SPANDIDOS PUBLICATIONS

Heat-directed suicide gene therapy mediated by heat shock protein promoter for gastric cancer

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Abstract. The prognosis of patients with metastatic gastric cancer, particularly peritoneal carcinomatosis, remains poor despite intensive interventions. Gene therapy and hyperthermia can be promising strategies for such advanced disease. The study was conducted to explore the possible effective therapeutic approach of suicide gene therapy with herpes simplex virus thymidine kinase (HSV-tk) in combination with hyperthermia for advanced gastric cancer. The heat shock protein (hsp) 70B gene promoter-oriented HSV-tk (HSP-tk)/ganciclovir (GCV) system directed by heat shock was developed. Hsp promoter activity under the control of heating was assessed by dual luciferase assay in gastric cancer cell lines and implanted tumors of nude mice. *In vitro* cytotoxic assay was performed using the HSP-tk/GCV delivered by the hemagglutinating virus of Japan (HVJ) liposome, with or without heating. Mice with subcutaneously xenografted tumors and peritoneal carcinomatosis were treated with hyperthermia and gene therapy using the HVJ-liposome-carrying HSP-tk. Assessment by luciferase assay demonstrated highly inducible and tumor-specific promoter activity *in vitro* and *in vivo*. Cytotoxic assays showed that cells transfected with HSP-tk became more sensitive to GCV with heating. A synergistic effect was also observed when treated with a non-heat-inducible cytomegalovirus (CMV) promoter-mediated HSV-tk/GCV and heating, indicating bystander killing. The HVJ-liposome-carrying HSP-tk/GCV combined with hyperthermia significantly inhibited the growth of subcutaneous tumors and prolonged survival of mice with peritoneal carcinomatosis. We conclude that the combination of suicide gene therapy with hyperthermia can provide a promising treatment modality for advanced gastric cancer.

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Introduction

Gastric cancer is a major worldwide cause of morbidity and mortality (1). Despite progress in early diagnosis and treatment of this cancer, aggressive surgical or chemotherapeutic interventions have not significantly improved the prognosis of patients with the advanced disease, particularly peritoneal carcinomatosis (2,3). Nevertheless, promising results have been reported for intraperitoneal hyperthermia with chemotherapy or cytoreductive surgery (4). Constant intraperitoneal hyperthermia devices by a closed continuous circuit are successfully developed in clinical settings. Thus, hyperthermia may offer a cure or palliation in this condition with few alternative options, albeit further prospective studies are warranted in carefully selected patients.

Gene therapy can be an innovative therapeutic modality for advanced gastric cancer (5). A promising approach widely used for malignant diseases, including gastric cancer, is the so-called suicide gene therapy with herpes simplex virus thymidine kinase (HSV-tk) followed by ganciclovir (GCV) administration (3,5). However, several limitations of this approach still need to be overcome, including tumor specificity and delivery (6).

To specifically target therapeutic gene expression, one approach is to use promoters of stress-inducible proteins that are highly expressed in tumor tissues as a result of environmental and pathophysiological stimuli, such as glucose deprivation, anoxia and acidic pH 6.0-8.0. Heat shock protein (HSP) 72, one of the most extensively studied stress proteins, not only serves as a molecular chaperone under normal conditions or in response to stress (i.e. heat shock), but is also involved in malignant transformation, tumorigenesis and tumor progression (10-14). In fact, we have demonstrated that HSP72 is overexpressed in gastric

cancer cells, but not in adjacent normal tissues (15). Since HSP72 expression is mediated by interaction of heat shock factor 1 with specific binding sites (heat shock elements) located in the promoter region of the hsp70B gene (11), incorporation of the hsp70B promoter into suicide gene therapy can potentially provide tumor-specific and inducible targeting for gastric cancer.

Although many viral and non-viral vectors have been developed (5,6), there are limitations. Hence, more effective and safer gene transfer should be established, preferably with the advantages of both viral and non-viral vectors. A unique non-viral/viral hybrid vector named 'HVJ liposome' was developed by combining synthetic liposome with fusion proteins derived from the hemagglutinating virus of Japan (HVJ), offering a new concept for gene transfer with high efficiency and low toxicity (16-18). Employing the HVJ liposome, we designed heat-directed suicide gene therapy with the HSV-tk/GCV system mediated by the hsp70B promoter, with the aim of providing a safe and effective treatment modality for advanced gastric cancer, particularly peritoneal carcinomatosis.

