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 HSV-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.

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 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) 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 ethylenediaminetetraacetate 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 nitroan membranes, and hybridization was performed using a probe labeled with 32P-dCTP. The probe was generated by oligolabeling the 2.5-kb BamHI/XhoI digest of HSP72/pcDNA. Labeled membranes were exposed overnight, and the autoradiographs were developed in an automated processor. As an internal control, cyclophillin mRNA was identified in the same way.

Construction of plasmids. The firefly luciferase plasmid, HSV-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 (HSV-tk) was constructed by replacement of the luciferase gene with the HSV-tk gene within HSV-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 [3β-N-(N’β-dimethylaminoethan-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
In vivo gene therapy. The 5-week-old female BALB/c nu/nu mice (Charles-River Japan, Tokyo, Japan) were injected intra-abdominally with $5 \times 10^6$ 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. Mice were sacrificed 24 h after heat shock, and small pieces (100 mg) of tissues were obtained and 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).
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).
LacZ β-galactosidase expression, was 22.7±5.4% (18).

The CMV-tk-transfected cells with heat treated were >1,000 times more sensitive to GCV than HSP-tk-transfected cells without heating when assessed by IC50 (Fig. 3B). Again, CMV-tk-transfected NUGC3 cells without heating (Fig. 3B). Cellular gene transfection by IC50 (Fig. 3B). Thus, HSP-tk-transfected cells that had been heat shock treatment. HSP-tk-transfected cells that underwent heat shock treatment. The HVJ liposome carrying HSP-tk or LacZ was intraperitoneally introduced on days 1, 4 and 8 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-β-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 IC50 (Fig. 3B). Again, CMV-tk-transfected cells with heat shock showed a 10-fold increase in IC50 for GCV compared to those without heating (Fig. 3B). Cellular gene transfection efficiency, which was assessed by flow cytometry based on LacZ β-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
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gene expression, the bystander effect is also important for
shock, while those with abundant HSP, which are therefore
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compared to either treatment alone. In a previous study, we demonstrated
combined treatment resulted in enhanced apoptosis compared
with this, quantitative analyses of apoptosis revealed that the
promoter, whose activity was not influenced by heating, had

Figure 5. Schematic strategy of ‘hyperthermic suicide gene therapy’. HSP,
heat shock protein; 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
either treatment alone. In a previous study, we demonstrated
that the combination of HSV-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 HSV-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 HSV-tk/GCV
plus heat shock showed a synergistic killing effect on gastric
cancer cells with high inducibility 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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