



# Gene expression and antitumor effects following direct interferon (IFN)- $\gamma$ gene transfer with naked plasmid DNA and DC-chol liposome complexes in mice

T Nomura<sup>1</sup>, K Yasuda<sup>1</sup>, T Yamada<sup>1</sup>, S Okamoto<sup>1</sup>, RI Mahato<sup>1,2</sup>, Y Watanabe<sup>3</sup>, Y Takakura<sup>1</sup> and M Hashida<sup>1</sup>

<sup>1</sup>Department of Drug Delivery Research; and <sup>3</sup>Department of Molecular Microbiology, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan

Gene expression was assessed in three types of mouse solid tumors after direct injection of naked plasmid DNA encoding the luciferase gene (pCMV-Luc) and its DC-chol liposome complexes. Intratumoral injection of 5 or 100  $\mu$ g naked pCMV-Luc into subcutaneously inoculated mouse colon tumor (CT-26), fibrosarcoma (MCA-15) and bladder carcinoma (MBT-2) resulted in significant gene expression. A DC-chol liposome formulation (5  $\mu$ g pCMV-Luc complexed with 25  $\mu$ g DC-chol liposome) showed lower level of gene expression in the tumor models. Based on the results using the reporter gene, we examined the antitumor effect after direct interferon- $\gamma$  (IFN- $\gamma$ ) gene transfer into CT-26 tumors. A significant IFN- $\gamma$  production and growth inhibition were obtained following direct intratumoral injection of IFN- $\gamma$  gene with naked plasmid DNA (pCMV-Mu $\gamma$ ). Interestingly, pCMV-Mu $\gamma$ /DC-chol liposome complexes

exhibited more pronounced growth inhibitory effect despite lower IFN- $\gamma$  production. Induction of CT-26 specific anti-tumor immunity by IFN- $\gamma$  gene transfer was confirmed by rejection of a CT-26 tumor challenge in the mice showing complete regression of CT-26 tumors after both treatments. Further analysis demonstrated that a significant cDNA-independent induction of IFN- $\beta$  and TNF- $\alpha$  occurred following injection with the liposome complexes, suggesting a nonspecific suppressive effect on CT-26 tumor growth by these cytokines. Thus, the present study has demonstrated that tumor tissue might be a promising target for direct IFN- $\gamma$  gene transfer with plasmid-based nonviral vectors. It is also suggested that immunomodulatory effects by various cytokines could be involved in antitumor effects after direct intratumoral injection of plasmid DNA formulations.

**Keywords:** interferon- $\gamma$ ; naked plasmid DNA; DC-chol liposome; gene therapy; intratumoral injection

## Introduction

In cancer gene therapy, direct DNA injection is currently a reliable, reproducible and simple technique for intratumoral gene transduction. A variety of specific gene therapy strategies have been demonstrated to be effective in both *in vitro* and *in vivo* tumor models.<sup>1–4</sup> For example, it has been reported that intratumoral injection of immunomodulatory genes can evoke a powerful immune response to tumor cells so that specific immune cells are activated sufficiently to destroy the tumor deposits.<sup>2–4</sup> The potential of several vector systems has been explored as a means of achieving *in vivo* direct gene transfer to target tumor cells. Most intratumoral gene delivery is viral involving retrovirus<sup>5–7</sup> and adenovirus systems.<sup>4,8–11</sup> These viral vectors are very efficient in delivering DNA to target cells. However, viral vectors also have various disadvantages such as immune recognition following adenoviral vector delivery and potential virus-associated

toxicity, including helper virus replication and insertional mutagenesis. In contrast, nonviral vectors, such as plasmid DNA and cationic liposomal systems, exhibit little immunogenicity and repeated administration is possible.<sup>12</sup>

Cationic liposomal systems are attractive nonviral vectors for *in vivo* gene transfer due to their favorable characteristics such as biodegradability, minimal toxicity, non-immunogenicity and simplicity of use.<sup>13–15</sup> Use of DNA/cationic liposomal complexes for transfecting cultured tumor cells has a clear advantage over naked DNA.<sup>16–18</sup> Their *in vivo* usefulness has also been established<sup>3,19</sup> and clinical trials by direct gene transfer to tumors have been carried out.<sup>20,21</sup> On the other hand, it has been reported that significant gene expression can be obtained by direct injection of naked plasmid DNA in some solid tumor models.<sup>1,19,22,23</sup> In mouse melanoma, gene expression with free DNA injection was inhibited by complex formation with cationic liposomes.<sup>22</sup>

Among immunomodulatory cytokines, interferon- $\gamma$  (IFN- $\gamma$ ) induces strong antitumor immunity via several distinct mechanisms.<sup>24</sup> IFN- $\gamma$  is able to activate a variety of host immune cells such as T lymphocytes, NK cells and macrophages. IFN- $\gamma$  is also a potent inducer of MHC class I and II antigens, which increases the antigen-presenting capacity of cells. These biological effects can

Correspondence: M Hashida, Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

<sup>2</sup>Present address: GeneMedicine Inc, 8301 New Trails Drive, The Woodlands, Texas 77381–4248

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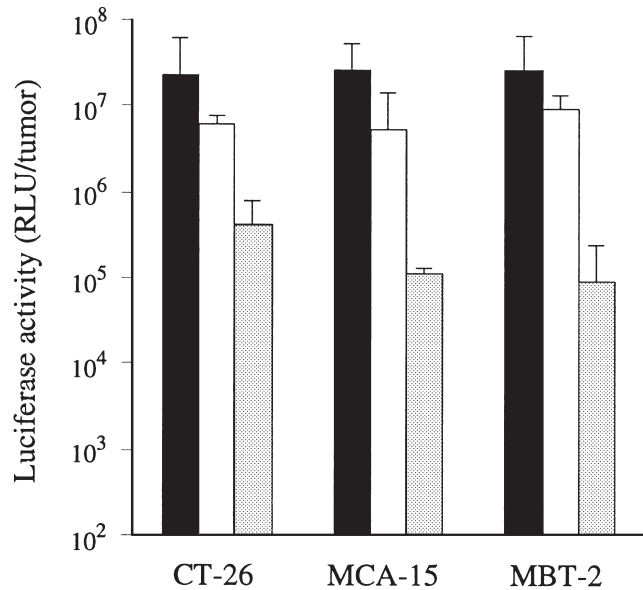
be induced at relatively low levels of IFN- $\gamma$  if the level is maintained in the vicinity of the tumor. We and others have demonstrated that transduction of tumor cells with the IFN- $\gamma$  gene by retroviral vectors abrogates their tumorigenicity through augmented antitumor immunity.<sup>25-27</sup> Recently, a phase 1 clinical trial has been conducted with IFN- $\gamma$  gene-modified autologous melanoma cells.<sup>28</sup> Direct *in vivo* IFN- $\gamma$  gene transfer with nonviral vectors could be a powerful alternative to this *ex vivo* approach.

