Gene therapy with TRAIL against renal cell carcinoma

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Abstract

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) induces apoptosis in cancer cells. However, TRAIL is not toxic against most normal cells. We have accordingly examined by *in vivo* electroporation whether TRAIL induces apoptosis in renal cell carcinoma. In addition, combination treatment with TRAIL and 5-fluorouracil (5-FU) against renal cell carcinoma was also investigated. The NC65 renal cell carcinoma line was used as a target. pCAGGS *TRAIL* was injected into the NC65 tumors in the right flanks of severe combined immunodeficient mice. Tumors were pulsed with the CUY21 electroporator. Electroporation was done once on day 0 or thrice on days 0, 2, and 4. Apoptosis was determined by terminal deoxyribonucleotide transferase–mediated nick-end labeling assay. When *TRAIL* gene therapy using *in vivo* i.t. electroporation was done once only, the growth of NC65 tumors was not inhibited. However, when *TRAIL* gene therapy was done thrice, growth suppression of the NC65 tumors was observed. Transfection of the *TRAIL* gene by *in vivo* electroporation induced apoptosis in NC65 tumors. When NC65 cells were treated with *TRAIL* gene therapy in combination with 5-FU, stronger growth suppression was obtained. *TRAIL* gene therapy did not induce liver dysfunction in severe combined immunodeficient mice. This study shows that *TRAIL* gene therapy induced growth suppression and apoptosis in NC65 tumors without severe side effects, and that combination treatment of NC65 cells with *TRAIL* gene therapy and 5-FU resulted in higher antitumor activity. These findings suggest that *TRAIL* gene therapy and/or 5-FU may be effective against renal cell carcinoma without harmful toxic effects. [Mol Cancer Ther 2006;5(9):2165–71]

Introduction

There are only a few effective therapies against metastatic renal cell carcinoma. Immunotherapy including IFNs against metastatic renal cell carcinoma is relatively effective (1–4). Many investigators have tried to enhance effectiveness of IFNs by combination treatment with other biological agents or chemotherapeutic agents (3, 4). However, these response rates were ~15% to 20% (1–4). Furthermore, systemic side effects and high cost have limited the clinical validity of immunotherapy using recombinant cytokine proteins. The development of new strategies for patients with metastatic renal cell carcinoma is therefore much to be desired.

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) belongs to the TNF family (5), and the corresponding cellular receptors have been identified (6). TNF-α is the first molecule in the TNF family to be tested for its anticancer activity, followed by Fas ligand. These two molecules are efficient in killing a variety of cancer cells. However, they cause significant damage to normal tissues, resulting in life-threatening toxicities (7, 8). TRAIL has been shown to selectively induce apoptosis in cancer cells and has minimal or no toxicity against normal tissues, as examined both *in vitro* and *in vivo* in mice (9, 10). Therefore, TRAIL may be effective *in vivo* as an anticancer agent, provided the cancer cells are sensitive to it. Unfortunately, most cancer cells, including renal cell carcinoma, are not sensitive to TRAIL-mediated apoptosis.

Electroporation was originally developed for taking anticancer agents into tumor cells. It is also a well-established laboratory technique for the transfection of genes *in vitro* for floating and adherent cells (11). *In vivo* electroporation is also an efficient method of gene transfer (12). Thus far, this technique has been widely applied to various tissues. For example, the cytotoxic effects on renal cell carcinoma of the combinations of anticancer drugs with electrical impulses were investigated both *in vitro* and *in vivo* (13). The efficacy of electroporation-mediated interleukin-12 gene therapy for hepatocellular carcinoma was studied (14).

Several anticancer chemotherapeutic drugs, as well as TRAIL, mediate apoptosis and may share common intracellular signaling pathway leading to apoptosis. We have reasoned that cancer cells that are resistant to TRAIL/drugs can be sensitized by combined treatment with TRAIL and anticancer drugs. Indeed, several studies tested the hypothesis and corroborated the drug-mediated sensitization of resistant cancer cells to TRAIL (15, 16). This study has investigated *in vivo* whether renal cell carcinoma is sensitive to *TRAIL* gene therapy, and whether the

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resistance of renal cell carcinoma to *TRAIL* gene therapy/5-fluorouracil (5-FU) can be overcome by a combination of *TRAIL* gene therapy and 5-FU.

