metastatic ability of a cachectic mouse adenocarcinoma cell line, colon 26 clone 20 in vivo (8).

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 at 9:00 - 11:00 a.m. All animal experiments complied with the standards set out in the Guidelines for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University and adhered to the principles of Laboratory Animal Care of the NIH.

Tumor Cells

Colon 26 clone 20 adenocarcinoma cells were grown as monolayer cultures in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine at 37°C with 5% CO_2 (8).

Plasmid Construction

A *Pst-I-Pst-I* fragment (0.4 kb) containing the entire coding region for human MCAF (9) was subcloned into a digested mammalian expression vector pHβAPr-3p-neo (pHβAPr-3p-neo/MCAF) (10) or an expression plasmid BCMGSNeo (BCMGSNeo/MCAF) (11). Both vectors have a neomycin resistance gene. pHβAPr-3p-neo/MCAF can express a foreign gene under control of the human β-actin pro-

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

Table I. In Vitro Growth Rate, Doubling Time, and MCAF Production of Clone 20, pHβAPr-3p-neo/Vector Transfected Clone 20 (C20/V), pHβAPr-3p-neo/MCAF Transfected Clone 20 (C20/V-MCAF1 and C20/V-MCAF2) and BCMGSNeo/MCAF Transfected Clone 20 (C20/V-BCMG)[a]

Cells	Growth rate, day^{-1} [b]	Doubling time, hr[b]	Level of MCAF[c]
clone 20	0.74 ± 0.11	22.9 ± 3.1	<0.185 ng/ml
C20/V	0.70 ± 0.09	24.1 ± 3.5	<0.185 ng/ml
C20/V-MCAF1	0.70 ± 0.04	23.8 ± 1.2	>2.5 ng/ml
C20/V-MACF2	0.77 ± 0.11	22.1 ± 3.6	>3.5 ng/ml
C20/V-BCMG	0.66 ± 0.11	25.5 ± 4.0	>1.9 ng/ml

[a] One million cells were cultured in vitro with 20 ml medium for 3 days before measuring. The result is representative of 3 independent experiments.
[b] Growth rate and doubling time were calculated by the following equation.

$$\text{Growth rate} = \ln(\text{number of final cell}/\text{number of initial cell})/3$$
$$\text{Doubling time} = 0.693/(\text{growth rate})$$

[c] Levels of MCAF were measured by ELISA.

moter. BCMGSNeo/MCAF contains a partial sequence of bovine papilloma virus and is a shuttle vector.

Transfection with MCAF cDNA

Colon 26 clone 20 cells were transfected with control- or MCAF-cDNA by a modified calcium phosphate co-precipitation method (12). 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 MCAF producing cells (>0.4 ng/ml) were used for the animal experiment.

In Vitro Cell Growth and the Secreted MCAF 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 MCAF levels using the ELISA (13). The concentrations were determined against the recombinant human MCAF expressed using an animal cell (14). Detection limit of the assay was 185 pg/ml.

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

MCAF mRNA expression was evaluated by RT-PCR analysis. Two μg total RNA prepared using RNAzol B (Biotex Laboratory Inc. Houston, Texas), was reverse transcribed in 25 μl reaction containing AMV reverse transcriptase (Seikagaku Corp., Tokyo) and the oligo d(T) primer (Pharmacia Biotech, 5 units). After 1 hr incubation at 42°C, 1 μl of cDNA product was amplified in 20 μl reaction containing 0.2 mM of dNTP, 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 by PCR is 284 bps (MCAF, 70-353). cDNA samples were size-fractionated on agarose gel. mRNA of MCAF were clearly detected in both primary and metastatic tumors of the MCAF-transfected clones.

Tumor Inoculation and Spontaneous Lung Metastasis

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 until the primary tumor was resected by amputation at 10 or 14 days after the inoculation (15). The mice were sacrificed 34 days after the inoculation to weigh both lungs and to count the number of metastatic nodules after fixation in Bouin's solution.