Materials and methods

Cell culture. Three gastric cancer cell lines, MKN45, NUGC3 and KATOIII, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The cell lines were maintained in RPMI-1640 medium containing 10% fetal bovine serum, 2 mmol/l L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (all from Invitrogen, Carlsbad, CA) in a humidified atmosphere of 5% CO_2.

Heat shock treatment. Cell culture Petri dishes were sealed and heat challenged by immersion in a calibrated water bath. For heat shock treatment, the desired temperatures ranging from 41-45˚C were maintained within ±0.05˚C for 30 min. After heating, the culture plates were immediately returned to the 37˚C incubator and maintained for the time stipulated.

Immunoblot analysis. To examine HSP expression, cells were incubated at 45˚C for 30 min, and further cultured at 37˚C for 0, 6, 12, 24 and 48 h. The cells were lysed in buffer containing 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES, pH 7.5), 1.5 M NaCl, 200 mM Tris-HCl, 5% Triton X and 0.25 mM dithiothreitol supplemented with protease inhibitors (20 μg/ml aprotinin, 20 μg/ml leupeptin, 40 μM pepstatin and 2 mM phenylmethylsulfonyl fluoride), then sonicated on ice. After centrifugation and denaturation by boiling for 10 min, the concentration of proteins was measured based on Bradford's method (Bio-Rad Laboratories, Hercules, CA). Each 25 μg of protein was loaded onto a 12% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis, then transferred onto polyvinylidene difluoride membranes. After blocking with 5% milk in TBST (137 mM NaCl, 25 mM Tris and 1 mM disodium ethylenediaminotetraacetate containing 0.1% Tween-20), the membranes were incubated with 1:1000 mouse monoclonal anti-HSP72 antibody (Oncogene Research Products, Cambridge, MA), followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (Biosource International, Camarillo, CA) at a dilution of 1:2000. Bound antibodies were visualized using enhanced chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL).

Northern blotting. Messenger (m) ribonucleic acid (RNA) coding for HSP72 was detected by Northern hybridization (19). At the time point of 0, 6, 12 and 24 h after heating at 45˚C for 30 min, total RNA from each cell population was extracted using a commercial kit according to the instructions provided by the supplier (Isogen, Nippon Gen, Toyama, Japan). The RNA (20 μg/lane) was separated by overnight electrophoresis on a 1% agarose/2.2 M formaldehyde gel. The 18S and 28S ribosomal RNA bands were of equal density when the gels were stained with ethidium bromide. The gels were transferred to nytran membranes, and hybridization was performed using a probe labeled with ^{32}P-dCTP. The probe was generated by oligolabeling the 2.5-kb *Bam*HI/*Xho*I digest of HSP72/pcDNA. Labeled membranes were exposed overnight, and the autoradiographs were developed in an automated processor. As an internal control, cyclophylin mRNA was identified in the same way.

Construction of plasmids. The firefly luciferase plasmid, HSP-Luc, was constructed from mammalian HSP expression vector p1730R by ligating a fragment of 2.3 kb containing the hsp70B promoter (Stressgen Biotechnologies, San Diego, CA) into the pGL3 basic vector (Stratagene, La Jolla, CA). The cytomegalovirus (CMV) promoter-oriented luciferase plasmid (CMV-Luc) was also constructed by ligating the CMV promoter and enhancer elements upstream of the luciferase gene. The HSV-tk mediated by the hsp70B promoter (HSP-tk) was constructed by replacement of the luciferase gene with the HSV-tk gene within HSP-Luc. The CMV promoter-oriented HSV-tk (CMV-tk) was constructed in a similar fashion.

Dual luciferase assay. The HSP-Luc plasmid DNA was transfected into the cancer cells using cationic liposome TFL2-0220, kindly provided by Daiichi Pharmaceutical (Tokyo, Japan). To correct for variations in transfection efficiency, the *Renilla* luciferase gene (Promega, Madison, WI) was co-transfected. After 24 h transfection, the cells were heat challenged at various temperatures for 30 min. After cell lysates were prepared, both firefly and *Renilla* luciferase activities were quantitated using the Dual Luciferase Reporter Assay System (Promega) according to the instructions provided by the manufacturer. The same experiment was also performed using CMV-Luc.