Materials and Methods

Tumor Cells

The NC65 human renal cell carcinoma line was used as a target. The cell line was maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin (Life Technologies), and 10% fetal bovine serum (Life Technologies, Inc., Bio-cult, Glasgow, Scotland, United Kingdom).

Reagents

5-FU was supplied by Kyowa Hakkou Co., Ltd. (Tokyo, Japan).

Plasmid

Human *TRAIL* cDNA was obtained by PCR with the use of a spleen cDNA library (Clontech, Palo Alto, CA). Amplified products were cloned into expression plasmid pCAGGS NEO containing the CAG promoter, forming pCAGGS NEO-*TRAIL*, and subjected to analysis to verify its sequence. The plasmids used in this study were purified with a Qiagen (Valencia, CA) plasmid Maxi kit. The amounts of endotoxin in purified plasmids were assayed by the *Limulus* test (Wako Pure Chemical Industries, Osaka, Japan). The plasmids contained endotoxin at <3.0 EU/μg.

Animal Care

Female severe combined immunodeficient (SCID) mice, ages 8 to 9 weeks, were purchased from CLEA Japan (Osaka, Japan) and housed in a specific pathogen-free animal facility. The animals were fed irradiated mouse chow and autoclaved reverse osmosis–treated water. The Committee for Animal Research in Kyoto Prefectural University of Medicine permitted the experimental procedure.

In vitro Electroporation Protocol and Cytotoxicity Assay

The target was the NC65 cell line. Electric pulses were delivered with an electric pulse generator (CUY-21; Bex, Tokyo, Japan). The electrodes consisted of tungsten needles, 4 mm in gap and 32 mm in diameter (CUY-510; Bex). The conditions for electroporation were as follows: 100 V, 10 ms on, 90 ms off, 10 times. The 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine tumor cell lysis as previously described (17, 18). Briefly, 100 μL of the target cell suspension (2×10^4) were added to each well of 96-well flat-bottomed microtiter plates (Corning Glass Works, Corning, NY), and each plate was incubated for 24 hours at 37°C in a humidified 5% CO_2 atmosphere. After incubation, 100 μL of drug solution or complete medium for control were distributed in the 96-well plates and each plate was incubated for 72 hours at 37°C. Following incubation, 20 μL MTT working solution (5 mg/mL; Sigma Chemical Co., St. Louis, MO) were added to each culture well and the cultures were incubated for 4 hours at 37°C in a humidified 5% CO_2 atmosphere. The culture medium was removed from the wells and replaced with 100 μL isopropanol (Sigma Chemical) supplemented with 0.05 N HCl. The absorbance of each well was measured with a microculture plate reader (Immunoreader, Japan Intermed Co., Ltd., Tokyo, Japan) at 540 nm. The percentage cytotoxicity was calculated by following formula: percentage cytotoxicity = [1 − (absorbance of experimental wells / absorbance of control wells)] × 100.

In vivo Study

Into the right flanks of the SCID mice, 6 million NC65 cells were s.c. injected with a mixture of 50 μL RPMI 1640 without antibiotics and serum, and 50 μL Matrigel (Becton Dickinson, Franklin Lakes, NJ). At 7 days following injection of the tumor cells, the maximum lengths of the NC65 tumors had developed to 5 to 7 mm in diameter.

Tumor sizes were measured at the indicated days after initial treatment. Tumor diameters were scaled with a digital scalper. Tumor volume was calculated as follows: volume = $a \times b^2 / 2$, where a is the long diameter and b is the short diameter.

