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Sequential Histopathological Changes *in vivo* after Suicide Gene Therapy of Gastric Cancer Induced by *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine in Rats

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Gastrointestinal cancer is the most important clinical target of gene therapy. Suicide gene therapy, such as with the herpes simplex virus type 1 thymidine kinase (*HSV-TK*) gene, has been shown to exert antitumor efficacy in various cancer models *in vitro*. We previously reported *in situ* gene transfer and gene therapy for gastric cancer induced by *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine (ENNG) in dogs. Here, we describe the sequential histopathological changes after suicide gene therapy of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)-induced gastric cancer in rats. Gastric tumors were induced by MNNG in 38/73 (52%) of Wistar strain rats. The suicide gene therapy group (14 rats) was subjected to *in situ* gene transfer with a recombinant adenovirus vector carrying the *HSV-TK* gene driven by CAG promoter (Ad.CAGHSV-TK) in gastric tumor, followed by the antiviral drug ganciclovir (GCV). To observe the histopathological changes at various times after HSV-TK/GCV gene therapy, groups of animals were sacrificed at 3, 8, and 30 days after gene transfer. Apoptosis in the gastric tumors was detected by the TUNEL method to assess the efficacy of HSV-TK/GCV gene therapy, and it was marked in the 8- and 30-day treatment groups compared to the sham operation controls (P<0.001). Various histopathological changes, degeneration of cancer tissue and fibrosis after necrosis and apoptosis were significantly greater in the 30-day treatment group. The *HSV-TK* gene was detectable in peripheral blood by PCR until 30 days after gene transfer. These results may be useful in devising a method of suicide gene therapy for humans.

Key words: Gene therapy — Suicide gene — MNNG — Rat

The incidence of gastric cancer in Japan is still high. Recently, early detection and progress in surgical treatment have improved the mortality of gastric cancer. However, aggressive treatment by surgery or chemotherapy has not sufficiently prolonged the survival time of patients with distant metastases of gastric cancer, such as liver metastasis and peritoneal dissemination.[1)] Thus, while stage-oriented surgery is useful, it is inadequate for stage IV gastric cancer. Gene therapy shows promise of becoming a treatment that will succeed against stage IV gastric cancer.

The transfer of suicide genes into tumor cells that have become sensitive to pharmacological agents is currently being studied as a method of treatment for various cancers, and the herpes simplex virus type 1 thymidine kinase (*HSV-TK*) gene has been the most widely studied suicide gene.[2)] Gene therapy has been shown to be effective *in vitro* in cell culture and *in vivo* in transplants of tumor cells. We reported suicide gene therapy for experimental gastric cancer induced by *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine (ENNG) in dogs, and the results showed that *in situ* gene transfer of a suicide gene followed by prodrug treatment may be applicable not only to the primary gastric tumor, but to lymph node metastasis. After HSV-TK/ganciclovir (GCV) therapy of canine gastric cancer, all of the dogs were killed in the short term (after 7 days) and there are no data on the medium-term therapeutic effect of suicide gene therapy.

In this study, we assessed the efficacy of gene therapy by *in situ* adeno-mediated transfer of the *HSV-TK* gene followed by GCV administration in gastric cancer induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) in rats, and we followed the histopathological changes for the gastric cancer and *HSV-TK* gene in peripheral blood for 30 days. The results showed that 1) apoptosis preceded tissue degeneration, 2) histopathological efficacy requires 30 days after suicide gene therapy, and 3) the *HSV-TK* gene persisted until 30 days after GCV treatment.

MATERIALS AND METHODS

Induction of gastric tumor All animal experiments were conducted according to the institutional guidelines of Nippon Medical School. Wistar strain male rats (n=104) 5 weeks old at the start of the experiment were used.[3)] An

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animal model of gastric tumors was induced with MNNG dissolved in distilled water for injection in light-shielded bottles and given as drinking water *ad libitum*. Concentrations of 100 μg/ml for 30 weeks have been shown to be effective in inducing gastric tumors.[4] Thirty-one rats died of small-intestinal sarcoma before the gene therapy experiment. Rats, weighing 295–536 g (average: 433 g) were used for the *in vivo* experiments at 60 weeks of age. The animals were anesthetized with 25 mg/kg pentobarbital (Dainippon Seiyaku Co., Ltd., Osaka), intraperitoneally.