In this study, we carried out gene expression experiments in tumor-bearing mice using the luciferase reporter gene. Gene expression efficiency was examined in three types of solid tumors after direct intratumoral injection of naked plasmid DNA and its DC-chol liposome complexes. Based on the results, we next evaluated the gene expression and antitumor effects following interferon- $\gamma$  (IFN- $\gamma$ ) gene transfer with direct injection of these two formulations in order to assess the possibility of their application in cancer gene therapy.

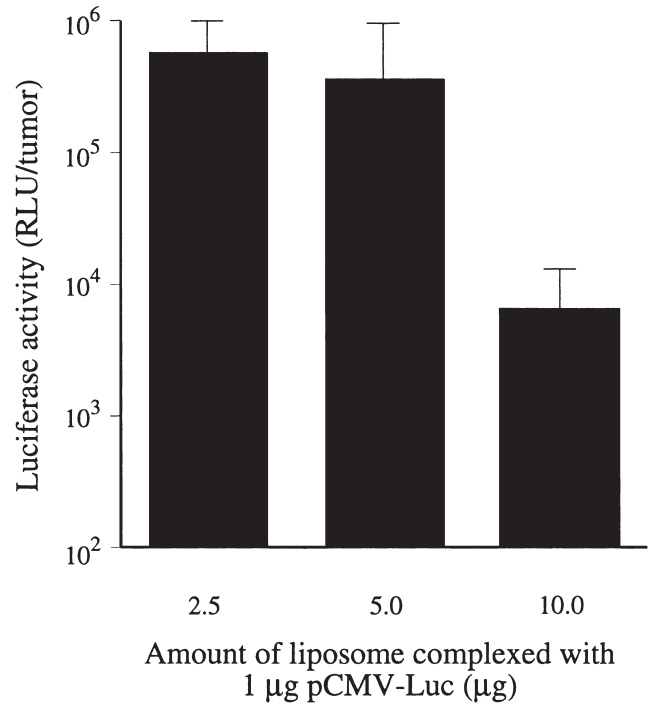
## Results

### Gene expression in mouse tumors

As shown in Figure 1, three types of tumor showed significant gene expression after intratumoral injection of 5 or 100  $\mu$ g naked pCMV-Luc. A DC-chol liposome formulation (5  $\mu$ g pCMV-Luc complexed with 25  $\mu$ g DC-chol liposome) showed a lower level of gene expression in these tumor models. Lipofectin, another commercially available cationic liposome, was less efficient than DC-chol liposomes (data not shown). Figure 2 shows the effect of DC-chol liposome amount on the luciferase gene expression produced by 1  $\mu$ g pCMV-Luc preparation.



**Figure 1** Luciferase expression in three types of tumors 48 h after intratumoral injection of naked pCMV-Luc and its cationic liposome complexes. CT-26, mouse colon tumor; MCA-15, mouse fibrosarcoma; MBT-2, mouse bladder tumor; black bar: 100  $\mu$ g naked pCMV-Luc; white bar: 5  $\mu$ g naked pCMV-Luc; gray bar: 5  $\mu$ g pCMV-Luc complexed with 25  $\mu$ g DC-chol liposomes. The level of luciferase expression (RLU/tumor) was determined 48 h after gene transfer. Each result represents the mean  $\pm$  s.d. of three tumors.



**Figure 2** Effect of the amount of cationic liposomes on gene expression after intratumoral direct gene transfer. One microgram pCMV-Luc complexed with 2.5, 5 or 10  $\mu$ g DC-chol liposomes in 100  $\mu$ l Opti-MEM was directly injected into s.c. CT-26 tumors. The level of luciferase expression was determined 48 h after gene transfer. Each result represents the mean  $\pm$  s.d. of five tumors.

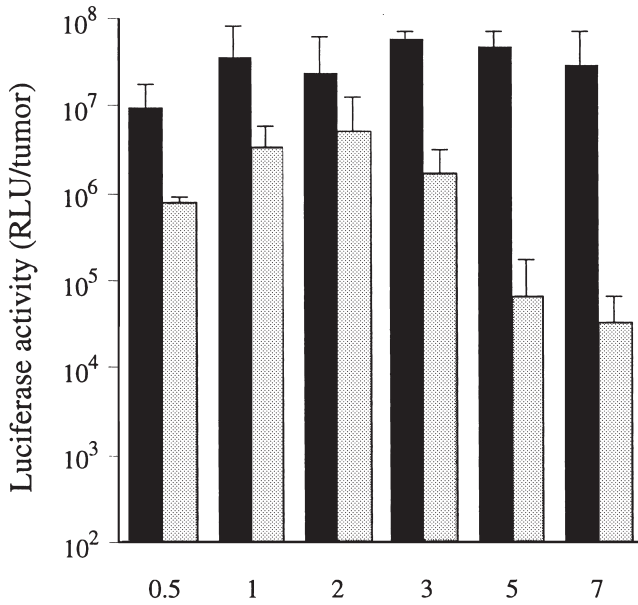
Gene expression was slightly higher at 1/2.5 (DNA/liposome, w/w) than that at 1/5 and decreased at 1/10. Agarose-gel electrophoresis showed that complex formation was not complete at 1/2.5 (data not shown), suggesting that free DNA might affect the gene expression at this ratio. The ratio of 1/5 (DNA/liposome, w/w) was used in the following experiments as optimum ratio *in vivo*.