I.t. or I.m. DNA Injection and Electroporation

Mice were anesthetized with pentobarbital sodium. Established s.c. NC65 tumors were injected with 50 μL saline, pCAGGS NEO (50 μL), pCAGGS *TRAIL* (50 μL), and/or 10 μmol/L/50 μL of 5-FU. Electrode needles were injected around the tumor, and electric pulses were delivered with an electric pulse generator (CUY-21; Bex). The shape of the pulse was a square wave. The electrodes consisted of tungsten needles, 10 mm in length and 0.4 mm in diameter. The conditions for electroporation were as follows: 33 V, 50 ms on, 950 ms off, eight times. Injections and electroporation were done once (day 0) or thrice (days 0, 2, and 4). In another experiment, 50 μL saline, pCAGGS NEO, and pCAGGS *TRAIL* was injected into thigh muscle six times (days 0, 2, 4, 6, 8, and 10).

Luciferase Assay

Tumors were homogenized in 200 μL reporter lysis buffer using a sonicator. After freezing and thawing twice, the extract was centrifuged at 10,000 rpm for 10 minutes. Luciferase activity in the supernatant was measured using a Luciferase assay kit (Promega, Madison, WI) according to the protocol of the manufacturer. Photoemission was measured during a 10-second period using a luminometer. Protein concentration in the supernatant was measured as described (19).

Evaluation of Liver Function

To evaluate the hepatotoxicity of *TRAIL* gene therapy in this mouse model, serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) were measured on day 7.

Apoptosis Detection by Terminal Deoxyribonucleotide Transferase–Mediated Nick-End Labeling Staining

For terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL) staining, the NC65 tumors were fixed in 4% paraformaldehyde at 4°C for 24 hours, embedded in optimum cutting temperature compound, and frozen at −80°C. Serial 10-μm sections were made, and TUNEL staining was done using the *in situ* apoptosis

detection kit (Takara, Kyoto, Japan) according to the protocol of the supplier. For fluorescent microscopic observation, tumors were fixed in 2% glutaraldehyde at 4°C for 2 hours, followed by washing with 0.1 mol/L phosphate buffer and immersion in 1% osmium tetroxide for 2 hours.

Dehydrated in a graded series of ethanol, the specimen was embedded in Spurr resin before staining of the sections with uranyl acetate and lead citrate. Observation was made with a Hitachi 7000 fluorescent microscope set at 75 kV.

Statistical Analysis

For tumor size *in vivo*, results were expressed as mean ± SE, and the Mann-Whitney *U* test was used for statistical analysis. For luciferase assay and differences in ALT and AST, the Fisher's protected least significant difference test was used to determine statistical significance. $P \leq 0.05$ was considered significant in all statistical evaluations.

Results

Luciferase Assay

At first, to confirm whether electroporation increases the gene transfection efficiency, luciferase assay was used in the experiment. Electroporation was set up at 33 V, 50 ms on, 950 ms off, eight times. The plasmid was directly injected into the NC65 tumors. Luciferase activity of the pCAGGS Luc with *in vivo* electroporation was 10 times higher than the pCAGGS Luc injection alone (Fig. 1). We also confirmed the effect in Luciferase assay *in vitro* (data not shown).

Cytotoxicity of *TRAIL* Gene Transfection against NC65 Cells by Electroporation *In vitro*

We investigated the cytotoxicity of *TRAIL* gene transfection using electroporation against NC65 cells *in vitro*, and the cytotoxicity was measured by MTT assay. The cytotoxicity of pCAGGS *TRAIL* + electroporation was 59.2 ± 10.1% (mean ± SD). The viability of the cells that get electroporated with a control plasmid was slightly reduced. Thus, a significant cytotoxicity of pCAGGS *TRAIL* + electroporation was observed. These findings suggested that electroporation is useful for *TRAIL* gene transfection *in vitro*.

