In situ Gene Transfer and Suicide Gene Therapy of Gastric Cancer Induced by N-Ethyl-N′′′′-nitro-N-nitrosoguanidine in Dogs

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Gene therapy could potentially revolutionize the treatment of gastrointestinal (GI) tract cancer. The aim of this study was to establish a practical method of gene transfer which would be applicable to human gastric cancer. Retrovirus or/and adenovirus vectors carrying the lacZ marker gene were transferred in situ by needle through an endoscopic biopsy channel into primary gastric cancer in six male beagle dogs that had been treated with N-ethyl-N′′′′-nitro-N-nitrosoguanidine (ENNG). In addition, an adenovirus vector carrying the herpes simplex virus thymidine kinase (Ad.CAGHSV-TK) gene was introduced in situ into cancer tissues in the stomach of three dogs, and the animals were treated with intravenous ganciclovir (GCV). Retrovirus-producing cells which expressed the lacZ gene were specifically localized to the injection site in the stomach. The lacZ gene was more widely transferred into the tumor by the adenovirus vector than by retrovirus-producing cells. Improvement of the needle used for gene transfer and the use of multiple injections per tumor led to more diffuse transfer of the vector into the tumor. The Ad.CAGlacZ gene was also transferred into regional lymph nodes of the stomach. Moderate to diffuse degeneration of the primary cancer tissues of the stomach was found after Ad.CAGHSV-TK/GCV gene therapy. Moreover, almost complete tissue degeneration was observed in the regional lymph nodes of the stomach. An adverse effect of HSV-TK/GCV gene therapy was acute hepatotoxicity, which was not found after Ad.CAGlacZ gene transfer, but was found after high-titer Ad.CAGHSV-TK gene transfer followed by GCV. These findings suggest that in situ gene transfer of a suicide gene followed by prodrug treatment may be applicable not only to primary tumors, but also to lymph node metastases of gastric cancer, though further study of both beneficial and adverse effects is required before clinical usage.

Key words: Gene therapy — Suicide gene — Adenovirus vector — Lymph node — Dog

Gene therapy provides a promising approach to the treatment of cancer,1, 2) and to date more than two thousand patients have undergone gene therapy or gene marking trials throughout the world. Initially, gene therapy was expected to serve as a treatment for genetic disorders, but cancer has now become the major target of gene therapy.3, 4) However, the specific targeting and killing of cancer cell remain difficult. Gastrointestinal (GI) tract cancers are the most common cancers worldwide, and therefore are an important target for gene therapy. GI tract carcinogenesis is a multi-stage process involving a series of defined genetic alterations.5) Phenotypic correction of cancer cells by the transfer of antisense oligonucleotides against certain oncogenes and of tumor suppressor genes is a promising approach,6) but there have been no reports yet of success against GI tract cancer in either animal models or humans. Suicide gene therapy may therefore provide a more promising approach to human GI tract cancer.7)

Suicide gene therapy involves the selective killing of cells expressing a transgene in the presence of a prodrug. A typical suicide gene is the herpes simplex virus thymidine kinase (HSV-TK) gene which can metabolize prodrugs such as ganciclovir (GCV) or aciclovir into phosphorylated forms. Such compounds act as chain terminators of DNA synthesis and specifically kill dividing cells in vitro8) and in vivo.9) A clinical advantage of the HSV-TK/GCV system is the “bystander effect,” which means that only a fraction of the tumor cells within the tumor mass need to express the suicide gene to kill the entire tumor.10) Moreover, we previously found a vaccine effect in tumor transplants in mice which received HSV-TK/GCV gene therapy.11)

Essential aspects of cancer gene therapy are specific targeting of cancer cells12) and high efficiency gene transfer.
Direct in situ in vivo gene transfer into the tumor is currently technically possible and may increase the specificity of exogenous gene expression. Most experimental gene therapy studies have used in vitro cell culture or transplants of tumor cells into small rodents, but have not examined cancers induced in animal models. In this study, we induced gastric cancer in dogs by administering N-ethyl-N’-nitro-N-nitosoguanidine (ENNG)\(^\text{13, 14}\) in drinking water and tumor growth in the stomach was followed by endoscopy. After diagnosis of cancer by biopsy, we examined the efficacy of retrovirus and adenovirus vector transfer using an Escherichia coli (E. coli) lacZ marker gene by in situ transfer with a needle through the biopsy channel of the endoscope into the tumors. Next we examined the targeting and efficacy of in situ suicide gene therapy in canine gastric cancer with an adenovirus vector containing the HSV-TK gene followed by intravenous administration of GCV.