### Duration of gene expression

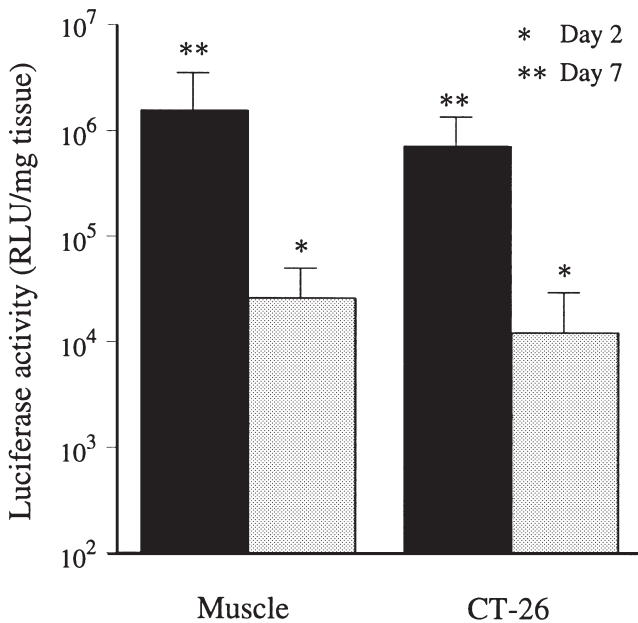
To determine the duration of expression, CT-26 tumors were removed at different times after injection of naked DNA and its DC-chol liposome complexes, and the luciferase activity was determined (Figure 3). A significant level of luciferase gene expression in tumors persisted for at least 7 days after injection of naked pCMV-Luc (100  $\mu$ g). DC-chol liposome complexes (5  $\mu$ g DNA, 25  $\mu$ g liposome) showed lower luciferase activity and the gene expression level decreased 3 days after injection (Figure 3).

### Comparison of naked DNA expression between tumor and muscle tissue

In the case of intramuscular injection, efficient luciferase expression was observed with 5  $\mu$ g naked pCMV-Luc as observed in tumors and it reached maximum level 7 days after injection (data not shown). Application of DC-chol liposomes significantly reduced gene expression in the muscle which was maximum 2 days after injection and decreased thereafter (data not shown). Figure 4 compares the level of gene expression in CT-26 tumor and muscle



**Figure 3** Time-course of luciferase expression in CT-26 after intratumoral injection of naked pCMV-Luc and its cationic liposome complexes. Black bar: 100  $\mu$ g naked pCMV-Luc; gray bar: 5  $\mu$ g pCMV-Luc complexed with 25  $\mu$ g DC-chol liposomes. The level of luciferase expression was determined at the indicated times after gene transfer. Each result represents the mean  $\pm$  s.d. of four tumors.



**Figure 4** Comparison of gene expression in tumors with muscle tissue after direct injection of naked pCMV-Luc and its cationic liposome complexes. The level of luciferase expression was determined at 48 h in the case of injection with DNA/DC-chol liposome complexes, and at day 7 in the case of injection with naked pCMV-Luc. Each result represents the mean  $\pm$  s.d. of three to five experiments.

tissue after intratumoral injection of naked plasmid DNA (at day 7) and DC-chol liposome complexes (at day 2). The level of luciferase activity per milligram tissue with naked pCMV-Luc was comparable with that in muscle tissue. Similar levels of gene expression were also

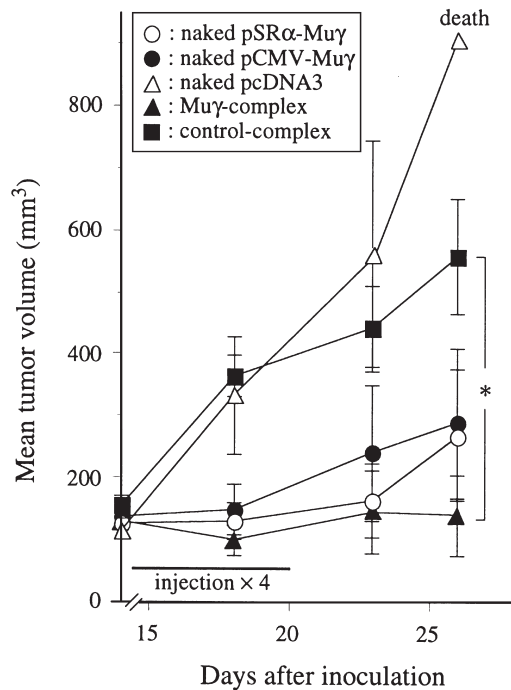
observed in both tissues after injection of pCMV-Luc/DC-chol liposome complexes.

*In vivo tumor growth after direct IFN- $\gamma$  gene transfer*

As shown in Figure 5, intratumoral injection of naked pCMV-Mu $\gamma$  or pSR $\alpha$ -Mu $\gamma$  (100  $\mu$ g  $\times$  4) inhibited growth of CT-26 tumors compared with the control group (100  $\mu$ g pcDNA3  $\times$  4) and complete tumor regression was also observed in 33% (2/6) of the treated mice in both cases (Table 1). On the other hand, more pronounced growth inhibition was observed in the mice treated with pCMV-Mu $\gamma$ /DC-chol liposome complexes (DNA/liposome, 5  $\mu$ g/25  $\mu$ g  $\times$  4) (Figure 5) and 50% (3/6) of the treated mice were cured of their tumors. All the mice, which showed complete regression of CT-26 tumors in both treatment groups, rejected s.c. rechallenged parental CT-26 cells ( $1 \times 10^5$  cells) which were tumorigenic to all the control naive mice (Table 1). This indicates that IFN- $\gamma$  gene transfer results in the induction of antitumor immunity specific to CT-26 cells.