Histological Analyses

Tissue from the site of tumor cell inoculation or meta-

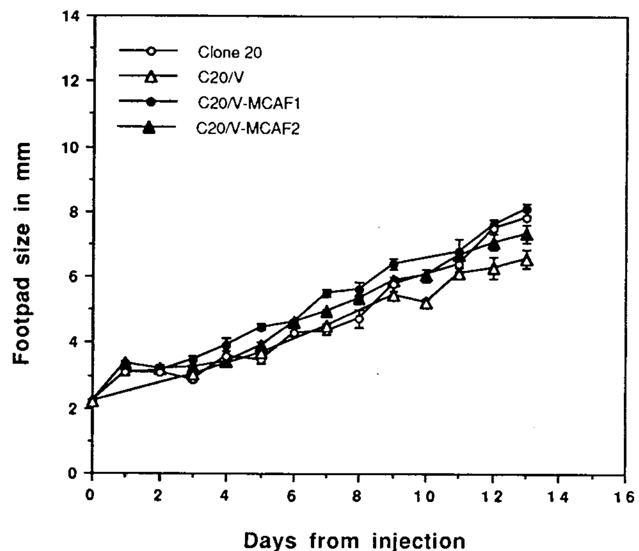


Fig. 1. Local tumor growth: 10^6 tumor cells were inoculated into the footpad of Balb/c mice; tumor size was measured as the height. Data presented are means with SEM of at least 9 mice. The combined results of three experiments with three to five mice per group are shown for C20/V, C20/V-MCAF1, and C20/V-MCAF2. Two experiments were performed for clone 20 cells. Similar growth of tumors was observed between the groups of clone 20 and C20/V.