Preparation of HVJ liposome. The HVJ-cationic liposome was prepared for *in vitro* gene therapy as described previously (17). Briefly, DC-cholesterol [3ß-N-(N',N'-dimethyl-aminoethan-carbamoyl)cholesterol], phosphatidylcholine and cholesterol (all from Sigma, St. Louis, MO) were mixed at a weight ratio of 1:8:4. The lipid mixtures (10 mg) were transferred to glass tubes (Iwaki Glass, Tokyo, Japan) and dried to a thin lipid film in a rotary evaporator (Iwaki Glass). The dried lipid was hydrated in 200 μl of a balanced salt solution (BSS; 137 mM NaCl, 5.4 mM KCl, and 13 mM

pH 7.6) containing vector DNAs (300 μg), agitated by vortexing for 30 sec, then left to stand for 30 sec. This procedure was repeated 8 times, followed by the addition of 800 μl of BSS and extrusion through a cellulose acetate membrane filter (pore size, 0.45 μm) and by sequential addition of 500 μl of BSS and extrusion via a 0.2 μm filter to obtain unilamellar liposomes. The liposome suspensions were mixed with 30,000 hemagglutinating units of HVJ (inactivated by ultraviolet irradiation at 1980 J/m^2), and incubated at 4˚C for 10 min, then 37˚C for 60 min with continuous shaking (120/min). Free HVJ was removed from the HVJ liposome solution by gradient centrifugation of sucrose density. The second layer of sucrose containing the HVJ liposome was collected.

For *in vivo* experiments, we selected the HVJ-anionic liposome method. The lipid mixtures consisted of phosphatidylserine, phosphatidylcholine and cholesterol at a weight ratio of 1:4.8:2. Otherwise, the procedures were performed as described above.

In vitro cytotoxic assay. Cells were plated at a density of 2x10^3 cells per well in a 96-well microtiter plate 16 h before transfection (day 0). The HSP-tk or *LacZ* plasmid DNAs were introduced to cells using the HVJ-cationic liposome method (day 1). The cells with or without heat shock at 45˚C for 30 min were treated with GCV (kindly provided by F. Hoffmann-La Roche, Basel, Switzerland) at 0 to 100 μg/ml in 100 μl of fresh medium (day 2). The medium in each well was replaced on days 3 and 5. On day 6, viable cell numbers were quantitated using the sulfonated tetrazolium salt, 4-[3-(4-iodophenyl)-2-4-nitrophenyl]-2H-tetrazolio-1,3-benzene disulfonate (WST-1) assay (Cell Counting kit; Dojindo, Kumamoto, Japan) according to the instructions provided by the manufacturer. The median inhibitory concentration (IC_{50}) was calculated using curve-fitting parameters. In some experiments, the same assay was carried out employing the CMV-tk/GCV system.

Detection of apoptotic cells. The cancer cells transfected with plasmid DNAs were heated at 45˚C for 30 min, 24 h after transfection. After 24 h, these cells together with the transfected cells without heating were treated with GCV (0.1 μg/ml). After a further 24 h, the cells were fixed with 2% glutaraldehyde, stained with 1 mM Hoechst 33342 (Wako Pure Chemical Industries, Osaka, Japan) and immediately observed using fluorescence microscopy.

In vivo gene therapy. The 5-week-old female BALB/c nu/nu mice (Charles-River Japan, Tokyo, Japan) were injected intra-abdominally with 5x10^6 MKN45 cells (day 0). When ascitic fluid was detected, the plasmid DNAs were intraperitoneally introduced using the HVJ-anionic liposome method on days 21, 23 and 25. Hyperthermia (42˚C for 30 min) was performed by immersion in a water bath on days 22, 24 and 26. Intraperitoneal GCV (25 mg/kg per day) administration was carried out from day 21 to day 35.

The cancer cells (5x10^6) were again implanted subcutaneously into the flanks of 5-week-old female nude mice. A week later (day 0), when the xenografts reached 7-11 mm in diameter, subcutaneous gene transfer (HSP-tk or mock vector) was performed 3 times (days 1, 4 and 8). GCV (25 mg/kg per day) was injected intraperitoneally until day 14. Hyperthermia was performed on days 2, 5 and 9 as described above. The perpendicular tumor diameter was measured with calipers, and tumor volume (V) was calculated using the formula for a rotational ellipsoid: $V= AxB^2/2$ (where A is the longer diameter and B is the shorter one). The results were expressed as percentages relative to tumor size on day 0.