*Secretion of IFN- $\gamma$  and other cytokines from tumor tissue after direct IFN- $\gamma$  gene transfer*

For the assessment of gene expression after *in vivo* direct IFN- $\gamma$  gene transfer, the amounts of IFN- $\gamma$  secreted from the excised tumor were determined in tissue culture (Figure 6). Tumors injected with naked pCMV-Mu $\gamma$  or pSR $\alpha$ -Mu $\gamma$  expressed and secreted a larger amount of IFN- $\gamma$  than those with pCMV-Mu $\gamma$ /DC-chol liposome complexes. Control naked plasmid (pcDNA3) and DC-

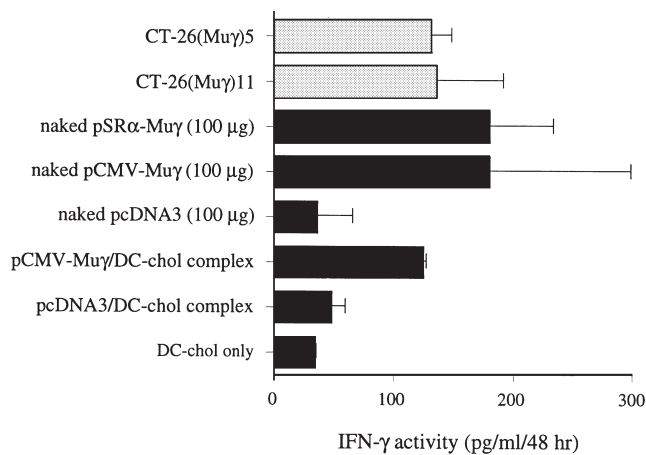


**Figure 5** *In vivo* gene therapy of established subcutaneous CT-26 tumors with naked pCMV-Mu $\gamma$  and its DC-chol liposome complexes. Tumors were injected with naked pCMV-Mu $\gamma$ , naked pSR $\alpha$ -Mu $\gamma$  or control plasmid DNA (pcDNA3) (100  $\mu$ g), and their DC-chol liposome complexes (DNA, 5  $\mu$ g; liposome, 25  $\mu$ g) at day 14, 16, 18 and 20 after s.c. inoculation of  $1 \times 10^5$  CT-26 cells. Mean tumor volumes  $\pm$  s.e. are shown for six mice per group. There is a statistically significant difference by ANOVA: \*,  $P < 0.05$ .

**Table 1** Immunity of mice that rejected CT-26 cells

Treatment	No. of cured mice	Rechallenge <sup>a</sup>	No. of mice with tumor
naked pCMV-Mu $\gamma$	2/6	parent CT-26	0/2
naked pSR $\alpha$ -Mu $\gamma$	2/6	parent CT-26	0/2
naked pcDNA3	0/6 <sup>b</sup>	—	—
pCMV-Mu $\gamma$ /DC-chol complex	3/6	parent CT-26	0/3
pcDNA3/DC-chol complex	0/6	—	—
control (naive mice)	—	parent CT-26	6/6
CT-26 (Mu $\gamma$ ) 5	3/5	parent CT-26	0/3
CT-26 (Mu $\gamma$ ) 11	3/5	parent CT-26	0/3
control (naive mice)	—	parent CT-26	3/3

<sup>a</sup>Naive mice or mice that rejected CT-26 cells were injected s.c. with  $1 \times 10^5$  parent CT-26 cells. <sup>b</sup>Five mice died of tumor 26 days after inoculation and mice in all the other groups survived up to the last day of monitoring.



**Figure 6** Secretion of IFN- $\gamma$  from s.c. CT-26 tumors after direct gene transfer and tumors of IFN- $\gamma$ -producing CT-26 cell lines. Tumors were injected with naked pCMV-Mu $\gamma$ , naked pSR $\alpha$ -Mu $\gamma$ , naked pcDNA3 and their DC-chol liposome complexes. pCMV-Mu $\gamma$ /DC-chol liposome complex and pcDNA3/DC-chol liposome complex; DNA/liposome, 5  $\mu$ g/25  $\mu$ g, DC-chol liposome only, 25  $\mu$ g. In the case of CT-26(Mu $\gamma$ )5 and CT-26(Mu $\gamma$ )11, palpable tumors (about 5 mm in diameter) were excised and subjected to tissue culture as described in Materials and methods. IFN- $\gamma$  in the culture medium was determined by ELISA. Each result represents the mean  $\pm$  s.e. of three to four tumors.

chol liposomes only resulted in lower IFN- $\gamma$  levels. In order to further analyze the correlation between IFN- $\gamma$  secretion and antitumor effects, we established two constitutively IFN- $\gamma$ -producing cell lines, CT-26(Mu $\gamma$ )5 or CT-26(Mu $\gamma$ )11, and determined IFN- $\gamma$  activities in the same experimental system. The IFN- $\gamma$  yields from excised tumors of these IFN- $\gamma$  producing cell lines were slightly lower than those obtained with naked plasmid DNA injection (Figure 6).

In order to explore the mechanisms of tumor growth inhibition after direct intratumoral IFN- $\gamma$  gene transfer, the amounts of other cytokines, ie, IFN- $\beta$  and TNF- $\alpha$ ,

secreted from the tumor were determined (Figure 7). We found that the secretion of IFN- $\beta$  and TNF- $\alpha$  was significantly promoted only in the tumors injected with plasmid DNA/DC-chol liposome complexes (Figure 7). Similar activities were observed for both pCMV-Mu $\gamma$  and control plasmid preparations, indicating that the cytokine secretion induced by DC-chol liposome complexes is cDNA-independent.

