Electroporation-mediated Interleukin-12 Gene Therapy for Hepatocellular Carcinoma in the Mice Model

Yo-ichi Yamashita, Mitsuo Shimada, Hirofumi Hasegawa, Ryosuke Minagawa, Tatsuya Rikimaru, Takayuki Hamatsu, Shinji Tanaka, Ken Shirabe, Jun-ichi Miyazaki, and Keizo Sugimachi

Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582 [Y. Y., M. S., H. H., T. R., T. H., S. T., K. S., K. S.]; Department of Immunology, Medical Institute of Bioregulation, Fukuoka 812-8582 [R. M.]; and Department of Nutrition and Physiological Chemistry, Osaka University Medical School, Osaka 565-0871 [Y. M.], Japan

ABSTRACT

Applications of nonviral vectors for gene transfer into tumors in vivo have been limited by the relatively low expression levels of the transferred gene. The aim of this study is to evaluate the efficacy of electroporation-mediated interleukin-12 (IL-12) gene therapy for hepatocellular carcinoma (HCC). First, we investigated the optimal conditions of electric pulses (voltage, pulsing duration, numbers of shocks) of in vivo electroporation for gene transfer into HCC established by s.c. implantation of MH134 cells to C3H mice. This process made use of plasmid DNA that express the luciferase gene. We concluded that the optimal conditions for the electric pulses are as follows: voltage at 150 V; pulsing duration at 50 ms; nonpulsing duration at 950 ms; and the number of shocks at 10. Second, we tried to treat s.c. HCC by electroporation using plasmid DNA that expresses the murine interleukin-12 (mIL-12) gene. Intratumor administration of the mIL-12 vector elevated serum IL-12 and IFN-γ and significantly inhibited the growth not only of HCC into which the mIL-12 vector had been directly transferred, but also of the distant HCC. In addition, intratumor administration of the mIL-12 vector inhibited spontaneous lung metastasis and delayed establishment of HCC injected 3 days after mIL-12 gene therapy. The IL-12 gene therapy induced more lymphocyte infiltration by NK cells, CD3+ cells, and Mac-1 positive cells into the tumor and reduced the number of microvessels. Therefore, more terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive tumor cells were found. These results demonstrate that gene therapy for HCC by electroporation in vivo using IL-12 is very efficient and is thus promising for further clinical trial.

INTRODUCTION

Despite advances in cancer therapy for HCC such as recent modifications in chemotherapy and modern surgical innovations, overall patient outcome has not substantially improved. The low rate of success is largely attributable to the high rate of multiplicity and intrahepatic recurrence (1). In addition, most HCCs develop in cirrhotic livers infected with hepatitis virus; therefore, liver dysfunction results in many inoperable cases. For these reasons, potential gene therapies for HCC are worthy of study. Successful gene therapies of HCC in mice models that use the herpes simplex virus thymidine kinase gene followed by ganciclovir have been reported (2, 3). But a new strategy of gene therapy is necessary that would be effective at introducing DNA into various types of cells in vitro (5, 6). Recently, gene transfer by electroporation in vivo was found to be effective at introducing DNA into mouse skin (7), mouse muscle (8), chick embryos (9), hearts of chick embryos (10), rat liver (11), murine melanoma (12), and rat glioblastoma (13). Like other nonviral methods, electroporation has a variety of advantages over viral vectors, because all of the tissues and cells could become targets, in theory. In addition, its handling is easy and quickly completed; no immunogenicity is expected, repeated administration of DNA is possible, no DNA size constraints are imposed, and no specialized process for DNA construction is required. However, electroporation is recognized as having disadvantages in common with other nonviral methods, i.e., gene expression is transient, and gene transfer efficiency is still low in comparison with that of viral vectors. To more closely consider the clinical application of gene therapy for HCC, we used an in vivo electroporation method of gene transfer into HCC. First, we examined the optimal conditions of electric pulses for gene transfer into HCC by luciferase activity to accelerate the efficacy of gene transfer.

