Suicide Gene Therapy for Chemically Induced Rat Bladder Tumor Entailing Instillation of Adenoviral Vectors

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The efficacy of an *in vivo* gene therapy protocol making use of an adenoviral vector in the treatment of bladder cancer was examined. Bladder tumors were induced in rats by oral administration of BBN (N-butyl-N-(4-hydroxybutyl)nitrosamine). Histologically, such tumors resemble those seen in human bladder cancer, and the cells can be selectively transduced using adenoviral vectors. The therapeutic protocol thus entailed instillation of an adenoviral vector containing the *HSV-tk* suicide gene into rat bladder followed by a regimen of intraperitoneal ganciclovir (GCV) injections. Histological examination after a short-term GCV regimen (3 days) revealed marked vacuolization of the tumor cells. Moreover, TUNEL assays showed that the cytotoxic reaction was mediated by apoptosis. Following a long-term GCV regimen (14 days), tumor growth was significantly inhibited and glandular metaplasia was observed. This is the first report demonstrating the efficacy of *in vivo* suicide gene therapy in a chemically induced transitional cell carcinoma like that seen in most human bladder cancer. Intravesical instillation is already a well established clinical technique. Our findings indicate that now there is a strong potential for its incorporation into new and useful gene therapies aimed at the treatment of human bladder cancer.

Key words: Gene therapy — Bladder tumor — HSV-tk — BBN — Adenoviral vector

Most bladder tumors are superficial transitional carcinomas and are generally treated by transurethral resection of the bladder tumor (TUR-BT) followed by intravesical instillation of bacillus Calmette-Guerin (BCG).[1, 2] However, intravesical BCG is sometimes ineffective, and recurrence is common even when tumors initially respond to treatment. Further, when tumors do recur some have become infiltrative carcinomas[3] that are highly resistant to treatment, including cisplatin-based, multiple-agent chemotherapy.[4] Therefore, to improve the quality of life for patients with recurrent superficial or infiltrative bladder cancer, it is necessary to develop a new treatment that is capable of preserving the bladder.

The approved gene therapy protocols for the treatment of cancer are diverse but can be broadly categorized as 1) potentiation of tumor immunogenicity by the transfer of cytokine genes[5]; 2) inhibition of cancer growth by transfer of tumor suppressor genes[6]; and 3) direct gene therapy involving suicide gene transfer.[7] Among these, considerable advances have been made in suicide gene therapy, which involves transfer of the thymidine kinase gene of the herpes simplex virus (*HSV-tk*) followed by administration of ganciclovir (GCV), and clinical trials have already been conducted in patients with brain tumors and mesothelioma.[8–11] Metabolites of GCV inhibit DNA syn-

thesis and thus cause cell death via an action of the transferred *HSV-tk* gene.[12–15] This method should, therefore, be effective against cancer if efficient gene transfer can be accomplished. However, since this type of therapy is based on gene transfer *in vivo*, a high rate of specific transfer to the tumor cells is required. This makes the bladder an attractive organ for gene therapy because transurethral instillation is available, and observation, injection and biopsy can be easily performed with a cystoscope. In fact, intravesical therapy for human bladder cancer (e.g., BCG instillation after TUR-BT) is already performed commonly.

We have recently examined the efficacy and safety of intravesical instillation of adenoviral vectors to develop gene therapy protocols for bladder cancer.[16] In this study, bladder tumors were induced in rats by oral administration of BBN (N-butyl-N-(4-hydroxybutyl)nitrosamine) and were instilled with an adenoviral vector containing the *β-galactosidase* gene. The surface layers of the tumors were efficiently transduced, while the normal bladder mucosa was highly resistant to adenoviral transduction. The preferential transduction of the bladder tumors was probably due to the lack of the glycosaminoglycan (GAG) layers on their surface. The GAG layers are thought to play an essential role in preventing infection with microorganisms in the normal bladder.[17] These results encouraged us to consider adenoviral-mediated gene therapy of bladder cancer.

In the present study, BBN-induced bladder tumors were treated by instillation of an adenoviral vector containing the *HSV-tk* gene and intraperitoneal administration of GCV. Since BBN-induced tumors are transitional cell carcinomas like the vast majority of human bladder cancers, they are considered to be an excellent model of clinical bladder cancer.[18–20] Using this model system, we demonstrated preferential *in vivo* gene transfer to tumor cells within the bladder as well as the efficacy of the suicide gene therapy.