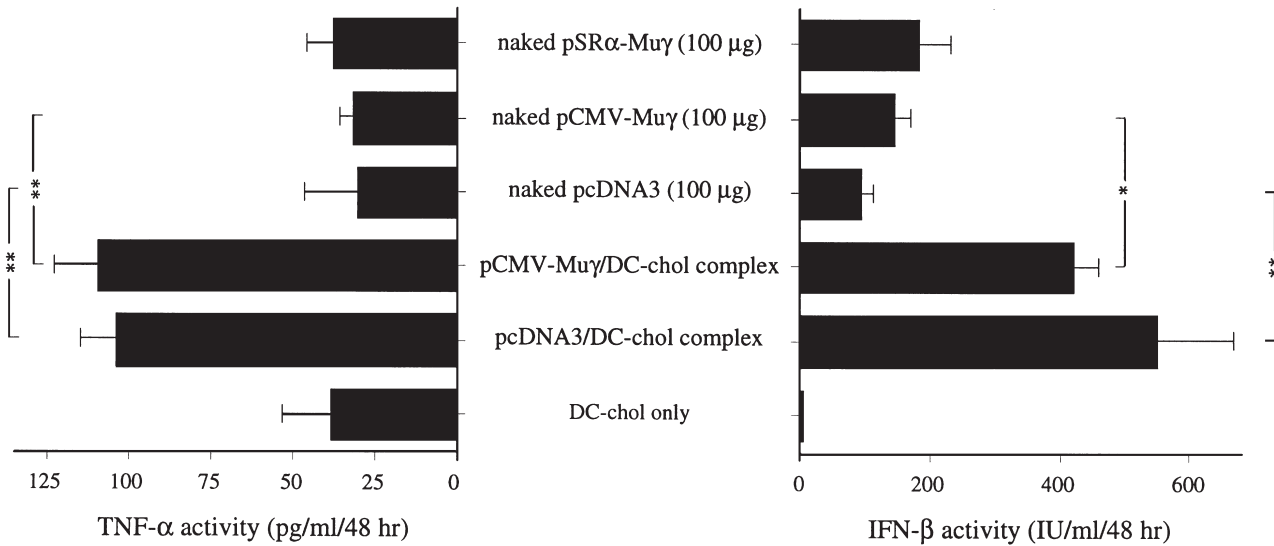
#### *In vivo tumorigenicity of IFN producer cells*

Effects of IFN- $\gamma$  and IFN- $\beta$  on the tumorigenicity of CT-26 cells were tested by s.c. transplantation of the gene-transferred cells, as well as the parent CT-26 cells. As shown in Figure 8, there was no significant difference in tumor growth between parent CT-26 and the IFN- $\beta$ -producing cell line, CT-26 (Mu $\beta$ ) 6 cells. In contrast, two IFN- $\gamma$ -producing cell lines, CT-26 (Mu $\gamma$ ) 5 and 11, showed significant growth inhibition and complete tumor regression was observed for both cell lines (3/5, 60%) (Table 1). In the mice that rejected CT-26 (Mu $\gamma$ ) 5 and 11 cells, s.c. reinjection of the parental CT-26 cells could no longer induce any tumor, as observed in cured mice after direct gene transfer (Table 1).

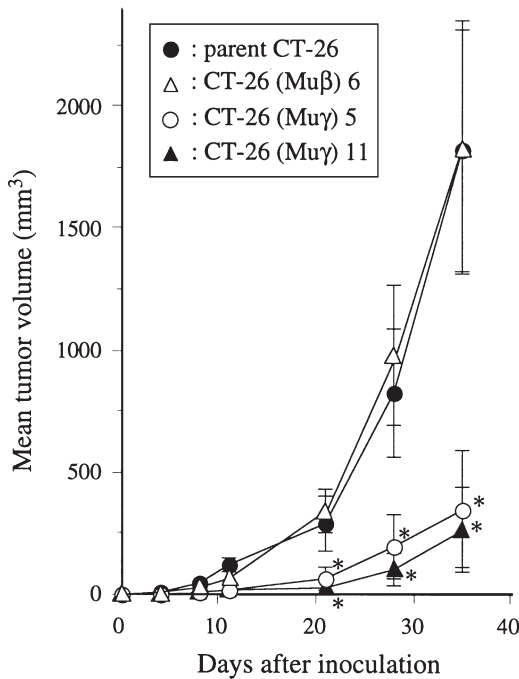
#### Discussion

It is well known that direct injection of naked plasmid DNA into muscle tissues leads to significant gene expression.<sup>29-33</sup> Similar results are also reported for liver<sup>34</sup> and brain.<sup>35</sup> However, information on direct gene transfer with naked DNA to solid tumors is limited and controversial. Yang and Huang<sup>22</sup> have shown that direct intratumoral injection of naked DNA into mouse melanoma BL6 can result in a high level of transfection which is inhibited by cationic liposomes. The authors also tested another six tumors, but the transfection efficiency was much lower than for the BL6 tumor. Egilmez *et al*<sup>36</sup> have examined direct injection of naked DNA encoding IL-2 in SCID mice bearing human tumor xenografts but failed to show any antitumor effect, whereas DNA/cationic liposome complexes exhibited significant effects. In our previous study, we also found that intratumoral injection of naked plasmid DNA resulted in a similar level of gene expression compared with DNA/liposome complexes in a rat tumor model.<sup>23</sup> In this study, at first, quantitative analysis of reporter gene expression was performed using three mouse tumor models in order to clarify the efficacy of direct intratumoral DNA injection and the effect of cationic liposomes on it.

DC-chol liposome is one of the most efficient cationic liposomes for *in vivo* intratumoral gene transfer.<sup>19,20</sup> Egilmez *et al*<sup>19</sup> reported that DC-chol liposome formulation was superior to other formulations (lipofectin, lipofectamine, cellfectin and DMRIE/DOPE) for *in vivo* intratumoral gene transfer. In our preliminary experiments, a higher reporter gene expression was also obtained with DC-Chol liposomes compared with lipofectin. Based on these findings, we used mostly DC-Chol liposomes in this study. As shown in Figure 1, in three different mouse tumors, efficient gene expression was observed after intratumoral injection of naked pCMV-Luc. A DC-Chol liposome formulation (5  $\mu$ g pCMV-Luc complexed with 25  $\mu$ g DC-chol liposome) showed a lower level of gene expression in these tumor models. The duration of gene expression also makes a difference between injections of



**Figure 7** Secretion of IFN- $\beta$  and TNF- $\alpha$  from s.c. CT-26 tumors after direct gene transfer. Injected formulations were the same as those shown in the legend to Figure 6. The tumors were excised and subjected to tissue culture as described in Materials and methods. IFN- $\beta$  and TNF- $\alpha$  in the culture medium were determined by bioassay and ELISA, respectively. Each result represents the mean  $\pm$  s.e. of three to seven tumors. There are statistically significant differences by ANOVA: \*,  $P < 0.05$ , \*\*,  $P < 0.01$ .