IL-12, a bimolecular glycoprotein that consists of a 35,000 and 40,000 subunit, was originally identified as a factor that stimulates natural killer cells (14, 15), promotes maturation of CTLs (16), and induces antiangiogenic effects (17). It has recently been demonstrated that local or systemic treatment with recombinant IL-12 protein mediates profound antitumor effects in vivo, causing regression of established tumors and their distant metastases (18). However, systemic administration of IL-12 protein has caused dose-dependent toxicity in mice (19) and in human trials (20). Alternative approaches, including gene therapy, for the delivery of IL-12 have been pursued. These include injection of genetically engineered fibroblasts for the secretion of IL-12 in an orthotopic melanoma model, direct transfer of IL-12 cDNA via gene gun-mediated transfusion of skin tissue overlaying s.c. melanomas, direct intratumoral injection of adenovirus that expresses IL-12 in breast, colon, and prostate cancer models, and intradermal injection of IL-12 cDNA and vaccination using irradiated IL-12-transfected tumor cells in a metastatic colon cancer model (21–28). These models suggest that IL-12 gene therapy results not only in tumor regression but also in the suppression of its metastases. In the present study, we demonstrated some effects of IL-12 gene therapy on the growth of s.c. HCC into which mIL-12 vector was directly transferred and untreated distant HCC using electroporation, and on its spontaneous lung metastasis in a s.c. HCC model in mice.

MATERIALS AND METHODS

Establishment of s.c. HCC in C3H Mice. MH134, a mouse hepatocellular carcinoma cell line, was induced by carbon tetrachloride in C3H mice. It is

Received 1/3/00; accepted 11/21/00.

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1 To whom requests for reprints should be addressed, at Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Phone: 81-92-642-5469; Fax: 81-92-642-5482; E-mail: yamashita@surg2.med.kyushu-u.ac.jp.

2 The abbreviations used are: HCC, hepatocellular carcinoma; IL-12, interleukin 12; mIL-12, murine interleukin-12; LU, light units; MVDopt, mean microvessel density; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; TIL, tumor-infiltrating lymphocytes; FACSTM, fluorescence-activated cell sorter.
moderately differentiated and grows in syngeneic recipients in both solid and ascitic forms (29, 30). Six-week-old female C3H/HEJ or C57BL/6J mice, which were purchased from Charles River Japan, Inc. (Tokyo, Japan), were used throughout this study. The mice were maintained under specific pathogen-free conditions in the animal facility at the Kyushu University Medical School. Under satisfactory diethyl ether anesthesia, the mice were challenged s.c. in the right or bilateral flank with 0.5 ml of a single-cell suspension containing 2.5 × 10^6 MH134 cells. About 7 days after tumor cell inoculation, s.c. HCCs were established. The Kyushu University Institutional Animal Care and Use Committee approved all of the animal protocols, which were designed according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy for Sciences and published by the NIH.

Plasmid DNA. The luciferase gene was isolated from the pGL2 promoter vector (Promega, Madison, WI) by HindIII and BamHI digestion. pcLuc and pActLuc were constructed by cloning the luciferase gene at HindIII and BamHI sites of pcDNA3. Mouse IL-12 expression plasmid, designated pCAGGS-mIL-12, was constructed as follows: Both mouse IL-12 p35 and p40 cDNAs were inserted into the EcoRI site of pCAGGS expression vector (31), resulting in pCAGGS-p35 and pCAGGS-p40, respectively. The expression unit for IL-12 p35 included the cytomegalovirus immediate early enhancer-chicken β-actin hybrid promoter. A rabbit β-globulin polyclonal signal was excised from pCAGGS-p35 and was inserted downstream of the IL-12 p40 expression unit of pCAGGS-p40.

Intratumoral DNA Injection and Electroporation. Mice were anesthetized with pentobarbital sodium. Established s.c. HCC, the volume of which reached about 0.5 cm³, was injected with 100 μg of closed circular DNA (pcDNA3Luc, control pCAGGS, and pCAGGS-mIL-12) at 1.0 μg/μl in saline using an insulin syringe with a 27-gauge needle. A pair of electrode needles was inserted into the tumor with a distance of 5 mm to encompass the DNA injection sites, and electric pulses were delivered using an electric pulse generator (CUTY-21; BEX Co., Ltd., Tokyo, Japan). The shape of the pulse was a square wave, i.e., the voltage remained constant for the duration of the pulse. Electrodes consisted of a pair of tungsten needles of 10 mm in length and 0.4 mm diameter. Pulses, at the rate of one pulse/s, of the opposite polarity were administered to each DNA injection site.

Luciferase Activity. Three days after pDNA encoding luciferase was transferred into HCC by electroporation under various conditions, HCC was dissolved. Twenty mIL-12 each tumor at five random areas was calculated using an image analyzer (MAC/BERTHOLD, Tokyo, Japan). Total protein content for each sample was determined by the Bradford method using BSA as a standard, and absorbencies were normalized to 100 μg BSA protein.