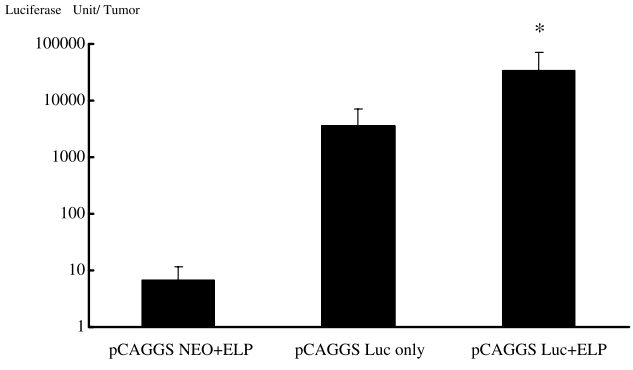

Figure 1. Luciferase assay. Luciferase activity of pCAGGS Luc with *in vivo* electroporation was 10 times higher than pCAGGS Luc only. *, $P < 0.05$, significant difference between the pCAGGS Luc and all of the other groups.

Antitumor Effect of *TRAIL* Gene Therapy by *In vivo* I.t. Electroporation on NC65 Tumors

Because *in vivo* electroporation increased the gene transfection efficiency, the growth-inhibitory effect on NC65 cells was investigated using *TRAIL* plasmid. We did *in vitro TRAIL* gene therapy by electroporation for NC65 cells and confirmed inhibition of cell proliferation. When *TRAIL* gene therapy using *in vivo* i.t. electroporation was done once (day 0), the growth of the NC65 tumors was not inhibited significantly. However, when *TRAIL* gene therapy was done thrice (days 0, 2, and 4), growth suppression of NC65 tumors was observed (Fig. 2A). The combination of the control plasmid and electroporation increased the tumor growth compared with the control, but the difference was not significant.

Antitumor Effect of *TRAIL* Gene Therapy and 5-FU by *In vivo* Electroporation on NC65 Tumors

In a previous study, we showed that treatment of established and freshly isolated renal cell carcinomas with a combination of TRAIL and 5-FU resulted in the potentiation of cytotoxicity and apoptosis and reversed their resistance (16). To obtain a more remarkable *in vivo* therapeutic outcome, we combined *TRAIL* gene transfer with an anticancer agent, 5-FU. When NC65 cells were treated with *TRAIL* gene therapy in combination with 5-FU, the antitumor effect of the combination therapy was more evident than either gene therapy alone or chemotherapy alone (Fig. 2B).

TUNEL Staining of *TRAIL* Gene–Transfected NC65 Tumors

To confirm the induction of apoptosis by *TRAIL* gene transfection, TUNEL staining was done. TUNEL staining revealed that the *TRAIL* gene–injected tumors had abundant apoptotic nuclei with double-strand DNA breaks (Fig. 3B), whereas apoptosis was not evident in the NC65 tumors treated with saline (Fig. 3A).

Effect of *In vivo* Electroporation of *TRAIL* Gene on Serum ALT and AST Levels of SCID Mice Bearing NC65 Tumors

The hepatic dysfunction due to TRAIL has been detected in some studies (20, 21). Effects of *TRAIL* gene transfection into NC65 tumors on serum ALT and AST levels were examined. *TRAIL* gene therapy had no effect on the serum levels of ALT and AST in SCID mice (Fig. 4).

Antitumor Effect of *TRAIL* Gene Therapy by *In vivo* I.m. Electroporation on NC65 Tumors

Aiming at clinical application, the *TRAIL* gene was introduced into thigh muscle, and the cell growth-inhibitory effect was investigated. It is clinically easier to do *in vivo* i.m. electroporation, compared with i.t. electroporation. When *TRAIL* gene therapy using *in vivo* electroporation was done six times (days 0, 2, 4, 6, 8, 10, and 12), growth suppression of the NC65 tumors was observed. On day 28, residual tumors receiving electroporation with the *TRAIL* gene were significantly smaller than those of tumors receiving PBS, pCAGGS NEO, or pCAGGS *TRAIL* only (Fig. 5). No muscular cell death, such as muscular atrophy and necrosis, was noted.