MATERIALS AND METHODS

Viral vectors An adenoviral vector containing the *HSV-tk* suicide gene under the control of the CAG promoter (Ad.CAGTK) was generated using the COS-TPC method.[21, 22] A control vector containing the *Escherichia coli lacZ* gene (Adex1CALacZ) was kindly provided by Dr. Saito of the Institute of Medical Science, University of Tokyo. The vectors used were purified and concentrated by two cycles of cesium chloride gradient centrifugation[21, 22] and had a titer of approximately 1×10^{10} pfu (plaque forming unit)/ml.

Animal model All animal experiments were conducted according to the institutional guidelines of Nippon Medical School.

Female Fischer 344 rats (seven weeks old) were purchased from Nihon CLEA Co., Ltd. (Tokyo). Bladder cancer was induced by adding 0.05% BBN to freely available drinking water for eight weeks.

Gene transfer by instillation of adenoviral vectors Rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg). For intravesical instillation, 1×10^{8} pfu of Ad.CAGTK or Adex1CALacZ dissolved in 0.5 ml of phosphate-buffered saline (PBS) was instilled transurethrally using a 4 Fr ureteral catheter for humans. After instillation, the external urethral orifice was ligated to prevent leakage, and the animals were only allowed to stand still. Ligatures were removed 2 h later.

Short-term experiment Initially, rats received intravesical instillation of 1×10^{8} pfu of Ad.CAGTK or Adex1-CALacZ vector as a control. Beginning two days later, 50 mg/kg GCV was administered intraperitoneally twice daily for three days. On the day after completion of the GCV treatment regimen, the animals were killed under deep ether anesthesia. A midline incision was then made in the lower abdomen to expose the bladder, the bladder neck was ligated and the organ was excised. The bladder was filled with 10% formalin for fixation, embedded in paraffin, cut into 2.5 μm sections and stained with hematoxylin-eosin to assess histologic changes.

Apoptotic cells were detected within specimens using the TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling) method, which recog-nizes the 3′ end of DNA fragments.[23–25] TUNEL was carried out with the *In Situ* Cell Death Detection Kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

Long-term experiment This experiment made use of four groups ($n=6$) of rats. Group A consisted of BBN-free normal rats. In group B, Ad.CAGTK (1×10^{8} pfu) was instilled intravesically 25 weeks after the start of BBN administration. Beginning three days later, 50 mg/kg GCV was administered intraperitoneally twice daily for 14 days. In group C, Adex1CALacZ (1×10^{8} pfu) was instilled intravesically; the animals were then subjected to the same GCV regimen as group B. Animals in group D were treated with BBN but received no anticancer therapy.

Two months after completing the GCV regimen (i.e., 36 weeks after the start of BBN administration), all rats were killed and their bladders excised, and the respective bladders and the bodies were weighed to determine the bladder weight (mg)/body weight (g) ratios. The bladders were then filled with 10% formalin for fixation, sectioned, photographed and subjected to hematoxylin-eosin staining for assessment of histologic changes. The response to therapy was evaluated by analysis of the bladder weight (mg)/body weight (g) ratio using Student's *t* test.

RESULTS

Short-term experiment Administration of 0.05% BBN to rats for eight weeks frequently induces superficial bladder tumors that occur after 20 weeks.[18–20] In our experiments, macroscopic tumors were detected in all animals. The previous work using an adenoviral vector containing the *lacZ* gene (Adex1CALacZ) showed that instillation of 1×10^{8} pfu of the vector could efficiently transduce bladder tumors with minimal inflammation reaction.[16] Accordingly we used the same amount of an adenoviral vector containing the *HSV-tk* gene (Ad.CAGTK) for gene therapy of bladder tumors. Rats received intravesical instillation of 1×10^{8} pfu of Ad.CAGTK followed by intraperitoneal injection of GCV.