ELISA of mL-12 and mIFN-γ. Serum samples obtained from the tail vein of mice were assayed for total mL-12 (Genzyme, Cambridge, MA) and mIFN-γ (Biosource International, Camarillo, CA) using an ELISA kit, according to the manufacturer’s instructions.

Morphological and Histopathological Analysis of HCC. In the bilateral s.c. HCC model, control pCAGGS (control group; n = 5) or pCAGGS-mIL-12 (IL-12 gene therapy group; n = 8) was transferred only to the right s.c. HCC. For a period of 28 days after gene therapy, tumor volume was calculated according to the following formula: V = A × B²/2 (cm³), where A is the largest diameter (cm), and B is the smallest diameter (cm). Fourteen days after gene therapy, another five sections of the bilateral s.c. HCC in both groups were resected and cut in the middle at the site of the original tumor inoculation. The tissue was then fixed in 10% buffered formalin and stained with H&E for histopathological analysis. The ratio of the viable area of MH134 cells in each tumor at five random areas was calculated using an image analyzer (MAC SCOP, Nagano, Japan), and the overall effect of IL-12 gene therapy was evaluated.

For CD 31 immunocytochemistry, paraffin-embedded sections that were 3 μm in thickness of bilateral s.c. HCC in both groups were incubated with 0.1% trypsin in water for 10–15 min at 37°C, then incubated overnight at 4°C with 0.5 μg/ml rat monoclonal antisemum against mouse CD31 (PharMingen, San Diego, CA). Primary antibody was detected with streptavidin-biotin using a Vectastain kit, according to the manufacturer’s instructions (Vector Laboratories, Burlingame, CA). CD31-positive microvessels within or surrounding the HCC were counted. Because microvessels are distributed heterogeneously throughout the tumor, the sections were examined at low magnifications (×40 and ×100) to identify the greatest vascular area of the tumor (hot spots), according to the method of Weidner et al. (32, 33). Within these areas, a minimum of 10 fields at ×400 magnification (total area 1.56 mm²) was examined. The average of all of the fields examined within the hot spot area (MVDmean) was then recorded and expressed as counts/mm².

TUNEL staining was performed using an Apop Taq in situ apoptosis detection kit (Oncore Inc., Gaithersburg, MD). The sections of bilateral s.c. HCC in both groups were deparaffin and digested in 20 μg/ml proteinase K (Nakarai Tasque, Inc., Kyoto, Japan) for 30 min at room temperature. Then, TUNEL staining was carried out using the working strength terminal deoxynucleotidyl transferase reaction mixture, working strength stop/wash buffer, and antidigoxigenin-dUTP were applied repeatedly. After staining with diaminobenzidine, the slides were counterstained with hematoxylin, dehydrated, and mounted. An apoptosis index (percentage) was calculated for each of the five samples at five random areas as the proportion of TUNEL-positive MH134 cells with respect to the total number of MH134 cells evaluated multiplied by 100. Only nonnecrotic MH134 cells were quantified to determine the apoptotic index. Approximately 2000 MH134 cells were evaluated for each sample.

Flow Cytometric Analysis of TILs. The TILs from the mice with bilateral s.c. HCC at the right flank into which pCAGGS-mIL-12 had been transferred or into which pCAGGS had been transferred at 14 days after gene therapy were processed as described earlier (34). In brief, the specimens were minced with scalpels into pieces smaller than 1 mm³ and underwent enzymatic treatment for 2 h. The cell suspensions were centrifuged on differential Ficoll-Hypaque gradients (Lymphocyte Separation Medium, ICN Biomedical Inc., Cleveland, OH; Ref. 35), and the TILs were collected from the lower interface, washed with HBSS three times, and checked for viability and cell counts with the aid of trypan blue dye. FACS analysis was performed on a FACS Caliber (Becton Dickinson, San Jose, CA). On the basis of staining with various leukocyte-specific antibodies and forward- and side-scatter analyses, CD45 positive TILs could be gated into different groups of infiltrating cell populations. To block the nonspecific FcγR binding of labeled antibodies, 10 μl of undiluted culture supernatant of 2.4G2 (rat antimeouse FcγR monoclonal antibody) was added to the first incubation. All of the antibodies used in this study were obtained from PharMingen. The biotin-conjugated anti-CD45 antibody 30F11.1 (IgG2b) was used, and streptavidin alophylocyanin was used for biotin labeling. Phycoerythrin-labeled anti-CD3ε antibody 145–2C11 (Armenian hamster IgG), anti-CD4 antibody H129.19 (IgG2a), anti-CD8 antibody 53–6.7 (IgG2a), anti-NK1.1 antibody PK136 (mouse IgG2a), and anti-CD 11C antibody HL3 (Armenian hamster IgGα) were used, as were FITC-labeled anti-CD3ε antibody 145–2C11 (Armenian hamster IgG), anti-B220 antibody (IgG2κ), and anti-Mac-1 antibody M1/70.15 (IgG2b). Propidium iodide staining was used to exclude dead cells.

