Trans-Tissue, Sustained Release of Gemcitabine from Photocured Gelatin Gel Inhibits the Growth of Heterotopic Human Pancreatic Tumor in Nude Mice

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ABSTRACT

Purpose: Although gemcitabine, a deoxycytidine analogue, recently demonstrated improvements in the response rate for pancreatic cancer, the median survival for patients is limited to 4–6 months. The purpose of the present study was to develop trans-tissue delivery of gemcitabine, which is based on photocured gelatin gel immobilized with gemcitabine, and to validate whether such a system inhibits the growth of the pancreatic tumor in vivo.

Experimental Design: The in vitro release profile of gel-embedded gemcitabine from a gel was examined based on in vitro chemosensitivity of AsPC1 cell (human pancreatic cancer cell line) for gemcitabine. The permeation of gel-embedded rhodamine B (used as a model drug) into tissues and inhibitory effect of tumor growth of photocured gelatin gel immobilized with gemcitabine were examined using in vivo s.c. tumor model of athymic mice.

Results: The release profile was characterized as an initial burst of release, followed by a gradual release, irrespective of gelatin concentration. Rhodamine B permeated into the tumor and retained for at least 10 days. Photocured gelatin gel immobilized with gemcitabine significantly reduced the tumor volume compared with gemcitabine injection. Therapeutic success was correlated with decreased cell proliferation and increased cell apoptosis in tumor cells, supported by proliferating cell nuclear antigen and terminal deoxynucleotidyltransferase-mediated nick end labeling staining. Blood analysis and body weight measurement showed that little side effect was observed in this therapy.

Conclusions: In situ trans-tissue gemcitabine delivery on the tissue with possibly remnant cancer cells using the drug-releasing matrix developed here is expected to reduce the rate of local recurrence for patients with pancreatic cancer.

INTRODUCTION

Pancreatic cancer has been a major unsolved health problem. It is estimated that only 1–4% of patients with pancreatic cancer will be alive for 5 years after diagnosis (1). This is largely attributable to difficulties in diagnosis, the progressive growth and metastasis even after extensive surgical operation, and the lack of effective systemic therapies (2–4). Recently, systemic administration of gemcitabine (2’-2’-difluorodeoxycytidine), a deoxycytidine analogue, demonstrated modest improvements in response rate (5–9). However, the median survival for patients with pancreatic cancer is limited to 4–6 months (6, 9). Although alternative gemcitabine schedules and chemotherapy combinations have been developed (6, 10), a novel administration strategy is required to improve the prognosis of the patients who have been surgically treated.

Our strategy is passive transport of gemcitabine, which is sustained-released from an in situ formed tissue adhesive matrix on a surgically resected tissue to targeted tissues for a prolonged period, resulting in the prevention of growth and metastasis of remnant cancer cells after extensive surgical operation. The in situ formable, drug-immobilized tissue adhesive matrix designed for this application is photocurable gelatin, which is derivatized with styrene groups in gelatin molecule, and photogelled under visible light irradiation (11). Our experimental research using Wistar rat showed that using this matrix, a proteinaceous drug, was able to permeate into the deeper region of tissue (12). The continuously permeated or diffusion-driven drug resulted in accumulation of high concentration of the drug at a targeted tissue and exhibited prolonged effectiveness while minimizing systemic side effect. Such a passive transport, trans-tissue drug delivery, may enable to realize the reduced local recurrence rate and concomitantly prolonged survival period for patients.

The present study is designed to determine whether gemcitabine-immobilized styrenated gelatin gel works effectively to inhibit the growth of pancreatic tumor heterotopically inoculated in nude mice. Such results will promise high efficacy of passive transport cancer therapy using the tissue adhesive, drug-immobilized, local DDS.3

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3 The abbreviations used are: DDS, drug delivery system; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCNA, prolif-
MATERIALS AND METHODS

Reagents. Styrenated gelatin (Mw 950,000) and water-soluble carboxylated camphorquinone [CQ: (IS)-7,7-dimethyl-2,3-dioxobicyclo(2.2.1) heptane-1-carboxylic acid] were synthesized according to the procedures previously reported (11, 12). Gemcitabine was commercially purchased from Eli Lilly and Co. (Indianapolis, IN).

