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Interferon-γ-inducing Factor Gene Transfection into Lewis Lung Carcinoma Cells Reduces Tumorigenicity *in vivo*

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To investigate the immunoregulatory effect of murine interferon-γ-inducing factor (mIGIF), we transfected Lewis lung carcinoma (LLC) cells with a mammalian expression vector containing the mIGIF complementary DNA. The culture medium of the transfectant cells stimulated interferon-γ (IFN-γ) production by spleen cells *in vitro* in the presence of anti-CD3 antibody and markedly potentiated the effect of interleukin-12 (IL-12) on IFN-γ production by spleen cells. mIGIF transfectant cells showed reduction of tumorigenicity and induction of an *in vivo* immuno-protective effect against the parental LLC cells. To examine the combined effect of systemic administration of recombinant IL-12 (rIL-12) and local mIGIF on the tumorigenicity, mice were challenged with LLC or transfectant cells on day 0, and the tumor-bearing mice were injected with 50 ng of rIL-12 intraperitoneally from day 7 to 11. Systemic rIL-12 showed an anti-tumor effect. However, mIGIF gene expression did not potentiate this effect of systemic rIL-12 *in vivo*.

Key words: Murine interferon-γ-inducing factor — Interferon-γ — Interleukin-12 — Lewis lung carcinoma — Transfectant

New immunization methods and new cytokines have led to advances in tumor immunology. Tumor cells engineered with genes encoding cytokines and costimulatory signals may potentially be useful as vaccines to activate specific anti-tumor immunity. One of the most promising cytokines is interleukin-12 (IL-12) because it has strong anti-tumor activity and is a strong inducer of Thelper 1 (Th1) cells.[1-3] Recently a new cytokine was identified and designated as murine interferon-γ-inducing factor (mIGIF). This cytokine is of particular interest because it has unique biological activities. mIGIF markedly stimulates interferon-γ (IFN-γ) production in spleen cells and Th1 cells, inducing much higher levels of IFN-γ than those induced by IL-12 *in vitro*.[4-6] Moreover, a synergistic effect of mIGIF and IL-12 on IFN-γ production *in vitro* has been reported.[4] We, therefore, focused our attention on the possible anti-tumor activity of mIGIF *in vivo*. To construct cell lines that constitutively produce mIGIF, we transfected murine Lewis lung carcinoma (LLC) cells with an expression vector containing *mIGIF* complementary DNA (cDNA). These cells were injected into syngeneic mice to determine the effect on tumorigenicity *in vivo*.

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MATERIALS AND METHODS

Cell lines and experimental animals LLC cells originated as a spontaneous carcinoma of the lung in a C57BL/6 mouse.[7,8] LLC cells and transfectant cells were cultured in Iscove medium (IBL, Fujioka) with 20% FCS (IBL) at 37°C in a humidified 5% CO_2/air mixture. Female C57 BL/6 mice, 5 weeks old, were purchased from Japan Charles River Co., Ltd. (Atugi) and were maintained under specific pathogen-free conditions in our laboratory.

Plasmid construction and transfection The full-length *mIGIF* cDNA was obtained from total RNA of C57BL/6 mouse spleen cells by reverse transcription-polymerase chain reaction (RT-PCR) using a Perkin-Elmer RT-PCR kit (Perkin-Elmer, Norwalk, CT). The primers for the RT-PCR were: 5′-AACCTCGAGACAATGGCTG-CCATGTCAGAAGAC-3′, and 5′-CATCTCGAGCC-CCACCTAACTTTGATGTAAGTTAGT-3′. The 0.6 kb PCR product was introduced into the *Xho I* site of the eukaryotic cDNA expression vector BMGNeo,[9] conferring neomycin resistance. The insert clone was sequenced on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems Division, Perkin-Elmer, Foster City, CA) and contained the complete mIGIF coding region.

BMGNeo containing *mIGIF* cDNA was designated BMGNeo-mIGIF. BMGNeo and BMGNeo-mIGIF were transfected into LLC cells using the Lipofectin reagent (Gibco BRL, Gaithersburg, MD) according to

the manufacturer's instructions.[10] The cells were cultured in Iscove medium containing 1 mg ml^{-1} G418 for selection. The resulting selected cell lines transfected with BMGNeo-mIGIF and BMGNeo were designated LLC-mIGIF and LLC-Neo, respectively.