Assay System for CTL Responses. In vitro sensitization and cytotoxicity assay were essentially the same as described previously (36). In brief, 5 × 10⁶ splenic cells from the mice with s.c. or i.t. pCAGGS-mIL-12 had been transferred or into which pCAGGS had been transferred at 7 days after gene therapy were sensitized in vitro to 1 × 10⁶ mitomycin C-treated tumor cells in a 2-ml volume in 24-well culture plates. Effector cells generated 5 days after culturing were assayed on the corresponding tumor target cells by ⁵¹Cr release assay. The percentage of specific lysis was calculated as described previously (37), and SE were excluded from the data for the sake of simplicity because they were consistently <5%. X5563 plasmacytoma derived from C3H strain was used to confirm the tumor specificity of cytotoxicity.

Distant Site Challenge with Parental Tumor Cells. Mice with s.c. HCC at the right flank into which pCAGGS-mIL-12 or pCAGGS had been transferred were challenged with a tumorigenic dose of MH134 parental tumor cells (2.5 × 10⁶ cells/mouse) that were implanted into the opposite flank at 3 days after intratumoral pDNA transfer. Tumor formation was macroscopically monitored; we defined the day of “distant tumor formation” as the day upon which the tumor volume reached 0.5 cm³.

Spontaneous Lung Metastasis. A few mice bearing right s.c. HCC 14 days after gene therapy were anesthetized with diethyl ether, and the tumor was removed through a 10-mm skin incision and careful dissection. A few mice
that eventually displayed local tumor relapse were excluded from the experiment. After 28 days (14 days after s.c. HCC resection), lungs were removed bilaterally. Tumor formation was macroscopically monitored. For further evaluation of spontaneous lung metastasis, the tissue was then fixed in 10% buffered formalin and stained with H&E.

**Statistical Analysis.** Statistical evaluations of numerical variables in both groups were performed using the Mann-Whitney U test. Differences in the qualitative variables were performed using Fisher’s exact probability test. Differences in tumor growth were statistically analyzed using the repeated measures ANOVA test. Differences in time to tumor development challenged at the distant site were statistically analyzed using the Breslow modification of the Wilcoxon test. Significance was defined as $P < 0.05$.

**RESULTS**

**Optimal Conditions of Electroporation for HCC in Vivo Luciferase Activity.** According to a previous report regarding electroporation gene transfer into rat liver (11), we set up the standard of electroporation for HCC as eight electric pulses (1 time/s) of 50-ms duration at 50 V. No detectable levels of luciferase activity were observed in s.c. HCC when the plasmid was directly injected.

The optimal voltage level of the electric pulses was determined when other conditions were fixed as follows: eight electric pulses, 50 ms in duration (Fig. 1A). The results indicate an increase in mean luciferase activity with an increase in voltage up to 150 V. HCC that was subjected to 50 V expressed a mean luciferase activity level of $5.2 \pm 3.8 \times 10^3$ LU/μg BSA, which increased to $7.0 \pm 1.0 \times 10^5$ LU/μg BSA in HCC that was subjected to 150 V ($P < 0.01$; mean ± SE). However, the mean luciferase activity in HCC that was subjected to 200 V decreased to $1.1 \pm 0.25 \times 10^5$ LU/μg BSA ($P < 0.01$).

The optimal duration of the pulses was determined when other conditions were fixed as follows: eight electric pulses at 150 V (Fig. 1B). The results indicate an increase in mean luciferase activity with an increase in duration up to 50 ms. But when the duration of the pulse exceeded 50 ms, the mean luciferase level decreased. A statistical difference was seen between luciferase levels of HCCs that were subjected to 50 ms ($7.0 \pm 1.0 \times 10^5$ LU/μg BSA) and those subjected to 70 ms ($2.4 \pm 0.6 \times 10^5$ LU/μg BSA; $P = 0.02$).

