A Candidate for Cancer Gene Therapy: MIP-1α Gene Transfer to an Adenocarcinoma Cell Line Reduced Tumorigenicity and Induced Protective Immunity in Immunocompetent Mice

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Purpose. To evaluate the possibility of cancer gene therapy by the gene delivery of chemokine, the effects of human macrophage inflammatory protein 1α (hu-MIP-1α), murine-macrophage inflammatory protein 1α (mu-MIP-1α), and human-interleukin 8 (hu-IL-8) on tumor progression and immunization were studied.

Methods. Cachexia-inducing and highly tumorigenic adenocarcinoma cells (cell line colon 26, clone 20) were transfected with either a control plasmid, hu-MIP-1α, mu-MIP-1α, or hu-IL-8 expression vector. The production of hu-MIP-1α reached >1.5 ng/ml in vitro when transfectant cells were cultured at a cell density of 2×10^5 cells in 7 ml for 3 days. Immunocompetent BALB/c mice were inoculated into the footpad with the tumor cells, and then primary tumor growth, morphological analyses, and tumor immunogenicity were studied.

Results. The secretion of hu-MIP-1α, mu-MIP-1α, and hu-IL-8 did not affect the growth rate in vitro. Reduced tumorigenicities in vivo were observed in transfected cells with hu-MIP-1α and mu-MIP-1α. Morphologic observation of the site of inoculation of cells transfected with hu-MIP-1α showed infiltration of macrophages and neutrophils on the 5th day after the inoculation. Mice that had rejected cells transfected with hu-MIP-1α gene were immune to a subsequent challenge with the parental cells.

Conclusions. The rejection of the cells depends on cytolysis and generates potent and long lasting antitumor immunity. These data suggest that tumor cells transfected with the MIP-1α gene might be useful as an effective therapy for the treatment of certain tumors.

KEY WORDS: gene transfer; MIP-1α; chemokine; protective immunity.

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ABBREVIATIONS: human macrophage inflammatory protein 1α (hu-MIP-1α); murine macrophage inflammatory protein 1α (mu-MIP-1α); human interleukin-8 (hu-IL-8); monocyte chemotactic and activating factor or monocyte chemotactic protein-1 (MCAF/MCP-1); enzyme-linked immunosorbent assay (ELISA).

INTRODUCTION

Novel cytokines with chemotactic activity against leukocytes are a superfamily of small proteins secreted by various types of tissue cells as well as leucocytes, and related by a conserved four-cysteine motif (1,2). The human monocyte chemotactic and activating factor or monocyte chemotactic protein-1 (MCAF/MCP-1) and the human macrophage inflammatory protein-1α (hu-MIP-1α), which is also known as LD78, are members of C-C chemokine which is thought to regulate the recruitment of monocytes/macrophages as well as their activation (3,4). The human interleukin-8 (hu-IL-8) has been shown to be a neutrophil and T cell chemoattractant. Both hu-MIP-1α and hu-IL-8 are active in mice, and seem to affect different, yet overlapping, leukocyte subsets (1). Since the chemokines show chemotactic activity against leukocytes to inflammatory or tumor sites, delivery of chemokine genes to tumors may be a potent candidate for cancer gene therapy to stimulate host immune responses that could reduce tumor size. In our previous studies (9,10), however, the gene transfer of MCAF/MCP-1 into a mouse cachectic and highly tumorigenic adenocarcinoma cell line, colon 26 clone 20 cells, augmented the metastatic potential of the cells (9). In contrast, the genes for hu-MIP-1α, mu-MIP-1α, and hu-IL-8 have been shown to reduce tumorigenicity in immunoincompetent nude mice in association with neutrophilic infiltration (6). These chemokines may regulate the chemotaxis and tumoricidal activation of leucocytes in immunocompetent mice, and might be an important mediator of tumor regression (7). However, it is still unclear whether the chemokines are useful for cancer gene therapy or not. We examined here the effect of hu-MIP-1α, mu-MIP-1α, and hu-IL-8 transfection on the tumorigenicity, metastatic ability, and response to systemic immunity of highly tumorigenic colon 26 clone 20 in immunocompetent mice (8).

MATERIALS AND METHODS

Mice

Female BALB/c mice were purchased 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 in air (5).

