Usefulness of Repeated Direct Intratumoral Gene Transfer Using Hemagglutinating Virus of Japan-Liposome Method for Cytosine Deaminase Suicide Gene Therapy

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Abstract

To investigate the feasibility of repeated gene transfection in suicide gene therapy against human solid tumors by a combination of 5-fluorocytosine (5-FC) and its converting enzyme, cytosine deaminase (CD), we repeatedly transfected the yeast CD gene into the human pancreatic cancer cell line BXPC3 using the hemagglutinating virus of Japan-liposome in a new gene transfer method. The in vivo growth of the s.c. transplanted BXPC3 tumor in nude mice given CD-gene transfection was significantly suppressed by i.p. injection of 5-FC when compared with tumors treated with the control vector. Furthermore, the tumor transfected with the CD gene during a 7-day interval was suppressed much more than that of a single transfection. These results suggest that repeated transfection of the suicide gene together with the combination of 5-FC and the yeast CD gene using the hemagglutinating virus of Japan-liposome gene transfer method may be useful for the treatment of human solid tumors, including pancreatic cancer.

Introduction

Pancreatic cancer, one of the most malignant neoplasms, is usually diagnosed at a relatively advanced stage; early detection is difficult because of the remote internal location. Furthermore, it is often difficult to resect curatively, which results in poor prognosis and the highest mortality rate among all gastrointestinal malignancies. At present, the only options for treating advanced pancreatic cancer are radiation and chemotherapy with anticancer drugs.

The most widely used chemotherapeutic agent is 5-FU, which has been used against various gastrointestinal malignant neoplasms including pancreatic cancer. It has proved to be of some value but is sometimes limited by unfavorable side effects, especially when a large dose is given systemically. To decrease the side effects, various strategies have been tried, including suicide gene therapy (1–3) using molecular biological techniques, which seems to be one of the more promising options. Basically, the strategy of suicide gene therapy involves the introduction into tumor cells of nonmammalian genes that encode enzymes that convert nontoxic prodrugs into toxic anti-metabolites, together with the systemic application of the prodrugs. Theoretically, this should result in high local concentrations of 5-FU in the target tumor tissue. In the case of 5-FU, the prodrug would be 5-FC, an antifungal drug that is relatively nontoxic in humans even at a high dose (4). The conversion enzyme would be CD, which is expressed in yeasts and bacteria, but not in mammalian organisms, and can catalyze the hydrolytic deamination of cytosine to uracil. This should result in the nontoxic 5-FC being converted to toxic 5-FU.

The possibility of suicide gene therapy using this CD and 5-FC combination has been the subject of several recent studies. First, in 1992, Mullen et al. (1) and Austin et al. (3) reported that mammalian cells transfected with the bacterial CD gene were selectively killed by the addition of 5-FC into their culture medium. In an in vivo trial, Mullen et al. (2) reported that s.c. tumors in nude mice given injections of CD-expressing cancer cells had increased sensitivity for 5-FC administered i.p. Huber et al. (5) also reported similar results. In considering practical gene therapy for solid tumors, one of the most important issues is how to deliver the CD gene effectively into the tumor tissue. In 1995, Hirschowitz et al. (6) showed effective gene transduction by direct injection of adenovirus vector-containing CD-expression plasmid into nude mice tumors. In trials for targeting cancer cells, Kanai et al. (7) showed the usefulness of the CD plasmid with an α-fetoprotein promoter for gene therapy against hepatocellular carcinoma. Freytag et al. (8) reported an interesting adenovirus vector system in which the induced gene could be expressed only in p53-deficient cancer cells. Interestingly, Hanna et al. (9) and Khil et al. (10) reported the increased radiosensitivity of CD gene-transfected cancer cells. Moreover, Kuriyama et al. (11) showed the remarkable cooperative effect of host immunity against induced gene products in CD gene therapy by using a syngenic animal model. This is important to note because the host immune response is thought to be very important in the clinical course of cancer patients. All these investigations clearly demonstrate the potential usefulness of CD suicide gene therapy against human solid malignancies. In clinical application, two important issues are the safety and the availability of repeated administration. This requires careful selection of vector systems for delivering the CD gene into cancer tissue. Among various gene delivery systems, the retrovirus vector, adenovirus vector, or liposome method has been used. However, the retrovirus vector and liposomes are expressed at only relatively low levels in vitro, and cationic liposomes have some toxicity to mammalian cells. At present, the most frequently used system for transfecting foreign genes into various types of cells or tissues is that of using the adenovirus vector (12, 13). The transfection efficiency of adenovirus vector systems has proved to be high in many organs compared with that of the retrovirus vector or the liposome method. However, there are some problems with this system, e.g., human pathogenicity and the unfavorable immune reaction of the host to the adenovirus itself. These problems are common to various gene transfer systems currently available. Therefore, a new gene transfer system needs to be developed for safe cancer gene therapy.