There was no macroscopic difference in the appearance of bladders from treated and control rats. In each case, several tumors approximately 1 or 2 mm in diameter were found, and histologic examination revealed mild neutrophil infiltration in the group receiving adenoviral vectors. Our earlier experiments showed that intravesical instillation of adenoviral vectors causes preferential gene transfer to bladder tumor cells.[16] Consistent with that finding, marked vacuolization was observed in the cytoplasm of tumor cells from the Ad.CAGTK/GCV group (Fig. 1A). Such histological changes were not observed in bladders treated with Adex1CALacZ/GCV, Ad.CAGTK alone or GCV alone (Fig. 1, B, C and D). It appeared, therefore, that HSV-tk was indeed transferred to the tumor cells by
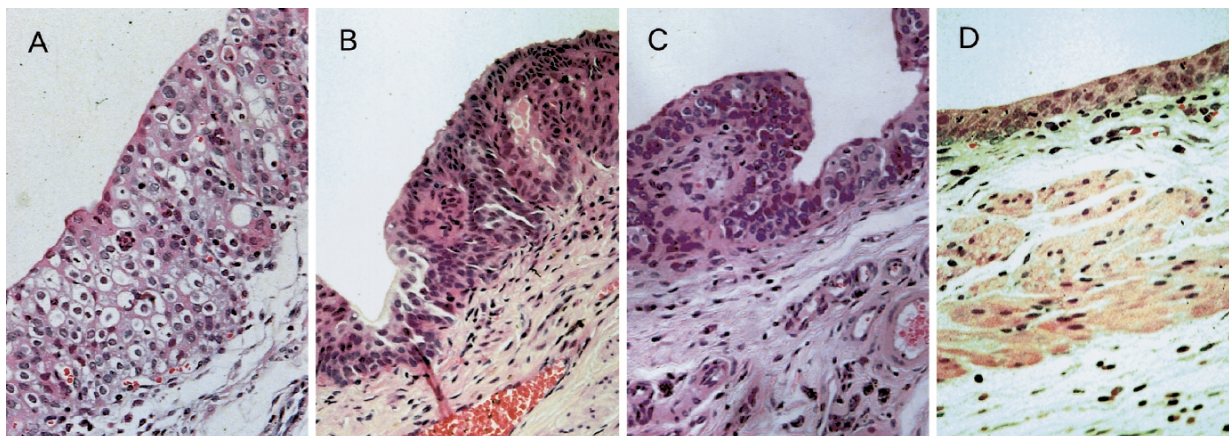
Fig. 1. Histologic findings of the short-term experiment (hematoxylin-eosin stain): (A), (B) and (C), BBN-induced bladder tumors; (D), normal bladder. Images are representative of tumor-containing bladders transfected with either Ad.CAGTK (A) or Adex1CALacZ (B) and subsequently treated with GCV; transfected with Ad.CAGTK alone (C); or normal bladders transfected with Ad.CAGTK and then treated with GCV (D). Magnification, 100×.
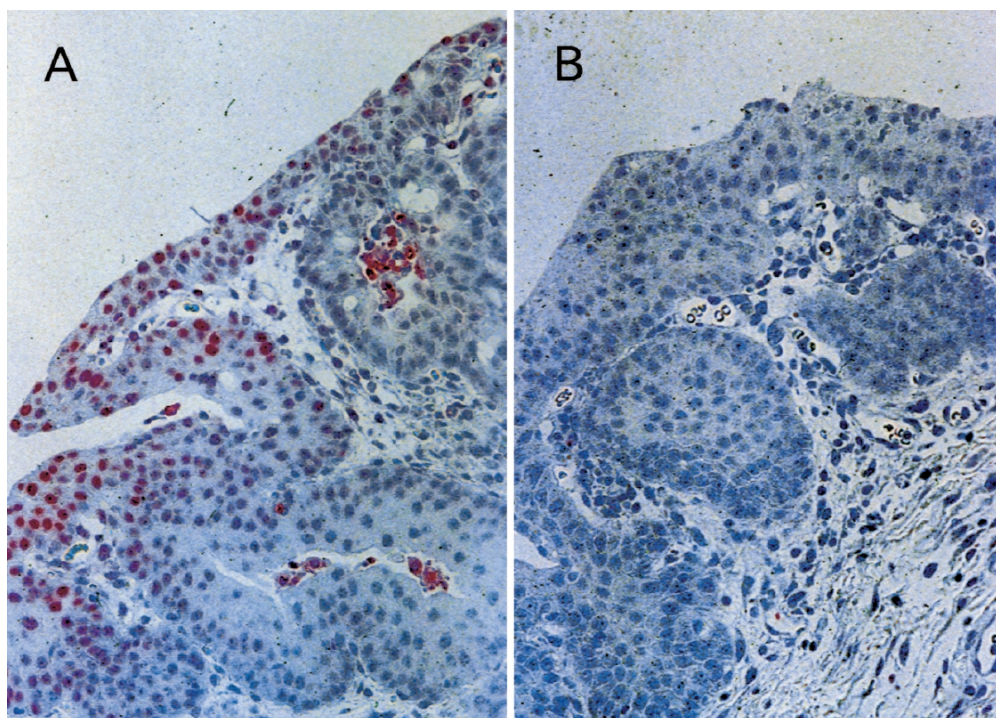