Interferon-γ production from spleen cells Spleen cells from C57BL/6 mice were collected and single cell suspensions were prepared by a cold water lysis method. Culture supernatants of LLC-Neo or LLC-mIGIF (1×10^6 cells ml^{-1} 24 h^{-1}, final; 25% v/v) were added to the cultures of spleen cells (1×10^6 cell ml^{-1} in Iscove medium with 20% FCS) in 24-well plates coated with/without 0.5 μg ml^{-1} anti-CD3 monoclonal antibody (mAb) (Pharmingen, San Diego, CA). For the combination assay of mIGIF and IL-12, we used mouse recombinant IL-12 (rIL-12) kindly supplied by Hoffmann-La Roche Inc. (Nutley, NJ). Culture supernatants of LLC-mIGIF (5×10^5 cells ml^{-1} 24 h^{-1}, final; 25% v/v) and/or rIL-12 (5 ng ml^{-1}) were added to cultures of spleen cells (5×10^5 cell ml^{-1}) in plates coated with 0.5 μg ml^{-1} anti-CD3 mAb.

After 40 h, the culture supernatants were assayed for IFN-γ activity using a "Cytoscreen" Immunoassay Kit (BioSource International, Camarillo, CA) according to the manufacturer's instructions.

***In vivo* evaluation of tumor growth** Tumor cells were harvested during exponential cell growth. Cell viability was determined by trypan blue dye exclusion. Cells were washed twice in Hanks solution (IBL) and 1×10^6 cells were injected subcutaneously into the right flank of C57BL/6 mice. Mice were inspected twice weekly and tumor sizes were calculated as the mean of the length and width. A tumor was defined as being >5 mm in mean diameter.

To examine the vaccine effect of LLC-mIGIF cells, C57BL/6 mice were immunized subcutaneously into the left flank three times at 7-day intervals, with 1×10^6 irradiated (100 Gy) cells. After the third booster injection, the mice were challenged subcutaneously with parental LLC cells (1×10^6 cells) into the right flank.

To examine the combination effect of systemic administration of rIL-12 and local mIGIF, mice were challenged with LLC, LLC-Neo or LLC-mIGIF cells and 50 ng of rIL-12 was injected intraperitoneally for 5 days, starting 7 days after tumor challenge.

RESULTS

***In vitro* characteristics of LLC-IGIF** An mIGIF expression plasmid, BMGNeo-mIGIF, was constructed by using a bovine papilloma virus-based expression vector containing a neomycin-resistance gene. LLC cells were transfected with BMGNeo or BMGNeo-mIGIF by lipofection. Transfectants were selected by using G418. By PCR, we confirmed that BMGNeo-mIGIF plasmids were present in the transfectants (data not shown). Doubling times of LLC, LLC-Neo and LLC-mIGIF cells were not significantly different at 13.7 h, 15.8 h and 13.0 h, respectively.

Table I. Interferon-γ Production by Spleen Cells

Culture medium	IFN-γ production (Mean$\pm$SD pg ml^{-1})	
	Anti-CD3 mAb (+)	Anti-CD3 mAb (−)
LLC-Neo	180.82$\pm$10.60	1.14$\pm$1.43
LLC-mIGIF	213.76$\pm$9.95[a)]	3.88$\pm$0.35

Spleen cells (1×10^6) were cultured for 40 h with 25% LLC-Neo or LLC-mIGIF culture medium (1×10^6 ml^{-1} 24 h^{-1}) in wells precoated with or without 0.5 μg ml^{-1} anti-CD3 mAb.
a) $P<0.05$, as compared with the control by unpaired Student's t test ($n=3$).

Table II. Enhanced Interferon-γ Production by mIGIF and IL-12 *in vitro*

Cytokines in culture medium	IFN-γ production (Mean$\pm$SD pg ml^{-1})
mIGIF	65.81$\pm$0.34
mIGIF+rIL-12	188.79$\pm$4.93[a)]
rIL-12	139.55$\pm$15.00

Spleen cells (5×10^5) were cultured for 40 h with 25% LLC-mIGIF culture medium (5×10^5 ml^{-1} 24 h^{-1}) and/or 5 ng ml^{-1} mouse rIL-12 in wells precoated with 0.5 μg ml^{-1} anti-CD3 mAb.
a) $P<0.05$, as compared with mIGIF or rIL-12 by unpaired Student's t test ($n=3$).