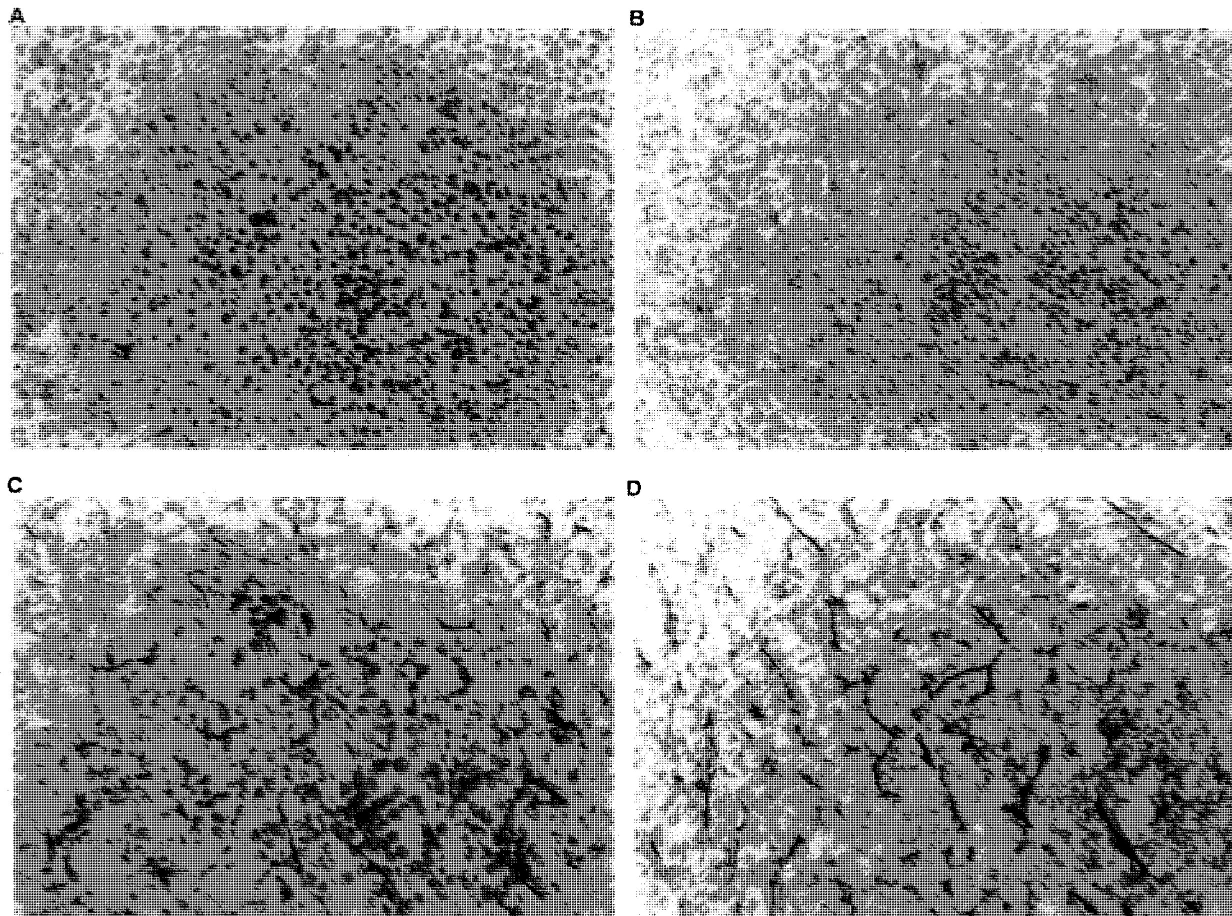


Fig. 2. Immunohistochemical stain for mouse endothelial cell in primary tumors of Colon 26 clone 20 carcinoma (A, C) and MCAF-secreting(B, D) cells, × 200. Angiogenesis in tumors was determined using an antibody to mouse endothelial cells for the tumors 10 (A, B) and 14 (C, D) days after footpad injection. The number of blood vessels increased progressively from the 10th to the 14th day.

static nodules in the lung were 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 endothelial cells (clone 5-34-3) (16). For immunoperoxidase, sections were incubated with the antibody before indirect immunoperoxidase staining by the avidin-biotin complex-(ABC) method.

Statistical Analysis

Data of the number of lung metastases were analyzed using Student's *t* test for comparison of unpaired sets of data, respectively. A *p* value of 0.05 or less was used to indicate a significant difference between the sets of data. The significance of difference in the survival experiments between groups was determined by the Wilcoxon rank sum test. Two tailed *p* values are presented for all experiments.

RESULTS

Characteristics of Transfected Colon 26 Clone 20 in Vitro

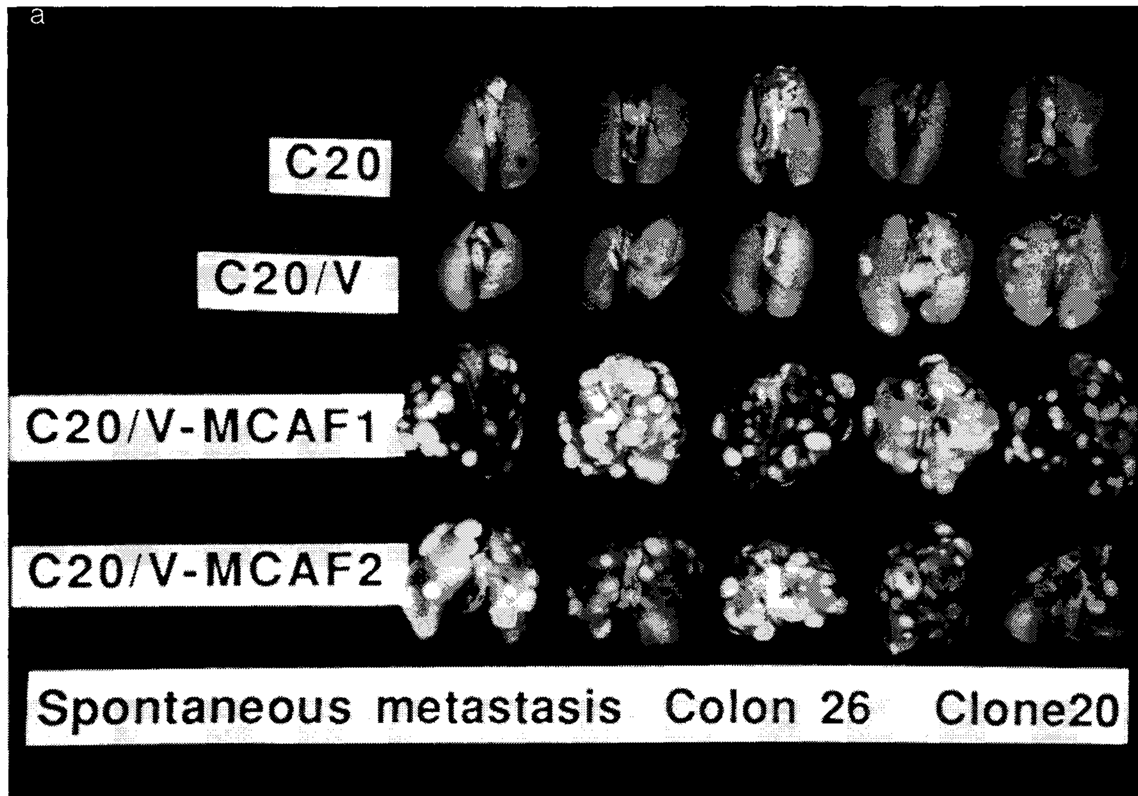
Two clones, designated C20/V-MCAF1 and C20/V-MCAF2, and a clone, designated C20/V-BCMG, were obtained after the transfection of pHβAPr-3p-neo/MCAF and BCMGSNeo/MCAF, respectively, into parental colon 26 clone 20. These clones produced more than 0.3 ng/ml of MCAF when cultured for 3 days at a cell density of 5×10^4 cells/ml, whereas neither parental nor control vector-transfected C20/V produced detectable levels of MCAF over 3-day culture in vitro (Table I). Transfection of MCAF cDNA did not affect the growth rate or the doubling time (Table I).