Cell Line. AsPC1, a human pancreatic cancer cell line, was generously provided by Dr. Haruo Iguchi (National Kyushu Cancer Center, Fukuoka, Japan) and maintained in DMEM (Life Technologies, Inc. Laboratories, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (CSL Co., Ltd., Victoria, Australia), 100 units/ml streptomycin, and 100 units/ml penicillin.

Chemosensitivity Assay for Gemcitabine. Chemosensitivity of AsPC1 cell for gemcitabine was evaluated using MTT (Sigma-Aldrich, St. Louis, MO) assay according to the method previously described earlier (13). The cells (2 × 10³ cells) were plated on the each well of a 24-well microplate (Corning Incorporated, Corning, NY). Twenty-four h after plating, the cells were exposed to indicated concentrations (10⁻⁷−10⁻³ μg/ml) of gemcitabine for 24 h. After gemcitabine-containing medium was gently aspirated and the cells were washed once with fresh DMEM, 1 ml of gemcitabine-free DMEM supplemented with 10% fetal bovine serum was added to each well. After incubation for 72 h, MTT assay was performed, and absorbance was measured with the microplate reader (Model 550; Bio-Rad, Hercules, CA) at 570 nm. All test points were measured 10 times, and the results are shown as percentage of corresponding untreated control.

In Vitro Gemcitabine Release Test. One hundred mg of a viscous mixture of PBS (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) of styrenated gelatin (30, 40, or 50 weight percent based on the total gel weight), carboxylated CQ (0.05 weight percent based on the styrenated gelatin weight) and gemcitabine (3 weight percent based on the total gel weight) was coated on the each well of a 48-well microplate (Corning Incorporated) and subsequently irradiated with visible light for 3 min using an 80-W halogen lamp (Tokuso Power Lite; Tokuyama Co., Ltd., Tokuyama, Japan) to form a gemcitabine-immobilized gel. Subsequently, 1 ml of DMEM was added to each well, and the solution was withdrawn at regular intervals. Chemosensitivity assay was conducted using the withdrawn solution in the same procedure as described above. The amount of gemcitabine released from the photocured gelatin gel was calculated from a linear relationship of concentration dependency of chemosensitivity of AsPC1. All test points were measured 10 times, and the results were plotted as percentage of cumulative release.

Drug Permeation Test. To examine whether gemcitabine (Mw 299) released from photocured gelatin gel permeates into the tissue in vivo, a red-color dye, rhodamine B (Mw 479; Sigma-Aldrich), was used as a model drug for assessment of some information on the drug permeation, although releasing and permeation characteristics of rhodamine B is not necessarily similar to those of gemcitabine but may provide some information on distribution and permeation of gemcitabine into a tissue. AsPC1 cells (3 × 10⁶ cells suspended in 50 μl of DMEM), which were preincubated with DiO cell-labeling solution (Molecular Probes, Eugene, OR) for 2 h, were s.c. inoculated in the right flank of 7-week-old BALB/c nude (nu/nu) mice (Kyudo Co., Ltd., Saga, Japan). Three days after inoculation, a surgical skin incision was made, and 100 mg of a viscous mixture of PBS of styrenated gelatin (50 weight percent based on the total gel weight), carboxylated CQ (0.05 weight percent based on the styrenated gelatin weight) and rhodamine B (0.3 weight percent based on the total gel weight) was coated on the pancreatic tumor s.c. inoculated in the nude mouse and subsequently irradiated with visible light to form a gel. Separately, rhodamine B solution (0.3 mg in 100 μl of DMEM) was s.c. injected near the tumor. Mice were sacrificed at days 1, 3, and 10 after gelation or injection and subjected to cryostat sections with a microtome (CM 1850; Leica, Nussloch, Germany) and then observed under confocal laser scanning microscopy (Radiance 2000; Bio-Rad) at the setting of excitation of 488 nm and emission of 515 ± 30 nm for DiO and at the setting of excitation of 514 nm and emission of 600 ± 50 nm for rhodamine B, respectively.