Fig. 2. *In situ* detection of apoptosis using the TUNEL method. BBN-induced rat bladder tumors were transfected with Ad.CAGTK (A), or Adex1CALacZ (B) and then treated with GCV.

intravesical instillation of the adenoviral vector and that cytotoxicity was induced by subsequent administration of GCV.

To determine the extent to which apoptosis was responsible for the cytotoxicity, specimens were examined using the TUNEL method. With this method, apoptosis is indicated by the fast red-stained nuclei of cells exhibiting DNA fragmentation.[23] Red-stained tumor cell nuclei were observed in bladders treated with Ad.CAGTK/GCV, primarily in the mucosa (Fig. 2A). In Adex1CALacZ/GCV-
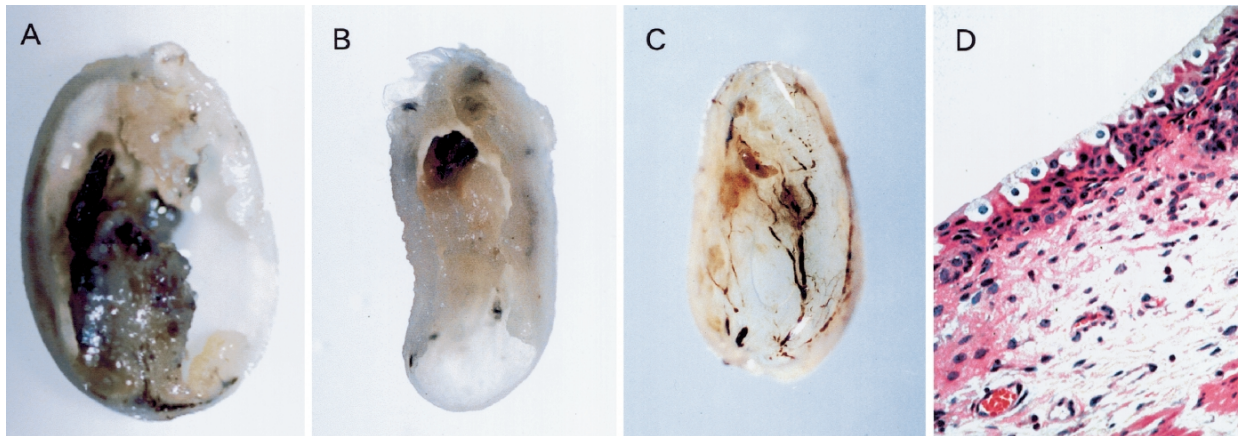
Fig. 3. Bladder tumors after the long-term experiment. Illustrated are representative bladders from an untreated rat (A; group D), a lacZ/GCV-treated rat (B; group C) and a HSV-tk/GCV-treated rat (C; group B). (D) Representative histologic findings from a HSV-tk/GCV-treated rat (group B). Sections were stained with hematoxylin-eosin stain; magnification, 100×.

treated bladders, by contrast, no red-stained nuclei were seen (Fig. 2B), nor were red-stained nuclei seen in the Ad.CAGTK or GCV controls (data not shown). Electrophoresis of DNA extracted from Ad.CAGTK/GCV-treated bladders revealed the ladder pattern characteristic of apoptosis (data not shown).[26]

Long-term experiment Because the results of the short-term study suggested that tumor cell death could be induced selectively by intravesical instillation of HSV-tk followed by GCV administration, we conducted additional experiments to evaluate the effect of long-term treatment on BBN-induced bladder tumors using the protocol described above in "Materials and Methods."