The secretion of mIGIF from transfectants could not be determined directly, because anti-mIGIF mAb and recombinant mIGIF were not available, and therefore the secretion of mIGIF from LLC-mIGIF was confirmed by examining the biological effect of culture supernatants on IFN-γ production by spleen cells (Table I). The supernatant of LLC-mIGIF significantly stimulated IFN-γ production by spleen cells in the presence of anti-CD3 mAb, as compared with the supernatant of LLC-Neo cells. Without anti-CD3 mAb stimulation, the supernatant of LLC-mIGIF could not stimulate IFN-γ production by spleen cells. These stimulation patterns were the same as previously reported for IGIF[4] and suggested that LLC-mIGIF was producing mIGIF. An interesting synergistic effect on IFN-γ production was noted in this previous report. We also examined the combined effect of the culture supernatant of LLC-mIGIF and rIL-12 on IFN-γ production by spleen cells. The supernatant of LLC-mIGIF markedly potentiated rIL-12-induced IFN-γ production by spleen cells as compared with stimulation by rIL-12 alone (Table II).

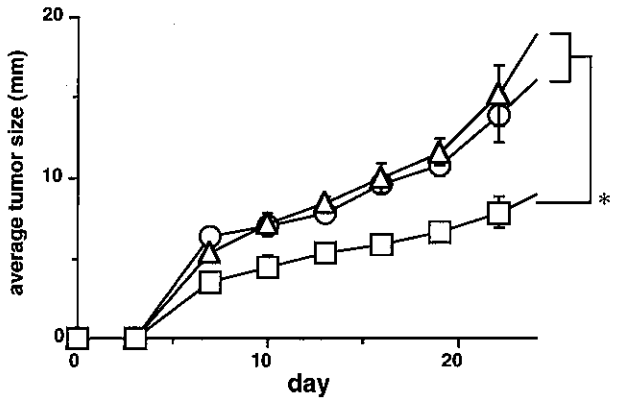

Fig. 1. One million cells were transplanted into the right flank of C57BL/6 mice. Each group consisted of 5 mice. Open circles, LLC; open triangles, mock-transfected; open squares, LLC-mIGIF. From day 10 to day 22, the size of LLC-mIGIF tumors was significantly smaller than those of LLC and LLC-Neo tumors ($*$ $P<0.05$ by unpaired Student's t test). Data are from one of 3 experiments which gave similar results.

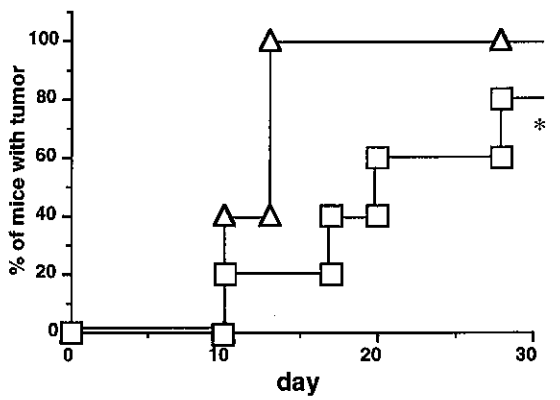

Fig. 2. Mice were injected 3 times into the left flank with 1×10^6 irradiated LLC-Neo or LLC-mIGIF cells at 7-day intervals as immunization before challenge with LLC. One million LLC were challenged in the right flank on day 0. Open triangles, irradiated LLC-Neo pre-injection; open squares, irradiated LLC-mIGIF pre-injection. A tumor >5 mm in mean diameter was classed as tumor formation. Each group consisted of 5 mice. $*$ P value was 0.032 by Cox-Mantel analysis.

***In vivo* tumorigenicity of LLC-IGIF** To determine whether constitutive and local mIGIF production from LLC cells might influence the tumorigenicity of these cells *in vivo*, parental LLC cells, LLC-Neo cells, and LLC-mIGIF cells were injected subcutaneously into the flank of syngeneic C57BL/6 mice. *In vivo* tumor growth curves, based on measured tumor size, are shown in Fig. 1. LLC, LLC-Neo and LLC-mIGIF resulted in tumor formation 7–10 days after injection. However, the growth rate of LLC-mIGIF *in vivo* was significantly slower than that of LLC or LLC-Neo (Fig. 1).

Immunization potential To compare the immunization potential of IGIF-producing and non-producing LLC cells against subsequent challenge with parental LLC cells, mice were injected 3 times with 1×10^6 LLC-Neo or LLC-mIGIF cells in the left flank before LLC challenge. Tumor formation (>5 mm) by parental LLC cells was significantly delayed in mice which had been immunized with LLC-mIGIF (Fig. 2). The median tumor formation time in mice immunized with LLC-Neo or LLC-mIGIF was 11.8 or 23.6 days, respectively, after LLC challenge (P value was 0.032 by Cox-Mantel analysis).