One promising new method uses the HVJ, an envelope virus belonging to the Paramyxovirus, which is approximately 300 nm in diameter and causes fusion with the cell surface (14). Kaneda et al. (15) and Nakanishi et al. (16) engineered the HVJ-liposome gene transfer method, which directly transfers exogenous DNA into the
cytoplasm by fused liposome and inactivated HVJ. This new gene transfer method has recently been shown to offer useful possibilities against various malignant diseases, e.g., hepatocellular carcinoma (17), glioma (18), and melanoma (19).

Here, we report our in vivo studies on the feasibility of suicide gene therapy for pancreatic cancer using 5-FC and the converting enzyme CD introduced via this new gene transfer method, the HVJ-liposome gene delivery system.

Materials and Methods

Cell Line. Human pancreatic cancer cell line BXPC3 was maintained in DMEM (Nikken Biomedical Laboratory, Tokyo, Japan) containing 10% fetal bovine serum and antibiotics-antimycotics consisting of 10,000 units/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B as fungizone R in 0.85% saline (Life Technologies, Inc., Grand Island, NY) at 37°C in 5% CO₂.

Expression Vector. The expression plasmid pEBActNII, which contains chicken β-actin promoter and enhancer and is 10.3 kb in size, luciferase expression plasmid pEBActNII/luci., and LacZ expression plasmid pEBActNII/LacZ were prepared at the Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan. Yeast CD cDNA (474 bp) was cloned and kindly presented by the Bristol-Myers Squibb Pharmaceutical Research Institute. CD expression plasmid pEBActNII/luci. was constructed by inserting yeast CD cDNA at the HindIII and XhoI sites of the pEBActNII plasmid.

Preparation of Cationic HVJ-Liposomes. Dried lipid mixture (20) was hydrated in 200 μl of BSS containing plasmid DNA (200 μg). The mixture was agitated intensely by vortexing for 30 s and then left to stand for 30 s. This procedure was repeated eight times. The liposome suspension was extruded three times through a 0.45-μm cellulose acetate membrane filter and then three times through a 0.25-μm filter. The liposome suspension was mixed with 30,000 inactivated hemagglutinating units of HVJ and left on ice for 10 min, then incubated at 37°C for 2 h with shaking (120/min) in a water bath. The HVJ-liposome complexes were then separated from free HVJ by sucrose density gradient centrifugation. The mixture was layered onto a discontinuous sucrose gradient (1 ml of 50% and 6.5 ml of 30% sucrose in BSS) and centrifuged at 62,800 g for 2 h in a swing-bucket rotor. The HVJ-liposome were then visualized in a layer between the BSS and the 30% sucrose solution and then collected.

X-Gal Staining. BXPC3 cells (5 × 10⁶) were inoculated s.c. into athymic BALB/c nu/nu mice (6–8 weeks of age, female; CLEA Japan, Inc., Tokyo, Japan). Thirteen days after inoculation, the HVJ-liposome solution containing pEBActNII/LacZ or pEBActNII/luci. (50 μl) with 1 mM CaCl₂ was injected directly into the tumor using a 29-gauge needle. Three or 7 days after transfection, the mice were sacrificed using diethyl ether, and the tumors were fixed with 4% paraformaldehyde at 4°C for 3 h then dehydrated with 20% sucrose at 4°C overnight and embedded in OCT compound. Five-μm sections were prepared and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside solution (1 mg/ml) was added before incubation at 37°C for 20 h. The number of β-galactosidase-positive cells was counted under a microscope. All animal procedures followed the established guidelines of Osaka University Medical School.

Expression of CD mRNA. To confirm the expression of CD mRNA, total RNA was extracted from the CD-transfected tumors at 3 or 6 days after transfection, and RT-PCR was performed as described previously (21) with yeast CD-specific primers: sense, ATGGTGACAGGGGGAATGGC; and antisense, TCCAAAGTGGAGATCTCACC. As an internal control, RT-PCR of the housekeeping enzyme GAPDH was performed in the same manner.

Confirmation of Exchange from 5-FC to 5-FU in Vitro. To confirm the exchange of 5-FC to 5-FU, 1 × 10⁶ cells were placed in 96-well microplates and incubated at 37°C with 5% CO₂ overnight, and then 0.33 μl HVJ-liposome solution containing pEBActNII/CD or pEBActNII/LacZ or neither was added to each plate. The samples were incubated for 12 h, then the medium containing HVJ-liposome solution was removed and changed to one containing various concentrations of 5-FC and incubated for 4 days. The supernatants were collected and the 5-FU concentration was measured by the enzyme immunoassay method (Otsuka Assay Laboratories, Tokyo, Japan).