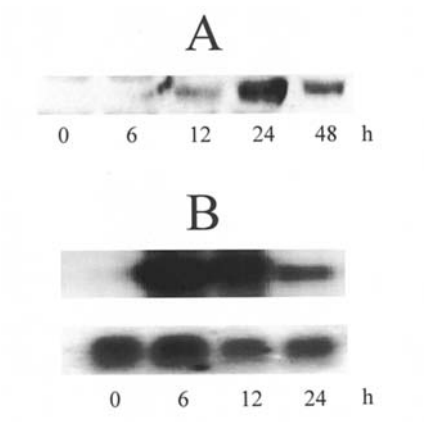

Figure 1. (A) Western blot analysis for heat shock protein (HSP) 72. (B) Northern blot analysis for messenger RNA coding for HSP72 (upper) and cyclophilin (lower) in MKN45 gastric cancer cells. H, hours after heat shock at 45˚C for 30 min.

For the assessment of gene transfer *in vivo*, the HSP-Luc gene was transferred into the peritoneal cavity of mice, which had been implanted into the MKN45 cells intra-abdominally and developed ascites, followed by heat shock at 42˚C for 30 min 2 days later. Mice were sacrificed 24 h after heat shock, and small pieces (100 mg) of tissues were obtained from implanted tumors and various abdominal tissues, then homogenized. The dual luciferase assay was performed as described above.

Care, treatment and euthanasia of the animals were performed in accordance with the Nagasaki University Institutional Ethics Committee guidelines. No mice showed signs of toxicity in response to the therapeutic approaches.

Statistical analysis. Data were expressed as mean ± standard deviation (SD). Statistical analyses were performed using a two-tailed Student's t-test. The Kaplan-Meier method was used to analyze survival rates. A p-value <0.05 was accepted as statistically significant.

Results

Induction of HSP72 protein and mRNA expression by heat shock. HSP72 protein and its mRNA expression, detected by Western blot (Fig. 1A) and Northern blot (Fig. 1B) analysis, respectively, were substantially induced by heat shock treatment in gastric cancer cells.

Promoter activity assay. Employing the HSP-Luc plasmid, the firefly luciferase activity relative to *Renilla* luciferase activity in all of the gastric cancer cell lines was markedly increased at temperatures of 42-45˚C, while negligible or small changes were noted at temperatures of 37-41˚C (Fig. 2A).