In untreated (group D) and lacZ/GCV-treated animals (group C), multiple papillomas developed which almost filled the bladder cavity (Fig. 3, A and B). Tumors also developed in HSV-tk/GCV-treated animals (group B), but they were substantially smaller than those in the control groups, suggesting that HSV-tk/GCV therapy caused tumor regression or growth inhibition (Fig. 3C).

Histologically, glandular metaplasia of the mucosa was observed in HSV-tk/GCV-treated bladders stained with hematoxylin-eosin. This change is generally seen after mucosal loss and was probably a response to therapy (Fig. 3D). No such changes occurred in the control groups.

The bladder weight (mg)/body weight (g) ratio is often used as an objective index to assess the efficacy of anti-cancer therapy against BBN-induced bladder cancer.[27] We observed that mean index of group B (HSV-tk/GCV group) was significantly smaller than the indices of either group C or D, which were themselves not significantly different. Thus, a significant therapeutic effect was only demonstrated in the HSV-tk/GCV-treated group (Fig. 4).
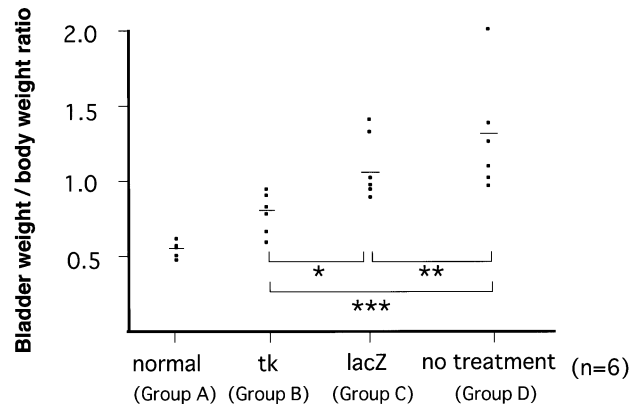

Fig. 4. Efficacy of suicide gene therapy in the treatment of BBN-induced rat bladder tumors. Symbols depict bladder weight (mg)/body weight (g) ratios. Horizontal bars indicate the mean ratio of each group. Means: group A, 0.52; group B, 0.70; group C, 1.04; and group D, 1.29. * $P=0.0147$; ** NS (not significant); *** $P=0.0099$.

DISCUSSION

Gene therapy is now considered to be an important therapeutic option for the treatment of bladder cancer.[16, 28, 29] Using a rat model of BBN-induced bladder cancer, we previously showed that genes can be preferentially transferred to tumors using intravesical instillation of adenoviral vectors.[16] In the present study, *in vivo* transfer of *HSV-tk* genes using an adenoviral vector followed by a regimen of GCV administration significantly reduced the size of bladder tumors, thereby demonstrating the efficacy of this gene therapy protocol.

The results of animal studies are important for investigating gene therapies eventually to be used in humans. Indeed, clinical trials of some gene therapies using protocols confirmed to be effective against cancer in mice and rats are already ongoing with the expectation of achieving similar responses in humans, and there have been several reports of clinical efficacy in the treatment of human cancer.[30] However, the beneficial effect of gene therapy has generally been far lower in humans than in animals, perhaps because of species differences or because of differences in the criteria used to choose candidates for therapy. At the present time, these differences in therapeutic efficacy between laboratory animals and humans have become a significant problem hindering further development of gene therapy protocols.[31]

Most of the important animal studies, which have formed the basis for several clinical trials of gene therapy, were performed on transplanted tumors produced by implantation of cell lines. Subcutaneous heterotopic graft models are often used because such studies are relatively easy to perform. Unfortunately, they completely ignore the histologic and anatomical specificity of the organs under study. Consequently, although they are useful in a limited fashion, these studies do not adequately serve as preclinical trials of human gene therapy. In their place, orthotopic graft models have been utilized. Orthotopic grafts are superior to the subcutaneous transplantation models with respect to the evaluation of the gene transfer methods and the assessment of the efficiency of *in vivo* gene transfer. Nonetheless, many differences from the actual tumor-bearing state in humans remain, especially with respect to the mechanism of carcinogenesis. It is thus questionable whether results from studies carried out using homogeneous cell lines selected *in vitro* will reflect the actual responses of histologically heterogeneous cancer in humans.