Plasmid Construction

A *Bam* HI-*Hind* III fragments containing the entire coding region of hu-MIP-1α, mu-MIP-1α, and hu-IL-8 cDNAs were subcloned into mammalian expression vector pHβAPr-3p-neo (pHβAPr-3p-neo/hu-MIP-1α, mu-MIP-1α, and hu-IL-8) (6).

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Transfection with hu-MIP-1α, mu-MIP-1α, and hu-IL-8 cDNA

Colon 26 clone 20 cells were transfected with control plasmid, or hu-MIP-1α, mu-MIP-1α, or hu-IL-8-expression plasmid by a modified calcium phosphate co-precipitation method as previously described (5).

In Vitro Cell Growth and Detection Method for the Secreted hu-MIP-1α and hu-IL-8

Cells were plated with 7 ml standard medium without G418, at a density of 2×10^5 cells per flask. Three days later, supernatants were collected and tested for hu-MIP-1α and hu-IL-8 levels using hu-MIP-1α ELISA kit (R & D Systems, Minneapolis, MN) and the IL-8 ELISA (10), respectively. Detection limits of the assay for hu-MIP-1α and hu-IL-8 were 40 pg/ml and 10 pg/ml, respectively.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis for mRNA in Primary Tumors

Hu- and mu-MIP-1α expressions were evaluated by RT-PCR analysis as previously described (5,6). Fragments by PCR are 351 bps and 373 bps for hu-MIP-1α and mu-MIP-1α, respectively.

Tumor Inoculation and Spontaneous Lung Metastasis

The adherent cells were collected after brief trypsinization. Unless otherwise stated, 9-week-old female BALB/c mice were inoculated into the footpad of the right hind leg with 10^6 tumor cells suspended in 40 μl of sterilized endotoxin-free PBS per mouse (5).

Histological Analyses

Tissues from the site of tumor cell inoculation were fixed with OCT compound (Miles, IN, USA) and frozen in liquid N_2. Immunohistochemical analyses were performed on the tumors at the inoculated footpad using a monoclonal antibody (U-RM2800, Caltag Lab. Inc., CA, USA) against Mac-1 antigen for mouse macrophages, neutrophils, and natural killer cells (5).

Statistical Analysis

Data of the tumor incidence rates were analyzed using Student's *t* test for comparison of unpaired sets of data. A *p* value of 0.05 or less was used to indicate a significant difference between the sets of data. Two tailed *p* values are presented for all experiments.

RESULTS

Characteristics of Transfected Colon 26 clone 20 *in Vitro*

Three clones (designated C20/hu-MIP-1α-1, C20/hu-MIP-1α-2, and C20/hu-MIP-1α-3), 3 clones (designated C20/mu-MIP-1α-1, C20/mu-MIP-1α-2, and C20/mu-MIP-1α-3), and a clone (designated C20/hu-IL-8) were obtained after the transfection of pHβAPr-3p-neo/hu-MIP-1α, pHβPr-3p-neo/mu-MIP-1α, and pHβAPr-3p-neo/hu-IL-8, respectively, into parental colon 26 clone 20. The clones transfected with hu-MIP-1α and hu-IL-8 produced more than 1.52 ng/ml of hu-MIP-1α and 1.43 ng/ml of hu-IL-8, respectively, when cultured for 3 days at a cell density of 2×10^5 cells in 7 ml culture medium. However, neither parental nor control vector-transfected C20/V produced detectable levels of hu-MIP-1α, and hu-IL-8 over 3-day culture in vitro. Transfection of hu-MIP-1α, mu-MIP-1α, and hu-IL-8 cDNA did not affect the in vitro growth rate or the doubling time (Table I).

Effect of Gene Transfer on Tumor Growth and Metastasis

To study the effects of hu-MIP-1α, mu-MIP-1α, and hu-IL-8 gene transfer on the tumorigenicity of the cells, we inoculated parental and transfected clones into the footpads of syngeneic BALB/c immunocompetent mice. Both parental and C20/V transfected clones readily formed tumors within 1–2 days after the inoculation. Table II shows tumor incidence as assayed at 14 days after transplantation. C20/hu-IL-8 showed the same growth rate as control cells (C20 and C20/V) in vivo, whereas some of the mice inoculated with hu-MIP-1α or mu-MIP-1α expressing clone showed no tumor growth. Tumor formation was totally prevented in 4 of ten mice in the case of C20/hu-MIP-1α-2 after the inoculation of 10^6 cells. When the number of tumor cells for the inoculation of C20/hu-MIP-1α-1 was reduced to one-tenth, the tumor incidence was decreased to 2 in 6 mice.