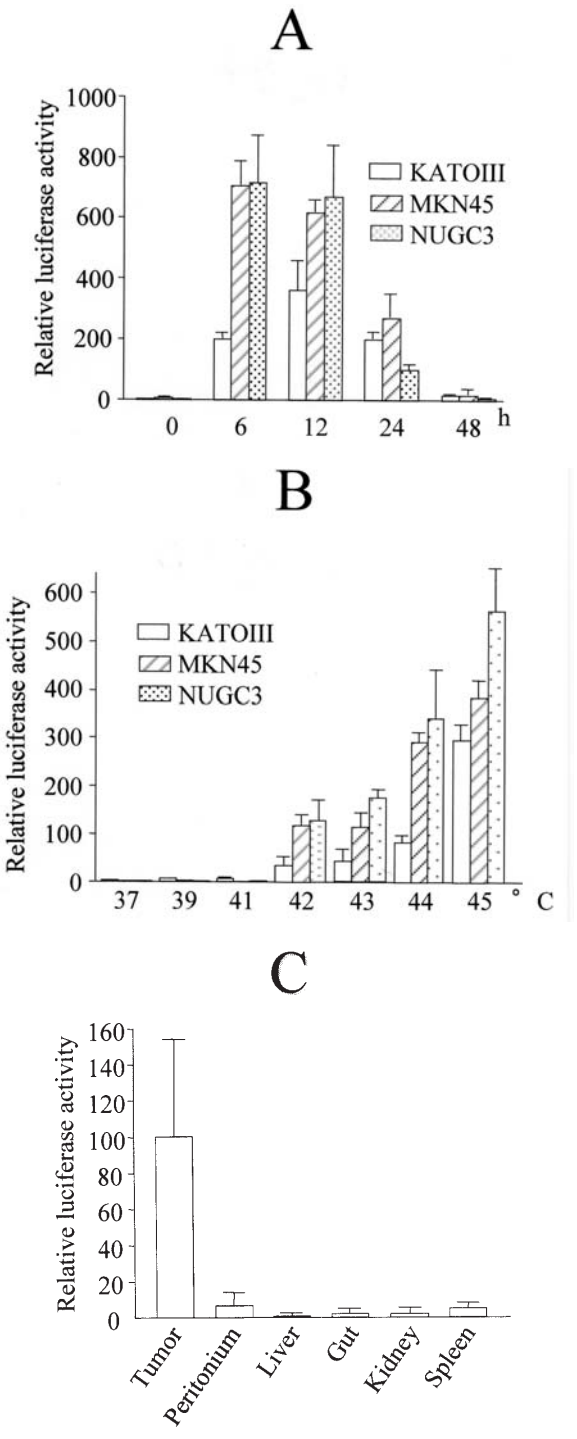

Figure 2. Dual luciferase assay showing temperature dependency of heat shock in firefly luciferase activity mediated by hsp70B promoter relative to *Renilla* luciferase activity 12 h after heating for 30 min in each gastric cancer cell line (A) and a time-dependent increase in promoter activity after heat shock at 45˚C for 30 min in the cells (B). Figures show representative data (mean ± SD, n=6). (C) Dual luciferase assay showing significantly increased relative luciferase activity in disseminated tumors relative to various abdominal organs in nude mice with peritoneal carcinomatosis due to intra-abdominal injection of MKN45 cells. The hsp70B promoter-controlled luciferase gene was introduced into the peritoneal cavity using the hemagglutinating virus of Japan (HVJ)-liposome method and heat treatment 24 h later. Figures show representative data (mean ± SD, n=3).