Adenoviral vector constructions The recombinant adenovirus vector was generated as previously described.[5] Briefly, the expression unit containing the CAG promoter,[6] and herpes simplex virus thymidine kinase cDNA (*HSV-TK*), and rabbit β-globin polyadenylation signal were inserted into E1- and E3-deleted adenovirus cosmid clone pAdex1w. This construct and *Eco*T221-digested adenovirus DNA-terminal protein complex were co-introduced into 293 cells. The recombinant adenovirus vectors, Ad.CAGHSV-TK, were isolated, purified, and then concentrated by two cycles of cesium-chloride gradient centrifugation.[7] An adenovirus vector containing the CAG promoter and the *lacZ* gene, AdexlCAlacZ, were kind gifts of I. Saito (University of Tokyo). The viral solution was stored at −80°C. The virus titer was determined by plaque assay on 293 cells as described.[8, 9]

***In vivo* gene transfer and protocol of the experiment** We used a total of 29 rats. Ad.CAGlacZ (5×10^8 pfu) or Ad.CAGHSV-TK (5×10^8 pfu) was injected into the stomach tumors via a 27 G needle and microliter syringe (Hamilton Co., Reno, NV) for about 3 min with 50 μl of phosphate-buffered saline (PBS), on the serosal side in 75% of the rats and the mucosal side in the other 25% after laparotomy and recognition of the gastric tumors. Two days after transfer of the *HSV-TK* gene, the rats were intraperitoneally injected with GCV (Tanabe Seiyaku Co., Ltd., Osaka) at a dose of 50 mg/kg, twice daily for 4 days. Rats were sacrificed 3 (n=4), 8 (n=5, 6 tumors), and 30 days (n=5) after gene transfer. Ad.CAGlacZ was given to 5 rats as the vector sham in a group. The controls (n=10) included a no treatment group (n=7) and a GCV group (n=3). The no treatment group was only sham-operated. The GCV group was sham-operated in addition to being intraperitoneally injected with GCV, 50 mg/kg, twice daily for 4 days. Rats were sacrificed with diethyl ether (Wako Pure Chemical Industries, Ltd., Osaka), and blood samples were collected. Stomach, small intestine, liver, lung, kidney, spleen, and testis samples were fixed in 4% paraformaldehyde and embedded in paraffin. All sections were stained with hematoxylin and eosin and examined macroscopically and microscopically.[10]

TUNEL method To evaluate the efficacy of the *in vivo* suicide gene therapy, we investigated apoptotic cells by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick-end labeling (TUNEL) method[11, 12] by using an *In Situ* Cell Death Detection Kit, POD (Nippon Roche K.K., Tokyo). Tissue samples were fixed in 4% buffered formaldehyde, embedded in 4 μm paraffin sections, deparaffinized and exposed to 20 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) in 10 m*M* Tris-HCl buffer (pH 7.6) for 30 min at room temperature (RT). The slides were then rinsed with PBS, and treated with blocking solution (0.3% H_2O_2 in methanol) for 30 min at RT. The sections were rinsed with PBS and distilled water, and immersed in permeabilization solution, 0.1% Triton X-100 in sodium citrate (Rohm & Haas, Philadelphia, PA) for 2 min on ice. The TUNEL reaction mixture (TdT and biotinylated dUTP in TdT buffer) was added until it covered the sections, and they were incubated in a humid atmosphere at 37°C for 60 min. The sections were then rinsed with PBS, covered with anti-fluorescein antibody, Fab fragment from sheep, conjugated with horseradish peroxidase (Converter-POD), and incubated in a humid atmosphere at 37°C for 30 min. The sections were rinsed with PBS, diaminobenzidine chromogen solution, was added, and the sections were incubated for 10 min at RT. We used an eyepiece micrometer disc 21 mm (Nikon Co., Tokyo) to count the apoptotic cells and counted a random sample of cells from each rat.

Detection of the *HSV-TK* gene by PCR in serum To analyze the target organs following *in situ* gene transfer infusion of the recombinant Ad, the genomic DNA of rats was extracted and subjected to PCR to determine the *HSV-TK* gene distribution in serum from peripheral blood. The *HSV-TK* DNA was amplified by using the primer pair: 5′-(GGCTATGCTGGCTGCGATTCG)-3′(sense) and 5′-(TCCCGGCAGCCGGCGGGCGAT)-3′ (antisense). The PCR conditions were: initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 5 min. A 10 μl sample of PCR product was analyzed by 1.2% agarose gel electrophoresis and stained with ethidium bromide to detect the DNA fragment of 1.2 k base pairs.