Combined effect of recombinant IL-12 and mIGIF *in vivo* To examine the combined effect of systemic administration of rIL-12 and local mIGIF on the tumorigenicity, mice were challenged with 1×10^6 LLC, LLC-Neo or LLC-mIGIF on day 0, and some mice were also inoculated intraperitoneally with 50 ng day^{-1} of rIL-12 from day 7 to day 11. Systemic administration of rIL-12 greatly reduced the growth rate of LLC and the transfectant cells. However, there was no apparent additive or synergistic anti-tumor effect of systemic administration of rIL-12 on LLC-mIGIF tumors (Fig. 3).

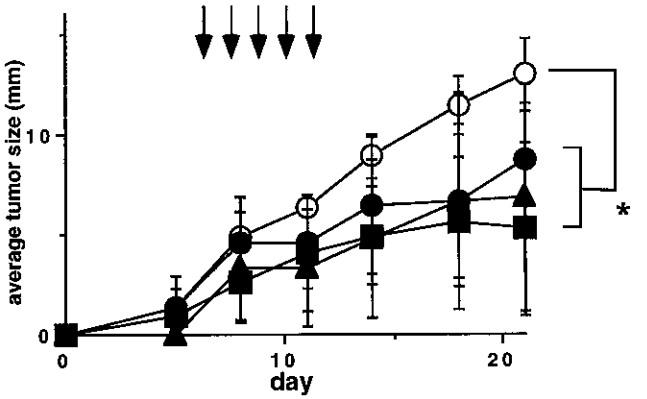

Fig. 3. One million tumor cells were injected into the right flank and 50 ng of IL-12 was injected intraperitoneally from day 7 to day 11 (arrows). Each group consisted of 5 mice. Open circles, untreated control; solid circles, LLC-challenged group with IL-12 treatment; solid triangles, LLC-Neo-challenged group with IL-12 treatment; solid squares, LLC-mIGIF-challenged group with IL-12 treatment. Recombinant IL-12 significantly inhibited tumor growth of LLC, LLC-Neo, or LLC-mIGIF ($*$ $P<0.05$ by unpaired Student's t test) but there was no significant difference between the IL-12-treated groups.

DISCUSSION

In this study, we demonstrated that mIGIF transfection of low-immunogenic LLC cells[11] significantly reduced tumorigenicity in syngeneic C57BL/6 mice. The phenomenon of reduced tumorigenicity after cytokine gene insertion into tumor cells has been demonstrated for several cytokines, but this is the first observation that *mIGIF* gene transfer to tumor cells has an anti-tumor effect. Moreover, we demonstrated that tumor formation by parental LLC cells was delayed in mice immunized with LLC-mIGIF. These results suggest that transfection of mIGIF was able to induce, in addition to a local immune response, a systemic anti-tumor immune response against parental LLC cells.

IL-12 is a particularly interesting cytokine in relation to anti-tumor immune response, because it may induce a primary Th1 response *in vitro* and *in vivo*.[1-3] The systemic administration of IL-12 improved the survival of mice bearing a great variety of tumors. However, in clinical use, administration of IL-12 induced strong side effects in a dose-dependent manner.[12]

Recently, the combined systemic administration of IL-12 and IL-2-secreting cancer vaccine was examined with the aim of enhancing the anti-tumor effect and reducing the side effect of IL-12, and some additive effect was reported.[13] For the same purpose, IGIF may be a more attractive cytokine, because IGIF induces IFN-γ through a different pathway from IL-12 and synergistically potentiates IFN-γ production in the presence of IL-12. Therefore, we examined the effect of combined systemic administration of rIL-12 and LLC-mIGIF. *In vitro*, the supernatant from LLC-mIGIF cells potentiated IL-12-induced IFN-γ production by spleen cells. *In vivo*, systemic administration of rIL-12 resulted in reduced tumorigenicity of LLC, LLC-Neo and LLC-mIGIF. However, we could not detect any additive effect of mIGIF transfection on the anti-tumor effect of rIL-12 systemic administration. The reason for this discrepancy between *in vivo* and *in vitro* results might be the low level of production of mIGIF by the LLC-mIGIF cells. In the previous report, a synergistic effect was observed[4] when over 1 ng ml^{-1} of mIGIF and 20 μg ml^{-1} rIL-12 were combined *in vitro*. However, no synergistic effect was observed with less than 1 ng ml^{-1} of mIGIF. We could not determine the level of mIGIF production from LLC-mIGIF cells in this study. However, production might not have been sufficient to induce a synergistic effect *in vivo*. Therefore, we are selecting for LLC-mIGIF clones which produce high titers of mIGIF to determine whether a high level of mIGIF production is able to potentiate the anti-tumor effect of IL-12 *in vivo*. In addition, to examine the anti-tumor effect of IGIF against other cell lines, we are planning to establish other transfectants.

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