Fourteen days after the inoculation, the mice inoculated with 10^6 cells of either clone exhibited a 10–24% weight loss (Table II). When the primary tumors were resected 14 days after the inoculation and lung metastases were analyzed 20 days after the amputation, no significant difference was observed in

Table I. *In Vitro* Cell Growth Rate, Doubling Time, and Chemokine Production

	Growth Rate (/day)[a]	Doubling Time (hr)[a]	Production (ng/ml)[b]
C20	0.719 ± 0.059	23.2 ± 1.9	—
C20/V	0.765 ± 0.025	21.8 ± 0.7	—
C20/hu-MIP-1α −1	0.689 ± 0.013	24.2 ± 0.5	1.52 ± 0.16
C20/hu-MIP-1α −2	0.819 ± 0.009	20.3 ± 0.2	2.24 ± 0.07
C20/hu-MIP-1α −3	0.771 ± 0.036	21.7 ± 0.9	2.23 ± 0.25
C20/mu-MIP-1α −1	0.669 ± 0.081	26.5 ± 3.4	—
C20/mu-MIP-1α −2	0.639 ± 0.021	26.2 ± 0.9	—
C20/mu-MIP-1α −3	0.660 ± 0.020	25.3 ± 0.7	—
C20/hu-IL-8	0.752 ± 0.048	22.3 ± 1.4	1.43 ± 0.39

[a] Each clone was seeded at 2×10^5 cells in 7 ml culture medium and after 3 days, the cells were harvested by trypsine and counted. Growth rate and doubling time were calculated by the following equation. Growth rate = ln (number of final cells/initial cells)/3. Doubling time = 0.693/(growth rate).

[b] Each clone was seeded at 2×10^5 cells in 7 ml culture medium and after 3 days, the culture supernatants were collected and the amount of huMIP-1 α and IL-8 in culture supernatants was measured by ELISA.

Table II. Tumorigenicity After Chemokine Gene Transfer

	Cell Number	Primary Tumor Incidence[a]	Lung Metastasis[b]	Lung Weight (g)	Decreased Body[c] Weight (%) (n)
C20	10^6	15/15 ——	3/7	0.201 ± 0.017	16.9 ± 3.7 (7)
C20	10^5	9/10 —	3/9	0.209 ± 0.014	12.4 ± 2.2 (9)
C20/V	10^6	9/9	3/9	0.247 ± 0.029	23.6 ± 2.9 (5)
C20/hu-MIP-1 α −1	10^6	9/11 * *	2/10	0.219 ± 0.006	11.7 ± 2.2 (9)
	10^5	2/6 —	1/5	0.241	4.2 ± 0.8 (2)
C20/hu-MIP-1 α −2	10^6	6/10 ——	4/10	0.190 ± 0.005	16.4 ± 4.7 (6)
C20/hu-MIP-1 α −3	10^6	9/11	2/10	0.221 ± 0.008	12.8 ± 3.6 (9)
C20/mu-MIP-1 α −1	10^6	8/9	5/9	0.213 ± 0.013	19.4 ± 1.3 (8)
	10^5	4/5	0/4	0.240 ± 0.022	0.5 ± 1.9 (4)
C20/mu-MIP-1 α −2	10^6	7/8	2/4	0.388 ± 0.197	19.5 ± 4.8 (7)
C20/mu-MIP-1 α −3	10^6	8/9	3/5	0.181 ± 0.018	11.4 ± 3.4 (8)
C20/hu-IL-8	10^6	10/10	3/9	0.199 ± 0.009	9.8 ± 2.8 (10)
	10^5	5/5	1/5	0.219 ± 0.008	6.4 ± 5.5 (5)

Note: BALB/c mice were inoculated with 1×10^6 or 1×10^5 tumor cells into the footpad. 14 days after inoculation the tumor-bearing leg was amputated below the knee, and mice were killed 34 days after inoculation. *: $P < 0.05$

[a] Tumor incidence (number of mice with tumor/number of mice inoculated) refers to 14 days after transplantation.