Therapy for Pancreatic Tumor in Vivo. An in vivo experiment to evaluate the therapeutic effect of photocured gelatin gel immobilized with gemcitabine (styrenated gelatin concentration: 50 weight percent) was carried out using the pancreatic tumor s.c. inoculated in nude mice, which was prepared in the same procedure as described above. Mice were separated into six groups as follows (10 mice/group): group I, no treatment; group II, treatment with 100 mg of photocured gelatin gel; group III, treatment with 300 μg of gemcitabine bolus injection near tumor; group IV, treatment with 3000 μg of gemcitabine bolus injection near tumor; group V, treatment with 100 mg of photocured gelatin gel immobilized with 300 μg of gemcitabine; and group VI, treatment with 100 mg of photocured gelatin gel immobilized with 3000 μg of gemcitabine. The diameter of the tumor was measured with caliper and tumor volume was calculated using the formula: tumor volume = 0.52 × a²b, in which a is the longest diameter, and b is the shortest diameter of the tumor (14).

Determination of Cell Proliferation and Apoptosis. The pancreatic tumor s.c. inoculated in the nude mouse was excised (3 mice/group) at day 10 after gelation or injection, fixed in 10% formalin, and embedded in paraffin. Tissue sections of specimens were deparaffinized in xylene and rehydrated in graded alcohol. Proliferating cells in the tumor and normal tissue were detected using an antibody (PC-10; Dako, A/S, Glostrup, Denmark) against PCNA and visualized by incubation with 3,3’-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) for 2 min. The sections were rinsed with distilled water and counterstained with hematoxylin. To detect apoptotic cells in the tumor and normal tissue, tissue sections were stained by TUNEL method (In Situ Apoptosis Detection Kit; Takara, Shiga, Japan) according to the manufacturer’s instruction. For quantification of PCNA and TUNEL expression, the number of positive cells was counted in 10 random high power fields (0.135 mm² fields at ×200 magnification) and divided by the total number of cells/fields.
Blood Biochemistry. To examine the side effect accompanied with therapy on bone marrow, liver and kidney, white blood cell count, glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, blood urea nitrogen, and creatinine of blood samples of nude mice (3 mice/group) were evaluated at day 10 after gelation or injection according to the routine procedure at SRL Co., Ltd. (Fukuoka, Japan). Separately, all nude mice were weighted using a scale (BJ 1500 S; Sartorius, Goettingen, Germany) at days 0, 3, 7, and 10.

Statistical Analysis. Statistical analysis was performed with the StatView 5.0 program (Berkley, CA). Data were shown as means ± SD. The values were subjected to statistical analysis using ANOVA. Differences among the groups were assessed by the Scheffe posthoc test.

RESULTS

Gemcitabine Release from Photocured Gelatin Gels in Vitro. In vitro concentration-dependent chemosensitivity of AsPC1 cell for gemcitabine was quantitatively determined using MTT assay to establish a gemcitabine concentration-cell proliferation relationship, from which the amount of gemcitabine released from a photocured gelatin gel in vitro can be calculated. The exposure of AsPC1 with gemcitabine for 24 h significantly induced dose-dependent growth inhibition (Fig. 1A). The IC$_{50}$, defined as the concentration of gemcitabine inducing 50% growth inhibition, was 0.009 µg/ml.

Three photocured gelatin gels (styrenated gelatin: 30, 40, or 50 weight percent based on the total gel weight, carboxylated CQ: 0.05 weight percent based on the styrenated gelatin weight) immobilized with gemcitabine (3 weight percent based on the total gel weight) were prepared, and their in vitro release profiles of gemcitabine were examined. The amount of gemcitabine released from the photocured gelatin gel into the medium was determined using the following equation obtained from the concentration dependency of the chemosensitivity of AsPC1 for gemcitabine (Fig. 1B), $y = 0.36e^{-7.0x}$, in which $y$ is the gemcitabine concentration, and $x$ is the relative cell proliferation rate (100% as defined for untreated control). Fig. 2 shows the time course of gemcitabine release from the photocured gelatin gels. The releasing profiles, regardless of gel concentration, were characterized as an initial burst of the drug, followed by gradual release for a prolonged period. The majority of gemcitabine (3.0 mg was embedded in each gel) was released within 6 h: ~80% (released amount: 2.4 mg) from 30 weight percent gel, 70% (2.1 mg) from 40 weight percent gel, and 62% (1.86 mg) from 50 weight percent gel.