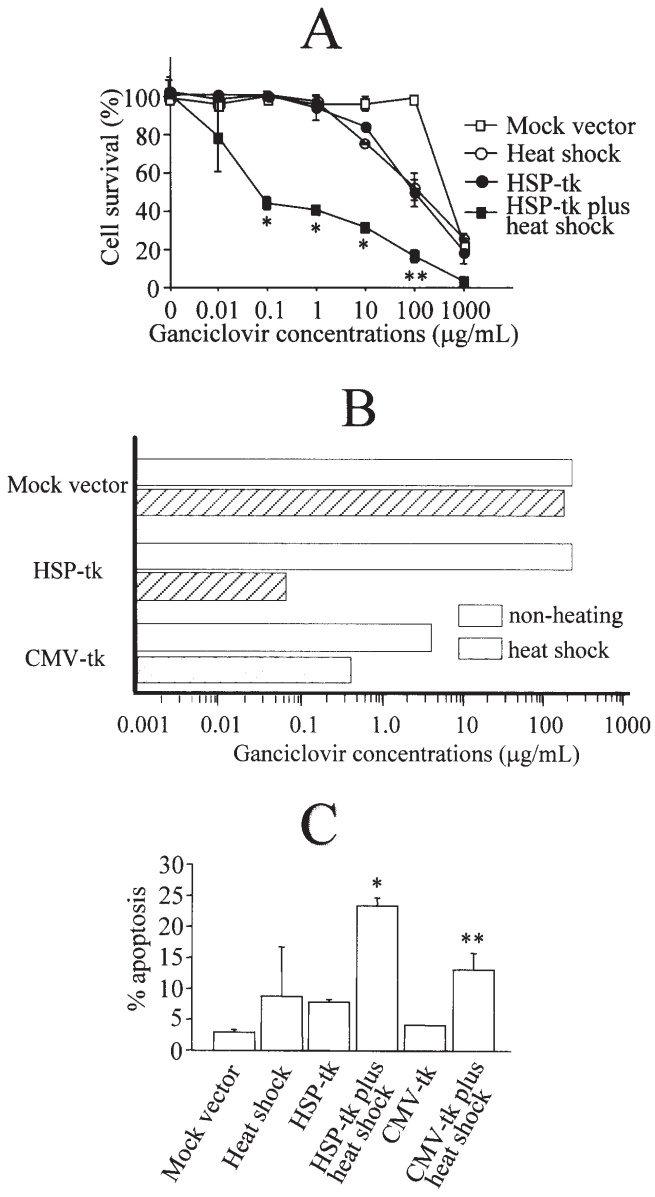

Figure 3. (A) Cytotoxic assay of ganciclovir (GCV) in NUGC3 cells transfected with hsp70B promoter-oriented herpes simplex virus thymidine kinase (HSP-tk) or *LacZ* SV-ß-galactosidase gene (mock vector) with or without heat shock. Data are representative of three separate experiments; each point represents mean ± SD and is expressed as a percentage relative to the *LacZ*-transfected control cells. $^{*}p<0.01$ and $^{**}p<0.001$, compared to mock vector. (B) Concentrations of GCV yielding 50% growth inhibition (IC_{50}) in each treatment group. CMV-tk, cytomegalovirus promoter-oriented herpes simplex virus thymidine kinase. (C) The percentages of apoptotic cells in various treatment groups, as assessed by Hoechst dye staining. $^{*}p<0.001$ compared to mock vector or the HSP-tk/GCV system without heating, $^{**}p<0.05$ compared to the CMV-tk/GCV alone. Data are expressed as mean ± SD (n=4).

The cancer cells exhibited a time-dependent increase in activity after heat treatment at 45˚C (Fig. 2B). In particular, the relative luciferase activity was markedly increased more than 700-fold between 6 to 12 h in NUGC3 cells, followed by MKN45 cells. Nevertheless, the promoter activity was rapidly decreased 24 h later and returned to baseline levels after 48 h heating. Thus, we employed NUGC3 cells for *in vitro* cytotoxic and apoptotic assays because of high inducibility and controllability. In contrast, the CMV promoter had little impact on the luciferase activity even in NUGC3 cells at the time point of 12 h post heating at 45˚C (only 1.43±1.52-fold over background at 37˚C).

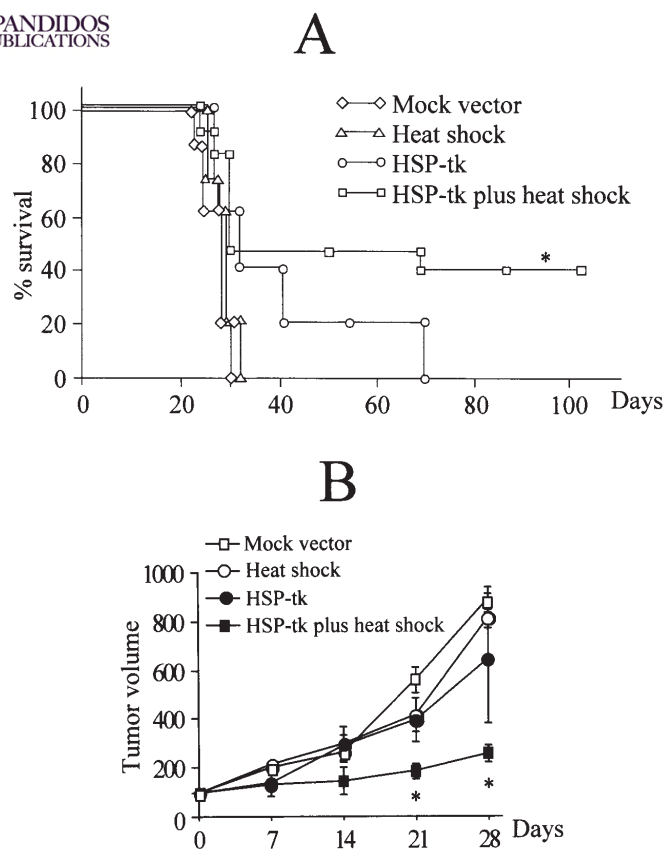

Figure 4. (A) Effect of gene therapy with the HSP-tk/GCV system and hyperthermia on survival of nude mice, which developed peritoneal carcinomatosis due to intraperitoneal inoculation of MKN45 cells (n=10). The HVJ liposome carrying HSP-tk or *LacZ* was intraperitoneally introduced on days 21, 23 and 25 with or without heat shock (42˚C for 30 min on days 22, 24 and 26). GCV was intraperitoneally administrated for 14 days. *p<0.01, compared to mock vector. (B) Effects of gene therapy with the HSP-tk/GCV system and hyperthermia on the tumor growth of implanted tumors into the flanks of nude mice (n=3). One week after the inoculation of cancer cells, the HVJ liposome carrying HSP-tk or *LacZ* was introduced to xenografts on days 1, 4 and 8 with or without heat shock (42˚C for 30 min on days 2, 5 and 9). GCV was intraperitoneally administrated for 14 days. *p<0.05, compared to mock vector. Each data point represents the mean ± SD and was expressed as a percentage relative to tumor size on day 0.