[b] Lung metastasis (number of mice with metastasis/number of mice killed) refers to 34 days after transplantation.

[c] Body weight was determined 12–14 days after transplantation.

the lung weight among clone 20, C20/V, C20/MIP-1α, and C20/IL-8 transplanted mice, although several mice showed small metastatic foci in lung 20 days after the amputation (Table II).

Histological Analyses on the Tumor Tissues at the Inoculation Sites

Morphologic observation of the site of inoculation of cells transfected with hu-MIP-1α, mu-MIP-1α, or IL-8 showed infiltration of macrophages and neutrophils on the 14th day after the inoculation (Fig. 1). The tumor tissue from the footpad of clone 20, C20/V, or C20/IL-8 transplanted mice showed neither necrosis nor apparent apoptosis on the 14th day after the inoculation. In contrast, the inoculation site of C20/hu-MIP-1α and C20/mu-MIP-1α showed necrotic destruction of tumor cells, and reduced tumorigenicities were observed in transfected cells with hu-MIP-1α and mu-MIP-1α.

Immunohistochemical analysis using a monoclonal antibody to mouse macrophages demonstrated that the number of macrophages and neutrophils progressively increased in the footpad up to the 5th day after the mice were inoculated with hu-MIP-1α transfected clones (Fig. 2). More staining was observed in the footpads of mice inoculated with hu-MIP-1α than those inoculated with parental clones.

Expression of mRNA in Tumor Sites

We performed RT-PCR analysis to investigate whether hu-MIP-1α and mu-MIP-1α were expressed in the primary tumor. Each mRNA of hu-MIP-1α and mu-MIP-1α was detected clearly in primary tumor after the inoculation of the transfectant cells (Fig. 3). A lower level of mRNA of mu-MIP-1α was expressed from parental cells and cells transfected with the vector alone than the cells transfected.

Immunotherapeutic Potential of C20/hu-MIP-1α Transfectants

We further tested whether C20/hu-MIP-1α transfectants can protect tumor-bearing mice against growth of the parental cells. The mice which rejected tumors inoculated intrafootpad with 10^5 C20/hu-MIP-1α cells were used in the following experiment. Starting on day 21 after the first inoculation, 10^6 C20/hu-MIP-1α cells were challenged at the same site; however, no tumor growth was observed in any mice. These mice were subsequently inoculated with 10^5 cells of parent cells at another site of the footpad (Fig. 4). No mice showed local growth of tumor cells until 3 weeks.

DISCUSSION

In the present study, we have demonstrated that hu-MIP-1α producing colon 26 clone 20 adenocarcinoma cells caused tumor suppression at the primary site (Table II). The mice that rejected C20/hu-MIP-1α cells showed further rejection of the parental cells when subsequently challenged intra footpad of another hind leg (Fig. 4). These results suggest that mice given inoculations of MIP-1α-producing cells develop a long-lasting tumor immune resistance.

It is known that vaccination with tumor cells that have been engineered to express cytokine genes has been developed in animal models as a novel form of tumor therapy (11). In most reports, the inhibition of tumor growth is mediated through infiltration of T lymphocytes and/or macrophages into the tumor sites (12). We found that the inhibition of tumor growth in immunocompetent mice by secretion of hu-MIP-1α was associated with the infiltration of both macrophages and neutrophils (Fig. 2) as previously observed in nude mice (6). The neutrophil and monocyte accumulation, seen in vivo immunocompetent mice as well as in nu/nu mice, suggests that the initial antitumor effect by hu-MIP-1α might be thymus independent. In addition to controlling the selective migration of monocytes/macrophages and neutrophils, the chemokine has been postulated to be also involved in controlling the trafficking of T cell subsets, although these points remain to be investigated.