**MATERIALS AND METHODS**

**Induction of experimental gastric cancer** Ten male beagle dogs, with an initial body weight of 5.0–6.5 kg (mean 5.8 kg), were used. ENNG was given to dogs in 500 ml of drinking water at a concentration of 100 µg/ml for 8 months or 150 µg/ml for 6 months. At the time of completion of ENNG administration, the body weights were 9.5–11.5 kg (mean 10.5 kg). The esophagus, stomach, and upper duodenum were examined by endoscopy (Olympus GIF-XQ 230; Olympus Corp., Tokyo) every 2–11 months from 6 months after the start of the ENNG administration under general anesthesia. Dogs were given 10 mg/kg ketamine HCl (Ketalar; Sankyo Co., Ltd., Tokyo) and 0.1 mg of atropine sulfate (Tanabe Seiyaku Co., Ltd., Osaka) intramuscularly and thereafter received intubation for general anesthesia with O\(_2\) and N\(_2\)O (3:2) containing 0.5–2% halothane (Halothane; Takeda Chemical Industries, Ltd., Osaka).

All of the vector transfer experiments were performed in a room with a P-2 level barrier system according to the safety guidelines of the Ministry of Education, Science, Sports and Culture of Japan.

**Efficacy of viral vector** Viral vectors were administered to tumors of the stomach via a 23G needle, which is clinically used for sclerotherapy for esophageal varices (Sumitomo Bakelene Co., Ltd., Tokyo). Viral vectors were injected in a 0.2–0.5 ml/volume into several points in each tumor. Retroviral vector-producing cells (0.5–1.2×10\(^6\) cells/ml) containing the lacZ gene of E. coli were injected into tumors of the stomach in 3 dogs (dogs 3, 5 and 7), and frozen sections of the gastric tumor were stained for X-gal activity 2 days after treatment. Adenoviral vector (2.5–9.8×10\(^9\) pfu/ml) containing the lacZ gene of E. coli driven by CAG promoter (Ad.CAGlacZ) was injected into tumors of the stomach in 5 dogs (dogs 2, 3, 5, 6 and 8) and stained for X-gal activity 2 days after treatment using frozen sections of the tumor or normal mucosa of the stomach.

**Generation of retroviral vector-producing cells and an adenovirus vector containing the lacZ gene, and X-gal histochemical assay** The retroviral vector-producing cell line, PA317/G1βgSvNa, was obtained from Genetic Therapy (Gaithersburg, MD). The vector contains the β-galactosidase gene and the simian virus 40 (SV40) early promoter-driven neomycin phosphotransferase gene.\(^\text{15}\) The adenovirus packaging system was the pAdex1CAwt cosmid cassette with a CAG promoter, which was kindly provided by Dr. I. Saito (Tokyo University, Tokyo). Distribution of the adenovirus vector containing the β-galactosidase gene, Ad.CAGlacZ, was detected by X-gal-based histochemical assay. Tissue was cut in 4-mm thick coronal sections, which were fixed with fixing solution (4% formaldehyde, 0.01% SDS, 0.02% NP 40) for 1 h. Sections were then rinsed thoroughly twice in phosphate-buffered saline (PBS; pH 7.4), and incubated with X-gal stain solution (3 mM K\(_4\)Fe(CN)\(_6\), 3 mM K\(_2\)Fe(CN)\(_6\), 1.3 mM MgCl\(_2\), 100 mM sodium phosphate pH 7.3, 1 mg/ml X-gal) at room temperature overnight. They were then rinsed thoroughly twice in PBS (pH 7.4), embedded in paraffin, cut into 5-µm sections and stained with Kernechtrot solution.