With *in vivo* settings, the relative luciferase activity in the implanted tumors from mice transfected with the HSP-Luc were markedly increased after heating, whereas other various tissues showed a modest increase in the promoter assay (Fig. 2C).

In vitro cytotoxic therapy. A low killing effect by GCV (0-100 μg/ml) was observed in the HSP-tk-transfected cancer cells without heat shock and SV-ß-galactosidase-transfected cells with or without heating (Fig. 3A). However, GCV administration resulted in significant growth suppression of HSP-tk-transfected cells that underwent heat shock treatment. Thus, HSP-tk-transfected cells that had been heat shock-treated were >1,000 times more sensitive to GCV than HSP-tk-transfected NUGC3 cells without heating when assessed by IC_{50} (Fig. 3B). Again, CMV-tk-transfected cells with heat shock showed a 10-fold increase in IC_{50} for GCV compared to those without heating (Fig. 3B). Cellular gene transfection efficiency, which was assessed by flow cytometry based on *LacZ* ß-galactosidase expression, was 22.7±5.4% (18).

Effect of heat shock and gene therapy on apoptosis. The percentage of apoptotic cells assessed by Hoechst dye staining was significantly increased in the HSP-tk/GCV system followed by heat shock compared to the HSP-tk/GCV without heating or the mock vector (each for p<0.001; Fig. 3C). Heating alone or the HSP-tk/GCV or CMV-tk/GCV system without heat shock showed a modest increase in cellular apoptosis. Following heating, however, the CMV-tk/GCV system exhibited a significantly enhanced cell killing effect (p<0.05, Fig. 3C).

Survival of nude mice with peritoneal carcinomatosis. Among mice challenged intraperitoneally with MKN45 cells, the mock vector-transfected mice and those challenged with heat shock alone died much earlier (Fig. 4A). Treatment with HSP-tk without heating tended to prolong survival, but insignificantly, whereas mice treated with HSP-tk gene therapy combined with hyperthermia showed significantly longer survival (Fig. 4A).

Effect of gene therapy and hyperthermia on the growth of subcutaneous tumors. Tumor growth of xenografts was significantly suppressed with the combination of the HSP-tk/GCV and hyperthermia on days 21 and 28 (p<0.05 and p<0.01, respectively). On the other hand, the HSP-tk/GCV without heating or heating alone had little impact on the growth of xenografted tumors (Fig. 4B).

Discussion

Our results demonstrated that the hsp70B promoter-oriented suicide gene therapy was effective in eliminating gastric cancer cells *in vitro* and *in vivo*, which is consistent with previous studies on other malignancies (21-23). In cytotoxic assays, the HSP-tk-transfected cells that underwent heat shock were >1,000 times more sensitive to GCV than cells that were not heat treated. After heating, the hsp70B promoter, which was incorporated upstream of the luciferase gene, increased the relative luciferase activity by no less than 700-fold over background activity, similar to the overexpression of HSP72 protein and mRNA in response to heat shock. Although the luciferase activity was undetectable at the baseline without heat treatment, it quickly reached a maximum value following heat shock (6-12 h), and dropped back within 48 h. This high level of inductivity under stringent heat control may be conducive to molecular targeting of gene therapy for gastric cancer.

In addition to high controllability of the hsp promoter in cancer cells by heating, HSP72 is selectively overexpressed in a variety of tumor tissues due to diverse intrinsic micro-environmental stresses and related to tumorigenesis, malignant phenotype, resistance to apoptosis and a poor clinical prognosis (10-14). We previously demonstrated enhanced HSP72 expression in gastric cancer tissues relative to the surrounding normal tissues (15). Moreover, the relative luciferase activity was exclusively increased with heating in the implanted tumors of mice, but not in the abdominal tissues. It is possible that such stress exposures *in vivo* milieu have already induced the hsp promoter-oriented transcription of targeted genes in tumor tissues even without heating and may augment the expression in synergy with heat shock. From a