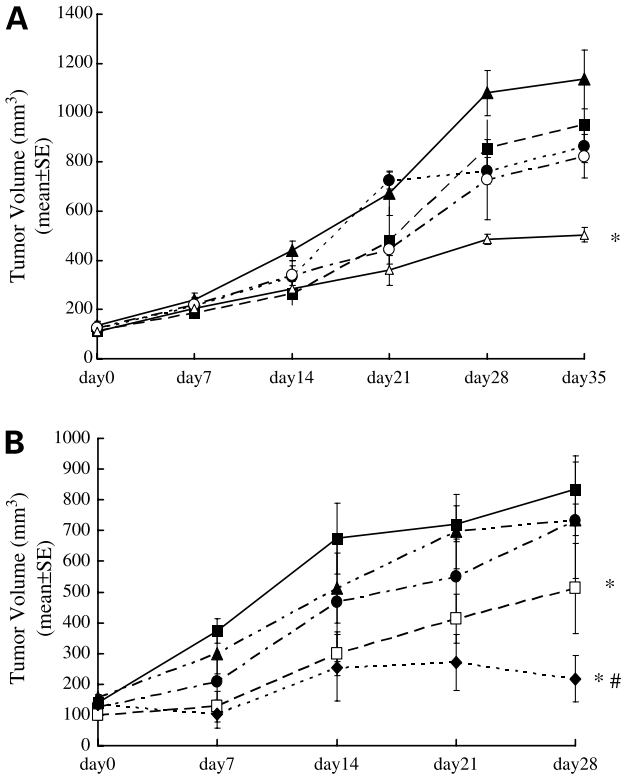

Figure 2. **A,** antitumor effect of *TRAIL* gene therapy by *in vivo* electroporation on NC65 tumors. *Y axis,* tumor volume, which was calculated as length × width^2 / 2; *X axis,* time of treatment. Measurement was done every 7 d and the results were compared between the following five groups: mice with tumors that received injection of PBS on day 0 (control; $n = 6$; ■); mice with tumors that received injection of pCAGGS TRAIL alone on day 0 ($n = 6$; ●); mice with tumors that received pCAGGS TRAIL injection plus electroporation on day 0 ($n = 6$; ○); mice with tumors that received injection of pCAGGS NEO, a control for the plasmid, plus electroporation on days 0, 2, and 4 ($n = 6$; ▲); and mice with tumors that were injected with pCAGGS TRAIL plus electroporation on days 0, 2, and 4 ($n = 6$; △). *, $P < 0.05$, significant difference in tumor volume at day 35 was observed between the pCAGGS TRAIL + electroporation (three times) and all of the other groups. **B,** antitumor effect of *TRAIL* gene therapy and 5-FU by *in vivo* electroporation on NC65 tumors. Electroporation and injections were done thrice on days 0, 2, and 4 in all groups, including the control ($n = 6$; PBS; 50 μL; ▲), pCAGGS NEO (50 μL; $n = 6$; ■), pCAGGS TRAIL (50 μL; $n = 6$; □), 5-FU (10 μmol/L/50 μL; $n = 6$; ●), and 5-FU (10 μmol/L/50 μL) + pCAGGS TRAIL ($n = 6$; ◆) groups. *, $P < 0.05$. Significant difference in tumor volume at day 28 was observed between the pCAGGS TRAIL group and the control, pCAGGS NEO, and 5-FU groups. #, $P < 0.05$. The 5-FU + pCAGGS TRAIL group showed more potent inhibition of cell proliferation than the pCAGGS TRAIL group and there was a significant difference in tumor volume at day 28 compared with all the other groups.

Discussion

Reports on gene therapy using *TRAIL* have been published (22–25), suggesting the great potential of this cytokine in the development of more effective anticancer drugs with few adverse reactions. This study showed that *TRAIL* gene therapy was effective against renal cell carcinoma. It also showed that combination treatment with *TRAIL* gene therapy and 5-FU was more effective against renal cell carcinoma and did not harm normal cells. These findings suggest the potential clinical application of *TRAIL* gene therapy and/or 5-FU in the treatment of renal cell carcinoma.