**Improvement of the needle for in situ transfer into the mucosal layer** Adenovirus vectors were introduced mainly into the submucosal layer by in situ injection via a sclerotherapy needle, which had a end hole. The virus vector could not be transferred effectively into the mucosal layer since the needle was fixed in the muscularis mucosa. We manufactured a new needle for in situ gene transfer into the mucosal layer, which had a blind end and 2 holes on the side (in association with Olympus Corp.). By using this improved needle, the adenovirus vector was transferred into the mucosal layer from the side holes in 5 dogs (dogs 1, 6, 8, 9 and 10).

**Generation of an adenovirus vector containing the HSV-TK gene** The recombinant adenovirus vector containing the HSV-TK gene, Ad.CAGHSV-TK, was generated as previously described.\(^\text{16}\) Briefly, the expression unit containing the CAG promoter,\(^\text{16}\) HSV-TK cDNA (provided by Dr. Tani), and the rabbit β-globin polyadenylation signal was inserted into an E1- and E3-deleted adenovirus cosmid clone, pAdex1w. This construct and EcoT221-digested adenovirus DNA-terminal protein complex were co-introduced into 293 cells. Ad.CAGHSV-TK was isolated, purified and concentrated by 2 cycles of cesium chloride gradient centrifugation.\(^\text{16, 17}\)

HSV-TK gene transfer with adenoviral vector followed by GCV Ad.CAGHSV-TK (1×10\(^2\)–1×10\(^3\) pfu/ml) was injected into tumors of the stomach in 3 dogs (dogs 1, 9 and 10), total 2–4 ml each and 2.4×10\(^9\)–4.4×10\(^10\) pfu/
animal, and animals were given GCV at 50 mg/kg twice a day intravenously (Denosin; Tanabe Seiyaku Co., Ltd., Osaka) from 2 days after HSV-TK gene transfer for 3–4 days, giving a total dose of GCV of 3.0–4.2 g/animal. Dogs were autopsied 7 days after the start of the experiment (Fig. 1).

Esophagus, stomach, regional lymph nodes, liver, spleen, kidney, lung and testis were fixed in 10% phosphate-buffered formalin for 48 h at room temperature. The whole area of the angulus-antrum of the stomach was cut along the longitudinal axis into three 5-mm thick sections, and the lesser curvature of the corpus of the stomach was cut into three 5-mm thick slices. Lymph nodes were cut into 2 slices and three to four slices of tissues were taken from the other organs for histological examination. Paraffin sections were prepared and stained with H&E or with alcian blue (pH 2.5)-periodic acid Schiff for the detection of mucin-containing cells.

Southern blotting for HSV-TK gene identification in the various tissues To evaluate dissemination of Ad.CAGHSV-TK following intratumoral injection of the gene and GCV treatment, tissues from stomach, regional lymph nodes, and liver were obtained at necropsy in a dog (dog 10) and DNA was extracted from these tissues. HSV-TK gene in these tissues was identified by the ordinary Southern blot hybridization using an HSV-TK probe following standard methods.18) Briefly, DNA was obtained by standard phenol/chloroform extraction and 10 µg of each DNA sample was digested with EcoRI, separated on ethidium bromide 0.8% agarose gel and then blotted onto Hybond-N+ membrane (Amersham Pharmacia Biotech, Buckinghamshire, England). The membrane was probed using a 32P-labeled portion of the HSV-TK corresponding to the 1.2-kb EcoRI-digested TK fragment.

Detection of HSV-TK gene by PCR HSV-TK gene was also detected by PCR amplification in the tissues from stomach, regional lymph nodes and liver, which were obtained at necropsy in a dog (dog 10) and DNA was extracted from these tissues. The HSV-TK DNA was amplified using a primer pair, 5′-(GGCTATGCTGGCT-GCGATTCCG)-3′ (sense) and 5′-(TCCGCGCAGCGG-CGGCGCAT)-3′ (antisense). 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 1 min (30 cycles) and additional extension at 72°C for 5 min. The amplified products were analyzed by electrophoresis on a 1.2% agarose gel and stained with ethidium bromide to detect the DNA fragment of 295 base pairs (bp).

Serum assay in dogs before and after gene transfer and therapy About 10 ml of blood was sampled from each dog for examination of general toxicity to various organs of retro- and adenovirus vectors carrying the lacZ gene and of HSV-TK/GCV gene therapy. Serum assay was performed by SRL Inc., Tokyo.