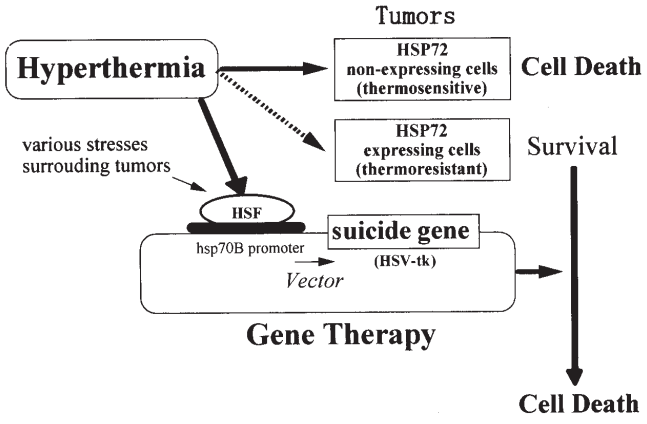

Figure 5. Schematic strategy of 'hyperthermic suicide gene therapy'. HSP, heat shock protein; HSF, heat shock factor 1; HSV-tk, herpes simplex virus thymidine kinase; GCV, ganciclovir.

clinical point of view, human tumors are heterogeneous, composed of HSP72-expressing and non-expressing cells. Cancer cells lacking this chaperone may be sensitive to heat shock, while those with abundant HSP, which are therefore thermotolerant, can be largely eradicated by the HSP-tk/GCV machinery (14). Fig. 5 represents this schematic strategy, named 'hyperthermic suicide gene therapy'.

Besides the controllability and selectivity of therapeutic gene expression, the bystander effect is also important for suicide gene therapy (24,25), since it is impossible for the existing delivery systems to transfer the target gene(s) into every tumor cell, especially in more advanced diseases with large solid tumors and/or distant metastases. There have been reports on the mechanisms of bystander killing through activation of tumor immunity by cytotoxic T cells in syngeneic animal models (26), but the nude mice in this study lack active T cell immunity, similar to patients who have fallen into an immunocompromised state due to advanced cancer. On the other hand, considerable bystander cell death can be mediated by intercellular communication. Fick *et al* reported that phosphorylated toxic GCV could be transferred through gap junctions to HSV-tk-negative cells (24). In our study, cytotoxic assays demonstrated that the combination of heat shock with suicide gene therapy directed by the CMV promoter, whose activity was not influenced by heating, had a synergistic effect on gastric cancer cell death. Concordant with this, quantitative analyses of apoptosis revealed that the combined treatment resulted in enhanced apoptosis compared to either treatment alone. In a previous study, we demonstrated that the combination of HSP-tk/GCV and heat shock upregulates both Fas and Fas ligand expression assessed by immunohistochemistry (22). Taken together, we believe that the hyperthermia-based approach can induce mutually potentiating bystander cell death when combined with the HSV-tk/GCV system, which is not dependent on T-cell-mediated tumor immunity, through synergistically apoptotic signaling, even if the target gene could not be transferred into all tumor cell populations.

In the present study, we employed the HVJ liposome method to deliver the suicide gene plasmids (16). This hybrid vector has a high capacity for gene encapsulation of non-viral vectors and, at the same time, has an acceptable transfection efficiency like viral vectors, as we demonstrated previously (16-18). Indeed, adenovirus- and retrovirus-mediated HSV-tk gene transfer plus GCV provides sufficient cytotoxic effects on digestive cancers, based on the ample ability to transfer the therapeutic gene (27,28). However, these viral vectors have several disadvantages in clinical applications in terms of safety, particularly immunogenicity, and the limited capacity of transgenic materials (6). The HVJ liposome was developed by combining liposome with fusion proteins derived from inactivated envelopes of HVJ, and is therefore much less immunogenic and cytotoxic than virus-mediated vectors (16-18), allowing repeated administrations (16). In fact, accumulating data showed the potential of the HVJ liposome method for gene therapy in a variety of diseases including cancer, cardiovascular disorders, arthritis and liver cirrhosis *in vivo (*21,22,29-31). In the present study, even mice with progressive peritoneal carcinomatosis could tolerate repetitive gene transfers by this hybrid vector without visible indications of toxicity, thus contributing to their prolonged survival. The same was true for subcutaneous xenografts, where the tumor growth was significantly suppressed with repeated HVJ-carrying HSP-tk/GCV plus heating. Thus, the HVJ liposome has several advantages over other delivery systems, particularly in *in vivo* settings.

In conclusion, the HVJ-liposome-carrying HSP-tk/GCV plus heat shock showed a synergistic killing effect on gastric cancer cells with high inductivity and selectivity. Repetitive treatment with this combined regimen resulted in growth inhibition of subcutaneously implanted tumors and prolonged survival of mice with peritoneal carcinomatosis, suggesting that hyperthermic suicide gene therapy can provide a safe and effective therapeutic option for advanced gastric cancer.

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