Table II. Pulmonary Metastasis at Different Resection Day[a]

Cells	Metastasis[b] after the resection of tumors at	
	10th day	14th day
clone 20	1/5	3/7
C20/V	1/5	3/9
C20/V-MCAF1	0/5	12/14
C20/V-MCAF2	1/5	12/15

[a] Mice were inoculated into the footpad with 1×10^6 cells/mouse and the tumors were resected 10–14 days after the inoculation. 34 days after the inoculation, lungs were excised.

[b] Number of animals with metastatic nodules/number of animals inoculated.



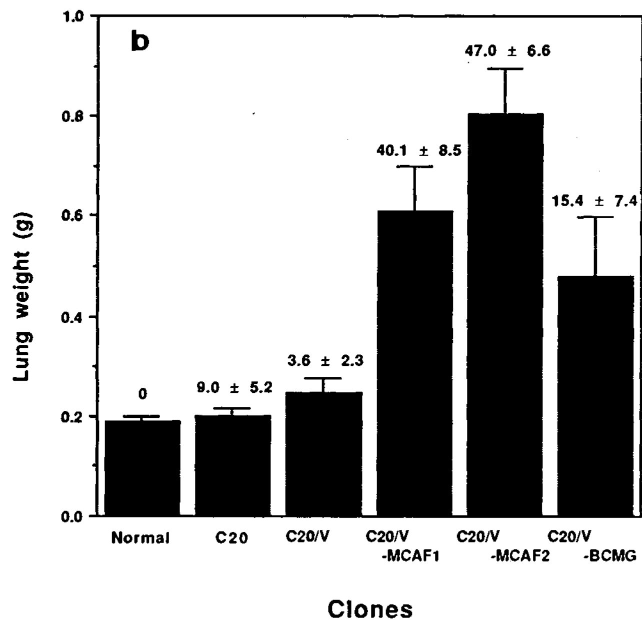


Fig. 3. Spontaneous metastasis of clone 20, C20/V, MCAF-transfected cells inoculated into Balb/c mice. Representative results are presented (a). The combined results of three experiments with three to five mice per group are shown for C20/V, C20/V-MCAF1, and C20/V-MCAF2 (b). Two experiments were performed for clone 20 cells and C20/V-BCMG transfected with BCMGSNeo/MCAF. Bars: mean ± SEM; numbers above bars indicate metastatic nodules, mean ± SEM. There was significant difference in the number of colonies and lung weight between the tumors of MCAF-producing cells and those of parental cells ($p < 0.05$).

To study the effects of MCAF 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. There was no significant difference in the tumor growth rate at the injection site among the clone 20, C20/V, C20/V-MCAF1, and C20/V-MCAF2 (Fig. 1).