**Figure 8** *In vivo* tumorigenicity of IFN-producing CT-26 cell lines. Parent CT-26, CT-26 (Mu $\beta$ ) 6, CT-26 (Mu $\gamma$ ) 5 and CT-26 (Mu $\gamma$ ) 11 cells ( $1 \times 10^5$  cells) were subcutaneously inoculated into Balb/c mice. Mean tumor volumes  $\pm$  s.e. are shown for five mice per group. There was no significant difference in growth rate between parent CT-26 cells and CT-26(neo) (data not shown). There are statistically significant differences from CT-26(Mu $\beta$ )6 by ANOVA: \*,  $P < 0.05$ .

naked pCMV-Luc and its DC-chol liposome complexes (Figure 3). The maximum level of gene expression in tumors with naked plasmid DNA was comparable with that in muscle tissue (Figure 4). These results are consistent with earlier observations in mouse melanoma.<sup>22</sup> Although we can not define the superiority of naked

DNA to cationic liposome formulations solely from these results, potential usefulness of naked DNA in direct intratumoral gene transfer was suggested.

Wolff *et al*<sup>31</sup> reported that naked plasmid DNA in skeletal muscle after direct injection is specifically located in T tubules and/or caveolae specific to striated muscle, and these structures may play an important role in the uptake rather than physical disruption of the membrane of myotubes by direct injection. On the other hand, specific uptake of naked plasmid DNA by tumor cells has never been described although there are a few reports, including our previous one, which describe naked DNA expression in tumor tissue after *in vivo* direct gene transfer.<sup>1,22,23</sup> Physical disruption by hydrostatic pressure might be one of the explanations why tumor cells can express naked plasmid DNA *in vivo* but not *in vitro*. Although the mechanism of this uptake and long-term expression in the tumor cells *in vivo* was not addressed in the present study, this important issue remains to be elucidated. In addition, it is reported that tumor cells are expected to be more susceptible to nuclear import and the uptake of transfected naked plasmid DNA at higher rates compared with terminally differentiated cells.<sup>37</sup> This factor may account for gene expression in tumor cells.

Based on the results regarding reporter gene expression, we next performed a series of experiments using plasmid DNA encoding IFN- $\gamma$ , one of the most potent cytokines for tumor immunotherapy, in an established CT-26 s.c. tumor model. Since IFN- $\gamma$  can exhibit various and strong biological activities even at relatively low titers, direct provocation of an antitumor immune response at the tumor sites could be expected by direct gene transfer with nonviral vectors. As shown in Figure 5, a significant antitumor effect was observed in both cases of treatment with naked pCMV-Mu $\gamma$  and its DC-chol liposome complexes compared with the control groups. In contrast to the results with reporter gene, tumors injected with pCMV-Mu $\gamma$ /DC-chol liposome complexes showed slightly more pronounced growth

inhibition compared with naked pCMV-Mu $\gamma$ . In order to evaluate the cDNA-dependent antitumor effect, the amount of IFN- $\gamma$  secreted was determined by culturing the excised tumor tissue after direct gene transfer. In our preliminary study, the IFN- $\gamma$  level in the blood circulation was under the detection limit after intramuscular injection of 100  $\mu$ g pCMV-Mu $\gamma$  presumably due to the short *in vivo* half-life of IFN- $\gamma$ . This culturing method enabled us to successfully determine IFN- $\gamma$  secreted from the tumors with relatively small variations (Figure 6). IFN- $\gamma$  secretion was higher in the case of injection with naked pCMV-Mu $\gamma$  or pSR $\alpha$ -Mu $\gamma$  compared with pCMV-Mu $\gamma$ /DC-chol liposome complex, which is in agreement with the results obtained with reporter gene (Figure 1). Slightly lower levels of IFN- $\gamma$  were also detected from the s.c. tumors of CT-26(Mu $\gamma$ )5 or CT-26(Mu $\gamma$ )11, constitutively IFN- $\gamma$  producing cell lines (Figure 6), which showed significant growth inhibition or regression of the tumors and resulted in induction of CT-26 specific antitumor immunity (Table 1). These results suggest that the low, but significant, level of IFN- $\gamma$  expressed in the tumor tissue by direct gene transfer has a powerful immunomodulatory effect on CT-26 tumors.<sup>25,26</sup>

We next sought to analyze the mechanisms of suppressive effects on tumor growth after direct gene transfer. As there was a discrepancy between the antitumor effects (Figure 5) and IFN- $\gamma$  expression (Figure 6), we considered that the superior antitumor effect by the plasmid DNA/liposome complexes might be due to IFN- $\gamma$ -independent immunomodulatory or cytotoxic effect on tumor cells. At first, we determined TNF- $\alpha$ , one of the cytokine candidates with such effects. TNF- $\alpha$  is produced predominantly by macrophages, has a direct antitumor activity and enhances T and B lymphocyte responsiveness.<sup>38</sup> Although the absolute levels of induced TNF- $\alpha$  were low, a significant increase in TNF- $\alpha$  secretion was found in the tumor after direct gene transfer with plasmid DNA/DC-chol liposome complexes (Figure 7). It is reported that TNF- $\alpha$  is induced in immune effector cells such as murine macrophages and T lymphocytes when they are transfected with nucleic acid/cationic lipid complexes *in vitro*.<sup>39</sup> Since these immune effector cells are considered to be the major site of localization of plasmid DNA/cationic liposome complexes after intratumoral injection, they might have contributed to the cDNA-independent suppressive effects on tumor growth.