RESULTS

Induction of experimental gastric cancer in dogs All tumors were induced in the angular region and antrum of the stomach. Experimental data are summarized in Tables I and II. Mucosal irregularity was examined 8–9 months after the start of ENNG administration. Gross examination indicated that many types of tumors were induced, including polyps and types 1, 2 and 3 advanced gastric cancer (according to the Japanese Classification of Gastric Carcinoma).19) Adenocarcinoma was identified microscopically 8–15 months after the start of ENNG administration. Histological diagnosis of the stomach tumors19) was adenoma, which showed a glandular structure with low structural and cellular atypia; papillary and tubular adenocarcinomas, which showed the presence of papillary or tubular structures with cellular atypia, respectively; poorly differentiated adenocarcinoma, which showed little tendency to form glandular structures with severe cellular atypia and only scant cytoplasmic mucins; and signet-ring cell carcinoma, which showed abundant mucins and depressed nuclei. These different carcinoma histologies coexisted in one tumor and multiple tumors were located in the angulus-antrum in one stomach. There was no lymph node, liver or peritoneal metastasis. No esophageal cancer was induced in this experiment. Dogs were used for gene transfer and gene therapy experiments at 17–33 months (mean 21.9 months).

Comparison of efficacy of in situ transfer of retrovirus and adenovirus vectors Vectors were introduced in the tumor by needle through the biopsy channel of an endoscope. Injection points were marked by 0.2 ml of black ink. In situ injection of retroviral vector-producing cells containing the lacZ marker gene into the tumors (1.7×10⁴ cells/animal), which could be identified by blue X-gal staining, indicated that the vector was transferred into tis-
Table II. Macroscopic and Histopathological Characteristics of Canine Gastric Cancer Induced by ENNG and Treated by Gene Transfer

| Dog | ENNG Concentration (µg/ml) | Duration (month) | Detection of carcinoma by biopsy (month) | Time of gene transfer (month) | Detection of carcinoma by biopsy (month) | Time of gene transfer (month) | Vector Gene | Titer (pfu/ml) | Dose (pfu/animal) | Prodrug Outcome (month) |
|-----|---------------------------|------------------|------------------------------------------|-------------------------------|------------------------------------------|-------------------------------|-------------|---------------|-------------------|-------------------------|
| 1   | 100                       | 8                | 8                                        | 33                            | adeno HSV-TK                            | 1.0×10^8–6.2×10^8           | adeno HSV-TK | 1.0×10^8       | GCV autopsy        | 20                      |
| 2   | 100                       | 8                | 12                                       | 17                            | adeno lacZ                              | 9.8×10^7–6.2×10^7           | adeno lacZ   | 5.0×10^8       | —                 | autopsy 17            |
| 3   | 100                       | 8                | 8                                        | 12                            | adeno/ lacZ                             | 2.5×10^8–4.0×10^7           | adeno/ lacZ  | 1.0×10^8       | —                 | autopsy 12            |
| 4   | 100                       | 8                | 12                                       | 20                            | adeno/ lacZ                             | 2.8×10^8–6.2×10^8           | adeno/ lacZ  | 1.2×10^8       | —                 | autopsy 20            |
| 5   | 150                       | 6                | 9                                        | 26                            | adeno lacZ                              | 5.0×10^8–1.5×10^9           | adeno lacZ   | 5.0×10^8       | —                 | autopsy 19            |
| 6   | 150                       | 6                | 9                                        | 19                            | retro lacZ                              | 0.5×10^8–1.7×10^9           | adeno lacZ   | 5.0×10^8       | —                 | autopsy 19            |
| 7   | 150                       | 6                | 15                                       | 19                            | adeno lacZ                              | 1.0×10^8–1.5×10^9           | adeno lacZ   | 5.0×10^8       | —                 | autopsy 19            |
| 8   | 150                       | 6                | 15                                       | 27                            | adeno HSV-TK                            | 1.0×10^8–1.6×10^9           | adeno HSV-TK | 5.0×10^8       | GCV autopsy         | 27                      |
| 9   | 150                       | 6                | 16                                       | 24                            | adeno HSV-TK                            | 2.5×10^8–4.5×10^9           | adeno HSV-TK | 2.5×10^8       | GCV autopsy         | 24                      |