Electroporation was used to introduce the *TRAIL* gene into NC65 cells in the present study. Reports on the use of this method of introducing genes into melanoma and hepatocellular carcinoma cells have been published (25, 26). In this method, cell membrane permeability is increased by applying electrical pulses to more efficiently incorporate drugs and plasmids into cells. It has been reported that the application of electrical pulses after the injection of plasmids into cells is more efficient than the reverse process. Therefore, we used this method in the present study. With this method, we investigated the inhibitory effect of TRAIL on the proliferation of NC65 cells transplanted into SCID mice. A more potent inhibitory effect was observed in the groups with *TRAIL* gene therapy than in the control group, and the effect was greater when *TRAIL* gene therapy was done thrice. These findings suggested that *TRAIL* gene therapy may be clinically applicable by electroporation.

The combination of TRAIL and chemotherapeutic agents, such as 5-FU, *cis*-diamminedichloroplatinum, doxorubicin, and CPT-11, was shown to augment TRAIL-induced apoptosis in some human cancer cells, including renal cell carcinoma (27–30). This synergy was also observed in multidrug-resistant cell lines (31) or TRAIL-resistant cell lines (26, 31, 32). It has been proposed that chemotherapeutic agents augment TRAIL-induced apoptosis by up-regulating DR5 (29, 30). Ionizing radiation may also enhance TRAIL-induced apoptosis by up-regulation of DR5 (33, 34). We then did combination therapy with *TRAIL* gene therapy and 5-FU, which has been used for renal cell carcinoma, to obtain a more potent antitumor effect. 5-FU enhanced the antitumor activity of *TRAIL* gene therapy. These findings suggest that the combination of *TRAIL* gene therapy and 5-FU is effective against renal cell carcinoma.

Previously, we have investigated the mechanisms of synergistic cytotoxicity of TRAIL and 5-FU *in vitro*. When Caki-1 renal cell carcinomas were treated with a combination of 5-FU and TRAIL, 5-FU and 5-fluoro-2′-deoxyuridine 5′-monophosphate accumulation inside the cells increased modestly. Because increased i.m. accumulation of 5-FU and 5-fluoro-2′-deoxyuridine 5′-monophosphate by TRAIL was observed, we examined the effect of TRAIL on the expression of enzymes involved in the metabolism of 5-FU. When Caki-1 cells were treated with TRAIL, thymidylate synthesis and dihydropyrimidine dehydrogenase expression was slightly reduced, whereas the expression of oratate phosphoribosyltransferase was up-regulated. However, the treatment had no effect on thymidine phosphorylase expression. Although immunocytochemical analysis failed to detect p53 in the Caki-1 cells, p53 expression was observed after treatment with 5-FU. Treatment of Caki-1 cells with 5-FU did not change the expression of bcl-2. However, bax expression in the Caki-1 cells was up-regulated following their treatment with 5-FU. These findings suggest that synergistic