The optimal number of shocks was determined when other conditions were fixed as follows: 50 ms in duration at 150 V (Fig. 1C). The results indicate an increase in mean luciferase activity that occurred with an increase in the number of shocks up to 10. No statistical difference was seen between HCCs that were subjected to 8 shocks ($7.0 \pm 1.0 \times 10^5$ LU/μg BSA) and those subjected to 10 shocks ($8.0 \pm 0.8 \times 10^5$ LU/μg BSA).

The optimal condition for electroporation gene transfer was shown to occur at 150 V, 50 ms in duration, and 10 shocks.

**Time Course of mIL-12 and mIFN-γ.** The serum mIL-12 level increased gradually after pCAGGS-mIL-12 transfer into HCC and peaked 5 days after electroporation ($4.4 \pm 0.4$ ng/ml). Thereafter, serum mIL-12 levels decreased gradually to approximately 57% of the maximum value ($2.5 \pm 0.4$ ng/ml) by 1 month after electroporation (Fig. 2A). The serum mIFN-γ level also increased gradually and peaked 7 days after electroporation (10.16 ± 4.2 pg/ml). This represented a few days of delay, as compared with the elevation of serum mIL-12 (Fig. 2B). On the other hand, in the mice with HCC-transferred pCAGGC, no elevation of mIL-12 was found, and mIFN-γ was not detectable.

**Morphological and Histopathological Analysis.** The growth of the HCC-transferred pCAGGS-mIL-12 by electroporation in vivo was significantly inhibited, as compared with HCC-transferred control pCAGGS ($P < 0.01$; ANOVA; Fig. 3). After 14 days, the volume of HCC-transferred control pCAGGS rapidly increased to more than 35-fold that of the amount on the day when the gene was transferred. However, the increase in volume of HCC-transferred pCAGGS-mIL-12 reduced to about 5-fold that amount. Suppressed growth was also recognized in the untreated distant left flank HCC that was inoculated and established at the same time. This finding was statistically significant when compared with that of the distant HCC in the control group ($P < 0.01$; ANOVA; Fig. 3).

Fourteen days after treatment, histopathological analysis of s.c.
HCC stained with H&E revealed that the distribution of viable MH134 cells not only in HCC-transferred pCAGGS-mIL-12 (25.2 ± 2.7%) but also in distant HCC at the opposite site (48.7 ± 3.1%) were reduced in comparison with that of the control group (93.7 ± 1.1%; P < 0.01; mean ± SE; Fig. 5A, B, and C).

The apoptosis index evaluated by TUNEL staining of the HCC sections revealed that more apoptotic MH134 cells were found in the pCAGGS-mIL-12-transferred HCC (2.42 ± 0.1%) and in the distant HCC at the opposite site (1.72 ± 0.3%) than those that were found in the control group (0.12 ± 0.1%; P < 0.01; mean ± SE; Fig. 6A, B, and C).

**Flow Cytometric Analysis of TILS.** Table 1 shows that more tumor-infiltrating NK cells, CD3⁺ T cells, and Mac-1 positive cells in the pCAGGS-mIL-12-transferred HCC (21.2/cm³-tumor, 14.5/cm³-tumor, and 20.3/cm³-tumor) and in the distant HCC at the opposite site (8.9/cm³-tumor, 7.8/cm³-tumor, and 10.1/cm³-tumor), respectively, were found when compared with the pCAGGS-transferred HCC (0.8/cm³-tumor, 1.0/cm³-tumor, and 3.4/cm³-tumor). The in-
creases of NK cells (IL-12: IL-12 distant: pCAGGS, 26.5: 11.1: 1) and CD3^+ T cells (IL-12: IL-12 distant: pCAGGS, 14.5: 7.8: 1) were dominant. A CD3^+ T-cell increase was found in both the CD8^+ T-cell population and in the CD4^+ T-cell population.

**Assay System for CTL Responses.** Effector cell activities that were generated by spleen cells from mice with s.c. pCAGGS-transferred HCC were marginal. However, spleen cells from mice with s.c. pCAGGS-miIL-12-transferred HCC resulted in potent cytotoxic responses (Fig. 7). Additional experiments revealed that such augmented cytotoxic responses are tumor-specific (Table 2).