Prolonged Tissue Permeation and Inhibition of Tumor Growth. Three days after inoculation of AsPC1 cells, mice were subjected to surgical skin incision. Then, a viscous-styrenated gelatin solution containing a red-color dye, rhodamine B (used as a model drug, $M_r$ 479), was coated and photogelled on the pancreatic tumor s.c. inoculated in nude mice. Fig. 3 shows the confocal laser scanning microscopic photographs of the excised pancreatic tumor at days 1, 3, and 10. No red fluorescence was observed in the control group (Fig. 3A). For the injection group in which rhodamine B solution was injected near the tumor, red color fluorescence of tissue was slightly observed at day 1 but disappeared at day 3 (Fig. 3B). In contrast, red color fluorescence of tissue was still observed at day 10 in the gel group (Fig. 3C), indicating that rhodamine B was continuously released from photocured gelatin gel and retained in the targeted tissue for a long period of time. This implies that gemcitabine ($M_r$ 299) immobilized in photocured gelatin gel permeated into and retained in the deep region of tissue for a long period of time.

Instead of rhodamine B, gemcitabine mixed with styrenated gelatin solution was coated on tumor tissues prepared
according to the method described above and photoirradiated (Fig. 4A), resulting in the formation of a tissue adhesive gelatinous matrix as shown in Fig. 4B. The time course of tumor volume after initiation of therapy and the appearance of the heterotopic pancreatic tumor at day 10 are shown in Fig. 5. The tumors in groups I (control: no treatment) and II (gel without gemcitabine) rapidly grew with time. In groups III and IV (injection groups: both of bolus injection with gemcitabine at
Gemcitabine Delivery Using Photocured Gelatin Gel

In contrast, in groups V and VI (gel groups immobilized with gemcitabine at 300 and 3000 µg, respectively) a significant reduction in pancreatic tumor volume was observed as compared with groups I, II, III, and IV (P < 0.01).

Histochemical Observation. PCNA and TUNEL staining were conducted at day 10 after gelation or injection (Fig. 6A). The percentage of PCNA-positive (proliferating) cells was significantly lower in tumors treated with photocured gelatin gel immobilized with gemcitabine (groups V and VI) compared with tumors of the other groups (P < 0.01; Fig. 6B). In contrast, the percentage of TUNEL-positive (apoptotic) tumor cells increased to ~40% in both groups V and VI, a value being >10 times higher than those of the other groups (P < 0.01; Fig. 6B).

Blood Biochemistry. Blood analysis and body weight measurement of nude mice were conducted to evaluate the side effect of therapy. Irrespective of group, no mice died of a series of these therapies within the observation period (10 days). Blood analysis indicated little significant difference in the data of bone marrow (Fig. 7A), liver (Fig. 7B), and kidney (Fig. 7C) functions between each group. Although body weight loss was statistically significant (P < 0.05) in group VI at day 3 after gelation, body weight increased with time, which indicated that severe side effect did not happen (Fig. 7D).

DISCUSSION

Recently, systemic administration of gemcitabine, a deoxycytidine analogue, demonstrated modest improvements in response rate (5–9). However, the median survival for patients with pancreatic cancer is limited to 4–6 months (6, 9). Therefore, a novel administration strategy is required to improve the prognosis of the patients who have been surgically treated. A DDS used in the preclinical and clinical treatments for a variety of cancers has been reported, e.g., nanoparticles, (15–17) microspheres (18, 19), and hydrogel-based DDS (20, 21).