The tumor lysis was observed by not only hu-MIP-1α but also mu-MIP-1α gene transfer (Fig. 1), whereas the transfer of hu-IL-8 did not cause tumor suppression (Table II). IL-8 is

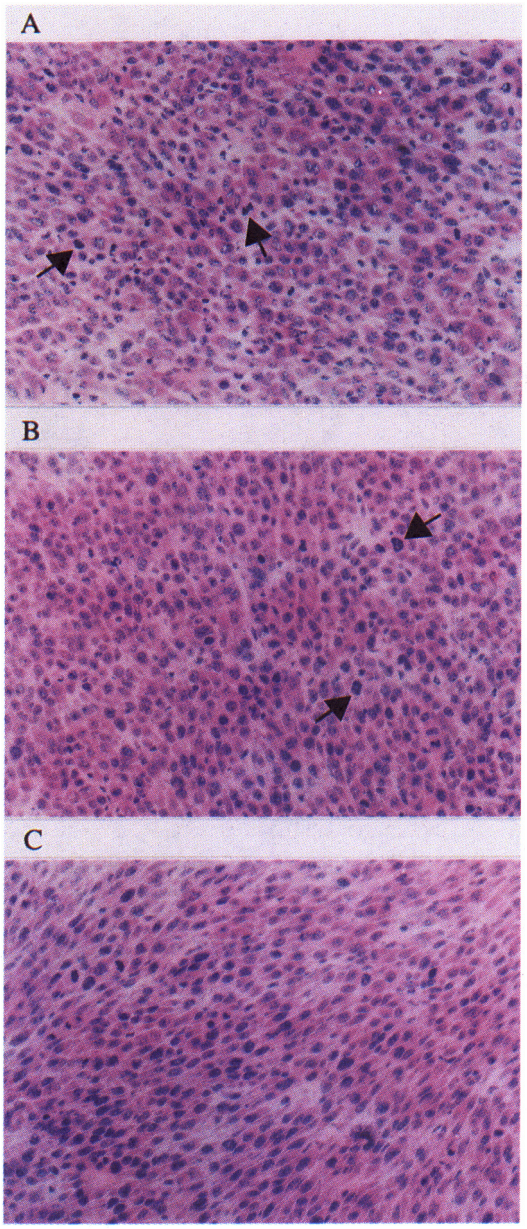

Fig. 1. Histology of hu-MIP-1α, mu-MIP-1α, and hu-IL-8-secreting tumors. Colon 26 clone 20 carcinoma cells were inoculated into the footpad of BALB/c mice, and then local tumors were resected 14 days after the inoculation and stained with hematoxylin and eosin, ×200. (A) A hu-MIP-1α producing tumor; (B) a mu-MIP-1α producing tumor; (C) a hu-IL-8 producing tumor. Note the characteristic appearance of the malignant tumor cells in hu-IL-8 producing cells with the absence of a host response (C). In contrast, the inoculation site of C20/hu-MIP-1α and C20/mu-MIP-1α contained a marked cellular infiltrate composed predominantly of macrophages and neutrophils, as well as necrotic destruction of tumor cells (A, B). Arrows indicate the infiltrated cells.