**Distant Site Challenge with Parental MH134 Cells.** All of the mice with s.c. HCC-transferred pCAGGS implanted with a tumorigenic dose of MH134 cells to a distant site developed s.c. HCC within 14 days. On the other hand, no mouse with s.c. HCC-transferred pCAGGS-miIL-12 implanted with the same dose of MH134 cells to a distant site developed a s.c. HCC within 14 days. The remaining mice with s.c. HCC-transferred pCAGGS-miIL-12 implanted with MH134 cells to a distant site developed a s.c. HCC on either day 18 (one mouse; 12.5%) or on day 24 (three mice; 37.5%). Furthermore, throughout the study, four mice (50%) did not develop distant HCC, which had been delayed, implanted at the opposite site. Thus, mice with s.c. HCC-transferred pCAGGS-miIL-12 demonstrated a significantly delayed development of HCC implanted at the opposite site.

<table>
<thead>
<tr>
<th>CD3^+ T cells</th>
<th>IL-12 (× 10^3/cm²)</th>
<th>IL-12 distant (× 10^3/cm²)</th>
<th>pCAGGS (× 10^3/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B220^+ B cells</td>
<td>4.5</td>
<td>3.4</td>
<td>0.9</td>
</tr>
<tr>
<td>NK1.1^+ CD3^+ NK cells</td>
<td>21.2</td>
<td>8.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Mac-1^+ cells</td>
<td>20.3</td>
<td>10.1</td>
<td>3.4</td>
</tr>
<tr>
<td>CD4^+ T cells</td>
<td>4.8</td>
<td>1.6</td>
<td>0.2</td>
</tr>
<tr>
<td>CD8^+ T cells</td>
<td>4.8</td>
<td>1.6</td>
<td>0.3</td>
</tr>
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^a Data represented have been confirmed in three independent experiments.
In a study of gene transfer into mouse testis, Muramatsu et al. (40) also reported that decreasing the voltage from 100 V to 25 V resulted in a remarkable reduction of chloramphenicol acetyltransferase activity, whereas increasing the time constant from 10 to 50 ms at the same 25 V, chloramphenicol acetyltransferase activity recovered almost to the level of activity observed at 100 V with 10 ms of exposure. However, in our study, decreasing the voltage from 150 to 100 or to 50 and concomitantly increasing the pulse duration or the number of shocks showed that luciferase activity in HCC could not recover to that found at 150 V (data not shown). One of the main reasons for this phenomenon would be that the cell density of the established HCC in vivo would be higher than that of other normal solid organs such as the liver. Furthermore, resistance to electric pulses was increased (HCC, 1.62 ± 0.09 kfd; liver, 0.42 ± 0.04 kfd; n = 8; P < 0.01) in our mouse model. Hence, the voltage necessary for the poration of cell membranes would be higher than 150 V. We examined the effects of different electrode voltage, time constants, and number of shocks, but we fixed other parameters, such as frequency of pulse (one time/s), DNA concentration (1 µg/µl), volume of injection fluid (100 µl), and solution type (0.85% NaCl; Ref. 41, 42). By optimizing these parameters, the efficiency of gene transfer might be improved.

IL-12 has been shown to enhance the cytolytic activity of NK cells and CTLs. IL-12 is also a very strong inducer of IFN-γ and appears to be a major determinant in the development of the Th1 immune response (43). Although previous studies (18, 19) on IL-12 protein therapy have generally shown that IL-12 is highly efficacious against a wide range of murine tumors, the present report is the first to describe the efficiency of IL-12 against this MH134 murine tumor model. When compared with IL-12, another well-known cytokine, IL-12 protein was found to be more effective and yet less toxic (44, 45). In similar studies, IL-12 also appeared to be more potent than IFN-γ (46). However, in the first clinical trial, IL-12 protein therapy resulted in the death of 2 patients and led to severe toxic effects in 15 others (20, 47). The absence of predosing was suggested to be a cause of this toxicity (47). The present study also tested IL-12 gene therapy strategy with the capacity to deliver easily regulated levels of transgenic protein at the site of the tumor. Previous studies (24) using an ex vivo gene transfer approach showed that murine tumor cells transduced by retroviral vectors containing an IL-12 cDNA were able to vaccinate mice successfully against tumor challenges. Moreover, injection of murine fibroblasts that were transfected with an IL-12 cDNA expression vector at the site of an intradermal tumor resulted in tumor regression (25). By using the direct transfer of IL-12 cDNA into skin or tumors, local IL-12 gene therapy resulted in the eradication of established murine tumors and their metastases, leading to the generation of tumor-specific immunological memory (23, 24, 27, 28).