Blood measurements To assess the general toxicity of Ad.CAGHSV-TK followed by GCV administration, blood chemistry measurements were performed by Mitsubishi Kagaku Biochemical Laboratory Inc., Tokyo.

Statistical analysis *P* values were calculated by using Student's *t* test. The statistical analysis was conducted by using the SPSS computer program.

RESULTS

Induction of gastric tumors in rats All tumors were observed in the glandular stomach of the rats (Fig. 1A). Gastric tumors were induced by MNNG in 38 (52%) of 73 rats, and 32 tumors were used in the experiment (Table I).

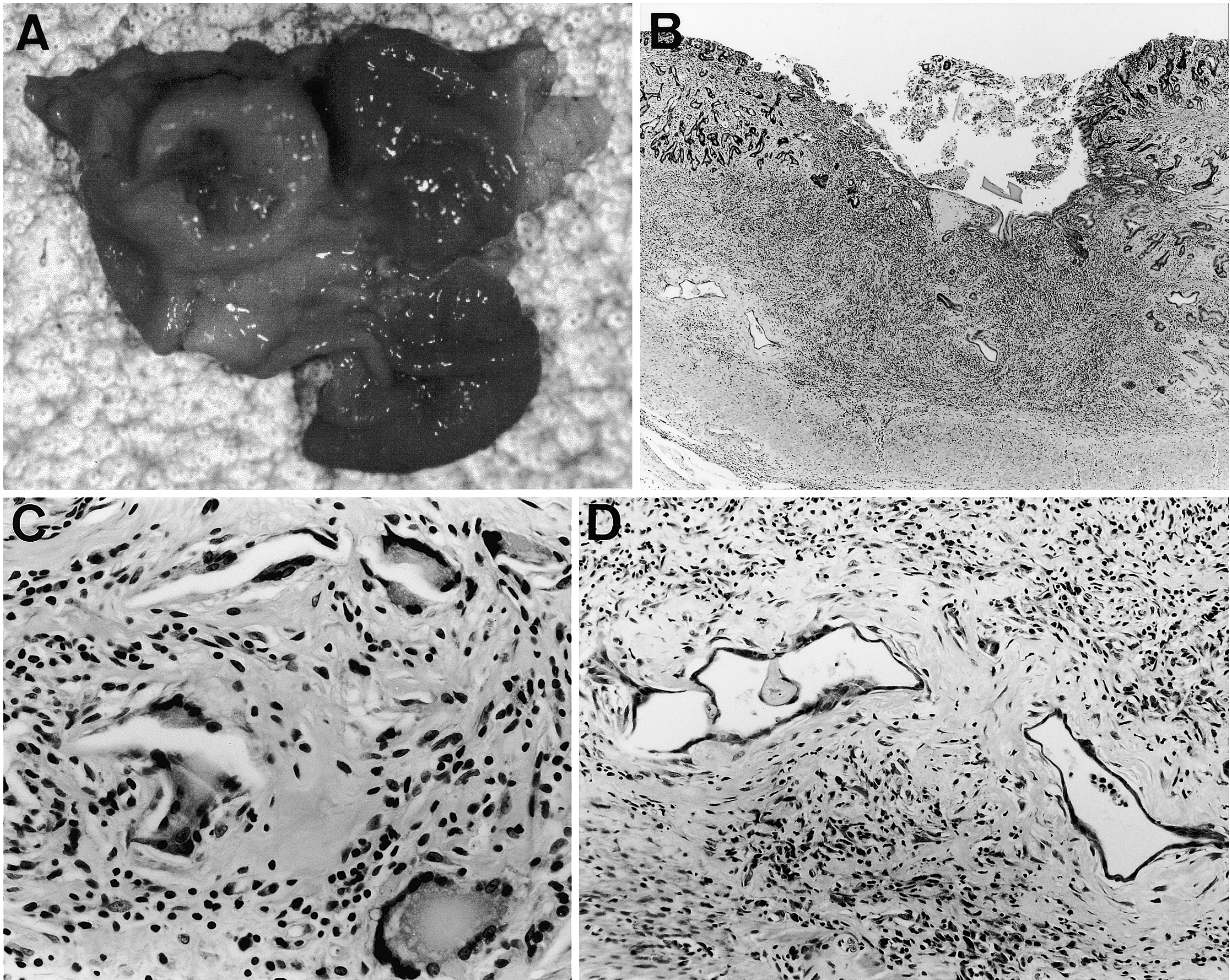

Fig. 1. (A) Gastric tumors were induced with *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine in 52% of the rats. All tumors were found in the glandular stomach. (B) Histopathological appearance of a gastric tumor at 30 days after suicide gene therapy. Significantly greater degenerative changes, i.e., scar formation, breaks in the muscle layer, giant cells, and glandular duct thickening, were observed in the 30-day treatment group than in the controls. (HE staining; original magnification ×10). (C) Giant cell after suicide gene therapy. (HE staining; original magnification ×50). (D) Glandular duct thickening after suicide gene therapy. (HE staining; original magnification ×20).

The tumors were 1 to 30 mm in maximum diameter (average: 6.9 mm). According to histological type, there were 26 adenocarcinomas (81%) and 6 sarcomas (19%). The adenocarcinomas included well and poorly differentiated adenocarcinomas. The classification of neoplastic glandular stomach lesions was based on histological criteria.[13, 14] Small-intestinal tumors formed in 48% of the rats and were fibrosarcomas. There were rats (15%) with both tumors. No metastasis to other organs, lung, liver, spleen, kidney and testis, or peritoneal cavity, was detected macroscopically.