a) N-Ethyl-N'-nitro-N-nitrosoguanidine (ENNG) was given to dogs in 500 ml of drinking water.
b) Adenovirus vector (adeno) or/and retrovirus vector producing cells (retro) were injected into the tumors of the stomach.
c) lacZ marker gene or herpes simplex virus thymidine kinase (HSV-TK) gene were introduced in situ into the tumor of the stomach.
d) Ganciclovir (GCV) was given intravenously at 50 mg/kg twice a day.
e) Retrovirus-producing cells and adenovirus vector were injected into different tumors in the stomach.
f) This dog died of pylorus stenosis induced by gastric cancer in the antrum.
g) Retroviral vector producing cells/ml.
h) Retroviral vector producing cells/animal.
sues only at the immediate injection site of the primary tumor and no lacZ marker gene was found in other organs in one dog (dog 7). Thus, the low efficacy of transfer of retroviral vector resulted in failure of transfer of the transgene to the entire tumor. Next, we compared the efficacy of lacZ gene transfer between retrovirus and adenovirus vectors using multiple cancers in the antrum in 2 dogs (dogs 3 and 5). After in situ injection of Ad.CAGlacZ (2.0×10^4 and 6.2×10^4 pfu/animal), the lacZ gene was more widely observed around the injection sites in the tumor in comparison with the retroviral vector, which was limited to the injection site (in dogs 3 and 5, respectively).

Injection of Ad.CAGlacZ into the normal gastric mucosa (1.2×10^4 pfu/animal) also showed transfer of the vector around the injection sites in one dog (dog 5).

**Efficacy of gene transfer by adenovirus vector for primary tumor of the stomach** In one dog (dog 6), a single injection of adenovirus vector (1.0×10^8 pfu/animal) into a tumor using the improved needle, which had a blind end and 2 holes on the side, facilitated gene transfer only around the injection site. Next, in a second dog (dog 8), use of both the sclerotherapy and improved needles, and multiple injections of adenovirus vector into the tumor (1.5×10^9 pfu/animal), resulted in diffuse lacZ gene transfer in the primary tumors as identified by X-gal staining. However, gene transfer was localized around the injection sites and no complete gene transfer within entire tumors was observed. No macroscopic or histological change in the tumors was observed.

**Effect of gene transfer by adenovirus vector on regional lymph nodes of the stomach and other organs** By in situ gene transfer of Ad.CAGlacZ into the primary tumor in one dog (dog 8), the lacZ gene was also introduced into peripheral areas of regional lymph nodes (subcapsular marginal sinus) of the stomach. lacZ gene expression was not detected in other organs, i.e. liver, lung, kidney and testis, as identified by X-gal staining. The regional lymph nodes showed no macroscopic changes and no histological degeneration.

**Efficacy of gene therapy for primary gastric tumors** Efficacy of gene therapy by in situ transfer of Ad.CAGHSV-TK followed by intravenous injection of GCV was examined in 3 dogs (Tables I and II). In one dog (dog 9), type 1 gastric cancer, which macroscopically appeared as a polypoid tumor sharply demarcated from the surrounding mucosa, was observed in the angulus of the stomach, and Ad.CAGHSV-TK (1.0×10^9 pfu/ml) was injected into the proximal side of the tumor (1.6×10^9 pfu/animal). This dog was treated with 50 mg/kg/day GCV twice a day for 3 days and autopsied after 7 days. Grossly, the proximal part of the type 1 carcinoma, in which the vector had been injected, was depressed. Histologically, papillo-tubular adenocarcinoma showed tissue degeneration with vacuolation of cancer cells with pyknotic nuclei, and hemorrhage was observed around the injection site of the tumor.