In the present study, it was shown that administration of mIL-12 cDNA into HCC mediated by the electroporation method significantly inhibited the growth of not only mIL-12 directly transferred HCC but also the growth of untreated distant HCC. In addition, spontaneous lung metastasis was inhibited, and the establishment of HCC injected 3 days after IL-12 therapy was inhibited or delayed. These findings support the results of previous reports. Elevations of IL-12 and IFN-γ serum levels were observed in our model. None of the mice subjected to IL-12 gene therapy died during the experiments. In a recent report, systemic elevation of IL-12 was shown to be more important than local IL-12 elevation in the cure of established tumors and spontaneous metastasis.

### DISCUSSION

To our knowledge, this is the first report of electroporation-mediated IL-12 gene therapy or IL-12 gene therapy for HCC. The efficiency of adenovirus-mediated IL-2 gene therapy caused by the infiltration of CD4+ or CD8+ T cells and natural killer cells into MH134 liver tumor has been reported previously (38), but this is the first report of the efficiency of IL-12 or IL-12 gene therapy for this MH134 tumor model.

In in vivo electroporation, square pulses should be used, because they are superior in inducing a higher level of gene expression than are exponential decay pulses. Unless the tissues or cells are irreversibly damaged, gene transfection efficiency is roughly proportional to the amount of heat generated (39). In the present study, optimal conditions for gene transfer into HCC appear to support this hypothesis, at least in part. Increasing the voltage to more than 150 V, the time constant to more than 50 ms, or the number of shocks to more than 10 resulted in a decrease of luciferase activity transferred into the HCC. In a study of gene transfer into mouse testis, Muramatsu et al. (40) also reported that decreasing the voltage from 100 V to 25 V resulted in a remarkable reduction of chloramphenicol acetyltransferase activity, whereas increasing the time constant from 10 to 50 ms at the same 25 V, chloramphenicol acetyltransferase activity recovered almost to the level of activity observed at 100 V with 10 ms of exposure. However, in our study, decreasing the voltage from 150 to 100 or to 50 and concomitantly increasing the pulse duration or the number of shocks showed that luciferase activity in HCC could not recover to that found at 150 V (data not shown). One of the main reasons for this phenomenon would be that the cell density of the established HCC in vivo would be higher than that of other normal solid organs such as the liver. Furthermore, resistance to electric pulses was increased (HCC, 1.62 ± 0.09 kfd; liver, 0.42 ± 0.04 kfd; n = 8; P < 0.01) in our mouse model. Hence, the voltage necessary for the poration of cell membranes would be higher than 150 V. We examined the effects of different electrode voltage, time constants, and number of shocks, but we fixed other parameters, such as frequency of pulse (one time/s), DNA concentration (1 µg/µl), volume of injection fluid (100 µl), and solution type (0.85% NaCl; Ref. 41, 42). By optimizing these parameters, the efficiency of gene transfer might be improved.

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### Table 2 Specificity of in vitro cytotoxic activity of effector cells (splenocytes) induced by the IL-12 gene therapy

<table>
<thead>
<tr>
<th>Target cell type</th>
<th>% specific lysis at effector:target ratio of 80:1</th>
<th>% specific lysis at effector:target ratio of 10:1</th>
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<td>MH 134</td>
<td>43.1</td>
<td>21.1</td>
</tr>
<tr>
<td>X 5563</td>
<td>12.8</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Fig. 7. Assay system for CTL response. In vitro lysis activity of lymphocytes (effector cells) from mice with s.c. HCC-transferred pCAGGS-mIL-12, from mice with s.c. HCC-transferred pCAGGS (mock), and from mice without tumor inoculation (naive) against MH134 cells (target cells). Effector cells generated by spleen cells from mice with s.c. pCAGGS-mIL-12-transferred HCC resulted in potent cytotoxic responses.