Apoptosis of gastric tumor cells after suicide gene therapy Apoptosis of gastric tumor cells was investigated to evaluate the efficacy of the *in vivo* suicide gene therapy, Ad.CAGHSV-TK was directly injected into the gastric tumors and was followed by GCV at various times after treatment (Fig. 2). Significantly more apoptosis was detected by the TUNEL method in the 8- and 30-day treatment groups than in the sham-operated controls ($P<0.001$) (Fig. 3).

Histopathological changes in gastric tumors after suicide gene therapy We assessed various histopathological

Table I. Suicide Gene Therapy of MNNG-induced Gastric Cancer in Rats

	Control		Vector	Ad.CAGHSV-TK/GCV[c]			Total
	No treatment[a]	GCV[b]		3 days	8 days	30 days	
Number of rats	7 (9[d])	3	5	4	5 (6[d])	5	29 (32[d])
Adenocarcinoma	9	2	3	3	5	4	26
Sarcoma	0	1	2	1	1	1	6
In situ via[e] Mucosa	0	0	3	2	0	0	5
Serosa	0	0	2	2	6	5	15

a) Sham-operation group.
b) Sham operation plus GCV (50 mg/kg) intraperitoneally, twice daily for 4 days.
c) Recombinant adenovirus vector carrying the herpes simplex virus type 1 gene driven by the CAG promoter.
d) Number of tumors.
e) *In situ* gene transfer on the mucosal or serosal side.

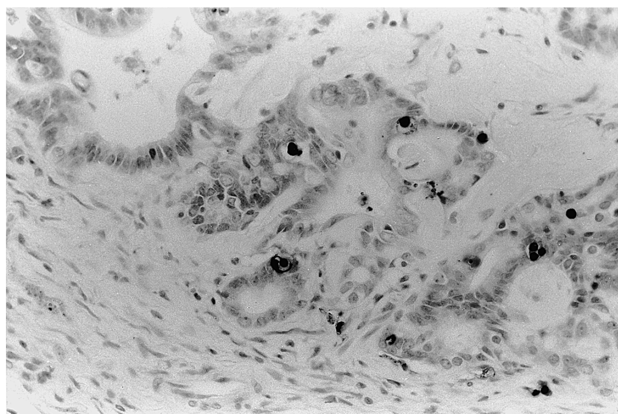

Fig. 2. Apoptotic cells following suicide gene therapy. Apoptotic cells detected by the TUNEL method in a gastric tumor. Some apoptotic cells in the tumor showed nuclear condensation and fragmentation of nuclei and shrinkage of the cytoplasm. (Original magnification ×100).