Our strategy is in situ construction of a tissue-adhesive, drug-releasing matrix on dissected tissue after extensive surgical operation. That is, a viscous solution of gemcitabine-containing styrenated gelatin is coated on the dissected tissues and subsequently irradiated with visible light to form a gel in situ on the tissue, which is expected to exert gemcitabine for a prolonged and site-specific anticancer effect, thereby increasing bioavailability of anticancer drug and preventing harmful systemic side effect. Such tissue adhesive gel formation immobilized with gemcitabine conducted immediately after operation might be a very effective strategy because the remnant cancer cells were activated to grow with the many kinds of cytokines induced by the surgical invasiveness, especially for a couple of days after operation (22).

In the present study, we demonstrated that photocured gelatin gel immobilized with gemcitabine is significantly effective in inhibiting the growth of pancreatic tumors s.c. inoculated in nude mice, whereas the bolus injection of gemcitabine did not inhibit the pancreatic tumor growth (Fig. 5). Therapeutic success correlated with decreased cell proliferation and increased apoptosis in the tumor cell, which was supported by PCNA and TUNEL staining (Fig. 6). Requirement for effective chemotherapy is that anticancer drug permeates to achieve a lethal concentration in all of the targeted tumor cells. Even gemcitabine, to which the constituent tumor cells are highly sensitive, will...
have limited efficacy if it only reaches some of the targeted tumor cells in low concentration. The appearance of nude mice s.c. injected with rhodamine B solution immediately turned to be red within 15 min, and the redness of skin disappeared within 12 h (data not shown). Confocal scanning laser microscopy revealed that rhodamine B, a model drug, released from photocured gelatin gel permeated and retained in the tissue for at least 10 days, whereas rhodamine B early disappeared from tissue in the injection group (Fig. 3). These results suggest that injected gemcitabine systemically spread via the vascular system and is rapidly converted to inactive metabolites, resulting in limited therapeutic efficacy to targeted tumor cells.

A previous article reported that gemcitabine exerts a cytotoxic effect against cancer cell in both dose- and time-dependent manner (23). The increase of gemcitabine exposure time from 2 to 24 h resulted in the increase in cytotoxicity up to 75-fold in IC$_{50}$, which indicates that the duration of gemcitabine exposure seems to be an important parameter for the anticancer effect of gemcitabine (24). Photocured gelatin gel continuously released gemcitabine (Fig. 2), which enables to prolong the contact duration of gemcitabine with tumor, resulting in effective exertion of anticancer effect.

Photocured gelatin gel immobilized with gemcitabine did not cause the local skin necrosis (Figs. 5 and 6). A high degree of tissue integrity was also confirmed with TUNEL staining (Fig. 6), showing the very small number of cells undergoing apoptosis.

**Fig. 6** A, immunohistochemical staining for pancreatic tumor (s.c. inoculation of AsPC1 cell in nude mouse) and normal tissue (skin of nude mouse) for H&E, PCNA (proliferation), and TUNEL (apoptosis) staining at day 10 after therapy. Representative pictures at ×200 magnification. B, quantification of PCNA staining- and TUNEL staining-positive cells in pancreatic tumor. C, quantification of PCNA staining- and TUNEL staining-positive cells in normal tissue. Data are shown as means ± SD [10 random high power fields (0.135 mm$^2$); group V compared with groups I, II, III, and IV (*, P < 0.01); group VI compared with groups I, II, III, and IV (**, P < 0.01).
apoptosis in normal tissue treated with gemcitabine. Gemcitabine, a deoxycytidine analogue, incorporates into DNA and causes a pause in DNA synthesis and subsequently induces cell apoptosis (8). Therefore, the S phase in the cell cycle is the most critical cytotoxic target for gemcitabine. PCNA staining showed that the percentage of proliferating cells in normal tissue (skin of nude mouse) was \( \frac{3}{10} \% \) (Fig. 6), indicating that the majority of the cells in normal tissue were nondividing cells. This may be the reason why normal tissue is not comparatively injured for the treatment of gemcitabine.

These in vitro and in vivo data supported the strategy of using photocured gelatin gel immobilized with gemcitabine to reduce or inhibit local recurrence while decreasing the risk of systemic side effect (Fig. 7). Although it is too early to judge the efficacy of this treatment, we hope that this treatment will be translated into clinical trial to improve the prognosis for patients with advanced pancreatic cancer in the near future.

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