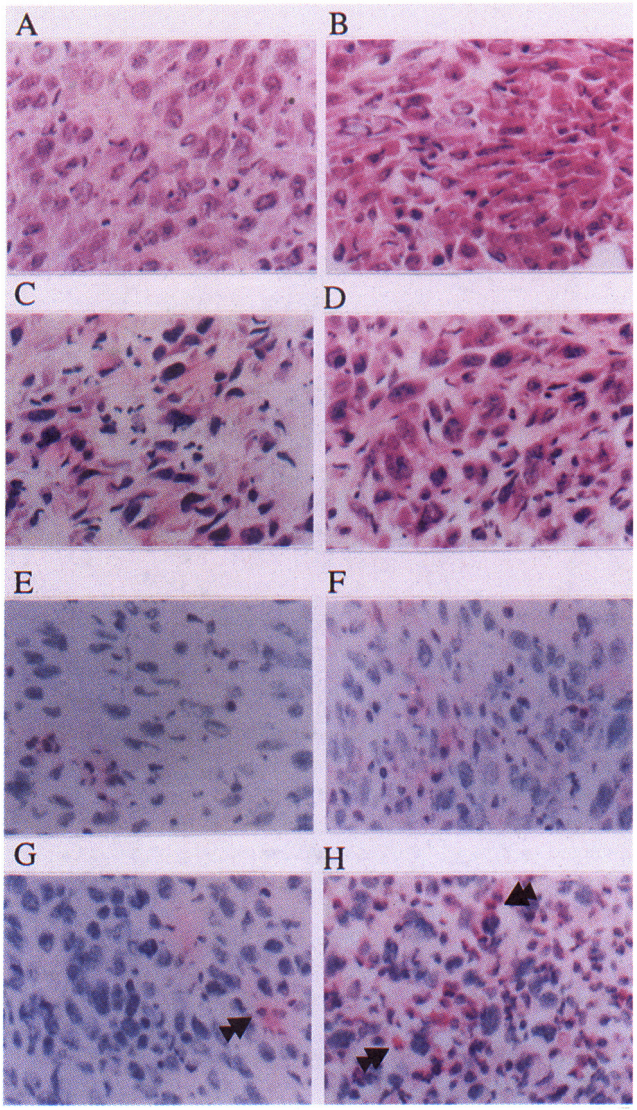

Fig. 2. Morphological and immunohistochemical stain for mouse macrophages and neutrophils in primary tumors of Colon 26 clone 20 carcinoma and hu-MIP-1α-secreting cells, ×400. (A) Colon 26 clone 20 carcinoma, hematoxylin and eosin stain, 3 days after the inoculation; (B) colon 26 clone 20 carcinoma, hematoxylin and eosin stain, 5 days after the inoculation; (C) a hu-MIP-1α producing tumor, hematoxylin and eosin stain, 3 days after the inoculation; (D) a hu-MIP-1α producing tumor, hematoxylin and eosin stain, 5 days after the inoculation; (E) colon 26 clone 20 carcinoma, immunohistochemical stain, 3 days after the inoculation; (F) colon 26 clone 20 carcinoma, immunohistochemical stain, 5 days after the inoculation; (G) a hu-MIP-1α producing tumor, immunohistochemical stain, 3 days after the inoculation; (H) a hu-MIP-1α producing tumor, immunohistochemical stain, 5 days after the inoculation. The number of macrophages and neutrophils increased progressively in hu-MIP-1α transfected cells. Arrows indicate the infiltrated cells.

specific for neutrophils and CD45RO+ memory T cells, and MIP-1α is specific for monocytes/macrophages, basophils, and mast cells (13). The cloned leukocyte chemokine receptors, IL-8 receptor (IL-8R) and the MIP-1α receptor, are related by sequence and chemokine binding to two herpesvirus products, and to the Duffy antigen that mediates erythrocyte invasion by the malaria-causing parasite Plasmodium vivax (14). The three-dimensional structure of MIP-1α has been modeled structurally, based on its sequence similarity to IL-8 and related proteins (15). Although the method employed for gene transfer in the present study is reported to achieve highly efficient transforma-