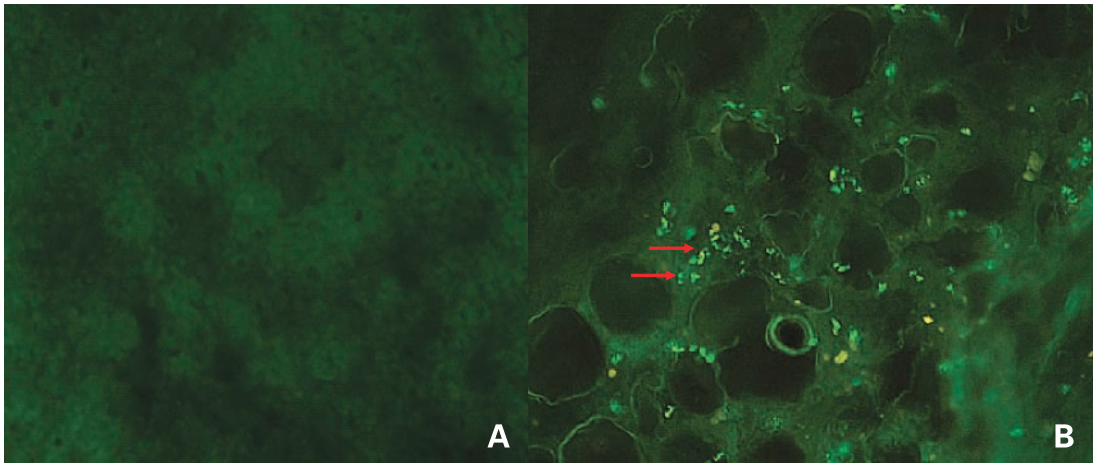

Figure 3. TUNEL staining of *TRAIL* gene transfected NC65 tumors. **A,** TUNEL-positive cells are not observed in the PBS-control group. **B,** TUNEL-positive cells can be observed in the pCAGGS TRAIL + electroporation (three times) group, which indicates that apoptosis has been induced. *Arrows,* TUNEL-positive cells.

cytotoxicity of TRAIL and 5-FU might be due, in part, to the modified expression of at least some of these enzymes by TRAIL and the enhanced expression of the proapoptotic p53 and bax proteins (16). The mechanisms responsible for enhanced cytotoxicity of TRAIL and 5-FU, however, remain unclear. Further studies are necessary to analyze the mechanisms.

We introduced the *TRAIL* gene directly into the tumors in the present study. However, this method might be difficult to be applied in clinical practice. Therefore, we also introduced the *TRAIL* gene into the thigh muscles of mice and investigated the outcome. The method of introducing genes into the thigh muscle was the same as that used for the tumors, but electroporation was done six times. There was a significant difference in the inhibitory effect on tumor growth between the thigh muscle gene incorporation group and the control group, indicating that this method also seems to be clinically applicable. We assume that TRAIL protein was produced by plasmids introduced into the muscle cells and then induced apoptosis by binding to the receptors on the tumor cells after being transported via the blood, thus inhibiting tumor proliferation. When TRAIL is administered to mice *in vivo*, TRAIL protein requires frequent administration (20). However, in gene therapy with plasmids, treatment effects were observed after direct injection into the tumor thrice or after i.m.

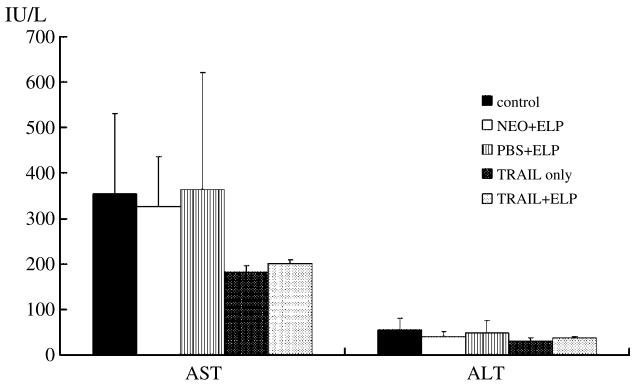

Figure 4. Effect of *in vivo* electroporation of *TRAIL* gene on serum AST and ALT levels of SCID mice bearing NC65 tumors. Hepatic dysfunction due to TRAIL has been reported; thus, we measured serum ALT and AST levels in mice (n = 6) with transplanted tumors treated with PBS (control), pCAGGS NEO + electroporation (*ELP*), PBS + electroporation, pCAGGS TRAIL, and pCAGGS TRAIL + electroporation to investigate hepatic dysfunction. As a result, no evidence of hepatic dysfunction was observed.