In the period since the report that oral administration of BBN induced bladder tumors in nearly 100% of rats,[32] BBN has become well established as a carcinogen useful for inducing experimental bladder tumors. Indeed, as a result of its selectivity, reliability and simplicity, this method is now commonly used for estimating clinical efficacy in humans.[18–20] This establishes the BBN-induced cancer model in rats as a reasonable model of human bladder cancer with respect to both biological and histologic properties, although the problem of species differences may still exist. This is the first report in which gene therapy was assessed using this model rather than a transplanted cancer, and our result confirming the effectiveness of HSV-tk/GCV is likely indicative of the protocol's potential for clinical application.

The HSV-tk/GCV protocol used in the present study has been the most intensively studied among a number of gene therapies and has undergone both basic and clinical investigations for various cancers. An aspect that makes this treatment very attractive is that *HSV-tk* gene-bearing tumor cells are killed directly and effectively by administration of GCV,[12–15] an effect not available with immuno-gene therapy. The mechanism of action is still open to discussion,[33, 34] but the bystander effect observed with this method, which may supplement the action of gene transfer that is not 100% efficient,[35–37] does not occur when apoptosis is induced by antioncogene transfer.[38] On the other hand, side effects caused by nonspecific transfer of *HSV-tk* genes have been reported,[39, 40] so this treatment requires the *HSV-tk* gene to be transferred specifically to the tumor cells. To accomplish this, the utilization of tissue-specific promoters[41] and targeting vectors with cell-specific, chimeric envelopes[42, 43] are being developed. However, such methods are not yet ready for practical use.

At present, mechanical targeting based on the histological and anatomical properties of organs, as is used for brain tumors,[8, 9] is the only method of specific transfer. Fortunately, the bladder is an organ that is easily approached from outside the body, and although it communicates with the kidneys via the ureters, it is separated from other important organs. Our method of gene transfer, based on the simple and well established technique of transurethral intravesical instillation, is less invasive than direct puncture of a tumor or organ and less invasive than systemic administration. RT-PCR analysis revealed that genes transferred in this way are specifically confined to the bladder, and that other structures including the ovaries and the kidneys are not involved,[16] most likely because the bladder is in the retroperitoneal space and separated from other organs. In addition, the anatomical function of the ureterovesical junction prevents distribution of vector to the kidneys via the ureter.

Previous study from our laboratory showed that instillation of adenoviral vectors resulted in selective gene transfer into bladder tumors, but only superficial cells were transduced. Therefore, it is somewhat surprising that marked vacuolization of the tumor cells and significant inhibition of tumor growth were observed in the present study. One possible explanation for this apparent discrepancy is that the bystander effect may be highly efficient in this experimental system. Another possibility is that transduction efficiency based on X-gal staining may be underestimated. Although the deeper cells in the tumor were X-gal negative, it may be possible that these cells were also transduced with the adenovirus vector and weakly expressed LacZ or HSV-tk.

We have recently demonstrated that treating tumors with HSV-tk/GCV *in vivo* induced antitumor immunity mediated by tumor cell-specific cytotoxic T lymphocytes in the tranplanted model.[44] Therefore, it is likely that the vaccination effect also contributes to inhibition of tumor growth in the present tumor model. However, because

established tumor cell lines originated from BBN-induced bladder tumor are not available, we do not yet know precisely how cyototoxic T lymphocytes are involved in our experimental system.

Clinically, adenovirus is known to cause hemorrhagic cystitis.[45] In our experiments, the intravesical instillation of adenoviral vectors caused mild neutrophil infiltration of the mucosa, but hemorrhagic cystitis was not observed either macroscopically or histologically.

In conclusion, the present study has demonstrated the efficacy of *in vivo* transfer of the *HSV-tk* gene in treating an established model of human bladder cancer. The results of this preclinical study suggest strong potential for this therapy to be incorporated into a new approach to the treatment of human bladder cancer.

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