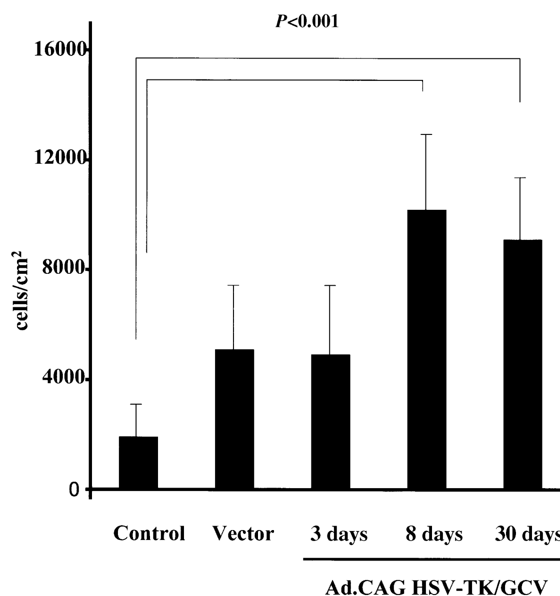

Fig. 3. Apoptotic cell counts in gastric tumors after suicide gene therapy. Apoptotic cells per cm^2 detected by the TUNEL method were significantly more numerous in the 8- and 30-day treatment groups than in sham-operated controls. ($P<0.001$).

changes in gastric tumors to evaluate the efficacy of *in vivo* HSV-TK/GCV gene therapy (Table II). Sequential histopathological findings in the gastric tumors of rats treated with Ad.CAGHSV-TK/GCV were examined in the 3-, 8-, and 30-day groups, the adenovirus vector sham group, and the sham-operated controls. The level of fibrosis was defined compared with the thickness of the normal muscle layer and that of inflammation was defined in terms of the number of neutrophils or lymphocytes. Inflammatory change in the gastric tumor was more severe in the 8- and 30-day groups than in the vector sham group and controls. Mild fibrosis or none was observed in 67% and 33%, respectively, of the controls, and in 20% and 80% of the vector sham group, but fibrosis was severe in the 30-day group. In addition, significantly greater degenerative changes, i.e., scar formation, breaks in the muscle layer, giant cells, and glandular duct thickening, were observed in the 30-day group ($P<0.05$) (Fig. 1, B, C and D). Complete tissue degeneration was observed in a mesenteric lymph node of one rat in the 8-day group.

Detection of *HSV-TK* gene by PCR The distribution of *HSV-TK* gene in serum was assessed to test the efficiency and safety of adenovirus-mediated gene transfer. The 1.2 kb *HSV-TK* gene was not detected by the PCR in the controls, but it was detected by PCR (Fig. 4) at 8 and 30 days

Table II. Sequential Histopathological Changes of Gastric Tumor after Gene Therapy

		Control	Vector	Ad.CAGHSV-TK/GCV		
				3 days	8 days	30 days
Fibrosis	(–)	8/12 (67%)	1/5	0/4	0/6	0/5
	Mild	4	4	3 (75%)	2	1
	Moderate	0	0	1	4 (67%)	1
	Severe	0	0	0	0	3 (60%)
Necrosis		0/12	0/5	1/4	1/6	1/5
Scar formation		0	0	0	0	4 (80%)*
Breaks in the muscle		0	0	0	0	3 (60%)
Giant cells		0	0	0	0	4 (80%)*
Glandular duct thickening		0	0	0	1 (17%)	2 (40%)
Inflammation	Mild	7/12 (58%)	2/5 (40%)	0/4	1/6	1/5
	Moderate	5	2	3	3 (50%)	2 (40%)
	Severe	0	1	1	2 (33%)	2 (40%)

* $P<0.05$.

after HSV-TK/GCV gene therapy. In this study, the *HSV-TK* gene was detected in injected sites of the tumors, but not detected in other organs.

Hepatotoxic effects of injected Ad.CAGHSV-TK and GCV Transaminase levels were mildly elevated in all groups (control: GOT 150±41.3 IU/liter, GPT 57±36.3 IU/liter; 8-day: GOT 194.2±53.4 IU/liter, GPT 35.2±14.5 IU/liter; 30-day: GOT 167±45.6 IU/liter, GPT 61.7±19.0 IU/liter), but the HSV-TK/GCV treatment groups were not significantly different from the controls (no treatment and GCV group). There were no significant differences among any of the groups in the following: lactate dehydrogenase, alkaline phosphatase, γ-glutamyltransferase, cholinesterase, leucine aminopeptidase, amylase, total protein, albumin, blood urea nitrogen, creatinine, sodium, potassium, chlorine, phosphorus, C-reactive peptide, total and direct bilirubin, total cholesterol, triglyceride, and uric acid.