Next, we attempted to induce complete tumor degeneration by Ad.CAGHSV-TK and GCV suicide gene therapy. In one dog (dog 1), type 3 gastric cancer was observed from the angulus to the lesser curvature of the antrum of the stomach. Histology of biopsy specimens showed papillary and tubular adenocarcinoma, poorly differentiated adenocarcinoma and signet-ring cell carcinoma. Histology of a polyp (1 cm diameter) located in the lesser curvature of the prepylorus showed adenoma. Ad.CAGHSV-TK (1.0×10^10 pfu/ml) was injected into the mucosal layer of the type 3 carcinoma 10 times using the improved needle and 10 times into the submucosal layer of the tumor using the sclerotherapy needle, and also 2 times into the polyp (4.4×10^10 pfu/animal). This dog was treated with 50 mg/kg/day GCV twice a day for 4 days, and autopsied at 7 days, immediately after death.

Gross examination of the injection site showed ulceration of the marginal wall of this type 3 carcinoma and blackening of the polyp (Fig. 2, A and B). Histologically, tumor tissues showed marked degeneration in the mucosal and submucosal layers and the layer of the proper muscle was also degenerated to a smaller extent (Fig. 3A). Histopathological findings of carcinoma tissue degeneration were vacuolation and eosinophilic coagulation of the cells with pyknotic nuclei and deterioration of tissues (Fig. 3B). Massive hemorrhage and inflammatory cell infiltration were also noted. The tissue degeneration covered all areas of the cancer and also of the polyp (Fig. 4, A and B).

**Effect of gene therapy on normal gastric mucosa** In dogs 9 and 10, no macroscopic change was observed in the normal gastric mucosa after HSV-TK/GCV therapy and histologically no tissue degeneration was observed in the antral and corpus mucosa. In dog 1, the surrounding normal mucosa of the type 3 gastric cancer showed marked degeneration of normal pyloric mucosa and the submucosa. Distant normal mucosa from the type 3 gastric cancer showed no abnormal macroscopic or histological findings.

**Efficacy and effect of gene therapy on regional lymph nodes and other organs** In dog 9, following injection of Ad.CAGHSV-TK into the primary tumor followed by GCV treatment, tissue degeneration showing vacuolation with pyknotic nuclei was also observed in the regional lymph nodes of the stomach. In dog 1, all seven regional lymph nodes of the stomach (groups 1 to 3 according to the lymph node groups in the Japanese Classification of Gastric Carcinoma[30]), which were extirpated at autopsy, showed almost complete degeneration with vacuolation, eosinophilia and dissolution of lymphatic cells with pyknotic nuclei and massive hemorrhage (Fig. 5, A, B and C). Histologically, no metastasis and no distinct abnormal
Fig. 2. (A) Gross features of the stomach in dog 1, in which an adenovirus vector carrying the HSV-TK gene was injected into the type 3 advanced gastric cancer in the lesser curvature of the angulus-antrum and an adenomatous polyp in the prepylorus followed by intravenous GCV treatment. Depression of the marginal wall of type 3 cancer is indicated with an arrowhead and a black polyp with arrow. (B) Scheme of histological area of tissue degeneration indicated by oblique lines. In the main tumor, four artificial ulcerations were induced by Ad.CAGHSV-TK/GCV treatment around ulcerated carcinoma infiltrating the surrounding wall. A polyp also showed degeneration.

Fig. 3. (A) Histological features of the main ulcerated carcinoma of Fig. 2. Marked tissue degeneration of cancer cells is observed in the mucosal and submucosal layers. The entire carcinoma tissue showed marked vacuolation and eosinophilia (H-E staining; original magnification ×100). (B) Higher magnification of (A) showing degenerated cells with vacuolation, eosinophilia and dissolution of carcinoma cells with pyknotic nuclei on the left and a few viable carcinoma cells on the right (H-E staining; original magnification ×200).
Histological findings were observed in the liver, spleen, kidney, lung or testis.

**Detection of HSV-TK gene in the tumor, normal mucosa and regional lymph nodes of the stomach and liver**

One dog (dog 10) showed multiple small type 3 and type 2 gastric cancers with macroscopic ulceration, in the antrum of the stomach. This dog was treated with Ad.CAGHSV-TK (2.5×10⁹ pfu/ml; 4.5×10⁹ pfu/animal) followed by 50 mg/kg/day of GCV twice a day for 3 days and autopsied after 7 days. Histologically, tissue degenera-
Japanese Classification of Gastric Carcinoma[20]) and two human stomach lymph nodes nos. 3, 4 (group 1 in the tumor. The regional lymph nodes, which correspond to the primary tumor, showed moderate hepatotoxicity after HSV-TK gene transfer and before GCV treatment, and severe hepatotoxicity after GCV treatment. No significant renal toxicity, as shown by blood urea nitrogen (BUN) and creatinine, was observed in any dog. There was no significant difference in nine dogs between before and after gene transfer or HSV-TK/GCV gene therapy in electrolytes, gastrin, amylase, total protein, total and direct bilirubin, total cholesterol, triglyceride and uric acid.