Histological Analyses on the Tumor Tissues at the Injection Sites

Histological examination of the tumor tissue from the footpad showed neither necrosis nor apparent apoptosis (data not shown). Immunohistochemical analysis using a monoclonal antibody to mouse endothelial cells (16) demonstrated that the number of endothelial cells progressively increased in the footpad from the 10th to the 14th day after

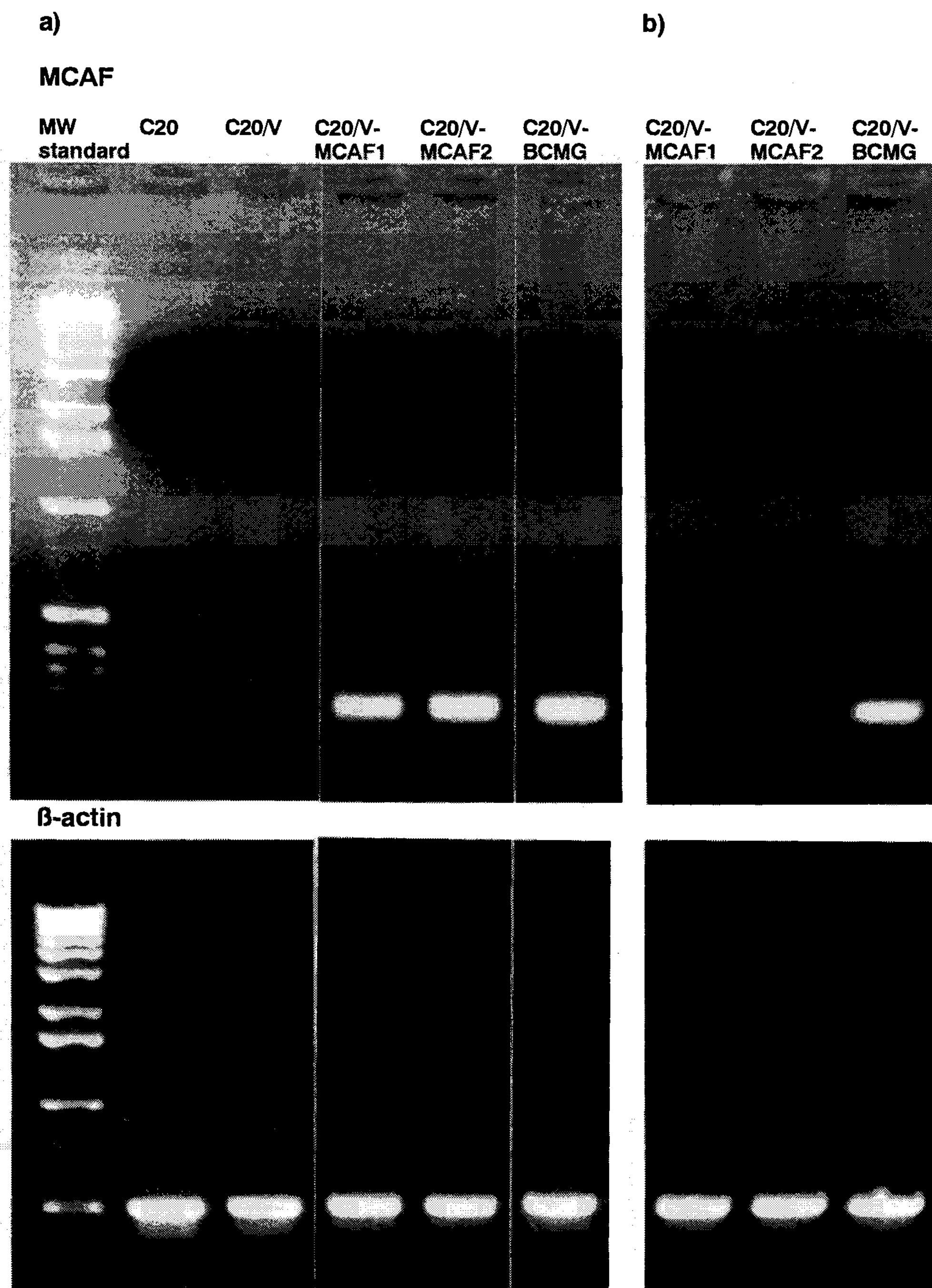


Fig. 4. RT-PCR analysis of primary (a) and secondary (b) tumors of parental (C20 and C20/V) and MCAF-transfectant (C20/V-MCAF1, C20/V-MCAF2 and C20/V-BCMG/MCAF) cells.

mice were injected with either parental or MCAF-transfected clones (Fig. 2). The number of endothelial cells tended to be higher in the footpads of mice inoculated with MCAF-transfectants than those inoculated with parental clones. These results raised the possibility that MCAF might augment angiogenesis in vivo.