Furthermore, a significant IFN activity was incidentally found in the supernatants of cultured excised tumors and it was proved to be derived from IFN- $\beta$  in typing experiments using monoclonal antibodies (Figure 7). This induction of IFN- $\beta$  was observed irrespective of the DNA content as well as TNF- $\alpha$ . IFN- $\beta$ , as well as IFN- $\gamma$ , has immunomodulatory and antitumor effects. We have already reported that s.c. tumor growth of a mouse bladder carcinoma (MBT-2) is partially suppressed by autocrine IFN- $\beta$ .<sup>40</sup> Yagi *et al*<sup>41</sup> reported the cytotoxic effect of IFN- $\beta$  on glioma transplanted into the brain following intratumoral injection of IFN- $\beta$  gene encapsulated into cationic liposomes.<sup>41</sup> In addition, growth inhibition of established CT-26 s.c. tumors was observed after intratumoral injection of double-stranded complexes of synthetic polyribonucleotides (polyinosinic-polycytidilic acid; pI:pC) known to be a powerful inducer of IFN- $\beta$ ,<sup>42,43</sup> and 400–500 IU/ml/48 h IFN- $\beta$  was detected in the tissue-culture supernatant (to be published elsewhere).

These results indicate that IFN- $\beta$  induced by plasmid DNA/DC-chol liposome complexes may partially contribute to the cDNA-independent growth-inhibitory effect on CT-26 tumors. This would also be supported by the fact that a slight growth inhibition was observed after intratumoral injection of control plasmid DNA (pcDNA3)/DC-chol liposome complexes but not after naked control plasmid DNA (Figure 5). The mechanisms of IFN- $\beta$  induction by plasmid DNA/DC-chol liposome complexes remains to be elucidated and induction of cytokines other than TNF- $\alpha$  and IFN- $\beta$  might occur.

The endotoxin level in the injected plasmid DNA is an important factor which may affect gene expression<sup>44</sup> and non-specific activation of the immune system. We have confirmed a negligible contamination of lipopolysaccharide (LPS) by LAL assay. As shown in Figure 7, the amounts of induced IFN- $\beta$  and TNF- $\alpha$  were significantly small in the case of injection of 100  $\mu$ g naked plasmid DNA or DC-chol liposome alone compared with 5  $\mu$ g plasmid DNA/liposome complexes. These results also indicated that secretion of IFN- $\beta$  and TNF- $\alpha$  would be specifically induced by the plasmid DNA/DC-chol liposome complexes and the effect of LPS may be negligible.

There may be other possible explanations for the non-specific suppressive effects of the plasmid DNA/DC-chol liposome complexes on tumor growth. One is involvement of the direct cytotoxic effect of cationic liposomes on tumor tissue. Another possibility is that bacterial plasmids themselves appear to be immunogenic, as reported recently and that there are immunostimulatory DNA sequences in them such as CpG motifs<sup>45</sup> and this immunostimulation might be promoted by the cationic liposomes.

In conclusion, the present study has demonstrated that tumor tissue might be a promising target for direct IFN- $\gamma$  gene transfer with plasmid-based nonviral vectors. It is also suggested that immunomodulatory effects by various cytokines could be involved in antitumor effects after direct intratumoral injection of plasmid DNA formulations.

## Materials and methods

### Chemicals

1,2-Dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) was purchased from Avanti Polar-Lipids (Birmingham, AL, USA). Lipofectin was purchased from Gibco BRL (Gaithersburg, MD, USA). 3 $\beta$ -(N-(N',N'-Dimethylaminoethane) carbamoyl) cholesterol (DC-chol) was synthesized as described.<sup>16</sup>

### Plasmid and cell lines

pCMV-Luc was constructed by inserting the *Hind*III/*Xba*I firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI, USA) into the *Hind*III/*Xba*I site of pcDNA3 (Invitrogen, Carlsbad, CA, USA). pCMV-Mu $\gamma$  was constructed by subcloning the *Bam*HI cDNA fragment of mouse IFN- $\gamma$  from WBE<sub>R</sub>. Mu $\gamma$  into the *Bam*HI site of pcDNA3. pSR $\alpha$ -Mu $\gamma$  was constructed by inserting the mouse IFN- $\gamma$  cDNA fragment from a retroviral vector, WB(KMu $\gamma$ ), into expression vector pGEM3SR $\alpha$ -Neo by blunt-end ligation. pCMV-Mu $\beta$  was constructed as described previously.<sup>46</sup> Plasmid DNA was amplified in *E. coli* strain *DH5 $\alpha$*  and then isolated

and purified using a Qiagen plasmid giga kit with an EndoFree plasmid maxi kit (Qiagen, Santa Clarita, CA, USA). Lipopolysaccharide (LPS) concentration in the plasmid DNA preparation was measured using a LAL assay kit (Limus F Single Test Wako, Wako; Pure Chemical, Osaka, Japan). The LPS concentration was negligible (less than 22 pg/ $\mu$ g DNA), which corresponds to the manufacturer's information. Purified plasmid DNA was redissolved in sterilized endotoxin-free saline and stored at  $-80^{\circ}\text{C}$  until use. The purity and integrity of the plasmids were determined by absorbance at 260 nm and 280 nm and gel electrophoresis on 1% agarose.

CT-26 (mouse colon tumor), MCA-15 (mouse fibrosarcoma) and MBT-2 (mouse bladder tumor) cells were cultured in RPMI 1640 containing 10% FCS and maintained in a 5%  $\text{CO}_2$  incubator at  $37^{\circ}\text{C}$ .

#### *In vivo direct gene transfer*

For intratumoral gene transfer, MBT-2, MCA-15 ( $2 \times 10^6$  cells, C3H/He), and CT-26 ( $1 \times 10^5$  cells, Balb/c) cells were inoculated into the back of 5-week-old female mice. One to 2 weeks after inoculation, tumors (about 5 mm in diameter) were directly injected with naked pCMV-Luc (in 100  $\mu$ l saline) and its cationic liposome complexes (5  $\mu$ g pCMV-Luc complexed with 25  $\mu$ g DC-chol liposome dissolved in 100  $\mu$ l Opti-MEM) using a 27 G  $\times$   $\frac{3}{4}$  Hamilton syringe. After injection, the needle was embedded in the tumor for 30 s to prevent back-diffusion.