DISCUSSION

Recently, scientists and clinicians are beginning to realize the limitations of cancer gene therapy, with no clinical trials to date having demonstrated marked effectiveness. However, the difficulty of the task should not dissuade us from developing phase I/II studies because it is only in this way that we will be able to achieve the desired goal.[20]

GI tract cancers are an important potential target for gene therapy, because of their high incidence worldwide, including colon cancer in the United States and stomach cancer in Japan. A clinical trial of HSV-TK/GCV treatment for brain tumor has been performed in the United States, but to date no clinical studies have applied this gene therapy approach to GI tract cancer. In the present study, we applied gene therapy to the treatment of gastric cancer in a canine model. This model has a number of advantages.[14] In dogs it is possible to induce cancer by using chemical carcinogens in vivo rather than by transplantation of tumor cells.

In addition, this animal model has the advantage of 100% induction of stomach cancer by oral administration of ENNG, with no induction of other tumors except for esophageal cancer at a high dose of ENNG.[13, 14] Macroscopic and histological features of the carcinomas induced in these dogs were more similar to human stomach cancer than is the case in small rodent models. We followed the mucosal changes and tumor induction every 2–11 months by endoscopy under general anesthesia. Grossly and histologically, multiple cancers in the stomach were induced in the antrum; some were located intra-mucosally, some invaded the submucosa, and some invaded the proper muscle to the subserosa. It was reported[14] that this canine gastric cancer in animals with a lengthy survival (more than 600 days) showed metastasis to lymph nodes and occasionally, to liver, lung, bones, as well as peritoni-
show high replication. Secondly, we used an adenovirus to transfer the lacZ gene. Cell division is not required for gene transfer and expression using adenovirus vectors. Adenovirus vector carrying the lacZ gene was injected into tumors in 3 dogs, and X-gal staining showed that the lacZ gene was widely transferred into the submucosa around the injection sites, but was only transferred into a limited intra-mucosal area. Use of both a needle for sclerotherapy and a new needle for in situ gene transfer with vector was successful in dogs 3, 5, and 7, although metastasis to the lymph nodes, peritoneal cavity, liver or distant organs was found in this study, although metastasis to the regional lymph nodes would have been difficult to detect histologically in dogs treated with Ad.CAGHSV-TK/GCV because of severe tissue degeneration. Finally, all of the instruments including the endoscope and injection needles used in this study were the same as those used in the clinic.

We transferred retrovirus and adenovirus vectors in situ by injection through the biopsy channel of an endoscope. This delivery approach has previously been applied to lung cancer. 22 In situ gene transfer by endoscopy should be easily applicable to all GI tract cancers, and the present study is the first trial of this method for gastric cancer.

We initially examined the efficacy of retrovirus vectors. Retrovirus-producing cells packaging the lacZ gene of E. coli were injected into tumors in 3 dogs (dogs 3, 5 and 7) and X-gal staining showed the gene was only transferred into tissue at the injection site in the stomach. Clearly, this efficiency was too low to cure all of the tumor of the stomach. Expression of retrovirus vectors is limited to cells undergoing replication at the time of infection, giving this approach a particular advantage for targeted gene delivery to brain cancer. 23 The ineffectiveness of retrovirus vector-mediated gene transfer to gastric cancer may be due to the fact that gastric cancer is not encapsulated and generative cell zones of normal gastric mucosa show high replication. Secondly, we used an adenovirus vector to transfer the lacZ gene. Cell division is not required for gene transfer and expression using adenovirus vectors. Adenovirus vector carrying the lacZ gene was injected into tumors in 3 dogs, and X-gal staining showed that the lacZ gene was widely transferred into the submucosa around the injection sites, but was only transferred into a limited intra-mucosal area. Use of both a needle for sclerotherapy and a new needle for in situ gene transfer with vector was successful in dogs 3, 5, and 7, although metastasis to the lymph nodes, peritoneal cavity, liver or distant organs was found in this study, although metastasis to the regional lymph nodes would have been difficult to detect histologically in dogs treated with Ad.CAGHSV-TK/GCV because of severe tissue degeneration. Finally, all of the instruments including the endoscope and injection needles used in this study were the same as those used in the clinic.