Fig. 8. Delayed establishment of s.c. HCC. Mice with s.c. HCC-transferred pCAGGS-mIL-12 showed a significantly delayed emergence of the HCC implanted at the opposite site 3 days after gene therapy, as compared with mice with s.c. HCC-transferred pCAGGS (P < 0.01).
ous metastases (48). Thus, the gradual elevation of systemic IL-12 by means of intratumoral gene transfer by electroporation would be an ideal model for IL-12 cancer therapy. According to histological analysis by H&E staining, more lymphocytic infiltration and a smaller distribution of viable cancer cells were observed not only in the section of pCAGGS-mIL-12-transferred HCC but also in the distant HCC, as compared with those of the control group. In this mice model, the acceleration of the infiltration of lymphocytes into the distant tumor in the IL-12 gene therapy group was because of the systemic mIL-12 elevation and the activation of the immune system. The lymphocytic infiltration of NK cells, CD3+ T cells (CD8+ T cells and CD4+ T cells), and Mac-1 positive cells revealed by FACS analysis of TILs was considered to induce HCC apoptosis. Thus, more TUNEL-positive cells were found in the pCAGGS-mIL-12-transferred HCC and the distant HCC than were found in the control group. Despite the scattered pattern of TUNEL-positive cells that could not explain the massive area of necrosis revealed by H&E staining, the antitumor effect of IL-12 gene therapy for this MH134 mice model was considered to depend largely or partly on the antiangiogenic effect revealed by the analysis of MVDmean. Because of local and systemic elevation of IL-12 and IFN-γ, which had antiangiogenic effects, fewer microvessels stained with antimouse CD31 antibody were found in the sections of HCC-transferred pCAGGS-mIL-12 and distant HCC than were found in the control group. Furthermore, the suppression of the establishment of s.c. HCC of the delayed injection on mice with pCAGGS-mIL-12-transferred HCC was recognized. This result was possibly attributable to the activation of macrophages, NK cells, and CTLs by systemic IL-12. In addition, the ratio of the formation of lung metastasis 14 days after resection of the pCAGGS-mIL-12-transferred HCC was smaller than that of the pCAGGS-transfected HCC. This phenomenon might be explained by the fact that the low number of microvessels rendered the MH134 cells more difficult to circulate from the primary HCC-transferred pCAGGS-mIL-12. In addition, it would be more difficult to attack and enlarge a tumor in the lung because of the acceleration of antitumor immunity of macrophages, NK cells, and CTLs with the elevation of systemic IL-12 and IFN-γ. But further experimentation is needed to determine the dominant mechanism of the antitumor effect of IL-12 gene therapy in this MH134 tumor model using C3H mice: the activation of the immune system or the antiangiogenic effect.

Because this is the first report of IL-12 gene therapy for HCC and the first use of this MH134 murine tumor model, the comparison of efficiency of the gene transfer and gene therapy against the previously established methods such as viral vectors is impossible. Serious concerns have been voiced about the use of viral vectors, especially when clinical trials are involved. To more closely consider the clinical application of gene therapy for HCC, the in vivo electroporation method of gene transfer has more critical advantages over viral vectors. However, electroporation has certain disadvantages in common with the viral vector method, i.e., gene transfer efficiency is still low, and gene expression is transient. Harrison et al. reported that in vivo electroporation was approximately equivalent to an adenovirus dose of 10⁸ transduction units but was less effective than a 10⁹ dosage evaluated by the percentage of heart tissue of chick embryos expressing green fluorescent protein (GFP) (10). But the efficiency of gene transfer mediated by electroporation should be accelerated by the examination of the optimal conditions of electric pulses, the type of electrode, and the plasmid concentration. In a recent report, Goto et al. (49) described the efficiency of repeated electro-gene therapy for s.c. tumors using herpes simplex virus thymidine kinase gene, and they reported that low efficiency of gene transfer should be dissolved by repeated use. But additional experiments are required regarding the comparison between electroporation-mediated IL-12 gene therapy and viral vectors against this MH134 tumor model.

In summary, electroporation-mediated IL-12 gene therapy for HCC was directly effective for mIL-12-transferred HCC but was also effective for distant HCC. Furthermore, the present study showed significant inhibition of lung metastasis and delayed establishment of HCC. Therefore, our data suggest that in vivo electroporation-mediated IL-12 gene therapy for HCC may be a promising treatment modality for advanced HCC with intrahepatic metastases in humans.

ACKNOWLEDGMENTS

We thank Dr. Ken-ichi Taguchi and Dr. Shin-ichi Aishima in the Second Department of Pathology, Kyushu University, for their critical comments and advice on the pathological evaluation in this study.

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Electroporation-mediated *Interleukin-12* Gene Therapy for Hepatocellular Carcinoma in the Mice Model


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