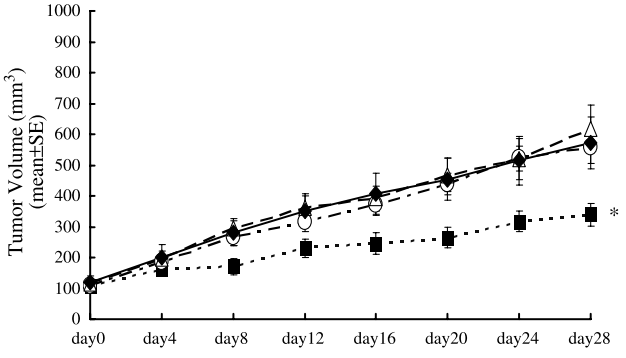

Figure 5. Antitumor effect of *TRAIL* gene transfer by *in vivo* i.m. electroporation on NC65 tumors. As would be done in clinical practice, we introduced genes into the thigh muscles. *Y axis,* tumor volume, which was calculated as length × width^2 / 2; *X axis,* time of treatment. Measurement was done every 4 d. Mice were treated with PBS + electroporation (n = 12; △), pCAGGS NEO + electroporation (n = 12; ◆), pCAGGS TRAIL (n = 12; ○), or pCAGGS TRAIL + electroporation (n = 12; ■) on days 0, 2, 4, 6, 8, 10, and 12, and the antitumor effect was evaluated. *, $P < 0.05$. The pCAGGS TRAIL + electroporation group showed a significant difference in tumor volume at day 28 compared with all of the other groups.

injection into a thigh muscle six times. Therefore, *TRAIL* gene therapy is the useful method.

Although i.m. electroporated *TRAIL* gene exhibited a significant inhibitory effect on NC65 renal cell carcinomas, its mechanism has not yet been clarified. It is considered that TRAIL protein may be synthesized in thigh muscle, released into the circulation, and inhibit the growth of renal cell carcinomas. Because TRAIL is a cytokine, TRAIL synthesized in muscle may stimulate host immunity and block the growth of renal cell carcinoma. We are planning to proceed experiments for elucidating this mechanism.

TRAIL has generally been reported to cause no adverse reaction, unlike other TNFs and Fas ligand, although hepatic dysfunction due to TRAIL has been detected in some studies (21). Therefore, we investigated hepatic dysfunction as a potential adverse reaction in the present study. Serum samples were obtained from mice after *TRAIL* gene introduction and hepatic enzymes were measured. As a result, no deterioration of hepatic function was observed compared with the control group. It was concluded that TRAIL did not cause any hepatic adverse reactions. Based on this result, TRAIL has the potential to become a superior anticancer drug.

We used electroporation as the gene transfer method. Limiting factor of retroviral vector may include access of the cDNA within the preintegration complex to the chromosomal target site and relatively low vector copy numbers that can be delivered (35). The inefficiency and poor fidelity of gene targeting by retroviral vectors have hindered its subsequent development. However, if these problems could be overcome, this technique could ultimately prove effective for gene targeting. Adenoviruses are linear, double-stranded DNA viruses, and vectors derived from them are able to transduce many cell types efficiently via episomal expression. Adenoviral vectors also have a low frequency of nonhomologous chromosomal integration, suggesting that they may be useful for gene targeting. The potential advantage of adenovirus vectors is their ability to package large fragments of homologous sequences. However, the absolute targeting frequencies were not markedly superior to those obtained by plasmid transfection or electroporation (36). The reasons why we selected electroporation for *TRAIL* gene transfection in this study were the absence of risk of viral infection and simplicity of electroporation.

Surgical treatment is the method of first choice for renal cell carcinoma, whereas immunotherapy is done when surgery is not applicable or when metastasis has occurred. However, the efficacy of these treatments is not satisfactory, so development of new anticancer drugs or treatments is required. TRAIL may contribute strongly to the treatment of renal cell carcinoma in the future. In the present study, we confirmed an antitumor effect of *TRAIL* by electroporation of genes not only into tumors but also into the thigh muscle. However, the actual mechanism of the action and of the method of applying electroporation to the thigh muscle in patients remain unclear. We will continue to perform further studies based on the present results, which suggest that TRAIL and/or 5-FU may be effective for the treatment of renal cell carcinoma.

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