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degeneration were compatible with those of apoptosis and we detected DNA ladder formation by gel electrophoresis in these tissues (data not shown). Moreover, we suspect a bystander effect in this HSV-TK/GCV system because massive tissue degeneration was observed in an area that extended beyond the injection sites. Tanaka et al. reported a bystander killing effect of the HSV-TK/GCV system in vitro in uninfected cells when only 20% of cells infected with adenovirus carrying the HSV-TK were mixed.

An interesting finding in this study was that the adenovirus vector was also transferred into the regional lymph nodes. Following HSV-TK/GCV treatment the regional lymph nodes examined were almost completely degenerated with hemorrhage. This degeneration of regional lymph nodes seems non-specific but this effect may be useful as a non-surgical procedure of lymphadenectomy. The systemic effect of complete degeneration of lymph nodes remains to be clarified.

In this study, normal gastric mucosa surrounding the tumor also showed tissue degeneration in dog 1, and therefore cancer tissue-specificity of the adenovirus vector was not observed in this experiment. The HSV-TK gene was identified by Southern blotting in dog 10 not only in the primary tumor, but also in regional lymph nodes of the stomach, but this gene was not detected in the normal gastric mucosa, which was distant from the primary tumor, or the liver. These results corresponded with the localization of the histological degeneration.

No significant hepatic or renal toxicity was observed by blood examinations after retrovirus or/and adenovirus vector transfer. Moderate hepatotoxicity was found after HSV-TK/GCV gene therapy in two dogs and one dog showed acute hepatotoxicity after Ad.CAGHSV-TK gene transfer and GCV treatment. Recently liver toxicity of the Ad.HSV-TK/GCV approach in mice was reported after injection of the vector into liver tumor. Moreover, liver dysfunction and mortality were seen in tumor-bearing and in tumor-free rats after intraportal administration of Ad.HSV-TK/GCV. The development of the liver damage (in terms of blood chemistry) suggests significant gene transfer to normal hepatocytes, which were subsequently killed by GCV, although the vector was not found by the ordinary Southern blot analysis. Indeed, HSV-TK gene was detected in the liver by PCR amplification. In this study, we examined toxicity at an early stage, but toxicity data after prolonged exposure are necessary before clinical usage of this gene therapy. Persistence of detectable expression of the viral vector after injection also remains to be clarified in the stomach and regional lymph nodes. It was reported that gene expression of adenovirus vector is low for the first 12 h after injection and is maximal at 24 to 36 h and that the half-life of phosphorylated GCV, the toxic metabolite, in the cell is 18 to 24 h. Therefore, acute toxicity may be important and other factors such as immunity to the adenovirus vector should be taken into consideration in further studies.

In this study, only 9 dogs with heterogenous gastric tumors were used to evaluate three types of vectors (retrovirus-lacZ, Ad.CAGlacZ and Ad.CAGHSV-TK) at different doses, so the data are essentially a case report. However, this animal model study showed that adenovirus vector is more widely distributed than retrovirus-producing cells, that high titers of vector and multiple injections/tumor were required for diffuse distribution in the primary tumors and that lymph nodes may be a good target for gene therapy. It remains to be clarified whether the tumor is finally destroyed or not, although we noted moderate to marked degeneration of cancer tissues. Acute hepatic toxicity should be taken into consideration in the combination of high dose of HSV-TK plus GCV. Given the hepatotropic nature of systemically administered adenovirus vectors, it will be essential to monitor liver functions of patients included in all gene therapy trials involving adenoviral vectors with the HSV-TK gene.

In combination with a tumor specific promoter, this in vivo, in situ method may become applicable to gene therapy for human GI tract cancer. In the future, a combined approach of both surgery and gene therapy may be applied to treatment of GI tract cancer.

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