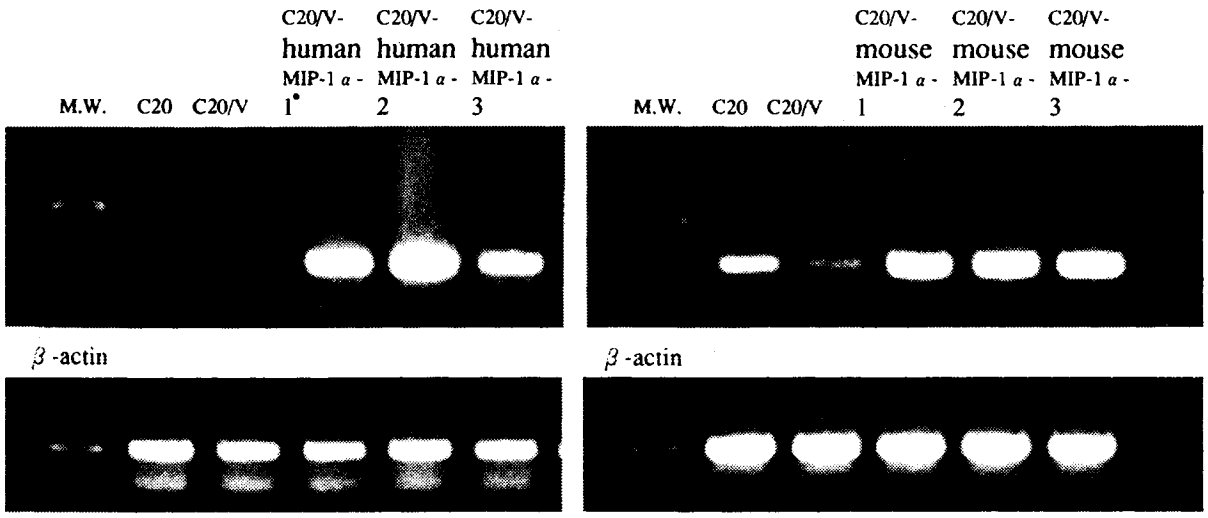

Fig. 3. RT-PCR analysis of primary tumors of parental (C20 and C20/V) and hu-MIP-1α and mu-MIP-1α cells.

tion of mammalian cells (9), the colon 26 clone 20 cells employed in this study produced relatively low levels of hu-IL-8 in the transfectants as compared to the hu-MIP-1α. Since the levels of hu-IL-8 expression may influence the tumorigenicity, we cannot rule out the possibility that the minimal cytocidal effect may be ascribed to the relatively low level of hu-IL-8 expression by the transfectants used in this study.

As seen in Fig. 3, a low level mRNA of mu-MIP-1α was detected in tumors 14 days after the inoculation with C20 and C20/V cells. Immunostain analyses for the tumors 5 days after the inoculation with C20 cells showed increased intensity of staining of macrophages compared to the tumors 3 days after the inoculation (Fig. 3). These results indicate that the immune responses increased greatly associated with the MIP-1α gene transfer. In order to obtain higher rates of tumor rejection with MIP-1α gene transfection, it will be necessary to monitor the level of secreted MIP-1α.

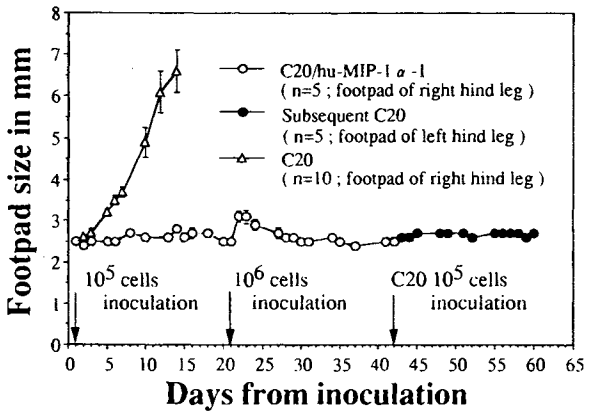

Fig. 4. Footpad size of tumor cells in BALB/c mice. 1×10^5 tumor cells (C20 or C20/hu-MIP-1α-1) were inoculated into the footpad of the right hind leg. Tumor size was measured as the mean of the height. Representative results are presented. On the 21st day after the inoculation, tumor free mice were inoculated with C20/hu-MIP-1α gene transfected cells into the right footpad with 1×10^6 cells. On the 42nd day after the first inoculation, tumor-free mice were inoculated with C20 parent cells into the left footpad with 1×10^5 cells. No tumor growth was observed thereafter.

The cell line employed in this study is a highly tumorigenic and cachexigenic clone. As listed in Table II, all mice inoculated with 10^6 cells of C20 or C20/V-transfectant showed tumor growth in the inoculation site. Both MIP-1α and IL-8-transfectant caused a degree of cachexia after intra footpad inoculation similar to that of the parental cells (Table II). In a previous study (5), we found that spontaneous lung metastases were augmented in the animals inoculated with MCAF-transfectants compared to those inoculated with parental cells with a concomitant increase of angiogenesis. We surveyed whether MIP-1α enhances the metastatic potency of the cells. In the present study, the incidence rates of spontaneous lung metastasis were similar in MIP-1α-, IL-8-transfectant, and the parental cells (Table II).

In conclusion, hu-MIP-1α gene transfer with relatively high level expression of hu-MIP-1α reduced tumorigenicity of colon 26 clone 20 cells and induced long-lasting immunity in immunocompetent mice. It is suggested that the clinical use of hu-MIP-1α may be of potential value for cancer gene therapy.

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