MCAF Production Accelerates Spontaneous Lung Metastasis

To clarify the effect of MCAF gene transfer on the metastatic process, a spontaneous metastatic assay was performed using primary tumors growing in mouse footpads. As shown in Table II, when primary tumors were resected at 10 days after the inoculation, few foci of lung metastasis were macroscopically observed at 34 days after the inoculation in mice injected with either parental or MCAF-transfected clone. When the primary tumors were resected 14 days after inoculation, no significant difference was observed in the lung weight among normal mice, clone 20 and C20/V transplanted mice. Metastatic lung nodules were macroscopically observed in some of the mice inoculated with either clone. In

contrast, the lung weight and the numbers of metastatic nodules increased markedly in mice transplanted with MCAF-producing cells (Fig. 3 a). MCAF gene transfer caused an increase of tumor cell colony formation on the lung surface, while the tumors with clone 20 and C20/V cells grew at more restricted regions in the lung. The enhancement was observed in the transfected clone with the expression vector of pHβAPr-3p-neo/MCAF and BCMGSNeo/MCAF (Fig. 3 b) (11).

We performed RT-PCR analysis to investigate whether MCAF was expressed in the primary and secondary tumor sites. MCAF mRNA was detected clearly in both primary and secondary tumor sites after the inoculation of the transfectant cells, whereas no MCAF mRNA was detected in tumors after the inoculation of the parental cells (Fig. 4). It is important to follow the relationship between the amount of gene expression and the tumor cytocidal activities. Our ELISA system is useful in the quantitative detection of MCAF in various fluids (13). We measured the expression of MCAF for the primary tumors 14 days after the inoculation. The MCAF level in medium after 3 days culture decreased from 3.5 ng/ml to 0.3 ng/ml, although mRNA of MCAF were detected clearly by RT-PCR for the MCAF-producing cells in both primary and pulmonary sites (Fig. 4).

DISCUSSION

In the present study, we have demonstrated that transfer of the MCAF cDNA gene into colon 26 clone 20 adenocarcinoma cells did not cause tumor suppression at the primary site (Fig. 1), but rather enhanced pulmonary metastasis (Figs. 3). It is known that an angiogenic phenotype of tumor cells can attract the nourishing vasculature needed for their growth (6) in both primary and metastatic sites. As listed in Table II, tumor metastases occurred between the 10th and 14th day after the inoculation of C20/V-MCAF. The significant increase of angiogenesis at this time suggests that the newly formed blood vessels may be an important factor in the metastatic process. Moreover, fourteen days after inoculation, the number of endothelial cells in MCAF-transfectants was higher than in the controls, suggesting that MCAF may also play a major role in the angiogenetic process.

The transfected DNA is still subject to many regulatory activities of endogeneous genes during the expression. Moreover, consecutive mutations usually occur in malignant progression. Although, less expression of mRNA of MCAF was observed in pulmonary sites than in primary sites (Fig. 4), it is reasonable to think that a secondary tumor focus may loose a part of the initial gene during the progression. We tried to induce a high level of expression of MCAF by using the two alternative vectors, pHβAPr-3p-neo/MCAF and BCMGSNeo/MCAF. However, both transfectants produced a similar level of MCAF in vitro, and showed augmented metastasis. A similar difficulty of gene expression in colon 26 cells has been already reported. Direct injection of DNA, encoding the β-galactosidase gene, into colon 26 tumors resulted in the disappearance of blue-staining cells 10 days after DNA injection, whereas blue-staining cells were seen in other tumor cell types tested (17).

In conclusion, MCAF gene transfer with relatively low level expression of MCAF augments the metastasis of colon 26 clone 20 cells. MCAF may participate in a cytokine network which regulates tumor invasion and metastasis directly as well as indirectly by inducing tumor neovascularization. Although a potential clinical role for infused MCAF in patients has been pointed out (4,5), our results suggest that the clinical use of MCAF may be limited because of the augmentation of the metastatic potential in some types of tumor. If MCAF generally promotes metastasis in the human system, the development of MCAF antagonists might be a useful approach to prevent this.

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