For intramuscular gene transfer, the quadriceps of 5-week-old female Balb/c mice were injected with naked pCMV-Luc and its cationic liposome complexes using the same formulation as for intratumoral injection. Injections were performed longitudinally as described.<sup>33</sup>

#### *Reporter gene assay*

At the indicated days after injection, the entire quadriceps or tumor tissue was excised and weighed. The tissue was minced and then homogenized with 150  $\mu$ l PBS, using an Omni EZ Connect Homogenizer (Omni, Gainesville, VA, USA). The sample was subjected to four cycles of freezing and thawing using alternating liquid nitrogen and  $37^{\circ}\text{C}$  water baths, centrifuged at 13 000  $g$  for 10 min, and the supernatant was transferred to another 1.5 ml tube. One hundred microliters luciferase substrate (PicaGene; Toyo Inki, Tokyo, Japan) was mixed with 10  $\mu$ l tissue extract and the cumulative relative light units (RLU) were measured for 10 s in a luminometer (Lumat LB 9507, EG&G Berthold, Bad Wildbad, Germany). Measurements of RLU were performed over the range where there was linearity between the amount of luciferase (pg) and the RLU value. The final luciferase activity was given as RLU per tissue or RLU per mg tissue.

#### *Secretion of IFNs in tumor tissue after gene transfer*

Naked pCMV-Mu $\gamma$  (100  $\mu$ g in 100  $\mu$ l saline) and its cationic liposome complexes (5  $\mu$ g pCMV-Mu $\gamma$  complexed with 25  $\mu$ g DC-chol liposomes dissolved in 100  $\mu$ l Opti-MEM) were directly injected into tumors twice at 1-h intervals. The treated tumors were subjected to tissue culture and cytokines secreted from the tumors were determined as reported previously.<sup>40</sup> Without standardization for cell number, cytokines secreted from the dissected tumor were successfully detected with relatively small variation in culture supernatants. Briefly, under sterile conditions, tumor tissues (6–8 mm diameter) were

excised 5 h after the second injection, minced and cultured in a 12-well dish with 1 ml medium (10% FCS-RPMI 1640). After incubation in a  $\text{CO}_2$  incubator for 48 h, supernatants were assayed for cytokines. IFN- $\gamma$  and IFN- $\beta$  secreted in the culture medium in this system were stable for at least 48 h (data not shown).

#### *Cytokine assays*

IFN- $\gamma$  and TNF- $\alpha$  yields of supernatant were analyzed using an ELISA kit (Interest- $\gamma$  and Factor-Test-X, respectively; Genzyme, Cambridge, MA, USA) according to the manufacturer's recommendations. The IFN- $\beta$  yield of supernatant was assayed by the antiviral activity, which was measured by the reduction in the cytopathic effect of vesicular stomatitis virus (VSV) on L cells as described.<sup>25</sup> Subtypes of IFNs were determined by combinatorial use of neutralizing monoclonal antibodies against IFN- $\alpha$  (4E-A1),<sup>47</sup> IFN- $\beta$  (7F-D3),<sup>47</sup> and IFN- $\gamma$  (R4-6A2).<sup>48</sup>

#### *Antitumor effect after direct IFN- $\gamma$ gene transfer*

CT-26 s.c. tumors (6–7 mm in diameter) were used for therapy experiments. Naked pCMV-Mu $\gamma$  (100  $\mu$ g) and its cationic liposome complexes (5  $\mu$ g pCMV-Mu $\gamma$  complexed with 25  $\mu$ g DC-chol liposomes dissolved in Opti-MEM) were directly injected into tumors four times at 1-day intervals. After the first treatment, tumor size, assessed by diameter (mm), was regularly measured twice a week. Tumor volume was estimated by determining the longest (a, mm) and shortest (b, mm) diameters of the tumors and calculating the size from the following equation:  $a \times b^2 / 2$  ( $\text{mm}^3$ ).

#### *IFN-producing cell lines and their in vivo growth*

CT-26 cells were transfected with neo plasmid (pcDNA3), pCMV-Mu $\beta$ , or pCMV-Mu $\gamma$  by lipofection, and transfectant cells were selected and cloned in the medium containing G418 (Geneticin; Gibco BRL, Gaithersburg, MD, USA) at 1 mg/ml. Out of several G418-resistant CT-26 sublines obtained, CT-26 (Mu $\beta$ ) 6, CT-26 (Mu $\gamma$ ) 5 and 11, were picked up as IFN- $\beta$ , high- and low-IFN- $\gamma$  producers, respectively. The IFN yield of these producers in the confluence culture fluid were 270 (IFN- $\beta$ ), 52 (IFN- $\gamma$ ) and 13 (IFN- $\gamma$ ) international units (IU)/ml/48 h, respectively. Alternatively, we obtained a G418-resistant CT-26 line, termed CT-26 (*neo*), as a control for gene-transferred cells which showed a comparable tumor growth rate to the parent CT-26 after s.c. inoculation (data not shown). There were no significant differences in growth rate *in vitro* among these gene-transferred and parent CT-26 cells. These producer cells ( $1 \times 10^5$  cells) were subcutaneously inoculated into 5-week-old female Balb/c mice. For the analysis of *in vivo* tumor growth, tumor size, assessed by tumor diameter, was measured at regular intervals. In addition, in order to determine the amount of IFNs produced from these s.c. tumor tissues, they were excised when their formation was confirmed by palpation (about 5 mm in diameter) and subjected to tissue-culture as described above.

#### *Rechallenge of tumor cells against CT-26 rejected mice*

The mice having rejected CT-26(Mu $\gamma$ )5 and CT-26(Mu $\gamma$ )11, or CT-26 cells by *in vivo* IFN- $\gamma$  gene transfer were reinjected s.c. with  $1 \times 10^5$  parent CT-26 cells and the tumor growth was monitored.

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