In Vivo Growth Inhibition. To examine the growth inhibition in vivo, BXPC3 (5 × 10⁶ cells) was inoculated s.c. into athymic BALB/c nu/nu mice (6–8 weeks of age, female; CLEA Japan, Inc., Tokyo, Japan). Thirteen days after inoculation, the HVJ-liposome solution containing pEBActNII/CD or pEBActNII/LacZ (50 μl) or neither, with 1 mM CaCl₂, was injected directly into the tumor using a 29-gauge needle. Beginning 1 day after transfection, 500 mg/kg body weight 5-FC was administered daily for 2 weeks by i.p. injection (single injection experiment). In the other group, the HVJ-liposome solution containing pEBActNII/CD or pEBActNII/LacZ (50 μl) or neither was injected again 1 week after the first injection, and 5-FC was administered in the same manner as the single-transfection group (double injection experiment). The tumor size was measured in a blind fashion with calipers and calculated as [length (mm) × width (mm)]²/2 (5).

Statistical Analysis. Statistical analysis was performed using the Mann-Whitney U test. P < 0.05 was considered to be statistically significant.

Results

Transfection Efficiency in Vivo. To elucidate the gene induction efficiency into BXPC3 by the HVJ-liposome method in vivo, the LacZ gene was the negative control. X-Gal staining was performed as described in the text. The X-Gal reaction time was ~20 h. The number of β-galactosidase-positive cells was counted. BXPC3 cell tumor transfected with the luciferase gene was the negative control. Glioma cells were transfected with the LacZ gene tumor as a positive control: BXPC3/Lac Z. BXPC3 cell tumor transfected with the Lac Z gene at 3 or 7 days after transfection.
gene or the luciferase gene was transfected directly into the tumors of BXPC3 nude mice. At 3 or 7 days after transfection, X-Gal staining was performed. At day 3, ~30% of the cells were β-galactosidase-positive, but at day 7, almost none were β-galactosidase-positive (Fig. 1).

Expression of CD mRNA. To confirm the expression of CD mRNA, RT-PCR was performed with yeast CD-specific primers. Fig. 2A shows that 206-bp PCR products could be recognized in CD-transfected cells at 3 days and at 6 days after transfection (Lanes 4 and 5). GAPDH gave 181-bp PCR products in all Lanes, showing the intactness of RNA. Although the amount of CD expression decreased day by day in the transient transfection assay, CD mRNA expression could be confirmed until at least 6 days after transfection.

Confirmation of Exchange from 5-FC to 5-FU in Vitro. To confirm that CD actually caused the exchange of 5-FC to 5-FU, BXPC3, BXPC3/LacZ, and BXPC3/CD were added with various concentrations of 5-FC, and after 4 days of incubation the supernatants were collected, and 5-FU concentrations were measured. White box, BXPC3; gray box, BXPC3/LacZ; dark gray box, BXPC3/CD.

In Vivo Growth Inhibition. To examine the growth inhibition in vivo, BXPC3 (5 × 10⁶ cells) was inoculated s.c. into nude mice, and HVJ-liposome solution containing pEBActNII/CD or pEBActNII/LacZ (50 µl) or neither was injected directly into the tumor once or twice. 5-FC was administered by i.p. injection daily for 2 weeks at the dose of 500 mg/kg body weight. The tumor growth of the single CD transfection group (n = 8) treated with 5-FC was remarkably suppressed in comparison with that of the control group (Fig. 3A). The tumor size was reduced ~55% at 28 days after transfection. Furthermore, the growth of tumors treated with the twice-transfected group (n = 9) was suppressed much more than that of the control group (Fig. 3B). The tumor size was reduced ~72% at 28 days after transfection. There was a statistical difference in tumor size at 28 days after transfection between the single-transfection group and the twice-transfected group (P = 0.0045). Fig. 4 shows the representative change in the tumors of nude mice at day 28 after transfection.

Discussion

5-FU is an antimetabolic chemotherapeutic drug that is very broadly used for various types of gastrointestinal malignancies. In patients whose tumors are sensitive to this agent, the anticancer cytotoxic effect mainly depends on the concentration of 5-FU in the tumor tissues. 5-FU is usually given i.v. or p.o., however, its systemic administration sometimes causes unfavorable toxic side effects because of its similar cytotoxic effects on normal cells, e.g., gastrointestinal mucosal epithelial cells or bone marrow cells. In such cases, 5-FU therapy must be stopped.
Several strategies have been reported to diminish the side effects of 5-FU. Also, in the course of related studies, several nontoxic prodrugs for 5-FU have been developed. Among them, 5-FC, which has been used as an antifungal drug because of its relative nontoxicity in humans, is a prodrug that can be converted to 5-FU by the enzyme CD. The enzyme activity of CD can be found in bacteria and in fungi but not in mammalian cells. In the strategy of suicide gene therapy, 5-FC administered systemically is converted to 5-FU by the enzyme CD. The fusion with the cell membrane, not by endocytosis, thus minimizing surface (14). Its mechanism of gene induction into the cytoplasm is by the fusion with the cell surface membrane. In this study, the transfection tumor. The rationale for this is that yeast belongs to the eukaryotes, as do humans, and the conversion efficiency of 5-FC to 5-FU of yeast CD is reported to be higher than that of bacteria (22). Thus, yeast CD is should be more appropriate for therapeutic application in humans.