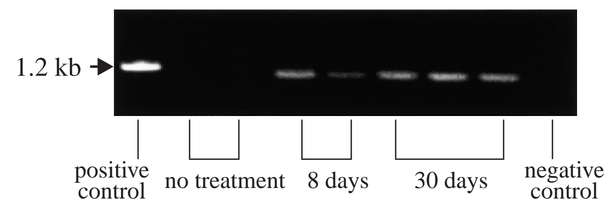

Fig. 4. *HSV-TK* gene was detected by PCR. The 1.2 kb *HSV-TK* gene was not detected by PCR in the controls, but was detected by PCR at 8 and 30 days after HSV-TK/GCV gene therapy.

DISCUSSION

No dramatic responses to human gene therapy for cancer have yet been reported, though clinical trials of gene therapy are now being conducted for diseases such as brain tumors. Moreover, attention has been focused on the side effects of gene therapy, because a case of death due to an adverse effect of gene therapy was recently reported.[15] The efficacy and safety of the vectors, including adenovirus vectors, are important basic issues. Observing the sequential changes after gene therapy in animal models may be important for protocol development and evaluation of human gene therapy. Histopathologically, the efficacy for HSV-TK/GCV gene therapy has been reported for inflammation and tissue necrosis using *in vivo* transplants of tumor cells, and differences of HSV-TK/GCV efficacy for colon cancer were reported among various *in vitro* cell lines.[16] Few reports are available on induced cancer[10] or histopathological changes, and gene therapy of induced digestive tract cancer has never been reported. In this study, we evaluated changes at various times after gene transfer and gene therapy for MNNG-induced experimental gastric tumors in rats. MNNG is effective in inducing gastric tumors in rats and has been used in many experiments. Three days after gene therapy, only mild fibrosis was observed, but degeneration of cancer tissue, fibrosis, and apoptosis were seen at 8 days, and these histopathological changes were more prominent at 30 days. Much greater apoptosis was observed at 8 days, and the degenerative changes were more severe at 30 days, indicating that apoptosis preceded the histopathological findings. Expression of adenovirus vector is known to be transient, but the ability of a replication-deficient adenovirus vector to transfer a foreign gene into the neural cells of rats, affording at least 45 days expression, was reported.[17] In an *in vivo* direct gene transfer using a replication-defective adenoviral vector containing a *β-galactosidase* gene to transduce

brain neurons, *lacZ* activity was evident for at least 8 weeks.[18] In the present study, the *HSV-TK* gene was demonstrated in peripheral blood at 30 days by PCR and Southern blotting (data not shown).

Degeneration of a mesenteric lymph node of one rat was also observed after suicide gene therapy in this study. If regional lymph nodes are suitable targets of *in situ* gene therapy, as shown in a canine gastric cancer model, this may be clinically useful for the treatment of stage IV gastric cancer. Adenovirus vector hepatotoxicity has been reported when the HSV-TK/GCV approach was used in mice[19] and in tumor-free rats after intraportal administration.[2] We have also demonstrated hepatotoxicity after *in situ* suicide gene therapy in canine gastric cancer.[20] Significant hepatotoxicity was not observed up to 30 days after adenovirus vector transfer under the present experimental conditions.

The problems to be overcome in gene therapy include specific targeting and high efficacy of gene transfer. Direct *in vivo* gene transfer using a replication-defective adenoviral vector containing the *HSV-TK* gene is necessary to demonstrate high levels of gene transfer *in vitro* and *in vivo*.[21, 22] Expression of the herpes simplex virus thymidine kinase gene renders cells susceptible to the inert nucleoside analog GCV, which becomes cytotoxic after phosphorylation by the viral enzyme.[23, 24] Cells transduced with the *HSV-TK* gene are efficiently killed by the direct cytotoxic effect of GCV-triphosphate, and the bystander effect kills adjacent non-transduced cells, although the mechanism of this is not fully understood.[25, 26] The bystander effect appears to rely on the presence of gap junctions between cells, or on components of the immune system.[27] However, it is difficult to transfer the gene to all of the cancer cells, and cell death away from the areas injected with the *HSV-TK* gene was not observed in this study. To control metastatic tumors, suicide gene therapy alone is thought to be insufficient, and the combination of suicide gene therapy and immunotherapy by cytokine transfer or gene therapy using replication-competent virus[28] may be preferable.

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