For various experiments on gene transfer, adenovirus vectors have been most frequently used. Their transfection efficiency is high compared with those of the retrovirus vector, liposome, or other vectors in many organs in vitro or in vivo. At present, the adenovirus vector is considered to be the best and most useful vector because of its satisfactory results without any serious side effects in various trials of gene therapy for cancer patients. However, several problems such as pathogenicity to humans or unfavorable host immunoreaction when administered repeatedly have been reported. For the present study, in the interest of safety, we selected a nonvirus vector, the hemagglutinating virus Japan (HVJ)-liposome, which has been used in gene therapy against several malignant diseases (17–19). The HVJ-liposome gene transfer method, which directly induces exogenous DNA into the cytoplasm by fusing liposome and inactivated HVJ, was originally developed by Kaneda et al. (15) and Nakashishi et al. (16). HVJ is an envelope virus belonging to the Paramyxovirus group that is approximately 300 nm in diameter and becomes fused to the cell surface (14). Its mechanism of gene induction into the cytoplasm is by fusion with the cell membrane, not by endocytosis, thus minimizing injury to the cell. Its most important feature is its complete lack of pathogenicity to humans. Hirano et al. (23) reported that the transfection efficiency was not affected by repetitive transfection by this method in rat liver, and the detected antibody response to HVJ-liposome was minimal and transient. Also, no CTLs were elicited. Hangai et al. (24) reported that when LacZ DNA was injected into the anterior chamber of the eyeball of rhesus monkeys by this method, there was no change in the operating site, no sign of eye irritation, nor a histological change in the treated tissues and apparent tissues. Hagihara et al. (25) reported the safety of this method for the central nervous system of the monkey, and the safety of i.v. continuous administration of HVJ-liposome in large quantities into the saphenous vein of the monkey has also been confirmed. However, the transfection efficiency for human pancreatic cancer cell BXPC3 used in the present study was ~30% in this system, which is relatively low compared with that of the adenovirus vector. Now, we are trying to increase the transfection efficiency of the HVJ-liposome method via several modifications, e.g., change of the lipid constitutions of the liposome, selection of either cationic or anionic liposomes depending on cell type, and the combined use of modifying enzyme molecules. Among these studies on this system, Yamano and Kaneda et al. have reported that CD expression could be increased in malignant cells if it were coupled with histone deacetylase inhibitor. Thus, if the transfection efficiency can be increased, this HVJ-liposome method could become a good candidate for a vector system for cancer gene therapy in addition to the adenovirus vector system.

From the viewpoint of clinical application, of utmost importance in using foreign genes is delivering them efficiently and safely only to the target organs. This is sometimes very difficult to do from outside the body, but it is important, particularly in suicide gene therapy, because of the possibility of systemic side effects. It is also difficult to insert foreign genes that are not originally found in humans into the chromosome of normal human cells. Therefore, the delivery of foreign genes directly into the target site was considered to be the most practical and safe option. This led us to try the direct injection method.

To examine the antitumor effect in vivo, BXPC3 cells were inoculated s.c. into nude mice, and after tumor formation had been confirmed, the CD gene was transfected into the tumor once or twice, at a 7-day interval, and 5-FC was administered i.p. The growth of the tumor transfected with the CD gene and then treated with 5-FC was suppressed compared with that of the control tumors. Furthermore, growth of the tumor transfected with the CD gene was suppressed much more than that of either the control tumor or the single-transfection tumor. Our finding that the repeated injection of CD

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5 Kaneda et al., unpublished data.
plasmid was remarkably effective should be noted. In clinical situations, the treatment against malignant neoplasms is usually performed continuously or repeatedly, and the repeated application without toxicity or host immunoreaction is one of the greatest advantages of this HVJ-liposome vector system.

In conclusion, our results clearly show that suicide gene therapy can be performed very effectively by repeated direct administration of the CD gene using the HVJ-liposome method. This new gene transfer system should be useful for gene therapy of solid tumors, especially surgically unresectable advanced cancers.

Acknowledgments

We thank Drs. Y. Kawabata and M. Tada for their valuable advice and R. Matsuyama for her excellent secretarial assistance.

References

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